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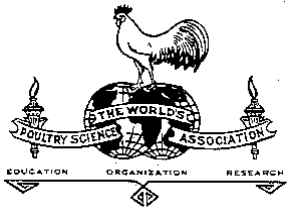

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THE WORLD'S POULTRY SCIENCE ASSOCIATION
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AUSTRALIAN POULTRY SCIENCE SYMPOSIUM
2024

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A **WISE MENTOR AND VISIONARY SCIENTIST:** E**MERITUS PROFESSOR E.F. ANNISON**

D. BALNAVE[1] and W.L. BRYDEN[1,2]

I. INTRODUCTION

On the 1st of June, 2023 the Poultry Research Foundation lost one of its most respected and honoured members with the passing of Emeritus Professor Ernest Frank Annison. Frank was 97 and is survived by his wife of 73 years, Dorine, by his sons Geoffrey and John, daughter Jennifer and by his grandchildren.

Frank Annison's name is known and highly respected internationally in the areas of animal science and education. During his time at the University of Sydney, Camden, he spent 20 years building and developing a progressive scientific research-orientated department which was recognised worldwide for its contribution to basic and applied animal science. For eleven of his twenty years at Camden he showed great vision and administrative skills while director of the Poultry and Dairy Research Foundations. This engagement with community and industry was an important part of the success of the University in these fields and reflected a deep understanding of the role that universities could play in the community.

Professor Annison was principally known for his pioneering research in quantitative animal nutrition and his research studies in nutritional biochemistry and physiology of ruminants (Bell, 2019). The many foresights and insights, gained from his own research experiences, were always freely available to his staff and this accessibility contributed in no small measure to the successful outcome of many research programmes of direct relevance to academia and industry. His wide knowledge and experience also resulted in Professor Annison being appointed to many government and industry committees and research panels.

Professor Annison graduated with a degree in Chemistry from the University of London in 1946 and in subsequent research gained a PhD from the same University in 1951. Subsequently, in 1967 the University of London awarded him the degree of Doctor of Science in recognition of his published scientific work. Frank Annison's abilities and achievements have received local and worldwide recognition. He was made a Fellow of the Nutrition Society of Australia in 1991, a Fellow of the Australian Society of Animal Production in 2002 and at the University of Sydney he was appointed Honorary Governor by the Senate in 1994. Internationally he was awarded the prestigious Roche Research Prize for Animal Nutrition in Switzerland in 1990 and his acceptance speech has been published (Annison, 1990). In 2004 he was awarded Membership of the Order of Australia.

II. CAREER AND RESEARCH INTERESTS

After the award of his higher degree in 1951 Frank Annison obtained a position at the Agricultural Research Council Institute of Animal Physiology in Cambridge, England, UK. There he commenced extensive research into volatile fatty acid and nitrogen metabolism in the rumen with another well known and respected scientist, Dyfed Lewis (Annison and Lewis, 1959). Frank had

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particularly fond memories of a one-year period of secondment he had at the Rowett Research Institute in Aberdeen, Scotland, early in this period of employment.

In 1958 Frank moved from the UK being appointed a Senior Lecturer in Biochemistry and Nutrition in the Faculty of Rural Science at the University of New England in NSW. There he continued his ruminant studies a major component of which was in world-leading research into lipid transport and metabolism in sheep (Bell, 2019). Along with two other academics and friends, Derek Lindsay and Ron Leng, Frank developed a research programme using radioactive isotopes in conjunction with arterio-venous differences to measure nutrient and metabolic turnover and utilisation in sheep. This programme established a rigorous, quantitative basis for understanding nutrient utilisation in ruminants (Annison and Leng, 1991).

Professor Annison was promoted to Associate Professor at the University of New England in 1961 but returned to the UK in 1964 to the Unilever Research Laboratory at Colworth House, Sharnbrook, Bedford. Here for nine years he directed research on the nutrition of ruminants and non-ruminants, including pigs and poultry. He became particularly interested in industry problems associated with predicting the nutritive value of feed ingredients from their chemical composition (Annison, 1974) and this was of major importance when he subsequently became Director of the Poultry Research Foundation and took a leading role in identifying the phenomenon of "low-ME" wheats for poultry (Mollah *et al.,* 1983), see below.

Professor Annison, on being appointed to the Chair of Animal Husbandry (later Animal Science) at the University of Sydney, returned to Australia in 1974. In this position and as Director of the Poultry and Dairy Research Foundations he has had a major impact on the research conducted in these two major agricultural industries. This impact has been exercised through the direction of research conducted under his guidance and through his presence and leadership of numerous research councils and advisory boards. He also undertook significant leadership roles in both the Faculties of Agriculture and Veterinary Science where he served terms as Pro (Deputy) Dean of both faculties. Frank also contributed in retirement through his appointment as Chair of the Animal Ethics Committee of the University of Sydney.

III. CONTRIBUTIONS TO POULTRY SCIENCE AND THE FOUNDATION

Professor Annison's research was centred on ruminant metabolism but he also collaborated in studies with other farm animals, including pigs and poultry. He had a particular interest in lipid metabolism and utilisation and published a number of papers in poultry (Annison *et al.,* 1969; Brickerstaffe and Annison, 1969a, b, 1970; Brickerstaffe *et al.,* 1970; Infield and Annison, 1973), including two perceptive reviews on avian lipid metabolism (Annison, 1971, 1983). In early studies with poultry he examined comparative aspects of glucose, acetate and fatty acid utilisation by sperm (Scott *et al.,* 1962 a, b), avian glucose turnover and oxidation (Annison *et al.,* 1966), and was able to demonstrate extensive microbial metabolism throughout the avian digestive tract (Annison *et al*., 1968).

When Professor Annison became Director of the Poultry Husbandry Research Foundation (now Poultry Research Foundation, PRF), he initiated research into the chemical composition of cereal grains as a means of predicting their energy content. This research (Annison and Mollah, 1977) was developed with the help of a PhD student, Yasin Mollah, (the late Dr Mollah worked for many years as a senior technical officer in the PRF laboratory at Camden). However, it was not possible to develop a satisfactory "feed concentration" equation for wheat due to the large variation in the apparent metabolisable energy (AME) values of this cereal (Mollah *et al.,* 1979). This was in accord with findings of a previous PRF Research Director, the late Charles Payne,

who had found that the AME value of wheat declined at high (>70%) dietary inclusion levels (Payne, 1976). The results of an extensive study of 22 samples of 13 wheat cultivars grown at four NSW locations in 1978 suggested that the underlying cause of the "low ME" wheat phenomenon was incomplete starch digestion as starch is the major energy component of the wheat grain (Mollah *et al.,* 1983). However detailed studies of wheat starch digestion both *in vitro* and *in vivo* by an American PhD student Anne Rogel demonstrated that wheat starch was completely digested (Rogel *et al.,* 1987). Detailed accounts of the nuances of the research to delineate "low-ME" wheat have been given by Frank (Annison *et al.,* 1987a,b) and his son Geoffrey (Annison, 1993). With the elimination of reduced starch digestibility as a possible cause of low ME the search turned to other aspects of wheat chemistry, especially non-starch polysaccharides (NSPs), and led to the contributions of Mingan Choct and Geoffrey Annison (Annison and Choct, 1991).

The other aspect of feed composition that Professor Annison was particularly interested in was the difficulties of relating the essential amino acid compositions of a feed ingredient with their availabilities (defined as that proportion of the dietary amino acid that is in a form suitable for digestion, absorption and utilisation). One obvious factor that influences amino acid availability is the extent to which an amino acid is absorbed. Measurement of digestion and absorption of amino acids appears to be the most practical way of determining the potential availability of amino acids. This can be accomplished by measuring the difference in amino acid content of the ingested meal and the amino acid content of undigested residue (Bryden *et al*., 1988). Measuring digestibility as an estimate of availability is often criticised because of microbial amino acid synthesis in the gut and the entry of endogenous protein into the gut. The influence of microbial metabolism can be minimised by measuring digestibility in the ileum or the end of the small intestine and the ileal site for digestibility measurement was adopted in PRF studies (Bryden *et al.,* 1990). Various approaches for measuring endogenous amino acids were examined but none proved satisfactory (Siriwan *et al*., 1993). Studies were expanded to examine the guandination of lysine to homoargine and using homoargine as a marker of endogenous secretion (Siriwan *et al*., 1994). This technique can be used in pigs and poultry (Bryden *et al*., 1996) and has been applied to quantify the effect of NSPs on endogenous amino acid secretions in meat chickens (Angkanaporn *et al.,* 1994). The digestibility concept was also used to determine the apparent and true ileal digestibility of dietary fats in broilers (Ajuyah *et al.,* 1996). Professor Annison, with PhD student Greg Heard, reverted to the use of radioisotopes to study the kinetics of the gastrointestinal absorption of vitamin B-6 (Heard and Annison, 1986) and with another PhD student, John Nell, to study aspects of avian molybdenum metabolism (Nell and Annison, 1980; Nell *et al.,* 1980).

The results of this extensive research programme involving a number of Foundation staff, post-doctoral and post-graduate students under Professor Annison's guidance resulted in feed formulation improvements of immense value to the commercial broiler industry worldwide. Another area in which Professor Annison had a major impact on the PRF and the industry was through the Australian Poultry Science Symposium (APSS). He, along with Derick Balnave, Bob Pym and Balkar Bains and the late Bruce Sheldon were instrumental in establishing the annual symposium as an internationally-acclaimed meeting with rigorous scientific standards.

In conclusion, Professor Annison will be remembered as a world-respected nutritional biochemist who made many outstanding contributions in his scientific career and academic profession. In addition, he was a man of great scientific vision who outlined this vision and his perspectives on the role of Animal Science and Nutrition in society in a paper that he presented (Annison, 1993) at a Festschrift held in his honour in 1991 at the University of Sydney. For those who worked with him he is remembered as a modest, wise and caring mentor.

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INTESTINAL INTEGRITY AND INFLAMMATION: HOW DOES THIS AFFECT THE MICROBIOTA COMPOSITION AND WHAT IS THE RELEVANCE FOR PRODUCTION?

F. VAN IMMERSEEL[1]

Summary

The intestinal tract of broilers is a key organ in health and disease, and optimal intestinal health is essential for animal performance. While the intestine is crucial for digestion of feed components, it is also a barrier for pathogens that can enter the bloodstream. Many pathogens can induce inflammation in the gut, costing energy to the host. While inflammation is essential to counteract pathogens, often inflammatory responses are excessive and should resolve rapidly to maintain optimal performance for the animals. The intestinal microbiota plays an important role in digestion and host health because of the breakdown of feed components and the production of a large panel of metabolites that can have a signaling function to other microbiota, but also to host cells. Because intestinal epithelial cells are the first host cells that sense these microbial metabolites, these cells are major drivers for transmitting bacterial signals to the host, and as such mucosa-associated microbial populations are main drivers of intestinal health. These microbial populations can either be (opportunistic) pathogens, such as *Escherichia coli* and *Clostridium perfringens*, or can consist of specific anaerobic genera that colonize the mucus layer and produce beneficial metabolites, such as butyrate.

Novel insights have been generated in the function of specific metabolites in intestinal inflammation, epithelial cell proliferation and differentiation, and in general physiological responses of the animal. This has led to novel methods of designing nutritional strategies to maintain health. In addition, the effect of classical feed additives, such as probiotics, prebiotics, and phytochemicals, can at least partly be explained by microbial metabolic shifts in the gut microbiota, although direct effects on host cells also have their role.

I. INTRODUCTION

Animals used for food production have been genetically selected for feed intake and muscle development and are therefore divergent from their ancestors. In addition, these animals are reared in conditions that favor fast spread of pathogens. The high uptake of feed and the fast growth make these animals prone to intestinal disorders. This has been neglected in the past because of the use of low doses of specific antimicrobial compounds, called antimicrobial growth promoters (AGPs). These were used worldwide to maintain the profitability of the broiler industry. These antibiotic substances, added at sub-therapeutic level as feed additives, increased animal performance.

A ban on the use of AGPs, mainly driven by consumer concerns about increases in antimicrobial resistance, was instigated in the EU in 2006, followed by global concerns that led to decreased use or ban, depending on the region. The mode of action of the AGPs is still under debate, but a variety of mechanisms have been proposed, including a reduction in total bacterial counts in the gut (and consequently less competition for nutrients), a reduction of specific pathogens (e.g. *Clostridium perfringens*), a decreased abundance of specific harmful bacterial properties (e.g., bile salt hydrolase activity and thus poor fat digestion), and reduced

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inflammatory reactions because of the decreased pathogen load, amongst others (Butaye et al., 2003; Knarreborg et al., 2004).

Also, direct immune-modulatory effects by AGPs have been suggested. Whatever the mechanism of action is, it is evident that host-microbiota interactions are involved. The gut-microbiota interactions are very complex since the gut is an organ that contains multiple cell types that fulfill many functions and hosts a diverse microbiota that carries out many functions as well, including breakdown of dietary molecules and consequently production of absorbable end products, and maturation and development of the (mucosal) immune system. The term 'gut ecosystem' is used to describe that the gut and the gut microbiota form one organ, with specific functions that are derived from both the gut microbiota's genetic potential (the microbiome), and the functions of the host gut wall. Novel technologies (-omics technologies) have been used recently to get a better understanding of host-microbiota interactions. More specifically, various studies using 16S rDNA sequencing led to the identification of microbial taxa that are associated with beneficial or harmful host responses, and metabolomics has been used to identify microbial metabolites that trigger these effects. The production of microbial metabolites can be steered using nutritional factors, creating an excellent opportunity to make animals more resilient against non-infectious and infectious challenges, using dietary additives.

II. THE HOST SIDE: EPITHELIAL CELLS AS MAJOR SIGNAL SENSORS

The luminal side of the intestinal wall is lined with absorptive epithelial cells, whose major task is water and nutrient uptake, and secretion of enzymes. They form a semi-permeable barrier between the outside world (the gut lumen) and the internal host tissues. The semi-permeable barrier is not only formed by the cell membranes of the epithelial cells, but also by tight junctions that connect neighboring epithelial cells (Piche, 2014). These connections are regulated at different levels (e.g., by cytokines). The permeability of the intestinal epithelial cell layer can be affected by epithelial cell death but also by luminal signals that increase the epithelial layer permeability by affecting the tight junctions or inducing cell death, and thus causing loss of integrity of an important barrier between the 'inside' and the 'outside' of the gut (Hooper, 2015). When epithelial cells are killed or when the tight junctions between epithelial cells are damaged, some opportunistic pathogens can benefit by gaining access to the basolateral side of the epithelial cells and induce inflammation.

Nutrient leakage and inflammation cost energy for the animal, and cause villus shortening or blunting, thus decreasing performance. Loss of intestinal epithelial integrity can cause losses of host proteins ('leaky gut') into the lumen and can allow luminal molecules (including toxins) and micro-organisms to reach the gut submucosa under the epithelial layer. If these components have pro-inflammatory properties, this can yield massive infiltration of immune cells, which is energy-demanding for the host. Inflammation is mediated by binding of pathogen associated molecular patterns (e.g., lipopolysaccharide, peptidoglycan lipoproteins, flagellin) to receptors (e.g., Toll like receptors) that transmit signals in a cascade ultimately leading to inflammatory cell infiltration in the mucosa (Brown et al., 2011). Although this is a protective response, this inflammatory cascade should be brought back to normal conditions when the trigger is eliminated. Also, intracellular receptors (NOD-like receptors) can sense bacterial compounds and can even induce tolerance (e.g., peptidoglycan-derived muramyldipeptides).

Apart from absorptive epithelial cells, also other epithelial cell types are present in the lining of the gut wall. These include mucin-producing goblet cells and antimicrobial peptide producing Paneth cells (in the crypts, not present in all animal species), important in innate defenses (Muniz et al., 2012). Entero-endocrine cells can secrete peptide hormones at the basal side of the cells that can reach the bloodstream. These peptide hormones have a variety of

functions, including effects on epithelial cell proliferation, inflammation, and consequently intestinal integrity, even at distant segments of the intestine. One of the key hormones is glucagon-like peptide 2 (GLP-2), a hormone that is important in maintaining epithelial integrity (Baldassano and Amato, 2014). Below the epithelial lining, many other cell types are present that form the lamina propria of the intestinal mucosa. These are immune cells, fibroblasts, nerve cells and muscle cells, amongst others. Intestinal integrity, inflammation, and gut function are all influenced by pathogens and their products (coccidia, toxins, bacterial pathogens, viruses) and by luminal signals, of which many are produced by the microbiota (Havenaar, 2011). The above-mentioned cells sense microbial signals and transmit these signals to other cell types and to other parts in the body of the animal. The microbiota composition and the metabolites produced by the bacteria are thus crucial for health and productivity.

III. THE MICROBIAL SIDE: THE MICROBIOTA AS SIGNAL PRODUCERS

The microbiota composition in the gut varies with age and with the gastrointestinal segment (Stanley et al., 2014; Song et al., 2017; Sun et al., 2019; Yang et al., 2019). In general, the diversity of the microbiota increases with age. In industrial animal production, often the birth or hatching environment is as sterile as possible. This situation can be considered as unfavorable because the establishment of a protective microbiota is delayed, and the young animals are more prone to colonization by pathogens.

In general, low numbers of bacteria are found in the proximal parts of the gut while the numbers increase towards the distal ileum, caecum, and colon. The diversity generally increases significantly towards the distal gut, and while in the small intestine a limited variability is found, with lactobacilli often dominant, the distal intestinal tract harbors a huge number of different bacterial groups. The distal intestinal tract of healthy subjects is mostly dominated by bacteria from the phyla *Bacteroidetes* and *Firmicutes* (together comprising more than 80% of the microbiota), the former containing many polysaccharide degrading bacterial species, while the latter contains a variety of bacterial families, including *Ruminococcaceae* and *Lachnospiraceae* families, that are considered important health-promoting populations, due to butyrate production. Also, members of the phylum *Proteobacteria* are usually present, although in lower numbers. These include *Enterobacteriaceae*, such as *Escherichia coli*, thus Gram-negative bacteria that contain opportunistic pathogens and often are associated with harmful inflammatory effects. The bacterial community has the genetic potential to carry out an enormous number of physiological functions. The number of microbial genes in the gut, the microbiome, exceeds the number of animal genes, and together they form a 'hologenome' (Rosenberg and Zilber-Rosenberg, 2011). The variety of bacterial functions includes degradation of complex substrates (polysaccharides, proteins, fat), fermentation of substrates to yield acidic compounds, immunomodulation, communication with other bacteria, and many more. The metabolites produced by the bacterial community are of vital importance for maintaining gut health and controlling pathogen colonization.

Polysaccharide breakdown is performed by the microbiota in a cascade in which different bacterial members take care of specific catalytic steps in degrading the substrates (Flint et al., 2012). Complex substrates (such as polysaccharides, including arabinoxylans, pectins, and cellulose) are converted to oligosaccharides by specific bacterial populations (e.g., lactobacilli, some *Bacteroides* species, and others), and these oligosaccharides (e.g., arabinoxylanoligosaccharides (AXOS)) are further used by other bacterial groups to produce short-chain fatty acids (SCFAs, i.e., acetic, propionic, and butyric acid), lactate and gases. The most important butyric acid producing bacteria belong to the *Ruminococcaceae* (Clostridial cluster IV) and *Lachnospiraceae* (Clostridial cluster XIVa) families (Pryde et al., 2002). These families contain strictly anaerobic bacteria that are highly abundant in the distal gut. Some of

the *Lachnospiraceae* consume lactic acid to produce butyric acid (Duncan et al., 2004). Butyric acid is a major energy source for enterocytes and has a variety of beneficial properties, including pathogen control, anti-inflammatory effects, increased mucin, and antimicrobial peptide production, strengthening of the epithelial barrier, etc. (Guilloteau et al., 2010).

Fermentation to butyrate in the distal gut can affect small intestinal function by stimulating GLP-2 secretion by entero-endocrine cells in the blood stream (Tappenden et al., 2003). This GLP-2 can have effects on various cell types in the small intestine, leading to anti-inflammatory effects, effects on the integrity of the epithelial barrier and increased cell proliferation (Rowland and Brubaker, 2011). Typically, inflammation is associated with a loss of anaerobes, including butyric acid producing bacteria, and an increase in oxygen-tolerant opportunistic pathogenic *Enterobacteriaceae*, such as *E. coli*. This exacerbates the inflammation, as the anti-inflammatory signal butyrate is decreased.

IV. INTERFERING WITH BACTERIAL SIGNAL PRODUCTION AND HOST SENSING, BY NUTRITIONAL INTERVENTIONS, RESULTING IN ANTI-INFLAMMATORY RESPONSES

A variety of feed additives are used nowadays as either antimicrobial growth promotor alternatives or gut health stabilizers. Some might inhibit certain bacterial groups, but most are supposed to steer the microbiota composition to a more favorable one, and have important host effects, either direct or indirect, the latter through the microbiota. As discussed above, feed formulas or feed additives should improve intestinal epithelial integrity, stimulate tolerance responses towards non-harmful bacteria, avoid excess inflammation, stimulate host antibacterial responses (mucin and antimicrobial peptide production), and bring the host to a steady state of mutualism with its microbiota. This means that these feed additives or formulae should favor beneficial microbes and inhibit the microbes that produce harmful metabolites or reduce pathogen colonization. This will result in reductions of inflammatory responses, and increased animal performance. Below a short overview is given on dietary additives that affect gut health and inflammation.

a) Feed composition and enzymes

Gut inflammation and villus shortening can be induced by feeding a diet containing high amounts of non-starch polysaccharides (NSP) without NSP-degrading enzymes (Teirlynck et al., 2009). AGPs are able to reverse the inflammatory changes and villus shortening induced by the high NSP containing diet, in association with a shift in the microbiota (Teirlynck et al., 2009). It appears that the use of AGPs in the past has masked the dysbiosis-inducing effects of many feed formulas used in monogastrics. Also, the feed structure, protein source and the choice of ingredients can affect gut health. Enzymes such as xylanases convert large polysaccharides to shorter oligosaccharides and thus perform one of the initial steps in the breakdown of these substrates, as is done in the gut by bacterial species in cross-feeding pathways. This also reduces viscosity and bacterial overgrowth in the small intestine. More information on the effects of feed constituents and gut health can be read in a review paper by Choct (2009).

b) Probiotics

Probiotics are defined as live micro-organisms that, when consumed in adequate amounts, confer a health effect on the host. The most widely used bacterial probiotics are bacilli, as they are stable in formulation (spores) and produce antibacterial compounds, apart from beneficial metabolites. As an example, recently it was shown that specific *Bacillus* species produce high

concentrations of niacin *in vivo*. Niacin is sensed by the receptor Gpr109a, that is also activated by butyrate, and activates anti-inflammatory responses (Singh et al., 2014). Apart from *Bacillus* species, other single strain probiotics are marketed, including lactobacilli. Multi-strain products are on the market as well.

Also, competitive exclusion products, containing a freeze-dried mixture of gut content, are marketed. In the scientific literature, reports on the effect of probiotics on intestinal inflammation and animal performance have been published, and reports on protection against pathogen colonization and disease are available. The question remains how many studies are not published because of inconsistent, no, or negative effects observed. Data from our laboratory show that the efficacy of probiotics is highly dependent on the model used and not all studies show clear, reproducible beneficial results. Instead of empirically developing and marketing probiotics only because of their genus name, we should rethink the system and develop probiotics based on their mode of action. For example, based on the above-described data, attempts could be made to evaluate strains that stimulate butyrate production by strains of Clostridial cluster IV and XIVa, or use these butyrate-producing strains as probiotics. These are, however, strict anaerobes and do not form spores consistently, making them difficult to formulate and use, while this is not a problem for *Bacillus* species, which are usually incorporated in feed as heat resistant spores (Shivaramaiah et al., 2011).

c) Prebiotics

Prebiotics are defined as natural or processed functional foods which contain biologically active compounds that have documented benefits on health by altering the interactions between beneficial and pathogenic bacteria (Gibson and Roberfroid, 1995). Most prebiotics are oligosaccharides such as, fructooligosaccharides, galactooligosaccharides, AXOS, and xylan oligosaccharides (XOS). Mannanoligosaccharides are often not considered as prebiotics because they may not be fermented but have direct immunomodulatory effects. Prebiotics are complex molecules because of the chain length, the nature of the sugar bounds, and the nature of the side chains on the saccharides. All this can affect function. The scientific literature reports various studies in which prebiotics have beneficial effects on broiler performance, inflammation, and pathogen control.

As with probiotics, it is difficult to estimate the bias that is present using data derived from scientific papers, because only beneficial effects are mostly reported, and no or negative effects are seldom published. It is anyhow the case that the prebiotics need to be converted by the microbiota to metabolites. Because prebiotics are saccharides, the end products will be SCFAs, lactate, and gases and thus the beneficial effect can theoretically be evaluated or predicted by measuring the ratio of beneficial versus harmful bacterial groups or metabolites. As such, prebiotics that increase colonization of butyrate-producing Clostridial cluster IV and XIVa bacteria are considered to be beneficial. Other parameters could include reductions in *Enterobacteriaceae*. Also, in the case of prebiotics we thus need to proceed in the future towards a science-driven development in which the mechanism of action plays a central role, instead of empirically developing prebiotics. For example, our group has shown that XOS administration to a broiler diet increased the number of lactobacilli and Clostridial cluster XIVa strains in the distal gut, hereby stimulating cross-feeding of lactate to produce butyrate (De Maesschalck et al., 2015).

d) Phytochemicals (essential oils, oleoresins, etc.)

Phytochemicals are also well-known feed additives in the broiler production industry. Biologically active constituents of plants include terpenoids (mono-and sesquiterpenes, steroids, etc.), phenolics (tannins), glycosides, and alkaloids (present as alcohols, aldehydes,

ketones, esters, ethers, lactones, etc.). Many of these, but not all, have antibacterial activity (Penalver et al., 2005; Barbosa et al., 2009). Effects on immune function have also been described. According to Adams (1999) the antimicrobial activity is rather weak for ginger and pepper, medium for cumin (p-cymene), coriander (lialol), oregano (carvacrol), rosemary (cineol), sage (cineol) and thyme (thymol) and strong for clove (eugenol), mustard (allylisothiocyanate), cinnamon (cinnamaldehyde) and garlic (allicin). Also, here the dosage, purity, extraction method from the plant (in case of mixtures, thus phytobiotics) or synthetic production method will determine the success of the products.

It is clear that the antibacterial essential oils will affect the gut microbiota composition, and there is a need to clarify which ones promote beneficial bacterial species, using *in vivo* studies. Resin acids have recently been studied and seem to alter matrix metalloproteinase activity in the gut mucosa, that could be highly relevant in restoration of intestinal damage (Aguirre et al., 2019). Indeed, matrix metalloproteinase upregulation has been shown in gut inflammation models and are likely involved in extracellular matrix breakdown.

f) Short chain fatty (and other) acids

Drinking water and feed additives containing SCFAs, medium chain fatty acids and even aromatic acids (e.g. benzoic acid) are widely used in the animal production industry. While drinking water acidification is mainly for sanitation purposes, feed additives are used mainly for optimizing animal performance and for pathogen control (Van Immerseel et al., 2006). It is difficult to compare the relative efficacy of commercial products because they differ in the nature of the acids used (often combinations are used), the concentration, and even more importantly, the delivery method (pure, on a carrier, encapsulated, etc.). The latter determines the site of release in the gut and can affect the outcome. Butyric acid has a strong anti-inflammatory effect in the gut. While SCFAs are more considered as signaling molecules for the microbiota and the host, the medium-chain and aromatic acids are more antibacterial.

V. FINAL CONSIDERATIONS

A huge number of experimental and field trials have been carried out in broilers, using a variety of feed additives. The most commonly measured outcome parameter is performance, either or not under challenge conditions. Some studies have been undertaken to determine the effect on pathogen colonization and inflammatory responses. The approach so far has been mostly empirical and the products are thus mainly developed without a clear understanding of the reasons for the expected beneficial effects. Many feed additives that are meant to replace AGPs have variable activities. The only way to develop a product with an enhanced activity as compared to the already existing products will be based on a thorough understanding of the intestinal ecosystem, and the way the gut wall responds to the microbiota and their metabolites. Identifying the microbiota components that are crucial for gut health is ongoing and is essential for proper development of additives that affect gut health. This needs to be done by identifying both the beneficial ones and the harmful ones. In fact, current knowledge indicates that butyrate-producing bacteria need to be boosted or maintained while *Enterobacteriaceae* and specific pathogens such as *C. perfringens* need to be suppressed, and the inflammation is reduced. These are easy to measure criteria and are well known to correlate with a good morphological structure of the gut. In fact, studies that have recently been carried out and future studies using –omics technologies will be of value to identify potential performance-related beneficial gut microbiota components and metabolites (Dehau et al., 2022a,b).

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CONTROLLED COLONISATION OF GUT IN BROILERS UNDER COMMERCIAL HATCHERY SYSTEM

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Summary

The Australian poultry industry faces challenges related to disease outbreaks and bird welfare. Maintaining healthy gut microbiota can help address these issues and improve bird health, quality of life and productivity. Ongoing research and innovative practices are helping address these challenges and advance the industry towards more sustainable and efficient poultry production. Here, we present the commercial trial data with the microbial community colonisation intervention performed at the commercial hatchery. Our data suggests that a rapid gut colonisation intervention at hatch can alter the microbial community and benefit the bird's welfare.

I. INTRODUCTION

The Australian poultry industry, consisting of over 800 commercial chicken farms, is a crucial part of the agriculture sector, contributing significantly to food production, meat processing, and the economy. The poultry meat sector continuously evolves through research and development efforts to boost productivity, enhance bird welfare, and meet the increasing demand for poultry products, especially meat (Poultry Hub Australia, 2023). Challenges faced by the industry include disease outbreaks (De Meyer et al., 2019), traditionally managed with antibiotics (Hafez and Attia, 2020), although concerns about antibiotic resistance have prompted a shift away from this practice. Additionally, high bird density in poultry farms can increase stress levels, making birds more susceptible to infections (Gomes, et al., 2014). To address this, the industry is adopting free-range and open-range production systems.

The gut microbiota in chickens plays a vital role in their overall health, influencing nutrient absorption, immune system development, and protection against harmful pathogens through competitive exclusion and strengthening of the intestinal lining (La Ragione and Woodward, 2003, Pan and Yu, 2014). Modern industrial hatching practices, which separate chicks from mother hens, result in variations in gut microbiota despite consistent feeding and environmental conditions. To promote robust gut colonisation, strategies such as fecal microbiota transplantation and early exposure to specific beneficial microbiota strains are gaining interest (Metzler-Zebeli, et al., 2019). These approaches aim to establish desirable microbiota dominance, enhancing bird health (Wilkinson, et al., 2020). This study conducted a large-scale commercial hatchery-based experiment, mimicking maternal inoculation using automated equipment to administer a diverse commercial product containing chicken caecal microbiota to newly hatched birds, with published findings providing insights into these practices (Kayal, et al., 2022).

II. MATERIALS AND METHODS

The animal trial was conducted in a commercial hatchery and broiler farm in Australia, and 164,000 Cobb-500 broiler chicks were involved. Chicks were divided into two groups: one control group of 82,000 chicks while the other group received a commercial inoculum of microbiota Aviguard® (AVG) using an automated spray system immediately after hatching. The inoculum was mixed with green gel food dye to create visible green droplets on the chicks,

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which were ingested within 2–5 minutes. The birds were then transported to temperature-controlled sheds for rearing, with 41,000 birds in each of the four sheds. The birds were provided with a commercial diet and water ad libitum. Sample collection involved euthanising ten randomly selected birds from each shed at the age of 28 days. Samples of jejunum, caecal, crop content and jejunum mucosal swabs were collected. DNA extraction, sequencing, and data analysis were performed, focusing on the V3-V4 region of the 16S rRNA gene. Alpha and Beta diversity analysis was assessed using the Shannon Entropy index and Principal Coordinate Analysis (PCoA). The animal study was approved by the Ethics Committee of Central Queensland University under approval number 0000020312

III. RESULTS

a) Animal performance

The animal performance was described in detail previously (Kayal, et al., 2022). Briefly, the final average body weight was higher in treated birds than in controls and adjusted FCR showed slight improvement, with 1.678 for the control and 1.656 for the treated birds.

b) Alpha & Beta Diversity

Alpha and beta diversity were investigated to compare the microbial communities between AVG and untreated groups in the different intestinal sections. Proventriculus and the caecum samples exhibited a microbial population with a higher diversity than the other gut sections, measured with the Shannon entropy index (Figure 1A). The plot also shows that the product inoculum had a diverse microbial population. The jejunum mucosal samples had the lowest richness and diversity values, but the AVG-treated group was significantly higher than the control group (Figure 1A).

The Principal Coordinate Analysis (PCoA) depicted that AVG is ecologically more similar to caecum samples than other sample types (Figure 1B). The PCoA plot demonstrates the clear distinction between caecal and AVG product samples from other gut sections.

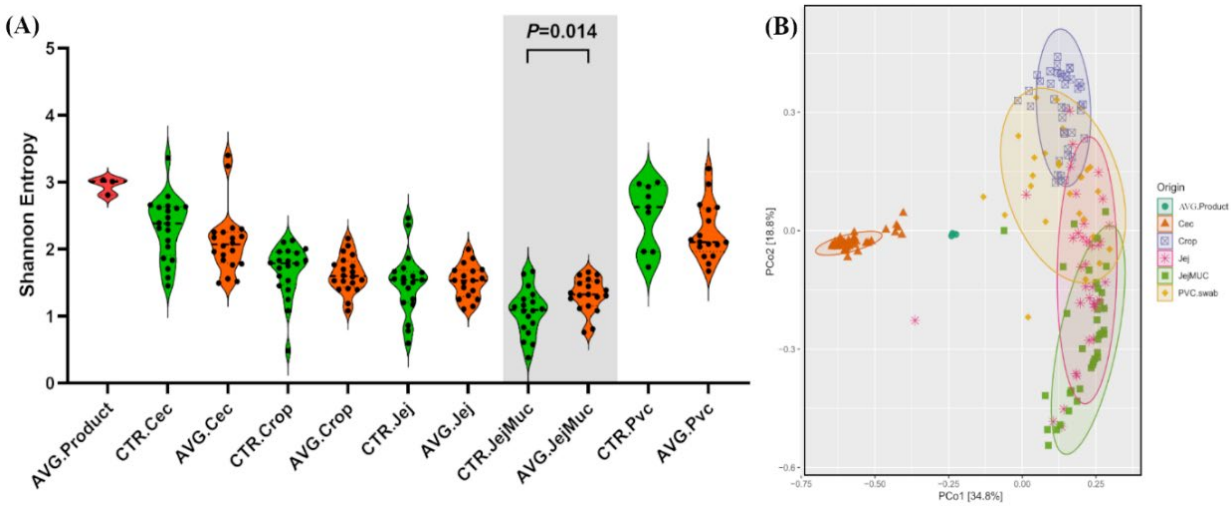

Figure 1 - Alpha diversity plot showing the observed species (A). PCoA plot based on Bray–Curtis dissimilarity showing separation and grouping of the samples based on their origin (B). Cec= cecum, Jej=jejunum, JejMuc= jejunal mucosa, Pvc=proventriculus.

c) LEfSe Analysis

LEfSe analysis was performed to determine the statistically significant differences in the microbial communities between the AVG-treated and the control groups. In the cecum samples, the treatment group was found to have a relatively high abundance of the genera *Olsenella*, *Bacteroides*, *Bacteroidales*, *Selenomodaceae*, *Provotellaceae* and others, while the control group was more abundant in *Bifidobacterium*, *Ruminococcus*, *Lachnospiraceae*, *Alistipes* etc. The crop samples from both groups showed the presence of different species of *Lactobacillus*. Proventriculus samples from the treatment were more abundant in *Staphylococcus*, *Brachybacterium*, *Micrococcales*, *Brevibacterium*, etc., while the control samples were abundant in *Escherichia-Shigella*, *Bacteroides* sp., *Streptococcus* and *Romboutsia*.

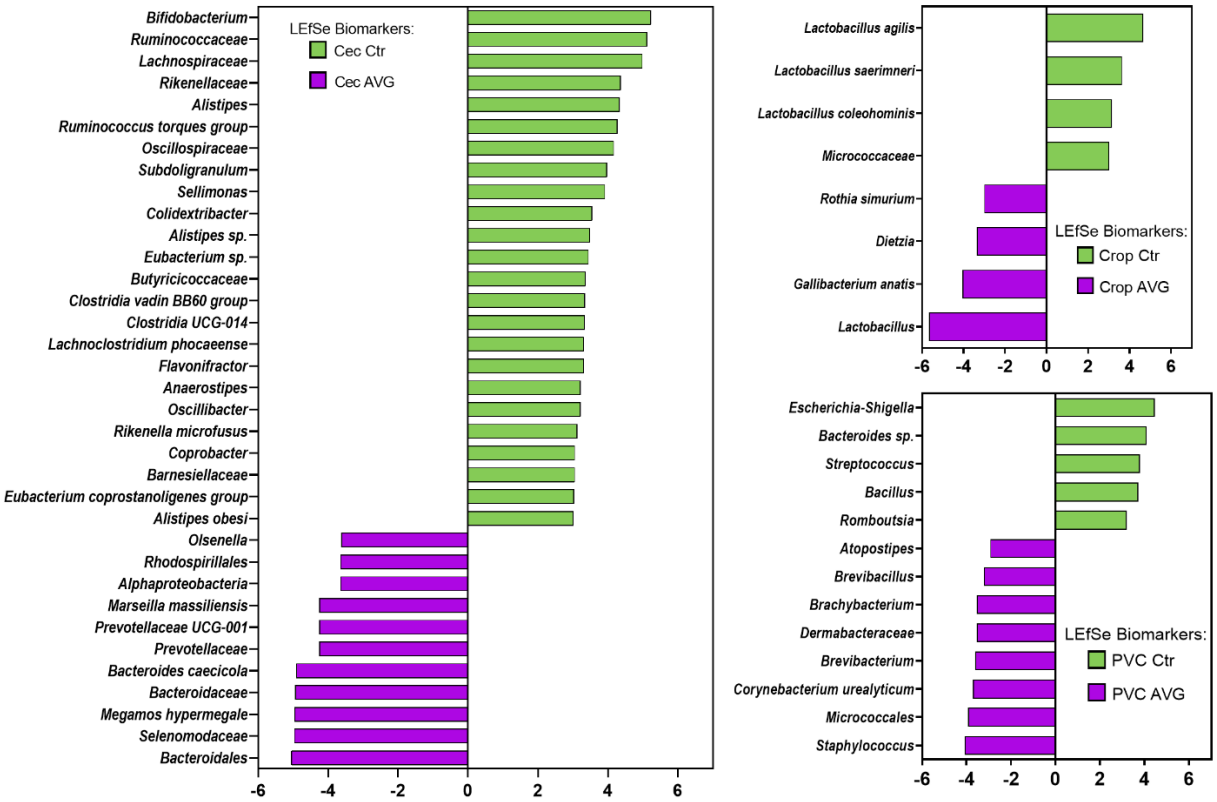

Figure 2 - LEfSe plots showing the abundant taxa in each section of the gut. Cec= cecum, Pvc=proventriculus.

IV. DISCUSSION

The concept of designer gut microbiota aims to manipulate and control the composition of microorganisms in the gut to benefit the host. Early intervention in the gut microbiota can lead to long-term changes in bacterial and metabolic composition, influencing gene expression (Yin, et al., 2010) and enhancing immunity against enteric pathogens (Kubasova, et al., 2019). Controlled colonisation and intervention methods, such as fecal transplants, have shown promise in reducing the abundance of pathogens like *Enterococcus* and *Escherichia-Shigella* while increasing the concentration of beneficial short-chain fatty acids (SCFAs) like acetate, propionate, butyrate, and isovalerate, improving gut health (Gong, et al., 2019).

Early microbial administration significantly affected the alpha diversity of microbial communities in the jejunum mucosa. While caecal diversity marginally decreased, the jejunal mucosa increased in diversity. This increase may be due to early exposure to rich microbial inoculum, with the mucosal layer being more likely to colonise rapidly. Beta diversity analysis

revealed significant alterations in the phylogenetic relationships between microbial communities in different gut sections due to the product administration. The upper gut sections, such as the crop and proventriculus, were not altered in abundance but showed differences in taxa presence or absence.

Colonisation intervention with rich native inoculum increased the abundance of several microbial genera associated with improved growth, immune response, intestinal morphology, and energy metabolism. These included genera like *Lactobacillus* (Amir Ebrahimi, et al., 2022), *Bacteroides* (Hou, et al., 2016) and *Olsenella* (Xiang, et al., 2021). However, it is important to note that the 16S microbiota data used in this study can not distinguish between species within these genera, and both beneficial and pathogenic effects are highly strain-specific.

V. CONCLUSION

The study demonstrated the large-scale application of AVG in commercial hatcheries, noting significant effects on gut colonisation dynamics across all sections. However, it emphasised the need for further research, including shotgun metagenomics, to explore functional capabilities and species transfer, as well as consider the influence of background microbiota in feed, sheds, and hatcheries on AVG-induced alterations.

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COMPETITIVE EXCLUSION IMPROVES THE PERFORMANCE OF BROILERS VACCINATED AGAINST COCCIDIOSIS

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Summary

Using vaccination to reduce the risk of coccidiosis, a major threat to poultry production, is becoming increasingly common in Australia and around the world. However, the vaccine itself has been reported to potentially have a negative effect on bird performance as it requires repeated cycling of coccidia within the bird to develop immunity. The current study investigates the impact of high levels of coccidiosis vaccines on bird performance and also the ability of using early, avian-specific microbiota development through a competitive exclusion product, to alleviate this negative impact on performance.

I. INTRODUCTION

Rapid development of an appropriate microbiota within a chicken gut is critical for its ongoing gut health and immunity (Meijerink et al., 2020). In natural circumstances the hen would pass her microbiota onto the chick, but in commercial conditions where eggs are set and hatched in a sterile hatchery this transfer is not possible and microbiota development is often from sources such as the environment (Dame-Korevaar et al., 2020). Development of a microbiota influenced by the environment rather than an avian-specific bacteria source has been shown to have detrimental effects on bird development and can result in colonisation of pathogenic bacteria (Meijerink et al., 2020). Application of a competitive exclusion product derived from a healthy adult hen has been shown to alleviate some of the risks (Meijerink et al., 2020).

Coccidiosis remains the major threat to the poultry industry in Australia and around the world (Mesa-Pineda et al., 2021). Added to this a recent concern of *Eimeria* isolates becoming resistant to anticoccidial drugs along with the societal demand for less antibiotic use have led to the development of alternative solutions, like live coccidiosis vaccines (Yu et al., 2023). Vaccination stimulates immunity to prevent coccidiosis, but several studies have reported the negative impact on zootechnical performances (Gautier et al., 2020; Repérant et al., 2022). In this context, in a pilot study, we have investigated a new way to mitigate the negative impact of a coccidiosis vaccine on performances using the early application of a competitive exclusion (CE) product.

II. METHOD

One hundred and fifty day-old, mixed sex ROSS 308 broilers were randomly allocated to 3 treatments: negative control (NC), vaccine group (VG) and vaccine group with a microbial solution (AviGuard® + Optiwall® - Lallemand SAS, CE). Each group consisted of 3 floor-pens of 15 chicks, except the NC with 4 replicates. Starter crumbles diet without anticoccidials were formulated and offered throughout the trial from 1 to 33 days.

Birds were vaccinated with EVALON® (HIPRA) at 5x the recommended application rate to get an accurate count of oocysts in droppings (Mathis et al., 2018). The vaccination was performed immediately after the allocation of the birds to their respective pens by individual

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gavage and to prior feed access. The CE group was also administered by gavage with the vaccine. The NC group received 1 ml of mineral water.

Chicks were individually weighted at d1, d5, d10, d25 and d33. Oocysts were counted in faeces at d10 & d25, as a proposed marker of vaccinal oocysts recycling (Repérante et al., 2022). Eimeria gut lesions were evaluated according to Johnson & Reid (1970) scales. Treatment group comparison was performed using a non-parametric Kruskal-Wallis test (SPSS Statistics 27.0, IBM). Pairwise comparison was established as an indication of the dietary effect even if a trend was depicted.

III. RESULTS

Detailed results are presented in Table 1. The vaccine induced a significant reduction in body weight (BW) at d33 of age (-6.6%), and even from d25 (-8.8%). The average daily gain (ADG) from d1 to d33 was also significantly altered (-7.5%). Feed intake was not influenced by any treatment. Consequently, the feed conversion ratio (FCR) was negatively impacted (+12.3%). Results indicated that CE vaccinated birds at d25 had significantly better performances (BW, ADG, FCR) than VG birds. Non-significant differences were found between non vaccinated birds and vaccinated birds inoculated with the competitive exclusion product except at d25 where CE birds' performances were intermediate between NC and VG group.

Oocyst shedding was identical between both vaccinated groups on d10 and d25, suggesting that a normal cycling of the vaccine oocysts had occurred. The average oocysts per gram of faeces (OPG) was 2,617 OPG at d10 and 767 OPG at d25. No oocysts were found in the NC. Coccidiosis-induced lesions were only score 1 at d5 and scores 1 and 2 at d33. No statistical difference between groups was depicted at d5 ($P = 0.312$) and at d33 ($P = 0.603$). Despite the low number of lesions at both time points, CE vaccinated birds was the group having the smallest number of lesions scored 1 at d5 and no lesions scored 2 at d33.

Table 1 - Zootechnical performance, mean and (standard deviation), per period for the 3 groups (A,B,C: $P \leq 0.10$; a,b,c: $P \leq 0.05$).

Mean (SD)	Day/ Period	Control	Control + Vaccine	Competitive Exclusion + Vaccine	P-value
Body weight (g)	1	39.2 (0.10)	39.3 (0.05)	39.4 (0.17)	0.26
	5	98.3 (2.5)	98.6 (1.2)	96.5 (1.7)	0.397
	10	234.4 (2.4)	232.0 (2.7)	230.6 (1.7)	0.153
	25	1221.6 (17.2) [a]	1114.4 (19.1) [b]	1157.9 (16.7) [c]	<0.001
	33	1891.5 (57.2) [a]	1767.4 (33.4) [b]	1849.4 (32.7) [ab]	0.025
Average daily gain (g/bird/ day)	1 – 5	11.8 (0.5)	11.9 (0.2)	11.4 (0.3)	0.361
	5 – 10	27.0 (0.6)	26.5 (0.3)	26.8 (0.3)	0.393
	10 – 25	65.8 (1.2) [a]	58.8 (1.5) [b]	61.8 (1.0) [c]	0.001
	25 – 33	83.7 (5.2)	81.6 (2.1)	86.4 (2.1)	0.344
	1 – 33	54.9 (2.0) [a]	50.8 (1.0) [b]	53.1 (1.0) [a]	0.029
Individual daily feed intake (g/animal/ day)	1 – 5	10.9 (0.7)	12.0 (2.3)	10.8 (0.5)	0.469
	5 – 10	36.1 (1.9)	38.5 (1.8)	35.9 (0.6)	0.145
	10 – 25	92.5 (10.8)	98.4 (7.6)	91.9 (1.4)	0.569
	25 – 33	147.7 (8.0)	151.5 (2.0)	154.8 (3.7)	0.328
	1 – 33	82.9 (4.8)	86.2 (3.4)	83.3 (1.5)	0.500
FCR	1 – 5	0.92 (0.019)	1.01 (0.168)	0.95 (0.036)	0.471
	5 – 10	1.34 (0.090)	1.45 (0.063	1.34 (0.014)	0.126
	10 – 25	1.41 (0.170) [A]	1.68 (0.149) [B]	1.49 (0.006) [AB]	0.095
	25 – 33	1.77 (0.031) [a]	1.86 (0.029) [b]	1.79 (0.021) [a]	0.010
	1 – 33	1.51 (0.100) [A]	1.70 (0.089) [B]	1.57 (0.005) [A]	0.052

IV. DISCUSSION

The CE tested in this study contributed to alleviate vaccination side effects. CE is based on a well-established concept aiming at reinforcing the immune status of the birds, especially through a faster mature and balanced microbiota establishment (Meijerink et al., 2020; Dame-Korevaar et al., 2020, Methner and Rösler, 2020).

Associating a live vaccine and CE has been already tested for *Salmonella* (Braukmann et al., 2016; Methner et al., 2017) with positive effect of the CE. Use of CE early in chick life shapes the gut microbiota, thus creating a favourable physiological environment for beneficial bacteria and reducing the possibility of opportunistic bacteria to develop. Another explanation is linked to the modulation of inflammation in the gut, which would minimize nutrients diversion towards an inflammatory response, to the benefit of performance. Here, in the context of cocci vaccine, this study presents an interesting strategy to compensate the previously reported negative effects of live cocci vaccines on performance. The OPG remained at the same level in both vaccinated groups, indicating that the CE did not alter the mode of action of the live vaccine while negating some of the vaccines impact on bird performance measured by weight gain and FCR.

From these results, it can be concluded that the CE is compatible with coccidiosis vaccines and could be a viable solution to alleviate the negative effect of coccidiosis vaccine on broiler performances.

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THE IMPACT OF A YEAST PROBIOTIC ON GUT MICROBIOTA IN BROILERS

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Summary

Yeast probiotics contribute to good gut health in poultry by enhancing gut morphology. Generally, supplementation with a yeast probiotic results in an increase in villus height and other morphological changes within the epithelium. Yeasts also have great ability to reduce inflammation in the gut and modulate immunity via epigenetic programming of immune cells. Such probiotics also modulate gut microbiota, shifting the population of the microflora towards one which is more diverse and robust. The presence of such a microbiome reduces inflammation within the gut and this in turn positively impacts bird performance.

In this study, it was shown that performance of broiler birds was enhanced when using a live yeast probiotic. It was also shown that supplementation with the live yeast probiotic shifted the microbial population, increasing the diversity of microbial species found in the gut of treated birds compared to non-treated birds.

I. INTRODUCTION

Yeast probiotics have the potential to reduce local intestinal inflammation by modulating cytokine gene expression, increase villi height and modulate the gut microbiota. This often reduces gastrointestinal distress and can impact performance. The aim of this study was to evaluate the effects of various doses of a yeast probiotic on the performance and gut microbiota of commercial broilers.

II. METHOD

A total of 1,200 (12 replicates per group) Ross 708 broilers were housed at day-old, challenged with coccidiosis vaccine (2x the recommended vaccination dose) and divided into 5 groups: negative control (NC), Bacitracin methylene disalicylate (BMD) (50g/T) and Actisaf® (yeast probiotic) at 250 g/T, 375 g/T, and 500 g/T respectively. Birds were reared on used litter up to 49 days of age and received a corn-soybean based diet with inclusion of 5% dried distillers grains and solubles. Body weight, feed consumption and mortality were measured and feed conversion ratio (FCR) was calculated. Ileum samples were collected at the end of the trial for evaluation of microbiota by 16S rRNA gene amplification and analysis. Performance data were analyzed using the GLM model of Minitab. Means were separated by Fisher's LSD. For α-diversity, a pairwise comparisons using Wilcoxon rank sum exact test was done. For β-diversity, PERMANOVA was used.

III. RESULTS

Birds fed the yeast probiotic were heavier than the NC at all time-points and this was significant at 42 days ($p \leq 0.05$). For all ages running cumulative FCR showed significant improvement ($p \leq 0.05$) compared to the NC and similar results to the BMD group. Birds treated with the yeast probiotic and BMD showed statistically significant higher α-diversity than NC birds with the former higher than the latter. The Principal Component Analysis showed 5 different microbiota communities which corresponded to the different treatment groups with an overlap

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of 95% between birds treated with Actisaf at 250 and 375 g/T, indicating the absence of major differences between these two treatments.

Actisaf treated birds showed a reduction of Firmicutes and an increase of Bacteroidetes when compared to the NC. This occurred mainly due to the increase of *Bacteroides* and *Butyricimonas* genera. Despite the overall decrease of Firmicutes, *Butyricicoccus*, a known producer of short chain fatty acids, was slightly increased on birds treated with Actisaf. Finally, the prevalence of *Sellimonas* was significantly higher in birds treated with Actisaf. Recent studies have shown that this genus can be a potential marker of intestinal homeostasis recovery which is relevant for challenging situations during production. The difference in gut microbial species and populations could explain the improvements in FCR especially because higher diversity is commonly associated with a more stable gut microbiota. Likewise, these improvements are most likely related to beneficial bacteria changes in the intestine.

IV. CONCLUSION

The yeast probiotic, at all concentrations, improved growth performance of broilers similar to traditional growth promoters suggesting that it can be used as an economical alternative.

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EFFICACY OF CLOPIDOL AND OTHER SYNTHETIC COCCIDIOSTATS IN FIELD CONDITIONS AGAINST *EIMERIA MAXIMA*

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Summary

Field data were analysed to compare the efficacy of 4 different synthetic coccidiostats: amprolium, clopidol (Coyden®), nicarbazin and zoalene. Data was collected in the US from 2019 until 2023 using Aviapp®. The efficacy of the coccidiostats was evaluated using a microscopic scoring system for *Eimeria maxima*. The system evaluates the severity of *E. maxima* lesions using microscopical evaluation of mucosal scapings and uses scores ranging from 0 to 4 depending on the number of oocysts found under the microscope. The results indicate that birds receiving clopidol had a lower score for *E. maxima* compared with birds receiving the other coccidiostats indicating better efficacy.

I. INTRODUCTION

Although a lot of experimental data and reports on coccidiosis control exist, the effects in field conditions are underreported. Whereas in experimental trials, the goal is to reveal causal relationships, conditions are often very different from the field. More specifically, often very high, single dose infections are applied and the time of follow up is limited, whereas in field conditions infection is gradual with increasing numbers of oocyst as the birds get older and the infection pressure rises. Field data provide a useful complement to experimental data. Field data has the advantage of providing a more realistic estimate. However, the disadvantage is that a lot more variables, like management, bird-specific factors, and environmental issues underly the effect. Hence the results originating from such analysis are referred to as associations.

II. METHOD

Data was obtained from Aviapp®, a tool for monitoring health, performance, diseases, and management in the broiler industry. The data used for the analysis included microscopic scoring for *E. maxima,* adapted from Goodwin et al. (1998), from the US from broilers on synthetic coccidiostats: i.e., clopidol (Coyden®), amprolium, nicarbazin and zoalene.

To evaluate the efficacy of the products, the *E. maxima* microscopic scores were analyzed as a function of age, with the resultant curve forming the basis for the statistical comparison of the products. The statistics evaluated were the area under the age curve (AUC), the age at which the peak occurs, and the maximum average score. The *E. maxima* microscopic score – age profile was estimated using a combination of 2 different models, taking into account the numerical characteristics of scoring data as well as to control for underlying differences in farms.

The first model was a generalized linear mixed model, modeling the prevalence of 0 scores. The second model, a linear mixed effects model, modeled all nonzero scores. Next both models were used to construct the average response by age by multiplying the prediction of the second model with the probability of observing a nonzero score of the first model. For both models, the fixed part included a polynomial of second degree for the age, the active and their two way interactions. Nonzero scores were log-transformed prior to fitting the model. To

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control for differences due to farms and year of observation a random effect of farm nested within the year was included as well. The AUC, peak average score, and age at which the peak occurred were derived from this curve. 95% Confidence intervals were constructed by bootstrapping the dataset stratified by the synthetic coccidiostat and repeating the above described procedure 500 times.

III. RESULTS

A summary of the data used for the analysis is shown in Table 1.

Table 1 - Information about the data used for the analysis.

Active	N° flocks	N° farms	Years	Minimum age (days)	Median age (days)	Maximum age (days)
Amprolium	219	29	2019-2023	15	28	43
Clopidol	639	41	2019-2023	6	26	49
Nicarbazin	205	37	2019-2023	12	16	28
Zoalene	850	45	2019-2023	12	27	50

From Table 1, it is clear that nicarbazin is only used until a maximum 28 days of age. This is much shorter than the other chemicals. Hence Figure 1 displays the age profiles of each compound ranging from their minimum to the maximum observed age in Table 1.

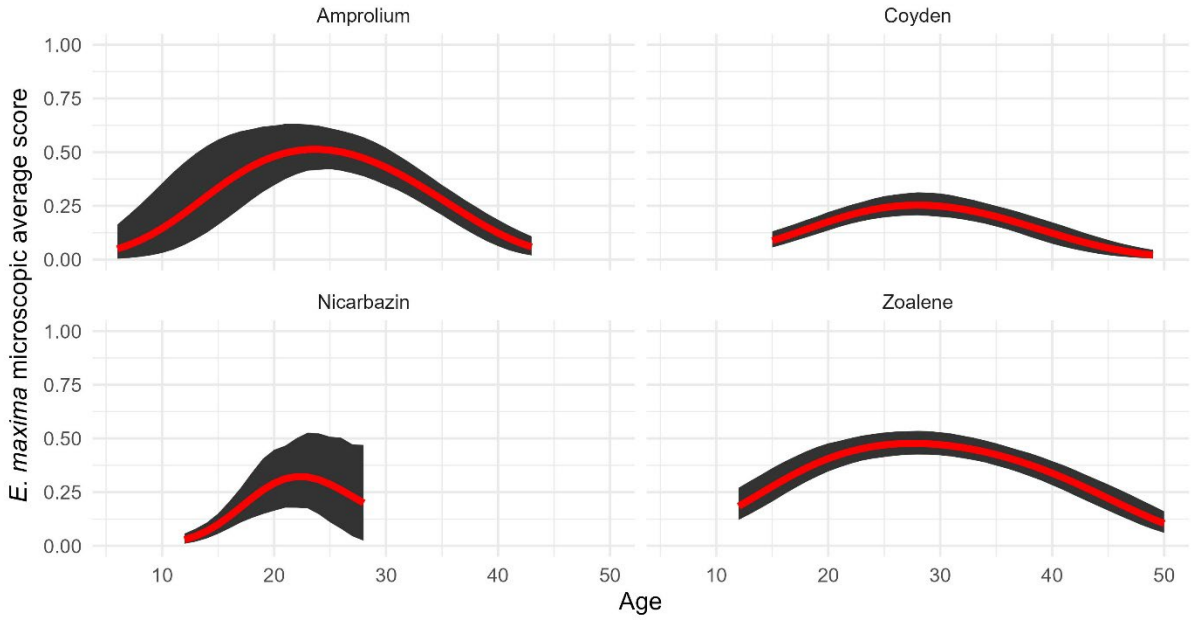

Figure 1 - E. maxima microscopic score – age profiles for different synthetic coccidiostats. The red line represents the average score of 500 bootstrap iterations, predicted by the model. The black regions represent the 95% confidence regions.

The derived statistics from these curves are provided in Table 2. Two estimates for the AUC are provided for amprolium, clopidol, and zoalene. The first estimate is a summary from 6-28 days of age, to enable comparison with nicarbazin. The second estimate is a summary from 12 – 49 days of age to make the comparison amongst amprolium, clopidol and zoalene.

Table 2 - Estimates derived from the age profile. Values are presented as the mean value [2.5%CL – 97.5% CL].

Active (day range)	AUC	Peak Score	Age at Peak
Amprolium (6-28)	0.34 [0.24 – 0.47]^{a}	-	
Clopidol (6-28)	0.13 [0.10 – 0.16]^{b}	-	
Nicarbazin (6-28)	0.16 [0.09 – 0.24]^{ab}	0.36 [0.19 – 0.62]^{a}	23.1 [20.0 – 30.0]^{a}
Zoalene (6-28)	0.30 [0.25 – 0.36]^{a}	-	
Amprolium (12-49)	0.35 [0.28 – 0.44]^{a}	0.52 [0.43 – 0.64]^{a}	23.3 [18.5 – 27.0]^{a}
Clopidol (12-49)	0.17 [0.14 – 0.21]^{b}	0.25 [0.21 – 0.31]^{b}	28 [26.0 – 30.0]^{a}
Zoalene (12-49)	0.39 [0.35 – 0.44]^{a}	0.48 [0.43 – 0.53]^{a}	27.5 [25.0 – 30.0^{a}]

Different letters in superscript mean that the results are significant ($P < 0.05$). Empty fields in the 6-28 day range mean that the estimates are identical to the 12-49 day range evaluation.

IV. DISCUSSION

Field data provide complementary information to experimental data. The results presented in this analysis provide information regarding the efficacy of 4 synthetic coccidiostats against *E. maxima* over different ages in broiler production. Due to the fact that products are used in different ways in the field, for example different days of inclusion, this has to be taken into account. For example, nicarbazin is only used until 28 days of age. When comparing the products from age 6 – 28 days, it is clear that clopidol had the lowest average *E. maxima* microscopic lesion score, being significantly different from zoalene and amprolium, but not from nicarbazin.

Similarly, the peak scores were also the lowest on clopidol, and significantly different from amprolium and zoalene. However, the age at which this peak occurs is numerically somewhat later compared to the others. Nevertheless, the intervals widely overlap, meaning that the age at which the peak occurs is highly variable and hence not significantly different amongst the products. The confidence regions for zoalene and amprolium tend to be wider. This is also evident from Figure 1. This is a direct result of the smaller number of observations for this group. Certainly for nicarbazin, the lower amount of sampling around the peak implies that these peak scores expand a wider range.

To conclude, this article summarizes the effect of 4 synthetic coccidiostats on the control of *E. maxima* in the field. Clopidol (Coyden®) displays the overall strongest control, with the lowest overall scores (measured by the AUC) and the lowest peak scores. Importantly, better control of coccidiosis also leads to better performance.

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PARTICLE SIZE DISTRIBUTIONS AND STRUCTURAL FEATURES OF UNDIGESTED FEED AT THE JEJUNUM AND ILEUM OF CHICKENS

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Summary

In Australia, chicken feeds are usually based on wheat with 10-25% whole grains that are ground in the gizzard prior to being digested by avian enzymes in the small intestine with residual undigested feed at the ileum passing to the caecum as a potential feedstock for the resident microbiota. To understand the consequences of gizzard grinding for subsequent digestion and fermentation outcomes, samples recovered from the jejunum and ileum of birds fed diets based on whole wheat, ground wheat and whole maize have been analyzed by particle size distribution, light microscopy and solid-state ^{13}C NMR. For all samples, there was a broad range of particle sizes, with a median in the range of 200 – 600 µm. Particles smaller than 100 µm were enriched in starch granules and represented a smaller proportion of particles at the ileum than the jejunum. Particles larger than 600 µm were enriched in multicellular tissues, represented a greater proportion of particles at the ileum than jejunum and included encapsulated and undigested starch. Ground wheat feed at the jejunum or ileum had only slightly smaller particle sizes than whole wheat, consistent with effective grinding of whole grains by the gizzard. The methods and approaches outlined here hold promise for understanding the mechanisms limiting the efficient digestion of feed in the avian small intestine.

I. INTRODUCTION

Whilst numerous studies have investigated the effects of feed formulation and processing on broiler chicken performance, there are still limitations to our understanding of the digestive mechanisms that are responsible for the findings obtained (Liu et al, 2015; Truong et al, 2016). By analogy with other monogastric species (pigs and humans), particle size distributions of residual feed in the small intestine may be a major factor. For pigs and humans, particle sizes are determined by food/feed processing, as these species have a limited capacity to break grain particles once ingested/swallowed. However, the gizzard is considered to provide chickens with the ability to effectively grind grain particles prior to small intestinal digestion. Knowledge of the particle size distribution, microstructure and size-dependent composition of feed particles from e.g., recovered duodenal or jejunal digesta may therefore provide useful information on the nature and effectiveness of gizzard grinding.

The enzymic digestion of, particularly, starch is influenced greatly by feed particle size (in the small intestine). This is because starch (and protein) can be effectively encapsulated within intact endosperm cells of cereals or cotyledonary cells of legumes. Individual cells are typically of the order of 100 µm in size, so particles >~500 µm are likely to contain intact cells in their interior with consequent slow digestion of encapsulated starch and protein (Gidley 2023). In vitro studies of size fractions from milled cereal grains show that the rate of starch digestion is inversely related to the square of the particle size, consistent with particle surface area being the rate limiting factor for enzyme digestion of starch (Al-Rabadi, Gilbert, and Gidley 2009; Ratanpaul et al. 2018). In the case of pigs, regrinding barley particles with sizes greater than 1.8 mm or sorghum particles larger than 0.9 mm resulted in an improved feed

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conversion ratio (Al-Rabadi et al. 2017), demonstrating the principle that larger particles limit overall porcine starch digestion in grain-based feeds. It is not known whether similar considerations apply to broiler chickens. Analysis of residual feed particle size distributions at the ileum compared with earlier parts of the small intestine would identify any size selectivity in particle digestion. Furthermore, compositional analysis of smaller and larger particle fractions at the beginning and end of the small intestine would shed light on the consequences of different particle sizes for (particularly starch) digestion. Analysis of residual feed in digesta typically focusses on the molecular composition, such as proteins (amino acids) and starch. However, particle structures could also be analyzed non-invasively by microscopy and solid-state compositional methods such as ^{13}C NMR to identify differences in composition and microstructure as a function of digesta particle size.

Broiler chicken feed normally contains large particles of grains that are considered to be effectively ground in the gizzard. Milled feed may only undergo limited particle size reduction in the gizzard and may restrict gizzard development (Svihus and Hetland, 2001; Hetland et al, 2002; Amerah et al, 2007a). Comparison of particle sizes at the beginning and end of the small intestine for both milled and non-milled grain-based feeds could help elucidate factors controlling particle size reduction and subsequent enzyme digestion.

In this study, the particle size distributions of residual feed at the jejunum and ileum of broiler chickens were characterized for feeds based on whole maize, whole wheat, and ground wheat. Small and large fractions from each digesta sample were further analyzed by light microscopy, to identify structural features of particles, and by solid-state ^{13}C NMR, to identify major carbohydrate components such as starch and cellulose. Taken together the combination of physical, microscopic and molecular analyses provides insights into the importance of characterizing particle size fractions for a more complete understanding of digestive processes in the avian small intestine.

II. METHODS

Materials - Samples of digesta from the jejunum and ileum of broilers fed diets based on whole maize, whole wheat and ground wheat were from the experiment described in (Chrystal et al. 2021). The selected samples were from formulations containing low crude protein.

Particle size distributions were determined by hand-sieving using multiple sieves (1.18 mm, 600 µm, 212 µm, 106 µm, 53 µm) and a pan. Specifically, the sieve is inclined at an angle of *ca*. 20°to the horizontal and is tapped by hand on the outer wall at a frequency of *ca.* twice per second for one minute, repeated three times after 90° rotation. Size distributions for all fractions were determined from percent weights retained on each sieve and in the pan.

Using a Carl Zeiss Axio 3.0 polarized light microscope (Zeiss, Okerkochen, Germany) the presence and morphology of starch in samples of two different particle size ranges, <106 µm and 106-600 µm, was observed. The images were processed using AxioVision 3.1.

Solid-state ^{13}C CP/MAS NMR spectra were acquired for size fractionated samples using a Bruker MSL-300 spectrometer (Bruker, Billerica, MA, USA) at 75.46 MHz. The samples were packed into a 4-mm diameter, cylindrical, PSZ (partially stabilized zirconium oxide) rotor with a Kel-F cap and spun at 7 kHz. A 90° pulse width at 5 μs and a contact time of 1 ms were used together with a 3 s recycle delay A minimum of 2000 scans were acquired, the transform size was 4 k, and 100 Hz line broadening was applied. Where possible, the intensity of the spectra were matched for the C-4 cellulose peak at 89 ppm.

III. RESULTS

Particle size distributions for jejunal and ileal digesta from the three feed types are shown in Fig 1. All distributions are bimodal with a minor fraction below 100 µm and a major fraction

centered on 200 – 600 µm and including a small amount of particles > 1.1 mm. The smaller fraction is relatively more abundant at the jejunum (typically ~40%) than the ileum (typically ~ 25%), with a correspondingly greater proportion of >600 µm particles at the ileum (Fig 1A). The same differences between jejunal (Fig 1B) and ileal (Fig 1C) samples was seen for each of the three feed types. Of particular note is the fact that ground wheat feed had similar particle size distributions at both jejunum and ileum as whole wheat feeds (Fig 1 B, C).

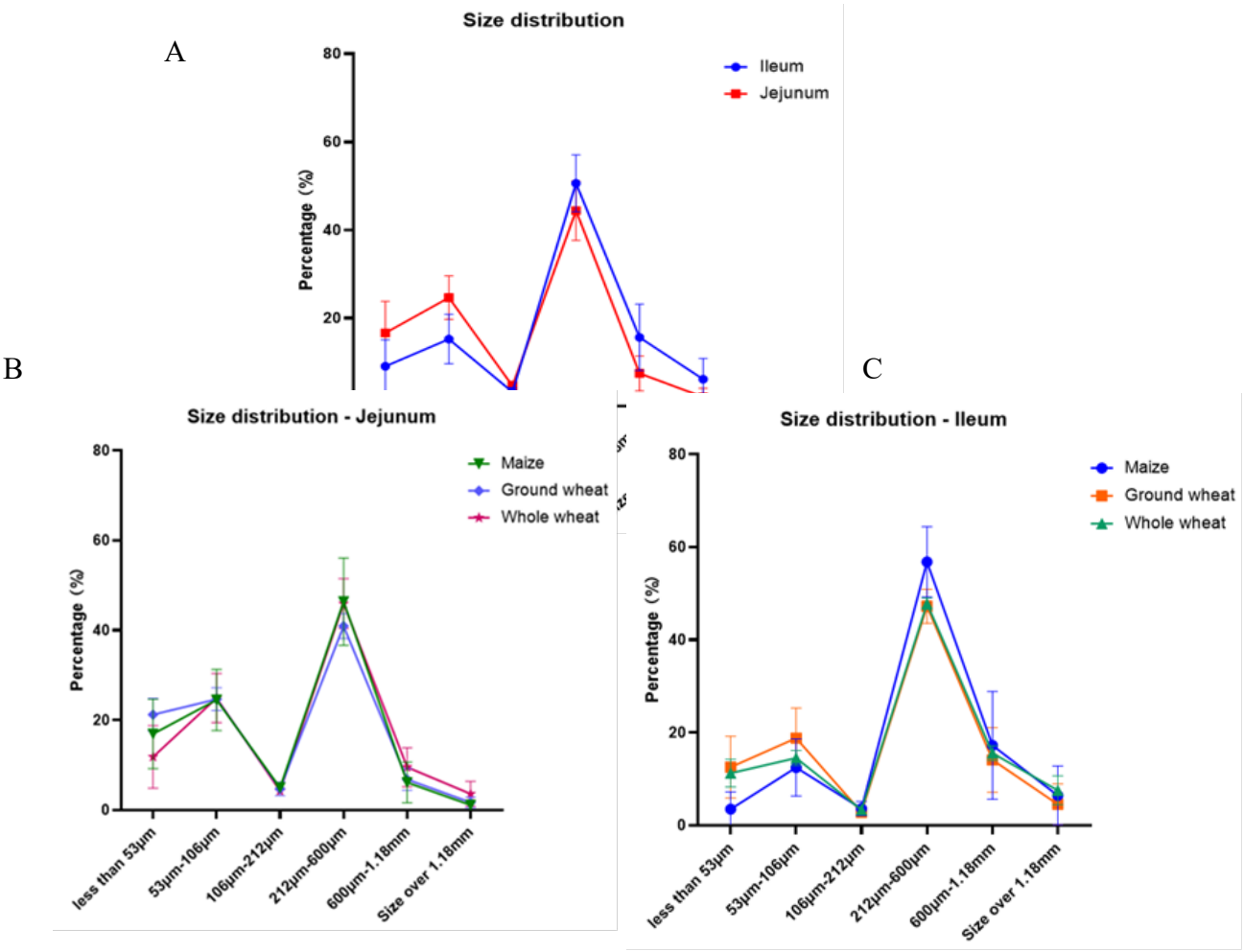

Figure 1 - Particle size distributions of digesta for A. All feed types at the jejunum and ileum, B. Each of the three feed types at the jejunum, and C. Each of the feed types at the ileum.

The structural features of particles were examined by polarized light microscopy. Illustrative micrographs are shown in Figs 2 and 3. One of the main features is small (~10 µm) starch granules that appear multicolored due to the birefringence arising from their semi-crystalline non-gelatinized state. These granules are either free (particularly in small size fractions and at the jejunum) or associated with cell wall material, and therefore potentially encapsulated and more resistant to enzyme digestion. Additional structures observed are likely to be fragments from fractured cell walls ('fibre') and other insoluble components in feed. In terms of factors controlling the rate and extent of digestion, it is the starch and cell wall fractions that are most abundant. In future work, protein could also be localized using specific stains.

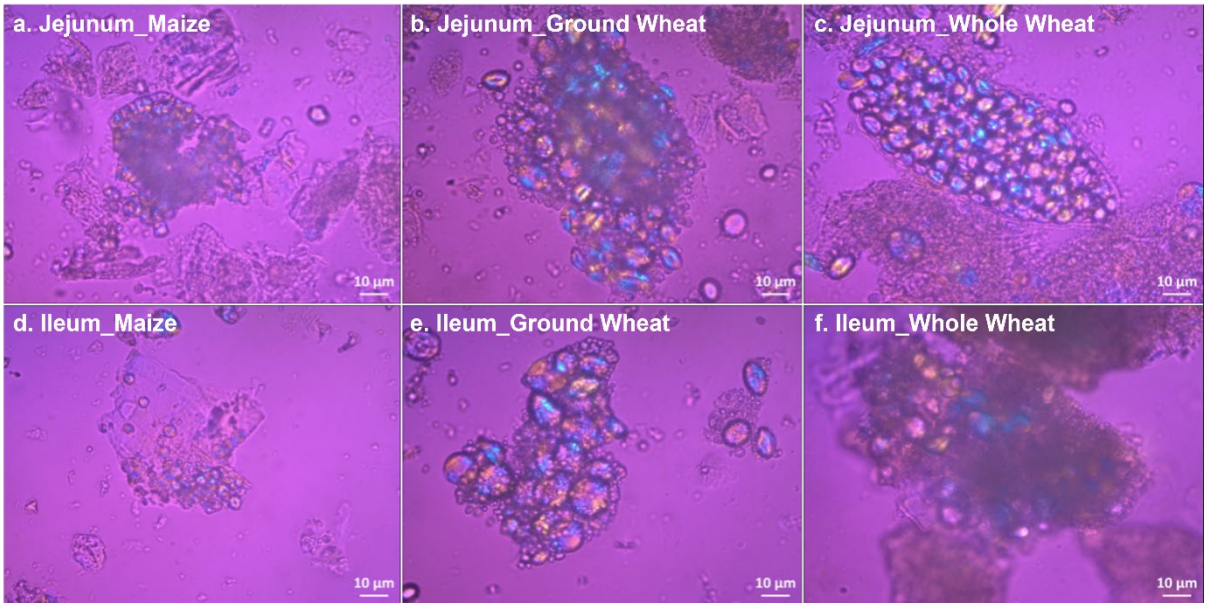

Figure 2 - Illustrative light micrographs of structures found in jejunal and ileal digesta for each of the three grain sources. Multi-colored objects are (birefringent) starch granules, in many cases enrobed by thin cell wall structures. Dark unfocussed areas arise from the three-dimensional nature of the particles limiting light transmission.

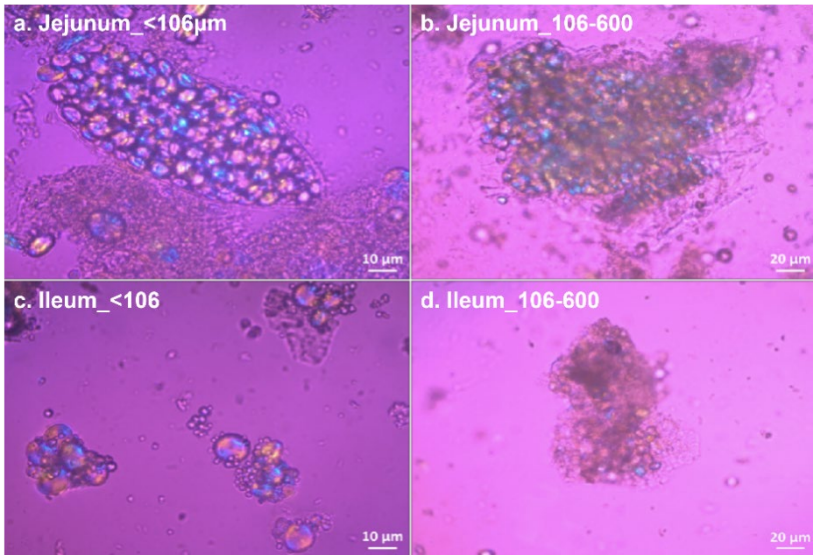

Figure 3 - Illustrative light micrographs of structures found at the jejunum and ileum for small (<106 µm) and medium-sized (106-600 µm) particles. Particles larger than 600 µm are too thick to obtain well-resolved light microscopic images due to limited light transmission. Images a, and c. are from ground wheat with b. and d. from whole wheat feeds.

To provide an overview of the molecular components present, solid state ^{13}C NMR was used. Most of the intensity was in the carbohydrate region at 60 – 110 ppm; representative spectra are shown in Fig 4. Although all features are broad, there are characteristic signals that can be associated with (particularly) starch and cellulose. In the C-1 region (95-110 ppm), starch intensity is greatest around 101 ppm, whereas cellulose intensity is centered on 105 ppm. In addition, intensity at ca 89 ppm is uniquely due to the C-4 of crystalline cellulose. The current data allow some qualitative conclusions to be drawn; further analysis combined with other structural techniques should result in future more quantitative analyses.

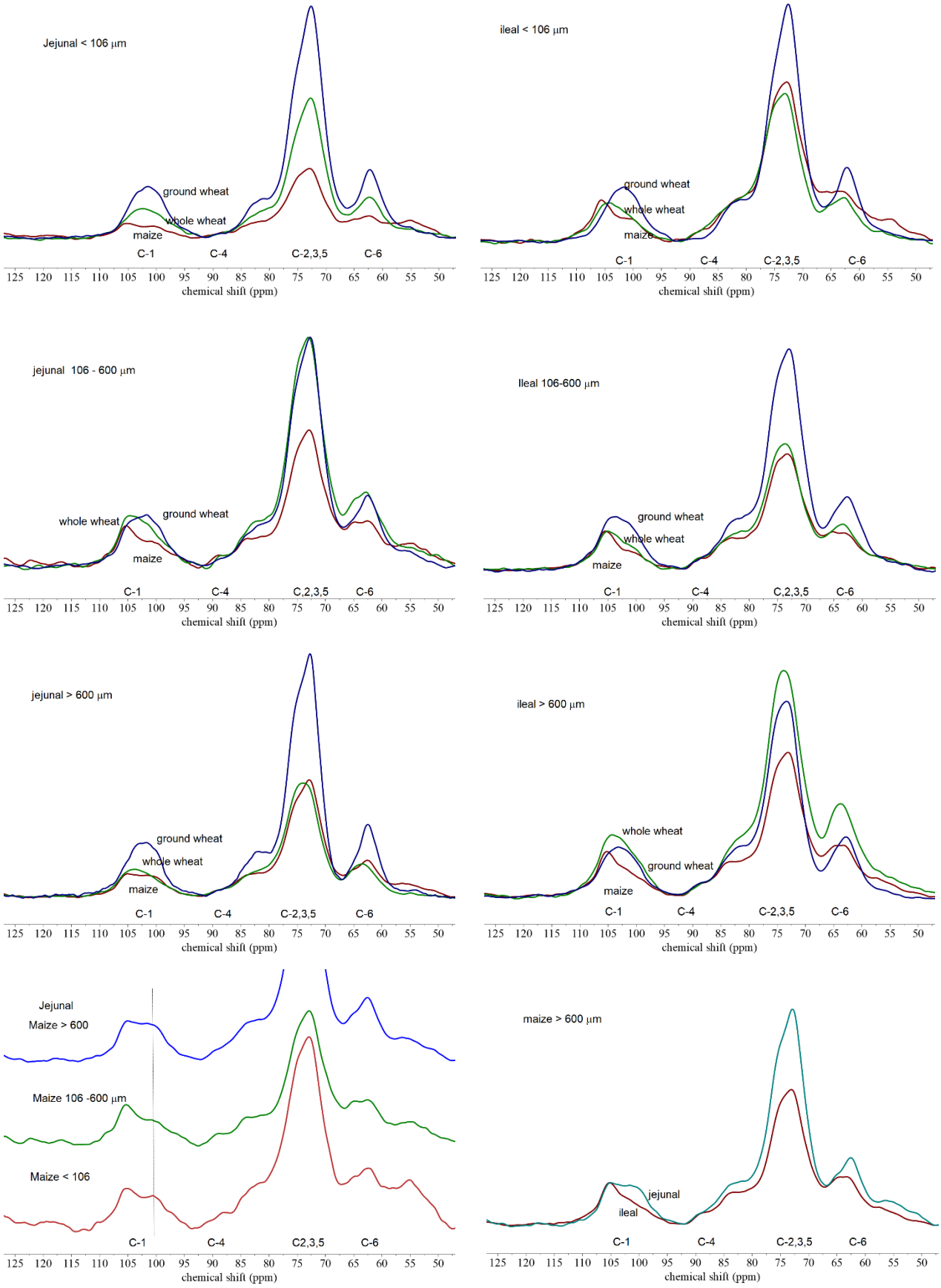

Figure 4 - Solid state ^{13}C NMR spectra of jejunal and ileal digesta for small (<106 µm), medium (106-600 µm) and large particles (> 600 µm), together with illustrations (lower spectra) of differences due to starch (C1 ~ 101 ppm) and cellulose (C1 ~ 105 ppm) levels.

Comparing intensities at 101 ppm (starch) and 105 ppm (cellulose), similar relative amounts of starch are found for the small and large fractions, with relatively less starch in the most abundant medium-sized (106 – 600 µm) fraction. This is particularly clear for maize as illustrated at the lower left of Fig 4. There is a decrease in the proportion of starch from the jejunum to the ileum for all samples, illustrated by a direct comparison for maize in the lower right of Fig 4. This is not surprising as starch is digestible in the small intestine whereas cellulose and other cell wall polymers are not. It is also interesting to note that there is a generally greater relative amount of starch in the ground wheat compared with whole wheat samples.

IV. DISCUSSION

This study has demonstrated an approach to combining characterization of the physical, microstructural, and compositional features of undigested fractions in the chicken small intestine that can complement the analytical chemistry measures more commonly used to identify ileal digestibility parameters. A broad bimodal size distribution of particles was found at both the jejunum and ileum, which is also apparent for the duodenal particle size data reported by Amerah et al (2007b, c; 2008) with major fractions at <75 µm and 250-500 µm and particles as large as 2 mm reported. The particle size distributions reported by Amerah et al (2008) at the duodenum had a greater proportion of particles of <100 µm for both wheat (60% for fine feed, 54% for coarse feed) and corn (43% for fine feed, 51% for coarse feed) diets than found in the current study at the jejunum (ca 40%). In another study, Amerah et al (2007c) reported between 44% and 56% of duodenal particles to be below 100 µm, and in a different study the same authors reported 48 – 49% of duodenal particles below 100 µm. It is possible that some of the very small particles present at the duodenum could have been digested by the jejunum, causing a greater proportion of larger particles at the jejunum than at the duodenum in the current study. This is consistent with the duodenum and proximal jejunum being major sites of starch digestion and glucose absorption (Riesenfeld et al, 1980). Hetland et al (2002) used a different method (laser diffraction) of size analysis that allowed greater discrimination in the <100 µm fraction and reported similar duodenal size fractions to Amerah et al (2008).

Through understanding the relationships between the physical structures in feed, processing in the gizzard and further transformation from the jejunum to the ileum, insights into the processes occurring in the avian small intestine can be obtained. From this study, we draw the following inferences:

1. Gizzard processing produces a wide range of particle sizes that include free starch granules of *ca*. 10 µm up to mm-scale pieces of grain tissue with cellularly-encapsulated starch (and protein) for both wheat- and maize-based diets.
2. The particle size distribution post-gizzard is comparable for whole wheat and ground wheat diets, although compositional features as a function of particle size are not identical.
3. There is a greater percentage of larger particles at the ileum, consistent with intact cellular structures being a limiting factor in starch (and possibly protein) digestion in the small intestine.

More generally, the wide range of particle sizes of undigested feed at both the jejunum and ileum means that measures of bulk chemical composition represent an average across all of the particles present, making it challenging to assign a structural rationale for measured digestion extents. Analyzing the composition of separated size fractions gives greater understanding of the mechanisms underlying the relative digestibility of important components at the end of the small intestine. A further consequence of any particle size-dependent

compositions is that undigested feed at the ileum is the primary source of nutrition for the cecal and large intestinal microbiota. Nourishing a healthy and resilient microbiome in the caeca and large intestine is important for bird health. In general, a carbohydrate-rich feedstock stimulates a healthy gut microbiota, but this can be modulated by particle characteristics such as size and water-holding capacity (Yao et al, 2023 a, b). A better appreciation of undigested feed at the ileum may provide more predictable outcomes in terms of microbial fermentation in the caeca and large intestine.

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COMPARING EFFECT OF A XYLOBIOSE-ENRICHED XYLO-OLIGOSACCHARIDE MIXTURE TO A COMMERCIAL XYLO-OLIGOSACCHARIDE MIXTURE ON BROILER CHICKEN PERFORMANCE

A. WALLACE[1], M.R. BEDFORD[2], G. GONZÁLEZ-ORTIZ[2] and N.K. MORGAN[3]

Numerous investigations have presented positive effects on performance when supplementing broiler chicken diets with commercially available xylo-oligosaccharide (XOS) mixtures. These mixtures encompass a range of XOS molecules with varying degrees of polymerisation (DP), typically DP2- DP6 (X_2 -X_6). X_2 is always the dominant product in commercial XOS mixtures, and from hydrolysis of dietary xylan by supplemental xylanases. *In vitro* studies have indicated that beneficial microbiota exhibit a preference for fermenting low DP XOS, in particular X_2 (Gullón et al., 2008). This implies that X_2 is the active ingredient in these mixtures, suggesting it may be advantageous to feed X_2-enriched XOS blends. This study compared performance responses in broilers fed a commercial XOS mixture to those fed an X_2-enriched XOS mixture. Cobb 500 mixed sex broilers (n =360 + 10 spare) were fed commercial-type wheat-, barley- and sorghum-based diets with either no supplement (Control) or 160g/t of either an X_2-enriched XOS mixture (X_2-Diet) or commercial XOS mixture (XOS Mix), either with or without xylanase (16,000 BXU/kg). Each dietary treatment was fed to 6 replicate pens, 10 birds per pen. Body weight gain (BWG), feed intake (FI) and feed conversion ratio corrected for mortality (cFCR) were determined from 0 to 35 days post-hatch. Univariate analysis and Tukey post-hoc test, using IBM SPSS Statistics 27, was used to evaluate effects of the XOS treatment and xylanase on day 0-35 performance. Statistical significance was declared at $P < 0.05$.

Table 1 - Main effects of supplementing commercial-type diets with an X_2 enriched XOS mixture (X_2-Diet) or a commercial XOS mixture (XOS Mix) on broiler day 0-35 performance.

XOS Treatment	Individual BWG day 0-35	Individual FI day 0-35	cFCR day 0-35
Control	2561	3999	1.564[a]
X_2-Diet	2542	3955	1.559[a]
XOS Mix	2692	3986	1.485[b]
P-value	0.079	0.777	0.015

Improved day 0-35 cFCR was observed in birds fed the XOS Mix compared to those fed the Control or X_2-Diet. This implies that X_2 is not the active ingredient in XOS mixtures. No interactions between xylanase and XOS treatment (BWG P = 0.376; FI P = 0.255; cFCR P = 0.337), or direct effects of xylanase (BWG P = 0.850, FI P = 0.691, cFCR P = 0.926) were observed in this study, possibly due to xylanase producing primarily X_2 and X_3. Results imply that larger DP XOS, not X_2, are required to enhance performance, suggesting future work should explore methods to enrich these higher DP XOS in supplemental XOS mixtures.

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MICROBIOME MODULATION IN BROILERS THROUGH PROTEASE-MEDIATED FEED PROTEIN DIGESTION

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Improving protein digestion is critical for profitable animal production not only because of the economic loss of undigested soy protein but also because of the deleterious effects of undigested protein on animal health. Exogenous proteases are known to increase the rate of intestinal protein degradation resulting in better animal performance. Furthermore, protease degradation products could potentially alter nutrient availability for the chicken microbiota, resulting in microbiome modulation. We hypothesized that protease supplementation would improve protein digestibility, lowering the availability of undigested protein and resulting in beneficial modulation of intestinal microbiota. Hence, we investigated the effect of multi-protease (KEMZYME^{TM} Protease) supplementation on the ileal and caecal microbiota of young broilers fed a protein-deficient corn-soybean diet. Male broiler (Ross 308) chicks were allocated to 36 floor pens (15 chicks/pen, 12 pens/treatment) and three dietary treatments consisting of a corn-soybean meal-based positive control (PC) diet, a low protein negative control (NC) diet (-1.0% CP and -4% digestible amino acids respectively) and multi-protease supplemented NC diet at 300g/t dosage. On day 21, selected birds were euthanized and dissected to obtain ileal and caecal samples for microbiome analysis by 16S rRNA sequencing using Illumina. Alpha diversity metrics in the caecum showed no significant differences ($p>0.05$) within each treatment, indicating no change in species diversity.

Significant differences ($p<0.05$) across all metrics were observed in the ileum. Evenness-based metrics, such as Shannon showed significant differences between NC and NC +Protease indicating differences in species abundance with protease treatment ($p<0.05$). Chao1 (richness metric) indicated a significant difference between PC and NC ($p<0.05$). Beta diversity analysis of caecum showed significant differences between NC and NC with Protease ($p<0.05$), whereas ileum showed differences between PC and NC+Protease. In the ileum, an increasing trend toward relative abundance of *Candidatus Arthromitus* spp and *Lactobacillus* was observed in the protease treatment group. *Candidatus Arthromitus* are segmented filamentous bacteria found attached to the intestine and have been found to positively correlate to bird performance at an early age, owing to their immunomodulatory capabilities[1]. ANCOM (Analysis of the composition of microbiomes) identified *Bacteroides uniformis* and *Sutterella* to be responsible for these differences. Protease treatment reduced the *B. uniformis* population in the caecum. *B. uniformis* is a major organism known to ferment protein to produce skatole and hence its reduction indicates lesser availability of undigested protein[2]. *Sutterella*, another proteobacteria was also found to be reduced by protease treatment indicating low availability of undigested protein. *Sutterella* is known to reduce production performance and negatively affect immune organs in broilers[3]. These beneficial modulations of microbiota were linked to better FCR ($p<0.05$). We conclude that protease supplementation effects were evident in the intestinal microbiota composition establishing the importance of efficient protein digestion in the ileum to reduce protein bypass from the ileum load to the caecum.

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IN OVO DELIVERY OF OREGANO ESSENTIAL OIL ACTIVATES XENOBIOTIC DETOXIFICATION AND LIPID METABOLISM IN HATCHED BROILER CHICKENS

S. NIKNAFS[1], M.M.Y. MEIJER[1], A.A. KHASKHELI[1] and E. ROURA[1]

In ovo interventions are used to improve embryonic development and robustness of one-day-old chicks. However, little is known on how bioactive phytochemicals such as carvacrol, the main compound of oregano essential oil (OEO), may impact the development and gastrointestinal tract of the chicken embryo. Previous data from our group showed that *in ovo* injection of 5 µL of OEO per egg at embryonic day (E) 17.5 was well-tolerated by the developing embryo with no negative impact on hatchability and other performance indicators (Niknafs et al., 2023). The aim of this study was to investigate the impact of *in ovo* delivery of oregano essential oil on gut function in broiler chickens. Ross 308 fertile eggs were injected with 5 µL/egg of OEO (emulsified with 5 µL of polysorbate 80 and 990 µL of saline) into the amniotic fluid at E17.5 (n = 48). To pursue a better understanding of mechanistic impacts of OEO, transcriptomic analyses of jejunal samples at hatch were performed, comparing the control (saline injected) and the group injected with OEO.

The transcriptomic analyses identified that 167 genes were upregulated and 90 were downregulated in the OEO group compared to the control group ($P < 0.01$). Functional analyses of the differentially expressed genes (DEG) using KEGG, REACTOME, and Gene Ontology showed that metabolic pathways related to the epoxygenase cytochrome P450 pathway associated with xenobiotic catabolic process were significantly upregulated ($P < 0.05$; Table 1). In addition, long-chain fatty acid metabolism associated with ATP binding transporters were upregulated in the OEO treated group ($P < 0.05$; Table 1). The results indicated that low *in ovo* doses of OEO have the potential to increase lipid metabolism in late stages of embryonic development.

In conclusion, *in ovo* delivery of OEO did not impact jejunum development and resulted in elevated expression of key enzymes and receptors involved in detoxification pathways and lipid metabolism in hatchling broiler chicks.

Table 1 - Effect of *in ovo* delivery of 5 µL of oregano essential oil (OEO) compared to saline injected group on metabolic pathways (P < 0.05) in the jejunum of broiler chicks at hatch.

Pathway Terms	P value	DEG enriching the pathways		
		Number of DEG	Upregulated genes	Downregulated genes
Peroxisome	0.002	7	*SLC27A2, DAO, SCP2, ACSL4, EHHADH, PHYH*	*HMGCLL1*
PPAR signaling pathway	0.005	6	*SLC27A2, SCP2, ACSL4, EHHADH, PPARα*	*FABP6*
Retinol metabolism	0.034	4	*SDR16C5, RETSAT, CYP2C18, CYP2C21L*	
ABC transporters	0.037	4	*ABCC2, ABCC6, ABCC10, ABCG8*	
Fatty acid metabolism	0.046	5	*ACSL4, GGT2, EHHADH, CYP4A22*	*ELOVL5*
Epoxygenase P450 pathway	0.000	4	*ENSGALG00000036831, CYP2C18, CYP2C21L, CYP2C23a*	

DEG: differentially expressed genes ($P < 0.01$).

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ENHANCING LAYING HEN PERFORMANCE AND GUT HEALTH IN HIGH-STRESS COMMERCIAL SETTINGS THROUGH TARGETED GLYCAN SUPPLEMENTATION

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Summary

In the dynamic landscape of commercial poultry production, there are persistent challenges related to disease control (especially emerging diseases), animal welfare, and the requirement to meet antibiotic-free standards. The effective management of gut microbiota stands as a crucial factor influencing poultry health and performance. The introduction of precision glycans as feed additives presents a promising dimension within this complex scenario. These glycans assume a vital role in strengthening gut health and immunity, thereby holding the potential to reduce antibiotic usage.

This study explored precision glycans as feed supplements for commercial layer hens within the Australian egg industry. The outcomes of this trial revealed significant improvements across various performance metrics, including a reduction in cumulative mortality, increased hen-housed egg production and improved feed conversion rates without significantly altering egg quality. Microbiota and histology analysis revealed significant alterations in the gizzard, ileum content, ileum mucosa, and the caecal and cloacal regions, with specific genera demonstrating noteworthy changes in abundance. In summary, the findings showed that precision glycans possess the capacity to strengthen poultry egg production, especially in the face of challenging farming conditions.

I. INTRODUCTION

Commercial poultry production faces persistent challenges related to disease and welfare, which are exacerbated by the resurgence of illnesses such as Spotty Liver due to the adoption of free-range systems (Van et al., 2017). The rising demand for antibiotic-free products (Zepeda et al., 2004; A.O.M.R., 2021) raises concerns regarding animal health and the potential for zoonotic diseases.

The complex interactions of gut microbiota assume an essential role in poultry health, which is significantly shaped by raw materials and feed additives (Pourabedin and Zhao, 2015; Ricke et al., 2020). While the antimicrobial effects are extensively documented, their impact on beneficial bacteria and disease susceptibility remains uncertain. A noticeable need exists for antibiotic alternatives that uphold microbiome equilibrium. In this context, polysaccharides, inclusive of glycans, can stimulate mucin production and contribute to gut health by forming protective layers along the gastrointestinal tract, serving as a physical barrier between the gut epithelial cells and the luminal contents (Bergstrom and Xia, 2013). Additionally, glycans play vital roles in immunological processes, influencing pathogen recognition and triggering immune system responses (Bergstrom and Xia, 2013). Recent improvements in biotechnology enable precision glycan synthesis tailored for specific in-vivo functions. The glycans employed in this study support the potential to enhance metabolic synthesis while mitigating amino acid degradation, thereby positively influencing poultry performance and protein utilisation (Bortoluzzi et al., 2023). Furthermore, these glycans modulate the microbiota, boosting the production of short-chain fatty acids, which are beneficial molecules for gut epithelial cells (Liu et al., 2021). The aim of this study was to examine the impact of precision glycans on

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intestinal health in layer hens, with a focal point on disease resistance, animal welfare, management, and sustainability.

II. METHOD

The study was conducted in a commercial aviary free-range system, using HyLine Brown layer hens. A single flock of pullets originating from the same rearing shed was used to ensure birds of the same age, management practices and feed diets were used. The layout of the two sheds at point of lay allowed for the division of the transferred pullet flock into two sides with independent silos and feed lines, enabling one side to receive the glycan treatment (PB) from 17 weeks of age, while the other served as the control group (CT), composed each of 20000 birds placed. A specific glycan product (a blend of gluco-oligosaccharide and silicon dioxide known commercially as Symphiome in Europe) was used.

The dose used of PB was 900 grams per tonne of feed (as per manufacturer recommendations). Identical diets were used in both groups; composed mainly of sorghum, wheat and soybean meal with nutritional levels recommended by breed standard in each stage and age of egg production.

Three sampling points were defined at 28 weeks (early lay), 50 weeks (mid-lay), and 72 weeks (late lay) of age. Each point sampled a total of 40 birds, 20 from each group (commonly accepted sample number for DNA analysis since requires euthanizing). Gut scoring was performed by a certified poultry veterinarian, intestinal samples were collected for 16S amplicon sequencing and ileum histology. Each sampling point analysed ileum content and ileum mucosa-associated microbiota, cecal and gizzard content microbiota, as well as microbiota from 100 cloacal swabs from both groups (higher sampling size compared to other gut sections given its non-invasive nature). Performance indicators such as mortality, feed conversion, egg quality, and egg production were collected daily and analysed using Paired Wilcoxon test.

III. RESULTS

At the end of the trial, PB outperformed CT in several key performance indicators. PB had a significant lower cumulated mortality, 0.36% lower compared to control ($P < 0.0001$). In both groups, there was a rise in mortality from 40 weeks until 50 weeks, predominantly due to smothering events randomly occurring per week. Hen-housed eggs (HHE) were significantly higher in the PB group at the end of the trial, producing 3.55 more HHE (Figure 1, $P < 0.0001$). There were significant differences in the cumulative feed conversion ratio (FCR; $P < 0.0001$). At the end of the experiment at 72 weeks, there was a reduction of 9 FCR points feed kg/dozen eggs (Figure 1) and 15 FCR points feed kg/egg kg produced in the PB. There were no significant differences between groups on the average rate of lay (ROL; $P > 0.22$) or egg quality indicators ($P > 0.52$) at the end of the trial.

Ileum histology showed at 28 and 50 weeks a significantly higher number of goblet cells in PB ($P = 0.005$ and $P < 0.0001$, respectively). No significant differences were detected at 72 weeks. Gut scoring showed significant differences with higher dysbiosis scores in CT at 28 and 50 weeks ($P = 0.001$ and $P < 0.0001$, respectively); CT birds had a higher presence of undigested feed and loss of gut integrity. No significant differences were found at 72 weeks.
Specific taxonomic variations were observed in different gastrointestinal sections; *Lactobacillus*, *Escherichia-Shigella* and *Campylobacter* showed notable changes in both CT and PB groups. *Lactobacillus* showed a consistently higher presence in PB at 28 weeks across all gut sections. At 50 weeks we observed similar results, except in ileum mucosa and cloacal swabs where CT presented a higher presence of *Lactobacillus*, and at 72 weeks, there were no apparent differences between groups.

Campylobacter levels were marginally altered at 28 weeks in both groups, but it was higher in ileum content in CT. At 50 weeks, there was a low occurrence of *Campylobacter* except in ileum content and mucosa, where CT had a notably higher presence. Variable results were observed in different origins at 72 weeks but considerably higher in ileum content and mucosa in CT and higher in PB in caeca.

It was observed that *Escherichia-Shigella* had a low presence at 28 weeks, except in ileum content and cloacal swabs, which were higher in CT. At 50 weeks, it showed a similar low abundance except in ileum content and cloacal swabs, where it was highly abundant and higher in CT than in PB. During late lay, *Escherichia-Shigella* had a higher abundance in PB cloacal swabs than in CT.

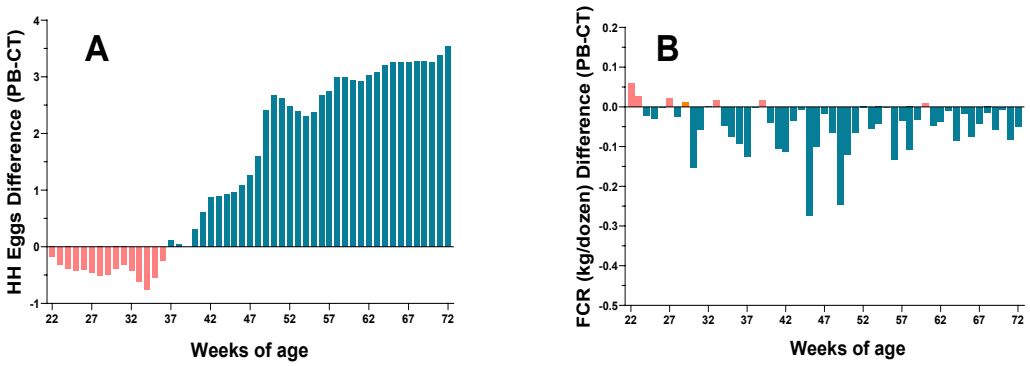

Figure 1 - Performance parameters summarising data collected from early lay at 22 weeks to 72 weeks (differences between PB and CT). Performance measures are shown as hen housed eggs (A) and feed conversion in kg/dozen (B). $P < 0.0001$ for both parameters.

IV. DISCUSSION

This study investigated the potential of precision glycans to influence the microbiota composition in commercial layer hens, ultimately enhancing their performance during the demanding peak production phase. Initially, both groups of hens exhibited expected performance levels described in the breed standard. However, as the trial progressed, significant differences emerged between the two groups.

The group receiving precision glycans displayed an improvement in feed conversion, a critical factor in poultry production economics. This improvement can be attributed to enhanced gastrointestinal tract function, as supported by several key observations. First, there was a clear macroscopic enhancement in gut functionality, characterised by a well-preserved intestinal structure, reduced undigested feed, and less abnormal content detected. Additionally, histological findings revealed improved gut health in the PB group, with a significantly increased goblet cell count observed at 28 and 50 weeks. Goblet cells play a vital role in producing mucin, a protective layer that can establish a favourable environment for commensal microbiota, which can utilise these glycoproteins as major nutrition source (Marcobal et al., 2013). This symbiotic relationship between goblet cells and commensal microbiota is crucial for maintaining gut health.

Interestingly, PB not only outperformed the CT in terms of feed conversion but also exhibited higher egg production, especially considering the increased mortality experienced by both groups due to sporadic smothering events, which impacted more the CT group. Smothering events are complex and not yet fully understood but can be influenced by factors such as age, time of day, temperature fluctuations, and litter conditions (Bright and Johnson, 2011). Nevertheless, none of these factors were reported and it can be assumed that both groups shared similar conditions for potential smothering occurrence. Furthermore, the performance

differences could not be related to these events, as it was found by (Herbert et al., 2021).When analysing 28 weeks results, clear differences emerged between PB and CT in various gut sections. PB exhibited a prevalence of lactic-acid bacteria, commonly associated with probiotics (Idoui, 2014; Fesseha et al., 2021). In contrast, CT showed a prevalence of potential pathogenic groups, such as *Escherichia-Shigella* and *Campylobacter* (Van et al., 2017; Kathayat et al., 2021).

In summary, this study demonstrated the potential of precision glycans as a practical and commercially viable tool to improve poultry egg production, even in challenging free-range environments. The use of precision glycans could help achieve the genetic potential of poultry breeds for optimum performance while offering economic benefits to poultry producers. However, further research is warranted to gain a deeper understanding of the underlying mechanisms and to optimise the application of precision glycans in poultry production for sustainable and efficient outcomes.

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TRANSGENERATIONAL IMPACT OF BROILER BREEDER NUTRITION

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Summary

Continuous genetic selection in meat-type chickens has led to rapid growth, increasing meat production, and reducing time to market weight. This selection has not only impacted nutrient metabolism during incubation but also influences gene expression and post-hatch growth performance and meat quality. The challenges in feeding and managing broiler breeders are discussed, emphasizing the importance of balanced nutrition for optimal reproductive performance and the transgenerational effects that influence the production of high-quality eggs and ensure chicks meet performance targets. It delves into the nutritional factors affecting broiler breeders, discussing the importance of proper dietary protein and energy intake, the role of amino acids, vitamins, and trace minerals in influencing progeny performance is highlighted, and emphasizing the significance of maintaining a balance to optimize reproductive performance. The concept of *in ovo* feeding, a technology that supplements soluble nutrients into the amnion of late term embryos, positively impacting various aspects of perinatal metabolism and development. This method has been shown to enhance glycogen reserves, improve early growth, and boost immune response, providing a comprehensive strategy for poultry production. This paper concludes with transgenerational epigenetic responses influenced by maternal nutrition. Recent research focuses on how nutrients, including methyl catalysts, selenium, vitamin D, and vitamin A, can modify DNA methylations and affect gene expression. The critical role of epigenetic mechanisms, such as microRNA activity, DNA methylation, and histone modification is explored, emphasizing their impact on the development and health of poultry progeny. In conclusion, this paper underscores the intricate relationship between genetic selection, nutrition, and reproductive performance in modern broiler breeders. It explores strategies like *in ovo* feeding (IOF) and delves into the emerging field of transgenerational epigenetic responses, providing valuable insights for optimizing poultry production and ensuring the health and performance of future generations.

I. INTRODUCTION

Continuous genetic selection in meat-type chicken for rapid growth resulted in increased meat production and decreased the time required for achieving the market weight. According to Havenstein et al. (2003), 42-day broiler weights have improved about 1% per year, and this trend has continued through to today. In 1957 a 42-day broiler weighed 540 g with and FCR of 2.35; in 2003 a 42 d broiler weighed 2800 g with an FCR of 1.70; and a recent study at NC State University demonstrated a 42 d broiler can weigh over 3500 g with an FCR of about 1.40. Hence, the incubation and neonatal period of modern commercial broilers can represent less than 50% of a bird's lifespan, depending on the age it is marketed. Because the chicken embryo undergoes development enclosed in an egg, it depends on the limited egg nutrients, particularly during the late-term embryonic developmental stage. Genetic selection has not only altered feed intake and meat production efficiency after hatch but also influenced gene expression and nutrient metabolism during incubation (Su et al., 2020). The differential between the growth potential of modern broilers and the target body weight of their parent stock to optimize reproductive performance has increased substantially over the past 60 years (Renema et al., 2007). Male breeder genetics positively affects the *in ovo* growth rate and nutrient demand that the female genetics and diet must supply (Schmidt and Figueiredo, 2009). Therefore, hen diets

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with insufficient essential nutrients may adversely affect embryonic development and consequently post-hatch performance. Intensity of broiler breeder feed restriction has consequently increased as genetic potential for growth rate and meat production increased so as to maintain reproductive performance (van der Klein et al., 2018 and Zuidhof, 2018); but limiting feed intake of broiler breeders can also influence favorable and unfavorable transgenerational effects. A lesser degree of growth restriction of broiler breeders during the prepubertal and early pubertal growth phase increased male progeny growth rate (Afrouziyeh et al., 2021). This paper discusses the challenges in feeding and managing modern broiler breeders, emphasizing the transgenerational effects that affect the production of high-quality eggs to maximize hatchability and ensure chicks can meet performance targets. Additionally, it explores key nutritional factors in broiler breeders that impact chick quality and offspring performance.

II. GENETIC IMPROVEMENT AFFECTS BROILER BREEDER NUTRITION

The nutritional strategy for breeder hens is crucial, as both excess protein and insufficient energy intake can have adverse effects. Excessive protein can lead to lower fat reserves and poor egg-shell quality, while inadequate energy intake affects the immune system, feathering, and overall reproductive performance (De Beer, 2010). Research has traditionally focused on studying nutrient requirements for maximizing egg production and hatchability in broiler breeders (Wilson, 1997). However, a slight increase in protein intake can positively impact egg size and chick weight, thus influencing broiler chicken growth at later stages (Bowmaker and Gous, 1991; Joseph et al., 2000; Afrouziyeh et al., 2021). Maintaining optimal egg size, hatchability, and body weight control in breeder hens poses challenges, especially with the use of higher protein diets after 40 weeks of age. Formulating breeder diets with minimum levels of crude protein is common, but careful consideration of digestible amino acids is necessary. Additionally, implementing a two-diet approach after 35 weeks, with lower protein and balanced amino acids in the second phase, is recommended to sustain production parameters and address issues like feed reduction post-peak (Leeson and Summers, 2000). Skilled management is crucial to understand the relationship between diet specification and feed allocation, ensuring overall flock health and performance (Leeson and Summers, 1991).

III. BREEDER NUTRITION AFFECTS CHICK QUALITY AND PERFORMANCE.

The quality of chicks in poultry farming is influenced by a myriad of factors, involving complex interactions. These factors include the physiological status of hens, hen nutrition, diet formulation, farm and hatchery management, transportation, and brooding efficiency. The interplay of these elements is crucial in determining the overall health and performance of breeder flocks, chick quality, and subsequent offspring performance. Poor flock uniformity, early photo-stimulation, improper feed allocation, and various stressors in the broiler house can significantly impact the outcomes (Chang et al., 2016).

Chick embryos rely on nutrients stored in the egg for normal growth and development, but the true nutritional requirements of the embryo remain largely unknown (Surai 2000). Egg composition, influenced by factors like nutrient allowances, diet fatty acid profile, hen age, health status, storage conditions, vitamin D metabolism, calcium allowances and sources, as well as mineral and vitamin supplementation, plays a crucial role. Flocks initiating egg production without meeting minimal development conditions may produce eggs of lower quality, characterized by lower weight, smaller yolk to albumen ratio, smaller fat content, and thicker shells (Ulmer-Franco et al. 2010). Although there are reviews on breeder nutritional factors of affecting chick quality and offspring performance (Kidd 2003, 2015; Calini and Sirri 2007), the applicability of these studies may be considered less relevant with the introduction

of modern genotypes and commercial conditions. Overall, a holistic approach considering various incubation conditions is essential in optimizing chick quality and ensuring successful poultry production and meat quality (Tona et al. 2003; Eusebio-Balcazar et al. 2014; Oviedo-Rondón et al., 2020).

a) Dietary protein and energy intake of broiler breeders affect progeny performance.

Aitken et al. (1969) first demonstrated the effects of protein and energy concentrations of broiler breeder hens on egg size and offspring performance. It was observed that offspring from parents fed a high-density diet had heavier hatching eggs, and their bodyweights were significantly greater at 42 and 63 days of age. The protein to energy ratio of the breeder diet was found to influence chick weight, with reduced chick size when the energy to protein ratio was low. Spratt and Leeson (1987) further investigated the impact of different concentrations of crude protein and energy on broiler breeders. Male chicks from hens fed high energy had improved early growth, while female chicks did not show the same result. Maternal diet effects on progeny were found to depend on the sex of the offspring, with high energy increasing male offspring carcass protein and decreasing carcass fat. Low-density diets can improve offspring growth and mortality, especially in older breeders. Enting et al. (2007) determined that low-density diets of broiler breeders can improve offspring growth rates, reduce mortality, and affect immune responses depending on breeder age and egg weight. Similarly, Hocking (2006) reported progeny of parents fed low-density diets diluted with oat hulls showed lower drinking behavior, improved litter quality, and differences in egg and chick size. In contrast, Moraes et al. (2014) found that when energy to protein ratio was increased to 18.25 kcal ME/g protein in the diets of young breeder hens, growth and breast-meat yield of progeny females increased. Similarly, van Emous et al. (2015) investigated the influence of dietary protein concentrations during rearing on embryonic and offspring performance. The study supported earlier findings that maternal diet effects on progeny performance depend on the sex of the offspring, and higher growth patterns during the rearing period had positive effects on fertility and offspring performance.

Energy and protein intake of male broiler breeders may also have transgenerational effects on offspring performance. Attia et al. (1993, 1995) observed broiler breeder males with varying daily energy intake levels demonstrated a significant increase in the 6-week bodyweight of their offspring when provided with high-energy diets. The authors proposed this outcome may be linked to the presence of supernumerary sperm in eggs laid by hens inseminated with sperm from males on high-energy diets. Another study reported a reduction in broiler male fertility due to inadequate feed allocation, resulting in a loss of mating activity in males and a subsequent 100g reduction in the bodyweight of the progeny (Romero-Sanchez, 2005). As a recommendation, it is advised to ensure breeder males receive sufficient cumulative energy (minimum 29,600 kcal metabolizable energy) and crude protein (minimum 1470 g) until photostimulation for optimal offspring growth (Bramwell et al., 1996; Romero-Sanchez, 2005; Attia et al., 1993, 1995).

b) Amino acid nutrition of broiler breeders affects the performance of their progeny.

Adequate levels and proper balance of amino acid in the diet of broiler breeders is crucial for optimal egg production, fertility, hatchability, and the health of the offspring. Dietary lysine of breeder-hen can affect progeny outcomes. Mejia et al. (2013) utilized corn-based distiller grains to decrease breeder-hen dietary lysine and found the progeny from young breeders exhibited low bodyweight and breast yield but higher dark-meat yields under the lowest lysine condition (600 mg lysine/bird.day). Ciacciariello & Tyler (2013) also observed a significant correlation between hen digestible lysine and offspring live performance on Day 21 and

concluded that changes in hen feed allocation to boost egg production over time could negatively impact offspring live performance. Additionally, another study by Kidd et al. (2005) suggested that breeder-hen dietary L-carnitine influences progeny carcass traits, with hens fed 25 mg L-carnitine/kg from 21 weeks of age showing decreased abdominal fat and increased breast meat in the progeny.

c) Transgenerational effects of dietary vitamins have been observed.

The impact of dietary vitamins of breeding poultry and effects on hatchability and progeny health has been extensively discussed in comprehensive reviews by Calini and Sirri (2007) and Oviedo-Rondón et al. (2023). Deficiencies of vitamins in breeder diets have shown to have substantial consequences. Vitamin A deficiency compromises the develop the normal blood system and causes embryonic malposition. Vitamin D3 deficiency causes improper calcification of eggshells, possible symptoms of calcium tetany in young breeders, rickets, stunted chicks with soft bones. Vitamin E deficiency reduces fertility, causes inadequate embryonic vascularization, early embryonic mortality, and exudative diathesis in chicks. Vitamin K deficiency prolongs embryonic blood-clotting time, embryonic hemorrhages, and extra embryonic blood vessels. Riboflavin deficiency increases embryonic mortality rates from 9 to 14 days of incubation, atrophied leg muscles, clubbed down, and curled toes. Vitamin B12 deficiency increases embryonic malposition with head between the legs, short beaks, poor muscle development, and high embryonic mortality rates from 8 to 14 days of incubation. Pyridoxine deficiency reduces hatchability. Biotin deficiency causes perosis, shortened or twisted bones, and excessive early embryonic mortality. Folic acid deficiency causes perosis and twisted toes and high mortality rate during pipping. Pantothenic acid deficiency causes abnormal feathering of chicks, subcutaneous hemorrhages of the embryo, weak hatchlings. Although dietary vitamin deficiencies rarely occur in commercial practice, premix supplementation errors do occasionally occur that result in marginal deficiencies because of low supplementation, imbalances, and excesses, poor quality of the vitamin source, and poor storage and feed manufacturing conditions. Sometimes the less dominant breeders consume less than their estimated feed allotment, and therefore may result in marginal vitamin deficiencies, particularly during peak of lay. Most likely, the progeny will not exhibit classical vitamin deficiency symptoms, but they will not perform at their genetic potential, especially during the challenges of the first 10 days post-hatch.

Among all the vitamins, Vitamin D and E nutrition of broiler breeders have the most significant transgenerational effect. Vitamin D3 status in hens is particularly important for optimal progeny development (Sunde et al., 1978). Higher maternal dietary concentrations of 2000 to 4000 IU vitamin D3/kg result in improved progeny weight gain and reduced incidence of tibial dyschondroplasia from hens during peak lay, but not after 45 weeks of age (Atencio et al., 2005a). The more bioavailable form, 25-OH-D3, has gained popularity for its positive effects on reducing embryo mortality and enhancing bone ash in progeny (Atencio et al., 2005b). The antioxidant status of broiler breeders and consequential effect on disease prevention in offspring is of increased commercial interest. Vitamin E has been associated with improved adaptive antibody transfer from parent to offspring (Boa-Amponsem et al., 2001).

d) Dietary trace minerals of breeders have significant transgenerational effects.

Although mineral requirements are well established for egg production of poultry, the trace minerals that have the greatest effect on progeny are selenium (Se), zinc (Zn), manganese (Mn), and perhaps iodine (I). The significance of Se, particularly in its organic form, as an antioxidant co-factor has been extensively studied. Jlali et al. (2013) demonstrated that dietary organic Se improves concentrations in eggs and enhanced the levels in the tissue of progeny (Surai, 2000;

Pappas et al., 2005; and Couloigner et al., 2015). Chicks from hens fed 0.5 mg/kg organic Se exhibited higher tissue concentrations than those from hens fed lower amounts (Pappas et al. 2006). Moreover, progeny from parents fed seleno-hydroxy-methionine showed a 1.25% improvement in feed conversion ratio (FCR) as compared to offspring from other Se sources (Couloigner et al. 2015). The higher muscle Se content at hatch suggested improved Se reserves, influencing the transition of the antioxidant system during the early days of chicks' lives (Surai, 2002).

The role of Zn in chick quality, feathering, progeny growth, and viability has been explored in studies by Turk et al. (1959), Edwards et al. (1959), and Kidd (2003). Higher concentrations of Zn supplements were found to enhance cellular immune function and early survival. When combined with organic Mn, maternal diets with these trace minerals improved progeny livability, immune parameters, and cardiac function (Virden et al. 2003, 2004). Progeny from hens fed organic Mn and Zn also tended to have improved breast-meat yield compared to those fed inorganic forms. Hocking (2007) suggests that significantly higher maternal dietary concentrations of Se, Zn, and Mn than typically recommended may positively impact immune function and livability when provided in combination. These findings highlight the importance of Se and Zn, especially in organic forms, in influencing the health and development of poultry progeny.

IV. EGG NUTRIENT SUPPLEMENTATION BY *IN OVO* FEEDING (IOF)

As discussed above, broiler breeder hen diet and nutritional status can have a significant effect on nutrient deposition in their eggs, especially during peak egg production of nutrient mobilization from the feed-restricted diet and body reserves are constrained. Alleviating these nutrient constraints is possible by IOF as defined by Uni and Ferket (2003). IOF technology supplements into the amnion of oviparous embryos soluble nutrients that play a crucial role in improving various aspects of perinatal metabolism and development. The glycogen stores, utilized as the primary energy source by the embryo, tend to be depleted by the end of the hatching process. IOF addresses this by enhancing glycogen reserves in the liver and muscles, serving as a vital energy source for the hatching process.

Studies conducted over approximately 20 years have delved into the efficacy of IOF with diverse nutrient supplements, including NaCl, sucrose, maltose, dextrin, and disaccharide, β-hydroxy-β-methyl-butyrate, eggwhite protein, and carbohydrate, glycerol and insulin-like growth factor, creatine monohydrate, linoleic acid, γ-aminobutyric acid, threonine, cysteine, arginine, methionine, L-leucine, vitamin E, vitamin B12, folic acid, Bacillus subtilis or raffinose, zinc and copper, manganese, and zinc-methionine (Peebles, 2018; Celik & Ferket, 2023), and IOF has become an excellent method of evaluating *in ovo* nutrition and epigenetic effects (Das et al., 2021). The positive effects extend across a spectrum of factors influencing early growth and development in poultry. Notable improvements include increased body weight at hatch, advanced morphometric development of the intestinal tract, enhanced expression of brush border digestive enzymes (such as sucrase-isomaltase and leucine aminopeptidase) and increased biological activity of these enzymes. Furthermore, there's enhanced expression of nutrient transporters, SGLT-1, PEPT-1, and NaK ATPase, contributing to improved nutrient absorption. The positive outcomes of IOF extend to various aspects, including increased breast muscle size at hatch, improved bone development, and enhanced immune response. Additionally, the technique has been associated with decreased cellular stress, improved oxidative status, and increased liver glycogen status. This multifaceted approach to supplementation not only influences the immediate post-hatch performance but also affects the development of critical tissues and bone of the neonate by approximately 2 days at the time of hatch. In summary, IOF emerges as a comprehensive strategy with far-

reaching benefits for poultry production, encompassing aspects of growth, development, immune response, and overall physiological well-being.

V. NUTRITIONAL AFFECTS TRANSGENERATIONAL EPIGENETIC RESPONSES

The most recent research related to the transgenerational impact of nutrition in poultry focuses on epigenetic mechanisms, which are genomic and metabolomic adaptations to maternal nutrient status and environmental stressors. Dunislawska et al. (2022) presents an excellent review of pre-hatching and post-hatching environmental factors related to epigenetic mechanisms in poultry. These authors present evidence that maternal nutrition and environmental factors may have transgenerational epigenetic effects. Using the quail as a model, Phillips (2020) demonstrated that maternal diets containing increased levels of methyl catalysts (choline, betaine, vitamin B12, folic acid, pyridoxine, and zinc) significantly modified specific DNA methylations at the cytosine residues of cytosine-phosphate-guanine dinucleotides (CpG) under the action of DNA methyltransferases. Other maternal dietary nutrients that can be transferred to the egg that affect methyl metabolism and gene expression include selenium, vitamin D, and vitamin A. Indeed, epigenetic programming is a new avenue of research.

The critical epigenetic reprogramming events occur during germ cell development in adolescent breeding stock, and chromatin remodeling due to events such as demethylation and remethylation of the embryonic genome during early embryogenesis (Wang et al., 2014). Increased methylation of CpG and histone acetylation can also occur during the early post-hatch period (Kisliouk et al., 2017). Key epigenetic mechanisms include microRNA (miRNA) activity, DNA methylation, and histone modification. Small RNA molecules encoded in the genome, miRNAs, play a crucial role in gene expression and epigenetic response (Chuang & Jones, 2007). They bind to the 3'-UTRs end of target gene mRNA, destabilizing it and preventing translation, thereby silencing target genes (Taganov et al., 2007). DNA methylation involves adding methyl residues to cytosines within CpG islands, inhibiting the transcription of genes from DNA into mRNA (Shen and Waterland, 2007). The methylation process is influenced by nutritional components and supplementation, as DNA requires methyl donors and cofactors from the external environment (Dunislawska et al., 2021). Histone modification, regulated by enzymes sensitive to endogenous small molecule metabolites, affects transcription and responds to environmental changes. For instance, changes in intestinal microbiota regulate histone methylation and acetylation in host tissues in a diet-dependent manner (Stoll et al., 2018).

In ovo feeding provides a valuable approach for early embryo support and allows for the assessment of nutrient effects on epigenetic changes in adult birds. A study administering folic acid to the yolk sac of broiler chicken embryos on the 11th day of incubation revealed induced methylation of histones in IL2 and IL4 promoters, with post-hatch effects on histone H3 lysine 4 (H3K4me2) enrichment and loss of histone H3 lysine 9 (H3K9me2) in growing chickens (Li et al., 2016). Conversely, the IL6 promoter showed decreased H3K4me2 and increased H3K9me2. H3K4me2 participates in euchromatin formation and ongoing gene expression; whereas H3K9me2 is a repressive histone mark that negatively regulates transcription by promoting a compact chromatin structure (Pekowska et al., 2011). Thus, folic acid administered with IOF impacts immune functions through epigenetic regulation of immune genes. Another investigation found that *in ovo* administration of Zn to Zn-deficient chicken eggs reduced embryo mortality and increased hatchability, with organic Zn showing higher efficiency in enhancing methylation and acetylation compared to inorganic Zn (Sun et al., 2018). Additionally, *in ovo* injection of betaine was shown to regulate cholesterol metabolism in chicken livers through epigenetic mechanisms, alleviating effects related to diet

and corticosterone exposure, and influencing gene expression and methylation modifications associated with CpG methylation in key genes (Hu et al., 2015; Hu et al., 2017).

The perinatal period is vital for programming the microbiota to facilitate the colonization of the embryo's intestines with beneficial bacteria before hatching. Notably, the administration of a single dose of prebiotic or synbiotic suspension to the egg's air chamber on the 12th day of incubation has enduring effects on the chicken's lifespan, with significant molecular changes observed in the liver and spleen (Dunislawska et al., 2017; Siwek et al., 2018). In a study by Dunislawska et al. (2020), synbiotics based on Lactobacillus strains were administered on the 12th day of egg incubation, resulting in hypermethylation of the ANGPTL4 gene in the liver. This hypermethylation was associated with a substantial decrease in gene expression, emphasizing the gene's role in lipid metabolism, insulin sensitivity, and glucose homeostasis. The epigenetic regulation of gene expression through early microbiota stimulation is also dependent on liver miRNA activity, suggesting miRNA is a crucial element in the molecular mechanism of host-microbiota interaction, particularly in the context of gene expression silencing (Sikorska et al., 2021). Maternal nutrition plays a crucial role in shaping the epigenome of future offspring through a process known as the maternal effect, involving non-genetic interference by the mother on the offspring's phenotype. In poultry production, maternal substances like antibodies, hormones, and antioxidants transferred through the yolk sac impact the immune response and microbiome in young birds (Paul et al., 2015).

V. CONCLUSION

The continuous genetic selection in meat-type chickens for rapid growth has significantly transformed the poultry industry over the past decades. The evolution in broiler weights and feed efficiency reflects the success of these breeding programs. However, this progress has introduced challenges in feeding and managing modern broiler breeders. The intricate interplay between genetics, nutrition, and environmental factors shapes the quality and performance of chicks. The essay delves into the nuanced realm of broiler breeder nutrition, emphasizing the transgenerational effects that influence egg quality, hatchability, and offspring performance. Furthermore, the exploration of IOF and its impact on early development, along with the emerging field of epigenetics, underscores the complexity of optimizing poultry production for both current and future generations.

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MATERNAL SUPPLEMENTATION OF GUANIDINO ACETIC ACID: INFLUENCE ON EGG COMPOSITION AND CHICK DEVELOPMENT

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Summary

Maternal supplementation of the creatine precursor Guanidino acetic acid (GAA) was investigated in commercial broiler breeder hens at two ages to determine the effects on chick hatch rate and growth. Birds were housed at a commercial broiler breeder farm complex (*n* = 6 sheds),and fed either a control commercial diet (*n* = 3 sheds) or control commercial diet + 0.08% GAA (*n* = 3 sheds) from 15 weeks of age. At 28 and 38 weeks of age a selection of eggs across all sheds were collected and incubated to compare the supplement effects on egg water loss (%) during incubation, residual yolk weight, chick hatch weight and fresh egg parameters. In the eggs from peak production, the fresh yolk was a larger (P = 0.016) portion of the egg, while the residual yolk sac in hatched chicks was smaller (P = 0.022). In eggs from both aged hens, the water weight loss (%) after 18 days incubation was higher (P = 0.020 28-week-old hens; P = 0.032 38-week-old hens) in eggs from supplemented hens. The results indicate that supplementation of GAA may increase the water content of the egg and in the hatched chick, which could be attributed to a decrease in metabolic water production as a by-product of residual yolk utilisation due to utilising the supplement instead, however this requires further investigation. If possible, increased chick hydration could be beneficial, particularly in the event of delayed chick placement from hatchery to farm.

I. INTRODUCTION

Young broiler breeder hens (< 30 weeks of age) produce smaller eggs contributing to a lower hatch rate and higher first week mortality (Yassin et al., 2009). The smaller eggs have proportionally smaller yolks, reducing the energy source the chick requires to grow and hatch (Yadgary et al., 2010). Physiologically, creatine is important for the storage and transport of energy to high energy demand tissues such as brain and muscle (Rackayova et al., 2017). The precursor to creatine, GAA is often used as a feed supplement due to its stability and is readily converted by the hen to creatine. Supplementation of GAA to hens has shown positive results, including increased creatine deposition in the albumen and yolk of fresh eggs from older hens (Reicher et al., 2020) and an increased hatch rate when administered *in ovo* to eggs from younger hens (Firman et al., 2023). This study was conducted to determine if maternal supplementation of GAA to hens during early and peak lay would improve chick hatch rate through efficiencies in energy utilisation and metabolism during incubation and at hatch.

II. METHOD

Commercial broiler breeder birds, housed at a commercial breeder farm complex, were fed either a standard commercial diet (Control; *n* = 3 sheds) or commercial diet supplemented with 0.08% GAA (Creamino™, Alzchem, Germany; *n* = 3 sheds) from 15 weeks of age. Fertile eggs were sampled when the flock was 28 weeks (*n* = 300) and 38 weeks (*n* = 240) of age.

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Eggs were numbered, weighed, and randomised for incubation at the University of Adelaide, Roseworthy. All procedures were approved by the University of Adelaide Animal Ethics Committee. Temperature, relative humidity, and incubator conditions including, rotation of eggs, were set as per industry specifications. Eggs were re-weighed at embryonic day18 to calculate the water weight lost during incubation then transferred to hatching trays. At hatch 96 chicks were humanely euthanised. Bodyweight was recorded and residual yolk sac removed and weighed. Fresh eggs (n = 30) per treatment were also sampled prior to incubation for egg yolk measurements at each timepoint. The yolk was separated from the egg and weighed to undergo future creatine and glycogen concentration analysis.

Water loss (%), fresh yolk weight (% egg weight) and residual yolk weight (% bodyweight) data were analysed using IBM® SPSS® Statistics 28 (Armonk, NY, USA). Hatchability was analysed using a Chi-squared test. Continuous data were checked for normality with a Shapiro–Wilk test. Data that failed the normality test were Log10 transformed for normality, which had been corrected. Data were then analysed with a generalised linear mixed model with treatment the only factor for water weight loss and sex, treatment, and sex by treatment factors for chicks at hatch.

III. RESULTS

The water weight loss (%) was higher in eggs from hens fed GAA both at 28 weeks (GAA 10.83%; Control 10.46%; P = 0.020) and 38 weeks (GAA 10.45%; Controls 10.04%; P = 0.032). There was an approximate 0.4% reduction in water loss for each group in the eggs from older hens. No significant differences in hatch rate or hatch weight were observed in eggs from either age group due to the maternal supplement. In hatched chicks, the proportion of residual yolk (% body weight) was lower in the chicks from 38-week-old hens fed GAA (13.25%) compared to the Controls (14.38%; P = 0.014). The chicks from the 28-week-old GAA supplemented hens also had a smaller residual yolk sack (12.60%) compared to controls (13.16%) but was not significant (P = 0.258). In the fresh eggs from the 38-week-old GAA supplemented hens, the yolk comprised a larger portion of the egg, 33.46% compared to controls 32.38% (P = 0.016). The yolk was also slightly larger in eggs from the 28-week-old supplemented hens, 29.73% compared to controls, 29.02%; P = 0.053). There was no sex by treatment effect in total chick or residual yolk sac weight at either time point (data not shown).

Table 1 - Mean ± SEM of parameters measured in eggs and hatched chicks from broiler breeder hens fed a commercial diet supplemented with 0.08% GAA or controls (commercial diet only) at 28 and 38 weeks of age.

	N	Control	N	GAA[1]	P-value
Egg weight (g) set at 28 weeks	131	52.68 ± 0.25	133	52.35 ± 0.25	0.348
Egg weight(g) set at 38 weeks	110	63.15 ± 0.30	107	63.47 ± 0.30	0.442
Egg weight loss (%) 28 weeks[2]	131	10.46 ± 0.14	133	10.83 ± 0.11	0.020
Egg weight loss (%) 38 weeks[2]	110	10.04 ± 0.15	107	10.45 ± 0.15	0.032
Chick hatch weight (g) 28 weeks	100	37.88 ± 0.27	104	37.40 ± 0.27	0.209
Chick hatch weight (g) 38 weeks	106	45.74 ± 0.28	103	45.96 ± 0.28	0.580
Hatch rate (%) 28 weeks	150	90.00	150	88.00	0.580
Hatch rate (%) 38 weeks	120	88.33	120	89.17	0.838
Residual yolk sac (%) 28 weeks[3]	43	13.16 ± 0.35	44	12.60 ± 0.35	0.258
Residual yolk sac (%) 38 weeks[3]	47	14.38 ± 0.32	47	13.25 ± 0.32	0.022
Fresh egg yolk (%) 28 weeks[4]	26	29.02 ± 0.32	29	29.77 ± 0.30	0.053
Fresh egg yolk (%) 38 weeks[4]	28	32.38 ± 0.31	29	33.46 ± 0.30	0.016

[1] Hens supplemented with 0.08% GAA:800g/ton Creamino (min 96% GAA) on top of feed from 15 weeks old.
[2] Percentage of water weight loss calculated from egg weight at set minus egg weight after 18 days incubation.
[3] Residual yolk sac at hatch as a percentage of hatched body weight
[4] Egg yolk as a percentage of whole egg weight in fresh fertile eggs

IV. DISCUSSION

Water loss during incubation occurs through the pores of the eggshell due to oxygen (O_2) – carbon dioxide (CO_2) exchange in which water vapor is lost along with expelled CO_2 (Mortola, 2009). Water needs to be lost during incubation for the air cell to form in the egg, this air cell provides the chick the first exposure to air when internal pipping occurs prior to external pipping and hatch (Ar, 2011). If the air cell is too small the lungs may not be able to fully inflate, if the air cell is too large the embryo can become dehydrated both resulting in unhatched or weaker chicks at hatch (Ar, 2011).

Water loss is replaced in small part by metabolic water, which is the water byproduct produced by the embryo when utilising and breaking down the protein and lipids from the egg yolk into active energy for growth (Ar and Rahn, 1980). Creatine provides a more readily available energy source which phosphorylates stored ADP to active ATP which does not produce metabolic water (Rackayova et al., 2017). The differences in water loss % were within commercial standard ranges and did not alter hatch rate. It's possible that both control and supplemented eggs lost the same amount of water, but supplemented eggs produced less metabolic water, accounting for the differences in total % loss.

While the albumen is the primary source of water for the growing embryo, comprising roughly 85% of the total egg water content, the fresh yolk is comprised of around 50% water, which accounts for around 15% of the water in the egg (Yadgary et al., 2010). Yadgary et al. (2010) further found that from incubation to hatch the amount of water in the yolk drastically decreased, and larger yolks from larger eggs that had more water weight in fresh yolks resulted in chicks that had the same water weight in residual yolk at hatch as chicks from smaller eggs. This indicates that the extra water is either lost as vapor or utilised by the chick.

The effects of creatine supplementation on tissue water content is an area of high interest and it is not fully understood, if creatine supplementation is increasing water content and tissue hydration there may still be post-hatch performance benefits even if hatch weight and rate are not improved.

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IN-OVO ENERGY ENRICHMENT WITH CREATINE MODULATES BROILERS' BREAST MUSCLE DEVELOPMENT THROUGH ALTERED EXPRESSION OF MYOGENIC GENES

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Summary

The pre-post-hatching is a critical phase of broiler chickens' lifespan. In-ovo feeding (IOF) with creatine monohydrate on embryonic day 17.5 (E17.5) was studied for its impact on energy levels (creatine and glycogen) and post-hatch muscle growth in this critical period. IOF with creatine increased creatine levels in the liver, breast muscle and yolk sac tissues, and increased breast muscle glycogen levels on E19. An automated histomorphology analysis of breast muscle samples from day 14 post-hatch revealed a higher number of myofibers with smaller diameters in the creatine in-ovo fed group compared to control groups (non-injected and IOF with NaCl). This was likely influenced by gene expression changes in the IOF creatine group at hatch; lower expression levels of MyoD which is related to myoblast proliferation and higher expression of myogenin (MYOG) and insulin-like growth factor 1 (IGF1) levels, both related to muscle cell differentiation. These data implicate a role for in-ovo enrichment with creatine in modulating breast muscle development in early stages post-hatch through altered expression of myogenic related genes.

I. INTRODUCTION

Throughout the broiler's lifespan, the pre- to post-hatching period entails the most dynamic transition from late-term embryo to hatchling and growing chick (Moran, 2007; De Oliveira et al., 2008). This period includes a dramatic change in energy metabolism, as energy production switches from oxidation of yolk-derived lipids to anaerobic catabolism by glycogenolysis and by gluconeogenesis (Decuypere et al., 2001; Tazawa et al., 1983). By the time the embryo hatches, almost all of its glycogen stores are depleted (Uni et al., 2005; Yadgary et al., 2012). As a result, gluconeogenesis becomes the dominant pathway for energy provision supplied primarily by breast muscle protein degradation to amino acids (Donaldson, 1995; Keirs et al., 2002). This suggests that adequate supply of energy pre-post-hatch is pivotal for muscle development and integrity as was previously reported (Halevy et al., 2000, 2003; Bigot et al., 2003; Kornasio et al., 2011). In this study, we examined whether the elevation of late-term embryos' energy stores by creatine will provide a jumpstart for breast muscle development and growth. For this aim, the method of in-ovo feeding (IOF) was applied by inserting creatine monohydrate solution into the embryo's amniotic fluid on E17.5, according to Uni and Ferket (2003). In chickens, creatine was shown to play a major role in energy metabolism, promoting the energetic status and post-hatch performance (Zhang et al., 2016; Zhao et al., 2017; Firman et al., 2023). A pre- dose-response test conducted in our lab showed that hatchlings receiving IOF with 1.5% creatine had significantly higher breast muscle weight and percentage compared to non-injected control. Here, we provide an inclusive view of creatine and glycogen dynamics during the pre- to post-hatching period in response to IOF with creatine in three essential tissues, breast muscle, liver, and yolk sac (YS), all of which are involved in energy supply and demand (Dayan et al., 2023a). To evaluate the effects of energy enhancement on breast muscle development during early post-hatch, a novel, high-precision image analysis tool for histological evaluation was applied (Dayan et al., 2023b). In addition, we examined a possible

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mechanism underlying how energy status may modulate muscle development through differential expression of genes involved in myoblast proliferation [myogenic determination factor 1 (MyoD)] and differentiation [insulin-like growth factor 1 (IGF1) and myogenin (MYOG)].

II. METHOD

Fertile eggs (n= 330; mean weight = 62.46 g, SD = 4.4 g) from 33-week-old broiler hens (Cobb 500) were incubated under standard conditions (37.8°C and 56% relative humidity). On E17.5, IOF was performed, according to three treatment groups: control (non-injected), IOF NaCl (injection volume: 0.6 ml containing 3 mg NaCl per embryo), and IOF creatine [injection volume: 0.6 ml containing 9 mg creatine monohydrate (AlzChem Trostberg GmbH, Germany) and 3 mg NaCl per embryo]. After IOF, eggs were transferred to hatching trays, and hatchability was monitored. Male chicks were reared for 14 days according to the breeder recommendations (Cobb-Vantress) with ad-libitum access to water and food. Tissue sampling was performed on various days, six embryos/birds per group were sampled. Body weight (BW), YS tissue weight, liver weight, and breast muscle weight were recorded. In addition, liver, YS tissue, and breast muscle samples were collected to determine creatine and glycogen content (from E17 to day 1) and breast muscle samples were collected for histological evaluation of myofibers on day 14 and gene expression analysis (from E17 to day 6). Statistical analyses were performed by JMP software (SAS Institute Inc., Cary, NC). Differences were evaluated by Tukey's HSD test and P-value lower than or equal to 0.05 ($P \leq 0.05$) was considered significant.

III. RESULTS

Our results show that hatchability was not affected by IOF; it ranged between 94.8% in IOF NaCl group to 95.04% in IOF creatine and 95.4% in the control group. As for energy levels in tissues (Table 1), results show that 48 h post-IOF, on E19, creatine levels were 45% higher, and glycogen levels were 30% higher ($P < 0.03$) in the breast muscle of the IOF creatine group compared to control and IOF NaCl groups. Analysis of liver and YS tissues on E19 revealed even a higher difference with more than 140% creatine amount ($P < 0.01$) in the IOF creatine group compared to the control and IOF NaCl groups.

Table 1 - Creatine and glycogen levels in treatment groups from E17 until day 1 post-hatch.

Day	Treatment	Total creatine amount in tissue (mg)			Total glycogen amount in tissue (mg)		
		Breast muscle	Liver	YS tissue	Breast muscle	Liver	YS tissue
E17	-	1.37 ± 0.15	0.023 ± 0.004	0.57 ± 0.06	2.63 ± 0.22	14.03 ± 0.8	104.5 ± 8.92
E19	Control	1.88 ± 0.1^{b}	0.030 ± 0.004^{b}	0.55 ± 0.16^{b}	3.66 ± 0.16^{b}	12.79 ± 2.76	69.92 ± 9.62
	IOF NaCl	2.08 ± 0.13^{b}	0.028 ± 0.006^{b}	0.59 ± 0.04^{b}	3.54 ± 0.34^{b}	11.38 ± 1.62	69.96 ± 6.21
	IOF Creatine	2.73 ± 0.11^{a}	0.075 ± 0.013^{a}	1.41 ± 0.17^{a}	4.89 ± 0.19^{a}	15.11 ± 2.4	75.57 ± 6.98
Hatch	Control	2.32 ± 0.24	0.05 ± 0.01	0.22 ± 0.04^{b}	1.74 ± 0.58	3.88 ± 1.33	12.41 ± 3.52
	IOF NaCl	2.21 ± 0.13	0.054 ± 0.01	0.21 ± 0.04^{b}	2.02 ± 0.56	3.82 ± 1.39	12.93 ± 3.3
	IOF Creatine	2.41 ± 0.26	0.075 ± 0.013	0.63 ± 0.11^{a}	2.44 ± 0.61	5.77 ± 1.62	19.55 ± 4.79
D1	Control	3.59 ± 0.36	0.028 ± 0.003	0.060 ± 0.01	8.01 ± 1.02	75.12 ± 12.16	12.83 ± 1.27
	IOF NaCl	3.78 ± 0.14	0.030 ± 0.003	0.040 ± 0.004	8.88 ± 1.49	76.49 ± 13.28	16.95 ± 2.27
	IOF Creatine	4.07 ± 0.22	0.033 ± 0.003	0.086 ± 0.037	7.51 ± 0.6	78.25 ± 4.84	11.74 ± 0.92

Total creatine and glycogen tissue amount (mg) of breast muscle, liver, and yolk sac (YS) tissue. Lowercase letters denote results that are significantly different between treatments at each time point, as derived from Tukey's HSD test ($P \leq 0.05$), n=6 per treatment and day.

No significant differences between treatments were observed for glycogen levels in the liver and YS tissues. At hatch, creatine amount in YS tissue remained significantly higher in the IOF creatine group with over 180% difference ($P < 0.004$). Overall, between E19 and the day of hatch there was a significant decrease in glycogen levels in all tissues of all examined treatments ($P < 0.01$).

Breast muscle weight and percentage as well as BW were comparable in all groups on day 14 post-hatch. To evaluate breast muscle myofibers' histomorphology on day 14 post-hatch, a novel deep learning-based automated image analysis tool was applied according to Dayan et al. (2023b). Results shown in Table 2, indicate that myofiber diameter average of the IOF creatine group was significantly smaller than that of other groups ($P < 0.0001$); the myofiber diameter and area were 21.4 μm and 577.4 μm^2, respectively, compared to 22.4 μm, 667.1 μm^2 of IOF NaCl and 24.9 μm, 787.2 μm^2 of control. The number of myofibers per mm^2 was significantly higher in the IOF creatine group compared to control and IOF NaCl ($P < 0.004$).

Analysis of IGF1 and MYOG genes revealed increased expression levels by more than twofold and fourfold, respectively in the IOF creatine group at hatch compared to control and NaCl groups ($P < 0.01$). For the expression of MyoD, significantly higher expression levels were found in the control group at hatch and on day 6 compared to the IOF creatine and IOF NaCl groups ($P < 0.04$).

Table 2 - Histomorphological analysis of treatment groups on day 14 post-hatch.

Treatment	Number of myofibers	Myofiber diameter (μm)	Myofiber area (μm^2)	Number of myofibers per mm^2
Control	19,610	24.97 ± 0.053^{a}	787.21 ± 2.84^{a}	126.85 ± 3.81^{c}
IOF NaCl	19,415	22.43 ± 0.052^{b}	667.13 ± 2.39^{b}	149.43 ± 4.26^{b}
IOF Creatine	20,103	21.14 ± 0.049^{c}	577.38 ± 2.36^{c}	179.78 ± 10.54^{a}

Summary of morphological analysis and comparison between the Control, IOF creatine, and IOF NaCl treatment groups in the pectoral muscle of 14-day-old broilers. Myofiber diameter and area are presented as mean size ± standard error mean. The number of myofibers per mm^2 was calculated for each image. The number of counted myofibers was normalized with the total myofiber area. Superscript letters denote the means that are significantly different between treatments, as derived from Tukey's HSD test ($P \leq 0.05$), n=84 (4 birds per treatment and 7 images per bird). Images comprised of 12 stitched fields of X60 magnification were generated using an EVOS FL Auto-inverted microscope.

IV. DISCUSSION

This study demonstrates that IOF with creatine monohydrate enhances the energy levels (creatine and glycogen) in the liver, breast muscle and YS tissues of late-term embryos. Surprisingly, despite the evidence of the highest energy levels, the IOF creatine group had a significantly higher number of myofibers per area with smaller size on day 14 post-hatch. This could be explained by a possible acceleration of differentiation with a slower rate of proliferation of muscle cells. Indeed, higher expression levels of IGF1 and MYOG genes in the IOF creatine group, which are related to the differentiation of myogenic cells, and lower expression of MyoD, related to their proliferation were found. Together, the results imply the potential of IOF with creatine monohydrate in modulating muscle growth and development of broiler chickens. Notably, the scope of this study was to evaluate the effect of IOF with creatine on short-term muscle development post-hatch. Therefore, it is important to explore the long-term effects of this intervention and its potential applications to overcome the current challenges in commercial broiler production.

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THE USE OF L-SELENOMETHIONINE IN BROILER BREEDER AND BROILER NUTRITION

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Summary

Genetic selection and stressors related to modern-day broiler production, such as high stocking density, pose challenges to the performance and health of broiler breeders and broiler offspring. Two trials were conducted to study the effects of L-selenomethionine in the diets of broiler breeders and broilers performance under normal or increased stocking density. When supplementing the diet of both broiler breeders and broiler offspring with L-selenomethionine, performance (body weight, feed conversion ratio, mortality) was improved. Drip loss was improved when L-selenomethionine was added to the diet of the broiler offspring, regardless of the maternal source. In the second trial, higher stocking densities did not seem to negatively affect broiler performance. However, L-selenomethionine improved performance of broilers compared to sodium selenite, independent of the applied stocking density. It can be concluded that L-selenomethionine in the diet of broiler breeders and/or broilers allows for improved bird performance.

I. INTRODUCTION

Genetic selection with the aim of achieving a higher growth rate, has led to an increase in body weight and appetite, and improved feed conversion in broilers and their breeders. However, this is often associated with reduced health and higher mortality. Poultry scientists have investigated many dietary supplements, including exchanging inorganic minerals for organic minerals in feed to improve the health and reproductive function of the breeders, along with better chick quality. Selenium is an essential trace element with functions in animal health (Biswas et al. 2006), reproduction (Khalili et al. 2018), performance (Khalifa et al. 2021) and reducing oxidative stress (Rahmanto and Davies, 2011), and can thus contribute to better health status of breeders. Selenium can be added to the diet in organic or inorganic forms. Organic selenium, in the form of L-selenomethionine, has the unique property, compared to other selenium sources, of being able to be stored in animal protein (De Marco et al. 2021). Therefore, it can be transferred to the offspring via the egg and supply a continuous source of selenium to the animal during high stress periods, such as high stocking densities (De Marco et al. 2021).

It was hypothesized that the use of L-selenomethionine, compared with inorganic sodium selenite, in the breeder's diet would have a positive impact on breeder health, incubation parameters and chick quality. Furthermore, it was expected that L-selenomethionine would improve broiler performance whilst stocking density increased.

II. METHOD

The first study was carried out at the facilities of the National University of Luján (UNLu), Argentina. The facility consists of 24 floor pens, 15 birds per pen, with a hopper feeder per pen and a nipple drinker. Cobb 500 birds were used. They originated from a breeder farm in the town of Luján. Two sheds with 45-week-old breeders were used for the trial. The breeders were

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fed two different diets consisting of two selenium sources; sodium selenite or L-selenomethionine (Excential Selenium 4000, Orffa Additives BV):

1. Control: regular diet + 0.3 ppm inorganic selenium (sodium selenite)
2. Treatment: regular diet + 0.3 ppm organic selenium (L-selenomethionine)

In week 55, eggs were collected and incubated. In total, 180 chickens from each treatment (sodium selenite vs L-selenomethionine) were selected. The chickens originating from the different groups were divided over four different treatments, distributed in 24 pens with a randomized block design, leaving 6 replications per treatment.

Chickens from mothers supplemented with L-selenomethionine were fed two different diets:

- T1: Regular diet + 0.3 ppm selenium (L-selenomethionine) → org/org
- T3: Regular diet + 0.3 ppm selenium (sodium selenite) → org/inorg

Chickens from mothers supplemented with sodium selenite were fed two different diets:

- T2: Regular diet + 0.3 ppm selenium (L-selenomethionine) → inorg/org
- T4: Regular diet + 0.3 ppm selenium (sodium selenite) → inorg/inorg

Weight was recorded weekly per bird and feed consumption and mortality were recorded weekly per pen. The chickens were reared up to 42 days of age, after which one chick per pen was selected to evaluate drip loss and selenium deposition in the breast muscle.

The second study aimed to investigate the effects of L-selenomethionine on improving performance in broilers, whilst increasing stocking density. This trial included 1630 broiler chickens (Ross 308), divided over 6 treatments, 5 pens per treatment. Treatments included a negative control with sodium selenite (0.3 mg Se/kg feed) and a treatment group with L-selenomethionine (Excential Selenium 4000, Orffa Additives BV) (0.3 mg Se/kg feed). The two treatments were applied for 3 different stocking densities; standard stocking density (50 birds/pen or 29.84 kg/m^2), +10% stocking density (55 birds/pen or 32.84 kg/m^2) and +16% stocking density (58 birds/pen or 34.63 kg/m^2). The birds were all fed the same corn and soybean meal-based pelleted diet, with the only difference being the type of selenium. The trial lasted 35 days (starter-finisher) and production parameters were recorded during the trial; body weight, feed conversion ratio (FCR), feed intake and mortality. After slaughter, meat quality and carcass composition were determined. Data were analyzed using ANOVA and Duncan's multiple-range tests. Differences between treatments were tested for significance at a significance level of 5% ($P < 0.05$) and confidence interval of 95%.

III. RESULTS

During the first trial, no significant differences in weight were observed until 28 days of age (figure 1). From day 28 onwards, birds receiving T1 (org/org) showed significantly higher body weights ($P = 0.0001$) compared to birds receiving T4 (inorg/inorg). At 35 days, birds receiving T1 continued with a higher body weight compared to the other three treatments ($P = 0.0001$). At the end of the trial, day 42, birds receiving T1 still showed significantly ($P = 0.0001$) higher body weights compared to T2 and T4. Lower drip loss percentages (Table 1) were observed in the treatments that received organic selenium in the broiler diets ($P < 0.05$). It was shown that meat from birds receiving T1 (org/org) and 2 (inorg/org) had significantly lower drip loss percentages compared to meat from birds receiving T4 (inorg/inorg).

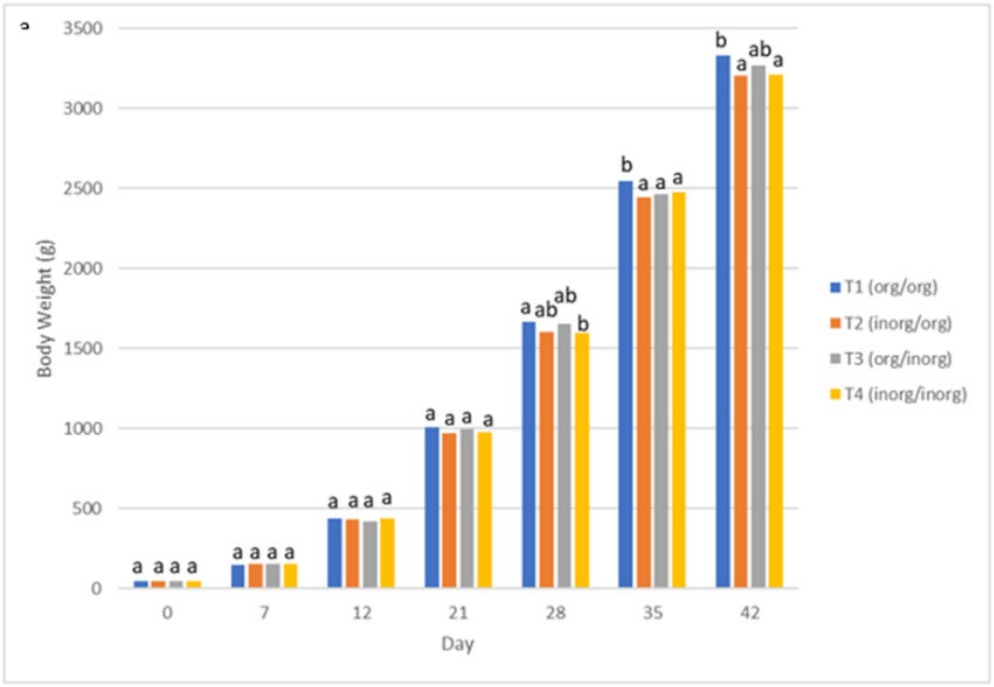

Figure 1 - Average body weight per treatment, letters (a, b) indicate significant differences (P < 0.05)

Table 1 - Results (Mortality, FCR, drip loss, selenium deposition).

	T1 (org/org)	T2 (inorg/org)	T3 (org/inorg)	T4 (inorg/inorg)
Mortality (%)	2	3	6	6
FCR	1.68	1.71	1.75	1.76
Drip loss (%)	1.04[a]	1.18[a]	1.67[ab]	2.02[b]
Selenium deposition breast muscle (mg/kg)	0.48	0.51	0.25	0.24

Letters (a, b) indicate significant differences (P < 0.05)

The second trial showed that replacement of sodium selenite with L-selenomethionine significantly increased body weight at all stocking densities applied (P = 0.002). Body weight uniformity (standard deviation of individual body weight in each pen / average body weight of the pen) was improved (P = 0.003), and FCR was significantly reduced (P = 0.04), for all stocking densities applied. L-selenomethionine decreased the shear force of breast meat from 4408.7 for sodium selenite to 3868.5 (P = 0.05).

Table 2 - Average results for sodium selenite and selenomethionine treatments (Body weight, FCR, body weight uniformity, shear force).

		P-value		
	Sodium selenite	L-SeMet	Selenium	Stocking density
Body weight (g)	2377.6[a]	2429.8[b]	0.002	0.32
Body weight uniformity (%)	85.32[a]	87.43[b]	0.003	0.93
FCR	1.35[b]	1.327[a]	0.04	0.23
Shear force (breast ; g)	3868.5[a]	4408.7[b]	0.05	0.92

Letters (a, b) indicate significant differences (P < 0.05)

IV. DISCUSSION

Body weight of broilers was shown to be the highest when theyreceived L-selenomethionine in their diet rather than sodium selenite, with optimal scores for chickens that also originated from mothers that were fed with L-selenomethionine. This indicates that supplementation with L-selenomethionine not only improves body weight in broilers, but also that it allows for long-lasting effects in chick progeny when fed to broiler breeders.

Drip loss percentage was reduced in broilers that received L-selenomethionine, again with best results in broilers originating from mothers also supplemented with L-selenomethionine. This indicates that L-selenomethionine allows for a greater reduction of oxidation reactions in the meat compared to sodium selenite, allowing for less drip loss.

Limitations of the current research include the lack of analysis regarding parameters related to fertility and hatchability, or enzymes related to redox-status and quality of the day-old-chicks. Future research could focus on investigating the effects of different selenium sources on those parameters.

In the second trial, it was shown that L-selenomethionine improved performance in broilers compared to sodium selenite, independent of the applied stocking density. Tenderness (shear force) and flock uniformity were also improved in the L-selenomethionine group.

In conclusion, regarding selenium supplementation in broiler breeders and broilers, L-selenomethionine allows for significant improvements in bird performance. The best results can be obtained when both broiler breeders, as well as broiler offspring, have a diet supplemented with L-selenomethionine.

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IMPORTANCE OF FIBRE AND FIBRE CHARACTERISTICS IN MODULATING FEED INTAKE IN BROILER BREEDERS

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Summary

An accurate management of feed intake is essential for the productivity of broiler breeders because it is the only way to control their body weight and ensure their reproductive performance. Currently, breeder feed intake is managed by limiting the amount of feed allocated, typically once a day feeding. There is no practical alternative to physical restriction but modulation of feed intake using nutrients, such as salt, tryptophan and fibre, has been explored. This review focuses and speculates on how fibre with specific physiochemical properties may be used to achieve gut fill and induce satiety in broiler breeders, which, in turn, helps moderate bird behaviour and alleviate welfare concerns.

I. INTRODUCTION

The significant genetic improvement to enable the modern-day broiler to achieve market weight by 30 days of age is due largely to the capacity of the bird to eat over 15% of its body weight on a daily basis. Such an extremely high growth rate, however, creates problems for the weight management of mature broiler hens and roosters, i.e., breeder birds, later in life.
Limiting feed intake and hence live weight, particularly during the rearing period, is essential for maximising the reproductive performance and longevity of broiler breeders (Leeson and Summers, 2000). But restricting nutrient intake without creating welfare and reproductive deficiency is not easy to manage. For instance, feed allocation has to be down to 20% of normal *ad libitum* feeding levels during 8 to 12 weeks of rearing (Riber and Tahamtani, 2020) to control body weight to 1.8kg at the point of lay around 25 weeks of age. Considering that the modern broiler can reach 1.8 kg at 30 days at *ad libitum* feeding, rearing and managing breeder birds poses difficult challenges.

The old practice of "skip a day" feeding is no longer an option commercially due to welfare concerns. Likewise, nutritional manipulation of feed intake and appetite suppression, for example, the inclusion of high levels of salt, sand and clay to depress and dilute feed intake or high tryptophan levels to increase serotonin to reduce aggression, particularly during rearing, is not commonly used. This is because individual birds react very differently to nutrient deficiencies and excesses and these practices create variation in egg production and weight uniformity, causing significant losses of settable eggs.

Another potential way to reduce feed intake is to use fibre (Nielsen et al., 2011; Hocking et al. 2004). Fibre can also modulate behaviour and change cannibalism outcomes in laying hens (Hartini et al., 2002; Hetland et al., 2004). There is significant evidence that dietary fibre, which is the sum of non-starch polysaccharides (NSP) and lignin, influences behaviour and satiety in pigs (de Leeuw et al., 2008). However, what is lacking is the characterisation of the particular forms and types of soluble and insoluble NSP that modulate voluntary feed intake as well as the sources of such fibre for use in commercial feed. This paper will describe dietary fibre within feed context, from chemical, physical and functional points of view and speculate on how certain NSP entities can be used to manage intake in broiler breeders without affecting egg production and hatchability.

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II. THE COMPLEXITY OF FIBRE

The word "fibre" is highly confusing to the nutritionist because: a) there are multiple terms that describe fibre in feed, and b) fibre is not set as a nutrient in feed formulation yet it is probably the single largest source of variation in the nutrient composition of feed. For instance, the Crude Fibre (CF) only captures a variable proportion of cellulose, hemicellulose, pectin and lignin, the four components of fibre (Figure 1; Choct, 2015). For some important vegetable ingredients, CF represents less than 20% of the total dietary fibre (DF) content (soybean meal: CF vs DF, 5 vs 25%; rapeseed meal, 14 vs 40%). Other fibre measures such as Acid Detergent Fibre (ADF) and Neutral Detergent Fibre (NDF) were introduced in the 1960s but both ADF and NDF still do not represent the total DF content of many ingredients, especially in vegetable proteins, because both ADF and NDF fail to account for most pectic polysaccharides and some of the soluble hemicelluloses present in these ingredients. Thus, we strongly advise nutritionists to use the total DF levels in feed formulation to account for all the fibre present in poultry feed. Regrettably, a comprehensive database on feed DF contents is yet to be widely available for nutritionists to use.

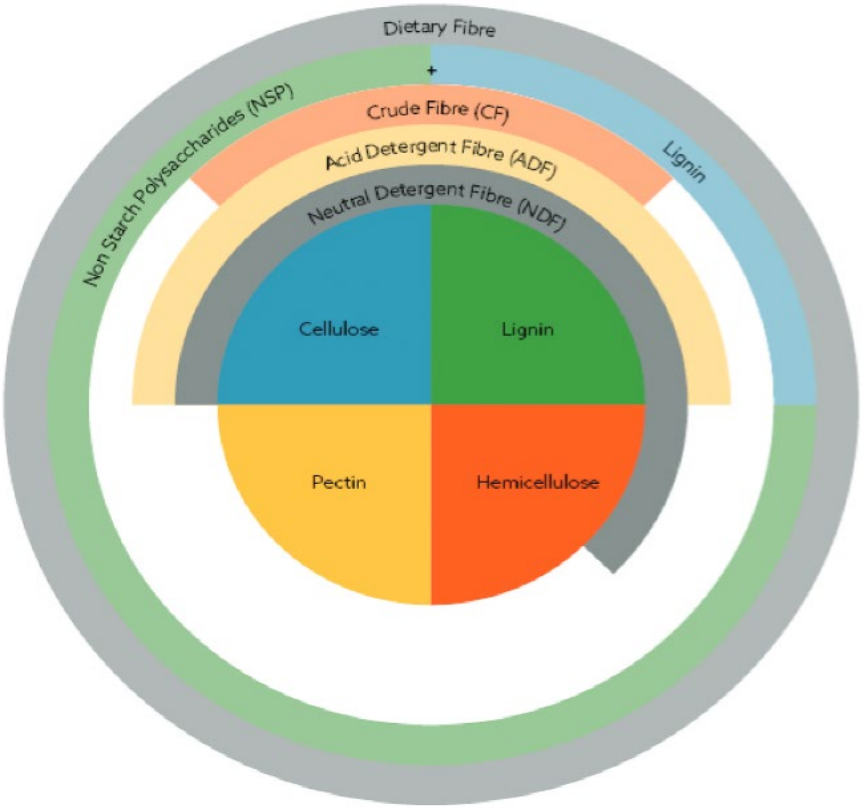

Figure 1 - Fibre composition. Adapted from Choct, 2015.

Knowing the total level of DF is only the first step towards understanding the nutritional roles of fibre in poultry diets, which depend on the functionality of the various chemical structures that make up fibre. These include the chemical characteristics (monosaccharide composition of a polysaccharide, the linkage type, degree of branching, molecular weight, cross-links, chemical structure), and physical properties (solubility, degree of crystallinity, ability to swell etc). These physicochemical properties determine the functions of a fibre entity in terms of its ability to: a) hold water in its matrix and swell in the foregut where some fermentation may occur; b) be retained in the gizzard to enhance its functions, such as grinding of food particles to a specific size and controlling substrate release into the small intestine (acts

as a pacemaker organ for nutrient digestion); c) regulate intestinal digesta transit time through increasing viscosity, and d) be susceptible to supplemental enzymes to release small molecular weight carbohydrates that can either be fermented or act as prebiotics to promote gut health.

There is a strong relationship between solubility and fermentability of DF, but the fermentability does not solely depend on solubility because some insoluble NSP entities are also fermentable depending on their molecular structures, sugar type and position in the cell wall architecture. Likewise, water holding capacity (WHC) of fibre also does not rely on solubility of the NSP. We will draw upon a few examples to illustrate how specific fractions of fibre can be used as a functional nutrient which alters the physiology of digesta movement and dynamics in the gut.

III. MODULATING GUT HEALTH, NUTRITION AND FEED INTAKE USING SPECIFIC FIBRE

a) Slowing Down Digesta Transit And Promoting Feed Retention In The Foregut

The size, structure and association with lignin all determine the solubility of dietary fibre and the properties which can either benefit or create negative effects in the gut of the bird. For instance, highly branched soluble arabinoxylans and beta-glucans can markedly elevate gut viscosity, leading to reduced nutrient digestion and absorption (Choct et al., 1992). There is also a link between feeding viscous grains and prevalence of necrotic enteritis outbreaks in broilers (Kalhusda1& Skjerve, 1996). Depressed feed intake followed by poor growth and wet droppings is often associated with high gut viscosity in broiler chickens. This function of soluble NSP, therefore, is worthy of exploring in breeder birds. Soluble NSP, especially the high molecular weight polymers, can certainly slow down digesta transit rate once solubilised (Annison, 1993). However, there is question as to whether enough solubilisation can occur in the foregut of chickens to increase crop content, considering that these NSP are well embedded in the cell wall matrix of feed ingredients and it requires some time for them to be processed and solubilised. Increased contents in the crop and gizzard may help birds to slow down feed intake, an effect that would alleviate breeder birds eating too fast, leading to an increased incidence of choking. In a recent study, Kim et al. (2022) compared the flow of NSP along the GIT of broiler chickens using a corn-soy diet and a wheat-soy diet. The accumulation of NSP along the gut differed widely between the diets. One noticeable difference was the accumulation of NSP in the gizzard of birds fed the corn-soy diet was highly pronounced and the main NSP fraction was pectin. Corn is significantly lower in protein than wheat and therefore a corn-soy diet contains larger levels of soybean meal and hence a higher level of pectin. But this difference diminished as the digesta moved down the GIT, suggesting that the pectin was increasingly fermented. This strongly suggests that the effects of pectin in influencing satiety and gut health are mostly occurring in the foregut.

The modern feeding practices focus on increasing feed intake and hence growth rate of the bird. This means, feed is formulated to give the best balance of nutrients in the most digestible manner. This is, however, not conducive to foregut development unless the feed contains certain degree of structural coarseness to stimulate crop holding and gizzard grinding. Indeed, the use of whole grain or fibre additives has become common practice today to promote gut health in poultry. Good gut health is optimised when the gut microbiota is modulated to maximise intestinal integrity, reduce inflammation and increase nutrient utilisation and absorption. Although physical structure of feed and fibre additives is often regarded as an obvious requirement for modulating gut development (Svihus, 2011), there is evidence that certain types of fibre additives can impart beneficial effects on gut health even when they are finely ground (Farran et al., 2017). Thus, it is important to understand the types of fibre that promote foregut development without relying solely on their physical structures.

b) Fibre and Satiety

Some fibre entities induce satiety by the distension of the crop, gizzard and, to a lesser extent, the small intestine and caeca, through promoting the production of satiety-inducing hormones like cholecystokinin (CCK), Glucagon Like Peptide 1 (GLP1) Glucagon Like Peptide 2 (GLP2), Peptide YY (PYY) and ghrelin (Bodnaruc et al, 2016).

Firstly, fibre sources rich in soluble NSP, such as pectins and beta-glucans, but low in lignin can exhibit medium to high affinity to absorb water and swell in the upper gastro-intestinal (GI) tract. The swelling or distension of the crop and gizzard stimulates the vagus nerve, sending satiety signals directly to the brain and reducing feed intake (Maljaars et al., 2007). By the time the fibre reaches the distal small intestine and caeca, 90% of it may be fermented, causing distension and triggering the release of GLP1, GLP, PYY and ghrelin. This also signals the brain to reduce feed intake and activates the ileal brake, which slows down the emptying of the stomach and reduces the rate at which feed moves through the small intestine in pigs (Ratanpaul et al., 2019). By delaying the emptying of the stomach and slowing down the digestion and absorption of nutrients in the small intestine, the ileal brake helps to regulate feed intake as well.

Secondly, fibre sources rich in insoluble NSP, such as insoluble arabinoxylan (e.g., from wheat bran) and lignified cellulose, are poorly fermented and maintain their physical structures down the GI tract. Such NSP, however, have a large capacity to absorb and hold water, with some forms of lignified cellulose having rapid rates of water absorption and holding up to 800 times their molecular weights in water. This also creates significant extension of the crop and gizzard as well as in the small intestine and caeca. The effect of gut fill may trigger negative feedback on the vagus nerve (Maljaars et al., 2007) and through the hormonal regulation as described earlier, inducing satiety.

Four main modes of action by which fibre induces satiety are postulated below:

i. Swelling and retention of digesta in the crop, gizzard and small intestine by ingredients high in fibre and WHC.
ii. Coarse physical structure such as whole wheat and addition of structural components (fibre high in lignocellulose content), that maximises gizzard function and fulness.
iii. Highly fermentable fibre (e.g., soluble pectins) producing a copious amount of gaseous end-products such as volatile fatty acids, causing distension of the crop, lower small intestine and the caeca.
iv. Distention of the upper and lower GI tract, plus the increased level of fermentation in the distal intestine and caeca increase, triggering the ileal brake and increased gut hormone release that induces satiety.

c) Water Holding Capacity

The classification of different sources of fibre based on their ability to hold water is not a new concept as it has been used to a limited degree in pig nutrition of over the past 25 years (Tsaras et al., 1998). Initially WHC in pig diets was seen as a negative trait which limited pig growth performance unless finishing pigs were depositing too much carcass fat in which WHC would be increased to restrict energy intake.

The use of minimum levels of WHC in poultry breeder diets is a relatively new concept that has high potential to improve satiety, welfare and reproductive performance of rearing birds as well as in mature egg producing birds. Table 1 shows the variation in WHC of common feed ingredients compared to specific dietary fibre components. While raw materials high in NDF and pectins tend to exhibit high WHC, there is no strong relationship with one particular dietary fibre fraction. This is not surprising as illustrated earlier in this review, chemical

structures alone cannot characterise the functions of fibre because of the complexity of physical properties of NSP and cell wall architecture of fibre in feed ingredients. It is also important to understand that each type of NSP, for instance, pectins, arabinoxylans and beta-glucans, represents a myriad of polysaccharides with the same name, differing in molecular weights, cross-links, side-chains and 3-D configurations. So, it is just names and does not convey a great deal of their functions. For instance, pectins isolated from sugar beet pulp (SBP) are soluble and they can hold up to 900% their own weight in water (Stephen and Cummings, 1979). SBP, as a potential ingredient for breeder diets, contains a very high level of soluble pectins and a negligible amount of lignin. It can be rapidly fermented into volatile fatty acids and other gaseous products in the presence of microbes in the distal ileum and caeca. Once the SBP is fermented it losses it WHC instantly. Another by-product readily available to the poultry industry is soybean hulls (SBH). Soybean hulls, whilst having a similar total fibre level to SBP, their WHC is 35-40% lower than SBP. The reason is, the NSP in SBH, despite containing an appreciable level of pectins, are not very fermentable nor soluble. Rapidly fermentable fibre can also lose its WHC once its physical structure is destroyed through fermentation, enzymatic degradation and treatments such as acid hydrolysis and mechanical processing. Thus, the degree of solubility of NSP, the main part of fibre, is likely to be directly related to their fermentability in the lower section of the gut in poultry.

Some ingredients contain a high amount of insoluble NSP in close association with lignin. These ingredients can hold high amounts of water (WHC 4-10%), in the meantime, the lignocellulose-dominated fibre in them remain largely intact in the distal gut and in the excreta. These types of fibre can maintain their beneficial effect on satiety throughout the gut (Sacranie et al., 2012), nutrient digestion and absorption (van Krimpen et al., 2008) and litter quality (Röhe & Zentek, 2021). The sugarcane co-product bagasse is also similar in water holding capacity to processed lignocellulose products once the waxy outer leaves are taken away. Bagasse has been reported to improve broiler performance (Kheravii et al, 2017), but its extremely low bulk density can be very hard to handle in the Feedmill. Wheat bran, canola and specially processed lignocellulose fibre additives sourced from softwoods belong to this group of ingredients that maintain their WHC throughout the gut in poultry. These ingredients are becoming increasingly popular in broiler breeder diets.

Table 1 - Water holding capacity of various raw materials compared to factor dietary fractions (adapted from the Premier Atlas ingredients matrix 2019; Stephen and Cummings, 1979).

Ingredients	Total DF %	WHC kg/kg	NDF %	Lignin %	Pectin %
Wheat	12.7	1.5	10	1.0	0.4
Sorghum	8.8	1.6	8.0	1.1	1.7
Barley	18.1	2.3	21.0	2.5	1.4
Soybean 48%	21.6	3.6	8.4	1.4	8.2
Canola meal 36%	36.2	3.4	27.0	7.0	9.9
Millrun (wheat bran/pollard	39.2	4.0	34.0	6.0	1.2
Wheat bran	60.1	5.0	45.0	7.0	1.5
Soybean hulls	66.5	5.0	58.0	1.5	21.5
Dried grass pellets	86.1	6.0	48.0	8.0	3.0
Sugar cane bagasse	89.5	7-8	62.0	11.2	0
Modified lignocellulose	89.0	7-10	70.0	8.0	1
Sugar beat pulp	76.9	6-10	37.0	1.9	30
Pure pectin	85.7	56.2	0	0	98

d) *In Situ* Production of Prebiotics to Prime the Gut Microbiota

The majority of broiler pullet and breeder diets contain an NSP-degrading enzyme, like xylanase and/or beta-glucanase as well as phytase. The benefits of these enzymes are well known to improve energy and nutrient digestibility, leading to improved performance. The added benefit of using xylanase and phytase in breeder feed is that they also significantly reduce the anti-nutritional effects that NSP and phytate can have, such as gut viscosity, osmotic balance, excessive exogenous secretions, proliferation of pathogens and poor litter quality.

One point of note is insoluble NSP from wheat bran can be a source of anti-nutritive soluble arabinoxylans once consumed and residing in the crop. A combination of hydration, natural microbial activity and lower pH can reduce the molecular size of the NSP, leading to the release of soluble NSP (Cadogan et al., 2003) or xylooligosaccharides, XOS (Morgan et al., 2020) depending on the type of xylanase added. The latter usually results from using endoxylanases, which are the most common type of xylanase used in pig and poultry feed today. XOS are known to be a highly effective prebiotic that encourages the growth of beneficial organisms. There is competition within the microbiota, whereas the host has evolved to keep the microbial ecosystem on a leash (Foster et al., 2017). What this implies is, under normal circumstances, the host and the microbiota exist in a symbiotic relationship with the microbiota often providing numerous benefits to the host. The advent of biotechnology makes it possible to intentionally manipulate a given species of the microbiota to enhance nutrient provision and energy supply, modulate immunity, prevent pathogen colonisation and improve gut functions. It is speculated that the earlier this process starts, the more beneficial the gut microbiota may be. For a long-life bird like a breeder hen, the early provision of prebiotics via *in situ* production may prime the microbiota to induce long-lasting changes.

Therefore, the benefits of adding higher levels of functional dietary fibre from wheat bran, barley or oat offal, rich in insoluble arabinoxylans and beta-glucans, can be enhanced if appropriate enzymes targeting the specific NSP entities are properly considered. Hence the role of NSP and phytate hydrolysing enzymes play a crucial role in maximising the benefits of functional fibre (Choct, 2015).

IV. THOUGHTS ON PRACTICAL USE OF FIBRE IN BREEDER DIETS

The poultry industry's challenge of addressing welfare concerns in breeder birds demands thinking outside the box and hence this review. It is not a new topic nor a new concept that nutritional modulation of bird behaviour through managing satiety is a worthy yet difficult area of research and practice.

Pecking and scavenging for food is an innate activity of birds that occupy the majority of their daylight time. However, in the modern poultry production, broiler breeders are allowed once a day feeding of a set amount of feed to control their body weight and maintain their reproductive performance. Thus, these birds spend a lot of time in a constrained space, getting frustrated and bored. As a consequence, several stereotypic behaviours, like pecking at drinker lines, overconsumption of water, and feather licking, occur (Hocking et al., 2004). At the highest feed restriction during rearing (e.g., 20% of *ad libitum* feed intake), it induces higher levels of stress and can cause cannibalism and aggression between birds. Increased spot pecking has been described as a coping mechanism for stress and birds under duress (Savory et al., 1993; Hocking at el., 1997). Hocking et al. (2004) found that either the addition of 5% SBP (soluble NSP) or 20% oat hulls (insoluble NSP) significantly reduced spot pecking and associated skin damage. This was associated with high wet weight of digesta in the crop and small intestine. Hartini et al. (2002) observed a significant reduction in cannibalism in layer hens when a high level of millrun (2/3 wheat bran, 1/3 wheat pollard) was included in the diet and suggested the beneficial nature of insoluble fibre in preventing aggressive pecking.

Moreover, the addition of 5% SBP significantly improved satiety in rearing and in broiler breeders and most measured welfare indicators (Hocking et al., 2004). The only reduction in welfare by offering SBP was a significant worsening in litter quality, which may have resulted from pectins being rapidly fermented in the distal gut releasing the previously bound water.

These studies, however, were unable to provide information on the physicochemical properties of the individual fibre fractions used, which makes it difficult to draw a clear conclusion about the types of fibre that may be most effective in modulating behaviour in breeder birds. But it could be inferred from the literature as well as our knowledge on various fibre entities presented in commonly used feed ingredients that it is possible to maximise satiety in rearing birds and broiler breeders through the use of adequate amounts of functional fibre high in NDF and WHC. Whilst more research is required to ascertain the minimum level of WHC and its relationship with the possible unwanted consequences on litter quality (eg., too much pectin and other soluble fibre) and faecal bulk, the literature suggests a mimimum of 2.5% WHC in mid to late rearer diets and a minimum of 2% for broiler breeder diets may be required to have an effect on satiety (Nielsen et al., 2011) to significantly increase breeder welfare and subequent performance. To insure there is enough structural fibre to enhance and optimise gizzard size and function, a minimum of 13% NDF and/or 15% total DF is recommended.

V. CONCLUSIONS

A great deal of work on fibre and its quantification, chemical structures and physical properties has occurred over the past three decades together with some in-depth investigation into the functional attributes of various fibre entities in gut health, nutrition, welfare and physiology in pigs and poultry. It is proposed whether certain types of fibre, not just any fibre, may be more effective in inducing satiety in birds, resulting in behavioural modification and better welfare outcomes.

Significant restrictions in nutrient intake for pullet rearing and broiler breeders is to optimise body weights and such a measure is essential to maximise the yield of settable ages and longevity of the bird. Using the types of fibre that have high WHC but have limited fermentable capacity in the hindgut may offer benefits in breeder birds by inducing satiety, moderating hunger and maintaining production performance.

This mini review has analysed the literature on the use of fibre as a tool to control feed intake and modulate behaviour in breeder chickens. A much deeper understanding of the types of fibre and setting minimum and maximum levels in diet formulation will only follow a comprehensive dietary fibre database together with a clear elucidation of the types of fibre present in various ingredients is widely available to the industry.

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EFFECT OF TRANSLUCENCY AND EGGSHELL COLOUR ON BROILER BREEDER EGG HATCHABILITY AND HATCH CHICK WEIGHT

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Summary

The aim of the current study was to describe the effects of shell translucency and colouration lightness (L* value) on shell thickness, hatchability, and chick weight. A total of 4320 eggs from four commercial Ross 708 breeder flocks (50 to 55-wk old) were selected for Translucency Score (TS) and L* value. A 3-point subjective scoring system was used for TS (1 = low, 2 = medium, 3 = high), and an electronic colorimeter for L* value, sorting the eggs as light (avg. L* = 80.7) or dark (avg. L* = 76.0). Results suggest that the colour of the eggshell was related to the egg weight at collection day (P = 0.006) and at transfer (P = 0.021), in both cases dark eggs were 0.6 g heavier than light eggs. Dark eggs had a 3.8% higher hatchability of egg set (P = 0.048) and yielded 6 µm thicker shells (P = 0.0019) when compared to light eggs. Egg weight at transfer was 0.8 g heavier for TS1 eggs compared to TS3 (P = 0.0358). The TS1 eggs had a 6.9% higher hatchability of set eggs (P = 0.013) and 0.7 g heavier chick weight (P = 0.038) compared to TS3. However, TS1 eggs had shells 28 µm thinner than the TS2 and 34 µm thinner than TS3 (P < 0.0001). An interaction effect was observed for eggshell thickness, L* value, and TS, where light colour eggs with TS1 had thinner shells compared to those that were dark with TS3 (P = 0.029). These results suggest that eggshell translucency and colouration lightness may be used as reliable noninvasive indicators of eggshell thickness, hatchability, and chick weight.

I. INTRODUCTION

The eggshell functions as a resistance barrier protecting egg's internal content from environmental hazards, allowing the proper embryo development during incubation. Egg quality parameters normally measured are specific gravity, vapor water conductance, weight, thickness, porosity, breaking strength, elastic modulus, static and dynamic stiffness, among others (McDaniel et al., 1979; King'ori, 2011; Liao et al., 2013). However, several of these quality measurements are destructive to the egg and require lengthy processes, whilst parameters such as shell translucency and colour don't require destruction of the egg and may be easier to measure. Shell translucency is described as a mottled appearance spotted in different sizes and shapes observed when candling eggs (Baker and Curtiss, 1957), and its generation is suggested to be caused by moisture accumulation in the shell and uneven drying after the egg is laid, leaving opaque and translucent areas (Talbot and Tyler, 1974). Eggshell colour has been significantly related to eggshell quality parameters (Sekeroglu and Duman, 2011).The aim of this project was to describe the effects of eggshell translucency and colouration intensity (dark and light) on eggshell thickness, hatchability, and chick weight.

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II. MATERIALS AND METHODS

A total of 4320 eggs from Ross 708 breeder hens between 50 and 55 weeks of age from a commercial hatchery were used. Eggs were collected over 4 consecutive days from different flocks each day (1080 eggs/d) and stored for 4 to 6 d at 15 °C and 70% relative humidity, prior sorting.

Pre-incubation and incubation measurements - Each day, 1080 eggs were categorized using the Zinpro® BlueBox™ methodology, which consists of classifying each egg with one of three Translucency Scores (TS1 = low, TS2 = medium and TS3 = high). The 3-point scoring system takes into consideration the amount, size and coverage of spot patterns or mottling areas in the eggshell (Figure 1). After scoring, colouration lightness (L* value) was evaluated using an electronic colorimeter (Nix Colour Sensor Pro2), sorting the eggs as light or dark and placed in a total of twelve 90-egg-incubator-trays. Eggshell thickness was determined using a non-invasive ultrasound gauge (Eggshell Thickness Gauge by Egg Tester). The average egg weight per tray was the initial egg weight prior to incubation. Eggs were set in 4 identical single stage incubators (Nature Form, model NMC 1080) with capacity of 1080 eggs. Relative humidity and temperature were maintained constant during incubation (37.7 °C and 55% relative humidity) and eggs were turned every hour.

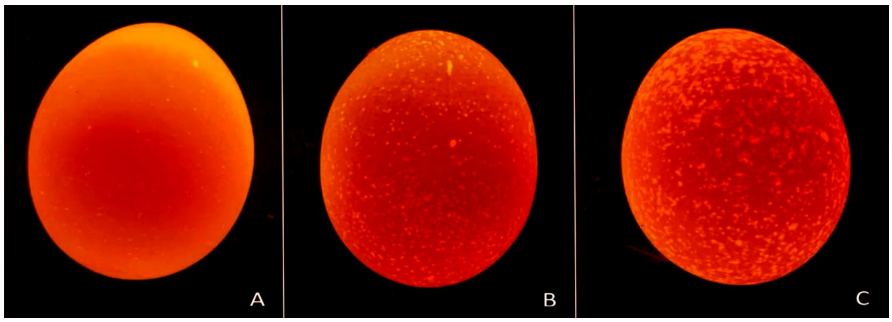

Figure 1 - Grades of eggshell Translucency Score. (A) TS1, (B) TS2, (C) TS3.

Egg transfer and post-hatch parameters - At d-18 of incubation, all eggs were candled to remove eggs that appeared to be infertile or with dead embryos. These eggs were then cracked to confirm the infertility and embryonic mortality by visual examination and counted for calculation of egg loss along with the cracked, contaminated, and exploded eggs. The fertile eggs placed in trays were weighed for the calculation of egg weight at transfer. Egg weight loss was calculated by the subtraction of transfer egg weight from the initial egg weight and then divided by the initial egg weight. Eggs were then transferred to hatching baskets and placed back into the same incubators at the same temperature and relative humidity. Hatchability was calculated based on the number of eggs hatched from the total of eggs set. Hatched chicks were weighed as an average of chicks per basket. Unhatched eggs were opened to visually confirm embryonic mortality and chicks that hatched but were weak and near death were culled and counted to calculate the unhatched+culls % based on the number of eggs set. Data of translucency and eggshell colour effects on initial and transferred egg weight, % water loss, % hatchability, % unhatched + culls, eggshell thickness, and chick weight was analyzed using the GLIMMIX procedure of SAS (V 9.4) and Tukey's HSD test was performed to separate means. Significant difference was considered between the means when $P < 0.05$.

III. RESULTS

The effects of eggshell translucency and eggshell colour are summarized in Tables 1 and 2, respectively. An interaction between colour and translucency was observed only for eggshell

thickness (P = 0.029), where eggs classified as light-colored and with TS1 had a thinner eggshell compared to those that were dark and had a TS3 egg.

Table 1 - Influence of translucency on initial egg weight, final egg weight, water loss %, egg loss %, hatchability, unhatched + culls %, chick weight and eggshell thickness.

	Translucency				
Parameter	TS1	TS2	TS3	SE	*P value*
Initial egg weight (g)	66.87	66.90	66.51	0.34	0.1962
Transfer egg weight (g)	60.74[a]	60.55[ab]	59.92[b]	0.46	0.0358
Water loss (%)	9.18	9.50	9.92	0.35	0.1188
Egg loss (%)	17.53[b]	19.29[ab]	23.32[a]	2.85	0.0282
Hatchability (% eggs set)	78.14[a]	75.83[ab]	71.23[b]	3.31	0.0127
Unhatched + culls (%)	4.33	4.95	5.45	0.71	0.3550
Chick weight (g)	45.79[a]	45.43[ab]	45.10[b]	0.64	0.0385
Eggshell thickness (µm)	393.8[c]	422.4[b]	427.6[a]	2.55	< 0.001

[abc] Different superscript letters represent statistically significant differences (P < 0.05) within rows.
TS1 = Translucency score of 1; TS2 = Translucency score of 2; TS3 = Translucency score of 3; SE = Standard deviation.

Table 2 - Influence of eggshell colour on initial egg weight, final egg weight, water loss %, egg loss %, hatchability, unhatched + culls %, chick weight and eggshell thickness.

	Colour			
Parameter	Dark	Light	SE	*P value*
Initial egg weight (g)	67.05[a]	66.49[b]	0.32	0.0056
Transfer egg weight (g)	60.72[a]	60.09[b]	0.44	0.0211
Water loss (%)	9.44	9.62	0.29	0.5389
Egg loss (%)	19.43	20.66	2.71	0.4828
Hatchability (% eggs set)	76.94[a]	73.19[b]	0.18	0.0481
Unhatched + culls (%)	3.68[b]	6.15[a]	0.64	0.0003
Chick weight (g)	45.53	45.35	0.63	0.4087
Eggshell thickness (µm)	417.5[a]	411.7[b]	2.40	0.0019

[ab] Different superscript letters represent statistically significant differences (P < 0.05) within rows.
SE = Standard error

IV. DISCUSSION

According to Liao et al. (2013), the length of the mammillary layer and width of mammillary cones are positively correlated with eggshell thickness. Chousalkar et al. (2010) observed that translucent eggshells have changes primarily in their mammillary layer and cones, suggesting that the increased thickness of the whole shell of the highly translucent eggs is caused predominantly by increasing the width of the mammillary cones, which leads to a higher mammillary layer ultrastructure. In this study, the thinner eggshells had the highest hatchability. The differences could be attributed to better uniform shell over the entire egg, which causes a greater strength of the eggs as suggested by Yan et al. (2014), who found that eggs with thin and uniform shells are stronger than those with thick yet less uniform shells.

Research has suggested that loss of weight during incubation could be attributed to water vapor exchange that can be influenced by eggshell porosity (Sousa de Araujo et al., 2017) and thickness (Roque and Soares, 1994) as these authors found that thinner shells can lose more weight during incubation. This is contrary to our observations, as we found that eggs with thicker shells, but high translucency lost more weight during incubation than eggs with thin shells. Translucent eggs have been reported to have thinner inner membranes, indicating reduced toughness and elasticity, and less protection to the egg content and embryo (Wang et al., 2017), which also negatively affects the flow of gases through the shell. The translucency impacted the % egg losses to d-18 of incubation, showing 5.8% higher egg losses in TS3

compared to TS1 eggs, and causes of embryonic death in translucent eggs could be related to poor resistance to water loss, altered embryo respiration rate and higher susceptibility to bacterial contamination. The TS1 eggs had a 6.9% higher hatchability of eggs set and greater chick weight in comparison to eggs with a TS3, agreeing with Burin et al. (2023) that reported hatch of fertile eggs were significantly impacted by translucency ($P < 0.0001$) with hatchability for TS 1, 2 and 3 being 92.3%, 91.4% and 86.3%, respectively.

In this study, dark-colored eggs had 3.8% higher hatchability, which agrees with previous research (Baylan et al., 2017), whom also reported that darker eggshells from broiler breeders have been related to a higher maternal antibody content in the yolks. Dark-colored eggs also had a thicker eggshell in this study, and as shell pigmentation and the calcification process are interrelated, with a significant deposit of pigment causing an increase in calcium deposition in the eggshell (Lang and Wells, 1987), which may explain why darker-colored eggs are thicker. The interaction between colour and translucency is consistent with the effect of translucency and colour on thickness when evaluated independently. Our study suggests that thickness can be best estimated by considering both translucency and colour of the eggshell, although these factors could be influenced by variation between flocks due to factors such as different farm management, environmental challenges, age, nutrition and vaccinations.

V. CONCLUSION

In conclusion, high translucent eggs (TS3) had reduced hatchability and day-old chick weight in comparison to TS1 and TS2 whereas high translucent eggs had the thickest eggshells. Regarding the impact of colour lightness, greater values for thickness and hatchability were found in dark-colored eggs. The interaction of both translucency and colour lightness only impacted shell thickness. These results suggested that eggshell translucency and colouration lightness may be good noninvasive indicators of eggshell thickness, hatchability, and chick weight in breeder flocks.

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EARLY IMMUNE FUNCTION IN THE SMALL INTESTINE IS MODULATED BY IN OVO CARVACROL DELIVERY IN BROILER CHICKS

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The phytochemical carvacrol has anti-inflammatory properties in the small intestine, which harbours the gut-associated lymphoid tissue (GALT) and may reduce the impact of enteric diseases in broiler chicks (Liu et al., 2019). In ovo delivery of carvacrol could influence early GALT development. This study aimed to explore how carvacrol affects GALT in the jejunum of broiler chicks around hatching. It was hypothesised that in ovo carvacrol could improve GALT development by influencing cytokine and immunoglobulin mRNA expression.

Jejunal samples were collected on embryonic day (E)19.5, day (d)0 and d14, (n = 10/treatment/d) to study the impact of two in ovo treatments: either 1mL of (1) 0.9% saline or (2) carvacrol (0.5% v/v) with polysorbate 80 (1:1 v/v) in 0.9% saline. Treatments were administered into the amniotic fluid of fertile Ross 308 eggs at E17.5. Relative mRNA expressions of IL1β, IL4, IL8, IL10, IFNγ, TNFα, TGFβ, NFκB, IgM, IgY and IgA were measured by qRT-PCR. Data were analysed using PROC MIXED (SAS 9.4).

Table 1 - Relative mRNA expression (fold change, FC) of immune-related genes in the jejunum, showing the main effects of in ovo treatment (saline or carvacrol) and age (E19.5, d0 and d14).

Treatment	Age	IL1β	IL4	IL8	IL10	IFNγ	TNFα	TGFβ	NFκB	IgM	IgY	IgA
Control		0.84^{b}	0.94	4.78	1.09^{b}	0.93	2.60	0.65	0.73^{b}	15.35^{a}	2.47	32.52
Carvacrol		1.31^{a}	1.14	5.25	1.46^{a}	1.10	2.72	0.62	0.84^{a}	10.40^{b}	1.55	28.24
SEM		0.13	0.24	1.20	0.14	0.15	0.13	0.03	0.02	4.51	0.49	13.57
	E19.5	1.54^{a}	2.05^{a}	1.13^{b}	1.70	0.98^{a}	1.11^{c}	1.05^{a}	1.12^{a}	0.94^{b}	1.35^{b}	1.35^{b}
	d0	0.87^{b}	0.29^{b}	6.30^{a}	0.99	0.60^{b}	2.07^{b}	0.41^{b}	0.76^{b}	0.51^{b}	0.37^{c}	0.38^{c}
	d14	0.81^{b}	0.77^{b}	7.62^{a}	1.02	1.47^{a}	4.80^{a}	0.43^{b}	0.48^{c}	37.17^{a}	4.30^{a}	89.41^{a}
	SEM	0.15	0.29	1.47	0.17	0.18	0.16	0.04	0.03	5.53	0.60	16.62
P-value												
Treatment		0.04	0.80	0.86	0.02	0.37	0.33	0.16	0.02	0.008	0.45	0.76
Age		<.001	<.001	<.001	0.08	<.001	<.001	<.001	<.001	<.001	<.001	<.001
Treatment x Age		0.19	0.76	0.92	0.49	0.41	0.78	0.17	0.57	0.86	0.16	0.38

E: embryonic day; d: day; a,b,c Means within a factor lacking a common superscript differ ($P \leq 0.05$).

There were no treatment and age interaction effects, but main effects were observed for relative mRNA expression of cytokines and immunoglobulins. Carvacrol increased the expression of pro-inflammatory cytokine IL1β and mediator NFκB, and anti-inflammatory cytokine IL10. Carvacrol decreased expression of IgM, the first antibody to appear in response to pathogens, indicative of suppressed immune function. Immune-related gene expression fluctuated over time independent of in ovo treatment. During late embryonic development (E19.5), genes with highest expression were IL1β, IL4, NFκB and TGFβ. On d14, expression of IL8, TNFα and IgM, IgY and IgA was highest. IFNγ decreased at d0 but rebounded at d14.

In conclusion, in ovo carvacrol delivery led to increased cytokine and immune mediator expressions, while reducing primary antibody responder IgM. The increased expression of IgM, IgY, and IgA on d14 may indicate a more functional and protective immune system.

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IN OVO DELIVERY OF ESSENTIAL OILS HAS THE POTENTIAL TO AFFECT GLUTAMATE TRANSPORT AND CARBOHYDRASE ACTIVITY IN THE JEJUNUM OF BROILER HATCHLINGS

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Essential oils (EOs) are secondary plant metabolites, some of which can stimulate feed intake, and digestion. The capacity of the small intestine to digest and absorb nutrients early in the life of the chick depends on an adequate embryonic development of the villi and crypts and overall, the integrity and functional properties of the epithelia including the expression and abundance of digestive enzymes and nutrient sensors and transporters. Some key transporters include the sodium-glucose transporter 1 (SGLT1), the excitatory amino acid transporter 3 (EAAT3), the peptide transporter 1 (PEPT1), and the fatty acid binding protein 1 (FABP1). Moreover, amino peptidase-N (APN) regulates catabolic enzyme digesting peptides, which release amino acids, while sucrase-isomaltase (SI) regulates a glycoprotein that plays an important role in the final degradation of carbohydrates in chickens. This study investigated the impact of *in ovo* injection of EOs on these nutrient transporters and digestive enzymes at hatch. We hypothesized that *in ovo* injection of EOs would improve the capacity of the small intestine to digest and absorb nutrients during the late stages of embryonic development in broiler chickens.

The study included 28 treatments (saline and 27 different EOs) selected based on their activity reported in the literature (Brenes & Roura, 2010). The eggs (24/treatment) were incubated under standard conditions. On embryonic day 17.5, 1 mL EO solution (5 µL EO+5 µL polysorbate-80+990 µL saline) was injected into the amnion. At hatch, jejunum samples from six hatchlings per treatment were collected for testing the expression of digestive enzymes APN and SI, and nutrient transporters SGLT1, EAAT3, PEPT1, and FABP1. Total RNA was extracted using RNeasy Mini Kit, QIAGEN. RNA quantity and purity were determined using a NanoDrop ND-8000 (Thermo Fisher, USA). The isolated RNA of each sample was reverse transcribed with the QuantiTect Reverse Transcription Kit. Quantitative PCR was performed using a SYBR Green with ABI QuantStudio 6 real-time PCR. The General Linear Model procedure in SAS 9.4 was used to compare the EO treatments with the control saline-injected group. Tukey test was applied for the multiple comparison and $P < 0.05$ was set as the threshold for significant differences.

The results showed that *in ovo* injection of spearmint, patchouli, and turmeric EOs significantly ($P < 0.05$) reduced the expression of EAAT3 compared to saline. This may affect the efficient transport of glutamate (Glu), a major metabolic fuel in enterocytes essential to the integrity and function of the jejunum, particularly in young chicks (Mott et al., 2008). In addition, bergamot EO significantly ($P < 0.05$) increased the expression of SI potentially improving carbohydrate digestion. No significant effects on the digestive biomarkers of interest were observed with the *in ovo* applications of the other 23 EOs.

It was concluded that *in ovo* delivery of spearmint, patchouli, and turmeric EOs may reduce the capacity to absorb Glu through the EAAT3 and that bergamot EO may increase carbohydrate digestion in the jejunum of broiler hatchlings.

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MITIGATING NECROTIC ENTERITIS IN BROILERS ON ANTIBIOTIC-FREE DIETS

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Summary

Necrotic enteritis is a major poultry disease caused by the bacterium *Clostridium perfringens*. In commercial poultry, subclinical necrotic enteritis induces significant economic losses due largely to reduced performance, greater mortality rates, treatment, and predisposition to additional stresses. With the continued reduced usage of prophylactic medication, alternative prevention methods have become the focus of recent research and development. In a series of studies, non-drug feed additives were assessed for their impact on broilers' performance and resilience to disease in the context of subclinical necrotic enteritis challenge models. These involved coccidiosis challenges as the chief predisposing factor to inducing necrotic enteritis upon introduction of *C. perfringens*. Among such potential non-drug dietary 'alternatives' were probiotics, synbiotics, phytogenic blends, and algal sulfate polysaccharides. While varying differential responses were observed, several additives could effectively mitigate necrotic enteritis by improving feed conversion, helping maintain the integrity of the epithelial barrier, enhancing immune competence, and modulating the expression of key structural and functional genes in the small intestine.

I. INTRODUCTION

Necrotic enteritis (NE), caused by toxins produced by the bacterium *Clostridium perfringens*, is a major enteric disease in commercial poultry with associated annual economic losses estimated at $6 billion worldwide (Wade and Keyburn, 2015). In broiler production, much of these costs are attributed to subclinical NE resulting in reduced performance associated with compromised integrity of the gut, higher mortality rates, and greater costs for disease treatment and prevention of subsequent infections (Van Immerseel et al., 2004; Timbermont et al., 2011; Emami et al., 2019; 2020; 2021). As *C. perfringens* is naturally occurring in the gastro-intestinal tract of poultry, the development of NE requires predisposing factors with *Eimeria* sp. challenge being the most commonly employed in research models. *C. perfringens* is a Gram-positive, spore-forming, opportunistic pathogen and is generally classified into 7 groups (A, B, C, D, E, F, and G) based on the production status of toxins: α, β, ε, ι, and NetB (Rood et al., 2018). In poultry, *C. perfringens* types A and G producing alpha toxin (CPA) and necrotic enteritis B-like Toxin (NetB) are most commonly associated with NE in broiler chickens (Rood et al., 2018; Emami and Dalloul, 2021). Such toxins bind to tight junction protein complexes of the intestinal epithelial layer, modulating permeability and allowing for pore formation (Saitoh et al., 2015). These complexes are dynamic barriers that can respond to external stimuli including nutrients, commensal bacteria, and pathogens/antigens present in the intestinal lumen (Emami et al., 2019; 2020). Therefore, the intestinal environment plays a critical role in maintaining the integrity of tight junction proteins and the structural damage caused by *C. perfringens* toxins compromise overall gut integrity. As a result, nutrient absorption is reduced and gut permeability increases, allowing traffic of macromolecules between the intestinal lumen and sub-epithelium (leaky gut) potentially leading to inflammatory responses and necrosis associated with toxin production (Awad et al., 2017; Ducatelle et al., 2018; Husta et al., 2023). Consequently, these reactions can trigger various

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signaling cascades initiating and/or activating immune response pathways that induce the production of cytokines and other immune-related factors including interferon (IFN) γ, interleukin (IL) 1β, IL10, IL12B, tumor necrosis factor (TNF) α, and annexin (ANXA) 1.

Traditionally, antimicrobial growth promoters (AGPs) were used as prophylactics to help mitigate enteric diseases such as NE. However, NE has become a larger problem for the poultry industry after the ban of AGPs in the European Union in 2006 followed by the implementation of the US Food and Drug Administration (FDA) Veterinary Feed Directive (VFD) in 2017 restricting the use of prophylactic AGPs in the United States. As a result, the industry use of therapeutic levels of AGPs has become less common with more poultry producers implementing no antibiotics ever (NAE) and antibiotic free (ABF) production schemes (Gaucher et al., 2017; Smith, 2019). AGPs such as virginiamycin, bacitracin methylene disalicylate (BMD), and lincomycin can promote poultry health and productivity by reducing pathogens, flock mortality, improving litter quality, and enhancing performance (Cervantes, 2015; Hofacre et al., 2018). Yet, consumer demands for drug-free production and legislative restrictions on AGPs have prompted an intense investment in developing effective alternative strategies mostly via nutritional interventions to mitigate the adverse effects of NE on broiler health (Calik et al., 2019; Emami et al., 2021; Blue et al., 2023). In this context, probiotics, synbiotics, phytogenics, and other industrial by-products such as yeast (e.g. beta-glucans) and algal derivatives are currently used in poultry production to mitigate the adverse effects of NE and other enteric challenges.

In summary, NE continues to be a leading complex enteric disease with a vast array of consequences, including compromised intestinal integrity and immune competence leading to significant economic losses. During pathogen (e.g. *C. perfringens*) invasion, a cascade of signaling events in the intestine leads to the secretion of various cytokines and other immunity factors that directly influence the integrity and function of the intestinal barrier, nutrient uptake, and epithelial cell energy metabolism (Emami et al., 2020). As a result, by altering the stability of the intestinal tract, NE is able to negatively impact production parameters and further predispose birds to stressors. With the removal of AGPs, maintaining homeostasis in performance and gut barrier integrity is critical for gut immune development, function and overall health. Achieving this goal requires a deep understanding of the changes that occur in tight junction proteins and gut immune responses during NE in broilers. Therefore, research should continue to investigate how targeted nutrition can help birds effectively prevent NE-induced damage, ultimately improving nutrient absorption, performance, and cell energy utilization and metabolism. Several groups across the globe continue to investigate and develop targeted non-drug dietary means with the collective goals of lessening the devastating impact of enteric diseases. Recently, our group has been investigating various such alternatives in controlled NE model studies employing *Eimeria* challenge as predisposing factor followed by *C. perfringens* introduction, and assessing their effectiveness on performance, pathology, and gut immunity and integrity of broiler chickens.

II. METHODS

Two challenge models were used in a series of studies assessing the effectiveness of dietary supplements on the broilers' responses during subclinical NE. In all studies, a basal diet (negative control) and an AGP diet (positive control) groups were included for comparative purposes. All studies were conducted according to the guidelines of the Institutional Animal Care and Use Committees.

The first study evaluated the effects of a saponin-based product on broilers' performance and carcass composition during an induced NE challenge. A total of 1,200 day-of-hatch male chicks were randomly assigned to four dietary treatments (10 pens/treatment; 30

birds/pen): treatment 1 (NC), a non-medicated corn–soybean basal diet; treatment 2 (PC), NC + 50 g/MT BMD; and treatments 3 (CQ15) and 4 (CQ30) consisting of NC + 15 and 30 g/MT of the test product Clarity Q, respectively. On the day (d) of placement, birds were challenged by a 10X dose of coccidia vaccine to induce NE. On d 8, 14, 28, and 42, performance parameters were measured. On d 8, three birds/pen were necropsied for NE lesions. On d 8 and d 14, jejunum samples from one bird/pen were collected for measuring mRNA abundance of tight junction proteins and nutrient transporter genes using qPCR. Data were subjected to a one-way ANOVA (JMP Pro, 16) except for lesion scores where a chi-square test was used. Fisher's LSD test compared separated means when statistical differences were noted and considered significant at $P \leq 0.05$.

In the second study, the NE model involved co-infection with *Eimeria maxima* and *C. perfringens* to assess whether an algal sulfate polysaccharide could mitigate the adverse effects of NE in broilers. A total of 600 d-old Ross 708 male chicks were randomly assigned to one of four treatment groups: the NC group (negative control, fed a corn-soybean meal diet); PC group (positive control, fed NC + 15 ppm Avilamycin and 125 ppm Amprolium); AGS (fed NC + algal product at 0.1% of the diet); and AGH (fed NC + algal product at 0.2% of the diet). On d 14, all birds were orally gavaged with 2,000 *E. maxima* sporulated oocysts, followed by one dose of approximately 1×10^8 CFU of *C. perfringens* on d 19. Performance parameters were measured on d 14, 21, 28, and 42. On d 21 the small intestines of four birds/pen were examined for necrotic lesions. On d 14, 21, and 42 jejunum samples (from one bird/pen) were collected to measure mRNA abundance of tight junction proteins. Data were analyzed using JMP and significance between treatments identified by LSD at $P \leq 0.05$.

In a similar setup, the third study evaluated the effects of phytogenic blends on performance, intestinal lesion scores, and mRNA abundance of tight junction proteins and immune response genes. It also consisted of 600 d-old Ross 708 male broilers allocated to one of four treatment groups (6 replicate floor pens, 25 birds/pen) including NC and PC as per the previous studies in addition to two phytogenic additive groups PHY1 (Alterna®) and PHY2 (Alterna® + Synbiotec®). PHY1 group was fed NC + Alterna at 0.4 kg/MT during the starter and grower phases and 0.3 kg/MT during the finisher phase. PHY2 was fed NC + Alterna® and Synbiotec® at an inclusion rate of 0.4 and 0.5 kg/MT, respectively, during the starter and grower phases, 0.3 and 0.25 kg/MT during the finisher phase. Performance parameters were measured on d 14, 21, 28, and 42. On d 21 the small intestines of four birds/pen were examined for lesions. On d 14, 21, and 42, jejunal samples were collected to assess mRNA abundance of IL1β, IL10, and IL12B, IFNγ, TNFα, and ANXA1. Data were analyzed using ANOVA and significance ($P \leq 0.05$) was determined by the LSD test.

III. RESULTS AND DISCUSSION

In evaluating a saponin-based product, the results revealed differential responses of broilers. Compared to PC and NC, CQ15 had slightly higher average daily gain (ADG) on d 8 ($P > 0.05$), and exhibited numerically lower NE lesions in the duodenum compared to all other treatments (Blue et al., 2023). Also at the peak of infection on d 8, mRNA abundance of CLDN1, CLDN5, AMPK, PepT2, and EAAT3 were significantly greater in CQ30 ($P < 0.05$) compared to both PC and NC. On d 14, considered as the recovery time in this particular model, mRNA abundance (Figure 1) of ZO2 and PepT2 was significantly lower in PC when compared to all treatments, while that of ANXA1, JAM3, and GLUT5 was comparable to CQ15. Additionally, OCLDN, CLDN1, JAM2, and ZO1 showed lower abundance in CQ15 and CQ30 compared to NC and PC. Under this subclinical NE challenge model when broilers were supplemented with Clarity Q, the data showed a numerical reduction in duodenal lesion scores on d 8 (CQ15) and a slightly improved FCR (CQ30) during the overall grow-out period.

The results also showed a positive modulation in mRNA abundance of several tight junction proteins and nutrient transporter genes. Overall, adding this lant-based product to broiler diets exhibited a potential to alleviate some of the adverse effects caused by this enteric disease. These were best manifested by improving performance, reducing intestinal lesions, and positively modulating the mRNA abundance of various tight junction proteins and key nutrient transporters during peak NE infection. As such, dietary supplementation of Clarity Q can potentially assist birds during an enteric disease challenge warranting further investigations under this as well as other disease/stress conditions.

Under a different NE challenge model, a sulfate polysaccharide extracted from marine algae could mitigate the adverse effects of NE in broilers. In this study, PC, AGS, and AGH had significantly lower mortality, ADFI, and FCR, and greater ADG compared to NC. Additionally, PC, AGS, and AGH significantly reduced NE lesions compared to the NC group on d 21. There were no significant differences in mRNA abundance of CLDN1, CLDN3, ZO2, and OCLDN on d 21 (peak infection) among all treatments. However, on d 42 (market age), AGS and AGH showed greater mRNA abundance of CLDN1, ZO1, and ZO2 ($P < 0.05$) compared to NC and PC groups. Collectively, the enhancements in performance, reduction in lesion scores, and increased post-infection expression of tight junction protein mRNA demonstrate the potential of this marine algae-derived dietary supplement as an effective alternative to AGPs. This approach has the potential to alleviate the negative impacts of disease in the context of this NE model, yet further investigations into its mode of action under various enteric challenges are warranted.

In the third study, supplementation of phytogenic blends resulted in interesting findings. During the challenge period of d 14-21, PHY2 and PC had a significantly lower mortality percentage compared to NC; the same trend was seen on d 0-21 and d 0-28. ADG was significantly greater in PC, PHY1, and PHY2 compared to NC during d 0-14, 21-28, and 0-28. Also, FCR was significantly lower in PHY1, PHY2, and PC compared to NC during d 14-21, 21-28, and 0-28 but not during 0-42 as the NC birds seemed to have compensated for growth during the last two weeks. On d 21, PHY1, PHY2 and PC significantly reduced NE lesion severity ($P = 0.0002$) compared to NC. As for qPCR results, mRNA abundance of CLDN1 was significantly greater in PHY2 compared to all other treatments on d 14, while that of OCLN was significantly greater in PC on d 21. mRNA abundance of TNFα, IL10, and ANXA1 was significantly lower in PHY2 compared to all treatments on d 21. Meanwhile, on d 42, PHY1 and PHY2 showed greater mRNA abundance of IFNγ, TNFα, IL10, ANXA1, and IL12B. Based on these results, the combination of Alterna® and Synbiotec® in the diet of broiler chickens has the potential to improve performance, reduce pathology, and have a positive effect on tight junction proteins similarly to an AGP and a coccidiostat. Further, during peak infection (d 21), a decrease in various inflammatory cytokines could enhance tolerance against infection, while the release of anti-inflammatory mediators can resolve inflammation and restore homeostasis. Taken together, these findings further demonstrated the usefulness of phytogenic blends in this NE model with their potential to diminish the intrusion of pathogens and enhance broilers' ability to counteract the adverse effects of NE.

In summary, necrotic enteritis is a costly enteric disease for the global poultry industry and requires continued research in order to meet the increasing demand of poultry products as the industry moves away from the use of AGPs. As these products continue to fall out of use in the animal industry, it is essential to identify intervention strategies that mitigate poultry health problems, including NE and associated enteric challenges. Several factors including *Eimeria* infection, environmental stressors, and feed ingredients can alter the intestinal tract structure and function in poultry in predisposing birds to NE. The outcomes of such factors can be harmful to varying degrees or at times beneficial, and delineating their impacts is critical to tailor effective mitigation under field conditions. To develop practical and efficient

applications, continued research into the mechanisms of the broiler's immune response to NE and its predisposing factors will be crucial.

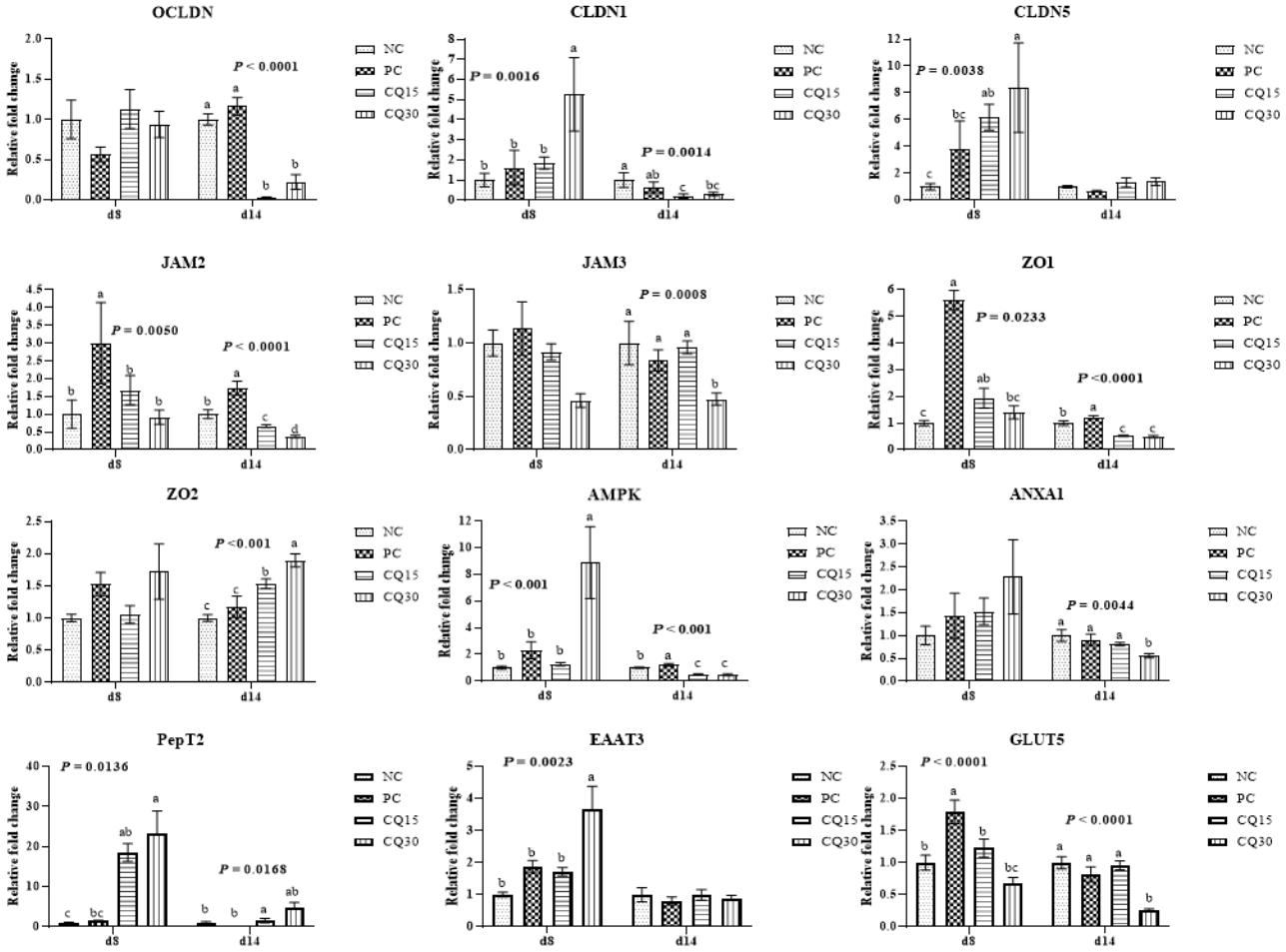

Figure 1 - Relative mRNA abundance of tight junction proteins, signaling pathway molecules, and nutrient transporters in the jejunum of broiler chickens on d 8 and d 14 during a 42 d NE challenge. Bars (among treatments for each day) with different letters (a-d) differ significantly ($P < 0.05$). Values are represented as n-fold difference relative to the calibrator (NC). Each bar represents the mean ± SE values of 10 replicate pens of 1 birds/pen. Occludin (OCLN), claudins (CLDN) 1 and 5, junctional adhesion molecules (JAM) 2 and 3, and zonula occludens (ZO) 1 and 2, adenosine monophosphate-activated protein kinase (AMPK), annexin 1 (ANXA1), excitatory amino acid transporter 3 (EAAT3), peptide trans-porter (PepT) 2, and glucose transporter (GLUT) 5. Treatments included negative control (NC) as corn-soybean meal basal diet; positive control (PC) as NC + 50 g/MT of BMD; and NC + 15 or 30 g/MT of Clarity Q (CQ15 and CQ30, respectively).

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CAN A SAPONIN-ALUMINOSILICATE BLEND PROMOTE RESILIENCE TO COCCIDIOSIS IN BROILERS?

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Summary

In broilers, coccidiosis leads to reduced growth and feed efficiency and it is often a predisposing factor to secondary diseases such as necrotic enteritis. Coccidiosis is commonly controlled with conventional control strategies such as prophylactic anticoccidial drugs and the use of vaccines. However, these strategies cannot fully prevent *Eimeria* infection and performance losses still occur due to subclinical coccidiosis. Natural solutions, such as saponins derived from the *Quillaja saponaria* Molina tree and aluminosilicates can bring added value to coccidiosis management strategies by reducing disease pressure and improving intestinal health.

I. INTRODUCTION

Coccidiosis is one of the main disease challenges affecting broiler production worldwide. This disease has a significant economic impact on the poultry industry and the global cost of coccidiosis in chickens linked to prevention, treatment, and performance losses is estimated at US$13 billion or US$0.20 per bird (Blake et al., 2020). Coccidiosis is caused by protozoan parasites of the genus *Eimeria*. Seven different *Eimeria* species are known to affect chickens, each with a different pathogenicity and targeting a specific location in the intestinal tract. In order to replicate, these parasites invade the intestinal cells of the host, which results in tissue damage, impaired nutrient digestion and absorption, and compromised well-being and growth performance in broilers (Mesa-Pineda et al., 2021). Furthermore, coccidiosis is also a predisposing factor to secondary diseases such as necrotic enteritis induced by *Clostridium perfringens* (Lee et al., 2011).

Current prevention and control of coccidiosis is mainly based on the use of anticoccidials and live vaccines. The extensive prophylactic use of anticoccidials has resulted in resistant *Eimeria* strains and loss of efficacy (Abbas et al., 2011). Vaccines, on the other hand, have a high relative cost and, if managed incorrectly, can predispose the animals to subclinical coccidiosis and necrotic enteritis. In broilers, vaccines often do not lead to a timely build-up of immunity (Mesa-Pineda et al., 2021).

With these drawbacks, broiler producers are looking for new tools to add to their global coccidiosis management strategy. Natural feed additives, such as saponin-rich plant extracts, are among the promising approaches used to control coccidiosis in broilers. Saponins are found in many plant species and are known to be antimicrobial, to inhibit mould, and to protect plants from insect attack (Francis et al., 2002). As a result, saponin extracts from plants such as the *Quillaja saponaria* Molina tree have a wide range of applications in livestock production and can be used as antibacterial, antiviral, and antiparasitic agents, as well as adjuvants (Fleck et al., 2019). The antiparasitic effect of saponins may be linked to their detergent action: the hydrophobic part of the saponin can integrate into the membrane of protozoa to form complexes with sterols, resulting in pore formation and cell lysis (Augustin et al., 2011; Fleck et al., 2019). Aluminosilicates are clay minerals which are also widely used as feed additives to improve growth performance and health of animals, mainly due to their ability to adsorb heavy metals, ammonia, mycotoxins and toxins, thereby protecting the integrity of the intestinal tract (Damato et al., 2022).

The objective of this study was to investigate the effect of a blend of *Quillaja saponaria* extract (a source of triterpenoid saponins) and aluminosilicate on oocyst excretion, intestinal lesions and productive performance in broilers raised on used litter seeded with coccidia oocysts.

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II. METHODS

One-day-old male Ross 708 broilers were obtained from a commercial hatchery (Elizabethtown, PA, USA) and, after sorting for health, 1152 chicks were assigned to 48 floor pens (24 broilers per pen). The trial consisted of four treatments that were randomly assigned to the pens, each with 12 replicates: a positive control with no anticoccidials and reared on clean pine shaving litter (PC); a negative control with no anticoccidials added to the diet and reared on used pine shaving litter (NC); a negative control with 60 mg/kg of an anticoccidial (salinomycin: Bio-Cox® 60g, Huvepharma Inc.) added to the diet and reared on used pine shaving litter (NC + sal); a negative control with a saponin-aluminosilicate blend (Excential Sapphire Q, Orffa Additives B.V.) providing 30 mg/kg of *Quillaja saponaria* extract in the diet and reared on used pine shaving litter (NC + sap-al).

The used litter containing *Eimeria* oocysts was generated as follows: In 48 separate floor pens, a total of 1200 one-day-old male Cobb chicks from a female broiler breeder line were housed (25 birds per pen) on clean pine shavings and received a 10x dose of a coccidiosis vaccine (Coccivac® B52, Merck Animal Health) via the feed for the first two days. The vaccine consisted of the following strains: *E. acervulina, E. maxima, E. maxima* MFP, *E. mivati,* and *E. tenella*. The birds were reared until 18 days of age to allow for at least three cycles of coccidiosis oocyst shedding into the litter. The used litter was then collected, mixed, and redistributed into the experimental pens. The broilers had free access to feed and water during the whole experimental period and were fed in three phases that is, starter (0 to 16 days), grower (16 to 29 days) and finisher (29 to 42 days). The diets were maize and soybean meal-based and were fed in a crumble form during the starter period and pellet form during the grower and finisher periods.

Mortalities were recorded daily. At 0, 17, 28, and 42 days of age (at the end of each feeding phase), body weight (BW) and average daily feed intake (ADFI) were determined to calculate the body weight gain (BWG) and feed conversion ratio (FCRm) (after correcting for mortalities) for the time periods 0 to 16, 0 to 29, and 0 to 42 days. At 16 days of age, three birds per pen were euthanised for macroscopic intestinal lesion evaluation using the methods described by Conway and McKenzie (2008) and Johnson and Reid (1970). On days 11 to 13, 17 to 19, and 22 to 24, fresh excreta samples were collected and pooled from each pen to determine the number of oocysts shed per gram of excreta. Oocysts were stored and processed as outlined by Long et al. (1970) and counted using procedures described by Dalloul et al. (2003), with the modification of correcting for total grams of excreta collected.

Broiler performance, intestinal lesion scoring, and oocyst shedding were analysed as one-way ANOVA using JMP Pro 16 (SAS Institute Inc., Cary, NC) with the significance level set at 0.05. Blocking within the house was used as a random variable within the model. Results were expressed as least square means (lsmeans) and the standard error of those means (SEM). If global ANOVA was significant, significant differences between lsmeans were determined using Fishers least significant difference test.

III. RESULTS

From 0 to 16 days, the NC + sal and NC + sap-al treatments had higher ADFI ($P = 0.004$) and BWG ($P = 0.009$) compared to NC and similar performance to PC ($P > 0.05$) (Table 1). From 0 to 29 days, there were no differences in ADFI ($P = 0.198$) among the treatments, but BWG was higher for the PC, NC + sal, and NC + sap-al treatments compared to the NC treatment group ($P = 0.018$). For the overall period from 0 to 42 days, NC + sal had a higher BWG compared to NC, with the PC and NC + sap-al treatments being intermediate but not significantly different from the NC and NC + sal treatments. For FCRm from 0 to 42 days the NC + sal and NC + sap-al treatments tended to improve the overall FCRm compared to the NC treatment ($P = 0.053$).

No differences in lesion scores were noted among treatments in the duodenum ($P = 0.509$), jejunum ($P = 0.101$) and ileum ($P = 0.579$) (Table 2).

From 11 to 13 days, the lowest oocyst shedding was observed for PC and the highest shedding for NC and NC + sap-al treatments, with the NC + sal treatment being intermediate ($P < 0.001$) (Figure 1). No significant differences in oocyst shedding were found between the treatments from 17 to 19 days ($P = 0.209$), although the PC group showed numerically the lowest oocyst counts on these days. From 22 to 24 days, a trend was observed for oocyst counts ($P = 0.063$). The NC + sal and NC + sap-al treatments tended to reduce the number of excreted oocysts compared to the NC treatment. The PC treatment showed numerically the lowest oocyst excretion on these days.

Table 1 - Effects of treatments on broiler body weight gain (BWG), average daily feed intake (ADFI) and mortality corrected feed conversion ratio (FCRm) from 0 to 16, 0 to 29, and 0 to 42 days of age.

Treatments	PC	NC	NC + sal	NC + sap-al	SEM	P-value
BW at day 0	43.1	43.0	43.4	43.4	0.2	0.632
0 to 16 days of age						
ADFI (g/broiler)	40.5[a]	37.9[b]	40.3[a]	39.4[a]	0.5	0.004
BWG (g/broiler)	468[a]	432[b]	473[a]	458[a]	8.6	0.009
FCRm	1.32	1.35	1.31	1.32	0.01	0.127
0 to 29 days of age						
ADFI (g/broiler)	72.3	69.2	71.1	70.2	1.0	0.198
BWG (g/broiler)	1491[a]	1407[b]	1499[a]	1469[a]	21.2	0.018
FCRm	1.47	1.48	1.44	1.44	0.02	0.225
0 to 42 days of age						
ADFI (g/broiler)	104.7	101.8	107.7	103.6	2.0	0.215
BWG (g/broiler)	2999[ab]	2869[b]	3110[a]	2997[ab]	53.9	0.030
FCRm	1.57[ab]	1.60[b]	1.55[a]	1.56[a]	0.01	0.053

[a,b]Values in a row with no common superscript differ significantly ($P < 0.05$).

Table 2 - Effects of treatments on intestinal lesion scores in broilers at 16 days of age.

Treatments	PC	NC	NC + sal	NC + sap-al	SEM	P-value
Duodenum	1.42	1.09	1.15	1.28	0.16	0.509
Jejunum	1.20	1.03	0.91	0.78	0.12	0.101
Ileum	0.89	0.70	0.88	0.69	0.13	0.579

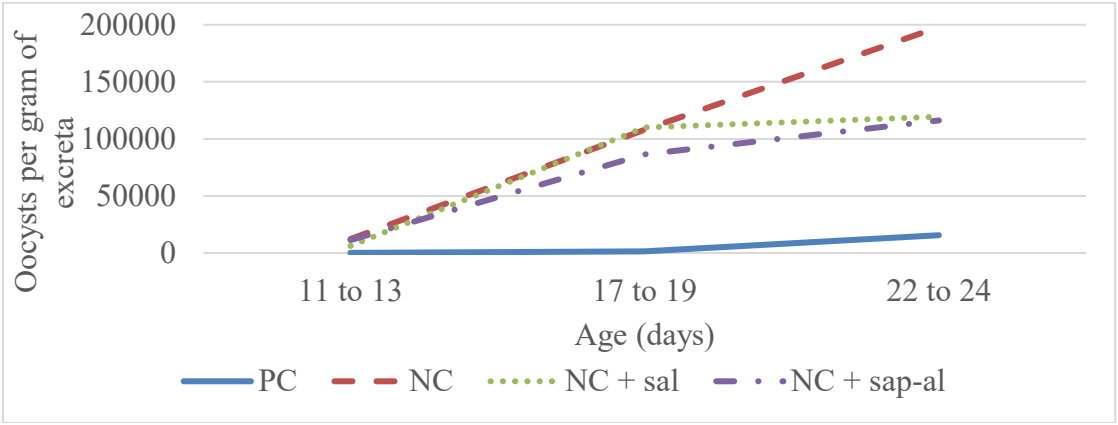

Figure 1 - Effects of treatments on oocyst counts in excreta of broilers during three collection periods.

IV. DISCUSSION

In the current study, the coccidia challenge was confirmed by lower oocyst counts for the PC and higher oocyst counts for the NC between 11 to 13 days. No significant differences in oocyst counts were observed between treatments for the collection periods 17 to 19 days and 22 to 24 days, possibly due to large variation within treatments. However, for the collection period between 22 to 24 days, oocyst excretion was reduced by 39.7% and 41.3% compared to negative control, when

salinomycin or the saponin-aluminosilicate blend was fed, respectively, indicating a direct anticoccidial effect of these additives. Saponins are amphiphilic molecules and are natural detergents because they contain a fat-soluble nucleus or sapogenin and water-soluble carbohydrate side chains (Francis et al., 2002; Augustin et al., 2011). The reduction in oocyst excretion seen for broilers fed the saponin-aluminosilicate blend was likely the result of the affinity for and ability of the sapogenin portion of the saponins to form complexes with cholesterol in the protozoal cell membrane. This would have affected the integrity of the parasite membrane by leading to pore formation and cell lysis and preventing the parasites from infecting intestinal cells and replicating (Augustin et al., 2011; Fleck et al., 2019). The saponin-aluminosilicate blend was able to improve the growth of coccidiosis-challenged broilers to a similar level as the unchallenged control and broilers fed salinomycin, an anticoccidial drug. A coccidiosis challenge generally occurs concurrently with necrotic enteritis which is caused by toxins produced by *Clostridium perfringens* (Lee et al., 2011). One beneficial effect of aluminosilicates is their ability to bind enterotoxins in the intestinal tract of animals (Damato et al., 2022). In this study, the improved performance seen for the NC + sap-al treatment compared to the NC treatment group may also have been due to the aluminosilicate which adsorbed toxins produced by opportunistic pathogens in the coccidiosis-challenged broilers, reducing intestinal damage and thereby improving growth.

The results of the current study indicate that the saponin-aluminosilicate blend can promote the resilience in broilers to coccidiosis. This blend can be implemented to further reduce the negative effect of subclinical coccidiosis on growth and feed efficiency in broilers and reduce the costs associated with these performance losses. If the societal demand for antibiotic-free animal products keeps increasing, this natural blend can provide a viable alternative for anticoccidial treatment in broilers.

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EVALUATION OF THE EFFECT OF A NOVEL DUAL STRAIN PROBIOTIC ON A CHICKEN INTESTINAL CELL MODEL CHALLENGED WITH THE CAUSATIVE AGENT OF NECROTIC ENTERITIS

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Summary

This work investigated for the first time the CHIC-8E11 chicken intestinal epithelial cells as a model for studying pathogenic traits of *Clostridium perfringens* (CP), the causative agent of Necrotic Enteritis (NE) and the potential efficacy of probiotic cell-free supernatant from *Lactobacillus acidophilus* AG01 and *Bifidobacterium animalis* subsp. *lactis* AG02. This study demonstrates the complementary potential of the secreted compounds produced by the two strains on adhesion potential, cytotoxicity, and epithelial integrity.

I. INTRODUCTION

Necrotic enteritis (NE) is a major intestinal disease in commercial poultry. It affects ~40% of broiler flocks and costs the industry through increased mortalities and reduced growth performances. The causative agent is *Clostridium perfringens* (CP) a Gram-positive member of the normal intestinal microbiota of poultry. A disturbance in the intestinal microbiome or a change in the behaviour of CP can result in the development of NE. CP can produce >18 different toxins (Revitt-Mills et al., 2015), including α, β, ε and Net-B toxins. The Net-B toxin, produced by type G strains (Emami and Dalloul, 2021), has been identified as a key virulence factor for NE development and can cause symptoms of NE even in the absence of the pathogen itself. No effective vaccines against NE are available. *In vitro* cellular models have a history of use in human biology for drug screening and elucidating infection processes for the subsequent identification of potential targets for future solution development. Until now an intestinal immortalized chicken intestinal cell line has not been available. A new chicken enterocyte cell line, CHIC clone 8E11 (Tentamus Pharma & Med Deutschland GmbH), has become available and is starting to be applied to the study of pathogen-host interactions (Ali et al., 2020). In this study, the CHIC-8E11 cell line was used to characterise pathogenic traits (adhesion to host cells, pathogen exclusion, effect on cell permeability and cytotoxicity) of 4 CP strains and of pure Net-B toxin. We then investigated *in vitro* the potential of probiotic *Lactobacillus acidophilus* strain AG01 and *Bifidobacterium animalis* subsp. *lactis* AG02 to reduce the negative effects of CP and Net-B.

II. METHOD

The chicken cell line was obtained from Brandenburg University of Technology, Germany. Four *C. perfringens* strains (CP1; CP10; CP21 and CP22) isolated from broilers and pure Net-B were used in this study. All *C. perfringens* strains carried CPA^+ while only CP21 and CP22 were $NetB^+$. *C. perfringens* cell free supernatants were collected from overnight liquid cultures

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grown in brain heart infusion (BHI) broth at 37°C/5% CO_2. Three assays were conducted to evaluate the use of the CHIC-8E11 cell model for NE infection purposes: adhesion; cytotoxicity and permeability, comparing challenged and unchallenged cell preparations, and Kruskal-Wallis H test was used for statistical analysis (Graph pad Prism 9). $P < 0.05$ was considered significant. Assays were repeated to investigate the effect of pretreatment of CHIC-8E11 by two probiotic cell-free supernatants (*Lactobacillus acidophilus* AG01 and *Bifidobacterium animalis* subsp. *lactis* AG02) obtained from cultures grown in MRS for 48 h at 37°C/5% CO_2.

For the adhesion assay, isolated CHIC-8E11 cells were seeded in 96-well cell culture plates at a density of 2.0×10^4 cells/well and grown for 48 h. Fresh cultures of 4 *C. perfringens* strains were grown overnight, and cells loaded on to the CHIC-8E11 cells at a dose level of 50 µl/ml ($OD_{600} = 1$). The plates were incubated for 1 h at 37°C/5% CO_2. Cells were rinsed, lysed and serially plated on TSB agar for *C. perfringens* enumeration. To determine the impact of probiotic cell-free supernatants, the initial culture media was replaced with fresh media plus cell-free supernatant from 1 of 2 probiotic strains and incubated overnight. Cells were PBS washed and the assay conducted as above. Cell culture media without cell-free supernatant acted as a control.

Cell permeability was assessed using the fluorescein isothiocyanate-dextran (FITC-Dextran-10 µg/ml) permeability assay. Cells were challenged and incubated for 2 h at 37°C/5% CO_2 prior to adding FITC-D for 3 h. To determine the impact of probiotic, the cell free supernatant was added to the apical compartment and incubated overnight. The challenge was then conducted as above.

The cytotoxicity of the 4 *C. perfringens* cell free supernatants added at 10, 20, 30, 40 or 50 µl/ml or pure Net-B toxin (added at 1 or 2 µg/ml), against CHIC-8E11 cells was assessed using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium cell viability assay (CyQUANT MTT™ Cell Viability Assay). Cytotoxicity was expressed as the percentage absorbance in the treatment (test) wells compared to the control. To determine the impact of probiotic, cell free supernatants was added at a volume of 10, 20, 30, 40 or 50 µl/ml prior overnight incubation at 37°C/5% CO_2. After incubation, *C. perfringens* cell free supernatant was added, and the assay conducted as above.

III. RESULTS

Using the cell model CHIC-8E11, adhesion, cell permeability and cytotoxicity of pathogenic *C. perfringens* strains were quantified (Table 1) and used as a challenge control then after to assess the potential of cell-free supernatants from *L. acidophilus* AG01 and *B. animalis* subsp. *lactis* AG02. Permeability effect of the probiotic cell-free supernatants were tested in the absence of pathogen challenge as quality control. AG01 cell-free supernatant had no effect on CHIC-8E11 permeability, while AG02 cell-free supernatant significantly improved the permeability ($P < 0.01$) compared to negative control.

Table 1 - Summary of results from the adhesion, cytotoxicity and permeability assays conducted using 4 *Clostridium perfringens* strains in a CHIC-8E11 cell model.

Clostridium perfringens strain I.D.	Adhesion (%, CFU based)	Cytotoxicity range* (%)	Permeability* (%, FD4 based)
CP1	0.05[a]	0.3–25	-
CP10	0.28[b]	1–24	-
CP21	0.48[c]	35–56	43.73[a]
CP22	0.33[c]	45–62	74.67[b]

** Percent change versus the control; - not evaluated; variables with same letters are not significantly different*

Overnight pre-treatment with cell-free supernatant from AG02 markedly reduced cell adhesion of CP10, CP21 and CP22 (respectively, by 84.8 ± 3.7, 77.4 ± 14.0, and 82.3 ± 15.6, vs control: $P < 0.001$; Fig. 1) but had no significant effect on CP1 adhesion. Cell-free supernatant from *L. acidophilus* AG01 had no statistically significant effect on adhesion.

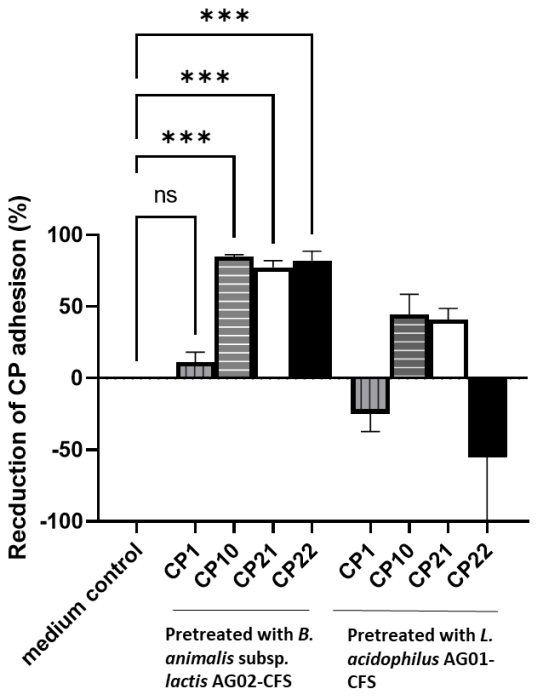

Figure 1 - The reduction in percentage adhesion in the probiotic cell free supernatant pre-treated groups was compared to the response in the medium control. Values represent means and associated standard deviations. ***, statistically significant at $P < 0.001$.

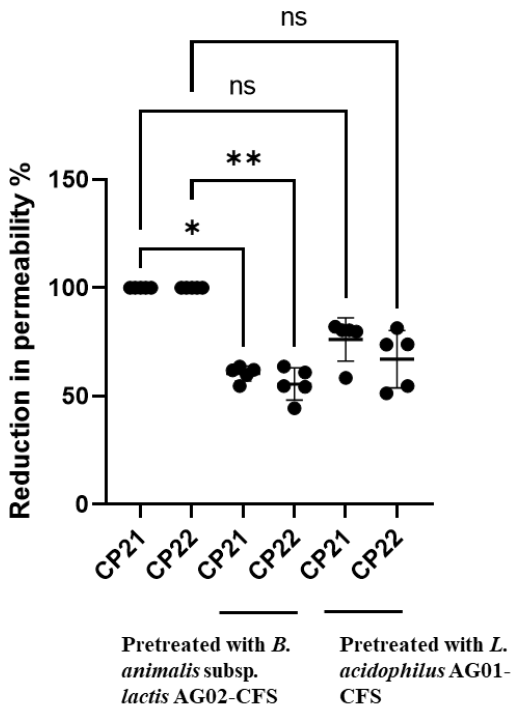

Figure 2 - The reduction in permeability (%) in the probiotic cell free supernatant pre-treated groups was compared to the response in the medium control. Values represent means and associated standard deviations. *, statistically significant at $P < 0.05$; **, statistically significant at $P < 0.01$; n.s., non-significant at $P < 0.05$.

Cell-free supernatant from AG02 significantly reduced the change (increase) in permeability of cells challenged with cell-free supernatant from CP21 or CP22 [respectively, by 40% points ($P < 0.05$), and by 44% points ($P < 0.01$), vs no probiotic preculture] (Fig. 2). The change in permeability of cells challenged with cell-free supernatant from strain CP21 or CP22 was numerically reduced by pretreatment of cells with cell-free supernatant from *L. acidophilus* AG01.

Overnight pre-treatment of CHIC-8E11 cells with cell free supernatants from *L. acidophilus* AG01 or *B. animalis* subsp. *lactis* AG02, prior to challenge for 4 h with cell free supernatant from *C. perfringens* strain CP01, CP22, or with pure Net-B toxin, numerically reduced the percentage cytotoxicity compared with the control treatment. The magnitude of reduction appeared to be dependent on the concentration of probiotic supernatant applied (greater with increasing concentration) and differed between the two probiotic strains. At the highest probiotic cell free supernatant concentration (50 µl/ml), both probiotic strains numerically reduced the cytotoxicity of CP21, CP22, and of Net-B against CHIC-8E11 cells, but with differing efficacy: cell free supernatant from *B. animalis* AG02 was numerically more effective than that from *L. acidophilus* AG01 at reducing the cytotoxicity of CP22 and Net-B (-21% compared with -16%, and -34% compared with -18%, respectively, vs control), whereas

the two probiotics were equally effective at reducing the cytotoxicity of strain CP21 (-24% and -24%, respectively, vs control).

IV. DISCUSSION

The cell adhesion assay results indicated that all 4 *C. perfringens* strains could adhere to CHIC-8E11 cells and levels aligned with previously reported pathogen adhesion values of 0.5-2% (Trejo et al., 2006) with significantly higher percentage adhesion of the Net-B-positive strains. The significant and marked reduction in the adhesion of three out of four *C. perfringens* strains (CP10, CP21 and CP22) to CHIC-8E11 cells following their pre-treatment with cell-free supernatant from *B. animalis* subsp. *lactis* AG02, suggests the presence of beneficial effector molecule(s) in the cell free supernatant from both probiotics that disrupted or blocked *C. perfringens* adhesion to cells.

In the present study, only cell free supernatant from *C. perfringens* strain CP22 significantly increased the permeability of CHIC-8E11 cells after 2 h incubation compared to control (BHI only) suggesting that substances within the supernatant secreted by the pathogen disrupted barrier integrity. The specific nature of these substances cannot be confirmed. Cell-free supernatant from *B. animalis* subsp. *lactis* AG02, but not from *L. acidophilus* AG01, significantly reduced the negative effect of *C. perfringens* cell-free supernatant on host cell permeability. Given the critical role of epithelial barrier integrity in regulating the passage of substances into the body from the gut lumen, and the knowledge that this permeability is impaired in NE (Latorre et al., 2018), a beneficial effect of cell-free supernatant from *B. animalis* subsp. *lactis* AG02 in reducing epithelial permeability would be expected to reduce toxin and bacterial translocation and CP pathogenesis.

The cell viability assay results demonstrated that CHIC-8E11 cells were permissive to cell-free supernatants from pathogenic CP strains. The effect of cell-free supernatant from each of the probiotic strains on CP induced cytotoxicity was suggestive of a beneficial effect of both, but the absence of statistical significance when compared to the response of the control treatment limits the ability to draw firm conclusions. The numerical reductions in cytotoxicity were of the order of 15 to 25% across both probiotics when applied to CHIC-8E11 cells at the maximum dose level of 50 µl/ml. Their differential degree of effect against individual CP suggests a complementarity between the two probiotic strains.

In conclusion, this study confirms that CHIC-8E11 cell line could be a useful model to study the implications of CP pathogenesis and used as a high throughput screening tool for solution development. These results support the combination of *L. acidophilus* AG01 and *B. animalis* subsp. *lactis* AG02 in future *in vitro* and *in vivo* assays to further assess their potential as alternatives to antibiotics for the prevention and control of NE in broilers.

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ANTIMICROBIAL RESISTANCE AND THE CONCEPT OF ONE HEALTH IN POULTRY

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Summary

Antimicrobial resistance (AMR) is a global One Health issue with implications for public and animal health. Urgently, efforts are needed to minimize AMR emergence and conserve vital antimicrobials through responsible use in human and animal health, emphasizing antimicrobial stewardship and adopting a "One Health" approach that considers human, animal, and environmental health needs. Surveillance programs for AMR are crucial in veterinary and food production industries to identify emerging threats, especially those linked to critically important antimicrobials (CIAs) - last line drugs needing to be reserved for human therapeutic use. Globally, there is much debate concerning antimicrobial usage in livestock and its proportional impact on public health. In recent decades, we have seen the emergence of CIA-resistant bacteria in food-producing animals in Asia, Europe and North America. This appearance predominantly includes resistance to critically important drugs such as fluoroquinolones and extended spectrum cephalosporins among *Escherichia coli* and *Salmonella* isolates from pigs, poultry and cattle. The emergence of resistance to CIAs in these regions is largely attributed to the direct use of such antimicrobials in food-producing animals.

Recent studies have suggested that the ecology of critically important AMR among key indicator (*E. coli* and Enterococci) and zoonotic pathogens (*Salmonella* and *Campylobacter*) isolated from Australian food-producing animals differs from that in other parts of the world. Cross-sectional studies have demonstrated that Australian livestock have low rates of carriage of critically important antimicrobial-resistant Gram-negative bacteria (*E. coli*, *Campylobacter jejuni* and *Campylobacter coli*). This is attributed to Australia's isolated geographical location, strict quarantine restrictions (restrictions on importation of livestock and fresh meat), and more importantly the tight regulations governing the use of CIAs in food-producing animals. These circumstances have delivered promising results in minimizing the occurrence of CIA resistant Gram-negative bacteria in food producing animals.

Australia's commendable One Health approach involves coordinated efforts across sectors and global collaboration. This article focusses on the key findings of AMR studies in Australian poultry and highlights key biosecurity challenges with regards to antimicrobial resistance in bacteria colonizing Australian poultry.

I. INTRODUCTION

In the last century, human ingenuity led to the identification and then exploitation of antimicrobial agents. Their use has had huge impacts by helping in the control of bacterial diseases, improving health, and enhancing productivity in agriculture. Unfortunately, this monumental success has eroded over time, with exposure of the microbiome to antimicrobials having selected for survivors that are inherently resistant or that have developed or acquired mechanisms to circumvent the activities of the antimicrobials. This antimicrobial resistance (AMR) continues to increase, and has become a serious, long-term concern for human and animal health worldwide [World Health Organisation 2019]. For example, in 2019, almost one million human deaths globally were attributed to the occurrence of AMR in just six bacterial species: *Escherichia coli*, *Staphylococcus aureus, Klebsiella pneumoniae, Streptococcus*

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pneumoniae, Acinetobacter baumannii, and *Pseudomonas aeruginosa* (Antimicrobial Resistance 2022). In the absence of a concerted global effort to curb the development and spread of AMR, and to develop new antimicrobials and alternative means to control infections, these figures are predicted to increase to more than 10 million deaths/year by 2050, with a cumulative economic impact of 100 trillion USD (O'Neill 2016).

Unfortunately, the development pipeline for new antimicrobials in human medicine is stagnating, due to high development costs and the extended timelines required to get new drugs to market. Worryingly, 15 of the 18 largest pharmaceutical companies in the world have discontinued their research and development programs on antimicrobials over the last three decades (Dutescu and Hillier 2021, Gotham, Moja et al. 2021). Consequently, it is more important than ever to minimise the emergence and spread of AMR, and to conserve our arsenal of medically important antimicrobials – through appropriate use of antimicrobials in human and animal health, and antimicrobial stewardship. To achieve this, it is essential to adopt a "One Health" approach that considers and balances human, animal and environmental health needs.

The use of antimicrobials to support animal and plant health and productivity has generated, and selected for, antimicrobial resistant organisms that pose significant potential risks to human health, and that may limit therapeutic options for the treatment of human disease (World Health Organisation 2019). These may include resistant zoonotic pathogens that can spread directly to humans, or resistant commensal or environmental organisms from which antimicrobial resistance genes may spread to pathogens. These considerations mean that surveillance programs for AMR are essential for identifying emerging AMR in the veterinary and food production industries, since their transmission to humans through the food chain would pose significant risks. Of particular concern is the identification of bacteria that are resistant to critically important antimicrobials (CIAs), including extended-spectrum cephalosporins (ESCs), fluoroquinolones (FQs) and carbapenems, which are considered to be the highest priority antimicrobials for therapeutic use in humans. This threat of resistance moving through the food chain has led to the use of CIAs being restricted to human therapeutic purposes in many countries (Tang, Caffrey et al. 2017).

Increasing levels of resistance to CIAs in isolates from food production animals, including indicator organisms such as *E. coli,* have been identified globally. For example, a 2022 French study reported the detection of colistin-resistant *E. coli* in 16.5% of veal calves (n=170) (Um, Dupouy et al. 2022), and the Danish Integrated Antimicrobial Resistance Monitoring and Research Programme (DANMAP) reported a 16% prevalence of FQ-resistant *E. coli* in broiler chickens (DANMAP 2020). Similarly, national monitoring data from the USA revealed 3.5% of pigs carried FQ-resistant *E. coli* (FDA, 2022). In contrast, data from the Australian livestock sector has shown more favourable results when it comes to the CIA-resistant colonisation of food producing animals (Abraham, Groves et al. 2014, Abraham, Jordan et al. 2015, Al-Habsi, Jordan et al. 2018, Kidsley, Abraham et al. 2018, Sodagari, Mohammed et al. 2019, Barlow, McMillan et al. 2022). Several factors have influenced this situation, including Australia's isolated geographic location, long-term regulatory constraints on the use of CIAs in food animals (Australian Strategic and Technical Advisory Group on Antimicrobial Resistance (ASTAG) 2018), and strict border control and quarantine programs, which have reduced the impact of AMR against CIAs in Australia (Turner 2011). The Australian livestock industry has a strong focus on vaccination and animal welfare programs, including a regulatory focus on eliminating chemical residues from animal products, so in some areas the impact of bacterial diseases is minimised. In the commercial layer industry, hens producing eggs for human consumption are completely spared exposure to antimicrobials that might otherwise result in accumulation of residues in eggs.

Eggs produced by commercial layer hens and chicken meat are two of the most frequently consumed animal products around the world. In Australia, the demand for eggs and chicken meat has been increasing steadily for the past decade, with on average each person consuming 246 eggs (Australian Egg Industry 2022) and 50 kg of chicken meat per year (The Australian Chicken Meat Federation 2023). Unfortunately, poor food handling and/or hygiene practices in some domestic and commercial establishments has led to outbreaks of foodborne illness associated with poultry products. Typically, infection is with zoonotic foodborne pathogens such as *Salmonella* and *Campylobacter*, and with non-pathogenic commensal bacteria, such as Enterococci and *E. coli*. (Chousalkar and Gole 2016). Where these organisms show AMR, then human treatment is made more difficult, and resistance traits may be spread to other potential pathogens, increasing the risk to human health via the food chain (de Mesquita Souza Saraiva, Lim et al. 2022).

II. SURVEILLANCE OF ANTIMICROBIAL RESISTANCE

The cornerstone of national and international efforts to address AMR is antimicrobial stewardship – programs and activities designed to halt the emergence and spread of resistance in animal and human populations (Food and Agriculture Organisation of the United Nations , World Organisation for Animal Health 2022, World Health Organization 2023a,b). Surveillance for AMR can help to identify emerging AMR and provide valuable feedback on how to ensure stewardship programs are effective. Surveillance for AMR in food producing animals and food is a well-established activity and the World Health Organisation has longstanding recommendations for performing 'integrated AMR surveillance' as part of the multifaceted management and control of AMR (World Health Organization 2019, 2023a, b). Surveillance of antimicrobial resistance is necessary to; *i) Assess trends in and sources of AMR, ii) Detect the emergence of new AMR mechanisms, iii) Support risk analysis, iv) Provide a basis for policy, v) Evaluate and inform antimicrobial use, and vi) Assess efficacy of interventions* (World Organisation for Animal Health 2022).

Countries such as the USA, Canada and Denmark have been proactive and at the forefront of AMR surveillance in livestock and food. A core component is the collection and analysis of data relating to the AMR profile of indicator bacteria (such as *E. coli* and Enterococci) and foodborne zoonotic pathogens such as *Salmonella and Campylobacter* from both livestock and food products. All these bacterial species may reside in the gastrointestinal tract of healthy animals and birds, although occasionally they may cause disease in these hosts. Australia does not have a formal AMR surveillance program in livestock yet; however, it has undertaken AMR surveys in livestock, including poultry. Some of the key findings from these surveys on indicator and zoonotic bacteria from Australian poultry are described below.

a) Escherichia coli

Escherichia coli is used as one of the key indicator organisms for AMR detection in animals, food and humans. This is due to its commensal nature and abundance in the intestines of warm-blooded animals (Aarestrup, Bager et al. 1998). Acquired antimicrobial resistance is common among *E. coli*, and mobile AMR determinants (transposons, plasmids and integrons carrying genes encoding AMR) are readily acquired and shared within *E. coli* and closely related commensal and pathogenic Gram-negative bacterial species (particularly in the gut) (Mukerji, O'Dea et al. 2017). As such, antimicrobial resistance among *E. coli* may be used as a marker to reflect overall resistance in animals (European Food Safety Authority, 2012).

Studies have indicated that resistance to CIAs among *E. coli* from Australian livestock is relatively low compared to other countries (Abraham, Groves et al. 2014, Barlow, McMillan et al. 2015, Mukerji, O'Dea et al. 2017, Barlow, McMillan et al. 2022), with a 2020 Australian

survey of healthy commercial laying hens supported this status (Australian Eggs 2021). In this study, *E. coli* isolates (n=296) from the faeces of healthy commercial laying hens were collected from 62 farms; 53% of the isolates were found to be susceptible to all antimicrobials tested, whilst all isolates were susceptible to the CIAs cefoxitin, ceftiofur, ceftriaxone, chloramphenicol and colistin. Relatively low frequencies of resistance were observed to amoxicillin-clavulanate (9.1%), ampicillin (16.2%), ciprofloxacin (2.7%), florfenicol (2.4%), gentamicin (1.0%), streptomycin (4.7%), tetracycline (37.8%) and trimethoprim/sulfamethoxazole (9.5%). Multi-drug resistance (MDR) was observed in 21 isolates (7.0%), with one isolate exhibiting resistance to four antimicrobial classes. This study confirmed that *E. coli* isolated from layer hens in Australia have low rates of AMR, and that strict control on antimicrobial usage – through regulation and voluntary measures – is likely to be contributing to this encouraging result.

Rates of AMR among isolates from Australian commercial meat chickens are also generally low in comparison to the situation in other countries (ACMF 2018). Using a robotic antimicrobial susceptibility platform (RASP) in a large-scale survey conducted in 2022, 56.8% of recovered *E. coli* isolates were susceptible to all antimicrobials tested, and no clinical resistance to third generation cephalosporins was detected (Australian Chicken Meat Federation 2022). Resistance to the fluoroquinolone (FQ) ciprofloxacin was detected in 96 isolates (3.25%), with 32 isolates (1.2%) showing clinical resistance. MDR was only present in 2.92% of *E. coli* isolates. Since FQs are not approved for use in the Australian commercial chicken meat industry, these isolates were investigated further. Sequencing identified mutations related to quinolone resistance in 25/32 clinically resistant isolates. *E. coli* belonging to sequence type (ST) ST354 (n=16) and ST773 (n=7) were the dominant FQ-resistant *E. coli* clones, and these clones are globally disseminated in different host species (Australian Chicken Meat Federation 2022). Considering the global prevalence of these FQ-resistant strains, and the fact that FQs are not used in the Australian chicken meat industry, it is likely that the strains have been introduced through an external source. This warrants further investigation into the potential origins of these resistant microorganisms, and the introduction of resistant indicator commensal bacteria into the Australian poultry sector.

b) Enterococcus sp.

Enterococci are commonly present bacteria in the gastrointestinal microbiota of mammals and birds, and generally act as harmless commensals. Nevertheless, certain Enterococci can act as opportunistic pathogens, leading to invasive infections of varying severity in both humans and animals (Byappanahalli, Nevers et al. 2012). In Europe, use of avoparcin (a glycopeptide antimicrobial similar to vancomycin) as a growth promoter in animal feed was implicated in an increase in vancomycin-resistant *Enterococcus faecium* (VRE) colonization in livestock (Bager, Madsen et al. 1997). At this time it was thought that a transfer of resistant strains to humans was occurring from livestock; however, while subsequent studies have identified some genetic similarities between Enterococci from human and livestock sources, and have explored the potential for zoonotic transmission, concrete evidence supporting this transmission has been limited. Rather, it is thought that the routine use of vancomycin in the public health system is responsible for the elevated prevalence of VRE in hospitals. Consistent with this, a 2016 study performed on Enterococci from Australian meat chickens found clinical resistance to a number of different antimicrobials, but it did not identify any resistance to vancomycin (O'Dea, Sahibzada et al. 2019). Moreover, *E. faecium* from meat chickens were found to be genetically distinct from hospital-adapted strains. Another survey across the Australian egg industry conducted in 2020 similarly revealed an absence of VRE among *Enterococcus* isolates (*E. faecium,* n=80; *Enterococcus faecalis,* n=135) (Australian Eggs 2021). In this study, 31.3% of

E. faecium and 39.3% of *E. faecalis* isolates displayed phenotypic susceptibility to all antimicrobials tested. Only one *E. faecium* and three *E. faecalis* isolates displayed an MDR phenotype. All tested *E. faecium* isolates from egg laying birds were susceptible to benzylpenicillin, chloramphenicol, daptomycin, gentamicin, linezolid, teicoplanin, vancomycin, and virginiamycin. Resistance was detected against ampicillin (5.1%), erythromycin (22.5%), and tetracycline (58.8%). Importantly, none of the resistant isolates belonged to the major STs associated with sepsis in humans in Australia in 2015-2017 (O'Dea, Sahibzada et al. 2019, Lee, Jordan et al. 2021) . The *E. faecalis* isolates showed resistance to tetracycline (57%), ampicillin (1.5%), chloramphenicol (1.5%), erythromycin (11.9%), linezolid (0.7%) and streptomycin (1.5%), but none demonstrated resistance to benzylpenicillin, daptomycin, gentamicin, teicoplanin, vancomycin or virginiamycin.

The most recent Australian chicken meat AMR survey on Enterococci (*E. faecium* n=147, *E. faecalis* n=24) conducted in 2022 also confirmed the absence of VRE and linezolid resistant Enterococci from chicken meat birds (Australian Chicken Meat Federation 2022). More than half of the *E. faecium* (64.6%) isolates were susceptible to all antimicrobials tested. In a similar trend to the 2016 survey (O'Dea, Sahibzada et al. 2019), resistance levels to erythromycin (5.4%) and quinupristin-dalfopristin (6.1%) continued to decrease while very low levels of resistance to virginiamycin (2.7%) were detected. The most common resistance was to tetracycline (30.6%), but this figure was also lower than in the 2016 study (40.3%). Among the *E. faecalis* isolates (n=24), the prevalence of tetracycline resistance was 87.5%, and erythromycin resistance was 41.7%. However, the low the number of isolates examined makes it difficult to make broad conclusions from these data.

c) *Salmonella enterica*

Salmonella enterica is one of the key zoonotic pathogens causing gastrointestinal diseases in humans and animals (Gupta, Fontana et al. 2003, Lan, Reeves and Octavia 2009). The emergence of MDR *S. enterica* in livestock is a major threat to animal and human health (Gupta, Fontana et al. 2003), especially when it involves serovars able to cause severe human illness. *Salmonella* from livestock regularly enter the food chain due to post-processing contamination and subsequently pose a risk of human illness due to their zoonotic potential. As a result, any emergence or magnification of AMR, particularly against CIAs, in the source population of animals represents an elevated threat to human health. Consequently, surveillance for *Salmonella* is routinely performed to evaluate the public health risk from both an AMR and a food safety (zoonotic pathogen) perspective.

Recent studies have demonstrated that there are very low levels of AMR in *Salmonella* from Australian livestock, including an absence of resistance to CIAs. To date, no resistance to colistin or carbapenems in *Salmonella* from Australian pigs, sheep or cattle has been reported, with resistance to FQs and ESCs being rare (0–3%) (Abraham, Groves et al. 2014, Barlow, McMillan et al. 2015, Kidsley, Abraham et al. 2018). A 2021 Australia-wide study of the commercial egg layer industry found that all 307 *S. enterica* subspecies *enterica* isolates obtained were susceptible to amoxicillin-clavulanate, azithromycin, ceftiofur, ceftriaxone, ciprofloxacin, colistin, florfenicol, gentamicin, kanamycin, and trimethoprim-sulfamethoxazole. Low levels of resistance to streptomycin (2.3%, n=7), sulfisoxazole (2.0%, n=6), chloramphenicol (1.3%, n=4), tetracycline (1.0%, n=3), ampicillin (2/307; 0.7%) and cefoxitin (2/307; 0.7%) were detected (Veltman, Jordan et al. 2021). Only two isolates were MDR, and no resistance to highest priority CIAs were detected. These extremely low levels of AMR reflect Australia's conservative antimicrobial registration policy in food-producing animals and low rates of antimicrobial use within the industry.

As with the results from the Australian layer bird study, *Salmonella* isolates from Australian meat chickens sampled in 2016 lacked resistance to most antimicrobials tested (Abraham, O'Dea et al. 2019, Australian Chicken Meat Federation 2022). The isolates (n=53) were susceptible to ceftiofur, chloramphenicol, ciprofloxacin, colistin, florfenicol, gentamicin and tetracycline. A few of the isolates exhibited resistance to streptomycin (1.9%), ampicillin (3.8%), or cefoxitin (11.3%). Of note, AMR was only detected among *Salmonella sofia* serovars. None of the *Salmonella* isolates exhibited an MDR phenotype. Whole genome sequencing did not identify any known resistance mechanisms for the *Salmonella* isolates that demonstrated resistance to cefoxitin. In a subsequent 2022 chicken meat survey, despite using the same sample size and bacterial isolation technique, only nine *Salmonella* isolates were recovered, and all were susceptible to all antimicrobials that were tested (Australian Chicken Meat Federation 2022).

d) Campylobacter sp.

Campylobacter infection is one of the leading causes of gastroenteritis in humans, often stemming from the consumption of inadequately cooked poultry. The two major species of concern are *Campylobacter jejuni* and *Campylobacter coli*, which have overtaken *Salmonella* as the key foodborne bacterial pathogens worldwide (European Food Safety, European Centre for Disease and Control 2022). While most cases of human illness resolve on their own, a small percentage of infections necessitate antimicrobial intervention, and a few patients may develop neurological complications. In instances where antimicrobials are required, antimicrobials such as FQs (ciprofloxacin) and macrolides (erythromycin) are recommended for treatment (Ruiz-Palacios 2007, Luangtongkum, Jeon et al. 2009). In the past decade global rates of resistance to first line antimicrobials (tetracyclines) and CIAs (FQs and macrolides) have been on the increase among *Campylobacter* sp. This increasing resistance has contributed to heightened concerns around antimicrobial efficacy for *Campylobacter* infections, resulting in major public health concern worldwide (Ruiz-Palacios 2007, Luangtongkum, Jeon et al. 2009).

An Australian national survey of AMR in *Campylobacter* isolates from meat chickens sampled in 2016 identified low rates of AMR (Abraham, Sahibzada et al. 2020). The survey revealed that most *C. jejuni* (63%) and *C. coli* (86.5%) samples were susceptible to all antimicrobials tested. However, this study uniquely reported the emergence of FQ resistance among *C. jejuni* (14.8%) and *C. coli* (5.4%) in the absence of direct FQ use in the Australian industry (Abraham, Sahibzada et al. 2020). Genomic analysis revealed that FQ-resistant isolates belonged to globally disseminated clones which had been previously reported in humans and animals in other international studies. These included ST7323, ST2083, and ST2343 for *C. jejuni* and ST860 for *C. coli.* Various factors, including the low level of resistance to other antimicrobials, the absence of FQ use in the Australian chicken meat industry, the adoption of measures for preventing spread of contagion between flocks, and particularly the genomic identities of isolates, all point to the hypothesis that the most plausible source of these resistant microorganisms in Australian chickens is external, possibly originating from humans, pest species, or wild birds.

A follow up chicken meat survey conducted in 2022 (Australian Chicken Meat Federation 2022) reported similar result to the 2016 study (Abraham, Sahibzada et al. 2020, Australian Chicken Meat Federation 2022). The majority of *Campylobacter* sp. isolated in this study were susceptible to all antimicrobials tested (68.7% of *C. jejuni* and 88.9 % of *C. coli*). All isolates were susceptable to azithromycin, chloramphenicol, clindamycin, erythromycin, florfenicol and gentamicin, with no MDR detected. The most commonly detected antimicrobial resistance was to the FQ ciprofloxacin, (24.4% *C. jejuni*; 3.2% *C. coli*), nalidixic acid (21.7% *C. jejuni*; 4.8% *C. coli*) and tetracycline (18.3% *C. jejuni*; 1.6% *C. coli*). All isolates showing

ciprofloxacin resistance had mutations known to confer resistance to quinolones. However, FQs are not used in the animal production industry in Australia, so the observed levels of resistance to ciprofloxacin (~25%) in *C. jejuni* are unexplained and concerning. This finding emphasizes the need for ongoing enhanced surveillance among indicator and zoonotic bacterial species: sampling along the food chain helps to understand the origin and dissemination of unusual forms of resistance, and it allows mitigation of risks of AMR to protect public health. Overall, the low AMR rates found in these zoonotic pathogens underscores the efficiency of Australia's AMR stewardship in the Australian chicken meat industry.

III. AMR BIOSECURITY CHALLENGES FROM WILD BIRDS AND HUMANS: A ONE HEALTH CHALLENGE

In recent years, studies have demonstrated that wild birds sharing proximity to humans may act as potential reservoirs for the amplification and transmission of resistant bacteria (Mukerji, Stegger et al. 2019, Mukerji, Gunasekera et al. 2020, Mukerji, Sahibzada et al. 2023). In Australia, studies on silver gulls identified the carriage of CIA-resistant *E. coli* across urban locations in various States (Mukerji, Stegger et al. 2019). High levels of CIA-resistance to ESCs (21.7%), and FQs (23.8%) were observed in isolates from the gulls, with carbapenem and colistin resistance observed at lower frequencies. Genomic analysis of the CIA-resistant *E. coli* identified them as predominantly clinically significant extra-intestinal pathogenic *E. coli* (ExPEC) clones with significant overlap with human clinical isolates. This indicated the existence of a potential bi-directional transmission or an undetermined reservoir connecting both species. The proximity of gulls to humans, their foraging habits (including feeding on human leftovers), and access to human waste, wastewater, and livestock waste were recognized as key factors predisposing gulls to act as major carriers of CIA-resistant *E. coli.* These findings have been validated by other Australian reports (Dolejska, Masarikova et al. 2016, Mukerji, Gunasekera et al. 2020, Wyrsch, Nesporova et al. 2022, Mukerji, Sahibzada et al. 2023), indicating that wild urban birds are a mobile potential ecological reservoir for *E. coli* isolates that are resistant to last-line drugs, and as such they represent a significant biosecurity concern for livestock and poultry, as well as humans.

Recent AMR surveys addressing various food animal systems have demonstrated recurring evidence of human to animal transmission of AMR (HAT-AMR) or bird to animal transmission of AMR (BAT-AMR), which may or may not involve passage through the environment (Abraham, Jagoe et al. 2017, Sahibzada, Abraham et al. 2017, Abraham, Sahibzada et al. 2020). Examples include the detection of human derived methicillin resistant *Staphylococcus aureus* (MRSA) ST93 in Australian pigs and cattle attributed to reverse zoonosis, and the detection of FQ-resistant *C. jejuni* and *C. coli* in Australian poultry in the absence of direct antimicrobial use.

These related issues challenge our understanding about how AMR to CIAs enters, evolves, and persists in food animals, particularly where those drugs have little or no use in the production animal system under question. The biology of both HAT-AMR and BAT-AMR needs to be clarified further due to their potential to impact on animal health, public health and perceptions about the safety of animal food products. We are already aware of instances where human-derived bacteria that are resistant to CIAs have become established in food producing animals, and then caused disease amongst in-contact humans (Groves, O'Sullivan et al. 2014, Sahibzada, Abraham et al. 2017, Abraham, Sahibzada et al. 2020).

AMR is a complex, multifactorial issue and it is fortunate that the advancement in genomic sequencing technology has improved our understanding of multi-directional movement of resistance between isolates from humans, animals, wildlife and the environment as summarised in Figure 1. This potential for transmission highlights the need for better

understanding of AMR from a One Health perspective. Management of these risks requires a broader understanding of the pathways and processes by which animals and products become infected (or contaminated) with isolates showing forms of AMR that would not normally be expected to be present (aberrant resistance).

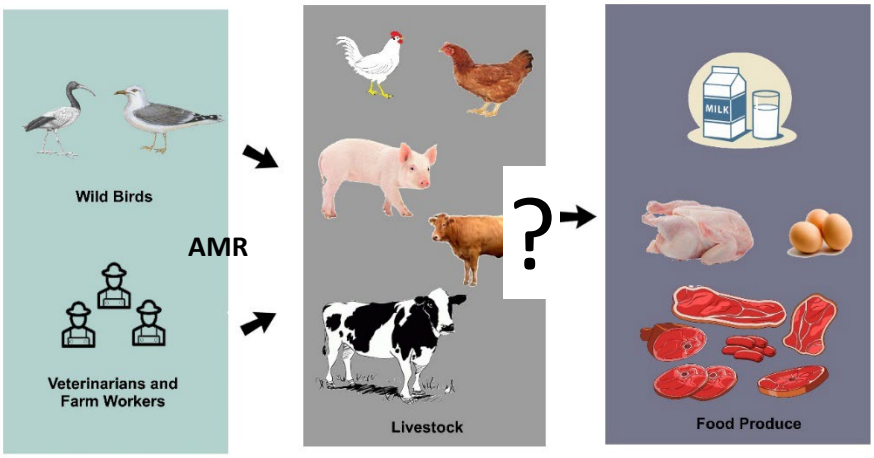

Figure 1 - Potential pathways for movement of antimicrobial resistant bacteria particularly those resistant to critically important antimicrobials (CIA) into livestock.

IV. CONCLUSIONS

The results of the above Australian studies have demonstrated that the responsible use of antimicrobials has helped to ensure that levels of AMR to high and medium importance CIAs remain low in the Australia egg layer and chicken meat industries. This reflects the commendable way that Australia has adopted a One Health approach in tackling the problems of AMR, taking coordinated action across all sectors where antimicrobials are used in the country, as well as coordinating closely with global action. Future work on AMR in poultry in Australia should focus on understanding antimicrobial usage in the industries, identifying the pathways driving AMR, including possible alternative routes for AMR transmission into flocks, and devising measures that can be taken to reduce the presence of resistant bacteria. Apart from regulated exclusion of CIAs from most aspects of livestock production, vaccination against key bacterial pathogens and stringent biosecurity are likely to have contributed to the favourable AMR status of the Australian chicken meat and egg industries. Nevertheless, industry and government agencies need to proactively monitor AMR and promote antimicrobial stewardship to ensure the long-term protection of both animal and human health.

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ENRICHMENT OF EARLY ENVIRONMENTS TO IMPROVE LAYING HEN RESILIENCE AND WELFARE

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Summary

The ability to successfully cope and thrive when faced with environmental disturbance, adversity, and stress is known as resilience. Across a hen's lifetime, they will be exposed to many stressors that require adaptation to maintain internal homeostasis against external variability and continue producing eggs. Birds that are able to successfully cope with environmental challenges will have better welfare. Individual birds from the same genetic background can vary in their personalities and the way they respond to stressors, but inherent responses can also be modulated by the bird's developmental conditions. A collection of studies conducted within Australia and internationally are detailed here to illustrate the positive benefits of environmental enrichment on adaptive capacity of hens when faced with challenges. Future research conducting measurements across a hen's life cycle such as body weight or egg production fluctuations could be applied to quantify lifetime impacts of rearing enrichment in facilitating the development of more resistant hens.

I. INTRODUCTION

Resilience is defined as the ability to successfully cope and thrive when faced with environmental disturbance, adversity, and stress. Across an animal's lifetime, they will be exposed to many stressors that require the individual to adapt and maintain internal homeostasis against external variability. For a laying hen, these stressors could include pathogens, vaccinations, extreme temperature variations, transfer from pullet to layer housing, and conspecific aggression as some examples. The challenges hens face could be greater in loose-housed systems such as free-range, where both the likelihood, and magnitude of daily fluctuations are often higher than more controlled indoor-only housing. In the face of these environmental fluctuations, regulation of daily biological rhythms such as feeding, drinking, activity, and resting to maintain the same functional outcomes of growth and egg production would be indicative of a resilient individual (Bedere et al., 2022; Berghof et al., 2019). Along the same lines, the ability of an individual to cope with compounded stressors is going to affect susceptibility to and recovery from, disease and infection. Thus, high resilience capacity of the individual birds in a flock is desirable for healthier individuals, optimised welfare, and efficiency of production.

It is well understood across laying hens (and other types of production animals) that there is variation among individuals within a flock. Birds of the same genetic history can vary in their physiological responses to stressors, personality traits (e.g., fear and boldness), and coping styles (Campbell et al., 2016; Cockrem, 2013). There is also a growing body of literature on the impacts of rearing environments on hens' subsequent physical health, gut microbiome, and behaviour, including propensity to develop undesirable behaviours such as feather pecking and floor egg laying (Bari et al., 2021; Campbell et al., 2019; Janczak and Riber, 2018). Early life conditions, including the incubation period, the hatching process, and pullet rearing period can all result in different outcomes depending on what the bird was exposed to. For example, high decibel noise during incubation can negatively affect neurotransmitter noradrenaline levels and impair spatial behaviour abilities in day-old chicks (Sanyal et al., 2013). Increased environmental complexity during rearing can improve spatial locomotion and navigation

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abilities of pullets (Rentsch et al., 2023). Commercial hatchery processing conditions versus quieter hatching without transport and delayed vaccination resulted in greater stress reactivity in pullets, increased feather damage, and reduced egg production (Hedlund et al., 2019; Hedlund and Jensen, 2022). It is also well established across many animal species that greater complexity during development will enhance neurological development (Campbell and Lee, 2021). Consequently, there is increasing interest in how environmental modifications, particularly in early life, can modulate and improve adaptive capacity and resilience of individual birds. Experiments with environmental enrichment as a means to enhance functional capacity of individuals are providing evidence of the benefits of developmental complexity (Colditz et al., 2023).

II. ENVIRONMENTAL ENRICHMENT TO ENHANCE ADAPTIVE CAPACITY

Globally, there is a small collection of studies that have been conducted over the past few years investigating the effects of environmental enrichment in enhancing adaptive capacity of hens. More projects around the topic are currently in progress as researchers seek to understand the best strategies for optimising the functioning of individual birds to cope with environmental disturbances which ultimately significantly improves their welfare and production efficiency. Further details of select trials to date are presented here.

The first study to highlight the impact of enrichment on adaptive capacity by Campbell et al. (2021) was conducted in Australia on free-range laying hens within an experimental housing system. This study aimed to understand how varying types of environmental enrichment during the entire pullet rearing period may affect the behaviour, adaptability, and welfare of laying hens at different stages across the production cycle. Hy-Line Brown day-old chicks were housed in an indoor floor-litter based rearing system. Pens with no additional objects served as a 'control' treatment. Two enrichment treatment groups were then also provided with either varying novel objects that were regularly changed throughout rearing as a 'novelty' treatment, or H-shaped perching structures that were fixed in place but had both open-frame and opaque sides to increase the complexity of the spatial environment ('structural' treatment). Within the larger experiment, different assessments were made on samples of the 1400 birds throughout their life cycle with some tests applied to specifically assess whether the enrichments affected the adaptability of the birds.

As stated earlier, individual chickens can differ in their personalities which dictate how they may react to environmental stimuli. They are also known to vary significantly in individual range use patterns. These ranging preferences may be related to personality differences. In this rearing enrichment experiment, it was predicted that the greater environmental complexity relative to control conditions would modulate personality by reducing fear, as well as increasing adaptive capacity (Campbell et al., 2021). A sample of birds were individually tested with a series of behavioural tests at 9 to 11 weeks and the same birds again at 20 to 21 weeks. These behavioural assessments included initial responses to a novel arena, adaptation across time to the novel arena with food present, an open field test, novel maze arena training to access food and finally maze completion testing. Additionally, individual range use was measured from 27 to 31 weeks using radio-frequency identification technology that detected movement in and out of pop-holes by individually tagged hens (Campbell et al., 2021). The results showed that the enrichment treatments reduced the latency to first step in some of the tests which is indicative of reduced fear in a new environment. When 16 correlations were assessed between behavioural test parameters across time, 11 were significant for control birds and only six to seven were significant for the enriched treatments. Furthermore, correlations between test parameters and subsequent range use were significant for only the control birds (Campbell et al., 2021, Table 1). These study results indicate enrichment during rearing may reduce fear and

increase adaptation. Fewer correlations among test parameters including with range use in the enriched hens suggest they developed a more plastic personality type that was flexible to their variable surroundings. A more plastic response strategy could have fitness benefits for hens, particularly free-range birds that encounter drastic environmental change across life stages.

Table 1 - The Spearman's correlations and *P*-values for comparisons between four behavioural test parameters conducted at 20-21 weeks of age and the mean daily time spent ranging as well as mean daily range visits as recorded from 27-31 weeks of age for hens from three rearing enrichment treatments (control: n = 29, novelty: n = 23, structural: n = 24).

Parameter		Rearing treatment		
Behavioural latencies (sec)	Daily ranging	Control	Novelty	Structural
Lat. vocal OFT	Mean duration	r_s = -0.37, P = 0.08	r_s = -0.09, P = 0.67	r_s = -0.13, P = 0.52
	Mean visits	r_s = -0.53, **P = 0.008**	r_s = 0.06, P = 0.77	r_s = -0.10, P = 0.60
Sum lat. train 2-5	Mean duration	r_s = -0.15, P = 0.50	r_s = 0.20, P = 0.37	r_s = -0.04, P = 0.85
	Mean visits	r_s = -0.20, P = 0.36	r_s = 0.19, P = 0.38	r_s = -0.04, P = 0.85
Lat. eat in maze	Mean duration	r_s = -0.32, P = 0.13	r_s = -0.08, P = 0.72	r_s = -0.07, P = 0.74
	Mean visits	r_s = -0.44, **P = 0.03**	r_s = -0.21, P < 0.34	r_s = -0.07, P = 0.71
Lat. leave HZ NO	Mean duration	r_s = -0.50, **P = 0.01**	r_s = -0.06, P = 0.77	r_s = -0.13, P = 0.51
	Mean visits	r_s = -0.66, **P < 0.001**	r_s = 0.11, P = 0.62	r_s = -0.13, P = 0.51

The behavioural test parameters were selected to be predictors of range use based on previous studies. These parameters included the latencies (lat) to vocalise in an open field test (OFT), the summed latency for training to reach food in an arena across the first day of training sessions, the latency to eat in a maze test, and the latency to leave the holding zone with a novel object present (HZ NO) in the maze test. Table adapted from Campbell et al. (2021) with further details on the behavioural tests in that paper. Significant P-values are indicated in **bold.**

Within the same rearing enrichment experiment, the adult free-range hens were exposed to a stressor at 44 weeks of age where the range area they had been accessing for several months was reduced by 80% for 11 days (Bari et al., 2020). Changes in ranging behaviour and albumen corticosterone concentrations were evaluated. Across all hens, ranging time decreased and the average number of range visits increased when the available area was reduced, but there was a lower increase in visit numbers for the structural treatment hens suggesting they were able to better adapt to the environmental change. The corticosterone concentrations also varied across treatments although the results were less clear to interpret. Both the control and novelty treatment hens' eggs showed increases immediately following the range shrinkage which decreased across the range shrinkage period. In contrast, the albumen corticosterone concentration in structural hens' eggs decreased immediately following the range shrinkage and then increased slightly toward the end of the range shrinkage period (Bari et al., 2020). These findings highlight the longer-term impacts of rearing environments and how they can modulate hens' responses to stressors they encounter across their lifetime.

Expanding our focus internationally, a collection of three studies conducted in Sweden investigated the impacts of rearing complexity on stress responses and adaptability in Bovans Robust white layers. In the first study, similar to the previous research with free-range hens, further support was found for early environmental enrichment improving adaptive plasticity (Campderrich et al., 2019). In a two-factorial design, small groups of one-day-old chicks were housed in either simple floor litter pens, or complex pens that included perches, a dark brooder, and wooden blocks. They were then also exposed to an acute 6-h cold stress treatment or not at two days of age. Across five days at four weeks of age, the four different treatment groups (enriched/non-enriched + acute stress/no acute stress) were exposed to intermittent and unpredictable stressors. These implemented stressors included, for example, changing the room temperature, changing out the bedding, random noise playback, and lighting schedule

modifications. Varying immunological parameters were assessed to measure the birds' immunocompetence. Results showed the enriched environment was able to mitigate the negative effects of the cold stress treatment. Similarly, the birds from the enriched rearing had improved physiological responses to the intermittent imposed stressors. This improvement may have been facilitated by increased resting behaviour observed in the enriched birds enabling greater recovery (Campderrich et al., 2019). This research contributes further evidence to the beneficial effects of early complexity and the ability to modulate a bird's phenotypes based on their rearing conditions.

In the second study, the effects of rearing environmental choice were assessed in small bird groups with the prediction that enrichment may have beneficial impacts through the mechanisms of allowing resource choice (i.e., increasing 'agency') in the young birds (Nazar et al., 2022). Treatments were rearing from day one with only a single litter type and a single perch, versus four different litter and perch types. Similar immunocompetence tests were performed at three weeks of age as well as behavioural tests of tonic immobility, novelty, and human-reward motivation conflict. Across all the measures, there was evidence that the rearing with environmental choice improved immunological parameters and indicators of fear and adaptability, although not every parameter showed statistical differences (Nazar et al., 2022). This study again supports that rearing complexity can alter the phenotype of the chick and enhance their coping with what they encounter in their environment.

In a third study by the same research group, Skånberg et al. (2023) built upon the previous findings to assess how both environmental choice, and environmental change affected chick adaptability. Different litter and perch types were provided simultaneously to chicks (static choice) or were swapped around multiple times each week (choice and change), and these treatments were compared with a single choice swapped out multiple times each week (change, no choice), and a no choice static rearing environment deemed to represent standard rearing conditions. When the chicks were four to five weeks old, behavioural tests were conducted including a novel arena test and a social detour test. The test results indicated that greater environmental change had impacts on reducing fear and greater environmental choice, increased exploration, and improved spatial skills. However, combining both change and choice did not have an additive effect on improving the chicks' behavioural adaptability. This indicated these environmental parameters both have positive impacts, but through different mechanisms (Skånberg et al., 2023).

As a final illustration of enrichment impacts, a study in Canada used small groups of young adult ISA Brown hens in floor pens (Ross et al., 2019; Ross et al., 2020). Comparisons were made between housing in control littered floor pens or an enriched floor pen environment with a larger area, and more spatial complexity including perches and platforms as well as foraging opportunities. When the birds were assessed in behavioural tests of judgement bias, restraint, and startle reflexes, including physiological measurement of stress-induced hyperthermia, the enriched hens fared better. Housing in a preferred enriched environment reduced both the behavioural and physiological responses to imposed stressors, including a more rapid return to prestress physiological levels which is indicative of improved resilience (Ross et al., 2020). These stress test measures did not covary with judgement bias assessments indicating different mechanisms of enrichment impact on affective states (Ross et al., 2019; Ross et al., 2020).

III. DISCUSSION AND CONCLUSIONS

The collection of studies detailed here illustrate the effects of early rearing complexity through different environmental enrichment strategies to beneficially modulate the phenotype of the bird. These modulations increase the bird's resilience and adaptability when faced with

environmental stressors and will ultimately improve their welfare. To date, there is some evidence of beneficial rearing effects still being present later in life, and enrichment can also still be beneficial when applied in young adults rather than the early rearing period. However, there is a need for more work to capture how this improved resilience may play out across the entire flock cycle. The studies described in this paper have reported significant beneficial effects on varying single-point measures that included behavioural as well as physiological assessments. However, not every measure showed significant impacts. This highlights the importance of multiple measurements to assess treatment impacts as the exact mechanism via which these improvements are happening is still under investigation. While single time point trait assessments are indicative of a modulated phenotype, there is a need for more continuous flock cycle assessments looking at fluctuations across time to wholly capture the longer-term resilience of the bird. For example, body weight variation in an individual bird across time may function as a heritable indicator of the bird's resilience capacity (Berghof et al., 2019). Furthermore, egg production traits (weekly deviations of an individual from the average) across a flock cycle may be indicative of individual resilience enabling selective breeding of more adaptive animals (Bedere et al., 2022). The holistic functioning of an individual organism across its lifetime and the ability to maintain functional performance in the face of environmental disturbances is a resilience individual that will better cope with challenges it may face (Colditz et al., 2023). Enhancing adaptive capacity through environmental complexity will provide the tools an individual bird needs to better perform throughout its life.

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VISION-BASED MULTI-BIRD TRACKING FOR ANIMAL WELFARE ON COMMERCIAL EGG FARMS

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Summary

Industry, consumers, and society call for continual progress in animal welfare worldwide. Continuous monitoring of animal behaviours conveys health issues, and it is crucial to observe the correlation between animal welfare and productivity, especially in intensive agricultural systems. This is because animal behaviours often reflect their health conditions related to the living environment factors, such as temperature and humidity. Yet, conventional monitoring techniques are laborious or intrusive. Precision Livestock Farming (PLF) leverages sensors, automation, and data interpretation for real-time individual animal supervision and this can boost animal welfare, health and efficiency. We introduce a non-intrusive flock tracking method based on video technology and computer vision for continuous, cost-efficient bird monitoring and recording on commercial egg farms. Our system's precise bird tracking offers accurate data on their activities, paving the way to clear reporting and supporting the investigation of improvements to health and welfare using data.

I. INTRODUCTION

In recent years, there has been a growing public interest in animal welfare in Australia (De Witte, K., 2009). Consumers expect continual and comprehensive improvements in poultry production (Li, Ren, Li, & Zeng, 2020). The health and product quality of poultry also is linked to their welfare. Healthy hens with better emotions tend to lay more eggs of higher quality (Eggs, 2023). The assessment of the welfare of poultry commonly complies with the Welfare Quality Assessment Protocol, which is a standardized method based on four main principles: good feeding, good housing, good health, and appropriate behaviour. These principles are highly correlated with the daily activities exhibited by animals. Therefore, one of the central features of welfare assessment is the emphasis on behaviour-based evaluations (De Jong et al., 2016).

Precision Livestock Farming (PLF) is a method that integrates sensors, automation, and data analysis to streamline and enhance livestock management. It facilitates real-time tracking of each animal, offering insights into individual bird's health, behaviour, and performance (Schillings, Bennett, & Rose, 2021). The goal of implementing PLF is to offer precise early warnings, enabling farmers to take timely actions during the initial stages of issues. It is necessary to monitor individual animals for PLF to provide truly effective early warning capability. Observing individual behaviours also informs management on the health, welfare, and production of each animal, which further enhances the accuracy of welfare assessments (Li et al., 2020).

Conventional individual identification and behaviour analysis typically rely on a radio-frequency identification (RFID) system. For example, to observe the range of laying hens, individual chickens are fitted with a silicone leg band, embedded with a unique ID microchip, to allow monitoring of activity patterns and duration (Larsen, H. et al., 2017). The benefit of applying RFID chips is they can provide individual animal data rather than the flock's behaviours. However, the RFID-based approach is intrusive because the attachment of RFID

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chips requires catching the birds and putting them on. Also, the leg band is only available for adult birds but not the very small ones, which restricts the age for monitoring. Thus, the data collected via RFID chips can only provide limited information for welfare analysis.

In this research, we introduce a novel approach based on machine learning and computer vision to automatically monitor and analyse the behaviour patterns of laying hens, focusing on eating, drinking and fast moving. In our prior work, we evaluated various tracking-by-detection methods to trace the trajectories of sheep simultaneously (Xu et al., 2020). However, compared to larger farmed mammals, commercial laying hens are both smaller in size and more densely populated, which can diminish the effectiveness of tracking systems. This research aimed to build algorithms that track chickens robustly in high-density and occlusion scenarios.

II. METHOD

We designed the monitoring system by mounting several video cameras in the top-down view to observe bird activity in the shed. The system tracks all the visible birds within the covered area using the multi-object detection technique. Specifically, it automatically identifies and tracks each laying hen within a continuous video sequence, so the birds' motions (such as wing flapping and running) and activities (such as eating and drinking) are recorded. To effectively record each bird's motion or activity, the system needs to track the individuals in the whole visible period, i.e., arriving and leaving the observation area, based on the visual appearance. Visual tracking is a challenging problem due to several factors such as occlusion, optical deformation, and illumination changes.

Tracking-by-detection involves first detecting each bird within the video frame sequences using an object detection algorithm. Then, a tracking algorithm is used to link the individual bird detections across frames thereby tracing each bird's trajectory. The Simple Online and Real-time Tracking (SORT)[*] algorithm is a commonly used tracking algorithm that can be used with a tracking-by-detection approach. SORT uses a Kalman filter to predict the object's location in the next frame and associates the detection with the predicted location using the intersection over union (IoU) metric. SORT then updates the Kalman filter's state using the associated detection and repeats the process for the next frame.

Visual tracking based on object detection and SORT is an effective approach that can be used across a variety of applications, such as robotics and self-driving cars (Li, R. et al., 2021). However, in a crowded visual environment, it is highly reliant on an accurate detection algorithm and is vulnerable to occlusion and deformation. Without accurate detection, there can be mistracking of individuals in the fixed observation area, i.e., many birds are re-allocated as new instances. To overcome the difficulties, DeepSORT (Wojke et al., 2017), an extension of SORT, uses a pre-trained model to extract the appearance features from detections, which are then used to compute the similarity score between the detections and existing tracks. However, DeepSORT has high computational complexity, which does not suit most real-time tracking and data recording applications. Since the individual bird tracking is based on the object detection technique, the detection confidence score (i.e., the current bounding box contains an identified real object with known accuracy), plays a key role in ensuring the correct positioning of the object to be tracked. Applying the two methods, objects with low detection scores are discarded, which brings the mis-tracking and fragmented trajectories. In this work, we instead use ByteTrack (Zhang et al., 2022), which implements the tracking by associating all detection bounding boxes instead of only the high-score ones. In the shed of laying hens on commercial egg farms visibility conditions are negatively impacted by illumination and occlusion. The (low) illumination issue is mainly caused by dust, while occlusion is mostly

[*] https://medium.com/@technomadlyf/an-introduction-to-object-tracking-algorithms-a-beginners-guide-877771d3a9cf

caused by birds crowding together and being covered by other birds and other objects (e.g., feeders). Consequently, when the birds are mis-detected due to the low scores, they are very likely to be lost in tracking. For the low-score bounding boxes, ByteTrack utilizes their similarities with 'tracklets' to recover true objects and filter out the background detections.

We implemented the system by deploying the bird detection and tracking system in the shed on a commercial egg farm in Wilberforce, NSW. We used 4 AXIS network cameras connected by PoE ports to a laptop. The object detection model was built with human-annotated instances. For data annotation, 140 frames were densely annotated with bounding boxes and activity labels for 16,991 bird instances engaged in eating, drinking, and other activities. These activities are directly observable by human vision. For example, when a bird's beak is touching the water tap, it is considered drinking. The detection model was trained using these annotated frames, achieving an average precision of 86.3% in identifying birds and activities. Equipped with the ByteTrack algorithm, the system has been running continuously for up to 9 weeks. Video data was exported for analysis as log files, which included the bird ID, time, location, and activity (eating, drinking and other activities).

III. RESULTS

Based on the detection and tracking model, we conducted a case study for bird activity recording and pattern analysis. The tracking visualization is illustrated in Figure 1, where we use a number to uniquely identify a laying hen, and the trajectories represent their movements. Each green or red dot indicates that the bird is eating or not eating at the time. Performance evaluation involved deploying the tracking model on pre-recorded videos and onsite cameras. Under such settings, the miss-tracking rate is 0.0127/second, i.e., within every second, about 1.27% of instances are lost by the trackers due to illumination, crowdedness, occlusion, and other factors. This means there are sufficient birds suitable tracked and monitored for any correlated association with welfare issues to be identified, such as illumination and humidity. The system demonstrated satisfactory robustness and stability, with visualizations depicting detected bird instances and their activities.

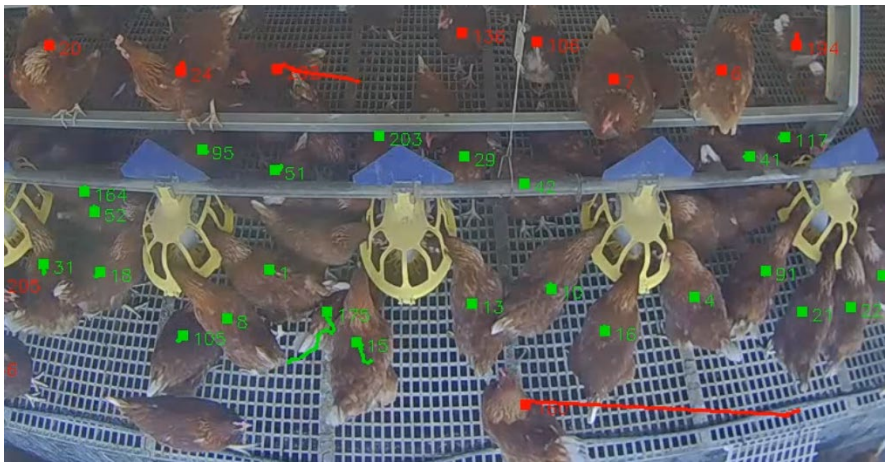

Figure 1 - The visualization of bird activity tracking

IV. DISCUSSION AND CONCLUSION

We have demonstrated a practical application of advanced computer vision and video techniques for bird tracking. Our system monitors individual bird movements and activities on commercial egg farms using a low-cost and robust setup because the cost of video cameras and a desktop is far less expensive than hiring an expert for continuous monitoring. The system is

suited to the automated monitoring of laying hens, which has applications for identifying a wide range of health, welfare and production risks using digital signals, statistical methods and correlation analysis. This data is available for further analysis, such as examining animal welfare status and impacts. The potential welfare issues reflected in bird tracking may be management (e.g., shed inspections) or environmental factors (e.g., humidity and temperature). This demonstrates that bird tracking using automated video camera monitoring has great potential to help improve animal welfare on large-scale commercial egg farms. Based on the feasibility of the current tracking method, the system considers temporal and spatial dependency, e.g., what activities are happening where and when — and what environmental, management or climatic changes are related to these changes, to motivate the research of animal science and contribute to the welfare improvement.

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DOES SMOTHERING OCCUR IN REARING?

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Recent research indicates that smothering accounts for up to 16% of mortality in adult laying hens in Australian commercial free range egg farms (Hemsworth et al., 2022). In addition, UK data indicates that piling (a precursor to smothering where hens press tightly together into dense packs) has implications not only for the risk of mortality due to smothering, but also may negatively impact egg production (Armstrong et al., 2023). Smothering research to date has been focused on identifying risk factors in adult flocks, but there is little available information on the incidence of, and possible risk factors for, smothering during rearing. This study aimed to investigate the incidence of smothering during rearing utilizing a limited data set collected within the research conducted by Hemsworth et al., (2022).

Surveys on management practices and flock behaviour were completed by rearing managers in relation to 50 pullet flocks across Victoria (n=21) and Queensland (n=29). The flocks were either Hy-line Brown (n=42), ISA Brown (n=7) or mixed (ISA Brown and Lohmann, n=1) and were housed indoors in either aviary (n= 17), jumpstart (n= 26) or floor rearing (n = 7) systems from day-old chicks. The surveys were completed by flock managers when the flocks were 16-17 weeks old.

Smothering-related mortality was reported in 44% of the flocks in the study, occurring between 3 to 15 weeks of age. Smothering occurred in 71% (n = 12) of aviary flocks, 35% (n = 8) of jumpstart flocks and 14% (n = 1) of floor-reared flocks. Reported locations for smothering were next to gates or partitions (36%, n = 4), at either the front or the back of the shed (27%, n =3), against walls (9%, n =1), in the system (18%, n =2), or near cooling pads or exhaust fans (9%, n =1). Preliminary analyses on some management practices were conducted using t-tests comparing the mean set temperature (temperature setting for the climate control) and lux (at the time of the survey) of flocks that smothered compared to those that did not. There was no significant difference in shed temperature between flocks with smothering compared to flocks with no smothering. However, in flocks where smothering occurred, the lux at the time of the survey was significantly lower ($\bar{x}_{Smother} = 14.1 \pm 2.1$ lux, $\bar{x}_{No\ smother} = 41.3 \pm 4.2$ lux, $p < 0.001$). Manipulation of lux is a common management practice to reduce problem behaviours (Janczak and Riber, 2015), and thus this association may simply reflect a management strategy to reduce smothering in problematic flocks.

These results are based on a limited data set and thus it is not possible to determine causal factors or risk factors; however, it does clearly indicate that smothering and piling occur during rearing as early as 3 weeks of age. Since early life experiences are known to influence the behaviour of the adult laying hen, there is a need to conduct further research into piling and smothering behaviours during rearing as these behaviours, which are known to be problematic in adult flocks, may actually develop during rearing.

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THE EFFECT OF AM/PM DIETS ON FEED EFFICIENCY, EGG QUALITY AND WELFARE PARAMETERS FOR FREE-RANGE LAYING HENS

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Laying hens have a cyclic reproductive physiology that requires high dietary protein and energy levels for yolk and albumen formation in the early morning and high dietary Ca levels for shell and membrane formation in the afternoon/evening. Therefore, feeding one diet throughout each day may be problematic as there is excess Ca in the morning and excess protein/amino acids and energy in the afternoon/evening. To minimise excess nutrients, there is increasing interest in alternative strategies such as AM/PM feeding (Cadogan and Bruerton, 2021); where a high energy and protein diet with lower Ca is provided in the morning (AM) and a lower energy and protein diet with higher Ca is fed in the afternoon/evening (PM, De Los Mozos et al., 2012). AM/PM feeding has been illustrated to improve feed efficiency, eggshell quality, and reduce environmental pollution (De Los Mozos et al. 2012; van Krimpen et al., 2018), by minimising excess nutrient and allowing the capacity for the hens to sequentially select feed. However, there are opportunities to investigate the potential effects of AM/PM diets on hen welfare, especially with feather pecking, which has been shown to increase with insufficient protein (Mens et al., 2020).

An experiment was conducted at UNE's free-range research facility, where two mash dietary treatments; conventional layer hen diet and AM/PM hen diets were offered to 9 replicate pens of 20 hens each, giving a total of 360 hens (18 pens) from 34 to 53 weeks of age. Diets were wheat-sorghum-soy based and the control contained 3688 kcal/kg gross energy, 17.46% crude protein, 4.53% Ca. Hens offered the AM/PM diets received the AM diet (3787 kcal/kg gross energy, 19.04% crude protein, 3.12% Ca) from 8 am to 4 pm and the PM diet (3500 kcal/kg gross energy, 16.09% crude protein, 5.10% Ca) from 4 pm to 8 am. Egg weight, egg production, daily feed consumption and feed conversion ratio (FCR) were measured weekly. Egg quality and bone quality were measured at week 53. Additionally, hen behaviour was assessed from 49 to 50 weeks of age with camera recordings and individual ranging behaviour was monitored by Radiofrequency Identification (RFID) technology from 39 to 48 weeks of age.

The results showed that AM/PM feeding tended to improve laying hen performance by increasing egg mass by 2.15% (60.4 vs 59.1 g/hen/day, $P = 0.086$) and improving feed efficiency by 8.34% (2.231 vs 2.436 kg feed/kg egg, $P < 0.05$) compared to the control feeding regime over 20 weeks of the study. Hens offered the AM/PM diet also had higher yolk colour score compared to the hens offered the control diet (12.3 vs 11.6, $P < 0.01$). AM/PM hens spent longer on the range (2.85 vs 2.47 hours/day, $P < 0.001$). Hens on the AM/PM treatment had higher tibia ash content (43.3% vs 41.6%, $P < 0.05$) and breaking strength (19.98 kg vs 17.13 kg of force, $P < 0.05$). Furthermore, AM/PM hens were observed to feather-peck less frequently than the control hens (0.39% vs 1.15%, $P = 0.01$). This study demonstrated the production, health, and welfare benefits of AM/PM feeding under Australian free-range conditions.

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FEATURES OF THE EXTENDED LAYING CYCLE OF HY-LINE BROWN HENS REARED UNDER DIFFERENT LIGHTING AND FEEDING REGIMENS

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Summary

The impact of two lighting and three feeding programs during rearing on hen egg-production characteristics across an extended laying cycle was evaluated. Hy-Line Brown chicks were housed in floor pens from day old under two lighting programs i.e. standard lighting (SL) of 10h light (L) /d from 7 weeks of age (WOA) or, rapid light reduction (RLR) of 9h L/d from 4 WOA. Feed was provided *ad libitum* until 4 WOA, when three feeding regimens i.e. *ad libitum* (*ad lib*), feeding to Hy-Line Brown breed standard weight (BSW), and feeding to 88% BSW (Managed) for age were introduced into each lighting program and continued to 16 WOA. Pullets were then transferred to individual cages in a cage layer facility with *ad libitum* feeding and gradual increase in lighting to 16 h L/d at 33 WOA. The feed intake (FI), egg production (EP), egg weight (EW), feed conversion ratio (FCR) and body weight (BW) of each hen was followed through until they were 100 WOA. An interim BW at 72 WOA is also reported. Between 96-100 WOA internal egg characteristics of Haugh unit (HU), relative albumen weight and yolk colour and, shell quality including relative shell weight, shell thickness and breaking strength, were assessed on 12 focal birds/rearing treatment. Lighting and feeding during rearing interacted to impact BW at 16 WOA i.e. pullets of SL and *ad lib* feeding were heaviest and Managed feeding with both SL and RLR were lightest ($P = 0.001$). When 100 WOA, birds that had been fed *ad lib* during rearing had similar BW to hens fed to BSW in rearing, both being heavier than birds from Managed feeding during rearing ($P < 0.001$). Hens from the former two treatments also consumed more feed ($P = 0.073$) across the laying phase. During their 100th WOA hen EP ranged 62-69%, which was not affected by the rearing treatment. Overall hens from all rearing regimens had produced 500 eggs by 100 WOA but, at 100 WOA hens reared under SL produced heavier eggs compared to rearing with RLR ($P=0.015$). Hens that had been fed to BSW and Managed feeding during rearing had lower FCR during their 100th WOA ($P= 0.004$), and numerically lower cumulative FCR throughout the laying phase. Eggs produced across 96-100 WOA by focal hens from Managed feeding during rearing had the highest HU ($P = 0.002$) and, *ad lib* feeding the lowest HU. Relative albumen weight was highest in eggs from hens reared under SL compared to RLR ($P = 0.037$), whereas the feeding regimens during rearing had no effect on relative albumen weight. There were no differences in relative shell weight, shell thickness nor shell breaking strength. Managing FI during rearing generated smaller hens with lower FCR but similar EP and eggs of higher HU compared to the larger sized hen that was achieved by *ad lib* feeding throughout rearing.

I. INTRODUCTION

The physiological patterns of hen FI and BW trajectory are established by early lay (Muir et al. 2023a). Careful management of lighting and feeding patterns during rearing can regulate bird size and feeding habits by the end of rearing (Muir et al. 2023b). Whether this influences persistency of lay, efficiency of production and egg quality during an extended laying period

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is unknown. The lighting program during rearing can alter pullet age at first egg (AFE), the number of eggs produced and EW (Santiago-Anadón and Latorre-Acevedo, 2004; Arango et al. 2007). We have shown that a more RLR during rearing, which provides fewer hours of light/day, slows chick growth and on light stimulation will initiate earlier AFE, compared to a SL schedule in current brown egg-laying hen strains (Muir et al. 2023b). But the ongoing impact of the lighting and feeding programs employed during rearing on Brown egg-laying hens held in an extended laying cycle has not been evaluated. Therefore, this longitudinal study followed hens reared under different lighting and feeding programs (Muir et al. 2023b) until they were 100 WOA, including measurement of their FI, EP, EW and FCR. The quality of the eggs produced towards the end of their laying cycle was also evaluated.

II. MATERIALS AND METHODS

As described in Muir et al. (2023b) 900 Hy-Line Brown day-old chicks, that had been beak trimmed and vaccinated at the hatchery were placed in groups of 30 in floor pens (7 m^2) at the Zootechny research facility, Austral, NSW, Australia. Each pen had a perch, automatic nipple drinkers and manually filled feed hoppers. The shed was brooded with space heaters, had side curtains, foggers, and dimmable lights with photoperiod control for each end of the shed. A light proof curtain traversed the centre of the shed separating 15 pens to each of the two lighting regimens. All birds were held under intermittent lighting during the first week (4hLight(L):2hDark(D)) then 20hL:4hD in the second week. For RLR the photoperiod was reduced as 16hL:8hD, then 12hL:12hD and finally held at 9hL/d from 4-16 WOA. Under SL program 20hL:4hD was maintained through to 3 WOA then reduced gradually to 10hL:14hD by 7 WOA and held there through to 16 WOA. All birds were fed *ad libitum* until 4 WOA when three feeding programs of five pens per lighting treatment were introduced. This was *ad libitum (ad lib)*; feeding to achieve breed standard weight (BSW); and feeding to achieve 88% BSW (Managed) for age. Hence the study was a 2 × 3 factorial arrangement of 2 lighting and 3 feeding programs. All birds receive the same commercial crumble pullet starter (0-5 WOA) and grower (5-12 WOA) (Barastoc, Australia), then a developer mash (12-16 WOA). When 16 WOA, 75 pullets/treatment were moved into individual pens in a high-rise layer shed under a common lighting program with gradual increase to 16 h L/d at 33 WOA. From 16-17.4 WOA all pullets received pre-lay diet and from 17.5-100 WOA diets changed through feeding phases 1-5 as per Hy-Line Brown breed recommendation (Hy-Line International 2018). From 16 WOA all diets were mash and fed *ad libitum*. Bird BW was measured at 16, 72 and 100 WOA, FI, EP, EW, egg mass (EM) and FCR were measured when hens were 100 WOA, and their cumulative measures were calculated from 17.5–100 WOA. Eggs from 12 focal birds/rearing treatment were assessed for EW, HU, relative albumen weight, yolk colour, relative shell weight, shell thickness and shell breaking strength, during 96-100 WOA. All data were analysed using a factorial ANOVA with lighting and feeding regimens during rearing as main effects.

III. RESULTS AND DISCUSSION

At 16 WOA BW was highest in pullets of *ad lib* feeding under SL and lowest for pullets of Managed feeding under both SL and RLR (Table 1). Similar trends in BW were evident at 72 WOA but BW was above breed standard for age (1.97 kg) in all treatments. At 100 WOA hens from Managed feeding during rearing had the lowest BW but FI was similar for all treatments. The 17.5-100 WOA cumulative FI was approaching significance as birds from Managed feeding during rearing had the lowest FI ($P = 0.073$; Table 1). Egg production at 100 WOA was similar, ranging from 62.3–68.9% and essentially all hens produced 500 eggs by 100 WOA. At 100 WOA hens fed *ad lib* during rearing had higher FCR at 100 WOA compared to

birds from restricted FI (BSW or Managed) rearing regimens (P = 0.004; Table 1). Further, cumulative FCR (17.5–100 WOA) was numerically lower in hens from Managed compared to *ad lib* feeding in rearing. Earlier in production, cumulative 17.5–61 WOA FCR had been significantly lower (data not shown) in lighter hens from Managed feeding during rearing, concurring with lower cumulative FCR of smaller hens from 18-69 WOA in a previous study (Muir et al. 2022) and then numerically lower FCR through to 89 WOA (Muir et al. 2023a).

Table 1 - Body weight at 16, 72 and 100 weeks, 100 week and cumulative (17.5-100 weeks) feed intake, egg production and feed conversion of Hy-Line Brown hens reared in different lighting and feeding regimens.

Treatment regimens during rearing	BW (kg) wk 16	BW (kg) wk 72	BW (kg) wk 100	FI (g/d) wk 100	CFI (kg) wks 17.5 -100	EP (%) wk 100	CEP wks 17.5-100	FCR (g/g) wk 100	CFCR (g/g) wks 17.5-100
Lighting									
SL	1.37	2.30	2.35	110	67.2	66.0	505	2.73	2.32
RLR	1.36	2.24	2.32	109	66.3	65.3	503	2.70	2.42
Feeding									
AD	1.50^{a}	2.32	2.38^{a}	109	67.4	62.8	503	3.18^{a}	2.47
BSW	1.37^{b}	2.31	2.38^{a}	113	67.1	68.0	502	2.53^{b}	2.53
M	1.22^{c}	2.16	2.24^{b}	108	65.9	66.1	506	2.43^{b}	2.29
Interaction									
SL*AD	1.52^{a}	2.41^{A}	2.44	114	68.7	63.2	507	3.26	2.36
SL*BSW	1.37^{c}	2.32^{A}	2.37	110	67.1	68.9	501	2.49	2.33
SL*M	1.21^{d}	2.16^{B}	2.25	108	65.9	65.7	506	2.45	2.27
RLR*AD	1.48^{b}	2.24^{AB}	2.33	104	66.0	62.3	499	3.11	2.57
RLR*BSW	1.37^{c}	2.31^{A}	2.38	115	67.0	67.0	504	2.57	2.36
RLR*M	1.23^{d}	2.16^{B}	2.23	109	65.8	66.5	506	2.41	2.32
P- Values									
Lighting	0.078	0.036	0.248	0.659	0.106	0.861	0.803	0.857	0.231
Feeding	< 0.001	<0.001	<0.001	0.332	0.073	0.549	0.897	0.004	0.183
Interaction	0.001	0.018	0.289	0.064	0.117	0.960	0.821	0.906	0.592

Lighting: Lighting program*;* SL*:* Standard lighting; RLR: Rapid light reduction; *Feeding*: Feeding program; AD: *Ad libitum*; BSW: Fed to achieve Breed standard weight for age; M: Managed feeding to achieve 88% BSW for age; BW: Body weight; wk: Week; FI: Feed intake; C: cumulative; EP: Egg production; FCR: feed conversion ratio.

Based on average EW from all eggs produced by hens of each treatment group, SL eggs were heavier compared to RLR (P = 0.015). Whereas, when based on EW of eggs from focal hens only, differences were approaching significance (P = 0.061; Table 2). Relative albumen weight was higher due to SL during rearing. Eggs from hens reared within the Managed feeding regimen had the highest HU (P < 0.01) though all HU met the >81 HU breed standard for age (Hy-Line International, 2018). Yolk colour was also above the recommended score of 11 (Roberts 2004). There were no differences in shell quality. Relative shell weight was >9% and shell thickness >0.35 mm for all treatments, minimizing the chances of shell cracks (Parkinson et al. 2008). Egg weight at 100 WOA remained around 65 g, aiding shell quality compared to larger (>70 g) eggs.

This study illustrated the opportunity to program hens to different sizes during lay by managing their FI during rearing. Smaller hens had lower FCR while producing a similar number of eggs with higher HU, compared to larger sized hens that have been fed *ad lib* throughout rearing. There may be opportunity to provide pullets of lower BW with a nutrient dense diet during early lay to provide additional nutrients in preparation for a longer laying cycle (Muir et al. 2023a).

Table 2 - Egg weight from all birds at 100 weeks * and characteristics of eggs produced by Hy-line Brown focal hens between 96-100 weeks of age following rearing in different lighting and feeding regimens.

Treatment regimens during rearing	Egg weight 100* wks (g)	Egg weight 96-100 wks (g)	Haugh unit	Album wt. (%)#	Yolk wt. (%)#	Yolk colour (1-15 score)	Shell wt. (%)#	Shell thick-ness (mm)	Shell breaking strength (kg)
Lighting									
SL	65.4	64.4	88.9	61.4	25.8	11.8	9.04	0.356	3.67
RLR	63.7	62.3	90.5	60.2	26.6	11.6	9.22	0.365	3.68
Feeding									
AD	64.1	63.7	84.2	60.9	26.3	11.8	8.99	0.365	3.67
BSW	65.0	64.2	90.2	60.9	26.0	11.4	9.02	0.360	3.58
M	64.5	62.0	94.8	60.6	26.3	11.7	9.38	0.370	3.78
Interaction									
SL*AD	65.1	64.6	81.1	61.2	26.2	12.0	8.90	0.360	3.57
SL*BSW	65.5	65.0	90.2	61.7	25.6	11.5	8.83	0.362	3.63
SL*M	65.4	63.6	95.5	61.4	25.7	11.8	9.39	0.373	3.81
RLR*AD	63.1	62.9	87.3	60.7	26.4	11.7	9.08	0.370	3.77
RLR*BSW	64.4	63.5	90.1	60.1	26.4	11.4	9.21	0.357	3.54
RLR*M	63.6	60.4	94.2	59.8	27.0	11.6	9.38	0.368	3.75
P- Values									
Lighting	0.015	0.061	0.498	0.037	0.079	0.192	0.319	0.939	0.895
Feeding	0.598	0.223	<0.01	0.880	0.782	0.113	0.153	0.289	0.219
Interaction	0.854	0.795	0.386	0.693	0.563	0.930	0.684	0.483	0.369

Lighting: Lighting program*;* SL*:* Standard lighting; RLR: Rapid light reduction m; *Feeding*: Feeding program; AD: *Ad libitum*; BSW: Fed to achieve Breed standard weight for age; M: Managed feeding to achieve 88% BSW for age; * egg weight for eggs from all birds within the treatment; wt: weight; # wt as a percent of egg wt.

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CIRCADIAN RHYTHMS AND ADAPTIVE DEVELOPMENT: HOW UNDERSTANDING CIRCADIAN RHYTHMS DURING MEAT CHICKEN INCUBATION COULD IMPROVE DEVELOPMENT AND TIMING OF THE HATCH WINDOW

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Adult chickens utilise external circadian cues to schedule their daily cycles. In other taxa, developing embryos can actively entrain the timing of life transitions to a circadian rhythm (Villamizar et al., 2013, Saunders, 2002), but we currently have a poor understanding of how circadian rhythms develop in meat chicken embryos. Commercial poultry are not exposed to diurnal light changes during incubation, removing the possible use of such cues for optimising hatch timing. Synchronising hatching is expected to reduce the hatch window and potentially improve both welfare and performance, as chicks would experience reduced wait times for access to feed and water (Careghi et al., 2005).

We hypothesised that circadian cues throughout incubation could benefit both hatching synchrony and post-hatch development. We tested the influence of two environmental circadian cues throughout incubation (light and temperature) on Ross308 broiler embryos using a 2 × 2 full factorial experimental design (n = 12 batches, 3 batches / treatment). Once hatched, chicks (n = 275) were individually tagged and raised in randomised mixed floor pens following breed guidelines until day 14. To quantify the hatch window, incubators were checked every 30 minutes and hatched chicks were counted. Lastly, to quantify differences in post-hatch development, all chicks were weighed, tarsus was measured, and a subset of chicks (n = 137) were scanned in a quantitative EchoMRI body composition analyser on days 1, 7 and 14. Hatch window and developmental variables were statistically analysed using one-way ANOVAs.

Initial data showed no significant effect for circadian light and temperature cues to increase the length of incubation before hatching of the first chick (n = 12 hatch windows, 277 eggs, $P > 0.05$). There were no significant treatment effects on chick weight or tarsus length (both n = 277 chicks, $P > 0.05$) at 1, 7 or 14 days post-hatch, and no significant effect on chick body composition (n = 137 chicks, $P > 0.05$). Whilst pilot data from our initial experiment provided no evidence that circadian light or temperature rhythms influenced the onset of hatching or development; we suggest that there is potential for other triggers to influence hatch timing and potential for the poultry industry to employ these to reduce the time before day-old chicks have access to feed and water. Future research should focus on improving the hatch window through means of replicating natural environmental conditions or focussing on particular light wavelengths.

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EFFECT OF ACTIVATED VITAMIN D3 ON EGGSHELL QUALITY AND PERFORMANCE IN OLDER BROWN LAYING HENS

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Summary

Calcitriol, the active form of vitamin D, may have beneficial effects on calcium utilisation and thus eggshell quality in older laying hens, as the hydroxylation reactions required to activate vitamin D_3 and 25-hydroxyvitamin D_3 ($25(OH)D_3$) are bypassed (Nys *et al*., 1999). In this study, hen production and egg quality were evaluated in older Hy-Line Brown laying hens fed a control diet, a control diet plus 75 g of activated vitamin D_3 /tonne (Treatment 1) or a control diet plus 125 g of activated vitamin D_3 /tonne (Treatment 2), over a 20-week trial beginning at 60 weeks of age (woa). At 68 woa, Treatment 2 had a significantly higher eggshell weight than the control group ($P = 0.02$). In addition, both Treatment 1 ($P < 0.001$) and Treatment 2 ($P < 0.001$) had significantly higher shell thicknesses than the control at 80 woa, indicating that activated vitamin D_3 supplementation may prevent the reduction in eggshell thickness observed in older hens. However, no significant difference in shell-breaking strength was found between the dietary treatments throughout the trial. Overall, dietary-activated vitamin D_3 supplementation in older laying hens was demonstrated to have some beneficial effects on eggshell quality.

I. INTRODUCTION

The mineralisation of calciferous eggshells is an extremely physiologically demanding process, having immense calcium requirements. As a result, laying hens have highly efficient, closely managed calcium homeostatic mechanisms involving a complex series of feedback loops primarily regulated by parathyroid hormone, calcitonin, and calcitriol (de Matos, 2008; Sinclair-Black *et al*., 2023). Calcitriol is the bioactive form of vitamin D, which increases calcium absorption from the small intestine (Chandra *et al*., 1990) and calcium reabsorption from the kidneys and bone (Schenck *et al*., 2012) during periods of hypocalcaemia.

A reduction in eggshell quality and breaking strength is frequently observed in older laying hens (Roland, 1979; Roberts *et al*., 2013). It is a current aim of the global egg industry to extend the production cycle of laying hens to 100 weeks to gain both financial and sustainability benefits (Dunn, 2013; Bain *et al*., 2016). However, for an extended production cycle to be adopted commercially, eggshell quality and laying rate must be maintained during the late stages of production (Molnár *et al*., 2016). One strategy being investigated to improve eggshell quality in older hens is the nutritional management of vitamin D. Calcitriol glycoside, an herbal form of vitamin D_3, is in development as an alternate source of vitamin D for poultry. By supplementing vitamin D in the bioactive form, the hydroxylation reactions required to activate vitamin D_3 and $25(OH)D_3$ are bypassed, potentially improving calcium utilisation in older hens.

A feed additive that contains calcitriol glycoside, ursolic acid and oleanolic acid has recently been released in the market. The product is said to improve eggshell quality and performance, particularly in laying hens over 50 woa although there has yet to be published data supporting this. Therefore, this study aimed to quantify the effect of activated vitamin D_3 supplementation on eggshell quality and performance in laying hens from 60-80 woa.

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II. METHOD

A total of 240 Hy-Line Brown layer hens, 55 woa, were purchased from a commercial laying farm and housed in the high-rise layer facility at The University of Sydney's Camden Campus. Each bird was kept in an individual cage (25 × 25 × 50 cm) with access to a common feeder trough and an individual nipple drinker. All hens were fed the control diet, *ad libitum*, for a 5-week acclimation period. At 60 woa each bird was weighed and randomly allocated to one of three dietary treatment groups (Control, Treatment 1, and Treatment 2) consisting of 80 hens each. Each group was further subdivided into 8 replicates comprised of 10 hens (24 replicates total). The Control diet was formulated based on wheat, soybean meal and canola meal (AME 2750 kcal/kg and digestible Lysine 0.69%) and contained 3 MIU of vitamin D_3/kg. Treatment 1 consisted of a control diet plus 75 g of activated vitamin D_3 product/ton, while Treatment 2 consisted of the control diet plus 125 g of activated vitamin D_3 product/ ton (Table 1). All diets were fed *ad libitum*. From 60-80 woa, egg production (EP), egg weight (EW) and feed intake (FI) were measured weekly, and egg mass (EM) and feed conversion ratio (FCR) were calculated. At 66, 68, 76 and 80 woa, 2 eggs from each replicate were randomly selected for quality testing, including Haugh unit (HU), yolk colour, eggshell breaking strength, eggshell thickness, and relative eggshell weight (%). Finally, at 80 woa each bird was re-weighed. Data was analysed using a one-way ANOVA for the three dietary treatments (Control, Treatment 1, and Treatment 2) when hens were 66, 68, 76 or 80 woa).

Table 1 - Nutrient composition of activated vitamin D_3 product (mg) per tonne of diet.

	Treatment 1	Treatment 2
Glycosidic calcitriol	0.5625	0.9375
Ursolic acid	1209	2015
Oleanolic acid	249.75	416.25

III. RESULTS

The effects of the dietary treatments on egg quality at 66, 68, 76 and 80 hen woa are presented in Table 2. Egg weight was not different due to dietary treatments at weeks 66 ($P = 0.65$), 72 ($P = 0.96$) and 80 ($P = 0.72$). At 68 woa, Treatment 2 had a lower EW than the control group ($P = 0.02$), but Treatment 1 EW was similar to control ($P = 0.50$) or Treatment 2 ($P = 0.26$).

At 66 ($P = 0.18$), 72 ($P = 0.61$) and 80 woa ($P = 0.60$), eggshell weight did not differ due to dietary treatments. However, at 68 woa, Treatment 2 generated a higher shell weight than the control ($P = 0.02$), but the shell weight of Treatment 1 was like the control ($P = 0.25$) and Treatment 2 ($P = 0.55$). Shell thickness did not differ at 66 ($P = 0.12$), 68 ($P = 0.13$) and 72 woa ($P = 0.54$). Nevertheless, at 80 woa, both Treatment 1 ($P < 0.001$) and Treatment 2 ($P < 0.001$) had higher shell thicknesses than the control. Shell breaking strength was not different between dietary treatments at 66 ($P = 0.68$), 68 ($P = 0.44$), 72 ($P = 0.49$) or 80 woa ($P = 0.42$).

Table 2 - Average egg quality data.

Hen age & dietary Treatments	Egg Weight (g)	Haugh Unit	Yolk Colour	Shell Weight (%)	Shell Thickness (mm)	Shell breaking strength (g)
Week 66						
Control	64.2±0.5	88.82^{ab}±1.20	10.80^{a}±0.26	10.55±0.13	0.47±0.007	4567±209
Trt 1	64.4±0.7	86.55^{a}±2.65	11.93^{b}±0.31	10.43±0.13	0.46±0.006	4521±125
Trt 2	65.0±0.6	93.53^{b}±1.56	11.87^{ab}±0.38	10.40±0.13	0.45±0.007	4353±197
P-value	0.081n.s.	0.039*	0.026*	0.680n.s.	0.124n.s.	0.683n.s.
Week 68						
Control	68.0^{a}±1.2	102.85^{a}±1.84	11.20±0.80	11.61^{b}±0.33	0.42±0.014	4421±165
Trt 1	65.9^{ab}±1.0	94.26^{b}±2.01	11.67±0.25	12.56^{ab}±0.31	0.46±0.014	4341±208
Trt 2	62.9^{b}±1.6	95.89^{b}±1.59	12.20±0.28	13.17^{a}±0.55	0.45±0.014	4095±168
P-value	0.034*	0.004**	0.097n.s.	0.034*	0.152n.s.	0.437n.s.
Week 76						
Control	66.4±1.0	98.60±4.15	11.94±0.30	10.97±0.29	0.45±0.007	4000±190
Trt 1	66.8±1.2	100.68±2.48	10.63±0.78	10.95±0.27	0.45±0.010	3832±154
Trt 2	66.4±0.9	104.22±1.61	11.25±0.27	10.62±0.26	0.44±0.012	4131±188
P-value	0.437n.s.	0.399n.s.	0.222n.s.	0.615n.s.	0.570n.s.	0.490n.s.
Week 80						
Control	68.5±1.0	97.40^{ab}±2.77	12.38±0.27	10.00±0.21	0.38^{b}±0.014	4468±256
Trt 1	69.6±1.3	92.68^{a}±1.95	12.00±0.20	9.74±0.10	0.45^{a}±0.010	4262±201
Trt 2	68.6±0.9	102.86^{b}±2.57	12.27±0.27	9.81±0.24	0.45^{a}±0.012	4048±187
P-value	0.284n.s.	0.020*	0.525n.s.	0.594n.s.	0.0001***	0.418n.s.

Trt 1: Treatment 1 (formulated with 75g of activated vitamin D_3 product/ton of control diet); Trt 2: Treatment 2 (formulated with 125g of activated vitamin D_3 product/ton of control diet. ^{a,b} Columns for age not sharing a common superscript differ at: *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; n.s., not significant.

IV. DISCUSSION

The supplementation of increasing concentrations of different sources of vitamin D in the diets of older laying hens has previously demonstrated inconsistent effects on eggshell quality. Some studies indicate that increasing dietary vitamin D in older hens improves shell quality (Plaimast *et al*., 2015; Wen *et al*., 2019; Jing *et al*., 2022), while other studies suggest that there are no effects (Mattila *et al*., 2004; Li *et al*., 2023). In this study, the supplementation of 125 µg of activated vitamin D_3 /ton of diet produced higher eggshell weight than the control diet when hens were 68 woa and had been on the supplemented diet for 18 weeks. However, 75 g of activated vitamin D3 / ton supplementation did not affect eggshell weight. This contradicts the results of Wen *et al*. (2019), which suggest that increasing vitamin D_3 supplementation does not affect eggshell weight.

In the activated vitamin D_3 supplemented treatments, shell thickness was maintained relatively consistently throughout the trial, but had dropped notably in the control birds at 80 woa. Concurrently, supplementation of both concentrations of activated vitamin D_3 increased eggshell thickness compared to the control diet, indicating that activated vitamin D_3 may prevent the reduction in shell thickness frequently observed as hens age. Similarly, Jing *et al*. (2022) observed an increase in shell thickness when Roman Grey hens, 60 woa, were supplemented with 125 µg vitamin D_3 or 125 µg 25(OH)D_3 /kg diet compared to the control of 62.5 µg/kg of vitamin D_3. Unlike Jing *et al*. (2022), an increase in eggshell-breaking strength in the vitamin D treatments compared to the control was not found in this study. This concurs

with the findings of Li *et al*. (2023), where supplementation of 69 and 125 μg/kg of 25(OH)D_3 did not affect eggshell breaking in hens at 70 woa. However, it should be noted that Li *et al*. (2023) found no significant differences in shell weight and shell thickness with increasing 25(OH)D_3 supplementation in contrast to this study. Additionally, the improvement observed in eggshell thickness at week 4 of supplementation by Jing *et al*. (2022) preceded the increased shell-breaking strength observed at week 8. Therefore, activated vitamin D_3 supplementation may improve eggshell-breaking strength after increases in shell thickness.

While no direct improvement in eggshell breaking strength was observed, significant improvements in relative shell weight and thickness indicate that supplementing activated vitamin D_3 in older laying hens may benefit eggshell quality. As calcitriol also plays a vital role in regulating bone mineralization, further investigations into the effects of activated vitamin D3 supplementation on bone strength in older laying hens is underway. Additionally, increased vitamin D_3 concentrations in the diets of laying hens have previously been found also to increase the vitamin D3 concentrations in eggs (Mattila *et al*., 2003; Plaimast *et al*., 2015). Therefore, it should be determined if similar effects in eggs occur due to the supplementation of activated vitamin D_3 in laying hens.

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WHAT MATTERS IN SPOTTY LIVER DISEASE?

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Summary

The epidemiology of Spotty Liver Disease (SLD - caused by *Campylobacter hepaticus*) in cage-free egg layers is only just beginning to be explored. Analytical epidemiological studies began with a cross sectional survey linking housing and management factors to SLD occurrence. This identified having a scratch area in the house as a major risk factor for SLD. Two further studies were then undertaken to search for other putative risk factors in houses that did or did not have a scratch area (i.e., partial or full floor coverage by plastic slats). These further studies identified natural ventilation systems and higher nest stocking rates (i.e., higher bird numbers / m^2 of available nest space) were risk factors for SLD occurrence or higher SLD severity. There were also associations of a slightly delayed onset of egg production and a slightly later age of transfer of the birds to the layer house with more likelihood of SLD occurring.

I. INTRODUCTION

Spotty Liver Disease (SLD) is a major problem in free-range layer farming (Courtice et al., 2018). The causative agent of SLD has been identified as *Campylobacter hepaticus* (Van et al., 2017). The epidemiology of SLD in cage-free layers is poorly understood. Three analytical epidemiological studies have been conducted in this series. The studies surveyed cage-free layer houses across Australia, searching for statistical associations between facility and management practices with the occurrence of SLD. Factors included house design and furniture, ventilation systems, floor space, feeder and drinker space and types, nest type and space allowance, slat design, range size, and many bird performance parameters. Cage-free housing varies within Australia, with older houses converted to free-range designs ("conventional free-range"), barn style, aviary system without ranging or aviary system with free-ranging. Ventilation systems varied between natural ventilation (open sided houses) or various mechanically ventilated houses (roof extraction or tunnel ventilation designs). Cage-free houses may have the entire floor area covered by slats or may have an exposed floor area (scratch area) to allow dust bathing inside the house.

II. METHOD

Several formal epidemiological surveys were conducted in Australia over 2019 to 2022 from flocks up to 40 weeks of age. These involved extensive housing and management questionnaires and collection of cloacal swabs, faeces and/or dust for detection of *C. hepaticus* by PCR from each flock. Recorded findings were cross tabulated against occurrence of SLD, or its severity. Initial comparisons used Pearson's Chi-square analysis for categorical factors and Student's t-tests for continuous data. Any factor showing an initial statistical association with SLD with a scanning P value of <0.20 (Hosmer et al., 2013) were then considered using multivariate technique (multiple logistic regression) where confounding could be reduced, and the most important putative factors studied in closer detail, while controlling for the effects of other factors.

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III. RESULTS AND DISCUSSION

Survey 1 identified the presence of a scratch area as a major risk factor for SLD (Table 1), with every such house being a clinical SLD case (Gao et al., 2023a). Transmission route for *C. hepaticus* is regarded as fecal-oral (Phung et al., 2022) and hence the greater exposure to faeces afforded by a scratch area explains this finding, while full slat coverage of the floor provides separation from much fecal material inside the house and thus can be somewhat protective against SLD. Of the fully slatted houses, 45% had clinical SLD, but the remaining sample size for the latter restricted further findings. Hence surveys 2 and 3 were conducted to evaluate other factors in either fully slatted houses or houses with a scratch area.

Table 1 - Survey #1: Distribution of SLD cases by slat coverage of floor.

Slat coverage	No. flocks with SLD	No. flocks without SLD	Odds ratio	P=
Partial	13	0	∞	0.003
Full	5	6		

Survey 2 (Gao et al., 2023b) examined only flocks in houses that had full slat cover of the floor areas. Several factors were found to be significantly associated with the occurrence of clinical SLD in this housing mode.

Houses with full slat coverage and with tunnel ventilation capacity appeared to be protected against SLD, compared with open-sided naturally ventilated houses (Table 2). This finding agrees with a field report of an ability to decrease SLD severity if house temperature can be lowered (Courtice, 2022). Within houses with natural ventilation, an association of SLD occurrence was found with bird numbers per m^2 nest space area, where for every extra bird per m^2 the odds of SLD increased by 17.2% (Table 3). The results suggested that maximum nest stocking densities to help avoid clinical SLD for brown egg layers in naturally ventilated houses would be 112 birds per m^2 of nest space. A further finding of interest was putative associations between several factors involved with the time lag for flocks to come into early egg production. All of these factors were related to each other, and they were statistically autocorrelated. Hence, a representative factor was selected for these, being the time between transfer and the flock reaching 60% HD production, as this had the least missing data points. Flocks that had longer lag times in reaching 60% HD production were at a higher risk of SLD occurrence. It is not clear whether this putative association reflects a cause or an effect. Arguably, flocks coming into lay slightly later may be more prone to SLD outbreaks. Conversely, flocks that were sub-clinically affected by SLD early in lay may have had their onset of lay delayed by this infection. We infer that this factor may be something of a predictor for SLD, with flocks beginning lay (i.e. reaching 5% HD) at 20 weeks of age being more likely to experience later clinical SLD than those beginning lay at 19 weeks of age. Cloacal swabs from flocks during lay revealed that flocks with clinical SLD outbreaks had significantly higher numbers of birds with positive detections of *C. hepaticus* than did the clinically unaffected flocks. It was of high interest that C. hepaticus could be detected in flocks which never broke with clinical SLD.

Table 2 - Survey #2: Distribution of SLD cases across ventilation systems in fully slatted houses.

Factor	Level	No. flocks with SLD	No. flocks without SLD	Odds ratio	P=
Ventilation	Mechanical	0	8	0	0.005
	Natural	18	23		

Table 3 - Survey #2: Logistic regression for nest stocking density as a risk for SLD.

Factor	Level	Estimate (β)	Std Err	Odds ratio	P=
Nest stocking density	Birds/ m^2	0.158	0.100	1.172	0.015

β = logistic regression coefficient

Survey 3 (unpublished data) was conducted in 48 houses across Australia which had a scratch area, in either conventional free-range, barn or aviary style housing. The occurrence of SLD was high in this survey (as predicted by Survey #1), hence a severity score, based on magnitude and duration of the mortality and egg production drop and whether antibiotic treatment was necessary, was used to categorize flocks with higher or lower disease levels. Many factors were evaluated, and confounding between factors was considerable. Hence multiple logistic regression techniques were used to distinguish the most statistically important effects. After multiple analysis controlling for the presence of all factors, the analyses reduced the statistically important factors to two – age at transfer to the layer house (later transfer compared to transfer one week earlier) and nest space allowance, where for every extra bird/ m^2 of nest space, risk of more severe SLD increased by 3.4%.

Survey 3 thus confirmed the effect of higher nest density (as seen in survey 2) where a higher number of birds placed per m^2 of available nest space increased risk of severe SLD The effect of nest density may increase SLD severity risk at a lower stocking rate in houses with a scratch area than that seen in fully slatted houses.

It is concluded that greater exposure of birds to fresh faeces in a scratch area inside the house is a strong risk factor for SLD. Further, a higher number of birds per m^2 of nest space increases the risk of SLD. The use of mechanical ventilation (roof extraction or tunnel ventilation systems) in houses may decrease the risk and severity of SLD occurrence. Later transfer to the layer house may also increase the risk of SLD severity.

These findings represent new information on the epidemiology of SLD and provide direction for prevention of the disease.

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GLYCERIDES OF LAURIC ACID SUPPLEMENTATION IN THE CHICKEN DIET ENHANCES THE HUMORAL AND CELLULAR IMMUNE RESPONSE TO INFECTIOUS BRONCHITIS VIRUS

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Summary

Pathogen's infections and medication reduction are important challenges in poultry industry. Many solutions are developed to enhance the immune response against pathogens such as the glycerides of lauric acid (GLA) supplementation which, besides its anti- microbial effect, can improve the immune response. In the present study, we aim to determine the effects of GLA supplementation in chickens' diets on humoral and cellular immune response to a pathogenic aggression, using an *in vivo* model of infectious bronchitis virus (IBV).

One-day-old Ross 308 broilers were vaccinated *via* eye-nose drops with live attenuated IBV and fed diets supplemented or not with GLA at 3 kg/ton. The levels of early (day 7) specific anti-IBV in broilers' *sera* (n = 12/group) significantly increased in broilers fed GLA supplemented diet compared to the control groups ($P < 0.05$) showing a better primary immune response. Basal T lymphocytes' cytokines secretions remained similar in the spleens of all experimental. Interestingly, the splenocytes of broilers fed with GLA, showed higher activation and effector abilities measured by IFN-γ ELISpot after 24h exposure to IBV antigens (N-261-280 peptide) or antigen independent mitogen (Con A). In response to the IBV N-261-280 peptide, GLA group showed a 2-fold increase of spot numbers ($P < 0.05$) and 3-fold increase of spot surfaces ($P < 0.01$) compared the control and non-vaccinated groups. Similarly, Con A stimulation showed a 2-fold increase of spot surfaces and numbers in the GLA supplemented group ($P < 0.01$).

In summary, our findings show that GLA supplementation in the feed improves the intensity of primary humoral immune response. Additionally, GLA supplementation enhanced the levels of specific cellular immune response mediated by T lymphocytes as well as the reservoir of effector T cells. Altogether, we show *in vivo* how GLA supplementation in the diet can enhance chicken resilience against pathogenic challenges by strengthening their immune response.

I. INTRODUCTION

The emergence of zoonoses, antimicrobial resistance, and more recently pandemics, has led to a rapid escalation of society demands regarding animal production and consumption. In addition to conventional practices like the genetic selection and vaccination strategies, numerous emerging solutions such as the supplementation of probiotics or short chain fatty acids in animal feed are being pursued to enhance animal health and resilience to disease. The glyceride of lauric acid (GLA) has demonstrated numerous notable direct antimicrobial and antiviral properties (Nitbani et al. 2022; Hornung, Amtmann et Sauer 1994; Nakatsuji et al. 2009; Lieberman, Enig et Preuss 2006; Matsue et al. 2019; Welch et al. 2020) which allows its use for disease prevention and medication reduction purposes. Furthermore, numerous studies conducted on human and rodent models described the potent immunomodulatory properties of lauric acid, and more generally medium chain fatty acids (MCFA, Bhutia et Ganapathy 2015). Lauric acid have been observed to enhance the differentiation, activation and antigen presenting capabilities of macrophages (Lee et al. 2003) and dendritic cells (Weatherill et al. 2005). Moreover, studies on inflammatory models demonstrated that the inclusion of lauric acid in diets promotes the differentiation of Th1 and Th17 T lymphocytes while disfavoring the regulatory T cells (Haghikia et al. 2015a; Bhutia et Ganapathy 2015; Hammer et al. 2017). Wong et al. (2009) showed that lauric acid also enhances B cell activation leading to increased secretion of

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IgG antibodies (Hammer et al. 2017). These immune modulations exacerbate clinical conditions of inflammatory diseases (Bhutia et Ganapathy 2015) but may improve the immunocompetence during viral infections. Hence, lauric acid sources such as GLA can be used to enhance the immune response against viral pathogens like infectious bronchitis virus (IBV). In this study, we hypothesize that the glycerides of lauric acid supplementation in chicken diets improves the kinetics and levels of humoral and cell mediated immune response to viral infections. To test this hypothesis, we used an *in vivo* model of broilers vaccinated with a live attenuated IBV strain.

II. MATERIAL AND METHODS

A total of 200, 1-day-old male Ross 308 were assigned randomly to 3 experimental groups: non vaccinated (NV), vaccinated and non GLA supplemented (VC) and vaccinated supplemented with GLA (VG) at 3 kg/ton (Adisseo NL, Ruisvoorn, Netherlands). Broiler from the vaccinated groups received, by eye nose drop, a dose of the attenuated live IBV strain 1/96 on day 1 (Ceva, Libourne, France). No other vaccine has been administrated to the birds. The body weight was measured individually on the 1st, 7th, 14th, and 28th day.

A) Antibodies quantification in chicken sera: Total IgY and IBV-specific antibodies were weekly quantified in *sera* of 12 broilers/group. The indirect ELISA were performed by Chicken IgY kit #ab189577 (Abcam, Cambridge, UK) and the Idexx IBV Ab kit 99-09262 (Westbrook, ME, US).

B) Chicken IFN-γ ELISpot assays: Chicken spleens, isolated and subsequently cut into pieces, were gently mashed, in RPMI culture medium (Thermo Fischer Scientific, Waltham, Massachusetts, US), to extract the cells. The splenocytes were filtrated through Falcon® 40 µm cell strainer then centrifuged at 150 g for 5 min then resuspended in RPMI medium. The cell suspension recentrifuged for 30 min at 400g in presence of the density gradient medium Histopaque® 1077 (Sigma-Aldrich). Splenic leukocytes were collected, suspended in the culture medium, supplemented with 8% of foetal calf serum and 2 % of chicken serum and 1% of L-glutamine (Thermo Fischer Scientific, Waltham, Massachusetts, US). 3×10^5 live cells / 200 µL of medium were distributed in wells of coated plates Mabtech® ELISpot Plus Chicken IFN-γ (Stockholm, Sweden) and subseauently incubated for 24 hours at 41°C and 5 % CO_2 in presence of three distinct stimulation groups: without stimulation (control), Concanavalin A (Con A) at 5 µg/mL (Sigma-Aldrich, Saint-Louis, Missouri, US), and the 261-280 peptide of IBV nucleocapsid protein (N 261-280) described by (Qin et al. 2021), at a concentration of 30 µg/mL (synthetised by Genscript Biotech, Piscataway, New Jersey, US). The IFN-γ ELISpot images were acquired using an NI-E® microscope and NIS Elements® software (Nikon, Tokyo, Japan). The numbers and sizes of IFN-γ spots were measured by ImageJ software.

C) Cytokines quantification: Protein lysates were prepared from 10^7 isolated chickens' splenocytes by using Invitrogen® cell lysis buffer II in presence of Pierce protease inhibitor (Thermo Fischer Scientific, Waltham, Massachusetts, US). Protein concentrations were measured by Interchim® BCA assay kit (Montluçon, France). The cytokines (IL-2, IL-6, IL-10, IL-16, IL-21, IFN-α, IFN-γ and M-CSF) concentrations were measured by Milliplex® chicken cytokine/chemokine panel 1 kit for multiplex ELISA (Merck-Millipore, Burlington, Massachusetts, US) according to the manufacturer's instructions.

D) Statistical analysis: All statistical analyses were conducted using JMP statistical software (SAS Institute, Cary, NC, USA). The measured parameters were analysed using either ANOVA with Tukey's test or an independent two-tailed t-test.

III. RESULTS

A) Vaccine administration reduced broilers performances: The performances results indicate that broilrs from the two vaccinated groups (VC: vaccinated control and VG: vaccine + GLA) showed a 15% lower average body weight compared to the non-vaccinated (NV) group starting from day 7 (average BW of 165 vs. 194g respectively, $p < 0.001$). The average body weight gain in the vaccinated groups was affected only during days 1-14 with a reduction of 19% and 12 % during days 1-7 and days 7-14, respectively ($p < 0.001$), but no significant differences were observed during 14-28-day period. The decline in broilers' performance can be attributed to the potential fever and

lethargy following the administration of the vaccine via eye/nose drops.

B) GLA supplementation reduced total IgY secretion in the blood: Sera from the vaccinated control (VC) group presented significantly higher levels of total IgY antibodies compared to the NV group on day 7 ($P < 0.05$) and the VG group on day 14 ($P < 0.01$). Overall, the VC demonstrated numerically higher levels of total IgY antibodies in the blood sera compared to the GLA supplemented groups, regardless the vaccine administration. These findings suggest that GLA supplementation in the diet may have an impact on total IgY antibodies secretion.

C) GLA supplementation increased primary humoral immune response: The kinetics of anti-IBV antibodies levels in chicken *sera* showed an overall pattern of anti-IBV antibodies titers with two distinct phases in both vaccinated groups. A first phase occurred during the first week, possibly indicating a primary anti-IBV humoral response, followed by a secretion phase starting at the third week. No significant differences were observed between the GLA-supplemented (VG) and the control group on the second phase, 21 and 28 d. However, the VG group presented significantly higher levels of anti-IBV antibodies at day 7 measured by ELISA OD ($P < 0.05$) and as ratio on total IgY. These results indicate a higher humoral primary immune response in presence of GLA in diets.

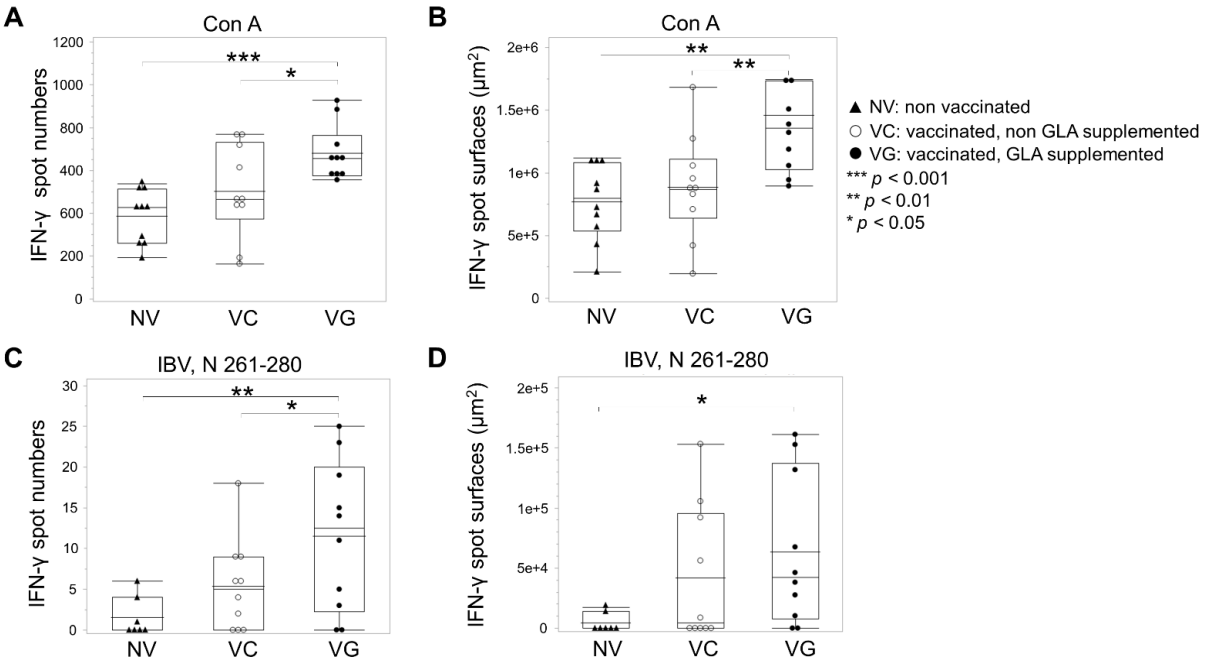

Figure 1 - Boxplot representing chicken IFN-γ ELISpot quantification of splenocytes stimulated with Con A or IBV N 261-280 peptide. Individual quantification of spot numbers (A and C) and sizes (B and D) of chicken splenocytes IFN-γ ELISpot after stimulation with Con A pan T cell (A and B) or with IBV specific N 261-280 peptide (C and D) antigens, for 24 hours. Data are individually presented by dark triangles (▲) for the non-vaccinated group and circles for the vaccinated groups supplemented (●) or not (○) with glycerides of lauric acid. Horizontal bars represent the mean and median values. Statistical differences were evaluated by impaired two tailed T-test.

D) GLA and IBV-vaccine administration did not affect the cytokines production spleens: The quantification of cytokines revealed no effect of GLA supplementation nor vaccination on T cell proliferation, as indicated by low levels of IL-2 in all experimental groups. Neither vaccination nor the GLA supplementation affected the basal levels of cytokines secretion by T lymphocytes subsets in the spleen like IFN-γ, IL-10 and IL-21 at day 21.

E) GLA supplementation in diet improved overall and IBV-specific T cell mediated response: To gain a deeper understanding of the specific and the overall cellular immune response, we conducted chicken IFN-γ ELISpot assays to assess the effects of GLA on Th1/CTL like responses after 24 hours of stimulation with Con A or IBV N 261-280 peptide. Following Con A stimulation, splenocytes from VG group showed a 2-fold increase of average number of IFN-γ spots (680 ± 41 *vs*. 388 ± 41 spots) and spot surfaces ($1.46\times10^6 \pm 1.8\times10^5$ *vs*.

$7.66\times10^5 \pm 0.96\times10^5$ μm^2) compared to the NV group ($P < 0.001$, Fig. 1A-B). They also demonstrated significantly higher spot numbers and surfaces compared to the VC group (504 spots/well and 8.85×10^5 μm^2, respectively). Upon stimulation with IBV N 261-280 peptide, splenocytes from VG group displayed significantly higher average spot numbers (12 ± 3 spots/well) compared to the NV (2 ± 0.9 spots/well, $P = 0.006$) and VC (5 ± 1.8 spots/well, $P < 0.05$, Fig. 1C). Moreover, a significantly higher average spot surface was observed in VG group (63569 μm^2) compared to the NV group (4482 μm^2, $P < 0.05$). Spot surfaces and numbers of splenocytes did not reveal any significant differences between the NV and groups (Fig. 1). These findings suggest that GLA supplementation in diet increased the proportions and activity of total and IBV-specific effector/memory Th1/CTL like lymphocytes.

IV. CONCLUSIONS

In summary our study suggests that GLA supplementation in chicken diet improved both humoral and cellular immune response against IBV. It increased the primary humoral response in the first week of broilers life which predict of a better secondary humoral response (Gussem et al. 2021) and higher survival rate (Wit, Swart et Fabri 2010a) after a IBV challenge. The effector/memory lymphocytes and the levels of specific T cell mediated response were enhanced in proportion and intensity insuring an efficient cellular response. Altogether, our results show that the GLA-induced modulations of immune response are adapted to intracellular infections like IBV and may enhance the efficiency of vaccines.

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MANAGING LITTER QUALITY THROUGH VENTILATION AND AIR MOVEMENT

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Summary

Litter quality, which is mostly determined by litter moisture, influences both the birds and the house environment they are raised within. Ventilation is an important part of controlling poultry house moisture and maintaining litter quality; however, ventilation alone will not keep litter dry and therefore air movement over the litter is required. A series of field studies were conducted to evaluate the impact of maintaining house relative humidity at 60% or lower and using high volume circulation fans to move more air over the litter. Litter moisture and footpad dermatitis evaluations were conducted weekly starting in the second week of the flock grow-out period. Litter moisture was lower and the incidence and severity of footpad dermatitis were reduced. The results of this work support the recommended practice of maintaining relative humidity below 60% and utilizing high volume circulation fans to move more air over the litter to keep it drier.

I. INTRODUCTION

Poultry house moisture contributes to many of the issues that poultry farms experience. As moisture builds in the litter/bedding, microbial populations thrive, more ammonia is generated, and the incidence and severity of footpad dermatitis increases. Much of the moisture in the house comes from the birds themselves, so in order to control moisture, one must understand the current moisture loads in the house. For example, broilers grow faster and convert feed more efficiently than 35 years ago. Broilers will drink 1.8-2.0 ml of water for every gram of feed consumed under typical rearing conditions (Czarick and Lacy, 2001; Fairchild and Czarick, 2006; Williams et al, 2013). As a result, birds are drinking more water and adding more water to the house environment through excretion and respiration, which means that ventilation rates should be increased proportionally. However, that is not what is practiced in many cases. Other things that have changed that affect house moisture levels include tighter housing, which has less air leakage. This means that proper ventilation rates are more important today because air exchange due to leakage is much less. It also means that the ability to remove moisture from the house has become more efficient. In order to maintain optimal bedding and air quality conditions for the birds, the houses have to be ventilated appropriately.

The use of circulation fans systems has been employed for several decades. These systems were developed and tested to provide more uniform house temperature and conserve energy (Czarick and Fairchild, 2003). While those systems were effective, the desire to have drier floors requires an air circulation system capable of moving more air across the floor. The objective of the current study was to evaluate the effect of moving 20% of the total house air volume every minute on the poultry house environment and the subsequent impact on litter moisture and footpad dermatitis.

II. METHOD

A series of 10 trials were conducted on poultry farms in Northeast Georgia during the months with cold weather (October-April). The farms utilized used litter (pine shavings was the original bedding) ranging from 2 to 5+ years and had an approximate depth of 15-20 cm. On each farm, one house served as the control and the other house was the treatment. The

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circulation fan system in these houses was replaced with 61 cm diameter, 1/3 horsepower fans, which move approximately 2.6 m^3/s (Munters CX24, Munters Corporaton, Amesbury, MA). Enough fans were installed to move approximately 20% of the total house air volume every minute, which generated an air speed of 0.1 - 0.8 m/s. The circulation fans were installed 30 cm to the side of the radiant tube heaters (which were installed in the center of the house and 15 cm from the ceiling). The fans were operated continuously starting when the house heating system was turned on to preheat the house and remained on for the entire grow-out period. The environment of both houses were managed according to the farm's normal operating procedures with the exception of minimum ventilation, which was manually adjusted as needed to maintain house relative humidity (Rh) at 60% or lower. All of the farms utilized were with the same integrator that was raising the broilers to a market weight of approximately 2.04 kg, which was typically achieved in 40-42 d.

Temperature and Rh were recorded using a temperature/Rh sensor. The temperature/Rh sensors (Onset HOBO External Temperature/RH sensor data logger- MX2302A; Bourne, MA) were connected to a datalogging system that recorded the data every five minutes. Footpad dermatitis and litter moisture were evaluated weekly. Footpad dermatitis lesions on 200 birds per house were quantified using a 3-point evaluation system where 0 = no lesions, 1= minor lesion (less than half the footpad area, and 2 = severe lesion that was larger than half the footpad area. Litter samples were taken across the width of the brooding area at four locations on both farms (30 cm off the inside drinker line towards the center and 30 cm off the outside drinker towards the sidewall) and down the length of the brooding area at four to six locations (n = 16-24 per house per sample day). Samples were transferred to a lab where a 200 g homogenized sample was oven dried over a period of 24 h at 70-75 °C. Samples were reweighed to calculate moisture content. No statistical analysis other than descriptive statistics were utilized.

III. RESULTS

All trials yielded similar results and the results from one of those trials are presented. These studies were replicated in four flocks on one farm and one flock on a second farm with a total of five flocks. The farms did a good job of maintaining the house Rh at 60% or lower (Figure 1) in both control and treatment houses. Litter moisture was lower and more uniform in the houses with more air movement compared to the control.

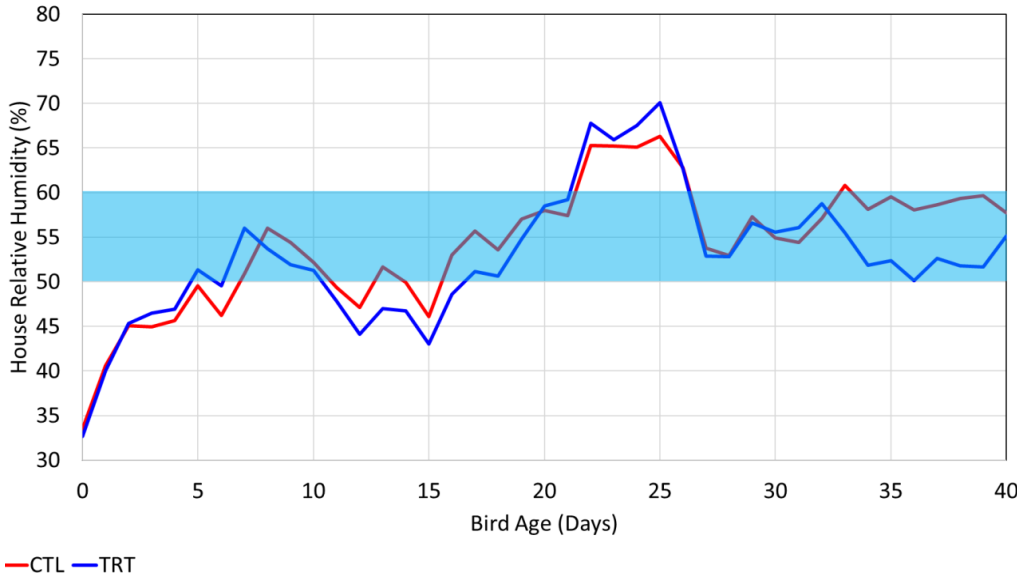

Figure 1 - House relative humidity. Note that the house Rh was 60% or less most of the time. The one period where it increased above 60% was due to a rainy weather.

Average house litter moisture and footpad dermatitis scores are presented in Figure 2. Houses with more air movement had lower litter moisture and subsequently less incidence and severity of footpad dermatitis than the control houses.

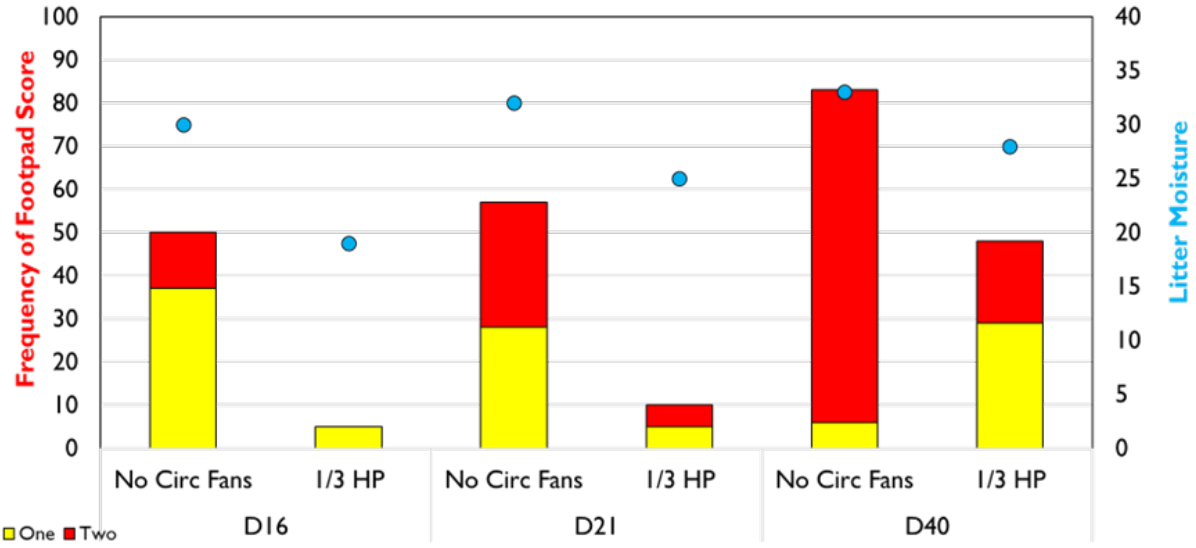

Figure 2 - Footpad lesion scores (%) and litter moisture (%) on days 16, 21 and 40 of the grow-out (Mou, 2020). The red bars are footpad scores of 2, the yellow bar are footpad scores of 1 and the difference between these two and 100 are the footpad scores of 0. The blue dot is litter moisture on the day that foot pad scores were recorded. No Circ Fans = houses with no circulation fans and 1/3 HP = houses with high volume air circulation fans.

IV. DISCUSSION

Wet litter is highly correlated with poor bird performance, health, and welfare (Dunlop et al., 2016). Moisture contributes to many issues that concern poultry growers such as litter quality, ammonia concentration, footpad dermatitis, coccidiosis, and microbial levels (Martland, 1985; Bilgili et al., 2009; Shepherd and Fairchild, 2010; De Jong et al., 2012; Kazuyo et al., 2013). Drier houses tend to provide better environments for poultry than those with high moisture, although dust concentrations may be higher. The removal of litter moisture is aided by heat and air movement. Air temperatures are typically warmer near the ceiling and circulation fans are used to not only break up the vertical stratification but can also move air from warmer areas of the house to cooler areas. The results of this study are similar to those observed by Weaver and Meijerhof (1991) where lower Rh was associated with less incidence and severity of footpad dermatitis and lameness due to leg problems. The results of this study demonstrated that the combination of maintaining relative humidity below 60% and increasing air movement, provided with high volume circulation fans, resulted in lower and more uniform litter moisture across the width of the house. This in turn resulted in lower frequency and severity of footpad dermatitis. Lu (2019) reported that birds with more air movement, in houses with proper brooding temperature, did not chill the birds. While not shown in the paper, birds were distributed more evenly across the width of the houses that had the increased air movement. This is due to more uniform temperatures from wall to wall, and is generally considered to be a sign that the chickens are at the correct temperature because they are not huddling in groups (a sign that they are cold) or gathering along the side-walls (a sign that they are too warm). The circulation fans not only distribute the warm air at the ceiling and warmer parts of the house, but also reduce the hot areas that can occur below the radiant heaters (Mou, 2020).

Utilizing a combination of ventilating to maintain Rh below 60% and increased air movement with circulation fans provided a better environment (demonstrated by lower incidence and severity of footpad dermatitis) for the broilers in these trials. This included drier litter and more uniform floor temperatures.

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IS INSECT PROTEIN A SUSTAINABLE OPTION FOR POULTRY DIETS?

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Summary

Sustainability comprises three distinct components, and any strategy adopted by the poultry industry needs to be considered in this light. Insects are often claimed as a sustainable protein source for poultry production. How it forms a part of the circular economy and reduces the environmental footprint bears testimony to this. Small-scale insect production is relatively simple, but commercial production will always be challenging because, with few exceptions, the setup costs of production facilities are high. In addition, the energy demand for running intensive insect production facilities is high. For insects to be transformed into acceptable feedstuff, some form of rendering is required, which is both energy-demanding and expensive. Using "cheap" waste streams as feedstock will be challenging from both food safety and production economic perspectives. Hence, the true sustainability of insect protein should probably be viewed in two dimensions. Small-scale production and the feeding of live insects to chickens that are deprived of protein is undoubtedly sustainable, but large-scale commercial production is less likely to be so.

I. INTRODUCTION

Sustainability comprises three principal components: the environment (demand for resources and potential for environmental pollution), ethical issues (welfare of man and his animals), and economic robustness (FAO, 2012). At first glance, insect protein is a sustainable alternative to traditional protein sources such as soybean meal. Insect production requires less land, water and other resources than traditional protein sources, and they can be raised on "upcycled" material from the human food supply chain (vegetable waste), or on decomposing organic matter not typically consumed by humans. The waste stream of insect production (frass) can be a fertilizer source for crop production (DiGiacomo and Leury, 2019).

Insects provide a favourable supply of AA for broiler chickens (Veldkamp and Bosch, 2015) together with adequate energy, although levels depend on the processing method. However, the costs associated with insect protein production can be high, bringing any benefits in terms of financial robustness into doubt. Insect production remains small and is faced with problems that beset most start-ups. These include a lack of investment in research and development, the problems associated with engineering controls and scaling up and an inconsistent regulatory environment. Despite this, the industry has the potential to provide insects to production animals (DiGiacomo, 2023). This paper explores insect production and will consider its possible role in sustainable poultry production.

II. INSECTS AS NUTRIMENT

Although several species of insect have been used as a source of insect protein, this paper will focus on the production of Black Soldier Fly (BSF) larvae and the meal produced from them. BSF is a source of protein, fat, metabolizable energy (AME), phosphorus, and fibre, which can be used as a replacement for other protein sources, such as soybean meal (SBM) and fishmeal (Facey et al.,2023). The protein quality of BSF was comparable with fish meal and SBM (Cheng et al., 2023). It is contented BSF can be used to replace fish meal in poultry diets, but it should be remembered that very little fish meal is used in modern poultry diets because of its

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cost. Of interest, BSF meal produced in East Africa is exported to Europe for use by the pet food industry. For a nutritionist to utilise any ingredient, details of its energy and digestible amino acid content are required. It has been established that insect meals contain high levels of energy and digestible AA compared with ingredients commonly used in poultry diets, and although excellent values for BSF have been published (Matin et al., 2021; INRAE, 2023), the nutritionist is still faced with the variability which exists in products from different production facilities, and indeed, on which feedstock was used in their production.

Several proteins expressed by insects serve as antimicrobial peptides and may serve as an alternative to antibiotics, enhancing immunocompetence and gut health in production animals (Li et al., 2012). Insects also contain high concentrations of chitin and medium-chain fatty acids (lauric and myristic acid), which are thought to improve both gut and immune health in broiler chickens through prebiotic and antimicrobial properties, reducing the reliance on antibiotics and coccidiostats in the poultry industry (Dörper et al., 2020; Bean-Hodgins et al., 2021).

Growth performance responses to the replacement of SBM with BSF are been variable, with some research finding that when less than 30% SBM was replaced, no change or improvement in broiler performance was measured (Dabbou et al., 2018; de Souza Vilela et al., 2021) In cases where replacement exceeded 50%, reduced performance was experienced (Dabbou et al., 2018; Murawska et al., 2021). Higher relative weights of the gizzard, small intestine, pancreas, and liver were observed at higher BSF inclusion, giving rise to health concerns (Facey et al.,2023). In laying hens, the substitution of fish meal with BSF did not affect the laying rate, feed intake, or FCR, although an increase in body weight was recorded (3% BSF meal) (Patterson et al., 2021; Zhao et al., 2022).

Approximately 2.5 billion people depend on small farms globally (FAO, 2013), many living below the poverty line (WHO, 2020). These small farmers, only contribute 8% of global egg and 2% of poultry meat production (Mottet et al., 2016). Despite the many benefits of intensive production, small-scale, local production is crucial in any move towards global sustainability and poverty alleviation. Small-scale farmers often face a challenge when trying to source protein for their poultry, and the protein provided by feeding insects will address this shortfall and improve performance. In addition, they will help reduce organic waste and pollution (Khusro et al., 2012; Józefiak et al., 2015).

III. INSECT PRODUCTION

Several aspects of insect protein production ought to be considered. First, insect production requires some form of production facility. These can range from small-scale, subsistence systems on farms to sophisticated modern climate-controlled ones. Second, before any product of animal origin can be used in poultry diets, rendering is required before a ‘safe’ product is available to be marketed. This involves high temperature and pressure (usually 3.5 Bar for 30 minutes), and then most of the remaining moisture needs to be driven off (Koutsos, 2021).

Commercial BSF production is high-intensity animal production, a methodology familiar to poultry producers. Climate control entails well-insulated growing rooms, which determine the quantity and quality of insect meal. Several parameters must be controlled, including food stock (substrate) and room temperature, humidity, ammonia and CO_2 levels. The process requires energy-efficient technologies and sophisticated climate control computers (www.insectengineers.com, 2023). An idea of the production scale is given by (Farrugia, 2022). A minimum viable level for onsite production would be approximately 2,000 tons of wet larvae a year (7 tons a day), representing about 2 tons on a dry matter basis. The use of non-conventional substrates is being explored for mass production of insects. These include food waste streams, agricultural by-products or manure from livestock farms. This application

of the circular economy reduces the environmental footprint and economic costs associated with insect production. However, edible insects can also be associated with several food safety hazards, including biological agents (bacterial, viral, fungal) and chemical contaminants (pesticides, toxic metals, pharmaceuticals). Farming insects under controlled hygienic conditions and implementing sanitary processing techniques reduces some hazards, but any production system should include mechanisms to prevent, detect, identify and mitigate such food safety concerns (FAO, 2021).

The nutrient content and the performance aspects of reared insects depend on the substrate used. Spranghers et al., (2017) offered BSF larvae three different vegetable waste substrates and chicken feed (17.5% CP) as a control. They found that the protein level and AA profile were constant regardless of the substrate fed, but that the fatty acid profile and mineral content differed. Despite the finding that they could effectively rear fly larvae on waste streams, the difference between feeding vegetable waste and chicken feed is insightful. The larvae fed chicken feed required 12.3 days to pupate, achieving a mass of 220 mg (17.9 mg/day), whereas larvae fed restaurant waste required 19 to pupate and weighed 154 mg (8.1 mg/day). In this authors opinion it should be questioned whether it is financially sustainable to throttle the output of an expensive animal production facility by using cheap inputs? Is it sensible to risk contamination in any waste stream when 'clean' feed could be used instead?

IV. SUSTAINABILITY

Measuring sustainability is complex because the entire value chain must be considered. This would include aspects such as the source of the substrate, any impact on the built environment, energy costs and the cost of logistics (Pelletier, 2015). In the case of feeding BSF, assessment is complicated by the multitude of different systems used, both to produce the insects and in terms of the poultry that will ultimately consume them. Where locally available waste streams are used on farm, and live insects fed on the same farm using insects as poultry feed is sustainable (van Huis & Oonincx, 2017; Veldkamp & van Niekerk, 2018).

The higher the bioconversion efficiencies, defined as the proportion of nutrients provided in the substrate which are incorporated into the insect biomass, the better the sustainability performance of a system will be. The efficiencies of nutrient conversion and gaseous emissions during BSF production were measured to quantify bioconversion efficiency (Parodi et al., 2020). Bioconversion efficiencies ranged from 14% (potassium) to 38% (nitrogen). Direct GHG emissions associated with BSF rearing were 16.8 ± 8.6 g CO_2eq per kg of dry larvae, without considering the energy used in its production. By comparison, a value of 0.580 g CO_2eq is given for locally produced SBM (INRAE, 2023).

V. CONCLUSIONS

The use of insect protein in the poultry industry is in its preliminary stages. Insects can convert waste streams, unfit for human consumption, into a nutrient and energy source for animal feeding, although the risk of contamination with rogue chemicals may be high. Insect protein is useful in subsistence poultry production, where it often forms the only dietary protein source. High-intensity, commercial insect production requires sophisticated facilities. Few proper assessments of the sustainability of commercial insect production have been published, however, the existent data would indicate that the carbon footprint of insect protein is likely higher than alternative ingredients. It has been shown that feeding insects a balanced feed, more than doubles their growth rate, so using low-density waste streams in high-intensity production systems may be questionable. Relative to the requirements of the poultry industry, the output of insect products is small. Perhaps the true sustainability of insect protein should be viewed in two dimensions. Small-scale production and the feeding of live insects to chickens that are

probably deprived of protein is most undoubtedly sustainable, but large-scale commercial production is unlikely to be so.

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SUGARCANE BAGASSE: A NOVEL INSOLUBLE FIBRE SOURCE FOR POULTRY

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In Australia, 35 million tonnes of sugarcane are harvested annually which produces approx. 10 million tonnes of a by-product called bagasse. Sugarcane bagasse is an insoluble fibre source and recent studies in broilers indicated that bagasse can be added at 2% in both normal and reduced protein (RP) diets to improve FCR by 2-3 points and weight gain by 30-40 g (Sharma et al., 2021a,b). Thus, sugarcane bagasse may be a novel insoluble fibre source for poultry. Follow up studies were conducted to investigate the chemical composition of various insoluble fibre sources as well as the mode of action of bagasse as it stood out.

The methods are reported in detail in previous study (Sharma et al., 2021a). In short, Ross 308 broilers (n = 672) were assigned to 6 treatments with 8 replicates of 14 birds each. The treatments were: a normal protein diet, a RP diet (-20 g/kg protein) and RP diets added with sugarcane bagasse at 20 g/kg, lignocellulose at 10 g/kg, oat hulls at 30 g/kg, or soy hulls at 30 g/kg. The basal diet was the same for all fibre sources and the formulations were adjusted by adding Celite, an inert indigestible component. On d 24, three birds/pen were sampled and digesta samples were collected from the distal jejunum, ileum and caeca to measure apparent digestibility coefficients of starch, protein and amino acids; and selected microbiota composition using qPCR. Tissue samples were collected from the jejunum and pancreas to measure the expression of genes related to digestive enzymes, tight junction proteins and nutrient transporters. Data were subjected to one way ANOVA using JMP v.14 (SAS Institute Inc, Cary, NC). Significance was determined at $P < 0.05$ using Tukey's HSD test.

Lignocellulose contained the highest concentration of crude fibre (657 g/kg) followed by bagasse (452 g/kg), soy hulls (377 g/kg) and oat hulls (310 g/kg). Bagasse contained the highest concentration of insoluble NSP (536 g/kg) followed by lignocellulose (489 g/kg), oat hulls (474 g/kg) and soy hulls (451 g/kg). Lignocellulose contained the highest concentration of lignin (268 g/kg) followed by bagasse (181 g/kg), oat hulls (152 g/kg) and soy hulls (38 g/kg). Soy hulls contained the highest concentration of crude protein (CP, 93 g/kg) followed by oat hulls (31 g/kg), bagasse (10 g/kg) and lignocellulose (3 g/kg). The birds offered a RP diet with insoluble fibres had no effects ($P > 0.05$) on water to feed intake ratio compared to those offered a RP control diet. The birds offered a RP diet with bagasse or oat hulls had a higher ($P < 0.05$) relative gizzard weight compared to those offered a RP control diet. The reduction in dietary CP increased ($P < 0.01$) the ileal starch digestibility coefficient. The birds offered a RP diet with either bagasse or soy hulls had a lower ($P < 0.01$) ileal starch digestibility coefficient compared to those offered the RP control diet. Bagasse addition in the RP diet also lowered ($P < 0.05$) the CP digestibility coefficient in the jejunum but not ($P > 0.05$) in the ileum. The birds offered the RP diet with either oat hulls or bagasse had the lowest counts of total bacteria in the ileal contents compared to others and lower *Lactobacillus* counts in the caecal contents compared to those offered the RP diet alone or with soy hulls. The reduction in dietary CP decreased ($P < 0.05$) the expression of a digestive enzyme AMY2A gene in pancreas. The addition of either of the four insoluble fibres to the RP diet had no effect ($P > 0.05$) on the expression of AMY2A compared to the RP treatment but bagasse, lignocellulose and soy hulls added RP diets had similar AMY2A expression as the normal protein treatment.

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LIFE CYCLE ASSESSMENT OF THE ENVIRONMENTAL IMPACTS OF THE AUSTRALIAN POULTRY INDUSTRY

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Summary

Current global environmental and consumer megatrends include the transparent reporting of environmental credentials as industries and corporations strive to achieve sustainable use of resources and net-zero production. The Australian poultry industry is highly efficient and produces low environmental impact products. There are many emerging and established options to achieve even lower carbon footprint products and this study uses life cycle assessment to quantify the effect of some of these on the carbon footprint of Australian eggs and chicken meat. Life cycle assessment (LCA) was used to evaluate the potential of investment or changes to management practices on the environmental impacts of poultry production. The assessment demonstrated that substantial reductions in emissions per kilogram of eggs and chicken meat (53% and 62%, respectively) can be achieved through dedicated procurement strategies, nutrition and renewable energy.

I. INTRODUCTION

Australian egg and chicken meat production share many similarities, not least that the systems are highly efficient and produce high-quality food products with a relatively low environmental footprint. Across both industries, the highest proportion of environmental impacts (Greenhouse gas (GHG) emissions, fossil energy, water consumption and stress, land occupation) are attributable to feed production. Both industries have growing free range production sectors, which (though not covered here) will also need to grapple with a nutrient management issue (eutrophication potential). Aside from the cost-of-production implications arising from the transition to less efficient free range production systems, there is also uncertainty regarding the emission factors from manure deposited on range areas – in some cases emissions may be lower and in others, higher.

Both industries will achieve some level of decarbonization (at least on a product basis) due to the planned increase in the renewable energy supply to the national grid but achieving reductions in Scope 3 emissions (from upstream feed production) will be challenging. Protein is a key component of feed but there are environmental challenges and uncertainties associated with staple sources, such as imported soybean meal, and major customers increasingly turning their attention to sustainable sourcing by eliminating or reducing 'high-risk' soy (e.g., connected to deforestation or land conversion) from their supply chains.

Life cycle assessment (LCA) is a useful methodology through which to assess the effect of changes in production, sourcing, emission factors, and energy. Whilst LCA may cover multiple environmental indicators, this study aimed to quantify how evolving industry practices will affect the carbon footprint of Australian egg and chicken meat production.

II. METHOD

Baseline performance and environmental impacts were previously determined by these authors for Australian egg (Copley et al. 2023) and chicken meat (Copley & Wiedemann 2022) production in 2020. The baseline studies assessed GHG emissions using the IPCC AR5 global warming potentials (GWP100) of 28 kg CO_2-e/kg CH4 and 265 kg CO_2-e/kg N2O as applied

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in the National Greenhouse Accounts Factors (Commonwealth of Australia 2023a). GHG emissions associated with land use (LU) and direct land-use change (dLUC) were included and reported separately, as recommended in ISO 14067 (2018).

In alignment with newly developed draft guidance for the Australian agriculture sector, LU and dLUC emissions and removals in Australian cropland for the ten years prior to the baseline analysis periods were determined. National and state datasets (from the National Greenhouse Accounts (2023): cropland remaining cropland, excluding woody perennials, and land converted to cropland) were used to determine emissions and removals for Australia (Table 1). The associated emissions per kilogram of product are reported with LU and dLUC emissions from imported soybean meal in the results.

Table 1 - Net LU and dLUC emissions (reported in million tonnes of carbon dioxide equivalent (Mt CO_2-e) by state from cropland, averaged over 10 years and excluding emissions from perennial woody crops.

	Units	2021*
NSW	Mt CO_2-e	2.00
QLD	Mt CO_2-e	1.21
VIC	Mt CO_2-e	-0.39
WA	Mt CO_2-e	-0.43
SA	Mt CO_2-e	-1.09
TAS	Mt CO_2-e	0.1
NT	Mt CO_2-e	0.02

*Data from Australia's National Greenhouse Accounts (2021)

To evaluate the effect of industry- and economy-wide trends and to determine the potential effect of various changes in key assumptions and sourcing, scenario analysis was conducted using the established LCA models. The scenarios were chosen to consider how Scope 1, Scope 2, and Scope 3 emissions may change over time. For Scope 1 emissions (direct emissions from sources owned or controlled by the producer (e.g., diesel & gas use, manure), we assessed the effect of a 10% reduction in dietary crude protein (from industry standard) on the carbon footprint of eggs and chicken meat (denoted as Scenario 1) noting that this can be achieved without compromising growth (Greenhalgh et al. 2020). For Scope 2 (emissions from purchased grid electricity), we assessed GHG emissions under the national renewable electricity target of 82% by 2030 (Scenario 2). For Scope 3 emissions (emissions from sources not owned or controlled by the producer but which are inputs to the farm (e.g., breeding & hatchery operations, production of feed commodities), we considered how the carbon footprint of poultry production may change under a sourcing strategy to minimize emissions from Australian-grown cereal grains and use of certified soybean meal (Scenarios 3 and 4). The scenarios were modelled separately and designed to be complementary and additive, e.g., Scenario 2 included Scenario 1, and Scenario 3 included Scenarios 1 and 2 etc.

III. RESULTS

Results are reported in Table 2 for egg production and Table 3 for chicken meat production. Results are reported as GHG (Scope 1, 2 and 3), LU and dLUC (Scope 3) and as totals per kilogram of the reference unit (kg eggs at grading floor-gate and kg chicken meat at primary processing gate).

Table 2 - Carbon footprint of Australian egg production (by housing system) for the 2020 baseline and for four scenarios.

	Cage			Cage-free			Free range		
	GHG	LU, dLUC	Total	GHG	LU, dLUC	Total	GHG	LU, dLUC	Total
Units	kg CO_2-e/kg eggs			kg CO_2-e/kg eggs			kg CO_2-e/ kg eggs		
Baseline	1.2	0.9	2.1	1.4	1.0	2.4	1.5	1.0	2.5
Scenario 1	1.2	0.9	2.1	1.4	1.0	2.4	1.5	1.0	2.5
Scenario 2	1.1	0.9	2.0	1.2	1.0	2.2	1.3	1.0	2.3
Scenario 3	1.0	0.6	1.6	1.1	0.7	1.8	1.2	0.7	1.9
Scenario 4	1.0	0.0	1.0	1.1	0.0	1.1	1.2	0.0	1.2

Table 3 - Carbon footprint of Australian chicken meat production (by housing system) for the 2020 baseline and for four scenarios.

	Conventional			Free range		
	GHG	LU, dLUC	Total	GHG	LU, dLUC	Total
Units	kg CO_2-e/chicken meat			kg CO_2-e/chicken meat		
Baseline	2.1	1.9	4.0	2.2	2.0	4.2
Scenario 1	2.1	1.9	4.0	2.2	2.0	4.2
Scenario 2	1.7	1.9	3.6	1.8	2.0	3.8
Scenario 3	1.6	1.7	3.4	1.7	1.8	3.5
Scenario 4	1.6	0.0	1.6	1.6	0.0	1.6

Scenario 1 achieved an average reduction of 1.5% in GHG emissions per kilogram of eggs and a 0.5% reduction in GHG emissions per kilogram of chicken meat. Scenario 2 achieved an average reduction of 13% per kilogram of eggs and a 19% reduction in GHG emissions per kilogram of chicken meat relative to the baseline. Scenario 3 achieved a 19% reduction in GHG emissions per kilogram of eggs and a 22% reduction per kilogram of chicken meat whilst Scenario 4 achieved a 21% reduction per kilogram of eggs and a 26% reduction per kilogram of chicken meat relative to the baseline. In addition, Scenario 3 achieved a 22% reduction in LU and dLUC emissions from eggs and an 8% reduction in LU and dLUC emissions from chicken meat. Scenario 4 achieved a 99% reduction in LU and dLUC emissions per kilogram of eggs and chicken meat relative to the baseline. This meant Scenario 4 resulted in a total reduction in emissions per kilogram of eggs of 54% from the baseline and a 60% reduction per kilogram of chicken meat from the baseline.

IV. DISCUSSION

Although by no means the greatest source of Scope 1 emissions from poultry production, uncertainty in relation to the emission factor for manure deposited on range areas is something to be considered as the free range sector grows. For one major egg producer, nitrous oxide emissions from free range areas were projected to approach 7% of business' Scope 1 & 2 emissions by 2035. This emission source has not been researched and may be over-predicted by as much as 10 times. In short, the emission source may be 0.7%, not 7%, and likely varies depending on soil moisture levels. In addition, emissions from composting and stockpiling are also associated with some uncertainty. To best knowledge, composting produces 0.01 kg of nitrous oxide emissions per kilogram of nitrogen (N) added to the compost system and stockpiling produces 0.005 kg of nitrous oxide emissions per kilogram of N added (Commonwealth of Australia, 2023b). Whilst there have been limited studies of emissions from composting of poultry manure, aerating and watering as part of the composting process creates a nitrogen rich, aerated environment from which nitrogen loss (as nitrous oxide emissions) is

likely to be high relative to stockpiling (the only study done in Australia on the topic (see Naylor et al., 2016) reported that nitrous oxide emissions from stockpiling were negligible).

Refinement or removal of uncertainty regarding these emission factors will be significant to industry achieving reductions in emissions from manure and manure/spent litter handling, as reductions to crude protein have only modest reduction on the carbon footprint of poultry products (per Scenario 1). Whilst reductions in Scope 1 emissions may arise in the future as a result of electrification of farm vehicles and trucks in the logistics sector, as yet these technologies are prohibitively expensive and/or not suited to many agricultural systems. Improving energy efficiency at the farm level, e.g., fans and lights, also has beneficial effects on the carbon footprint, however, again these were found to be modest (a 10% improvement corresponded to a 1% improvement in the carbon footprint).

Addressing Scope 2 emissions in egg and chicken meat production systems is most important for emission reduction ambitions out to 2030. Thereafter, reducing grid electricity consumption is less critical because of the level of renewable generated electricity in the supply chain, i.e., decreasing marginal abatement from uptake of on-farm renewable electricity. A passive Scope 2 strategy (e.g., relying on decarbonization in the energy market) yielded nearly a 20% reduction in GHG emissions per kilogram of chicken meat and 12% reduction per kilogram of eggs. This creates a significant decision for farmers and companies motivated to achieve emission reduction – investing in on-site renewable energy generation now will lead to a short-term advantage (in terms of carbon footprint) over competitors but eventually they will achieve the same reduction at the market price. Currently, there are several broiler and layer farms where a large portion of the electricity demand is met by on-site solar, however, at the processing plant level biogas remains the most cost-effective and utilizable source of renewable energy.

There are, however, challenges that may confound the potential emission reduction. In egg production systems, no improvement in feed conversion ratio (FCRs) was observed for farms assessed in both the 2020 industry baseline LCA (see Copley et al. 2023) and an earlier 2010 study (Wiedemann & McGahan 2011). Pursuit of a larger egg produced for longer and attempts to reduce instances of cannibalism in non-cage systems were reportedly the cause. Given retailer and consumer demands and welfare concerns, this challenge is likely to remain for some time. For the Australian chicken meat industry, low levels of antimicrobial resistance are a strength and policies are established to continue and strengthen antimicrobial stewardship over time. However, as therapeutic antibiotics may improve feed conversion and promote growth (Mehdi et al. 2018), any reduction in use will likely be detrimental to productivity. For these reasons, FCR improvement was not assessed here, however, it is the single most significant determinant of carbon footprint (and other environmental impacts) of poultry production. Previously, we found a 0.1 improvement in FCR resulted in a 3% reduction in the carbon footprint of eggs (see Copley et al. 2023) and a 5% reduction in the carbon footprint of chicken meat (see Copley & Wiedemann 2022).

Aside from the emission reduction potential associated with improving genetics and FCRs, the leading edge of Australian poultry producers are beginning to develop insights into their feed grain suppliers. Scenario 3 is the ultimate representation of that – a procurement strategy that sources low environmental impact grains to achieve substantial reductions in Scope 3 emissions. Traceability in the feed grain sector (and engagement with third-party suppliers) is in its infancy and further work is needed in the grains sector to expand the existing availability of benchmarking data (e.g., Simmons et al. 2019).

The LU and dLUC emissions reported in this study are higher than those reported in the baseline assessments due to application of new methods which considered impacts from Australian grain on soil carbon loss over the past 10-year baseline period. LU and dLUC emissions from Australian cropland vary considerably between states (see Table 1) and in

some, e.g., Victoria, Western Australia and South Australia, cropland represents an emission removal. Developing traceability in Australian grain production systems that can demonstrate management practices that do not result in soil carbon loss, and providing certified grain, would remove this emission source. Scenario 3, for example, demonstrates that improved traceability (and sourcing) to determine that grains were sourced from well-managed land substantially reduced LU & dLUC emissions.

The effect of a procurement strategy for soy was also assessed in Scenario 4 and achieved substantial further reductions in LU and dLUC emissions per kilogram of product. Note that whilst the strategy here assumed soy was sourced through certified supply chains with no deforestation/land conversion, a similar reduction could be achieved through sourcing soy from alternative markets (e.g., the US) or through replacement with alternative proteins. In most cases, changes such as this would increase cost-of-production, however, given targets set by major customers, industry should look to leverage opportunities to secure co-funding for this type of emission reduction strategy as they reduce retailers' Scope 3 emissions too.

As the 'low-hanging fruit' are addressed, harder to abate sources that currently represent small proportions of the carbon footprint will become more significant. Uncertainty in emission factors from range areas and manure stockpiling are one example discussed here. Another (not considered here) is optimised transport and logistics at the distribution and retail level. Transport and distribution may add up to 1kg of emissions to the carbon footprint and whilst this is a marginal addition to the carbon footprint of red meat products, it may result in a 50-75% increase in the carbon footprint of poultry. Regardless, egg and chicken meat products would still have a significantly smaller carbon footprint than red meat.

In short, LCA has confirmed that Australian poultry production has strong environmental credentials and that there are significant opportunities to further improve environmental performance through renewable energy, diet and optimised sourcing of feed ingredients. The whole of the supply chain (cropping through to retail) should be engaged so that this potential can be realised.

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SUPPLEMENTING CARBOHYDRASES TO BROILER CHICKENS OFFERED DIETS CONTAINING WHEAT DRIED DISTILLER'S GRAINS WITH SOLUBLES

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Although there is growing interest in employing biofuel by-products, such as wheat dried distiller's grains with solubles (wDDGS), in broiler feed, their use has been limited due to the high-fibre contents. In this study, a 3 × 2 factorial arrangement of treatments with eight replicates per treatment (16 birds per pen) was conducted using 768 Cobb 500 mixed-sex broiler chicks. Factors were the inclusion levels of wDDGS: no, mid or high; and supplemental carbohydrases (Natugrain® TS; xylanase, 560 XTU/kg and β-glucanase, 250 TGU/kg): no or yes. All diets were formulated based on maize and soybean meal, and were isocaloric and marginally low in essential amino acids (10% downspec). The mid-wDDGS diet contained 6%, 12% and 12% of wDDGS, whereas the high-wDDGS diet 12%, 18% and 20%, in the starter (d 0 – 10), grower (d 10 – 21) and finisher (d 21 – 35) feed, respectively. The percentage of male birds in a pen was used as a covariate. The analysed soluble non-starch polysaccharides (NSP) levels were 3.2, 7.5 and 11.1 g/kg DM in the finisher control, mid- and high-wDDGS diets, respectively. Overall (d 0 – 35), birds fed the high-wDDGS diet showed lower weight gain (WG; $P < 0.001$) compared to those fed the control or mid-wDDGS diet. Supplemental carbohydrases improved the WG ($P < 0.001$) compared to non-supplemented birds. There was a trend in an interaction ($P = 0.095$) between wDDGS levels and supplemental carbohydrases on overall WG such that greater improvement by carbohydrases was observed in birds fed the control or mid-wDDGS diet than those fed the high-wDDGS diet. The inclusion of wDDGS impeded the overall feed conversion ratio (FCR; $P = 0.011$) compared to the no-wDDGS diet. Supplemental carbohydrases tended ($P = 0.097$) to improve the FCR compared to non-supplemented birds. Compared to the no-wDDGS diet, the high-wDDGS diet resulted in increased ileal digesta viscosity ($P = 0.002$) and litter moisture ($P = 0.001$) at d 35. Supplemental carbohydrases reduced the ileal digesta viscosity ($P = 0.001$) and litter moisture ($P = 0.047$) measured at d 35 when compared to non-supplemented birds. Collectively, a main driver for reduced growth performance by wDDGS was its high NSP level, especially the soluble fraction, which can interfere with efficient nutrient utilisation. These anti-nutritive characteristics of wDDGS could be mitigated by carbohydrases via successful degradation of soluble NSP, alleviating viscous gut environment and wet litter problem.

Table 1 - Effects of wheat dried distiller's grains with solubles and supplemental carbohydrases on growth performance, ileal digesta viscosity (mPa·s) and litter moisture (%)[1].

		d 0 – 35			d 35	
Main effects		Weight gain, g	Feed intake, g	Feed conversion ratio, g/g	Ileal digesta viscosity	Litter moisture
wDDGS	No	2,388[a]	3,305	1.38[b]	2.99[b]	41.1[b]
	Mid	2,332[a]	3,328	1.49[a]	3.16[ab]	44.7[ab]
	High	2,098[b]	3,100	1.48[a]	3.53[a]	47.2[a]
Carbohydrases	No	2,234[b]	3,199	1.48	3.43[a]	45.6[a]
	Yes	2,311[a]	3,290	1.43	3.03[b]	43.0[b]
SEM		20.9	58.0	0.017	0.073	0.84
P-value	wDDGS	<0.001	0.213	0.011	0.002	0.001
	Carbohydrases	<0.001	0.429	0.097	0.001	0.047
	Interaction	0.095	0.310	0.874	0.083	0.489

[ab]Different superscripts within a factor of analysis (wDDGS and carbohydrases) are statistically different ($P < 0.05$), n = 8 (growth performance, 16 birds/pen from d 0 – 21 and 12 birds/pen from d 21 – 35; ileal digesta viscosity, 4 birds/pen)
[1]wDDGS, wheat distiller's grains with solubles; SEM, standard error of the mean.

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NET ENERGY SYSTEMS FOR POULTRY POTENTIAL FOR LOW PROTEIN DIET

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Summary

A meta-analysis of 28 studies on low protein diets for broilers was conducted, considering Net Energy (NE) content instead of Metabolisable Energy (ME) content. The analysis showed that reducing crude protein (CP) content in feed improves protein retention efficiency and reduces the dependence on vegetable protein sources. The inclusion of synthetic amino acids, such as methionine, lysine, and threonine, along with a focus on animal requirements, allows for the formulation of broiler diets with reduced CP.

The results showed that decreased CP content in broiler feed diets decreased body weight and increased Feed Conversion Ratio (FCR) ($P < 0.001$). Net energy calculations based on diet composition revealed a positive correlation between CP content and the digestible lysine/NE ratio. CP reduction was associated with NE increase resulting in an imbalance between energy and digestible amino acids not detectable in the ME system. Body protein and lipid deposition were also correlated with the digestible lysine/NE ratio, explaining increased fat deposition and worsened FCR for low CP diets. This suggests that the NE system can better balance energy and protein, enabling more sustainable protein production with low protein diets.

The discussion highlights the importance of accurate dietary energy assessment, how the NE system changes the ranking of raw materials compared to other energy systems, and its relevance to hot topics in poultry nutrition like low protein diets. Further research is recommended to explore the potential of the NE system and animal requirements under challenging conditions to estimate energy requirements for maintenance.

I. INTRODUCTION

The global animal nutrition industry is moving rapidly towards precision livestock feeding to manage natural resources more efficiently. Sustainability is all about retrieving the most accurate nutritional values to i) better match the animal's nutritional needs and ii) better predict animal responses to the formulated diets. Having the most reliable data available is crucial in times with high raw materials prices but also to increase the use of by-products and alternative feed ingredients in poultry diets. Wasting or overfeeding nutrients only makes the feed costs rise. Energy is one of the most important and expensive components of poultry diet. This article aims to highlight the benefits of switching from ME to NE values to optimise broiler diets and better predict poultry performance. *Figure 1* presents energy decomposition from gross to net energy. To be more precise and not overfeed valuable nutrients, it is recommended to make the next accuracy step and use the NE values. NE values do not have to be measured; they can be calculated from the ME values (RSD = 0.21 MJ/kg DM; Wu et al., 2019). The transformation of gross energy (GE) to NE can be described by three steps as shown in *Figure 1*.

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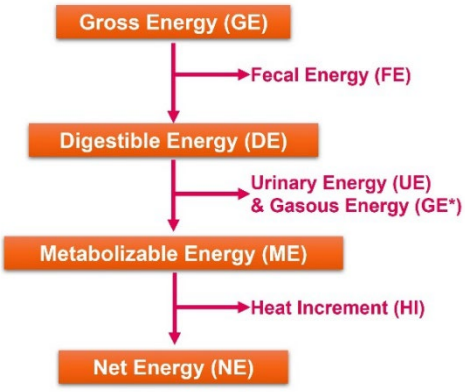

Figure 1 – The energy flow diagram.

II. METHODS

A meta-analysis was performed on 28 studies evaluating low protein diets (range from 14 to 40% protein) in iso-ME diets in broilers from 1985 to 2021, for a total of 75 dietary treatments. The objective was to evaluate the potential of NE and amino acids to NE balance to explain the difference in animal performance. Net energy content of the diets was calculated based on the equation developed by Wu et al. (2019). Data were analysed using SAS (2008) using the MIXED procedure considering experiment as a random effect significant variable were reported. The relationship between diets' nutrient contents (CP, ME, NE, digestible lysine), performance and interaction were established.

Table 1 - NE values provide a new hierarchy for feed ingredients.

Ingredients	GE	ME	NE	NE/ME
Wheat	3780	2257	1629	72
Barley	3810	2390	1782	75
Corn	3860	2842	2088	73
Sorghum	3900	2861	2164	76
Corn DDGS	4080	2865	2166	76
Soybean meal	4130	3324	2527	75
Fish meal	4530	3351	2560	76
Feather meal	5276	3354	2565	77
Fat	9380	9000	7920	88

III. RESULTS

Raw materials NE content were presented Table 1. Ranking of Raw materials change depending of Energy system. Protein rich raw materials were step back in NE system compare with ME system. It explained partly increase of NE content for low CP diets. Reduction of CP content in feed increases animal protein retention efficiency and would reduce dependency on plant protein sources. Inclusions of non-bound amino acids particularly methionine, lysine and threonine, together with good knowledge of animal requirements allow nutritionists to formulate reduced CP diets for broilers. Therefore, in ME system, greater reductions of dietary CP (40 to 50 g/kg) invariably compromise broiler performance. On average, reducing CP content in broiler feed was associated with a decrease of body weight (19 g) and an increase in FCR (+0.04 per unit CP) ($P < 0.01$). No variation in feed intake was observed. Net energy values were calculated based on the chemical composition of experimental diets. Data suggested (Figure 2) a positive correlation between CP content and digestible lysine/NE ratio ($r^2 = 0.83$; $P < 0.001$). In other word, CP increase was associated with increase of digestible lysine/NE ratio. This result suggested that an imbalance between energy and digestible amino

acids may not be detectable in ME system. Body protein and lipid deposition were also significantly correlated with digestible lysine/NE ratio ($r^2 = 0.98$ and $r^2 = 0.77$; respectively; Figure 2). Correlations were positive and negative for protein and lipid deposition, respectively. Unfortunately, few studies exhibited body composition data. The correlations were positive for body protein deposition but negative for body fat deposition. This result explains the increase of fat deposition and deterioration of FCR for low CP diet. Thus, the NE system might allow better energy to protein balance and allow higher decrease of CP content in poultry diets for more sustainable protein production.

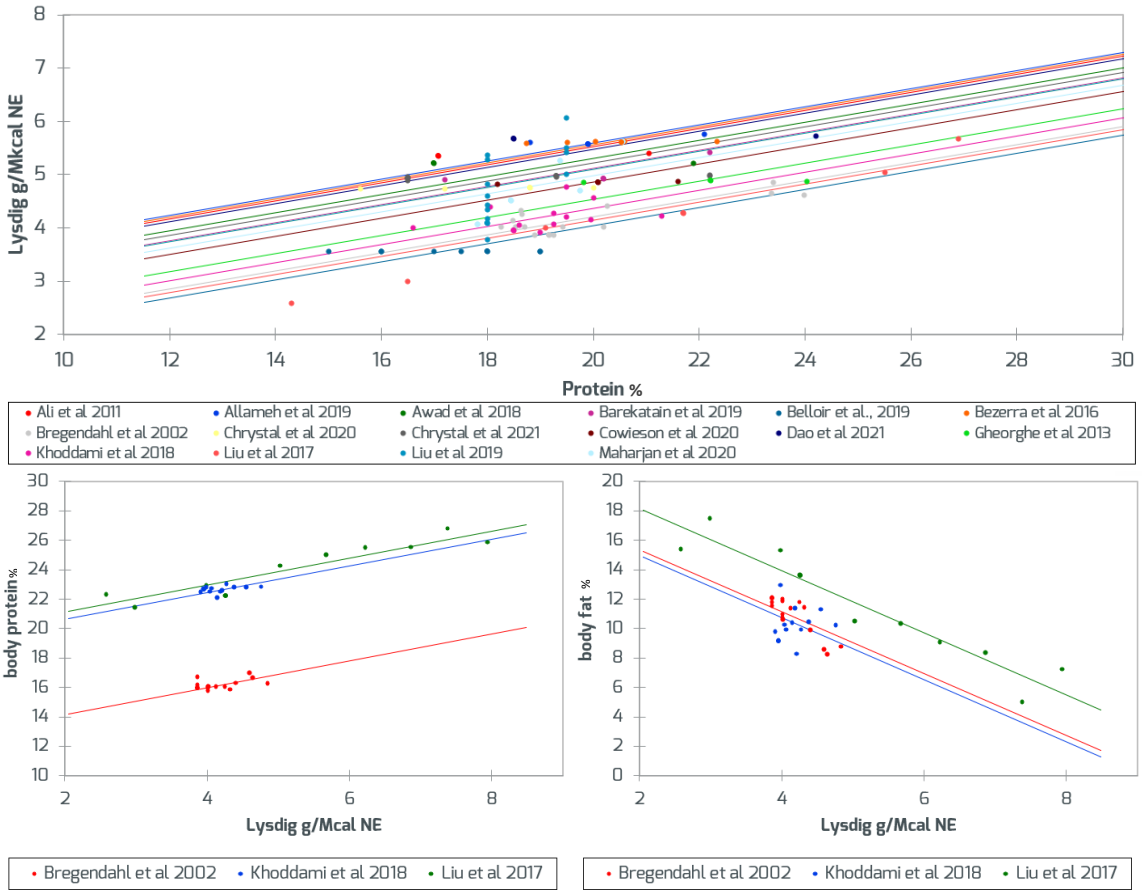

Figure 2 - Decrease of CP content associated with change in digestible lysine/NE ratio and body deposition.

IV. DISCUSSION

Correct evaluation of dietary energy is crucial for optimum feed formulation and ingredient selection in poultry diets. The more robust an energy system is, the more accurately it will predict animal performance and feed efficacy. Net energy system changes raw materials ranking compared with other energy systems. The relative price of ingredients and the derived feed formulations differed in connection with NE energy ranking. Closer to animal performance balance between NE and digestible lysine give good prediction for animal deposition and new insight into poultry nutrition hot topics such as reduced. Digestible amino acids to net energy value should be defined precisely optimize animal production efficacy. In addition, future research is needed to investigate the relationship between NE and digestible amino acids nutrition under standard and challenging conditions.

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SUPPLEMENTATION OF GUANIDINOACETIC ACID TO REDUCED PROTEIN DIETS FED TO BROILER CHICKENS WITH OR WITHOUT HEAT STRESS

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The arginine sparing benefits of guanidinoacetic acid (GAA) as the precursor of creatine in broilers fed reduced protein (RP) diets has been documented (Sharma et al., 2022). However, the effectiveness of GAA in RP diets on performance and gut health of broilers under heat stress is largely unknown. A 35-d experiment was conducted using four dietary treatments: a standard protein diet (SP, 221 and 207 g/kg CP in grower and finisher), a RP diet (201 and 187g/kg in grower and finisher), a RP diet with 0.92 g GAA per kg diet substituting 50% of supplemented arginine (GAA50) at one to one ratio and a RP diet with the same amount of GAA added on top (GAAtop). From d 0 to 10, all birds received the same diet as per Ross 308 requirements. Day-old male chicks were assigned to 64 pens (10 birds each) in two rooms. In each room, each diet was replicated 8 times. From d 25 to 35, birds in one room were subjected to a cyclic heat stress (32±1 ˚C for 8 hours). To assess intestinal permeability (InP), on d 27, an oral gavage of fluorescein isothiocyanate-dextran was used (4.16 mg/kg BW). There was no interaction between diets and heat stress for any of the performance parameters (Table 1). Body weight gain (BWG) was unaffected by dietary treatments. From d 10 to 24, feed intake and FCR were not affected. GAA50 followed by GAAtop significantly decreased the feed intake during the finisher phase (P<0.01) and from d 10 to 35 (P<0.001), compared with SP diet. At the same time, birds fed SP diet had the highest feed consumption. Heat stress reduced (P<0.0001) feed intake and BWG at any stage of the study but did not impact FCR. The GAA50 tended to reduce FCR from d 24 to 35 (P=0.086) and d 10 to 35 (P=0.082) compared with SP and RP. Heat stress increased (P<0.05) InP whereas diets had no effect. The results indicate that replacing 50% of supplemented arginine with GAA tends to improve FCR by reducing the feed intake under both thermoneutral and heat stress conditions. Additional analysis is underway to investigate association between GAA and gut health considering the involvement of creatine in energy balance of enterocyte and indirect effects on gut integrity.

Table 1 - Growth performance of broilers fed experimental diets with or without heat stress.

	Feed intake (g/bird)		BWG (g/bird)		FCR	
	d24-35	d10-35	d24-35	d10-35	d24-35	d10-35
Diet						
Standard protein	2151[a]	3549[a]	1346	2472	1.602	1.437
Reduced protein (RP)	2101[b]	3505[ab]	1310	2433	1.604	1.441
RP-GAA50	2043[c]	3423[c]	1315	2425	1.556	1.412
RP-GAAtop	2096[b]	3481[bc]	1326	2450	1.583	1.421
Room temperature						
Thermoneutral	2233[a]	3628[a]	1419[a]	2546[a]	1.575	1.426
Heat stress	1962[b]	3351[b]	1229[b]	2344[b]	1.597	1.429
SEM	7.8	10.4	5.2	7.1	0.0069	0.0042
Diet	0.0004	0.002	0.12	0.15	0.086	0.082
Room temperature	<0.0001	<0.0001	<0.0001	<0.0001	0.13	0.66
Diet × temperature	0.87	0.81	0.31	0.77	0.65	0.90

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THE POTENTIAL OF DIETARY GLUTAMINE AND GLUTAMATE IN REDUCED CRUDE PROTEIN BROILER DIETS

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Summary

The aim of this review is to explore the potential of dietary glutamine (Gln) and glutamate (Glu) fortification in reduced-crude protein (CP) broiler diets. Supplementing conventional broiler diets with Gln has shown promise, but the dietary concentrations of Gln specifically were not reported. However, just which amino acids are the dominant energy substrates in avian enterocytes remains an open question. Both amino acids play an important role in gastrointestinal integrity but the relative extent of their catabolism in enterocytes for energy provision is debatable. An important post-enteral role of Gln and Glu is the detoxification of ammonia arising from amino acid deamination. It may be more rewarding to fortify reduced-CP broiler diets with dietary Gln than Glu as Glu may be more extensively catabolised in enterocytes.

I. INTRODUCTION

Glutamine and glutamate are important and interchangeable amino acids in poultry (He et al., 2021). Glutamine and Glu are related to gastrointestinal tract development of broiler chickens, serving as energy substrates and for the maintenance of gastrointestinal tract integrity (Ebadiasl, 2011). Dietary supplementation of Gln has shown promising outcomes in broiler chickens (Han et al., 1992; Bartell and Batal, 2007; Xue et al., 2018). However, the analytical differentiation of protein-bound Gln and Glu is not straightforward in relevant feedstuffs; therefore, concentrations of Gln and Glu are usually expressed as the sum of Gln and Glu (Gln+Glu) in broiler diets. However, dietary supplementation of Gln and Glu should be advantageous in reduced-CP broiler diets because CP reductions axiomatically depress Gln+Glu concentrations in broiler diets. For instance, in 13 broiler feeding studies completed at the Poultry Research Foundation, when dietary-CP was reduced from 211 to 172 g/kg, concentrations of Gln+Glu decreased from 41.1 to 31.7 g/kg as shown in Table 1. Therefore, it is possible that either Gln or Glu are deficient in broilers offered reduced-CP diets and contribute to the deteriorated performance observed.

II. GLUTAMINE AND GLUTAMATE CONCENTRATIONS IN COMMON FEEDSTUFFS

Relevant feedstuffs contain relatively high concentrations of Gln+Glu as they are the most abundant amino acids in living organisms. However, there is a lack of data for the individual concentrations of Gln and Glu in feedstuffs. Instead, Gln+Glu is generally presented simply as Glu in the published literature. The reason for the lack of data is that the standard analytical procedure of determining amino acids in feedstuffs converts Gln into Glu by acid hydrolysis. Interestingly, a combined method of determining Gln and Glu was described by Wu et al. (2009), where Gln:Glu ratios were determined by a bioassay method involved porcine digestive enzymes following acid hydrolysation. Based on the porcine enzymes bioassay method, individual concentrations of Gln and Glu in common feedstuffs were reported by Li et al. (2011) as tabulated in Table 2. The data reveals that maize contains 59% higher concentration of Gln than Glu, whereas sorghum, soybean meal, meat and bone-meal and fishmeal have higher Glu concentrations than Gln by 38.8, 9.7, 44.1 and 52.5%, respectively. Therefore, it is quite evident that the concentrations of Gln+Glu in broiler diets do not reflect their individual concentrations as Gln:Glu ratios are influenced by the specific feedstuffs used in broiler diets.

III. IMPORTANCE OF DIETARY GLUTAMINE AND GLUTAMATE IN BROILER DIETS

Dietary Gln and Glu concentrations are considered to be adequate in conventional broiler diets, and therefore, supplementation with non-bound Gln or Glu is not practised. However, promising

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performance outcomes have been reported when using Gln supplementation in conventional broiler diets (He et al., 2021). Moreover, it has been reported that supplementation of dietary Gln and Glu is essential for broiler chickens under heat stress (Porto et al., 2015) and gut infections (Souba et al., 1990). Several studies have reported that dietary Gln supplementation to conventional broiler diets at 10 g/kg significantly improved growth performance of broiler chickens (Bartell and Batal, 2007; Namroud et al., 2017; Xue et al., 2018). There are few recommendations for dietary requirements of Gln+Glu for broiler chickens; however, Wu et al. (2014) recommended 306 of Gln+Glu:Lysine ratio whilst 269 of Gln+Glu:Lysine ratio was recommended by Maharjan et al. (2021). Thus, reducing dietary-crude protein may lead to Gln+Glu deficiencies in broilers where average dietary-CP reduction from 211 to 172 g/kg has decreased Gln+Glu concentrations from 41.1 to 31.7 g/kg in 13 feeding studies completed at Poultry Research Foundation. If the Gln+Glu:Lys requirements for broiler chickens set at 288 based on mean recommendations of Wu et al. (2014) and Maharjan et al. (2021), it can be deduced from the linear equation ($Y = -15.81 + 0.269 \times CP$; $r = 0.731$; $P < 0.001$) generated using aforementioned 13 studies ($n = 44$) that dietary-CP reduction more than 193 g/kg may lead to Gln+Glu deficiencies in broiler chickens. Therefore, dietary supplementation of Gln and Glu maybe pivotal in reduced-CP broiler diets but the relative importance of Gln and Glu requires further investigation. Interestingly, Han et al. (1992) demonstrated that supplementing 23.1 g/kg of Glu to 190 g/kg CP diet significantly improved the FCR by 5.00% (1.445 versus 1.513), in contrast, Glu supplementation did not ameliorate poor performance associated with reduced-CP diets in Corzo et al. (2005) and Awad et al. (2014). Nevertheless, there is a paucity of studies evaluating Gln supplementation in reduced-CP broiler diets.

IV. ROLE OF GLUTAMINE AND GLUTAMATE IN ENTEROCYTES

The series of studies provided evidence that Gln and Glu have different but closely related functional roles in the intestinal mucosa of rats, humans, pigs (Reeds et al., 2000) and poultry (He et al., 2021). However, there is uncertainty as to which amino acid is primarily used as the dominant enterocyte fuel. Lacey and Wilmore (1990) reported that Gln appears to be a unique amino acid that serves as a respiratory fuel for enterocytes, while Fleming et al. (1997) concluded that glucose and Gln are the main energy substrates in rat enterocytes. However, contrasting findings were reported that Glu is catabolised to a greater extent than Gln in enterocytes as stated in Reeds et al. (2000). According to the rate of CO_2 production in broiler enterocytes, it has been found that Glu catabolism is 10 times higher than that from glucose but the rate of CO_2 production from Gln was much lower than Glu (He et al., 2018). This extensive Glu catabolism in broiler enterocyte is also reflected in plasma amino acid concentrations, where Glu concentrations are usually low (<100 μM) compared to Gln (Watford and Wu, 2005). Additionally, in portal (anterior mesenteric vein) circulation of broiler chickens, the mean Gln concentration was found to be 81.7% higher (109 versus 60 μg/mL) than Glu (Moss et al., 2018). The role of Gln in enterocytes is also important, though they may not be used extensively as energy substrates in enterocytes but, are essential to the integrity, function, and health of chicken small intestine (He et al., 2021).

V. ROLE OF GLUTAMINE AND GLUTAMATE IN LOW PROTEIN BROILER DIETS

Dietary-CP reductions in broiler diets are achieved by partially replacing soybean meal with non-bound amino acids (NBAA) as alternative protein sources. However, the lack of bioequivalence between protein-bound amino acids and NBAA may cause postprandial amino acid imbalances, leading to increased toxic NH_3 production from the deamination of surplus amino acids. There is recent evidence that dietary NBAA inclusions were linearly related to plasma NH_3 concentrations in Macelline et al. (2023). The detoxification of NH_3 principally takes place in the liver where NH_3 generated from amino acid catabolism is condensed with Glu via glutamine synthetase and synthesised to uric acid by the Krebs cycle with glycine. The relevant condensation reaction may depicted as follows as in Minet et al. (1997).

$$[NH_{4+} + Glu + ATP + Mg^{2+} \longrightarrow Gln + ADP + Pi.]$$

The retro-analyses in Table 3 support this rationale as the overall Gln concentration in systemic plasma was significantly higher than that in portal plasma by 21% (132 versus 109 μg/mL) while Glu

concentrations were reduced by 65% (21 versus 60 μg/mL) with the transition from portal to systemic circulations. Therefore, it would appear that dietary Glu supplementation in broiler diets maybe crucial in reduced-CP broiler diets than conventional diets to facilitate NH_3 detoxification. However, supplementation of broiler diets with Glu may be confounded by its extensive catabolism in the gut mucosa, which does not apply equally to Gln (He et al., 2022). Therefore, it should be more instructive to fortify reduced-CP broiler diets with dietary Gln than Glu where Gln can be converted to Glu via phosphate-activated glutaminase.

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Table 1 - Dietary Gln+Glu concentrations in conventional and reduced-CP diets across 13 feeding studies completed at Poultry Research Foundation.

Study	Feed grain	Dietary-CP (g/kg)		Dietary Glu + Gln (g/kg)		Difference
		High	Low	High	Low	(%)
Moss et al. (2018)	Maize	213	178	35.6	32.2	-9.55
Chrystal et al. (2020a)	Maize	210	165	38.2	27.4	-28.9
Chrystal et al. (2020b)	Maize	197	163	33.3	29.1	-24.7
Chrystal et al. (2020c)	Maize	200	156	35.7	22.7	-36.4
Chrystal et al. (2021)	Maize	222	165	38.2	22.6	-40.8
Greenhalgh et al. (2022a)	Maize	195	175	31.9	27.1	-15.2
Greenhalgh et al. (2022b)	Maize	220	180	38.0	30.0	-22.2
Yin et al. (2020)	Wheat	215	165	42.5	34.3	-19.3
Greenhalgh et al. (2020)	Wheat	215	180	45.9	37.6	-18.1
Chrystal et al. (2021)	Wheat	222	165	40.4	26.1	-35.4
Macelline et al. (2023a)	Wheat	210	180	46.7	40.0	-14.4
Macelline et al. (2023b)	Wheat	210	180	45.7	38.9	-14.9
Greenhalgh et al. (2022a)	Wheat	220	180	45.4	34.5	-24.0
Macelline et al. (2023c)	Wheat	210	170	46.7	36.7	-21.4
Macelline et al. (2023c)	Wheat	210	170	51.7	36.9	-28.6
Mean		211	172	41.1	31.7	-23.4

Table 2 - Concentrations of Gln and Glu in common feedstuffs (adopted from Li et al., 2011).

Item (g/kg)	Maize	Sorghum	Soybean meal	Meat and bone meal	Fishmeal
Crude protein	93.0	101	436	520	634
Glutamate	6.40	11.8	41.7	40.5	60.1
Glutamine	10.2	8.50	38.0	28.1	39.4
Glu:Gln	0.63	1.39	1.10	1.44	1.53

Table 3 - Free concentrations (µg/mL) of Gln and Glu in portal and systemic plasma in broiler chickens offered three dietary treatments [retrospective analysis from Moss et al. (2018)].

Treatments		Glutamine	Glutamic acid	Gln + Glu
Plasma	Dietary CP (g/kg)			
Portal	213	109	52	161
	192	109	60	169
	178	110	66	177
Systemic	213	126	20	146
	192	129	26	155
	178	141	20	160
SEM		9.1	6.0	12.8
Main effects: Plasma				
Portal		109[a]	60[b]	169
Systemic		132[b]	21[a]	154
Dietary-CP (g/kg)				
213		118	36	154
192		119	43	162
178		126	43	169
Significance (P =)				
Plasma		0.004	<0.001	0.157
Dietary-CP		0.648	0.438	0.518
Plasma × Dietary-CP		0.736	0.441	0.598

INCREASING THE DOSE OF A NOVEL CONSENSUS BACTERIAL 6-PHYTASE VARIANT IMPROVED ILEAL DIGESTIBILITY OF Zn, Cu, Fe, AND Mn IN BROILERS

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Summary

A meta-analysis was performed on data from two studies that had evaluated the effect of increasing the dose level of a novel consensus bacterial 6-phytase variant (PhyG) on ileal digestible zinc (Zn), copper (Cu), iron (Fe) and manganese (Mn). The analysis of 360 datapoints from 12 datasets showed that increasing the PhyG dose level increased ileal digestible Zn, Cu, Fe and Mn above the level achieved by an unsupplemented control diet, in an exponential manner. The data indicate that in diets containing PhyG, the level of Zn, Cu, Fe and Mn supplementation can be reduced. This would reduce the excretion of these trace minerals into the environment and improve sustainability in broiler production.

I. INTRODUCTION

Phytate (myo-inositol hexakisphosphate, IP_6) is the major storage form of phosphorus (P) in plant ingredients. In the pH environment of the small intestine of broilers (pH 4–6; Selle and Ravindran, 2007; Selle et al., 2009), phytate has a strong affinity to bind mineral cations including Ca^{2+}, Zn^{2+}, Fe^{2+}, Mn^{2+}, Cu^{2+}, forming insoluble complexes that are not readily absorbed. Using an *in vitro* digestion model, Yu et al. (2018) showed that exogenous phytase can improve the bioavailability of trace minerals (TM) in common feedstuffs. However, *in vivo* studies have shown inconsistent responses. A recently developed novel consensus bacterial 6-phytase variant (PhyG) has been shown to exhibit high relative activity in the upper gastrointestinal tract (pH 1.5–3.5) and to efficiently break down phytic acid at this pH (Christensen et al., 2020). It was hypothesised that such a phytase could reduce the binding of phytic acid to TM and thereby improve TM digestibility. The objective of this study was to evaluate the effect of increasing PhyG dose on ileal and total tract Zn, Cu, Fe and Mn digestibility in broilers.

II. METHOD

Data from two studies were analysed, both carried out at the University of Sydney. Both experiments employed a completely randomized design in which day-old Ross 308 male birds were randomly allocated to cages with 20 birds/cage and 6 cages/treatment. Both employed a basal diet based on wheat, corn and soybean meal with added rapeseed meal and rice bran and both tested the addition of PhyG at five doses: 0 (NC), 500, 1,000, 2,000 and 4,000 FTU/kg. Diets were formulated in three phases [0–10, 10–21 and 21–35 days (d) of age].

Experiment 1 (Exp. 1) used a 3 x 5 factorial arrangement of treatments comprising three formulated levels of dietary phytate-P (PP) [2.45 g/kg (low), 2.95 g/kg (medium) and 3.45 g/kg (high)], each formulated to contain PhyG at each of the five doses. A total of 1,800 birds were tested across the 15 treatments. The average analysed PP content of the diets across phases was 2.9, 3.4 and 3.9g/kg for ‘low’, ‘medium’ and ‘high’ PP level diets, respectively.

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Experiment 2 (Exp. 2) employed five treatments comprising the basal diet supplemented with PhyG at each of the five doses. A total of 600 birds were tested. A single formulated PP level was used in all diets. The analysed PP content across diets was 3.3, 3.1 and 2.8 g/kg in starter, grower and finisher phase, respectively. In both studies, Zn, Cu, Fe and Mn in sulphate form were supplemented to diets via a premix. The analysed content of these trace minerals in the treatment diets is presented in Table 1.

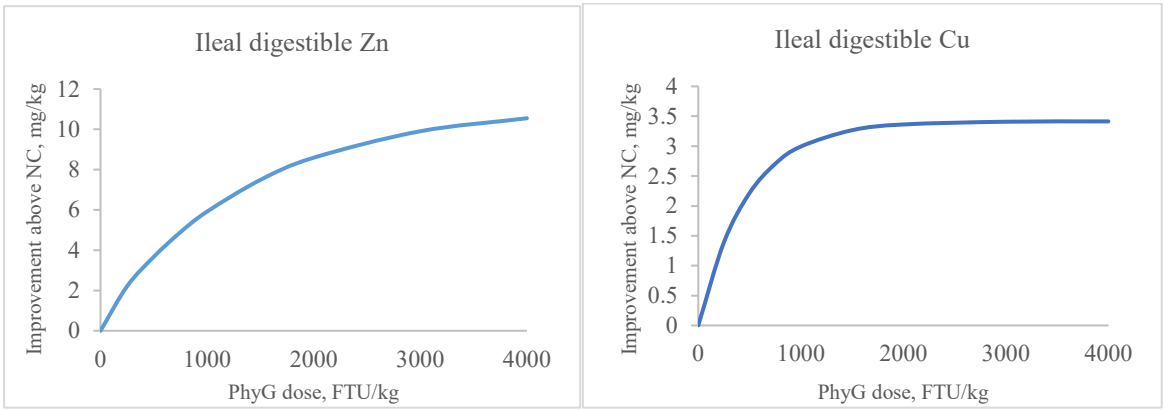
Table 1 - Analysed Zn, Cu, Fe and Mn levels (mg/kg) in the diets (average, across treatments).

	Zn			Cu			Fe			Mn		
Days of age	0-10	10-21	21-35	0-10	10-21	21-35	0-10	10-21	21-35	0-10	10-21	21-35
Exp. 1 low PP	99	105	97	31	31	36	221	193	167	136	139	136
Exp. 1 med PP	106	107	92	35	30	33	230	199	161	149	143	119
Exp. 1 high PP	103	104	94	33	30	35	209	162	166	147	141	127
Exp. 2	86	103	82	33	24	34	259	287	292	123	113	120

In both studies, celite, a source of acid insoluble ash, was added to all diets as an indigestible marker, at 20 g/kg. Diets were steam-pelleted at 80°C. On d 10, eight birds per cage and on d 21 and 35, six birds per cage were euthanised, ileal digesta collected and pooled per replicate for the determination of Zu, Cu, Fe and Mn. Total excreta samples were collected from all cages during 6–9, 17– 20 and 22–35 d of age for TM determination. These determinations produced a total of 360 datapoints drawn from 12 datasets across the two studies. Ileal digestible TM in mg/kg was calculated based on the determined digestibility coefficient and the analysed TM content of the diet. The improvement in ileal digestible TM above the average response in the NC was calculated, values obtained checked for outliers and then fitted to an exponential curve. The relationship between apparent total tract digestibility (ATTD) of individual TM and increasing phytase dose was also analysed by exponential curve fitting.

III. RESULTS

Increasing PhyG dose exponentially improved digestible Zn, Cu, Fe and Mn content expressed as the increase above the response of the NC (Figure 1).



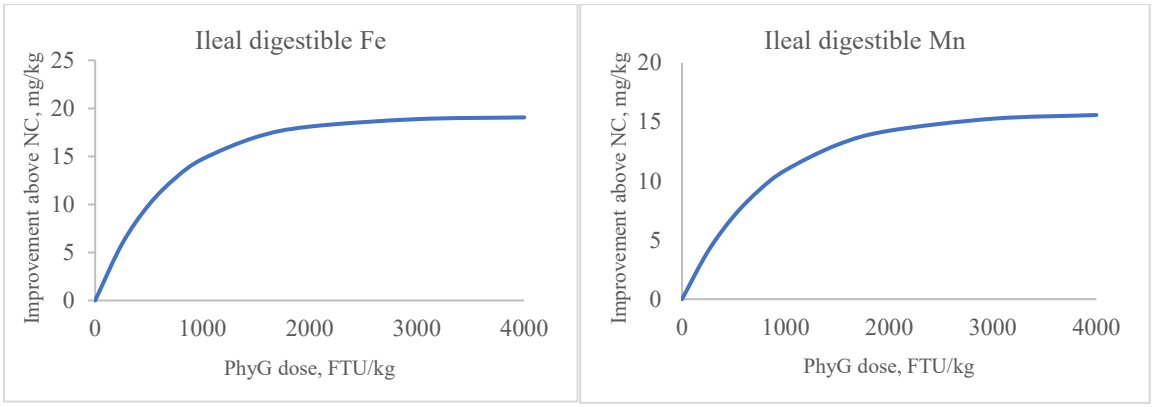


Figure 1 - Improvement in ileal digestible Zn, Cu, Fe and Mn (mg/kg feed) above the response of the NC, with increasing PhyG dose, modelled by exponential curve fitting.

Apparent total tract digestibility of Zn and Mn exponentially increased with increasing PhyG dose (Figure 2), whereas ATTD of Fe and Cu were unaffected.

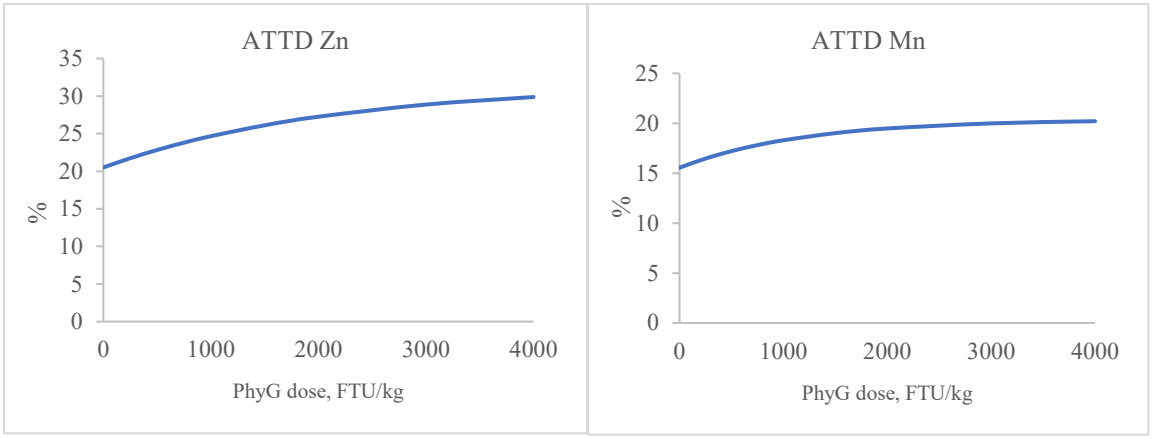


Figure 2 - Relationship between apparent total tract digestibility (ATTD) of Zn and Mn (%) with increasing PhyG dose.

IV. DISCUSSION

The modelled data from these two experiments demonstrated a consistent *in vivo* improvement in the (ileal) digestible Zn, Cu, Fe and Mn content of the diet when PhyG was added. This suggests that the phytase increased the bioavailability of these TM to the birds and therefore that it could potentially 'replace' some of the added TM in the diet. These data complement the *in vitro* findings of Yu et al. (2018) in which a different phytase added at a dose of 5,000 U/g to a simulated stomach and small intestine digestion increased the 'release rate' (defined as the percentage of soluble TM) of Cu and Zn from corn, Cu, Zn and Mn from wheat, and Zn and Mn in soybean meal, and also reduced the abundance of intact phytic acid. As these three cereal ingredients formed the main base of the test diet in the present studies, it is hypothesized that the increased TM bioavailability effected by PhyG was due (at least in part) to TM release from wheat, corn and soybean meal. The capacity of the PhyG phytase to rapidly and extensively degrade phytate in the low pH of the upper GIT that has been demonstrated both *in vitro* and *in vivo* (Christensen et al., 2020; Dersjant-Li et al., 2022) would explain this effect as it would lead to reduced availability of phytate to complex with (supplemental or feedstuff-derived) TM.

The improvements in ileal digestible TM above the NC plateaued somewhere between 1000 and 2000 FTU/kg for Cu (an improvement of ~3 mg/kg) and Fe (an improvement of ~18

mg/kg) but continued up to the highest dose (4000 FTU/kg) for Zn and Mn (improvements of ~11 and ~16 mg/kg respectively). For Cu and Fe, the greater digestibility and absorbance of these TM at ileal level (with increasing PhyG dose) was not followed by improved digestibility at total tract level, which could indicate that the requirement for these TM was met and therefore the increase in digestible Cu and Fe in the ileum was not retained by the birds. However, for Zn and Mn, incremental improvements in the digestibility of these TM with increasing PhyG dose were evident also at total tract level, suggesting that the beneficial effect of the phytase may have contributed towards meeting the requirement for these TM. Phytate has a strong binding capacity to Zn which may explain the continued response with increasing PhyG dose for this TM. These results are in agreement with recent findings from a study of the TM 'replacement' capacity of PhyG in which PhyG supplemented in a tiered dosing strategy by phase to a basal diet deficient in Zn, Mn and Cu improved tissue utilization of Zn (in bone, liver and plasma) and the utilization of TM for growth (Bello et al., 2023). The latter was indicated by improved final body weight and bodyweight gain in birds supplemented with the phytase, to levels equivalent to or improved compared with the effect of TM supplementation, regardless of TM source or dose (Marchal et al., 2023).

In summary, the meta-analysis has shown that the novel PhyG phytase can improve the bioavailability of TM from basal ingredients to broilers. In practice, a trace mineral matrix could be applied when using this phytase which would help to reduce feed costs and improve the sustainability of production.

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INTERACTIVE EFFECTS OF DIETARY ENERGY LEVELS WITH AMINO ACID DENSITY ON GROWTH PERFORMANCE AND CARCASS CHARACTERISTICS OF BROILER CHICKENS

M. TOGHYANI[1,2], S.P. MACELLINE[1,2], P.H. SELLE[2] and S.Y. LIU[1,2]

Growth is a function of nutrient intake, and further increasing nutrient density can be expected to linearly improve feed efficiency. However, meat chicken response to nutrient density, particularly key nutrients such as metabolisable energy (ME) and amino acids (AA), is curvilinear, exhibiting a phenomenon known as diminishing returns. Therefore, meat chicken diets must be formulated to contain precise adequate ME and AA to meet the bird's requirements for maintenance and production, in order to maximize the protein accretion potential of the diet and therefore feed efficiency. The current trial was conducted to determine the interactive effect of diet AA density with dietary ME levels on growth performance and carcass characteristics of broiler chicks raised to 42 days of age.

The feeding study consisted of 12 dietary treatments designed as a 4 × 3 factorial arrangement, which included four levels of ME (standard, -50, -100 and -150 kcal/kg) and 3 levels of AA densities (standard, +3 % and +6 %) for each of the four phases of the study. The diets were formulated to Ross 308 nutrient specifications (Aviagen 2022), and the reduction of ME and increase in AA density was applied to the base levels recommended by the breeder. Each treatment was replicated 8 times with 25 Ross 308 off-sex male chicks per replicate pen. The overall performance results showed that ME reduction at standard AA density had no significant effect on final body weight (BW) (d 42), however, increasing AA density improved BW when birds were fed diets with standard, -100 and -150 kcal ME, but at -50 kcal ME only high AA density improved BW compared to standard AA density, resulting in an interaction between ME and AA levels ($P < 0.05$). Reducing ME density and increasing AA density, independently increased the overall feed intake (FI) ($P < 0.01$). An interaction between ME and AA resulted in improved feed conversion ratio (FCR) in response to increased AA density at both +3 and +6 % only at standard ME density, but with reduced ME diets FCR significantly improved only when birds received the high AA density diets ($P < 0.01$). Decreasing ME density by 100 and 150 kcal enhanced breast meat yield and reduced abdominal fat pad weight irrespective of diet AA density ($P < 0.01$). Increasing AA density, at +6 % enhanced breast meat yield and at +3 and +6 % decreased abdominal fat pad weight ($P < 0.01$). The dietary ME density did not have any significant effect on woody breast, however, decreasing ME density significantly reduced white striping compared to standard ME levels ($P < 0.05$). Increasing AA density at +6 % increased both white stripping and woody breast ($P < 0.01$). In conclusion, the findings of the current study suggest that overall, despite the interactions between ME and AA density for some performance responses, decreasing dietary ME does not have any significant effect on body weight gain or growth rate. However, every 50 kcal/kg reduction in ME increased feed intake by approximately 66 g/bird (0-42 d). This increased feed intake in response to lower ME levels increases FCR by around 2 points for every 50-kcal reduction of ME across all AA levels. Increasing dietary AA density by 3 % increases bodyweight gain and feed intake by approximately 90 and 85 g/bird, respectively (0-42 d). Increasing AA density by another 3 % results in a further 50 g/bird increase in bodyweight and 30 g/bird increase in feed intake. Each 3 % increase in AA density improves FCR by approximately 3 points.

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EXOGENOUS PHYTASE AND INTESTINAL UPTAKES OF AMINO ACIDS

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Summary

The impacts of exogenous phytase on intestinal uptakes of amino acids, which are pivotal to protein utilisation, are reviewed. The likelihood is that phytases enhance amino acid intestinal uptakes as oligopeptides conducted via the peptide carrier, PepT-1, and as monomeric amino acids via Na^+-dependent transport systems. Phytate and phytase have profound reciprocal effects on sodium (Na) which may impact the function of the sodium pump (Na^+,K^+-ATPase). The functionality of Na^+-dependent amino acid transporters, directly, and PepT-1, indirectly, depend on the sodium pump.

I. INTRODUCTION

Exogenous phytases increased apparent ileal digestibility coefficients of 17 amino acids by an average of 4.13% (0.833 versus 0.800) in one meta-analysis (Cowieson et al., 2017); however, the scale of responses varies. Phytases increased apparent ileal digestibility coefficients of 16 amino acids by averages of 12.7%, 7.24% and 9.15%, respectively, across three selected assays (Amerah et al., 2014; Truong et al., 2015; Martínez-Vallespín et al., 2022) with a pronounced overall increase of 9.58% (0.853 versus 0.779). Although the other two assays reported greater distal ileal responses, 500 FTU/kg phytase increased proximal jejunal digestibilities of 16 amino acids by an average of 49.7% (0.720 versus 0.481) in Truong et al. (2015). The differences in these outcomes merit closer examination and the likelihood is that small intestinal uptakes of amino acids are more rate-limiting on broiler growth performance than protein digestion by endogenous proteolytic enzymes in the gut lumen (Croom et al., 1999). Increases in digestibility of protein/amino acids generated by phytase are consequences of the corresponding anti-nutritive effects of the substrate, phytate (*myo*-inositol hexaphosphate; IP_6).

II. PROTEIN DIGESTION

Pepsin and peptide end-products of pepsin digestion initiate and regulate protein digestive processes (Hersey, 1987). However, an additional 7.80 g/kg dietary phytate significantly depressed pepsin activity in the proventriculus of broiler chickens by 6.31% (14.84 versus 15.84 nmol/mg) in Liu et al. (2009). The genesis of this observation is that protein bound to phytate in binary protein-phytate complexes is refractory to pepsin digestion (Vaintraub and Bulmaga, 1991). Indeed, under *in vitro* conditions, IP_6 phytate must be degraded to IP_2 and IP_1 phytate for complete alleviation of pepsin inhibition (Yu et al., 2012). The likelihood is that the refractory properties of phytate-complexed protein promote compensatory hypersecretions of pepsin and HCl and support for this has been generated in rats (Mitjavila et al., 1973) and pigs (Decuypere et al., 1981). Protective hypersecretions of mucin and sodium bicarbonate ($NaHCO_3$) are generated to counteract the 'internal aggressors', pepsin and HCl (Allen and Flemström, 2005). Accordingly, Onyango et al. (2009) found that Mg-K-phytate increased crude mucin excretion by a 2.6-fold factor (8.00 versus 3.05 g/bird) over a 54-hour period in broiler chickens. Additionally, Selle et al. (2009) found that phytase increased apparent ileal Na digestibility by 92.3% (-0.04 versus -0.52) in association with an average increase of 5.08% (0.806 versus 0.767) in the digestibility of 17 amino acids. The negative Na digestibility coefficient in the control birds is indicative of endogenous Na flows, probably arising from

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pancreatic secretions of $NaHCO_3$ into the duodenum (Case et al., 1970), but phytase attenuates the need for protective hypersecretions of $NaHCO_3$ and mucin.

III. INTESTINAL UPTAKES OF AMINO ACIDS

The following approximations are instructive in respect of amino acid intestinal uptakes. About 10% of amino acids are absorbed as single entities via several Na^+-independent transport systems, but a further 15% are absorbed via numerous Na^+-dependent transport systems. Pivotally, some 75% of amino acids are absorbed as di- and tri-peptides, or oligopeptides, via the peptide transporter, PepT-1 (Krehbiel and Matthews, 2003). However, PepT-1 is a proton-coupled carrier and functions in tandem with the Na^+/H^+ exchanger, NHE. Therefore, PepT-1 is indirectly Na^+-dependent as it is reliant on the provision of protons from NHE, which is in turn reliant on the activity of the 'sodium pump' (Na^+,-K^+-ATPase). The intestinal uptakes of amino acids and glucose, as outlined, are illustrated in Figure 1.

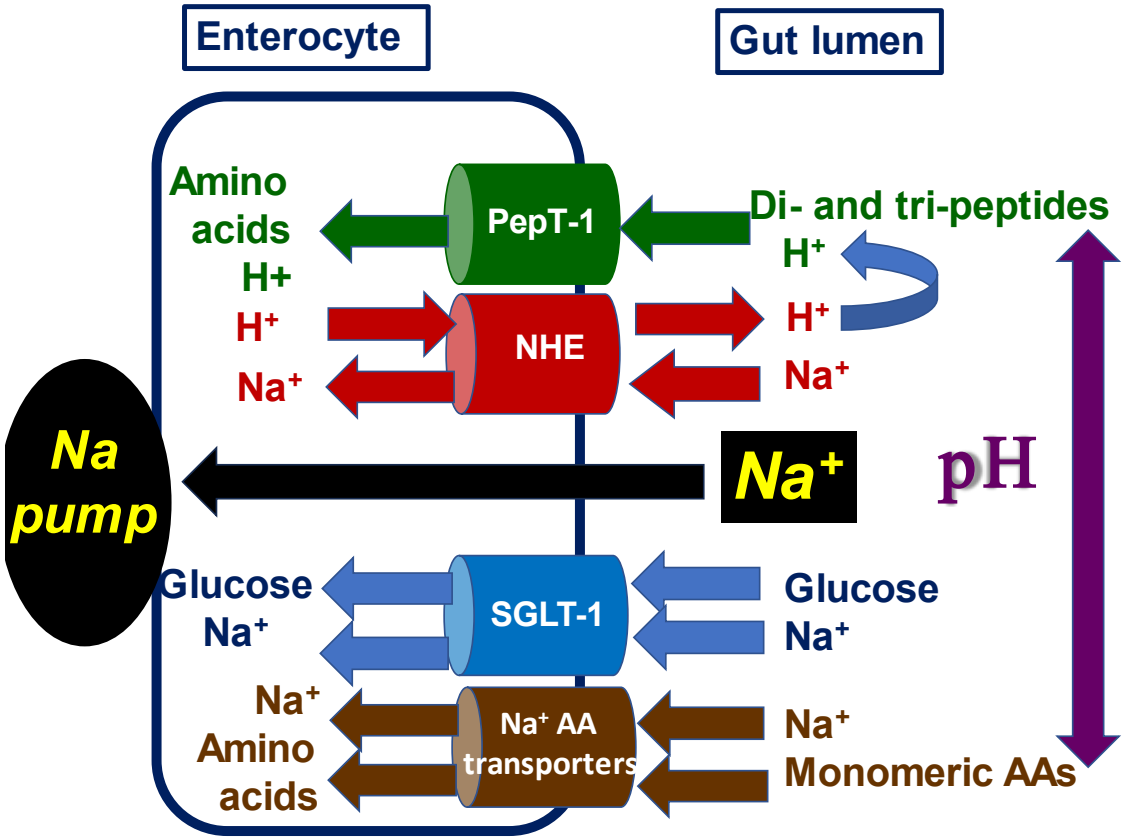

Figure 1 - A schematic diagram of the intestinal uptakes of amino acids and glucose that are directly, or indirectly, dependent on the activity of the sodium pump, Na^+,K^+-ATPase.

Interestingly, Dilworth et al. (2005) reported that sodium phytate or phytate extracted from sweet potato reduced small intestinal Na^+,-K^+-ATPase activity by approximately 70% in rats. Therefore, it is relevant that 500 and 1000 FTU/kg phytase has been shown to increase Na^+,K^+-ATPase concentrations by a collective 18.2% (11.52 versus 9.75 µmol/mg) in the jejunal mucosa of broiler chickens (Liu et al., 2008). Also, Akter et al. (2019) reported that phytase increased Na^+,K^+-ATPase activity by 42.9% (89.88 versus 62.88 nmol/mg protein/minute) in the jejunal mucosa of broilers offered diets with three tiers of Na concentrations. Clearly, the reciprocal impacts of phytate and phytase on the activity of Na^+,K^+-ATPase carry huge implications for the Na^+-dependent intestinal uptakes of nutrients which

includes amino acids and glucose absorption, which principally takes place via the Na^+-dependent transporter, SGLT-1 (Wright, 1993).

IV. PEPT-1 AND PHYTASE

It has been proposed that the functionality of PepT-1, the transporter responsible for intestinal uptakes of most amino acids, is advantaged by exogenous phytase (Selle et al., 2023). One plank for this proposal was that the prime factor influencing PepT-1 activity is the presence of substrates in the gut lumen; di- and tri-peptides (Wang et al., 2017). It is plausible that phytase, by rendering proteins more vulnerable to pepsin digestion, is facilitating the conversion of polypeptides to oligopeptides along the digestive tract, which would up-regulate PepT-1 activity. In support, elevated dietary protein levels have been shown to increase mRNA abundance for PepT-1 in poultry (Osmanyan et al., 2018). A second plank was that PepT-1/NHE activity is favoured by relatively low small intestinal pH levels (Kennedy et al., 2002). However, phytase P and Ca matrix values allow reduced levels of limestone and dicalcium phosphate in broiler diets, both of which have very high acid binding capacities (Lawlor et al., 2005). This should favour PepT-1/NHE activity by lowering dietary acid binding capacities and depressing pH levels along the small intestine. In support of this, increased dietary limestone inclusions from 3.0 to 18.7 g/kg was associated with a decrease of 7.95% (0.718 versus 0.780) in mean ileal digestibilities of 17 amino acids in Amerah et al. (2014). Finally, PepT-1 is, effectively, a Na^+-dependent transporter given that it requires proton donation from the Na^+/H^+ exchanger, NHE, for the co-transport of oligopeptides (Spanier, 2014). The unequivocal impact of phytase on apparent Na digestibility coefficients in broiler chickens was demonstrated by Truong et al. (2014, 2015, 2017). Collectively, exogenous phytase increased Na digestibility by 30.5% (–2.443 versus–3.517) in proximal jejunum, 30.2% (–1.425 versus –2.043) in distal jejunum, 42.7% (–0.839 versus –1.465) in proximal ileum and 25.5% (–0.327 versus– 0.439) in distal ileum at 28 days post-hatch.

V. SODIUM PUMP (Na^+,K^+-ATPase) AND PHYTASE

Phytase increased apparent Na digestibility coefficients along the small intestine by a pronounced average of 32.5% (-1.259 versus -1.866) in the above data. The genesis of this response may be a combination of attenuated endogenous $NaHCO_3$ secretions and enhanced function of Na^+,K^+-ATPase (Liu et al., 2008). Importantly, sodium pump activity is highly dependent on cytoplasmic Na concentrations within enterocytes (Therien and Blostein, 2000). This raises the possibility that if Na is partitioned to the pancreas to provide endogenous secretions of $NaHCO_3$, then Na concentrations within enterocytes could be depleted to the extent that Na^+,K^+-ATPase activity is depressed and Na^+-dependent intestinal uptakes of amino acids are compromised. This is not established and there may be other causes of depressed Na^+,K^+-ATPase activity, including the possibility that phytase facilitates the re-phosphorylation of the sodium pump by liberating phytate-bound phosphorus , as suggested by Martinez-Amezcua et al. (2006).

While speculative, the pronounced improvements in amino acid digestibilities in response to exogenous phytase may stem from increases in amino acid intestinal uptakes as oligopeptides driven by PepT-1. Certainly, further research into the phytate-phytase axis in relation to the functions of Pept-1, NHE and Na^+,K^+-ATPase in broiler chickens is justified. This could provide strategies for chicken-meat producers to take greater advantage of the ‘protein effect’ of exogenous phytases.

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HIGH DOSES OF PHYTASE CAN COMPLETELY REMOVE INORGANIC PHOSPHATE FROM BROILERS DIETS FROM 6 TO 21 DAYS OF AGE

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Summary

An experiment was conducted to evaluate the effects of graded levels of phytase supplementation in phosphate-free diets on performance, bone parameters and plasma mineral concentration of broilers from 6 to 21 days of age. Three hundred and sixty male Cobb 500 broilers were randomly distributed to 9 treatments with 8 cages per replicate. Treatments consisted of 5 dietary available P (avP) levels (4.2, 3.5, 2.7, 2.0 and 0.12 g/kg) and 4 dietary phytase (250, 500, 1000, and 2000 FTU/kg) levels added on top of the 0.12 g/kg basal diet. On average, phytase was capable of sparing 1.11, 1.53, 1.99 and 2.49 g/kg of avP, for 250, 500, 1000 and 2000 FTU/kg, respectively. The results of this study concluded that higher doses of dietary phytase can be used to completely remove inorganic phosphate from broilers diets from 6-21 days of age without impairing performance or tibia ash.

I. INTRODUCTION

Phosphorus (P) is an essential mineral incorporated into broiler diets in the form of an inorganic phosphate. In broilers it is involved in growth, bone development, protein synthesis, and energy metabolism (Li et al., 2016). The supply of P is limited, hence the poultry industry and nutritionists are investigating alternative avenues to reduce inorganic phosphorus supplementation, while supporting the reduction of their carbon footprint effectively, in broilers. Therefore, the objective of this study was to evaluate the effect of graded levels of phytase supplementation in phosphate-free diets on performance, bone parameters and plasma mineral concentration of broilers from 6 to 21 days of age.

II. MATERIALS AND METHODS

Three hundred and sixty male Cobb 500 broilers were randomly distributed to 9 treatments with 8 cages per replicate. Treatments consisted of 5 dietary avP levels (4.2, 3.5, 2.7, 2.0 and 1.2 g/kg) and 4 dietary phytase levels (250, 500, 1000 and 2000 FTU/kg, Quantum Blue, AB Vista, UK). The 4 levels of dietary phytase were added on top of the 1.2 g/kg avP diet. Monocalcium phosphate was used as source of dietary inorganic phosphorus. Dietary calcium was also decreased providing 8.4, 7.6, 6.8, 5.9 and 5.1 g/kg, respectively. All birds were raised from day 1 to day 5 on a 4.2g/kg avP and 8.4 g/kg Ca prior to going on the dietary treatments on day six to avoid excessive mortality and comply with welfare standards. Diets were corn and soybean-meal based and contained 20g/kg of rapeseed meal and 22g/kg of rice bran to increase dietary phytate-P content to 3 g/kg to ensure adequate substrate for the phytase (Table 1). Diets were steam pelleted followed by crumbling and fed *ad libitum* to 21 days. Performance parameters measured were average daily gain (ADG), average daily feed intake (ADFI) and feed conversion ratio corrected for mortality (mFCR). At 21 days of age all birds were sacrificed, left tibias excised, and tibia ash percent (TAP) measured. Additionally, blood was sampled via cardiac puncture, plasma separated, with phosphorus (P) and calcium (Ca) analyzed. A one-way ANOVA was performed (JMP Pro 16.2) and means separated using

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Student's T-Test ($P \leq 0.05$). Polynomial orthogonal contrasts (linear, logarithmical, and quadratic) were assessed and P equivalency of phytase calculated.

Table 1 - Basal diets composition.

	Available P	
Ingredient composition, %	4.2	1.2
Maize (corn)	57.77	60.52
Rice bran, defatted	2.200	2.200
Rapeseed/Canola meal	2.000	2.000
Soybean meal	31.08	30.84
Soya oil	2.710	1.793
Limestone	1.247	0.982
Monocalcium phosphate	1.310	0.000
Salt	0.289	0.286
Sodium bicarbonate	0.131	0.135
Titanium dioxide	0.300	0.300
Lysine HCl	0.258	0.260
DL Methionine	0.320	0.316
L Threonine	0.095	0.093
L Valine	0.049	0.047
Choline chloride; 60%	0.058	0.055
Trace Mineral Premix	0.080	0.080
Vitamin Premix	0.100	0.100
Calculated nutrient composition		
Crude Protein, g/kg	200	200
Ash, g/kg	53.8	40.1
Ca, g/kg	8.40	5.10
Total P, g/kg	7.00	4.10
Available P, g/kg	4.20	1.20
Phytate P, g/kg	3.00	3.00
Na, g/kg	1.60	1.60
Cl,g/kg	3.00	3.00
dLys, g/kg	11.2	11.2
dM+C, g/kg	8.50	8.50
dThr, g/kg	7.30	7.30
dVal, g/kg	8.50	8.50
Choline, mg/kg	1600	1600
AME, MJ/kg	12.7	12.7

III. RESULTS AND DISCUSSION

A reduction of P and Ca by reducing MCP and limestone suppressed ADG, ADFI, TAP, and plasma mineral content of broilers ($P < 0.01$, Table 2) to day 21. For ADG, ADFI and TAP, there was a linear, logarithmical, and quadratic reduction ($P < 0.01$, Table 2), with the logarithmic regression yielding the best fit. Plasma P was also affected linearly, logarithmically, and quadratically ($P < 0.01$, Table 2), with the best fit being with linear regression. However, for Plasma Ca, the only significant fit was quadratic ($P < 0.01$, Table 2).

Table 2 - Performance, percent tibia ash and plasma mineral content of broilers fed diets with graded levels of phosphate and phytase from 6 to 21 days of age.

Parameter		ADG, g/b/d	ADFI, g/b/d	mFCR, g:g	Liv-ability[1] %	Tibia ash, %	Plasma P, mmol/kg	Plasma Ca, mmol/kg
Dietary avP, g/kg	4.2	62.2[a]	78.8[ab]	1.28[b]	97.5	36.5[a]	1.92[a]	2.64[cd]
	3.5	61.7[a]	80.3[a]	1.30[b]	100	36.2[ab]	1.57[bc]	2.69[bcd]
	2.7	59.0[ab]	76.7[ab]	1.30[b]	100	33.4[bc]	1.14[def]	2.92[ab]
	2.0	49.5[c]	67.6[d]	1.41[b]	97.5	31.6[cd]	0.82[f]	3.06[a]
	1.2	31.6[d]	48.5[e]	1.65[a]	82.5	24.5[e]	1.18[de]	2.64[cd]
Phytase, FTU/kg	250	51.8[c]	69.9[cd]	1.39[b]	97.5	29.8[d]	1.10[def]	2.81[bc]
	500	56.8[b]	74.7[bc]	1.32[b]	100	31.7[cd]	1.03[ef]	2.80[bc]
	1000	61.5[a]	79.4[ab]	1.32[b]	97.5	35.2[ab]	1.40[cd]	2.56[cd]
	2000	62.1[a]	79.4[ab]	1.28[b]	100	35.9[ab]	1.76[ab]	2.47[d]
Pooled SEM[2]		1.63	1.76	0.047	-	0.88	0.122	0.103
P-Values	ANOVA	< 0.01	< 0.01	< 0.01	0.09	< 0.01	< 0.01	< 0.01
	avP	L, LN, Q	L, LN, Q	L, LN, Q	-	L, LN, Q	L, LN, Q	Q
	Phytase	L, LN, Q	L, LN, Q	L, LN, Q	-	L, LN, Q	L, LN	L

[a-f] Means having different superscripts within the column are significantly different (P < 0.05); [1]Data analyzed using Chi-Square (not normally distributed); [2]Pooled standard error of mean; Linear (L), logarithmic (LN) and quadratic (Q) significance of Orthogonal Polynomial Contrasts (P < 0.05).

Phytase analysis was 28% on average lower than expected. Despite being lower than expected, phytase inclusion improved performance, TAP, and plasma mineral content (P < 0.01, Table 2). The addition of phytase improved performance and TAP (P < 0.01, Table 2) linearly, logarithmically, and quadratically, with the best fit being attained with logarithmic regressions. For plasma minerals, phytase dose improved plasma P content in a linear and logarithmical fashion (P < 0.01, Table 2) while plasma Ca was improved in a linear fashion (P < 0.05, Table 2). For plasma Ca and P the best fit was the linear regression.

Table 3 - Phosphorus equivalency and sparing effect of monocalcium phosphate and limestone for graded levels of phytase supplemented to broilers fed phosphate free diets from 6 to 21 days of age.

FTU/kg	P Equivalency, g/kg				MCP, g/kg feed	Limestone, g/kg feed
	ADG	ADFI	TAP	Average		
250	1.31	1.32	0.71	1.11	5.0	1.0
500	1.68	1.69	1.23	1.53	6.9	1.4
1000	2.07	2.09	1.81	1.99	9.0	1.8
2000	2.49	2.52	2.45	2.49	11.2	2.3

To determine P equivalency, ADG, ADFI and TAP were used. A best-fit regression for P dose-response and phytase dose response was employed, which was logarithmical. On average, phytase spared 1.11, 1.53, 1.99, and 2.49 g/kg of avP for 250, 500, 1000, and 2000 FTU/kg, respectively (Table 3). This corresponded to a reduction in dietary monocalcium phosphate of 11.2 g/kg, and 2.3 g/kg of limestone when 2000 FTU/kg was utilized in the diet (Table 3).

To sense check the P equivalencies, we determined the maximum response of P for ADG, ADFI, and TAP using a vertex of quadratic regression. The vertex was observed at 3.5, 3.4, and 3.8 g/kg of avP for ADG, ADFI and TAP, respectively. This demonstrates the avP response was in fact, limited to a range of 2.2 to 2.6 g/kg, and caution needs to be exercised when looking at the P equivalence of 2000 FTU/kg phytase.

In conclusion, this study demonstrates that higher levels of phytase can be used to completely remove inorganic phosphate from broilers' diets from 6-21 days of age without impairing performance or tibia ash of broilers. Available phosphorus requirement (expressed

as 95% of maximum response or vertex) ranged from 3.1 to 3.4 g/kg of avP, which is below the breeder guideline recommendations and may limit the correct assessment of P-sparing effects of phytases, especially for dose rates above 1000 FTU/kg.

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BROILER CHICKENS OFFERED REDUCED CRUDE PROTEIN DIETS BASED ON SORGHUM OUTPERFORM THEIR WHEAT-BASED DIET COUNTERPARTS

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Summary

The study compared growth performance in straight-run broiler chickens offered standard (205 g/kg) or reduced (175g/kg) crude protein diets based on wheat or sorghum from 14 to 35 days post-hatch. Treatment interactions ($P < 0.001$) were observed for crude protein concentrations and grain type for weight gain and FCR. Reducing crude protein in sorghum-based diets did not influence weight gain and FCR; in contrast, weight gain and FCR were compromised in broilers offered wheat-based diets. This outcome was consistent with previous studies where wheat-based diets were inferior to maize-based diets following crude protein reductions.

I. INTRODUCTION

Wheat and sorghum are the most common feed grains for chicken-meat production in Australia. In two direct comparisons birds offered maize-based diets were better able to accommodate dietary crude protein (CP) reductions than wheat-based diets (Chrystal et al., 2021; Greenhalgh et al., 2022). One underlying reason for this difference appears to be the higher protein contents of wheat than maize and, similarly, wheat (n = 27) contained higher protein levels (115.5 versus 101.9 g/kg CP) than sorghum (n = 17) in one Australian survey (Bryden et al., 2009). The higher wheat protein content results in elevated non-bound (synthetic, crystalline) amino acid inclusions to meet dietary amino acids specifications. This may exacerbate post-enteral imbalances between protein-bound and non-bound amino acids. Relative to wheat, sorghum and maize are similar in respect of lower CP contents, slower starch digestion rates, and lower soluble NSP contents (Liu et al., 2015). The objective of this study was to determine the impact of dietary CP concentrations and grain type on growth performance and relative fat-pad weights in straight-run broiler chickens. The hypothesis is that reduced-CP sorghum-based diets will maintain better broiler growth performance than wheat-based diets.

II. MATERIALS AND METHODS

A common starter diet was offered to birds to 14 days post-hatch. 288 mixed-sex Ross 308 broiler birds were weighed, tagged, and allocated to four dietary treatments with 12 replicates of 6 birds per cage at 14 days post-hatch. The experimental design was a 2×2 factorial array of treatments, incorporating two concentrations of dietary CP (205 vs 175 g/kg) and two feed grains (wheat vs sorghum). The experimental diets were offered to the birds from 14 to 35 days post-hatch. The composition of the experimental diets is shown in Table 1. A total of 14 non-bound amino acids were incorporated into the 175 g/kg CP diets in order to match their amino acid concentrations in the 205 g/kg CP diets. Both wheat and sorghum were mediumly ground (4.0 mm hammer-mill screen) prior to being blended into the complete diets which were steam-pelleted through a Palmer PP330 pellet press (Palmer Milling Engineering, Griffith, NSW, Australia) at a conditioning temperature of 80°C with a conditioner residence time of 14 seconds and were then cooled. All experimental diets contained both phytate- and NSP-degrading enzymes. At the end of the study the gender of birds was identified, and a two-way

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analysis of covariance (ANCOVA) was employed to determine the impact of dietary treatments in which the percentage of male birds in each caged replicate was used as the covariant.

Table 1 - Composition of experimental diets.

Feed ingredient (g/kg)	Starter	W 205	W 175	S 205	S 175
Wheat 12%	320	647	822	0.00	0.00
Sorghum 10%	320	0.00	0.00	623	745
Soybean Meal 46.0%	295	259	66	282	131
Canola Oil	18.0	40.2	10.2	39.4	21.3
d,l-Methionine	4.13	3.40	4.11	3.45	3.92
Glycine	0.00	0.00	5.08	0.00	4.88
l-Arginine	1.85	1.11	6.34	1.03	5.31
l-Histidine	0.00	0.00	1.67	0.00	1.47
l-Isoleucine	0.70	0.40	3.43	0.00	2.43
l-Leucine	0.00	0.00	4.60	0.00	1.04
l-Lysine-HCl	4.79	2.87	7.30	2.72	6.31
l-Phenylalanine	0.00	0.00	3.06	0.00	2.28
l-Threonine	2.27	1.71	4.19	1.44	3.44
l-Tryptophan	0.00	0.00	0.43	0.00	0.44
l-Valine	1.22	0.61	3.63	0.16	2.59
l-Cysteine	0.00	0.00	0.81	0.00	0.83
l-Glutamine	0.00	0.00	1.00	0.00	8.83
l-Proline	0.00	0.00	1.73	0.00	2.11
Celite	0	20.0	20.0	20.0	20.0
Limestone	13.8	11.7	12.1	11.4	11.0
Sodium bicarbonate	3.41	2.82	5.80	3.51	6.06
Sodium chloride	1.91	2.01	0	1.68	0
Vitamin-mineral premix	2.00	2.00	2.00	2.00	2.00
Choline chloride 75%	1.00	0.62	1.18	1.24	1.83
Xylanase	0.20	0.20	0.20	0.20	0.20
Phytase	0.10	0.10	0.10	0.10	0.10
Total non-bound amino acids	13.91	10.1	47.4	8.80	45.9

III. RESULTS

The effects of dietary crude protein concentration and grain type on broiler growth performance and relative fat-pad weights are presented in Table 2. A treatment interaction ($P < 0.001$) between CP level and grain type was observed for weight gain because there was a 10.1% decline in weight gain (1964 versus 2184 g/bird) in birds offered wheat-based diets but sorghum supported statistically similar weight gains. A similar interaction ($P < 0.001$) was observed for FCR, which was compromised by 9.68% (1.575 versus 1.436) in birds offered wheat-based diets as opposed to a numerical increase of 0.97% (1.464 versus 1.450) with sorghum-based diets. A third interaction ($P < 0.001$) was observed relative fat-pad weights as in reduced-CP diets, sorghum-based diets generated 26.0% (10.90 versus 8.65 g/kg) heavier abdominal fat-pads than wheat. There were not any significant treatment effects for feed intakes.

IV. DISCUSSION

Birds across all treatments outperformed 2022 Ross 308 performance objectives by 18.7% (2092 versus 1763 g/bird) in weight gain, by 15.3% (3093 versus 2682 g/bird) in feed intake and by 2.63% (1.481 versus 1.521) in FCR. Wheat supported faster weight gain than sorghum by 2.92% (2184 versus 2122 g/bird) and improved FCR by 0.96% (1.436 versus 1.450) in 205 g/kg CP diets; in contrast, sorghum supported 6.72% (2096 versus 1964 g/bird) faster gains

and an improvement in FCR of 7.05% (1.464 versus 1.575) in 175 g/kg CP diets. This turnaround demonstrates that sorghum is a more suitable feed grain than wheat in the context of reduced-CP diets; thus, the hypothesis was established.

Table 2 - The effects of dietary treatments on growth performance and relative abdominal fat-pad weights from 14 to 35 days post-hatch.

Treatment		Growth performance (g/bird)			Fad-pad
Crude protein (g/kg)	Feed grain	Weight gain	Feed intake	FCR (g/g)	weights (g/kg)
205	Wheat	2184[a]	3135	1.436[c]	8.86[b]
	Sorghum	2122[b]	3076	1.450[bc]	8.12[b]
175	Wheat	1964[c]	3095	1.575[a]	8.64[b]
	Sorghum	2096[b]	3067	1.464[b]	10.9[a]
SEM		18.9	24.2	0.0089	0.35
Main effects: Crude protein					
205		2153	3106	1.443	8.49
175		2030	3081	1.520	9.76
Feed grain					
Wheat		2074	3115	1.506	8.85
Sorghum		2109	3072	1.457	9.50
Significance (P=)					
Crude protein (CP)		< 0.001	0.321	< 0.001	< 0.001
Feed grain (FG)		0.036	0.075	< 0.001	0.035
CP x FG interaction		< 0.001	0.522	< 0.001	< 0.001

[abc] Means within columns not sharing a common suffix are significantly different at the 5% level of probability.

The shortfalls of reduced-CP, wheat-based broiler diets were given consideration in Selle et al. (2022a). The digestion-rate constant of wheat starch (0.036/minute) is more rapid than both sorghum (0.018/minute) and maize (0.017/minute) under *in vitro* conditions (Giuberti et al., 2012). Similar patterns have been reported in broiler chickens (Selle et al., 2021), where the value for wheat starch (0.117) was again more rapid than sorghum (0.075) and maize (0.086). Slowly digestible starch is seen as an advantage in broiler diets (Herwig et al., 2019) and may spare amino acids from catabolism in the gut mucosa (Enting et al., 2005). Moreover, the digestion of slowly digestible starch and absorption of glucose may be better aligned with protein digestion and amino acid absorption resulting in a more harmonious provision of glucose and amino acids at sites of protein synthesis to drive efficient growth (Liu and Selle, 2017). Curiously, the difference in relative fat-pad weights, where sorghum generated greater fat deposition, may stem from the difference in starch digestion rates. It seems likely that the gradual digestion of sorghum starch results in more glucose being converted to glycogen and then fat via *de novo* lipogenesis, whereas glucose from rapidly digested wheat starch is directly oxidised more readily (Selle et al., 2022b). Additional factors inherent in wheat that may be contributing to its lack of suitability in reduced-CP diets may include soluble non-starch polysaccharides, amylase trypsin inhibitors and gluten (Selle et al., 2022a). Also, any impacts of starch on growth performance in reduced-CP diets would be amplified by the greater increase in wheat inclusions (647 to 822 g/kg – 27%) than sorghum (from 623 to 745 g/kg – 20%) pursuant to dietary CP reductions.

Usually a reduced-CP, wheat-based diet will contain more non-bound amino acids than equivalent diets based on sorghum or maize because of wheat's higher protein content. However, this was not the case in the present study where the sorghum- and wheat-based diets contained 45.9 and 47.4 g/kg non-bound amino acids, respectively. These high total inclusion levels are effectively identical and the inclusions of individual non-bound amino acids are similar with the exception of glutamine. Glutamine was added at 8.83 g/kg to the reduced-CP sorghum diet as opposed to 1.00 g/kg in the wheat-based diet; wheat typically contains more

glutamic acid than sorghum (Bryden et al., 2009). This difference in glutamine supplementation to reduced-CP diets is probably important. This is because 10 g/kg non-bound glutamine inclusions in adequate protein diets generated an average 7.36% (range: 3.07 to 10.6%) increase in weight gain across seven studies as reviewed by Selle et al. (2024). For example, significant responses of 9.33% (Soltan, 2009) and 10.6% (Moghaddam and Alizadeh-Ghamsari, 2013) were recorded in two of these studies. Given that diets in the seven studies contained adequate protein levels, glutamine concentrations would have been notionally sufficient; nevertheless, positive weight gain responses were consistently reported. The positive impacts of glutamine probably involved the effects of this amino acid on both protein turnover and acid-base balance.

The interchangeable amino acids, glutamine and glutamate, are vital metabolites as they play a central role in cell metabolism and function (Newsholme et al., 2003). The positive relationship between intramuscular glutamine concentrations and protein synthesis in chickens, indicates that intracellular glutamine is involved in promoting protein synthesis (Watford and Wu, 2005). Earlier, Wu and Thompson (1990) investigated the effects of glutamine on protein turnover in isolated avian skeletal muscle tissue and concluded that glutamine appeared to have an overall anabolic effect. Amino acid metabolism and acid-base homeostasis are inextricably related (Patience, 1990). In a recent broiler study, it was shown that glutamine and asparagine had positive impacts on acid-base balance (Ibrahim et al., 2023).

Finally, sorghum-based diets exhibited promise following the dietary CP reduction of 30.0 g/kg by attenuating declines in weight gain and feed conversion efficiency compared to wheat-based diets. The additional glutamine in the sorghum-based diets may have been a factor in this comparison and glutamine merits further investigation.

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XYLO-OLIGOSACCHARIDES AND ARABINOXYLAN SUPPLEMENTATION ENHANCES ILEAL LACTATE PRODUCTION IN BROILER CHICKENS

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Xylo-oligosaccharides (XOS) are fermented in the gut by the microbiota leading to the production of short-chain fatty acids (SCFA). A previous study showed that XOS (0.5%) and arabinoxylan-rich fraction (AXRF; 1%) supplementation can improve feed efficiency and gut health in broiler chickens (Castro *et al.*, 2023). This study aimed to evaluate microbiome composition and fermentation patterns following XOS/AXRF supplementation. The hypothesis is that XOS/AXRF supplementation would increase SCFA production and modulate the microbial community by promoting specific beneficial bacteria.

One-day-old broiler chickens (mixed-sex) were provided with a corn/soybean meal-based mash diet either with or without supplemental XOS (0.5%) and AXRF of wheat (1%). Feed and water were provided ad libitum from 1 to 42 days of age. Each treatment was fed to 8 replicate pens, with 8 chickens per pen. On day 42, one male chicken of average weight per pen was euthanised. Ileum and cecum digesta contents were collected for SCFA and microbiome analysis, respectively. QIAamp PowerFecal Pro DNA Kit was used to extract DNA from the cecum digesta, and MinION Oxford Nanopore Technology was used for long-read sequencing. The dataset was analyzed in Galaxy EU platform. Identification and quantification of SCFA was performed using ^{1}H NMR spectroscopy. R was used to analyze SCFA data using Mann–Whitney-Wilcoxon test, and microbial diversity and relative abundance. The results showed no significant ($P > 0.05$) differences in microbial alpha and beta diversity between supplemented and control birds. *Faecalibacterium* was the most abundant genus across both groups; mean relative abundance was 12% and 15% for XOS/AXRF supplemented and controls, respectively. Ileal lactate concentration was significantly ($P < 0.05$) higher in XOS/AXRF birds compared to the control group (Table 1). Studies have shown that lactate can be converted to butyrate production – known for its positive impact on gut health - by bacterial cross-feeding mechanisms. No significant differences ($P > 0.05$) in SCFA concentrations were observed in the cecum between the two groups.

Table 1 - Concentration of SCFA in the ileum of chickens supplemented with xylo-oligosaccharides and arabinoxylan-rich fraction (XOS/AXRF) compared to control birds.

Treatment	SCFA µmol/g					
	Acetate	Propionate	Butyrate	i-Butyrate	Lactate	Succinate
Control	60.3	0.5	1.0	13.8	25.4^{b}	0.9
XOS/AXRF	82.6	2.1	13.3	58.4	205.9^{a}	5.7
P value	0.3823	0.0650	0.2345	0.5054	0.0047	0.5054

SCFA= short-chain fatty acids. ^{a,b} Means within the same column with no common superscript differ significantly ($P < 0.05$).

In conclusion, XOS/AXRF supplementation enhances lactate production in the ileum in broiler chickens. Thus, XOS/AXRF may potentially contribute to improve gut health.

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EFFECTS OF DIETARY METHIONINE SOURCES AND LEVELS ON THE INFLAMMATORY RESPONSES OF LIPOPOLYSACCHARIDE-CHALLENGED BROILERS

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Summary

Poultry are often facing inflammatory challenges due to sanitary pressure which accompanied by immune stress that impaired growth performance, thereby changing the nutrient demand in such environment. Oral challenge with lipopolysaccharides (LPS) is often used as an immune stress model for evaluation of nutrient needs under stressful conditions. This study investigated the effects of dietary methionine (Met) sources and levels on growth and immune response of broilers challenged with LPS. We used 792, one-d-old Arbor Acre broiler chicks in a 2 × 3 × 2 factorial arrangement of treatments with two Met sources (DL-Met and DL-2-hydroxy-4-(methylthio)-butanoic acid (OH-Met)), three TSAA levels (Low:80%, Middle:100%, and High:120% of the recommended TSAA Level) and two immunological states (LPS challenge or saline). The trial included starter phase (1 to 14 days), and stress period (15 to 21 days). During stress period, broilers were injected intra-abdominally either with LPS solution (one mg/kg) or with equal sterile saline. Relative to saline injection, lower feed intake (FI), lower body weight gain (BWG), increased relative spleen weight, and decreased relative thymus weight were found in broilers after LPS challenge ($P < 0.05$). Significant main effects of LPS, Met sources and levels on serum ovotransferrin (OT) were also observed ($P < 0.05$). Alpha-1-acid glycoprotein (α1-AGP) and IgA were increased by Met source and LPS challenge ($P < 0.05$) with a significant interaction effect of Met sources and LPS challenge on serum-amyloid-A and IgA ($P < 0.05$). Compared with the DL-Met group, dietary OH-Met supplementation increased BWG in broilers at middle-TSAA level ($P < 0.05$). The mRNA expressions of *IL-1β* and *IL-6* and *TNF-α* in the spleen were also affected by LPS challenge, and some of those responses varied with the Met sources or Met levels. Additionally, interactions among Met sources, Met levels and LPS challenge were specifically found in the *IL-6* expression. In summary, Met sources and levels played a role in the development of responses to LPS infections.

I. INTRODUCTION

It is widely known that Met is not only an essential amino acid but also the first limiting amino acid for broilers fed corn-soy diets and plays an important regulatory role in many physiological processes (Estevez et al., 2020). To meet the nutritional needs of Met, synthetic sources such as DL-Met and OH-Met are routinely added in broiler diets. LPS is a cell wall component of Gram-negative bacteria and is released in increased amounts when the bacteria die, or its cell wall is broken down. LPS stimulates the production of pro-inflammatory agents such as TNF-α, IL-1, IL-6 by activating TLR4/MyD88/NFκB signaling pathway reaction to exacerbate inflammation (Cohen, 2002). Hence, LPS is widely used as an immune stress model. The present study was designed to determine the effects of Met sources and levels on growth performance and immune responses in chickens subjected to an LPS inflammatory challenge.

II. METHOD

A total of 792, one-d-old male Arbor Acre broilers were assigned randomly to 12 treatments with six replicate pens with 11 broilers in each pen. The birds were distributed in a factorial arrangement with two Met sources (DL-Met vs. OH-Met) × three TSAA levels (80%, 100%, and 120% of the breeding company recommendation) × two immunological states (LPS challenge or saline) factorial arrangement. The experiment was divided into two stages i.e., starter period: 0-14 days,

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and stress period: 15-21 days. At 15, 17, 19 and 21 days of age, broilers were intraperitoneally injected with LPS at a level of one mg/kg of body weight or an equal amount of sterile saline solution. Three hours post LPS injection on 21 d, one broiler close to the average body weight was selected from each replicate, shocked to stun and killed. The blood was collected to harvest serum by centrifugation at 3000 rpm for 10 min at 4℃ and stored at -20°C for subsequent analysis. The body cavity was quickly excised to collect and weigh spleen, liver, and thymus. Tissue samples from the spleen were collected in RNA-free centrifuge tube, snap frozen in liquid nitrogen and stored at -80°C for mRNA analysis.

Total RNA was isolated using a Trizol reagent (TaKaRa Bio Inc., Kyoto, Japan) and reverse transcribed into cDNA using the PrimeScript RT reagent (RR047A, Takara Bio Inc., Kyoto, Japan) according to the manufacturer's guidelines. The concentration and purity of RNA were determined using a NanoDrop 2000 spectrophotometer (Thermo Scientific, Massachusetts, America). SybrGreen based quantitative PCR was performed with a quantitative real-time PCR master mix (RR420A, Takara Bio Inc., Kyoto, Japan) in a 7500 real-time PCR system (Applied Biosystems LLC., Massachusetts, America). *β-actin* was used as a housekeeping gene and the relative gene expression level was calculated by the $2^{-\Delta\Delta Ct}$ method. The primers used for quantifying selected genes are listed in Table 1. All serum samples were thawed and homogenized before analysis. The contents of serum serum-amyloid-A, OT, α1-AGP, and IgA were determined by enzyme-linked immunosorbent assay (ELISA) according to the manufacturer's instructions (Shanghai Enzyme-linked Biotechnology Co., Ltd., Shanghai, China).

The statistical differences were determined by three-way ANOVA in a 2 × 3 × 2 factorial arrangement with Duncan's test for multiple comparisons. And one-way ANOVA and Duncan's multiple comparisons were used when a significant interaction was observed. A value of $P < 0.05$ was considered significant. $0.05 < P < 0.10$ was viewed as a trend.

Table 1 - Primer sequences used in real-time quantitative PCR analysis.

Genes	Orientation	Sequence 5'→3'	Product size (bp)	Series number
β-actin	Forward	CAACACAGTGCTGTCTGGTGGTAC	199	NM_205518.1
	Reverse	CTCCTGCTTGCTGATCCACATCTG		
IL-1β	Forward	ACTGGGCATCAAGGGCTA	131	XM_046931582.1
	Reverse	GGTAGAAGATGAAGCGGGTC		
IL-6	Forward	CGCCCAGAAATCCCTCCTC	152	NM_204628.2
	Reverse	AGGCACTGAAACTCCTGGTC		
TNF-α	Forward	GAGCGTTGACTTGGCTGTC	64	XM_046927265.1
	Reverse	AAGCAACAACCAGCTATGCAC		

III. RESULTS

Performance data are presented in Table 2. During the challenge period (15-21 days), LPS challenge reduced FI of chickens ($P < 0.01$), while there was no significant change in FCR ($P > 0.05$). The interaction between Met sources and levels was significant on BWG during the challenge ($P < 0.05$, Table 5). At middle TSAA level, BWG was significantly higher in chickens fed the OH-Met diet. Moreover, there was a trend of interaction between the Met sources and LPS challenge on BWG ($P = 0.057$). The results of relative weight of immune organs are shown in Table 3. The broilers in the LPS-challenged group exhibited an increased relative spleen weight ($P < 0.01$) and a decreased relative thymus weight ($P < 0.01$). In addition, the broilers in the low- and high-TSAA groups exhibited an increased relative thymus weight ($P < 0.05$). Middle TSAA group also significantly increased relative liver weight ($P < 0.05$).

Splenic gene expression data for the broilers are also shown in Table 3. Dietary supplementation with OH-Met significantly reduced the mRNA expressions of *IL-1β* ($P < 0.05$). The broilers in the LPS-challenged group exhibited increased mRNA expression of *TNF-α* ($P < 0.01$). There was a significant interaction between Met sources and levels for *IL-6* expression ($P = 0.032$, Table 5). The DL-Met levels did not affect the gene expression of *IL-6,* but the *IL-6* expression has a tendency of linear increase with increasing level of OH-Met ($P = 0.057$). There

was a significant interaction between Met sources and LPS challenge for *IL-6* expression (P = 0.015, Table 6). The Met source did not affect the expression of *IL-6* under normal conditions. Nonetheless, under LPS challenge, broilers fed OH-Met diet exhibited lower *IL-6* expression. The *IL-1β* expression was influenced by the interaction between Met levels and LPS challenge (P < 0.01, Table 7). The Met level did not affect the expression of *IL-1β* under normal conditions. However, broilers fed low-TSAA level showed higher *IL-1β* expression with LPS challenge. We also observed interaction among Met sources, levels and LPS challenge for the expression of *IL-6* (P < 0.05). The *IL-6* expression was upregulated by LPS challenge, regardless of Met level in DL-Met group while *IL-6* expression was increased with LPS challenge at high-TSAA level in OH-Met group.

Table 2 - Effects of Met sources, levels and LPS challenge on growth performance from 15-21 d.

Items		FI, g	BWG, g	FCR
Source	DL-Met	442.0	293.4[b]	1.509
	OH-Met	451.8	304.9[a]	1.486
Level	Low	448.2	295.4	1.520
	Middle	448.5	304.9	1.474
	High	444.4	297.6	1.498
LPS challenge	-	472.3[a]	317.8	1.490
	+	420.9[b]	280.1	1.505
P value				
Source		0.387	0.024	0.420
Level		0.906	0.196	0.545
LPS		< 0.001	< 0.001	0.674
Source × Level		0.308	0.044	0.805
Source × LPS		0.334	0.057	0.544
Level × LPS		0.921	0.594	0.632
Source × Level × LPS		0.529	0.706	0.220

Note: FI, feed intake; BWG, body weight gain; FCR, feed conversion ratio. Means within a column without a common superscript differ significantly (P < 0.05).

Table 3 - Relative organ weight (%) and relative gene expression related to inflammation in spleen at 3 h post LPS challenge at 21 d.

Items		Spleen	Liver	Thymus	*IL-1β*	*IL-6*	*TNF-α*
Source	DL-Met	0.095	3.788	0.295	1.92[a]	3.75	1.96
	OH-Met	0.097	3.892	0.314	1.22[b]	2.94	1.89
Level	Low	0.103	3.892[ab]	0.319[a]	2.67	3.44	1.86
	Middle	0.091	3.963[a]	0.273[b]	1.20	3.29	2.00
	High	0.095	3.669[b]	0.321[a]	0.79	3.31	1.93
LPS challenge	-	0.084[b]	3.822	0.330[a]	0.64	1.51	1.05[b]
	+	0.109[a]	3.915	0.280[b]	2.52	5.08	2.75[a]
P value							
Source		0.747	0.185	0.209	0.038	0.122	0.618
Level		0.302	0.042	0.027	< 0.001	0.870	0.692
LPS		< 0.001	0.537	0.005	< 0.001	< 0.001	< 0.001
Source × Level		0.490	0.597	0.931	0.135	0.032	0.535
Source × LPS		0.978	0.345	0.326	0.209	0.015	0.198
Level × LPS		0.761	0.662	0.135	< 0.001	0.885	0.530
Source × Level × LPS		0.054	0.250	0.747	0.370	0.036	0.795

Note: Means within a column without a common superscript differ significantly (P < 0.05).

The results of serum acute phase proteins and IgA contents are shown in Table 4. We observed significant main effects of LPS, Met sources and levels on serum OT which was increased by LPS challenge, Met source and level (P < 0.05). α1-AGP and IgA were increased by Met sources and LPS challenge (P < 0.05). In addition, there was a significant interaction effect of Met sources and LPS on serum-amyloid-A and IgA (P < 0.05).

Table 4 - Serum acute phase proteins and IgA contents post 3 h LPS challenge at 21 d.

Items		SAA μg/mL	OT, g/L	α1-AGP, mg/L	IgA, μg/mL
Source	DL-Met	14.04	9.93[b]	1106.73[b]	440.14[b]
	OH-Met	13.97	10.28[a]	1156.08[a]	456.90[a]
Level	Low	13.80	9.74[b]	1108.06	443.00
	Middle	13.98	10.22[a]	1134.76	458.05
	High	14.24	10.34[a]	1150.33	444.33
LPS challenge	-	13.85	9.61[b]	1082.93[b]	429.13[b]
	+	14.15	10.55[a]	1175.16[a]	465.76[a]
P value					
Source		0.594	0.047	0.009	0.050
Level		0.157	0.006	0.151	0.175
LPS		0.083	< 0.001	< 0.001	< 0.001
Source × Level		0.653	0.325	0.278	0.491
Source × LPS		0.009	0.100	0.355	0.032
Level × LPS		0.907	0.317	0.288	0.602
Source × Level × LPS		0.979	0.839	0.803	0.428

Note: SAA: serum-amyloid-A; OT: ovotransferrin; α1-AGP: alpha-1-acid-glycoprotein; IgA: immunoglobulin A. Means within a column without a common superscript differ significantly ($P < 0.05$).

Table 5 - Interaction effect of methionine sources and levels.

Source	Dosage	BWG, g	*IL-6*
DL-Met	Low	291.2[b]	4.55
	Middle	291.2[b]	3.72
	High	298.4[ab]	2.92
OH-Met	Low	298.1[ab]	2.22
	Middle	319.0[a]	2.91
	High	297.0[ab]	3.70
SEM		3.44	0.33
P value		0.044	0.032

Note: *IL-6*: *P* value (DL-Met, Linear) = 0.260; *P* value (OH-Met, Linear) = 0.057. Means within a column without a common superscript differ significantly ($P < 0.05$). SEM means standard error of the mean.

Table 6 - Interaction effect of methionine sources and LPS challenge.

LPS challenge	Source	*IL-6*	SAA μg/mL	IgA, μg/mL
no	DL-Met	1.29[c]	14.13[a]	429.68[b]
	OH-Met	1.72[c]	13.54[b]	428.57[b]
yes	DL-Met	5.94[a]	13.95[ab]	449.44[b]
	OH-Met	4.17[b]	14.35[a]	482.08[a]
SEM		0.33	0.09	4.61
P value		0.015	0.009	0.032

Note: Means within a column without a common superscript differ significantly ($P < 0.05$). SEM means standard error of the mean.

Table 7 - Interaction effect of methionine levels and LPS challenge.

LPS challenge	Dosage	*IL-1β*
no	Low	0.68[b]
	Middle	0.86[b]
	High	0.36[b]
yes	Low	4.66[a]
	Middle	1.54[b]
	High	1.26[b]
SEM		0.24
P value		< 0.001

Note: Means within a column without a common superscript differ significantly ($P < 0.05$). SEM means standard error of the mean.

IV. DISCUSSION

Our study demonstrated that LPS challenge decreased FI and BWG, whereas there was no effect on the FCR of broilers from 15-21 days of age, which was consistent with the results of previous study (Yang et al., 2019) and indicated that LPS challenge was successfully induced. Importantly, our findings suggested that OH-Met supported better BWG compared with DL-Met during stress period. The spleen is a secondary lymphoid organ and plays a crucial role in immune response. The LPS-induced immune response increased the production of proinflammatory cytokines (IL-1β and IL-6 and TNF-α) in the spleen, and in turn, led to compensatory splenomegaly (Yang et al., 2008; Pozo et al., 2009), which is consistent with our study findings, further validated by an increase in relative spleen weight in broiler chickens post LPS challenge. Also, we observed a significant Met level effect on liver weight. Combined with the findings on serum acute phase proteins which were increased with OH-Met, it can be postulated that LPS challenge triggered the demand for cysteine to produce acute phase proteins. In this context, OH-Met would respond more efficiently due to its better transsulfuration (Martin-Venegas et al., 2006). We further tested the expression of inflammatory cytokines in spleen and found that LPS challenge stimulated the expression of inflammatory cytokines. In addition, OH-Met downregulated the expression of *IL-1β*, and the mRNA expression of *IL-6* was reduced in OH-Met-fed broiler chickens when challenged with LPS, which suggested that OH-Met was able to exert advantageous effects on homeostatic mechanisms associated with the splenic immune response. In conclusion, OH-Met ameliorated the inflammatory damage caused by LPS challenge and improved the BWG during the challenge condition.

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ACID BINDING CAPACITY OF POULTRY FEED INGREDIENTS AND DIETS

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Summary

The objective of the present work is to determine the acid binding capacity (ABC) of feed ingredients (inorganic, animal, and plant sources) and diets (layer mash and pelleted broiler diets) used in poultry nutrition. The measurements were made at three pH points (4, 3 and 2) to examine the best pH point for the ABC estimation which was a secondary objective of the study. The results showed that the limestone and oyster shell had the highest ABC at all pH points. The ABC of limestone was variable depending on the source. The ABC of 10 limestone samples ranged from 8,261-13,459 mEq/kg at pH 4; 9,836-15,566 mEq/kg at pH 3 and 16,010-22,393 mEq/kg at pH 2. Next to inorganic sources, animal-based ingredients had higher ABC with an average of 632 1,122 and 2,449 mEq/kg, at pH 4, 3 and 2, respectively. Among the plant-based sources, cereals had the lowest ABC (960 mEq/kg at pH 2) compared to the plant-protein sources (1,590 mEq/kg at pH 2) and cereal-by products (1,216 mEq/kg at pH 2). Layer mash had a higher ABC (5,846 mEq/kg at pH 2) when compared to broiler diets (1,275-1,446 mEq/kg at pH 2). In conclusion, limestone was the most consequential ingredient in terms of increasing ABC. The variability in ABC among different limestone sources may have implications for nutrient utilisation and absorption. The most suitable pH point was pH 2, as the readings were more stable at this pH and it is more relevant to the pH in the foregut of poultry.

I. INTRODUCTION

Acid binding capacity (ABC) of an ingredient, through its effect on gastric pH, plays an important role in the digestion and absorption of nutrients in poultry. The ABC is defined as the resistance of a feed ingredient to the pH reduction by gastric acid. Limestone, the major Ca source in poultry diets, has a high ABC (Lawlor et al., 2005), which can increase the digesta pH and, influence the solubility and digestibility of nutrients, including minerals. The average pH in the segments of proventriculus and gizzard ranges from 0.5 to 4.8 (Ravindran, 2013; Lee et al., 2018). For optimum digestion, a low pH must be maintained in the gizzard. Therefore, the inherent buffering capacity of the ingredients must be considered for the effective use of ingredients, especially of the mineral sources, in feed formulations. Lawlor et al. (2005) examined the ABC of some feed ingredients used in pig feeds. Similar studies for ingredients and diets used for poultry feeding are scant. The objective of present study was to determine the ABC of feed ingredients and diets used in poultry nutrition. A secondary objective was to examine the influence of pH (4.0, 3.0 and 2.0) on the measurement of ABC.

II. MATERIALS AND METHODS

Ingredients and poultry diets were obtained from various commercial sources in New Zealand and Australia. All samples were ground to pass through a 0.5 mm screen using a laboratory rotor mill and were stored in air-tight plastic containers at 4˚C until analysis. The samples examined were mineral sources (limestone, oyster shell, dicalcium phosphate and monocalcium phosphate), cereals and by-products (barley, maize, sorghum, triticale, wheat,

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and wheat bran), plant-protein sources (canola meal, peas, soybean meal, sunflower meal), animal protein sources (fish meal, meat and bone meal [MBM], meat meal and blood meal) and diets (layer mash and broiler starter, grower and finisher diets). A modified procedure of Lawlor et al. (2005) was used to determine the ABC. Instead of measuring at two pH points (3.0 and 4.0), the present work used three pH points (4.0, 3.0 and 2.0). All pH measurements were made using a laboratory pH meter which was calibrated using certified pH of 4.0 and 7.0 buffer solutions. A 0.5 g sample of ingredient or diet was suspended in 50 mL of deionised water and continuously stirred with a magnetic stirrer at 37° C for one hour. However, for the ingredients having extremely high buffering capacity namely, limestone, oyster shell and layer mash, a sample amount of 0.1 g was used which was determined based on preliminary experiments. Titrations were performed by addition of 0.1N hydrochloric acid in variable increments (0.1 to 45 mL depending on the ingredient type and the stage of titration). Initial pH and all further readings taken during the titration were recorded after equilibration for three minutes. The ABC was calculated as the amount of hydrochloric acid in milliequivalents (mEq) required to lower the pH of 1 kg of sample to respective pH point. Calculated ABC values of each sample were means of 3 replicates.

III. RESULTS AND DISCUSSION

Table 1 summarises the initial pH and average ABC of test ingredients and diets. Limestone and oyster shell had extremely high ABC at all pH points. The ABC of limestone was highly variable among the different sources. The ABC of the 10 samples ranged from 8,261-13,459 mEq/kg at pH 4; 9,836-15,567 mEq/kg at pH 3; and 16,010-22,394 mEq/kg at pH 2 with standard deviations of 1,657, 1,812 and 1,723, respectively. Among the animal protein sources, MBM (878-3,061 mEq/kg) had higher ABC followed by fish meal (785-2,876 mEq/kg) at all pH points. Among the plant-based sources, ABC of cereals were very low with an average of 101, 223 and 960 mEq/kg, respectively, at pH 4, 3 and 2. The ABC of cereal by-product (wheat bran) was higher than that of cereals (231-1,216 mEq/kg). Among the plant protein sources, soybean meal had the highest ABC while peas had the lowest ABC. Among the diets, layer mash had higher ABC when compared to broiler diets.

Most of the current estimates are in general agreement with those of Lawlor et al. (2005). The current work shows that the inorganic mineral sources (except for monocalcium phosphate) had higher ABC when compared to other categories, which is in agreement with previous results (Lawlor et al., 2005; Gilani et al., 2013; Lu et al., 2016). The average ABC of 13 limestone samples has been reported as 12,932 and 15,044 mEq/kg at pH 4 and 3, respectively, by Lawlor et al. (2005), which is 16% higher than the current estimates. In contrast to the current work, Lawlor et al. (2005) reported a relatively higher ABC for fish meal (738-1,457 mEq/kg) when compared to MBM (595-920 mEq/kg) which could be due to the difference in the chemical composition among various sources. For instance, the calcium concentration of MBM samples may vary from 71 to 118 g/kg (Anwar et al., 2016), which might have an influence on the ABC of different MBM samples. The former study also reported slightly higher values for cereals and plant protein sources which again may reflect differences in nutrient composition within ingredients depending on the source. Higher ABC of layer mash when compared to broiler diets was as expected because of the high inclusion of limestone in layer mash to meet the high calcium demand for egg production.

Among the pH points examined, pH 2 is most applicable to poultry nutrition when compared to pH 3 and 4. According to Duke (1986), the pH of proventriculus and gizzard is around 2. The readings were also more stable at pH 2 compared to the other pH points because determining the amount of hydrochloric acid required to increase the pH to 4 and 3 was found

to be demanding. This was particularly an issue for the high-ABC materials (limestone, oyster shell and layer mash).

Table 1 - Acid binding capacity of feed ingredients and diets at pH points, 4, 3 and 2.

Ingredient	Number of samples	Initial pH[1]	pH 4	pH 3	pH 2
Inorganic sources					
Limestone	10	9.4	10,966 (15.1%)[1]	12,698 (14.3%)	19,567 (8.8%)
Oyster shell	1	9.9	11,355	12,517	18,858
Dicalcium phosphate	1	7.6	2,866	4,661	8,686
Monocalcium phosphate	1	4.1	26.6	686	4,182
Animal-based sources					
Meat and bone meal	2	6.7	878	1,477	3,061
Fish meal	1	5.8	785	1,411	2,876
Blood meal	1	6.9	193	445	1,535
Meat meal	1	6.3	669	1,152	2.323
Plant-protein sources					
Soybean meal	3	7.0	516	820	1,762
Canola meal	1	6.5	423	739	1,865
Sunflower meal	2	6.6	389	727	1,576
Peas	1	6.9	186	343	1,158
Cereals and by-products					
Wheat bran	2	6.8	231	416	1,216
Wheat	5	6.7	68	180	930
Sorghum	2	6.8	90	208	1,014
Barley	3	6.2	79	186	888
Triticale	1	6.9	70	190	852
Maize	1	6.3	67	156	859
Diets					
Layer mash	1	7.7	726	1,453	5,846
Broiler starter	1	7.2	303	486	1,424
Broiler grower	1	7.4	266	439	1,275
Broiler finisher	1	7.3	293	483	1,446

[1] Coefficient of variation.

Other than pH, factors that may influence the ABC of ingredients are temperature of the solution, type of processing (pelleting, extrusion) and particle size. According to Gilani et al. (2013), increasing temperature from 21 to 41˚ C reduced the pH and ABC for selected inorganic (calcium carbonate, oyster shell, dicalcium phosphate and monocalcium phosphate) and plant-based (wheat, rice bran, soybean meal) feed ingredients. It must be noted that a temperature of 37˚ C was used in the current work. The ABC of ingredients is affected by their particle size as the particle size greatly influence their solubility.

The extraordinarily high ABC of limestone has implications for digesta pH along the gastrointestinal tract. This is reflected in increased digesta pH in the crop (5.32 vs. 4.89; $P < 0.05$) and ileum (7.39 vs. 6.62; $P < 0.01$) pursuant to the addition of 40 g/kg limestone to broiler diets in Shafey et al. (1991). The digestion of protein and absorption of amino acids are paramount to the growth performance of poultry. Intestinal uptakes of amino acids principally take place as constituents of di- and tripeptides (oligopeptides) via the peptide transporter, PepT-1, rather than as monomeric entities. Interestingly, there are indications (Kennedy et al. 2002) that intestinal mucosal surface pH values of less than 6.1 to 6.8 would advantage the functionality of PepT-1 (Steel et al., 1997). Therefore, it is relevant that increasing limestone

inclusions from 3.0 to 18.7 g/kg in maize-soy broiler diets depressed average apparent ileal digestibility coefficients of 17 amino acids by 7.95% (0.78 vs 0.72) as reported by Amerah et al. (2014). However, in the same study, increasing limestone inclusions did not significantly influence pH of ileal digesta. This suggests that the ABC of limestone was not influencing digesta pH along the digestive tract and other factors stemming from limestone and/or calcium were influential. Accordingly, considerable attention is now being paid to the calcium content, particle size and solubility of limestone used in poultry diets (Gilani et al., 2022). Moreover, the use of phytase may also impact the ABC. Phytase matrix values (1000 FTU/kg) for calcium and phosphorous of 2.09 and 1.97 g/kg, respectively, are often applied to the formulation of broiler diets (Moss et al., 2022). Given the high ABC of limestone, dicalcium phosphate and monocalcium phosphate reported herein, the corresponding reductions in their dietary inclusions would translate to tangible depressions in dietary ABC, which should be to the advantage of broiler growth performance.

In conclusion, determining the ABC at pH 2 is most suitable for poultry ingredients when compared to pH 3 and 4, because (i) it represents the gastric pH on birds and (ii) the readings during the titration process were more stable at pH 2. Limestone was the most influential ingredient in terms of ABC of poultry diets. Substantial variability in the ABC of the 10 limestone samples determined in the current work is noteworthy and, this may cause differences in the digestion and utilisation of nutrients and on bird performance. This is a research topic that has not been previously explored and future studies are warranted.

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POSITIVE EFFECT OF A *Q. SAPONARIA* AND *Y. SCHIDIGERA* COMBINATION PRODUCT, A FOUR-STRAIN *BACILLUS* BASED PROBITIC AND THEIR COMBINATION ON BIRDS EXPOSED TO INTESTINAL CHALLENGE

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Summary

The current study aims to assess the effects of a *Quillaja saponaria* and *Yucca schidigera* combination product (QY) and a four-strain *Bacillus*-based probiotic (PRM) when applied separately or in combination in feed for broilers subjected to a non-specific enteritis challenge model in comparison to infected and uninfected untreated controls (IUC and UUC respectively). The model successfully mimicked field enteritis causing a significant increase in mortality and intestinal inflammation scores, a deterioration in performance and an increase in intestinal pathogen isolation in the IUC compared to UUC. Both products provided significant and complete alleviation of the challenge regarding mortality, body weight and weight homogeneity when used separately. In addition, PRM provided complete alleviation of the challenge regarding feed conversion, EPEF and carcass yield, while QY treatments were not different from UUC in regard to intestinal inflammation scores. Both products provided significant improvement regarding pathogen isolation from the intestinal tract in comparison to IUC but were not equal to UUC. When used together the two products provided further benefits in regard to breast meat yield and reduction of *Escherichia coli* in the intestinal content to a level not different to UUC and improved processing weight uniformity to a level significantly better than UUC.

I. INTRODUCTION

Numerous scientific publications and commercial field experience indicate positive effects of *Quillaja saponaria* and *Yucca schidigera* combination product (QY), containing a minimum of 3.5% triterpenoid (Quillaja) saponins and typically 0.8-1.0% of total polyphenols expressed as gallic acid equivalent (Magni-Phi®) on broilers' performance when exposed to enteritis challenge from specific pathogens (Bafundo et al., 2020, Bafundo et al., 2022) or stress factors such as feed deprivation (Osho et al., 2022). Furthermore, there is evidence of the beneficial effect of a four-strain *Bacillus*-based probiotic, MicroLife / Provia Prime® (PRM), containing a minimum of total of 4 x 10^9 CFU/g viable spores of *B. subtilis*, *B. licheniformis*, *B. amyloliquefaciens* and *B. coagulans* on broiler performance and pathogen control of broilers exposed to an enteritis challenge (Osho et al., 2023), and thus providing a valid alternative to the use of certain antibiotics such as virginiamycin and bacitracin methylene disalicylate (Costello et al., 2023). The current study aims to compare the effects of QY and PRM and identify potential synergies between the two products in broilers subjected to a subclinical enteritis challenge model.

II. METHOD

A total of 2 860 as-hatched day old Ross 708 broilers were allocated to 5 treatments: T1 - uninfected untreated control (UUC); T2 - infected untreated control (IUC); and three infected

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treatments: T3 - supplemented with QY (commercially available product Magni-Phi®) at 250 g/t; T4 - supplemented with PRM (commercially available four-strain *Bacillus*-based probiotic, MicroLife / Provia Prime®) at 0.5 x 10^9 CFU/kg feed; and T5 – supplemented with QY+PRM at the above doses. Birds were vaccinated for coccidiosis at day 0 with a commercial vaccine Coccivac® B52. Each treatment had 11 replicates, 52 birds each, in floor pens. The UUC group was placed on fresh litter, while all other treatments were challenged by being placed on used litter known to contain coccidia, *Clostridium perfringens*, *Escherichia coli*, *Salmonella* spp. and other pathogens. Additionally, before the bird placement for these treatments, the litter was supplemented with 100 000 occysts/bird, primarily *E. acervulina* and *E. maxima*, to mimic natural field enteritis infection. Birds were provided with three phase diets: starter 0-21 days of age; grower 22-35 days of age and finisher 36-42 days of age.

Mortality, body weight (BW), daily weight gain (DWG), feed conversion ratio (FCR) and the European Production Efficiency Factor (EPEF) were measured at day 42. Final body weight coefficient of variation (CV), carcass yield and breast yield (%) at 42 days were measured. At day 21 and 42, four birds per pen were humanely sacrificed and scored for intestinal inflammation as follows: 0 – no lesions found; 1 – mild hyperemia, but no cellular sloughing or mucous; 2 - moderate hyperemia and /or mild cellular sloughing; 3 – severe hyperemia and/or severe cellular sloughing and 4 – actual necrosis or bleeding observed. The same birds were also sampled for cecal *C. perfringens* and *E. coli* count and *Salmonella* spp. incidence. Statistical analysis using Fisher LSD test was applied and treatments were considered significantly different at $P \leq 0.05$.

III. RESULTS

An overview of the performance results is provided in Table 1. Mortality was significantly increased in IUC compared to UUC, evidencing successful enteritis challenge model. Both QY and PRM reduced mortality to a level not significantly different from the UUC. BW at day 42 was significantly reduced in IUC compared to UUC, and all supplemented treatments provided similar final weights compared to non-challenged control. FCR was also significantly compromised by the challenge. Both QY and PRM provided significant improvement of FCR compared to the challenge control. QY+PRM provided the lowest numerical FCR among all infected treatments and was not different from UUC. A similar pattern was exhibited regarding EPEF. Weight homogeneity was also deteriorated by the challenge, while both QY and PRM alleviated this effect and were not different from the UUC, when used together they provided a CV of BW at 42 days significantly lower compared to UUC. Both carcass and breast meat yield were significantly affected by the challenge, while QY and PRM alleviated this effect to different extend with best results achieved when the two products were used together.

Table 1 - Overview of life and processing plant performance parameters: mortality, body weight (BW), feed conversion ratio (FCR), European Production Efficiency Factor (EPEF), processing weight coefficient of variation (CV), carcass and breast meat yield for the 0-42 days period.

Treatment	Mortality(%) 0-42 d	BW (g) d 42	FCR 0-42 d	EPEF	Weight CV d 42	Carcass yield (%)	Breast yield (%)
UUC	0.95^{a}	$2\,856^{b}$	1.786^{a}	367^{a}	14.25^{b}	71.57^{a}	16.48^{a}
IUC	7.20^{b}	$2\,546^{a}$	1.893^{c}	283^{c}	15.88^{c}	67.65^{b}	14.49^{d}
QY	1.71^{a}	$2\,826^{b}$	1.840b	350^{b}	14.50^{b}	68.39^{b}	15.48^{c}
PRM	2.08^{a}	$2\,863^{b}$	1.817^{ab}	357^{ab}	13.64^{b}	70.33^{a}	15.87^{b}
QY+PRM	2.08^{a}	$2\,881^{b}$	1.794^{a}	363^{a}	8.20^{a}	71.60^{a}	16.52^{a}

Means with different superscripts are significantly different ($P < 0.05$) (LSD Fisher test).

An overview of the effects on pathogen isolation and intestinal inflammation is provided in Table 2. The IUC group had significantly higher *C. perfringens* and *E. coli* counts, *Salmonella* spp. incidence and intestinal inflammation scores compared to UUC, demonstrating the impact of litter contamination on pathogen abundance and intestinal inflammatory state. Both QY and PRM significantly lowered intestinal inflammation scores in comparison to IUC. Moreover, in the groups supplemented with QY intestinal inflammation score was not different from the UUC. Both QY and PRM reduced pathogen counts and incidence in comparison to IUC, though were not able to meet the levels in UUC. PRM provided significantly better effects than QY regarding *E. coli* counts at both age points. The lowest incidence and counts were recorded in the QY+PRM treatment.

Table 2 - Overview of pathogen isolation and intestinal inflammation score per treatment group.

Treatment	*C. perfringens* (log_{10})		*E. coli* (log_{10})		*Salmonella* incidence (%)		Intestinal inflammation	
	21 d	42 d	21 d	42 d	21 d	42 d	21 d	42 d
UUC	4.64^{a}	4.75^{a}	4.92^{a}	5.15^{a}	6.82^{a}	3.64^{a}	0.205c	0.318^{c}
IUC	6.76^{d}	6.74^{d}	7.03^{d}	6.77^{d}	72.73^{c}	83.64^{c}	1.795^{a}	1.709^{a}
QY	5.54^{c}	5.73^{c}	5.96^{c}	5.75^{c}	29.55^{b}	47.27^{b}	0.272^{bc}	0.418^{bc}
PRM	5.78^{c}	5.79^{c}	5.53^{b}	5.44^{b}	36.36^{b}	41.82^{b}	0.500^{b}	0.464^{b}
QY+PRM	5.10^{b}	5.13^{b}	4.83^{a}	5.11^{a}	20.46^{ab}	39.09^{b}	0.364^{bc}	0.427^{bc}

Means with different superscripts are significantly different ($P < 0.05$) (LSD Fisher test)

IV. DISCUSSION

Due to the content of certain phyto-molecules such as Quillaja saponins QS-21, QS-7, QS-17, QS-18, piscidic, vanillic, p-coumaric, ferulic acid, resveratrol and different yuccaols that can boost cell-mediated immune response (Marciani et al., 2000; Lacaille-Dubois, 2019) and reduce oxidative tissue stress and inflammation (Maier et al., 2015; Piacente et al., 2005), QY is able to support bird intestinal health and performance. On the other hand, probiotics provide multiple benefits to the host by different modes of action such as: 1) maintaining intestinal barrier function by tight junction upregulation and preventing cytokine-induced cell apoptosis; 2) competing with pathogens by creating a hostile microecology, producing certain antibacterial substances while competing for nutrients and attachment sights on the mucosa; 3) stimulating beneficial microflora by cross-feeding and quorum sensing; 4) providing direct beneficial effects for the host by producing certain beneficial metabolites such as enzymes, vitamins and butyric acid; and last but not least, 5) interacting with the host immune system to produce desirable antimicrobial immune responses with minimal inflammatory tissue damage (Bermudez-Brito, 2012).

The current study model successfully mimicked a field enteritis challenge. The results of the work confirm previous findings (Bafundo et al., 2020; Bafundo et al., 2022; Osho et al., 2023) of the beneficial effects of QY and PRM on broilers intestinal health and performance, when exposed to intestinal challenge related to either specific infections or stress from a nutritional or management standpoint causing dysbacteriosis or non-specific enteritis. QY demonstrated anti-inflammatory properties alleviating intestinal inflammation completely with scores being not different from UUC. PRM on the other hand demonstrated an ability to modulate microflora, especially regarding *E. coli* isolations. However, when used together, QY and PRM provided additive or synergistic effects regarding *C. perfringens* isolation, probably due to the different and non-overlapping mode of action of the two products. The effects on intestinal inflammation and pathogen isolation were well correlated with significant improvements of zootechnical performance with both products used separately when compared to IUC, with highest improvement realized when the two products were used simultaneously.

In the latter case, all performance parameters were not different from the UUC, demonstrating a complete offsetting effect on the intestinal challenge. In conclusion, the results of the study suggest that both QY and PRM when used on their own provide a valid tool to reduce the impact of intestinal challenges on bird health and performance. The trial results suggest that there is a room for synergism between the products that will potentially allow a complete offset of intestinal challenges without using other intervention tools such as antibiotics. This would contribute to improved performance and sustainability of the poultry industry, improved bird health and welfare which can also lead to a reduced need of antibiotic treatments and a lesser risk of antibiotic resistance development.

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EFFECTS OF FEEDGRADE ANHYDROUS BETAINE AND PHYTOGENICS COMBINATION ON PERFORMANCE OF BROILERS EXPOSED TO A CHALLENGE

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Summary

The present experiment was conducted in order to evaluate the effect of betaine and a phytogenic blend on performance and lesion scores in broilers subjected to a coccidiosis and *Clostridium Perfringens* challenge. The combination was able to significantly reduce *Eimeria* lesion scores and Necrotic Enteritis lesion scores in the guts of challenged birds and as a result reduce the negative performance effects of the challenge.

I. INTRODUCTION

The animal production industry's move towards raising animals with reduced or no antibiotic use whilst still preventing disease outbreaks and maintaining, or even improving animal performance is driving the need to look for alternative strategies. Such strategies can include combining existing feed additives with established modes of action such as phytogenics and betaine. Phytogenics have been shown to inhibit non-beneficial, potentially pathogenic bacteria and positively impact gut integrity (Ouwehand et al., 2010, Bento et al., 2013; Putaala et al., 2017), positively influencing the gut microbiome and inhibiting *C. perfringens* populations. Betaine, through its osmoregulatory effects, has been shown to help reduce performance losses of broilers exposed to coccidiosis (Tiihonen et al., 2001) and to reduce lesion scores reducing the impact of coccidiosis and positively affecting digestibility and performance (Amerah and Ravindran., 2015). It is expected that the modes of action of phytogenics and betaine may be complementary and that using the two products together may lead to reduced lesion scoring and improved performance of broiler chicks during challenge situations.

II. METHODS

A total of 1080 male Cobb 500 day old chicks were randomly assigned to 3 treatments with 9 replicates per treatment and 40 birds per replicate. Birds were vaccinated at the hatchery with VAXXITEK® HVT + IBD (in ovo, for Infectious Bursal Disease and Marek's disease) and NEWHATCH-C2-M® (spray, for Newcastle disease and Massachusetts type infectious bronchitis). Birds were fed corn/soybean meal-based diets which were fed *ad libitum* as crumble or pellets. All diets contained 750 FTU/kg of a *Buttiauxella* phytase. Diets were fed in 3 phases: starter (1-14 days), grower (14 to 28 days) and finisher (28-42 days). Built up litter was used in the pens. On day 14, birds were challenged by oral gavage with a 10x dose (0.1 ml per bird) of COCCIVAC®-B52 (Merck Animal Health) to cause tissue damage and predispose the birds to Necrotic Enteritis (NE). Control birds were given 0.1 ml of sterile phosphate buffered saline (PBS) containing no *Eimeria* oocysts. On days 18-20, birds were challenged via oral gavage once per day with 1.0 ml of fluid thioglycolate (FTG) broth containing approximately 1 x 10^8 CFU/ml *Clostridium perfringens* (netB-positive strain) to induce NE. Control birds were inoculated once daily from 18-20 days of age with sterile FTG. Challenged birds were either fed diets with no additives or diets containing BEO (feed-grade betaine anhydrous at 1 kg/t and a phytogenic blend of Cinnamaldehyde and Thymol at 100 g/t).

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Bodyweights and feed intakes were measured on days 0, 14, 21 and 42 and FCR calculated. On days 21 and 28, five birds were selected per pen, sacrificed and evaluated for coccidia intestinal lesions consistent with *E. maxima* and *E. acervulina* standards according to the 0-4 scoring method put forth by Johnson and Reid (1970). All data were analyzed using the linear regression model (the lm() function) in R (version 4.1.2) to identify statistically significant differences in response measures between the three treatment groups, using group as a fixed effect. Tukey's adjustment was used for multiple comparisons. Significance was considered at $P < 0.05$ and a trend was considered at $P < 0.1$.

III. RESULTS

The current NE challenge model was considered mild, as daily gain and final BW were moderately reduced in the CC treatment compared to the UC treatment (Table 1), but liveability was not significantly different between treatments and average NE lesion score was still less than 1 in the CC (Table 2). This model represents commonly seen subclinical NE challenges in commercial flocks. Performance of the challenged birds was numerically improved with the BEO treatment. The BEO treated challenged birds had significantly lower lesion scores due to *Eimeria acervuline* and *Eimeria maxima* oocysts at 28 days of age. *Eimeria maxima* lesion scores were also significantly lower at 21 days of age, immediately following the *Eimeria* vaccine challenge. Challenging the birds with *Eimeria* and *C. perfringens* resulted in a significant increase in NE lesion scores versus the control treatment and the BEO treated birds had significantly lower NE lesion scores than the challenged control birds. The lesion scores of birds that broke with NE were significantly improved versus the challenged control and restored to the level of the unchallenged control birds.

Table 1 - Performance (1-42 days).

Parameter	Unchallenged control (UC)	Challenged control (CC)	CC + Betaine and EO
Daily gain (g/b/d)	60.7[a]	58.1[b]	59.3[ab]
Daily feed intake (g/b/d)	108	106	107
Final bodyweight (g)	2,592[a]	2,439[b]	2,534[ab]
FCRc	1.77	1.83	1.80

Table 2 - *Eimeria* and NE lesions scores in guts of birds.

Parameter	Unchallenged control (UC)	Challenged control (CC)	CC + Betaine and EO
Eimeria Acervulina (lesion score, 21 days)	-	0.91	0.78
Eimeria Maxima (lesion score, 21 days)	-	1.13[a]	0.82[b]
Eimeria Acervulina (lesion score, 28 days)	-	1.09[a]	0.67[b]
Eimeria Maxima (lesions score, 28 days)	-	1.47[a]	0.91[b]
Average NE lesion score (21 days)	0.22	0.36	0.33
Average NE lesion score (28 days)	0.16[c]	0.89[a]	0.64[b]
NE lesion score – NE+ birds only (28 days)	1.00[b]	1.38[a]	1.12[b]

IV.DISCUSSION

Results of the current study show clear effects of the feed-grade anhydrous betaine and phytogenic combination on both *Eimeria* lesion scores and NE lesions scores in birds challenged with both coccidiosis and *Clostridium perfringens*. This reduction in lesion scores is likely due to firstly improved gut integrity and therefore greater resilience of the gut during times of challenge. Previous studies with both betaine and the phytogenics Cinnamaldehyde and Thymol have demonstrated positive effects on gut integrity and/or gut lesion scores

(Amerah et al., 2015; Putaala et al., 2017). Secondly the combination is likely reducing the persistence of *Eimeria* and *C. perfringens* in the guts of the bird. It has previously been suggested that betaine may partially inhibit coccidial invasion and development (Augustine et al., 1997) and Cinnamaldehyde and Thymol have been shown to have anti-microbial activity against *C. perfringens* (Ouwehand et al., 2010). In this study, these combined effects of the additives resulted in reducing the performance losses seen due to the applied coccidial and Clostridial challenge. In conclusion, this study demonstrated an alternative strategy with feed-grade anhydrous betaine and phytogenics can aid to reduce antibiotic use by reducing the negative impacts of an NE challenge.

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POTENTIAL OF GLUCOSE OXIDASE TO IMPROVE PERFORMANCE OF BROILERS UNDER NECROTIC ENTERITIS CHALLENGE

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The withdrawal of antimicrobials as growth promoters in the broiler industry may increase the risk of enteric diseases including necrotic enteritis (NE). The *netB* positive *Clostridium perfringens* is the bacterial pathogen compromising gut integrity during NE infections. Glucose oxidase (GOD) is an aerobic dehydrogenase enzyme, which oxidizes β-D-glucose into gluconic acid and produces hydrogen peroxide. By exhausting oxygen, GOD may help to maintain intestinal microcosmic ecological balance, improve the intestinal digestive environment, protect against oxidative stress, reduce pathogen invasion, maintain intestinal pH and ultimately improve the growth performance (Gao et al., 2022, Wu et al., 2019). Therefore, this study investigated the effect and optimal dose of glucose oxidase (VTR-GOD®) on the performance of broiler chickens under a subclinical NE challenge.

A total of 720-day-old Cobb-500 chicks were randomly allocated into 6 treatments with 8 replicates each. The details of treatments are included in Table 1. Birds were challenged with *Eimeria* spp. on d-9 and *C. perfringens* on d-14. All birds and feed were weighed on d 0, 8 and 19 and average body weight gain (WG), feed intake (FI) and feed conversion ratio (FCR) were calculated. Data were analysed by one-way ANOVA with Tukey's test.

Table 1 - The effect of dietary treatment on growth performance.

	day 0-8			day 0-19			
Treatment	FI [1]	WG [2]	FCR[3]	FI [1]	WG [2]	FCR[3]	Mortality (%)
NC-non-challenged	186	146^{bc}	1.278^{a}	1060^{a}	770^{ab}	1.376^{c}	8.33
NE challenged	182	143^{c}	1.272^{a}	987^{b}	671^{c}	1.471^{a}	5.83
NE+ antibiotics[4]	183	144^{c}	1.270^{a}	1059^{a}	801^{a}	1.322^{d}	10.0
NE+ GOD 100g/T	187	159^{a}	1.174^{b}	1018^{ab}	715^{bc}	1.424^{b}	5.00
NE+ GOD 200 g/T	189	158^{a}	1.191^{b}	1027^{ab}	710^{c}	1.448^{ab}	10.8
NE+ GOD 300g/T	186	154^{ab}	1.206^{b}	1016^{ab}	711^{c}	1.431^{ab}	3.33
SEM[5]	2.79	2.15	0.01	14.7	13.2	0.01	2.66
P-value	0.583	<0.001	<0.001	0.009	<0.001	<0.001	0.309

[1,2,3] FI: feed intake; WG: weight gain; FCR: feed conversion ratio. [4]antibiotics in Treatment 3 includes Salinomycin +Zn bacitracin, 500 gm/T each; [a-d]values within a column with different letters differ significantly ($P < 0.05$).[5]SEM: standard error of means.

Results from this study show that, up to day 8, supplementing diets with the 3 different levels of GOD, improved FCR ($P < 0.001$) compared to the non-supplemented and antibiotic groups. However, during the NE challenge period (d 9-19), all challenged groups, except antibiotic supplemented birds, showed higher FCR compared to the non-challenged group ($P \leq 0.001$) (data not presented). The overall period, d 0-19, results show antibiotic supplemented birds, had significantly lower FCR compared to all groups ($P < 0.001$). Interestingly, in this period, bird's fed with the lowest dose of GOD, showed a significantly improved FCR compared to the non-supplemented challenged birds ($P \leq 0.001$).

The study concludes that supplementation of GOD at a low dose (100g/T) may help improve feed efficiency in broilers under a subclinical NE challenge. Further investigation is required to verify whether improved feed efficiency is associated with improved gut health.

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LIVER TRANSCRIPTOMIC PROFILE OF BROILER CHICKENS UNDER DIFFERENT CRUDE PROTEIN AND ENERGY DIET REGIMENS

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Variations in crude protein (CP) and energy levels, the primary macronutrients in poultry diets, directly affect growth performance, income, and the environment. In view of that, poultry researchers are working to adjust these nutritional components without compromising the growth performance of chickens to overcome economic and environmental challenges (Strifler et al., 2023). However, the molecular mechanisms underlying the response of growth performance to CP and energy levels have been less explored.

This study aimed to reveal differentially expressed genes (DEGs) and regulatory pathways affecting the growth of Cobb 500 broiler chicks fed different levels of CP and energy through liver transcriptomic analysis. Four dietary treatments, normal protein-normal energy (NPNE; 18% and 10.4 MJ/kg), normal protein-low energy (NPLE; 18% and 9.9 MJ/kg), low protein-normal energy (LPNE; 16% and 10.4 MJ/kg), and low protein-low energy (LPLE; 16% and 9.9 MJ/kg) diets were offered to birds during the finisher stage (day 19-35). All dietary treatments were fortified with free amino acids to meet the standard breed requirements. Reducing NE densities in the low-energy diets was achieved by diluting the diets with fillers. Illumina HiSeq 2000 platform was used to produce pair-end reads (150 bp) that were cleaned and aligned to the *Gallus gallus* reference genome (GRCg7b) and assembled to generate the gene count matrix. The transcriptomic profile of these groups was compared with the edgeR package in R software (version 4.1.2), making six contrasts as NPNE vs NPLE, NPNE vs LPNE, NPLE vs LPNE, NPNE vs LPLE, NPLE vs LPLE, and LPNE vs LPLE to identify DEGs at a cut-off of absolute fold change >1 and adjusted false discovery rate < 0.05.

The performance results showed no differences among birds fed the NPNE, NPLE, and LPNE diets. Notably, only the LPLE group showed significantly ($P<0.05$) lower body weight gain, feed intake, and higher feed conversion ratio than the other groups. No significant DEGs were identified when NPNE, NPLE, and LPNE were compared among themselves. However, in NPNE vs LPLE, NPLE vs LPLE, and LPNE vs LPLE groups, 561, 526, and 493 DEGs were found, respectively. Interestingly, a total of 304 overlapping DEGs were identified between the three contrasts. Overall, the top five DEGs (*SYNRG, ALDH1L2, DUSP14, PPP1CC,* and *HSPA9*) and growth-related genes (*GHR and IGF1*) were respectively upregulated and downregulated in LPLE-fed birds. The Gene Ontology and Kyoto Encyclopedia of Genes and Genomes functional enrichment analysis showed that the DEGs were generally associated with amino acid biosynthesis and metabolism. These results showed that the similar performance of birds fed NPNE, NPLE, and LPNE diets activated similar genes, while the expression profile of those genes was different in LPLE-fed birds. It is concluded that the poor performance observed in the birds fed the LPLE diet is partially a consequence of transcriptomic responses to both protein and energy levels, at least in the liver. Furthermore, the transcriptomic responses in the liver are consistent with the performance responses. Further transcriptomic study in the digestive system may be useful to understand low-protein-fed bird responses.

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GENE EXPRESSION OF AMINO ACIDS AND FATTY ACIDS SENSORS IN THE GASTROINTESTINAL TRACT OF BROILER CHICKENS

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During the first week after hatch, broilers face significant challenges regarding feed intake, digestion and nutrient absorption affected by an immature gastrointestinal tract (GIT). These involves a dietary shift from the internal yolk to feed. The mechanisms orchestrating feed intake are based on chemosensory mechanisms. The existence and role of amino acid (AA), and fatty acid (FA) sensors has been previously described in chickens (Niknafs et al., 2023). However, how this network of chemosensory receptors orchestrates feed intake requires an understanding of tissue specificity and of the dynamic adaptations to different life stages which was lacking, thus, becoming the aim of this work. It was hypothesized that the expression of the chemosensory network would be dependent on tissue type and function, and responsive to nutrient requirements and feed composition.

Sixteen broilers (Ross 308) were selected on days 7 and 26 of age, with the feeding program including a starter diet (ME: 2950 kcal – CP: 22.9% – dLYS: 2.2%) up to 23 days and a grower diet (ME: 3100 kcal – CP: 18.1% – dLYS: 1.6%) until sacrifice. Ten sections of the GIT (upper palate, tongue base, crop, proventriculus, gizzard, duodenum, jejunum, ileum, cecum, and colon). Relative expressions of AA (T1R1, T1R3, mGluR1, mGluR4, GPR92, and CaSR) and FA (FFAR2, FFAR3, and FFAR4) sensors were measured by qPCR. Statistical analysis was performed using Kruskall-Wallis test. The results showed a higher expression of AA and FA sensors on day 7 compared to day 26 ($P<0.05$) associated with a higher CP content (and nutrient density) of the diet. The tissue effects showed that the lower tract (i.e., cecum and colon) had higher AA and FA sensor abundance than the middle and upper tracts ($P<0.01$) which may indicate the relevance of the microbiome in gut sensing, thus, the hunger-satiety cycle.

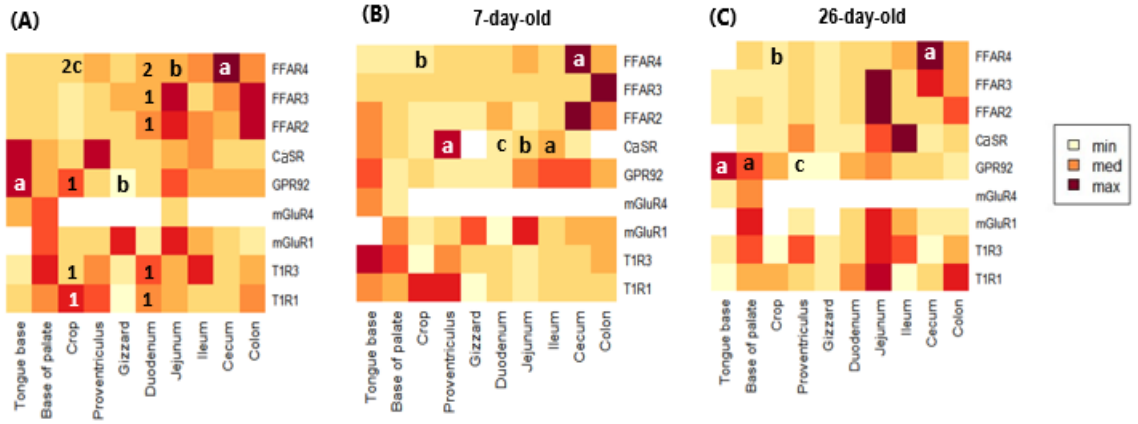

Figure 1 - Heatmaps of nutrient sensors in ten sections of the GIT at 7 or 26 day-old broiler chickens. ([a,b,c] Cells within the same row with different letters show significant difference ($P \leq 0.05$) for the gene across tissues.[1,2]Cells within the same column with different numbers ($P \leq 0.05$) show significant differences for the tissue across genes.)

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UPDATE ON FOOD SAFETY IN THE AUSTRALIAN POULTRY INDUSTRY

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Summary

Globally, *Campylobacter* and *Salmonella enterica* subsp. *enterica* are among the most common causes of bacterial foodborne illness in humans. Contaminated food products of poultry origin are frequently implicated in outbreaks of human salmonellosis. *Salmonella* Enteritidis and *Salmonella* Typhimurium are frequently involved in egg and egg product-associated foodborne outbreaks. On the other hand, campylobacteriosis is primarily associated with the consumption of contaminated chicken meat. In Australia, *Salmonella* Typhimurium has been a predominant cause of local foodborne outbreaks attributed to poultry products. Recently however there have been some outbreaks of *Salmonella* Enteritidis in free range layer flocks. This paper provides an overview of food safety challenges in the Australian Poultry industy.

I. INTRODUCTION

In Australia *Salmonella* spp. are frequently involved in egg and egg product-associated foodborne outbreaks. On the other hand, campylobacteriosis is associated with the consumption of contaminated chicken meat (Sexton, 2016). The *Salmonella* and *Campylobacter* shedding in poultry flocks can be highly variable across different flocks and farms; as a result, the level of product (chicken meat and/or eggs) contamination is largely attributed to the flock management. The refrigeration of the product in supply chain and handling can also influence the safety of the poultry products. For the past several decades, both in Australia and other parts of the world, campylobacteriosis has been the most notified foodborne infection. The consumption of inadequately cooked or undercooked chicken meat and chicken meat products is one of the primary sources of human campylobacteriosis (Bryan & Doyle, 1995). The industry and regulatory agencies are often focussed on achieving the best practices to reduce *Campylobacter* contamination during processing to ensure food safety for the consumers. Different intervention strategies have been applied across the supply chain, from bird rearing in the poultry farms through to the poultry meat production in the processing plants, to reduce the level of *Campylobacter* in the food chain. Strict biosecurity measures on farms prevent the spreading of *Campylobacter* within the farm and between different farms, while the processing plants apply multiple decontamination methods to prevent bacterial cross-contamination in chicken meat (Umaraw et al., 2017). Worldwide, contaminated eggs and egg products are frequently implicated in outbreaks of human salmonellosis (Chousalkar & Gole, 2016). *Salmonella* Enteritidis (*S.* Enteritidis) and *Salmonella* Typhimurium (*S.* Typhimurium) serovars have dominated the epidemiology of *Salmonella* and are the most common causes of human salmonellosis (Hendriksen et al., 2011). Thorough cooking of eggs can destroy most, if not all, bacteria present, including *Salmonella,* and will pose a low risk to human health. If egg products or food items are prepared from raw or lightly cooked egg contents however, this will not destroy all *Salmonella (*if present). A rough estimate of the presence of *Salmonella* (all species, not just pathogenic) on eggs is greater than 1 in 20,000 (Arnold et al., 2014). Considering the estimated production of eggs in Australia and per capita consumption, the risk of foodborne illness in general is very low for humans consuming eggs.

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II. FOOD SAFETY IN AUSTRALIAN CHICKEN MEAT INDUSTRY

Chicken meat remains the most consumed animal protein in Australia (50.1 kg/person/year), comparable to the consumption of pork, sheep, beef and veal combined (ACMF, 2023). The chicken meat industry is dominated by large vertically integrated companies, with operations including breeder farms, hatcheries, grower farms, processing plants and feed mills.

There have been multiple longitudinal studies conducted in the egg layer industry due to high-risks of *Salmonella* outbreaks associated with eggs and egg-based products, however published studies are limited within the chicken meat industry (Abraham et al., 2019; Ford et al., 2018; N.-L. Willson & K. K. Chousalkar, 2023) with most surveillance conducted in-house. In Australia, *Salmonella* Typhimurium has been the leading cause of salmonellosis for over two decades, representing 84% of serovars linked to outbreaks between 2001-2016 (Ford et al., 2018). For *Campylobacter* spp., however a large proportion of isolates from notified Australian cases are not speciated (Cribb et al., 2022). The prevalence of *Campylobacter* spp. on raw poultry products at retail is high (Walker et al., 2019) and annual notifications of illness are increasing. *Salmonella* infection notification rates however are decreasing (NNDSS, 2022).

A recent study in Australia investigating the prevalence of *Salmonella* in broiler breeders and hatcheries found low detection rates for *Salmonella* (8% positive during rearing and 1.9% positive during production) over a 40 week surveillance period (N.-L. Willson & K. K. Chousalkar, 2023). There was also no association with serovars found in the hatchery and from the breeder flock they originated from, supporting studies in the UK that demonstrate contamination of eggs likely occurs from established infections within the hatchery rather that continued infection from breeder flocks (Withenshaw et al., 2021). Risk factors associated with *Campylobacter* colonisation include increasing animal age, number of sheds on farm, production type, stocking density, flock size, presence of other animals, partial depopulation and the type of nipple drinkers (Alter & Reich, 2021). Similar risk factors are associated with *Salmonella(Chousalkar et al., 2017)* and transmission vectors for both are heavily influenced by adequate biosecurity.

Gut health plays an integral role in the colonisation of foodborne pathogens, with the overarching aim in production to reduce the prevalence of carriage in poultry, thereby mitigating disease risk to the community. The first three days post hatch is a critical window for chick intestinal health as the microbiota is in early development and susceptible to colonisation, particularly with *Salmonella* (Ijaz et al., 2021). Although infection can be induced early in experimental chicks, *Campylobacter* will not generally be observed prior to 2-3 weeks in commercial flocks with a colonisation 'lag-phase' observed for multiple *Campylobacter* spp. but the rapid spread throughout the flock (Sahin et al., 2002). Once colonisation is established, bacterial shedding occurs, particularly in response to production stressors (N.-L. Willson & K. K. Chousalkar, 2023).

III. CONTROL OF FOOD BORNE PATHOGENS IN THE CHICKEN MEAT INDUSTRY

Campylobacter and *Salmonella* often establish persistent colonisation in the gastrointestinal tract of poultry (Hermans et al., 2012). Bacterial shedding from birds can be intermittent on farm but proliferation of both bacterial species has been linked with transport stress (Whyte et al., 2001) representing a significant risk for the downstream chicken meat supply chain. During processing, chicken carcasses can become contaminated with bacteria from feathers, skin, and ruptured intestinal tracts and cross-contamination of carcasses can also occur during the different stages of processing (Cox & Pavic, 2010). Thus, controlling bacteria throughout the chicken meat supply chain requires a multi-faceted approach that involves interventions at various states from farm to fork.

The source of *Campylobacter* and *Salmonella* on a farm can include residual litter, rodents, insects, feed, or water that can result in flock-to-flock infection (Battersby et al., 2016). Thus, the first line of defence against bacterial introduction is at the farm level. The Australian chicken meat industry has impleted strict biosecurity measures and several interventions to minimise the risk of bacterial contamination. This includes maintaining proper hygiene, regular cleaning and disinfection of poultry sheds and equipment in between batches, and clean water sources. In addition, all Australian commercial broiler breeder flocks are vaccinated with a live, attenuated *Salmonella* vaccine (N.-L. Willson & K. Chousalkar, 2023). Implementing a comprehensive vaccination program can reduce the prevalence of bacteria in individual flocks thereby minimizing on farm contamination as well as in the chicken meat supply chain. Currently, there is no commercially available poultry vaccine for *Campylobacter*.

Controlling bacteria in the chicken meat supply chain also involves strategies such as feed management. While *Campylobacter* is not commonly detected in feed components, *Salmonella* can be present in components of poultry feed (Ricke et al., 2020). Implementing measures such as proper storage, handling, and processing of feed can help minimise the risk of bacterial contamination. Feed supplements such as biochar, zeolite, and organic acids have been shown to reduce loads of foodborne bacterial pathogens in the poultry gastrointestinal tract (Prasai et al., 2016; Ricke et al., 2020).

Australian poultry meat processing plants use several interventions to reduce bacterial contamination of chicken carcasses including an inside/outside wash with water, chilling carcasses to 4°C, and immersion chilling in chlorinated water (Cox & Pavic, 2010). The efficacy of sodium hypochlorite, however, is dependent on organic load and total bacterial load (Lillard, 1980; Muhandiramlage et al., 2020). The Australian New Zealand Food Standards code permits the use of peroxyacetic acid (PAA) and acidified sodium chlorite (ASC) for use as food sanitizers (FSANZ, 2005) but chlorine remains the most commonly used sanitizer in the Australian poultry industry. Both PAA and ASC have been shown to reduce microorganisms linked with foodborne gastrointestinal disease on naturally contaminated bird carcasses obtained from Australian processing plants (Chousalkar et al., 2019; McWhorter et al., 2022; Sexton et al., 2007). The processing plants use strict hygiene protocols, such as regular cleaning and sanitisation of processing equipment and facilities. Regular testing and monitoring of chicken meat is also carried out to detect and control bacterial contamination.

IV. FOOD SAFETY IN EGG INDUSTRY

In Australia, longitudinal studies were conducted to study the shedding of *Salmonella* in cage (V. C. Gole, C. G. Caraguel, et al., 2014) and free range production systems (Gole et al., 2017; McWhorter & Chousalkar, 2019) and found the load and the prevalence of *Salmonella* is dependent on the individual farm and or flock management. There are a number of bacteria found on egghsell surface (Chousalkar & Roberts, 2012); however, from food safety perspective, *Salmonella* plays a more important role. As reviewed in Gantois et al., (Gantois et al., 2009), not all chicken-associated *Salmonella* serotypes have the potential to transmit vertically. For example, *S.* Enteritidis transmits both vertically and horizontally, while *S.* Typhimurium predominantly contaminate eggs through horizontal route of transmission. During horizontal transmission, *Salmonella* can survive on the eggshell surface, in shell pores and egg internal contents at various temperatures (Khan et al., 2021). At room temperature *Salmonella* can penetrate into the egg internal contents (Lin et al., 2021), which results in the ultimate contamination of albumen and yolk.

Egg white has antibacterial properties and the alkaline pH and reduced iron level in albumen make it harsh environment for *Salmonella* replication. The antimicrobial activities of albumen is significantly reduced at higher temperatures (e.g. 45°C) (Baron et al., 2020). In

poultry production, intact eggs typically will have less than 10 Colony Forming Units/egg of *Salmonella* (Humphrey et al., 1989); however, 10^6 CFUs/egg has also been reported on the shell surface (V. C. Gole, V. Torok, et al., 2014) . The load of *Salmonella* within albumen or yolk depends upon multiple conditions including the egg storage temperature, level of contamination shell quality characteristics. In the egg, *Salmonella* up-regulates various genes involved in cell metabolism and virulence (Clavijo et al., 2006); however, at lower storage temperatures (e.g. 4°C), *Salmonella* will not replicate exponentially, due to its metabolic arrest. To overcome the antimicrobial properties of albumen, *Salmonella* regulates genes, such as ybgC, yoaE and cpxR for its survivability (Huang et al., 2020; Qin et al., 2019). Once *Salmonella* translocates from the albumen to yolk within an intact egg, its growth can be significantly increased at higher storage temperature (Khan et al., 2021). Yolk favours the upregulation of metabolic pathways in *Salmonella* involved in type II secretion system, infection process and epithelial cell invasion of host (Xu et al., 2022). Therefore, it is recommended to store eggs at lower temperatures in the food supply chain in some countries.

V. CONTROL OF FOOD BORNE PATHOGENS IN THE EGG INDUSTRY

The Australian egg industry use many intervention strategies such as flock vaccinations, probiotics, in-feed organic acids, egg washing, farm hygiene and biosecurity etc. The effectiveness of each intervention such as vaccination (McWhorter & Chousalkar, 2018), use of probiotics (Khan & Chousalkar, 2021), and egg washing (Vaibhav C Gole et al., 2014) have been tested, however it is important to note that these biosecurity measures do not offer complete elimination of *Salmonella* spp. Stress can influence the *Salmonella* shedding in a flock and ultimately affect the load of *Salmonella* on eggs (V. C. Gole, V. Torok, et al., 2014). Egg washing reduces *Salmonella* on the shell surface but viable cells can still survive in the shell pores (McWhorter & Chousalkar, 2020). Therefore, it is paramount to store the washed eggs at appropriate temperature. It is important to note that egg washing is not practised in some parts of the world, such as Europe.

Major commercial egg producers in Australia are represented by Australian Egg Corporation Limited (AECL). The AECL has a voluntary egg quality program which provides guidelines for food safety, biosecurity, environmental use, hen health, welfare and labelling for the national egg industry. The Australia Eggs has voluntary Codes of Practice for assisting egg producers. These codes provide guidance on hygienic egg production, storage, packaging and distribution of shell eggs and egg products for human consumption. The recommended temperature for egg storage on farm, during transport and at the retail outlet is below 15 °C (+ /- 3°C). There is a *S.* Enteritidis monitoring and accreditation programme for commercial egg producers exporting eggs to overseas market. In Australia *S.*Typhimurium has been a predominant cause of local foodborne outbreaks attributed to the poultry egg products (Ford et al., 2018) . Recently, there have been some outbreaks of *S.* Enteritidis in some free range layer flocks (Collins et al., 2023). The efficacy of current *S.* Typhimurium vaccine has been tested against *S.* Enteritidis in experimental pen trials but currently there is no *S.* Enteritidis vaccine in Australia.

VI. CONCLUDING REMARKS

Despite several improved interventions, reducing the level of *Salmonella* and *Campylobacter* spp. remains a significant challenge for the industry. Communication between regulators and industry is paramount to control poultry product related foodborne outbreaks, and collaborative efforts are required to design and implement the control strategies. Continious education of the general public on safe handling of eggs and chicken meat is also necessary. Foodborne illnesses are frequently linked to poor poultry handling practices and subsequent cross-contamination in

the kitchen environment. For example, washing or rinsing raw meat and poultry products can result in cross-contamination because the juices and wash water may come into contact with other foods, surfaces, and utensils (Shumaker *et al*., 2022). Previous studies found that food-borne pathogens such as *Salmonella* and *Campylobacter* are partially preventable through improvements in consumer preparation of Poultry products (Luber et al., 2006). It has been found that using hot water and detergent to clean hands and utensils after chicken preparation/handling in the kitchen achieved a 50% reduction in bacterial contamination (Cogan *et al*., 2002).

Previous studies concluded that despite guidance from food safety agencies, adherence to current recommended chicken meat and egg handling practices is low. The industry peak bodies and state regulatory authorities have included factsheets and resources on their websites to educate consumers on safe chicken meat and egg handling practices (Kosa *et al*., 2015). The impact of these educational materials on consumer awareness remains unclear.

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ANTIMICROBIAL RESISTANCE PROFILES OF *ENTEROCOCCUS* SPP. ASSOCIATED WITH POULTRY DISEASES AND THEIR SUSCEPTIBILITY TO PROBIOTIC *BACILLUS*

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Summary

This work investigated the antimicrobial resistance patterns of clinical *Enterococcus* spp., commensal bacteria and opportunistic pathogens that can cause outbreaks in poultry, and the potential efficacy of cell-free supernatant (CFS) prepared from 4 *Bacillus* probiotic strains. This study showed the high prevalence of *E. cecorum* isolates resistant to gentamycin, erythromycin and tetracycline, while their growth kinetics were negatively affected by the compounds produced by some of the tested *Bacillus* strains.

I. INTRODUCTION

Enterococci are Gram-positive bacteria commonly found in the environment and are part of the normal microbiota of the intestinal tract of humans and animals including poultry (Aarestrup et al., 2002). They are also opportunistic bacteria that cause disease in humans and animals. Multiple species are associated with diseases in poultry - *E. faecium, E. faecalis, E. durans*, *E. hirae* with *E. cecorum* being the dominant species (Souillard et al., 2022). Clinical manifestation includes locomotor syndromes, septicemia and omphalitis leading to significant economic losses (Jung et al., 2018). There are currently no available vaccines against *Enterococcus* spp. and protective measures mainly include biosecurity and good management practices since antimicrobial agents are ineffective for symptomatic birds (EFSA, 2022). Furthermore, *Enterococcus* spp. (especially *E. cecorum, E. faecium* and *E. faecalis*) trigger public health warnings for the risk of antimicrobial resistance and resistance transfer (EFSA, 2022). The aim of this study was to determine the antibiotic resistance profiles of 28 clinical *Enterococcus* spp. isolates collected throughout different production systems, geographical regions, and a variety of infection sites. Then, this study assessed the potential of probiotic *Bacillus* secretions to interact with the growth kinetics of a wider collection of 123 clinical *Enterococcus* spp. isolates.

II. METHOD

One hundred and twenty-three *Enterococcus* spp. isolates were collected from birds suffering from clinical symptoms characteristic of *Enterococcus* spp. infections over 3 different continents: North America, Europe and Middle East after 2 campaigns in 2013 and 2023. Samples originated from breeders (43.91%), hatchery (4.06%), broiler flocks (46.34%) and layers (5.69%) and infection sites were recorded. *Enterococcus* were identified by 16S ribosomal RNA sequences. The strains belonged to 9 different species: *E. avium* (n=3), *E. casseliflavus* (n=4), *E. cecorum* (n=97), *E. durans* (n=3), *E. faecalis* (n=4), *E. faecium* (n=4), *E. gallinarum* (n=4), *E. hirae* (n=4), *E. mundtii* (n=1).

The broth microdilution method (BMD) was used to determine the minimal inhibitory concentrations (MICs) of 8 antimicrobial agents for a subset of 28 *Enterococcus* isolates (26 *E. cecorum*, 1 *E. gallinarum* and 1 *E. avium*) according to the method described by Laurentie et al. (2023). The MICs were read after 18h of incubation at 35°C and 24 h more for streptomycin. In the absence of cut off values specified for *E. cecorum*, the susceptibility profiles were determined according to EUCAST recommendations for *Enterococcus* spp. (EUCAST 2023) for ampicillin (AMP), gentamycin (GEN), streptomycin (STR) and vancomycin (VAN) and according to

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specified EFSA cut off values (EFSA, 2012) for a *E. faecium* feed additive for the remaining agents - kanamycin (KAN), erythromycin (ERY), tetracycline (TET) and chloramphenicol (CHL).

The inhibitory potential of 4 different commercial strains of probiotic *Bacillus* were tested*:* 3 *B. velezensis* strains BS8, B. 15AP4 and B. 2084 used as a blend - Enviva®PRO (Danisco Animal Nutrition & Health, NL) and one single commercial strain *B. licheniformis* DSM 27810. CFS were prepared according to the procedure described by Medina-Fernandez et al. (2019). The CFSs were stored at -20◦C until further use.

Enterococcus inhibitory assays were conducted as described by Medina-Fernandez et al. (2019). Briefly, fresh *Enterococcus* cultures were inoculated in brain-heart infusion (BHI) broth in 96-well UV-treated microtiter plates with flat-bottomed wells, at a final concentration of 10^4 colony forming units (CFU)/ml. CFS samples were added to the treated wells (10% v/v) containing BHI and *E. cecorum* isolates (1% v/v). Non-treated wells contained BHI and *E. cecorum* isolate (1% v/v) only (PC). BHI only was used as a negative control (NC). All microtiter plates were covered and incubated at 37℃ for 15 h and optical densities (OD) measured at 595 nm every 15 min. All assays were conducted in biological duplicates (corresponding to 4 technical replicates). The average OD across replicates were used to calculate the percentage growth inhibition vs PC. The kinetics of *E. cecorum* growth in the presence/absence of the *Bacillus* CFSs was also studied. The delay in pathogen growth in the presence of *Bacillus* CFS was calculated as the difference in time (minutes) to reach the OD captured at the middle of the exponential growth phase between the PC and CFS plus *E. cecorum* supplemented wells (Figure 1).

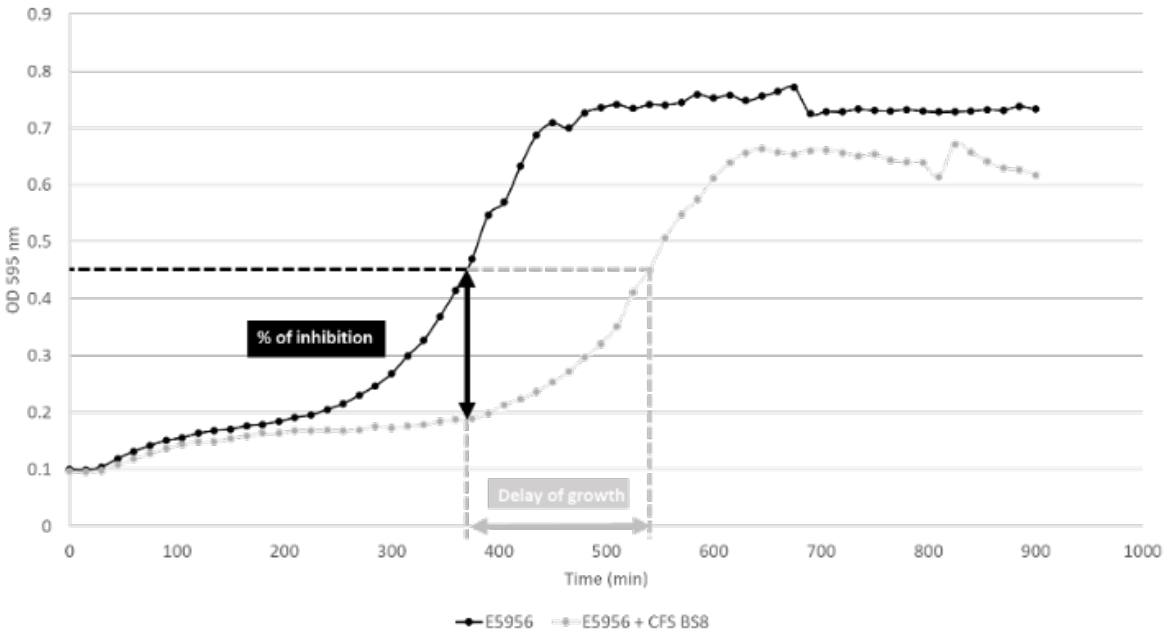

Figure 1 - Example of *E. cecorum* E5956 growth kinetics seen during exposure to *B. velezensis* BS8 CFS and illustration of critical OD and time points used to determine percentage of inhibition and delay of growth.

One way ANOVA was used to compare inhibitory potentials of the probiotic strains. Post-hoc means separation was achieved using Tukey's HSD test. Differences were considered significant at $P < 0.05$. Data analyzed used the Fit X by Y function of JMP 16.1.

III. RESULTS

All *Enterococcus* isolates were susceptible to AMP whilst 10.7% and 7.1% demonstrated resistance against vancomycin and chloramphenicol respectively. Most of the isolates showed resistance profiles to GEN (82.1% - a critically important antibiotic for human medicine), ERY (78.6%) and TET (71.4%). EUCAST determined MICs for High Level of Resistance (HLR) for the *Enterococcus* spp. against GEN (MIC ≥ 128 mg/L) and STR (MIC HLR ≥ 512 mg/L). Among the

23 isolates showing resistance to GEN, 6 exhibit a HLR and were predominately from Belgium. Among the 13 isolates showing resistance to STR, 69.3% showed HLR but their geographic origin was spread.

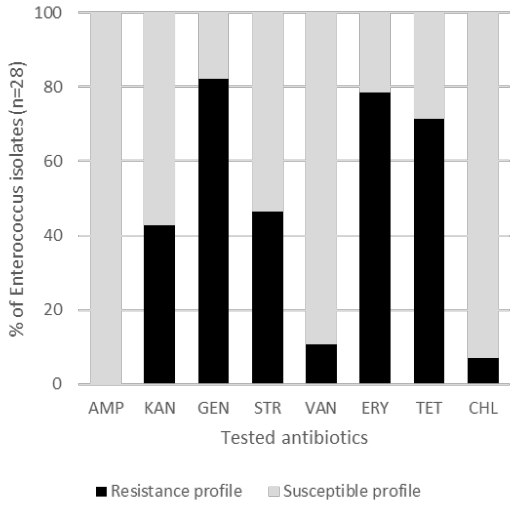

Figure 2 - Percentage of *Enterococcus* isolates (n=28) showing susceptible or resistance profiles to tested antibiotics (AMP-ampicillin; KAN-Kanamycin; GEN-Gentamycin; STR-Streptomycin; VAN-Vancomycin; ERY-Erythromycin; TET: Tetracycline and CHL-Chloramphenicol).

Figure 3 shows the mean percentage growth inhibition of the 97 clinical *E. cecorum* isolates by 3 *Bacillus* CFSs (BS8, 15AP4 and 2084) and a subset of 88 by *Bacillus* DSM28710 CFS. All 3 CFSs from BS8, 15AP4 and 2084 consistently inhibited the growth of *E. cecorum* isolates, respectively 80.15% (±3.67), 73.14% (±2.92) and 68.67% (± 3.21). The CFS from *B. licheniformis* 28710 showed a positive effect that was significantly different from the effect of the *B. velezensis* strains ($P < 0.0001$), resulting in the growth promotion of the clinical *E. cecorum* isolates (translating into a negative mean of inhibition - 28.4% ± 8.76).

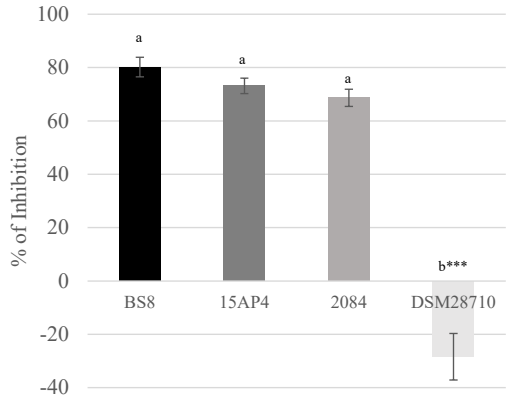

Figure 3 - Percentage growth inhibition of clinical *E. cecorum* isolates by 4 *Bacillus* CFSs, measured at a time-point equivalent to the middle of the exponential growth phase of the PC ($P<0.05$ and *** $P < 0.0001$).

Beyond the anti-*E. cecorum* activity, the CFSs of the *B. velezensis* strains tested as a blend (ratio 1:1:1) also displayed, but in a lesser extent, inhibition categorized as moderate (40-60% inhibition) against *E. avium, E. casseliflavus, E. faecalis* and *E. gallinarum* or strong (inhibition > 60%) against *E. durans, E. faecium* and *E. hirae*. The CFS blend obtained from the *B. velezensis* cultures also delayed the growth of clinical isolates from 1 to 10 hours. Unlike the promoting effect of *Bacillus* 28710 CFS against *E. cecorum*, the secreted compounds of *B.* 28710 showed moderate inhibitory effect against *E. casseliflavus* isolates (44.79 %) or no effect (0 -25% inhibition) against 7 other *Enterococcus* spp. tested.

IV. DISCUSSION

The antibiotic resistance profiles of the tested *E. cecorum* isolates collected from the Northern Hemisphere and the high prevalence of resistant strains for erythromycin, tetracycline and gentamycin are in alignment with existing literature for the *E. cecorum* and other *Enterococcus* spp. of poultry origin (Stępień-Pyśniak et al., 2021). Interestingly, isolates collected in EU showed multiple and very high levels of resistance to gentamycin, tetracycline and erythromycin, 3 major antibiotics used in broiler production as therapeutic agents. Gentamycin is applied against *E. coli* omphalitis, tetracycline against *E. coli* (airsacculitis and arthritis), *Clostridium perfringens* (necrotic enteritis), and *Staphylococcus* (arthritis) while erythromycin is mainly used against chronic respiratory diseases (Agunos et al., 2012). The combination of the commensal origin of *Enterococcus* spp., their status of co-infection with *E. coli* and other poultry diseases (Souillard et al., 2022) and their ability to acquired easily antibiotic resistance genes, may explain the high prevalence of these acquired AMR profiles. The increasing clinical importance of *E. cecorum*, combined with the wide distribution and the high levels of resistance found for certain antimicrobials, supported the EU statement to consider *E. cecorum* eligible to be listed and categorized within the Animal Health Law framework (EFSA, 2022).

Probiotic strains of *Bacillus* are known to secrete a large variety of natural compounds (e.g. subtilin, subtilosin, amylosin, lichenicidin) which can either support, limit or prevent the growth of other microbes (Vaca et al., 2022). This study demonstrated that the 3 selected strains of *Bacillus velezensis* produced compounds which either prevent or delay the growth of clinical *Enterococcus* isolates with a more specific effect on *E. cecorum*, the dominant species encountered in enterococcal poultry diseases. These interactions were superior to those produced by the *Bacillus licheniformis* probiotic strain, highlighting the species and strain-specificities of overall probiotic properties and the need for in depth screening and characterization. This consistent and robust *in vitro* direct interaction against a large and diverse collection of *Enterococcus* spp clinical isolates established the rational to pursue *in vivo* the assessment of the preharvest potential of *B. velezensis* BS8, 15AP4 and 2084 blend as a long-term and sustainable alternative to antibiotics for enterococcal poultry diseases.

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OREGANO ESSENTIAL OIL IS CYTOTOXIC TO CHICKEN EMBRYO FIBROBLASTS ABOVE 8,000 PPM

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Several essential oils have shown significant functional activities that may help keeping gut health (Brenes and Roura, 2010). Carvacrol, the main bioactive compound of oregano essential oil (OEO), has been shown to improve gut health and immunity as a feed supplement. Recent results show promising potential for the *in ovo* application of OEO to improve embryonic development. However, it has also been shown that carvacrol has the potential to induce apoptosis (Llana-Ruiz-Cabello et al., 2013). In addition, little is known about the safety margins of *in ovo* applications of OEO. This study aimed to evaluate the cytotoxic effect of OEO on chicken embryo fibroblast cells (CEF). It was hypothesized that OEO is a safe essential oil with low cytotoxicity (above 5,000 ppm).

Cell cytotoxicity assays were used to study a range of OEO concentrations by looking at the NADPH-associated conversion of yellow tetrazolium salt (MTT) into purple formazan crystals occurring only in viable cells. Primary chicken embryo fibroblast cells were obtained by dissecting 10-day old chicken embryo. Polysorbate 80 (Tween 80®, Sigma Aldrich) was used (in a ratio of 1:1) as an emulsifier to solubilize the OEO with media homogenously. For cytotoxicity assay, a plate of 96 wells was used, and cells were seeded at a rate of 5×10^4 cells/well. Each column was considered as a treatment having eight replicates of wells. A column of cells+media acted as the negative control whereas addition of geneticin (G418, 300 mg mL^{-1}), an antibiotic, acted as the positive control. Two cytotoxicity assays were conducted. The absorbance of the crystals was measured using a multi-well gas chromatography/mass spectrometry (GC-MS) (Epoch) at 550 and 600nm. Subsequently, the half-maximal inhibitory concentration (IC50) was determined to identify the maximal concentration of OEO applications *in ovo*. Data were analysed using SAS software version 9.4 (SAS Institute Inc 2013) and Microsoft Excel.

The GC-MS analysis of the OEO identified 40 compounds accounting for 99.97% of the total area. Phenolic compound, carvacrol, had the largest percentage (74.23%) of the chromatogram, thus confirming it was the predominant active compound within the sample. In the first cytotoxic assay, doses between 10,000 ppm-0.001 ppm, the addition of the OEO to the media at 10,000 ppm had a significant negative effect on cell viability compared to the control group ($P < 0.05$). In the second one, between 10,000 ppm-1,000 ppm, again a clear cytotoxic effect of OEO was observed at 10,000 ppm ($P < 0.05$). A negative relationship was found between the concentration of the OEO media and the presence of healthy CEF cells above 8,000 ppm. In contrast, no cytotoxic effect of OEO was detected at concentrations lower than 8,000 ppm. This is within the range of other IC50 values obtained from other cell lines using OEO, so future *in ovo* studies can focus on optimising the concentration below 8,000 ppm to promote healthier embryonic development.

In conclusion, *in vitro* application of OEO is safe in doses below IC50 of 8,000 ppm.

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POTENTIAL OF SURFACTIN TO IMPROVE PERFORMANCE AND GUT HEALTH OF BROILERS UNDER NECROTIC ENTERITIS CHALLENGE

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Probiotics have promising effects in improving performance and gut health in broilers and reducing the incidence of diseases in the post-antibiotic era. However, the results are inconsistent under necrotic enteritis (NE) challenge. Therefore, this study was conducted to evaluate the effects of surfactin (SURFA TID, Enhalor), a lipopeptide-type biosurfactant derived from probiotic bacteria strain *Bacillus subtilis*, on performance and gut health of broilers under NE challenge. A total of 512 d-old Cobb 500 mixed-sex broiler chicks were randomly distributed into four treatment groups, with eight replicates of 16 birds per pen in a completely randomised design. The treatments were: UC (T1) - Unchallenged control; CC (T2) - NE challenged control; Surfactin (T3) - CC+surfactin 100 g/t and Antibiotics (T4) - CC+ zinc bacitracin and salinomycin at 267 and 500 g/ton in the starter, grower, and finisher phases. Birds were fed a wheat-soybean meal-sorghum-based diet supplemented with phytase (1000 FTU/kg). Challenged birds were gavaged with *Eimeria* spp. on d 9 and *Clostridium perfringens* EHE-NE18 on d 14 and 15 according to Rodgers et al. (2015). Intestinal NE lesions, serum immunoglobulins and fluorescence isothiocyanate dextran (FITC-d), a leaky gut marker on d 16 and growth performance on d 0 to 35 data were analysed to evaluate to efficacy of additive. Data were subjected to a one-way analysis of variance using JMP 17.0 where the female percentage was used as a covariate for the performance analysis to reduce the pen variation and increase the statistical power in nutrition-based trials (it is necessary as male/female ratio varied across pens due to the random initial allocation to groups), and significance was determined at $P < 0.05$ by the Tukey HSD test.

During the challenge period (d8-19), birds in the CC group had a lower body weight gain (BWG), higher feed conversion ratio (FCR) and intestinal lesions compared to the UC group ($P < 0.05$) indicating a successful sub-clinical NE challenge of the birds. Supplementation of surfactin showed similar performance compared to the CC group on d8-19 ($P > 0.05$), but had improved FCR in the later finisher phase on d28-35 ($P < 0.05$) indicating better feed efficiency in the recovery phase. In the overall period (d0-35), birds fed surfactin had similar performance compared to CC group but the BWG in the surfactin group was not different from UC group ($P > 0.05$). Birds fed surfactin had similar jejunal lesion scores, serum FITC-d, serum IgA and IgM concentrations compared to CC and antibiotics groups ($P > 0.05$) where jejunal lesion scores, FITC-d and IgA were significantly different in the CC group compared to the antibiotics group ($P < 0.05$). Overall, these results suggest that surfactin has the potential to improve feed efficiency in the challenge recovery phase compared to CC group and to maintain similar BWG compared to the UC group across the overall period of the study. This could be a result of the lower jejunal lesions and enhanced gut integrity, and immunity in the birds fed the surfactin supplemented diet.

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THE EFFECT OF DIETARY INCLUSION OF DUCKWEED (*LEMNOIDEAE*) ON PRODUCTION PERFORMANCE, IMMUNE SYSTEM, AND MEAT QUALITY IN COMMERCIAL BROILER CHICKEN

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Summary

This study aimed to evaluate the effect of the dietary inclusion of Duckweed as a protein-enriched ingredient in commercial broiler chicken production. Growth performance, meat quality, and immune system status of chickens were studied for 42 days using 192-day-old male broiler chickens (ROSS 308) with Duckweed included in the diet at three inclusion rates (0, 2.5, and 5%) as a partial replacement for soybean meal. The inclusion of duckweed at 2.5% in the diet had no adverse effects on broiler growth, feed efficiency, immune response, and meat quality ($P>0.05$). Dietary inclusion of 5% Duckweed decreased L* (lightness) breast meat color, and increased b* (yellowness) Chroma and Hue meat color indices compared to the diet with 0% (control) group ($P<0.05$). Duckweed can therefore be integrated into broiler diets at 2.5% substitution with soybean meal without compromising production performance or meat quality, offering a sustainable and cost-effective alternative feed source.

I. INTRODUCTION

Duckweed (*Lemnoideae*), a small aquatic plant developed over or below the water surface, has been exploited as animal feeds for hundreds of years in Asian production systems (Escobar and Escobar AC, 2017). Fresh duckweed is reported to contain dry matter ranging from 3% to 14%. Duckweed's nutrients may vary substantially depending on the growing environment. The protein content ranges from 7% to 45% (mostly 20% to 45%), the fat content accounts for 2% to 9%, the fiber content accounts for 12% to 28% and the carbohydrate content accounts for 14% to 44% of the dry matter. In addition, duckweed can absorb a variety of macro- and micronutrients (Ca, Cl, K, Na, Si, N, H, C, Fe, Mg, Mn, Al, Si, B, P, Cu, and Zn) from the water/growth media. It also contains vitamins A, B, and E, which improve the biological value of duckweed. Numerous studies have been conducted to investigate the use of duckweed as a part of poultry diets (Ahammad et al., 2003; Haustein et al., 1994; Zakaria and Shammout, 2018). Inclusion of duckweed to a broiler's feeding regimen at a level of 15% of the dietary protein led to higher body weight and lower feed cost in comparison to a diet without duckweed (Paguia et al., 2022). This study aimed to evaluate different dietary inclusion rates of duckweed as a partial replacement for soybean meal on growth performance, and meat quality, immune response in commercial broiler chickens.

II. METHOD

A total of 192 one-day-old male broiler chickens (ROSS 308) were purchased from a commercial hatchery (Aviagen) and transferred to the Queensland Animal Science Precinct (QASP) Facility at Gatton Campus, University of Queensland after obtaining Animal Ethics Clearance (Cert. No. 2022/AE000504). The chickens were weighed at arrival and randomly

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allocated to 24-floor pens with eight birds per pen (n=64 chicks per experimental group). The grow-out trial followed the management guidelines of the breeder. The birds had *ad libitum* access to feed and fresh water for the total experimental period. Experimental diets were formulated to meet all the nutrient recommendations for the three growth phases (starter, 1 to 10 days of age; grower, 11 to 28 days of age, and finisher, 29 to 42 days of age). During the starter phase, a basal soybean meal-corn-wheat diet was used. On day 11 of age, the birds were randomly allocated to one of the three experimental groups for the grower and finisher periods. Duckweed (*Lemnoideae*), sourced from the Schenk lab, was employed as the nucleation source. Duckweed was cultivated at the Pinjarra Hills campus of the University of Queensland between November 2021 and September 2022. After harvesting, the biomass was subjected to 24 hours of drying in a food dehydrator at 50°C, followed by collection and storage in a cold room. The experimental diets included different levels of duckweed substituted for soybean meal in the basal diet (control 0%, 2.5% and 5%). All diets were formulated to be isocaloric and isonitrogenous and to meet all nutrient recommendations following breeders' guidelines. Feed intake (FI), body weight (BW) gain, and feed conversion rate (FCR) were recorded for d 1-14, d 15-28, d 28-42 and d 1-42 periods. On day 42, five birds per pen were humanely euthanized. Blood and meat samples (only one bird was used for meat quality analysis) to study meat quality and blood immunoglobulins (IgG, IgA, and IgM) were collected. Additionally, samples for ongoing analysis of nutrient digestibility and gut morphology were also collected. Data was subjected to analysis of variance using Jamovi software and results were presented as means and standard error of the mean (SEM). Turkey's test were conducted to multiple comparisons of the means and $P \leq 0.05$ were considered as the level of statistically difference.

III. RESULTS

Table 1 - The effect of dietary inclusion of duckweed substituting soybean meals on the production performance of broiler chickens.

Parameters	Experimental groups[1]			SEM[3]	*P* Value
	0%	2.5%	5%		
Average Body weight (g)					
d 1	36.95	36.92	37.02	0.39	0.99
d 11	313.45	315.53	314.69	2.99	0.89
d 28	1622.30^{a}	1576.31^{ab}	1550.69^{b}	18.78	0.04
d 41	3197.30^{a}	3101.53^{ab}	3036.04^{b}	31.54	<0.01
Average daily weight gain (g)					
d 1-10	27.65	27.86	27.77	0.30	0.88
d 11-28	95.43^{a}	92.72^{ab}	91.22^{b}	1.10	0.04
d 29-41	112.50^{a}	108.94^{ab}	106.10^{b}	1.64	0.04
d1 – 41	75.25^{a}	72.97^{ab}	71.41^{b}	0.75	<0.01
Average daily feed intake (g)					
d 1-10	30.53	30.89	30.94	0.54	0.85
d 11-28	102.57	99.69	99.92	1.21	0.20
d 29-41	174.88	170.72	173.73	1.97	0.33
d 1-41	109.52	106.78	107.93	1.12	0.25
FCR					
d 1-10	1.11	1.11	1.12	0.02	0.96
d 11-28	1.08	1.08	1.10	0.01	0.21
d 29-41	1.55^{b}	1.57^{b}	1.64^{a}	0.02	<0.01
d 1-41	1.46^{b}	1.46^{b}	1.51^{a}	0.01	<0.01

[1] Duckweed inclusion rate substituting soybean meal during the growing and finishing phases.
[2] Means with different superscripts differ (P≤0.05)
[3] SEM: Standard error of the mean.

As expected, BW, ADG, FI and FCR were not differ on 11 days of age (P>0.05; Table 1). However, BW decreased in the diet with 5% duckweed compared to the control diet at d 28 and 42 (P<0.05). A similar trend was noted for average daily gain (ADG), where no significant difference was observed between 2.5% duckweed inclusion and the control diet, but the ADG decreased in the diet with 5% duckweed compared to the control diet at the grower and finisher phases and for the overall growth period (P<0.05). Average daily feed intake was not affected by duckweed inclusion at either level during the experimental periods (P>0.05). Although FCR did different among groups during the grower phases (11-28 days of age), it was significantly higher in the diet with 5% duckweed compared to the two other diets during the finisher and overall period (P<0.05).

The treatments showed no impact (P>0.05) on breast pH, cooking water loss, shear force (N), and breast colour a* (Table 2). However, with an increasing substitution level of duckweed, there was a decrease (P<0.05) in breast lightness (colour L*) and an increase (P<0.05) in Hue at a 5% duckweed inclusion level when compared to the control group (0% duckweed). Furthermore, breast colour b* and chroma exhibited a linear increase (P<0.05) with increasing duckweed inclusion levels.

Table 2 - The effect of dietary inclusion of duckweed substituting soybean meals on breast meat quality in broiler chickens.

Parameters	Experimental groups[1]			SEM	*P* Value
	0%	2.5%	5%		
Breast pH	5.73	5.65	5.75	0.04	0.19
Cooking water loss (%)	31.04	34.05	28.09	2.35	0.23
Shear force (N)	24.43	25.50	25.52	5.44	0.99
Breast colour , L*	50.98^{a}	49.48^{ab}	46.89^{b}	0.75	<0.01
Breast colour , a*	2.58	2.67	2.16	0.28	0.41
Breast colour , b*	6.20^{c}	8.87^{b}	10.92^{a}	0.55	<0.01
Chroma	6.75^{c}	9.29^{b}	11.20^{a}	0.51	<0.01
Hue	1.18^{b}	1.27^{ab}	1.36^{a}	0.04	0.02

Means with different superscripts differ (P≤0.01).

There was no difference between experimental groups on blood IgA, IgM, IgG and corticosterone (P>0.05; Figure 1).

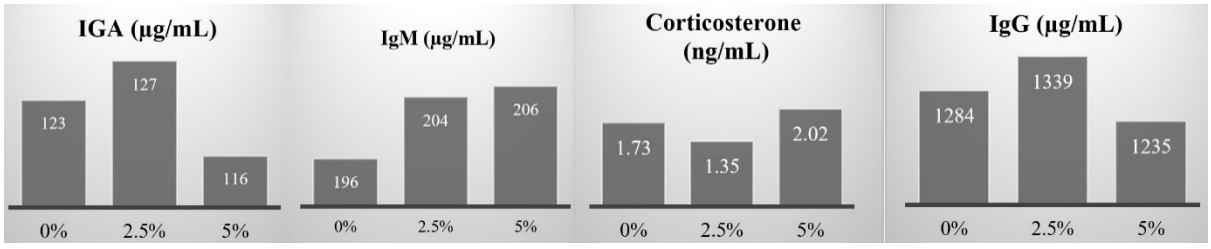

Figure 1 - The effect of dietary inclusion of duckweed substituting soybean meals on the immune response in broiler chickens.

IV. DISCUSSION

This study investigated the potential of duckweed as a feed ingredient for broiler chickens. In the present study, the 5% duckweed diet reduced BW compared to the control at days 28 and 42. This observation deviates from findings by Haustein et al. (1992), who reported that birds (Male Titan and Arbor Acres breeds) fed diets containing 5% *Lemna gibba* exhibited slightly but not significantly greater weight gain compared to those fed standard diets. Conversely, (Hamid et al., 1993) reported minimal variations in body weight gain when duckweed meal

was included in the diet of ducklings, a trend similarly observed by Zakaria et al. (2018) when incorporating *Lemna minor* meal into the diet of laying hens.

In this study, the average daily feed intake remained unaffected by the duckweed diets throughout the experimental period. In contrast (Kabir, et al., 2005) reported reduced feed intake (2.50 kg/broiler vs. 2.31–2.46 kg/broiler) in comparison to the control group when administering diets containing 4%, 8%, or 12% of dried duckweed meal to Vencobb broilers for 42 days. While no significant differences in FCR were observed between groups during the starter and grower phases, the 5% duckweed diet exhibited a notably higher FCR compared to the other two groups during the finisher phase and over the total period. The cause of this decline might be the presence of high fiber, tannin content, and reduced protein digestibility of the Duckweed; factors that are known to likely contribute to diminished live weight (Islam et al., 1997). In addition, the effect of 5% Duckweed in lowering L* and enhancing b*, Hue and chroma meat colour indices could be explained by the duckweed pigments and its potential in absorption minerals. Although no significant changes were noted in chicken's productivity, meat quality and immune system status between experimental groups at 2.5% duckweed inclusion, reduction of productivity and colour quality in 5% dietary Duckweed substitution for soybean meal infer a kind of nutritional limitation levels for inclusion Duckweed in broilers' diet. Further exploration of optimal inclusion rates, processing and enzyme supplementation of duckweed-included diets could provide additional insights into harnessing the full potential of duckweed in broiler nutrition, thereby supporting more environmentally friendly and sustainable poultry production systems.

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THE IMPACT OF REDUCING THE LEVELS OF CA AND P ON BROILER PERFORMANCE, CA AND P DIGESTIBILITY AND BONE PARAMETERS

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The release of P into the environment is a target that must be achieved not only for economic and environmental reasons, but also to increase the sustainability of animal production. The impact of dietary Ca on P digestibility, and consequently the level of the inclusion of inorganic P sources in the feed, plays an important role by hindering the phytase to hydrolyse phytate-P (Sommerfeld et al., 2018). The aim of this trial was to investigate the impact of lowering the inclusion of limestone (and consequently Ca) combined with lowering P on broiler performance, Ca and P digestibility and bone quality.

Eighteen pens with 24 male Ross 308 male per pen were fed a feed containing an intrinsically heat stable 6-phytase (OptiPhos® Plus) at 1000 FTU/kg (only considering a 1.76 g/kg available P (aP) matrix value) and an endo-1,4- beta-xylanase (Hostazym® X) (on top; 1500 EPU/kg) and were split over 2 treatments. Birds of one treatment were fed a starter feed (day 1-10) containing 0.85 % Ca and 0.45 % aP, a grower feed (day 10-21) containing 0.70 % Ca and 0.36 % aP and a finisher feed (day 21-35) containing 0.60 % Ca and 0.30 % aP. Birds of the other treatment were fed the same feed except with Ca and aP lowered to 0.65 and 0.40 %, 0.50 and 0.31 % and 0.40 and 0.29 % in starter, grower and finisher respectively. As a result, the grower and finisher feed did not contain any added inorganic P. Technical performance was measured for every feeding phase. At day 21, the tibiae from 3 birds per pen were removed and analysed for bone strength. Afterwards, the 3 tibiae were pooled into 1 sample for the determination of ash after fat extraction and drying. At day 35, a mixed faecal sample was taken from 5 birds per pen for the determination of the total tract digestibility of Ca and P (TiO_2 as marker).

The birds outperformed the breed standard (close to 2.7 kg bird weight at 35 days with an FCR of 1.4). Lowering the Ca and P level in the feed did not have a negative effect on performance. On the contrary, it increased end weight by 22 g numerically (from 2675 to 2697 g, $P > 0.05$). However, the bone ash decreased slightly from 47.5 to 46.8 %, while bone strength decreased from 548 to 532 N (in both cases numerically). On the contrary, lowering the Ca and aP levels in the feed significantly increased the Ca digestibility (51.7 to 66.4 %) and P digestibility (77.3 to 83.2 %). Based on feed intake and analysed Ca and P levels in the feed, this yielded a significantly lower digestible Ca uptake (0.49 vs 0.62 g per bird per day) but increased the digestible P intake significantly from 0.53 to 0.57 g per bird per day. This trial demonstrated that lowering the Ca and P levels in the feed with 0.2 % and 0.05 % in starter, grower and finisher which contains 1000 FTU/kg phytase does not have a negative impact on performance, increases Ca and P digestibility, and has a limited impact on bone ash and bone strength. However, it indicates that there are different Ca and P requirements for technical performance and for bone development as suggested by David et al. (2023).

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EFFECT OF ENZYME SUPPLEMENATION ON BROILER DIETS FED WITH CASSAVA MEAL WITH DIFFERENT INCLUSION RATE

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Summary

A 28-day feeding trial, with 432-day old male Cobb birds, was conducted to determine the performance of broilers fed diets containing different levels of cassava meal, with and without enzyme treatment. Dietary treatments consisted of a corn-soybean meal (SBM)-rice bran (RB) diet control, and 5 experimental diets: a diet (T2) containing moderate inclusion levels of cassava meal (CM) for booster (5%), starter (10%), and finisher (20%), a diet (T3) using enzyme (200 g/t) on top of T2; a diet (T4) containing high inclusion levels of CM for booster (10%), starter (20%), and finisher (30%) and two diets using enzyme of 200 g/t (T5) and 400 g/t (T6), respectively. Performance parameters including feed intake (FI), body weight (BW), body weight gain (BWG), average daily gain (ADG), and feed conversion ratio (FCR) were measured. The effect of enzyme supplementation on the digestibility of CM was conducted *in vitro*. Inclusion of CM at both moderate and high levels tended to compromise FCR (P = 0.053); however, this was partially improved with enzyme supplementation. Higher dosage of enzyme did not generate additional improvement on growth performance for the high CM inclusion treatment. Enzyme supplementation did not influence *in vitro* digestibility, possibly due to the low fiber content of cassava that was used in the study.

I. INTRODUCTION

Cassava is abundant in certain regions of the world that could be used as an alternative energy source in poultry feed to reduce feed cost (Hossain et al., 2014). It has a high energy (around 3,200 kcal/kg as fed) due to its high starch content (70-74% as fed), despite low level of fat (less than 1%) (Natalie et al., 2016). CM was reported to compromise growth performance in corn-SBM diets (Akinfala et al., 2002), but nutrient utilization of cassava may be improved by enzyme supplementation (Midau et al., 2011 and Bhuiyan et al., 2012). Hence, this study was designed to determine the performance of broilers fed diets containing varying levels of CM and to determine the efficacy of exogenous feed enzymes on nutrient utilization.

II. MATERIALS AND METHODS

This trial was carried out in the experimental farm of Central Luzon State University (CLSU, this study was previously approved by the Ethics and Biosecurity Committee of the CLSU). A total of 288-day-old male Cobb 500 broiler chicks were randomly allotted to 6 treatments, 6 replicates (cage) per treatment, 12 birds per cage using a randomized complete block design. Broilers were raised in an open-sided broiler house equipped with cooling fans.

Dietary treatments consisted of a corn-SBM diet control, and five experimental diets (Table 1). The multi-enzyme preparation (MEP) used in this study was Rovabio Advance (Adisseo SAS, France), which contains mainly xylanase (1,250 vu/g), beta-glucanase (850 vu/g) and arabinofuranosidases. The dietary treatments were formulated to be isocaloric (2,950 kcal/kg for booster, 3,025 kcal/kg for starter and 3,100 kcal/kg for finisher) and isonitrogenous

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(21% crude protein for booster, 20% for starter and 19% for finisher) to meet Cobb 500 Nutrient Specifications. Birds were fed with the booster crumble, starter pellet, and finisher pellet diets from d 0 to 10, 11 to 24, and d 25 to 28, respectively.

Table 1 - Dietary treatment design.

Treatment[1]	Chick Booster (Day 1-10)	Broiler Starter (Day 11-24)	Broiler Finisher (Day 25-28)
1		Corn-SBM-RB	
2	Corn-SBM-RB-cassava (5%)	Corn-SBM-RB-cassava (10%)	Corn-SBM-RB-cassava (20%)
3		T2 + MEP (200 g/ton)	
4	Corn-SBM-RB-cassava (10%)	Corn-SBM-RB-cassava (20%)	Corn-SBM-RB-cassava (30%)
5		T4 + MEP (200 g/ton)	
6		T4 + MEP (400 g/ton)	

[1]All treatment diets contained phytase of 1000 FTU/kg feed.

Birds, experimental diets, and feed leftovers were weighed at the end of each feeding period to calculate for periodic and cumulative FI, BW, BWG, ADG and FCR.

In vitro dry matter digestibility (DMD) of CM was carried out at the Research and Innovation Center in China Adisseo (RICA). The MEP used in this trial was same as the broiler feeding trial. A simulated digestive system called SDS III (Zhongben Intelligent Technology Development Co. Ltd., China) was used in this trial. The *in vitro* digestion procedures followed the previous report (Liu et al., 2021). In details, 3-step *in vitro* digestion assay (stomach phase, small intestine phase I and small intestine phase II) was used in this study. In the stomach phase, the simulated gastric fluid was made of 141 U/mL pepsin to match the in vivo activity of pepsin in gastric fluid of broilers. The pH was at 2 and this procedure lasted 4 hours. In the small intestine phase I, the concentrated simulated small intestinal fluid used for *in vitro* intestinal digestion was prepared with 401 U/mL of amylase, 49U/mL of trypsin and 11 U/mL of chymotrypsin. The pH was at 6.5 and lasted 7.5 hours. Last phase was the small intestine phase II where the pH is at 7.99 for 7.5 h. Temperature was maintained at 41C during the process. After simulated digestion, undigested residues were transferred to a pre weighed vessel and dried overnight at 65°C, after which they were dried at 105°C for 5 h to constant weight for subsequent analysis.

III. RESULTS AND DISCUSSION

Growth performance results for booster (0-10 day), starter (11-24 day) and finisher (25-28 day) periods are presented in Table 2. The body weight and average daily gain of broilers did not differ significantly among diets in any of the growing periods. There was a significant difference ($P = 0.004$) in feed intake during the booster stage. The lowest FI came from T3 and T6, both of which had enzyme treatment, indicating the potential effect to boost the feed efficiency in both groups from MEP. The addition of enzyme improved the digestibility and absorption of nutrients in the immature birds so that even at a lower feed intake the birds were able to convert the nutrients to body weight as the FCR were statistically the same among all the groups. No significant difference in feed intake and FCR was observed in the starter phase. In the finisher phase, there was a significant difference in feed intake ($P = 0.002$) wherein control group had a lower feed intake among all the groups.

The above results are consistent with previous report (Chang'a et al, 2020) where feed intake was increased when birds fed with cassava and feed enzyme supplementation, but with no difference of FCR. Cassava starch is regarded to be more digestible than maize starch due to lower amylose content in cassava root (17% to 19%) compared to maize (20% to 30%).

Therefore, diets containing cassava could have less retention time in the gut to result in the increase in the feed intake of the birds.

Table 2 - Dietary effects in broiler performance parameter in each feeding phase[1].

Treat-ment	Booster 0-10 day				Starter 11-24 day				Finisher 25-28 day			
	BW, g	ADG, g/d	FI, g/d	FCR	BW, g	ADG, g/d	FI, g/d	FCR	BW, g	ADG, g/d	FI, g/d	FCR
T1	343.88	30.08	27.61[a]	1.15	1374.35	73.60	100.51	1.37	1648.05	68.43	116.62[a]	1.71
T2	333.98	29.14	27.32[a]	1.18	1362.47	73.33	101.07	1.38	1648.09	66.51	120.11[bc]	1.80
T3	331.28	28.87	25.24[b]	1.10	1374.39	73.94	102.93	1.38	1628.25	71.49	117.11[c]	1.70
T4	353.32	31.01	27.71[a]	1.11	1359.05	71.84	102.84	1.43	1642.62	70.89	123.83[b]	1.75
T5	361.67	31.86	28.61[a]	1.14	1373.62	72.28	102.27	1.42	1670.05	74.11	128.85[b]	1.74
T6	347.23	30.39	25.34[b]	1.05	1343.27	71.14	99.63	1.40	1627.75	71.12	127.37[b]	1.80
SEM	4.083	0.405	0.360	0.043	7.362	0.453	0.747	0.011	8.515	0.976	1.362	0.019
P-val.	0.457	0.437	0.004	0.076	0.417	0.284	0.405	0.119	0.384	0.249	0.002	0.384

[1]The live weight of Day 1 chicks was 43 g/b without statistical difference among treatments.

As shown in Table 3, throughout the whole feeding phase (0-28 day) there were no significant difference among all the treatment groups for BW, ADG, and FI. T2 and T4 with cassava at both moderate and high inclusion level had the significantly poorer performance when compared to the control diet (P=0.053).

The observation in current trial is in line with the previous investigation (Chang'a et al., 2020, that growth performance was negatively affected when above 50% of corn was replaced by cassava in the diet. However, the impact of high cassava content in the feed can be restored by using feed enzymes containing α-amylase, β-glucanase, and other non-starch polysaccharides enzymes (NSPases) targeting on various antinutritional factors (ANFs) from other plant-based feed ingredients. Therefore, it is believed that the MEP used in this study could deliver the similar function to improve the digestion of nutrients deactivating ANFs in cassava and other cereal ingredient. Although either single or double dose of enzyme could only partially reverse the negative impact from both moderate and extreme cassava inclusion in the diet, with numerical improvement of FCR but not at the significant level. Our findings in current trial are also supported by a similar trial wherein cassava could be used to replace corn in broiler diets up to 50% without affecting production performance with enzyme supplementation (Bhuiyan et al., 2012).

Table 3 - Dietary effects in broiler performance parameter throughout the whole phase, 0-28d[1].

Treatments	BW, g	ADG, g/d	FI, g/d	FCR
T1	1,648.06	57.32	76.77	1.34[a]
T2	1628.55	56.64	77.43	1.37[ab]
T3	1660.34	57.71	77.21	1.34[a]
T4	1,642.61	57.12	79.00	1.38[b]
T5	1,670.05	58.10	79.76	1.37[ab]
T6	1,627.75	56.59	77.06	1.36[ab]
SEM	8.515	0.302	0.509	0.006
P-value	0.384	0.379	0.091	0.053

[1]The live weight of Day 1 chicks was 43 g/b without statistical difference among treatments.

DMD is the foundation of nutrient availability of Feed RMs, hence the animal performance. As shown in Figure 1, MEP had no impact on the *in vitro* DMD of cassava used in this study, consistent with our observations in the broiler feed trials that MEP had limited impact on overall animal performance, especially when the cassava inclusion rate is high. However, we recorded a significant DMD improvement ($P < 0.01$) by MEP treatment in the RB samples tested by the *in vitro* digestion procedures. Alternative ingredients enriched in

arabinoxylan such as wheat bran and rice bran are the favorable substances for MEP (Cozannet et al., 2017), resulting in the significant DM improvement. The *in vitro* DMD results can partially explain in T3 when CM inclusion at the moderate level, MEP had the trend to reverse its negative impact, probably through the enhanced digestibility of other plant based RMs, such as rice bran, in the diets, although we only observed the numerical changes in FCR between T2 and T3. This hypothesis is also supported by the previous report that an improved digestibility can be achieved by NSPases treatment when formulating the broiler diet containing cassava peel meal with around 18% crude fiber (Midau et al, 2011).

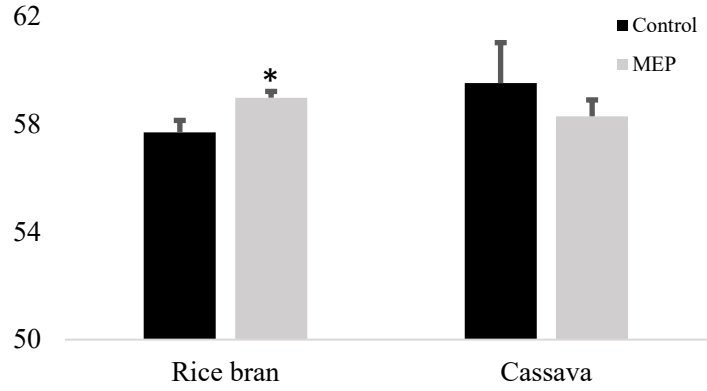

Figure 1 - In vitro dry matter digestibility of cassava. "*" indicates significant differences.

This study concluded that the CM inclusion in the broiler feed had negative impacts on the birds' performance, especially when the inclusion rate is high. This deficiency brought by CM could be marginally improved by enzyme supplementation, especially when moderate CM inclusion is diluted by other plan based RMs in the diet (T3 vs. T1). However, increasing the dosage of enzyme did not render any further improvement in the performance when CM inclusion at the high level. Enzyme supplementation *in vitro* did not have any significant effect on digestibility of CM due to low fiber content of cassava but deliver significant improvement on fibrous RM like rice bran. The result of current study can be used as a reference to facilitate CM in broiler diet for cost-saving purposes. It is recommended to formulate the broiler diet with moderate CM levels, sufficient crude fibers, and appropriate feed enzymes without affecting broiler performance.

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NET ENERGY FOR FEED ADDITIVES WITH EMPHASIS ON ENZYMES IN POULTRY

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Summary

The study aims to explore the effects of enzymes (enz) on metabolisable energy (ME) and net energy (NE) values in broiler diets with different raw material and nutrient content. Data were collected from digestibility trials and respiratory chambers encompassing various diets with and without Enz (Rovabio Advance; Adisseo France). Experiments were carried out on a broad range of diets differing in raw material and nutrient content. Data analysis showed that enz supplementation significantly increased ME/GE ratio by 2.7% and NE/ME ratio by 2.0%, leading to increased the dietary energy utilization in broiler chickens. As a consequence, ME and NE content of the diets increased by 93 and 117 kcal/kg DM, respectively. Enzymes break down anti-nutritional factors and enhance overall feed and nutrient digestibility in broilers. Enzyme supplementation reduces heat production and also improves nitrogen retention and subsequently increases NE/ME ratio. Further research is warranted to establish relationship between enz and substrate arabinoxylan (AX).

I. INTRODUCTION

The cost of feed represents an important part of the total cost in swine and poultry production (>60%) and, in the feed, energy is the most expensive component accounting for 70% of feed cost (Noblet and van Milgen, 2004). Exogenous enz can improve energy and protein utilisation, nutrient digestibility and growth rates (Cozannet et al., 2017). The main mechanism behind this is that these exogenous enz improve nutrient availability by reducing intestinal digesta viscosity, through the degradation of non-starch polysaccharides (not for all enzymes). For instance, they can hydrolyse arabinoxylan polymers, thereby increasing the ability of endogenous enz to access to nutrients present in digestive tract (Tahir et al., 2008). In addition, numerous work have been performed to determine the health effect of exogenous enz in broilers in relation to potential probiotic effect (Yacoubi et al., 2018) which is associated with improvement of animal performance (Raza et al., 2019). These results might be associated with a change in animal metabolic savings allowing more energy for production. Therefore, change might only be observable on animal heat increment (HI) and efficiency beyond digestibility. The main purpose of this review is to describe enzyme effect in NE and ME systems. the current paper present the key factors affecting exogenous enz efficiency and effect of exogenous enz on ME/GE and NE/ME ratios in different diets which were decomposed as digestion effect and health effect of exogenous enz.

II. METHODS

Data were gathered from digestibility and respiratory chambers trials performed for exogenous enz evaluation (Rovabio Advance). Data were selected trials performed simultaneously ME and NE content. Trials were published giving overview of experimental methods used for ME (Cozannet et al., 2017) or for NE (Musigwa et al., 2021). Methods were described in publications. As a result, the database included 17 diets evaluated for ME and NE content in-

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vivo. Diets could be associated in pairs with and without exogenous enz. Measurement were carried out on broilers from 20 to 28 days.

Table 1 - Composition and nutrient characteristics of experimental diets (as-fed basis).

	Average	Min	Max
Composition, %			
Wheat	23.9	0.0	67.9
Barley	8.2	0.0	63.5
Rye	0.7	0.0	14.2
Maize	19.4	0.0	53.5
Sorghum	0.5	0.0	14.0
Wheat bran	3.6	0.0	20.0
Oat bran	0.5	0.0	16.6
Maize distiller	1.3	0.0	33.2
Extruded soybeans	0.9	0.0	12.0
Soybean meal	28.7	7.4	45.0
Sunflower meal	0.4	0.0	7.6
Rapeseed meal	0.5	0.0	7.6
Animal meal	2.2	0.0	12.6
Vegetable oil	3.7	0.0	11.4
Animal oil	1.3	0.0	13.5
Amino acids, minerals and Premix	4.1	1.9	9.2
Nutrients analysis, %			
Starch	32.9	21.8	50.1
Crude protein	21.9	17.2	31.0
Crude fat	7.4	3.1	15.8
NDF	13.4	10.2	18.1
Ash	5.5	2.7	8.0
Residue[1]	19.6	8.6	27.1
Arabinoxylan			
Soluble	1.1	0.6	1.7
Insoluble	4.9	3.1	7.1

Diet compositions were described Table 1. A large number of raw materials were used to fit with different situations with a wide range of nutrient contents to establish the most robust equation. Nutrient content remained adequate for broiler acceptability and avoided abnormal animal metabolism.

Exogenous enz were produced by fermentation of Talaromyces versatilis (Rovabio Advance, Adisseo France S.A.S., Antony, France) and applied in liquid form. The enz producing strain was genetically modified via self-cloning to enrich the product in Xyn (+14%) and Abf (+65%), with the aim of enhancing its efficacy in breaking down highly substituted arabinoxylan (AX; Lafond et al., 2011; De la Mare et al., 2013). The enz was sprayed over the pelleted feed to provide a minimum of 1,250 visco-units of endo-β-1,4-xylanase, 9,250 visco-units of α-L-arabinofuranosidase, and 860 visco-units of endo-1,3(4)-β-glucanase. Exogenous enz content were considered adequate for experiment when activities recovery between 80 and 120% were achieved.

Extracted data were analysed using the MIXED procedure of SAS (SAS, 2008) considering the experiment as a random effect (St-Pierre, 2001). Dataset included experiment (n=8), diets (n=34) and enzyme level (n=2). The effects of all independent variables on ME, ME standardized for zero nitrogen balance (MEn) and ratio were evaluated using a mixed model. Qualitative enzyme effects on different energy systems were evaluated using diets and enzyme inclusion as discrete variables.

III. RESULTS

Diet compositions is described in Table 1. A large number of feed ingredients were used to ensure representativity of current practices. Inclusions rates of ingredients varied in large extent. Finally analyzed nutrient contents such as starch, crude protein, crude fat and NDF was changed to measure individual effect of each parameter on NE or ME value of the feed. Enzyme effects were analysed using variance analysis (Table 2). Metabolisable energy content represented 74% of GE in average. Consequently, 26% of energy losses occurred in feces and uric acids. Corresponding values with enzymes supplementation were 76% of GE metabolised and 24% of energy lost as feces and uric acids. Net energy content represented 71% of ME with 29% of ME lost as heat. Values varied for control diets from 61 to 79% and from 64 to 78% for ME/GE and NE/ME ratio, respectively. Average (Min – Max) energy content of the diets was 3439 (2971 – 3935) and 2497 (2087 – 2964) kcal/kg for ME and NE, respectively. Enzyme significantly affect all parameter (ME/GE, MEn/GE, NE/ME, ME, MEn and NE; $P < 0.01$). Ratio ME/GE and NE/ME were improved ($P < 0.01$) by 2.7 and 2.0 %, respectively. As a consequence, ME and NE content were significantly increased by 93 and 117 kcal/kg DM by enzyme supplementation. Improvement was 2.7 and 4.6% with enzyme supplementation relative to control. Broilers offered diets with supplemental enzymes had lower HI than those offered the unsupplemented diets ($P < 0.01$).

Table 2 - Effect of enzyme supplementation on energy utilisation in broilers.

	Enzyme		Improvement, %	Statistic[1]		
	without	with		Trial	Enzyme	R^2
Energy ratio, %						
ME/GE	74.0	76.0	2.7	< 0.0001	0.003	0.72
MEn/GE	70.1	71.9	2.6	< 0.0001	0.006	0.72
NE/ME	70.7	72.1	2.0	< 0.0001	0.001	0.91
Energy content, kcal/kg DM						
ME	3452	3545	2.7	< 0.0001	0.014	0.67
MEn	3275	3361	2.6	< 0.0001	0.015	0.70
NE	2592	2709	4.5	< 0.0001	< 0.0001	0.90

[1]Variance analysis using proc Mixed of SAS with Trial as random effect and diet (n=17) and enzyme (n=2) as fixed effect.

IV. DISCUSSION

Net energy is a rather old concept that has been used in domestic animals, rodents and humans (Armsby and Fries, 1915). It is mostly based on the development of calorimetry methods, either direct or indirect techniques (McLean and Tobin, 1987). Net energy is defined as the ME content minus the HI associated with feed ingestion, digestion, and metabolic utilisation of energy. Measuring heat production through indirect calorimetry and calculating NE provides a more accurate measure of energy partitioning, because it allows for the separation of energy used for production and energy lost as heat (Council, 1981). Therefore, NE measurement is considered as complex and laborious.

Effects of exogenous enz supplementation, especially exogenous enz, on the ME values of feed ingredients and diets been well reported previously (Cozannet et al., 2017). Degradation of anti-nutritional factor resulted in increased starch, fat, protein, fat and NDF digestibility, and subsequently ME improvement. It has been shows that exogenous enz remove the nutrient encapsulating effect of Non Starch Polysaccharides in broiler diets (Van Campenhout, 2007), thereby improving nutrient access for endogenous enzymes and enhancing overall feed digestibility (Meng et al., 2005). The enhancement of nutrients availability observed in previous studies was related to the increased accessibility of the nutrients to the endogenous proteolytic, amylolytic, and lipolytic enzymes. It might therefore affect the use of added

enzymes in feed formulation and the way to reformulate diet with such additives (Cozannet et al., 2017). These effects were associated with substrate content and especially AX content.

Enzyme supplementation increased NE to ME ratio by decreasing maintenance energy cost (Cowieson et al., 2019). These authors reported reduction of 9 kcal HP/bird/day and 6 kcal HI/kg $BW^{0.70}$. These results further confirm that enzyme supplementation can improve energy utilisation by reducing HP and HI. In studies evaluating fiber effect without exogenous enz inclusion, Jimenez-Moreno et al. (2019) found that moderate amounts of insoluble fiber, especially oat hulls, increased gizzard weight, reduced gizzard pH in young broilers with subsequent higher maintenance cost. Exogenous enz improvements are not likely attributable to improvements in nutrient digestibility per se, but rather not to digestive physiology improvement. Fiber had been evaluated for their effect on digestive tract development and microbiota composition (Amerah et al., 2009). Rezaei et al. (2011) found that supplementation of micronized insoluble fiber, corresponding in some extent to hydrolyzed fibers with exogenous enz, resulted in dose-dependent increases in daily weight gain and feed conversion ratio, as well as improvements in intestinal morphology and litter moisture. Similarly, Yacoubi et al. (2018) found beneficial effect of enzyme hydrolysis end products on animal performance. Authors concluded in prebiotic properties of products obtained by fiber hydrolysis with exogenous enz.

In conclusion, the benefits of exogenous enz extend beyond direct effect on gross energy digestibility, but also their effect on reducing HP and HI and improving N retention in broiler chickens. Further research is needed to quantify the net effects of enz and predict effect in connection with substrate.

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EFFECT OF ZINC SOURCE AND ITS INTERACTION WITH GRADED LEVELS OF PHYTASE ON BROILER PERFORMANCE AND CARCASS TRAITS

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Summary

The effect of zinc (Zn) source and Zn level were evaluated against varying doses of phytase on broiler performance and carcass traits to 42 days of age. A total of 1680 male day-old Cobb 500 broilers (49 g at placement) were allotted to one of 84 pens and split into 21 treatments in nested factorial design with 3 basal diets devoid of any Zn supplementation fed either 0, 500 or 1500 FTU/kg of phytase. The remainder of treatments comprised 3 Zn levels (50, 100 and 150 ppm), 3 phytase doses (0, 500 and 1500 FTU/kg), and 2 Zn sources (Zn-sulphate, 35% Zn; and Zn-glycinate, 26% Zn), which were used as nesting factor for the statistical analysis. Based on performance and carcass traits, the requirement for Zn-sulphate in this evaluation was between 50-100 ppm when fed at least 500 FTU/kg of phytase. Based on the same criteria (performance and carcass traits), the requirement for Zn-glycinate fell closer to 50 ppm when fed a minimum of 500 FTU/kg of phytase. Overall, phytase effect was not influenced by Zn source, and the magnitude of response of broilers to phytase was superior to that to Zn supplementation, with optimal performance and carcass traits observed at 1500 FTU/kg of phytase when supplemented to the diets ($P < 0.05$). Interestingly, diets containing 1500 FTU/kg of phytase without supplemental Zn showed better performance when compared to a standard dose of phytase (500 FTU/kg) and 150 ppm regardless of Zn source.

I. INTRODUCTION

Zinc (Zn) is an essential trace element with several roles in animal metabolism involving numerous metalloenzyme systems (Gaither and Eide, 2001). Most recently, attention has been brought to Zn utilization in Europe, after its ban at high dose levels. The concern was around higher Zn accumulations in soil and water bodies. Phytate present in the vegetable ingredients can be complex with divalent cations (including Zn) and render these unavailable to monogastric animals. Xu et al. (1992) showed that lower esters of phytate (IP4 and IP3, specifically), can still reduce Zn availability. In this context, the objective of this study was to evaluate the interactions of Zn level (ppm) and form (inorganic sulphate or organic glycinate) when fed with varying doses of phytase (FTU/kg), especially at higher phytase doses which degrade IP6 beyond the lower esters, on broiler performance and carcass traits to 42 days of age.

II. MATERIALS AND METHODS

The experimental protocol was approved by the Federal University of Paraiba Animal Ethics Committee. A total of 1680 male day-old Cobb 500 broilers (49 g at placement) were allotted to one of 84 pens and split into 21 treatments comprising of 3 Zn levels (50, 100 and 150 ppm), 2 Zn sources (Zn-sulphate, 35% Zn; and Zn-glycinate, 26% Zn), and 3 phytase doses (0, 500 and 1500 FTU/kg, Quantum Blue, AB Vista, Marlborough, UK). This study was a nested factorial design with 3 extra treatments devoid of any Zn supplementation fed either 0, 500 or 1500 FTU/kg of phytase. All other trace minerals were supplemented as recommended by the

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breed guidelines. All diets were offered as mash, corn and soybean-meal based, and fed *ad libitum* in three phases, from 1-10 d (starter), 11-25 d (grower) and 26-42 d (finisher). Basal diets were formulated taking into consideration the available phosphorus (avP) and calcium (Ca) contribution of 500 FTU/kg of phytase (0.15 and 0.165, respectively) and avP levels were formulated 10-15% higher than breed guidelines to avoid a severe P deficiency in treatments devoid of phytase. Body weight (BW) and feed intake were measured and feed conversion ratio corrected for mortality (mFCR) was calculated. At 42 d of age 5 birds per pen were sacrificed for determination of carcass weight and breast weight, with carcass yield being expressed as percentage of live weight (LW) and breast yield expressed as percentage of carcass weight (CW). Data was analyzed as a nested factorial design (JMP Pro 16.2) with Zn source used as the nesting factor {((Phytase, FTU/kg [0, 500, 1500] x Zn, ppm [50, 100, 150]) x Zn Source [Zn-sulphate, Zn-glycinate]) + No Zn added (0, 500 and 1500 FTU/kg phytase)}. Two-way nested ANOVA was first performed, and whenever an interaction was significant ($P \leq 0.05$), means were then separated using Student's t-test. Additionally, orthogonal polynomial contrasts (linear and logarithmical for both phytase and Zn, and quadratic for Zn levels) analysis was performed to determine the response behavior for phytase and Zn supplementation.

III. RESULTS AND DISCUSSION

No interaction for BW was observed at either 25 or 42 d of age ($P > 0.10$, Table 1). Phytase improved the BW of birds in a linear and logarithmical fashion, irrespective of Zn source ($P < 0.01$, Table 1). Although ANOVA showed significant differences for Zn levels at both 25 and 42 d, the source of Zn did not. For Zn-sulfate, BW was improved to 25 d in both a quadratic ($P < 0.05$) or linear ($P < 0.10$) fashion, while Zn-glycinate improved the BW of broilers at 25 and 42 d in a linear and logarithmical fashion ($P < 0.01$, Table 1). Zn-glycinate improved the BW of birds compared to that of Zn-sulphate fed birds and birds not supplemented with Zn at either age ($P < 0.01$).

Table 1 - Body weight (g/bird) of broiler chickens fed diets with graded levels of zinc sulphate or zinc glycinate (0 to 150 ppm) and graded levels of phytase (0, 500 or 1500 FTU/kg) from 0-25 or 0-42 days of age.

Zn Source	Zn, ppm	Phytase, FTU/kg			Phytase, FTU/kg		
		0	500	1500	0	500	1500
		25 days			42 days		
None	0	1247	1294	1378	2955	3004	3082
	50	1254	1301	1338	2890	2991	3139
Sulphate	100	1249	1305	1360	2881	3026	3171
	150	1273	1310	1361	2893	3028	3144
	50	1266	1311	1383	2945	3001	3164
Glycinate	100	1265	1321	1388	2930	3005	3148
	150	1273	1326	1390	2954	3026	3168
SEM			7.0			10.9	
		P-Values					
Phytase FTU/kg [Zn Source]		< 0.01 (N: L*, LN*; S: L*, LN*; G: L*, LN*)			< 0.01 (N: L*, LN*; S: L*, LN*; G: L*, LN*)		
Zn ppm [Zn Source]		0.01 (S: L‡, Q†; G: L*, LN*)			0.02 (G: L*, LN*)		
Phytase FTU/kg * Zn PPM [Zn Source]		0.56			0.32		
Zn Source		< 0.01 (G > S = N)			< 0.01 (G > S = N)		

Orthogonal Polynomial Contrasts for every zinc source on the trial: None: N; Sulphate: S; Glycinate: G; Linear (L), logarithmic (LN) and quadratic (Q) significance of Orthogonal Polynomial Contrasts .* $P \leq 0.01$; † $P \leq 0.05$; ‡ $P \leq 0.10$.

From 0-25 d of age, an interaction based on Zn source for mFCR was observed ($P < 0.05$, Table 2). In essence, the mFCR was improved when diets were supplemented with Zn-sulphate in the absence of phytase only, while Zn-glycinate improved mFCR independently of phytase supplementation. Phytase, from 0-25 d, improved mFCR irrespective of Zn source ($P < 0.01$).

From 0-42 d, no significant interaction was observed for phytase ($P > 0.10$, Table 2). A significant main effect was observed on mFCR due to phytase dose ($P < 0.01$, Table 2). No effect was observed for Zn level on mFCR from 0-42 d($P>0.10$), however, when comparing Zn sources Zn-glycinate improved the mFCR beyond that of the Zn-sulfate fed birds, while Zn-sulfate fed birds had better mFCR than those birds fed diets devoid of supplemented Zn at either age ($P < 0.01$, Table 2).

Table 2 - Mortality corrected feed conversion ratio (g:g) of broiler chickens fed diets with graded levels of zinc sulphate or zinc glycinate (0 to 150 ppm) and graded levels of phytase (0, 500 or 1500 FTU/kg) from 0-25 or 0-42 days of age.

Zn Source	Zn, ppm	Phytase, FTU/kg			Phytase, FTU/kg		
		0	500	1500	0	500	1500
		0-25 days			0-42 days		
None	0	1.49[a]	1.37[cd]	1.24[f]	1.71	1.66	1.58
	50	1.45[b]	1.39[c]	1.25[f]	1.74	1.64	1.50
Sulphate	100	1.44[b]	1.34[d]	1.24[f]	1.72	1.63	1.50
	150	1.38[c]	1.37[cd]	1.24[f]	1.72	1.63	1.54
	50	1.42[b]	1.36[cd]	1.19[g]	1.72	1.60	1.50
Glycinate	100	1.44[b]	1.35[d]	1.19[g]	1.71	1.63	1.50
	150	1.37[cd]	1.30[e]	1.18[g]	1.65	1.61	1.50
SEM			0.013			0.017	
		P-Values					
Phytase FTU/kg [Zn Source]		< 0.01 (N: L*, LN*; S: L*, LN*; G: L*, LN*)			< 0.01 (N: L*, LN*; S: L*, LN*; G: L*, LN*)		
Zn PPM [Zn Source]		0.01 (S: L*, LN*; G: L*, LN*)			0.34 (S: LN†, Q‡; G: L*, LN*)		
Phytase FTU/kg * Zn PPM [Zn Source]		0.02			0.56		
Zn Source		< 0.01 (G > S > N)			< 0.01 (G > S > N)		

Orthogonal Polynomial Contrasts for every zinc source on the trial: None: N; Sulphate: S; Glycinate: G; Linear (L), logarithmic (LN) and quadratic (Q) significance of Orthogonal Polynomial Contrasts .* $P \leq 0.01$; † $P \leq 0.05$; ‡ $P \leq 0.10$.

No interaction was observed for carcass yield as a g/kg of LW (CY) in birds at 42 days of age ($P > 0.10$, Table 1). Phytase improved CY of birds in a linear and logarithmical fashion, with Zn source ($P < 0.01$) significantly improving CY compared to diets devoid of supplemental Zn ($P < 0.10$, Table 3). Zn supplementation was significant for CY ($P < 0.05$) with linear and logarithmic improvements for both Zn-sulphate and Zn-glycinate ($P < 0.01$) and quadratic for Zn-glycinate alone ($P < 0.01$, Table 3). An interaction was observed for breast yield, g/kg CW, (BY, $P < 0.05$). Phytase dose significantly improved BY in diets devoid of Zn in a linear and logarithmic fashion ($P < 0.01$). In the absence of phytase, Zn appeared to have a clearer response, while 1500 FTU of phytase muted Zn response from 50 ppm onwards, irrespective of Zn source ($P < 0.05$, Table 3).

In summary, based on performance and carcass traits, the Zn-sulphate requirement for the current trial was around 50-100 ppm in diets with at least 500 FTU/kg of phytase, while for Zn-glycinate the requirement to obtain the best performance and carcass traits were usually attained with supplementation levels of around 50 ppm, when birds were fed at least 500FTU/kg of phytase. In general, phytase effect was not influenced by Zn source, and the magnitude of response of phytase was superior to that of Zn supplementation, with optimal performance and carcass traits observed when 1500 FTU/kg of phytase was supplemented to

the diets (P < 0.05). In fact, 1500 FTU/kg of phytase in diets devoid of supplemental Zn showed better performance than that of broilers fed 500 FTU/kg with either 150 ppm of Zn-sulphate or Zn-glycinate.

Table 3 - Carcass traits of 42d-old broiler chickens fed diets with graded levels of zinc sulphate or zinc glycinate (0 to 150 ppm) and graded levels of phytase (0, 500 or 1500 FTU/kg).

Zn Source	Zn, ppm	Phytase, FTU/kg			Phytase, FTU/kg		
		0	500	1500	0	500	1500
		Carcass Yield, g/kg LW			Breast Yield, g/kg CW		
None	0	750	757	765	349^{f}	355^{ef}	358^{def}
Sulphate	50	747	765	777	350^{f}	370^{ab}	372^{ab}
	100	757	766	781	358^{cdef}	367^{bcd}	370^{abc}
	150	762	771	795	366^{bcde}	374^{ab}	381^{a}
Glycinate	50	771	758	783	379^{a}	357^{def}	373^{ab}
	100	771	770	791	373^{ab}	372^{ab}	372^{ab}
	150	759	779	785	372^{ab}	379^{a}	373^{ab}
SEM			5.3			4.2	
		P-Values					
Phytase FTU/kg [Zn Source]		< 0.01 (N: L‡, LN‡; S: L*, LN*; G: L*, LN*)			< 0.01 (S: L*, LN*)		
Zn PPM [Zn Source]		0.02 (S: L*, LN*; G: L*, LN*, Q*)			0.02 (S: L*, LN*; G: L*, LN*, Q*)		
Phytase FTU/kg * Zn PPM [Zn Source]		0.19			0.04		
Zn Source		< 0.01 (G > S > N)			< 0.01 (G > S > N)		

Orthogonal Polynomial Contrasts for every zinc source on the trial: None: N; Sulphate: S; Glycinate: G; Linear (L), logarithmic (LN) and quadratic (Q) significance of Orthogonal Polynomial Contrasts .* P ≤ 0.01; † P ≤ 0.05; ‡ P ≤ 0.10.

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DETERMINATION OF EXTRA PHOSPHORIC EFFECT OF PHYTASE AT DIFFERENT DOSAGE IN DIETS OF BROILERS

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Extra-phosphoric effects of phytase deliver economic benefits in diet formulation and enhances phytase value. This study was to determine the extra-phosphoric effectiveness of Kemin's thermostable phytase (Phygest^{TM} HT) in increasing the performance, carcass traits and tibia characteristics of low-phosphorous (P)-diet-fed broilers. A total of 336 Ross308 chicks were divided into (1) Positive control with standard diet (PC), (2) 0.10% less available P from PC (NC1), (3) 0.15% less available P from PC (NC2), (4) 0.20% less available P from PC (NC3), (5) NC3 + 500 FTU/kg phytase (NCP1), (6) NC3 + 1000 FTU/kg phytase (NCP2), (7) NC3 + 1500 FTU/kg phytase (NCP3) groups. Data was analyzed using one-way ANOVA in SPSS. As shown in table 1, compared to PC group, all NC group birds had lower average body weight (BW) ($P<0.05$) from day 14 to 28 and this was overcome with phytase supplementation at ≥1000 FTU/kg (NCP2 & NCP3; $P<0.05$). Lower average daily gain and higher feed conversion ratio ($P<0.05$) was observed only in NC3 group, and this was undone with phytase supplementation at all concentrations ($P<0.05$). At day 21 and 35, carcass traits such as breast meat, and drumstick weights, tibial calcium and phosphorus, and AID of energy were lower in NC3 group ($P<0.05$) in comparison to PC group and these effects were reversed in NCP2 and NCP3 groups ($P<0.05$). Interestingly, at day 35, NCP2 group exhibited higher AID of energy ($P<0.05$) relative to PC group. Associated with this IP_6 degradation in duodenum/jejunum of NCP2 and NCP3 group was higher as manifested by lower IP_6 ($P<0.05$) and higher IP_3 ($P<0.05$) availability. In conclusion, for most parameters analyzed, phytase inclusion at a minimum of 1,000 FTU/kg was able to improve bird performance in a low-P diet.

Table 1 - Effect of phytase inclusion in diets on growth performance and digestibility in broilers.

Items	Dietary Treatment							P-value
	PC	NC1	NC2	NC3	NCP1	NCP2	NCP3	
Body weight (g)								
Day 14	323.56^{c}	270.75^{ab}	263.01^{ab}	245.72^{a}	290.68^{abc}	301.69^{bc}	304.35^{bc}	0.001
Day 21	745.74^{d}	620.50^{abc}	565.17^{ab}	537.61^{a}	670.74^{bcd}	716.24^{cd}	710.18^{cd}	0.001
Day 28	1336.75^{d}	1148.91^{abc}	1087.05^{ab}	1046.89^{a}	1232.90^{abcd}	1326.34^{cd}	1302.83^{cd}	0.001
Average daily gain (g/d)								
Day 1-35	55.67^{bcd}	51.00^{abc}	49.45^{ab}	46.73^{a}	55.27^{bcd}	57.73^{d}	56.76^{cd}	0.001
Feed Conversion ratio (g/g)								
Day 1-35	1.32^{a}	1.54^{ab}	1.54^{ab}	1.68^{b}	1.38^{a}	1.37^{a}	1.37^{a}	0.003
Nutrient Digestibility Day 21								
Energy	79.32^{d}	75.22^{bc}	72.70^{ab}	70.03^{a}	76.03^{c}	77.06^{cd}	77.59^{cd}	< 0.001
IP_6	0.79^{c}	0.70bc	0.73bc	0.69bc	0.50ab	0.36a	0.40a	< 0.001
IP_3	0.03^{ab}	0.01a	0.02ab	0.02ab	0.05bc	0.08cd	0.14e	< 0.001
Nutrient Digestibility Day 35								
Energy	82.82^{d}	79.07^{b}	80.76^{c}	77.43^{a}	81.42^{cd}	84.51^{e}	82.79^{d}	< 0.001
IP_6	0.69^{b}	0.64b	0.60b	0.66b	0.36a	0.36a	0.35a	< 0.001
IP_3	0.01^{a}	0.01a	0.02a	0.01a	0.21b	0.18b	0.17b	< 0.001

Values are mean of 6-7 replicates/treatment. Values with different superscripts in a row differ significantly ($P<0.05$)

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IMPROVEMENTS IN NUTRIENT DIGESTIBILITY AND GROWTH PERFORMANCE FOLLOWING THE DIETARY INCLUSION OF A NATURAL EMULSIFIER IN BROILERS FED A CORN-WHEAT-SOY-BASED DIET

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Summary

This study aimed to investigate the effects of a natural emulsifier on the performance and nutrient digestibility of broiler chickens. The emulsifier was added to the diet at dose rates of 0, 250, 500, and 750 ppm. Average daily gain and feed conversion ratio, during all dietary phases, improved linearly ($P<0.05$) with the level of emulsifier in the diet. Average daily feed intake was not affected ($P>0.05$). Apparent digestibility of crude protein, and crude fat during the grower phase (day 21 to 28) improved linearly ($P<.05$). The study revealed that supplementation of natural exogenous emulsifiers in diets containing moderate quantities of added fats may substantially improve broiler performance and nutrient digestibility.

I. INTRODUCTION

Fats and oils contain at least twice the available energy of carbohydrates and protein and are widely used to increase the energetic density of poultry diets (Tancharoenrat and Ravindran, 2014). Besides supplying energy, the addition of lipids also improves the absorption of fat-soluble vitamins and increases the palatability of the diets (Baião and Lara, 2005). However, the quantity of fat included in a diet should be restricted to 5% and a higher fat content could negatively impact both the feed manufacturing process and feed quality (Cheah et al, 2017). Several studies have indicated that the digestion and absorption capacity of lipids in young birds is poor. Young animals have a physiological limitation to absorb fat because of a low level of natural lipase production and bile salt synthesis (Ravindran et al.,2016; Tancharoenrat et al., 2014). Therefore, this forced the nutritionist to focus on better utilization of the energy in the diet for younger birds. The amount of energy that an animal can obtain from dietary lipids mainly depends on its digestibility. The higher fat digestion and absorption results in more available energy from the diet. In other words, this higher digestibility means that from the same amount of crude fat and gross energy, birds can obtain more energy from the diet. The digestion and absorption of fat is a highly complex process that requires a functioning liver, pancreas, and small intestine, as well as numerous digestive enzymes. Ingested lipids undergo intestinal emulsification, micellar solubilization, and incorporation into lipoproteins before release into the interstitial fluid (Krogdahl, 1985). Emulsification is one important step among several stages in fat digestion. This process is naturally mediated by endogenous emulsifiers, such as bile salts. It has been reported that endogenous emulsifiers alone do not support high-fat digestion, therefore, exogenous emulsifiers are used in poultry nutrition to improve poultry performance. We hypothesized that the supplementation of emulsifiers in broiler diets may contribute to the efficient utilization of energy and in increasing fat digestibility, thereby improving performance.

II. MATERIALS AND METHODS

This experiment received prior approval from the local ethics committee for animal experiments at Poulpharm Belgium (EC = C21384-EC).

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Experimental birds and diets – A total of 864 1-d-old Ross mixed-sex broilers (Ross 308) were obtained from a local supplier and allocated to the 4 experimental treatments with 6 replications per treatment housed inside a commercial production facility. Starter, grower 1, 2, and 3 diets were offered to the birds from 0 to 10 d, from 11 to 21 d, 22 to 28 d, and 29 to 35 d of age, respectively. Feed and water were provided ad libitum throughout the experiment. Experimental diets were formulated using a corn-wheat soy-based diet. The formulated ME in the diets were 3030, 3120, 3200, and 3250 kcal/kg, Crude proteins (CP) were 21, 20, 18.6, 18.4 % and added fat were 2.2, 2.9, 4.1 and 4.8% for starter, grower 1,2 and 3 respectively. This study was carried out in a completely randomized design consisting of four doses 0, 250, 500, and 750 g/ton of a natural emulsifier derived from hydrolyzed soy lecithin (Maxilys® Plus) consisting of a complex mixture of phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylinositol (PI), phosphatidic acid (PA), and their lysophospholipid derivatives (LPC, LPE, LPI, LPA). Any birds showing signs of ill health, injury, or being in poor condition were discarded. The experiment was carried out in 24 pens (each 2 × 1.5 m) with 18 random male and 18 random female broilers per pen. The stocking density after subtracting the area occupied by feeders and drinkers was approximately 14 birds/m^2. The temperature was controlled with heaters under the initial condition of 35°C at the chick level, followed by a reduction of 0·5°C each day until 22°C was reached at the 4th week of age. The chicks were exposed to the following lighting schedule: day 1 to day 4, 23·30 h; day 4 to day 35, 18 h.. All diets were presented to the birds as 3-mm pellets. For each feeding period, diets were calculated to be iso-nutritive to meet or exceed the nutrient requirements recommended by the NRC (1994) for broilers.

Growth Performance and Nutrient Digestibility – Average daily gain (ADG), average daily feed intake (ADFI) , and feed conversion ratio (FCR) (pen basis) were determined for each dietary phase (starter, grower 1,2, and 3). Nutrient digestibility (dry matter (DM), crude protein (CP), and ether extract (EE)) was calculated. For this purpose, Celite was used as an inert marker and mixed at the rate of 1 % of feed in the feed from day 21 to day 28 (Grower 2) of the trial. For the collection of excreta, a polythene sheet was spread in each pen. Excreta samples were collected for two days (d 27 and 28) on the polythene sheets. After sample collection, the sample was made free from contaminants such as feathers, down, and scales. Two-day samples were mixed, and the composite sample was obtained for the determination of nutrient digestibility. Dry matter analysis of diets was performed after oven drying and for excreta after freeze-drying. Determination of the nitrogen (N), and crude fat (ether extract) contents of oven-dried feed and freeze-dried excreta was done according to the methods described by the AOAC (2000). Acid insoluble ash (marker) concentrations in the diets and excreta samples were determined using the method described by Vogtmann et al. (1975). Separation of the N content of excreta into N of urinary and N of excreta origin was done following the method of Terpstra and De Hart (1974). Crude protein was calculated from corrected N × 6.25 (AOAC, 2000). Digestibility of CP and CF were calculated using the following equations (Stefanello et al., 2019): Digestibility = (1 - (% marker in feed ÷ % marker in excreta) × (% nutrient in excreta ÷ % nutrient in feed)).

Statistical analysis – The data were analyzed using the GLM procedure of SAS (SAS Institute, 2002) in a randomized complete block design. For growth performance (body weight, feed intake, and feed conversion ratio) the replicate was the average for all broiler chickens in each pen and the pen served as the experimental unit. Linear regression analyses were used to examine the dose response to the dietary emulsifier. Collected excreta from each pen were considered as experimental units for the nutrient digestibility parameters. Linear regression analysis was performed to assess the correlation between the dose-response of the emulsifier and the level of crude protein (CP), and crude fat (CF). Variability in the data was expressed as the standard deviation (SD) and $P < 0.05$ was considered to be statistically significant.

III. RESULTS

The effects of the four dietary treatments on ADG, ADFI, and FCR are summarized in Table 1. No cubic relationships were found to be significant for all measured parameters (P > 0.05). The supplementation of the basal diet with 250, 500, and 750 g/ton of the natural emulsifier linearly increased the average daily gain (ADG) on d 21, d 28 and as well as during the overall rearing period (d 35) (P = 0.012, P = 0.023 and P < 0.001 respectively). The emulsifier improved the FCR significantly in a linear manner over 0–4 and 0–5 weeks (P = 0.0303, and P = <0.001, respectively). Concerning the average feed consumption rate the emulsifier had no effect on ADFI. Mortality was not significantly (P > 0·05) associated with treatment but was mostly associated with the typical symptoms of coccidiosis. Quadratic effects were also observed for ADG on days 28 and 35 (P = 0.018, and P = 0.003 respectively), with the lowest value being observed at 250 ppm, while the 500 ppm of emulsifier had the highest ADG, and 750 ppm was in the middle. Regarding the FCR a quadratic effect (P = 0.032) was also observed over the 5-week period where FCR was lowest for the 500 g/ton of the emulsifier, followed by 750 g/ton, and 250 and 0 g/ton having the highest FCR. In addition, the emulsifier linearly increased (P < 0.05) crude protein (CP) and crude fat (CF) digestibility studied on day 27 to 28.

Table 1 - Effect of a natural emulsifier on performance and digestibility coefficients of uric acid corrected crude protein (CP), crude fat (CF), on DM basis of broilers.

Variable	Days	Natural emulsifier (g/ton of feed)				P[2] Li[3]
		0	250	500	750	
Performance						
ADG (g)	10 d	27.70	28.20	28.23	28.15	0.053
	21 d	47.21	47.88	48.54	48.30	0.012
	28 d	58.31	59.34	60.59	59.66	0.023
	35 d	65.01	66.42	67.32	66.91	<0.001
ADFI (g)	0-10 d	34.36	34.50	34.69	34.56	0.362
	0-21 d	59.92	60.42	60.67	60.43	0.066
	0-28 d	81.71	82.38	82.22	82.08	0.639
	0-35 d	98.83	100.10	99.27	99.13	0.345
FCR	0-10 d	1.152	1.149	1.149	1.149	0.050
	0-21 d	1.218	1.218	1.205	1.206	0.0991
	0-28 d	1.402	1.381	1.357	1.376	0.030
	0-35 d	1.520	1.508	1.475	1.482	<0.001
Digestibility coefficients						
Crude Protein %	27-28 d	75.97	77.96	78.82	80.87	0.001
Crude Fat %		88.16	89.32	90.01	92.75	< 0.001

[2] P values were obtained from the Linear Mixed Model (Polynomial); [3] Li = Linear

IV. DISCUSSION

It is well-documented that emulsifiers are capable of improving fat digestibility and subsequently sustaining or enhancing the growth performance of broiler chickens (Siyal et al 2017). Emulsifiers are molecules that have two parts. One part of the molecule is called hydrophilic, and the other part is called hydrophobic. The emulsifier molecule dissolves with its hydrophilic part in the water and its hydrophobic part in the oil (Krog, 1977). Thus, emulsifiers can keep the oil droplets in the emulsion distributed which is good for the digestion and absorption of lipids. In this study, the tested natural emulsifier significantly improves

broiler performance by enhancing feed utilization and nutrient digestion. Therefore, from the results obtained in this experiment, the use of this emulsifier based on lysophospholipids represents a potential solution to improve the feed efficiency of broiler chickens.

V. CONCLUSION

The natural emulsifier improved the digestibility of crude protein and fat which reflected in improved body weight gain and FCR. It was economically beneficial to apply the LPL-based a natural-emulsifier in broiler corn-wheat soy-based diets. Therefore, the emulsifier may be added to feed formulation to decrease the usage of costly added dietary fat or to maximize growth performance.

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THYME, ROSEMARY, AND OREGANO EXTRACTS IMPROVED THE PERFORMANCE OF BROILER CHICKENS REARED IN TROPICAL CONDITIONS

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Global warming is increasing the challenge of chicken meat production in sub-tropical climates by creating conditions typical of the tropics. It is imperative to find strategies that may help maintain broiler performance in these conditions. Thyme, rosemary, and oregano whole plants powder have been extensively reported to have significant antimicrobial and antioxidant activities and stimulate appetite and digestion (Yang et al., 2009; Brenes and Roura, 2010). However, very little is known of the effectiveness of these three extracts on the performance and meat quality of broiler chickens raised in a tropical environment. It was hypothesized that thyme, rosemary, and oregano extracts would improve the performance of broilers raised in tropical conditions.

A total of 480-day-old Indian River broiler chicks were allocated to four feeding treatments, with six replicates of 20 birds per treatment. The birds were reared in an open sided shed with average maximum and minimum temperatures of 34.71°C and 30.36°C, respectively, and average humidity of 82.53%. The control group (T_1) was fed corn-soya mash diets as starter (0-21 days; ME=2958 kcal/kg, CP=21.77%, Lys=1.35%), and grower (22-35 days; ME=3046 kcal/kg, CP=20.31%, Lys=1.26%). The remaining three treatments consisted of the T_1 diet supplemented with 5g/kg of thyme (T_2), rosemary (T_3) or oregano (T_4) extracts, supplemented as powder. Growth performance data was recorded weekly. On day 35, five birds of average weight per pen were euthanized. Breast meat quality, serum cholesterol, and hemoglobin levels were analyzed in each bird. A one-way ANOVA and Duncan Multiple Range Test (DMRT) were conducted using SAS Computer Package Program (version 9.1).

Table 1 presents that the three extracts tested significantly improved ($P < 0.05$) growth performance parameters, breast meat water holding capacity, cooking loss and redness, and total cholesterol, and hemoglobin level. These results are comparable with previous reports presenting that these plant extracts improve appetite and performance. In conclusion, supplementation of thyme, rosemary, and oregano powder enhances performance & meat quality, and decreases cholesterol level of broilers raised in a tropical environment.

Table 1 – Effect of dietary thyme, rosemary, and oregano extracts at 5g/kg feed on broiler performance, meat quality measures, and blood profiles at day 35of age.

Treatments	ADG (g)	ADFI(g)	FCR	WHC (%)	CL (%)	a*	TC (mg/dl)	Hb (g/100ml)
T_1	46.71^{a}	78.97^{a}	1.69^{b}	90.87^{a}	25.83^{b}	4.92^{a}	106.54^{b}	12.40^{a}
T_2	49.93^{b}	80.30^{b}	1.61^{a}	92.72^{b}	22.49^{a}	6.37^{b}	86.97^{a}	1353^{b}
T_3	51.17^{b}	80.69^{b}	1.57^{a}	93.84^{b}	21.97^{a}	6.29^{b}	88.80^{a}	1493^{c}
T_4	50.46^{b}	80.43^{b}	1.60^{a}	92.64^{b}	22.62^{a}	6.51^{b}	88.54^{a}	1427^{bc}
SEM	14.77	7.59	0.01	0.38	0.58	0.22	2.91	0.30
P value	0.001	0.012	0.001	0.017	0.037	0.004	0.020	0.001

T_1=control feed; T_2=control+thyme (5kg); T_3=control+rosemary (5kg); T_4=control+oregano (5kg); ADG=average daily gain; ADFI=average daily feed intake; FCR=feed conversion ratio; WHC=water holding capacity; CL=cooking loss; a*=redness; TC=total cholesterol; Hb=hemoglobin. SEM=standard error of mean. ^{a,b,c}Different superscripts within a column are significantly different ($P < 0.05$)

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A NOVEL PHYTASE COMPLETELY REPLACED INORGANIC PHOSPHATE IN BROILERS FED WITH A HIGH PHYTATE DIET

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Since commercial phytase products have evolved to hydrolyze phytate more efficiently, the need to add inorganic phosphate to poultry diets has been reduced. A recent study reported that a high dose (4,000 FYT/kg) of a third generation phytase derived from *Citrobacter braakii* successfully reversed the negative impacts on broiler performance and bone conformation associated with removal of inorganic phosphate from grower and finisher diets (Ribeiro et al., 2019). Achieving total replacement of inorganic phosphate with phytase remains especially challenging during the starter phase, because of the high P requirements of young birds. Therefore, the objective of this study was to evaluate if a novel phytase could completely replace inorganic phosphate in the starter diet of broilers fed with a high phytate diet.

A total of 1,040 Cobb 500 male broilers (day-old) were weighed and randomly assigned to one of five dietary treatments, which consisted of: 1) a positive control diet (PC, formulated with 16.0, 13.5 or 11.5 g/kg DCP in the starter, grower or finisher, respectively); 2) a negative control 1 diet (NC1, formulated with 2.5 g/kg DCP in the starter and no DCP in the grower or finisher); 3) the NC1 diet supplemented with a novel phytase (HiPhorius^{TM}, DSM Nutritional Products, Switzerland) at 1,000 FYT/kg across all three phases (NC1+1000/1000/1000); 4) the NC1 diet supplemented with the novel phytase at 2,000, 2,000 or 1,000 FYT/kg in the starter, grower or finisher, respectively (NC1+2000/2000/1000); 5) a negative control 2 diet (NC2) formulated with no DCP across the three phases, and supplemented with the novel phytase at 4,000, 2,000 or 1,000 FYT/kg in the starter, grower or finisher, respectively (NC2+4000/2000/1000). The experimental diets were formulated to contain 0.34% phytate P with corn, SBM, wheat, rice bran and rapeseed meal as the main ingredients. The total Ca and nPP of PC, NC1 or NC2 in the starter were 0.90 and 0.44%, 0.71 and 0.21%, or 0.69 and 0.17%; in the grower were 0.80 and 0.39%, 0.61 and 0.16% or 0.61 and 0.16%; in the finisher were 0.70 and 0.35%, 0.54 and 0.16% or 0.54 and 0.16%, respectively. The novel phytase (a fourth generation product) was encoded by a 6-phytase gene from *Citrobacter braakii* with great improvement in intrinsic temperature and pH stability. There were 26 birds/cage and 8 cages/treatment. At 42 d of age, the right tibia or breast meat was taken from 2 or 4 birds/pen. Data from all treatments were analyzed by one-way ANOVA with Fisher's LSD test.

Compared to PC, the birds fed NC1 significantly decreased body weight gain (BWG) and increased body weight corrected feed conversion ratio (BWcFCR) at 0-42 d of age ($P < 0.05$), decreased breast meat yield and tibia ash weight at 42 d of age ($P < 0.05$). Birds in the group of NC1+1000/1000/1000 showed significant improvements on all these observations compared to NC1 ($P < 0.05$), and the improved values were comparable to those of PC ($P > 0.05$). Birds in the treatments of NC1+2000/2000/1000 and NC2+4000/2000/1000 showed significantly higher BWG and lower BWcFCR at 0-42 d of age ($P < 0.05$), numerically higher breast meat yield and tibia ash weight at 42 d of age ($P > 0.05$) when compared to PC. The additional benefits of the latter two treatments may be derived from the extra-phosphoric effect of phytase, referring to the provision of myo-inositol and the release of energy and amino acids beyond minerals. In summary, the results demonstrated the effectiveness of the novel phytase to completely replace inorganic phosphate during an entire growth cycle of broilers, which required its 4×, 2× and 1× recommend dose in the starter, grower and finisher phases, together with diets formulated to be particularly rich in phytate substrate.

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A SYNERGISTIC BLEND OF ORGANIC ACIDS ENHANCES GUT FUNCTIONALITY AND IMPROVES THE GROWTH PERFORMANCE OF BROILERS

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Organic acids (OA) have been used worldwide in animal feed and/or water to support health and promote growth (Nguyen and Kim. 2020). They may inhibit the growth of pathogenic bacteria, alter gut microbiota, boost immune responses, and improve animal health and performance (Kumar *et al*., 2022). Today, several products based on single, or a blend of OA are available in the market. Compared to individual acids, the combination of two or more acids appears to have a more synergistic effect in supporting gut health and enhancing growth rate (Samudovska *et al*., 2018). This study aimed to evaluate the efficacy of a water acidifier based on a synergistic blend of free and buffered short-chain fatty acids (SPH) (Selko-pH, Selko Feed Additives) on growth performance, gut histology, and nutrient digestibility.

A total of 646 one-day-old mixed sex Ross 308 broilers chicks with an initial average body weight (BW) of 48.26 ± 0.40 g were allocated into two treatment groups with 17 replicates of 19 birds/pen. The test treatments included a basal diet (NC) and a basal diet plus 1 L SPH/1000 L water (SPH). Birds had *ad libitum* access to water and a two-phase (starter: days 0-14 and grower: days 15- 35) antibiotic-free corn-soya-based diet throughout the experimental period. Growth performance [BW, average daily feed intake (ADFI), average daily gain (ADG), average daily water intake (ADWI), feed conversion ratio (FCR), mortality] was recorded from d1-35. On d14, a section of the duodenum was examined for gut morphology (villus height [VH] and crypt depth [CD]), and at d35, nutrient digestibility was assessed. Data were analyzed using the MIXED procedure in SAS and Tukey's range test was used to determine the significance between treatment means (P<0.05).

The supplementation of SPH considerably enhanced the growth performance of broilers during the whole production period. Compared to the control treatment, broilers supplemented with SPH had significantly greater BW (+5.1 %, 1.64 vs 1.56 kg) and ADG (+5.3 %, 45.5 vs 43.2 g/d), and a lower FCR (-9.3 %, 1.607 vs 1.772) (P<0.05). In addition, broilers supplemented with SPH had significantly higher VH (1260.6 vs 1050.8 µm, P=0.002) and VH:CD ratio (8.5 vs. 7.2, P=001). Furthermore, SPH supplementation increased the digestion coefficient for dry matter (78.6 vs. 73.3%, P=0.05), gross energy (75.6 vs. 71.5%, P=0.03) and nitrogen (71.7 vs 61.6%, P=0.10) compared with the NC. The supplementation of SPH, however, did not affect ADFI, ADWI, and mortality rate (P>0.10). In conclusion, the current findings suggest that the addition of a synergistic blend of free and buffered short-chain fatty acids in water (SPH) can be used to enhance gut functionality and support the growth performance of broilers.

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PYRUVATE SUPPLEMENTATION MITIGATES THE NEGATIVE IMPACT OF HIGH DIETARY OXALATE IN BROILER CHICKENS

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Oxalate is a toxic compound found in a wide range of feedstuffs and forages particularly abundant in tropical and subtropical areas (Rahman and Kawamura, 2011). In chickens it has been shown to cause hypocalcemia by binding trace minerals (mainly Ca and Mg) in the gut and circulatory systems, potentially causing severe kidney damage. Clinical signs of oxalate toxicosis include weakness and tremors resulting from impaired energy metabolism. Oxalic acid inhibits glycolytic enzyme activities including pyruvate kinase and carboxylase essential in glycolysis and the Krebs cycle in liver and muscle. Once absorbed, oxalate is detoxified in the liver through the alanine (Ala) - glyoxylate aminotransferase (AGT) pathway. However, our current understanding on the impact of dietary oxalate and oxalate catabolic pathways in broiler chickens is very limited. This study was developed to test the hypothesis that supplemental alanine or pyruvate would increase the activity of the oxalate detoxification pathway, thus, mitigating the negative impact on growth performance in broiler chickens.

A total of 432 broilers (Ross 308) were allocated to 6 treatments resulting from a 2 by 3 factorial design with two levels of dietary oxalate (0 or 1%) and the mitigating supplements with 1% alanine, 1% pyruvate, or without supplements (control). The data were statistically assessed by ANOVA using the GLM procedure of SAS 9.4 software. The results (Table 1) showed that dietary oxalate significantly decreased body weight gain (BWG), feed intake (FI) and final body weight (FBW) ($P < 0.001$) compared to the control diet. There were highly significant interactions between oxalate and the dietary supplements for BWG ($P < 0.001$) and feed conversion ratio (FCR) ($P < 0.018$). The supplementation of pyruvate with 1% oxalate diet significantly increased BWG and FI compared to alanine supplementation or no supplements in the 1% oxalate diet. In contrast, Ala supplementation showed a significantly reduction in performance parameters independent of the inclusion of dietary oxalate. These results indicate that an increase in the availability of pyruvate is crucial in the detoxification pathway of dietary oxalate.

In conclusion, high oxalate levels in broiler diets adversely affected performance by decreasing FBW, BWG, and FI. Pyruvate supplemented into 1% oxalate diet alleviated the oxalate toxicity in broiler chickens.

Table 1 - Effects of alanine and pyruvate on growth performance in broilers fed oxalate (0-42 days).

Diet	Supplement	IBW (g/bird)	FBW (g/bird)	BWG (g/bird)	FI (g/bird)	FCR
Without Ox	No Sup	42	3,414^{a}	3,368^{a}	4,891^{a}	1.453^{bcd}
Without Ox	1% Ala	42	3,279^{b}	3,238^{b}	4,721^{b}	1.460^{abcd}
Without Ox	1% Pyr	42	3,380^{a}	3,338^{a}	4,939^{a}	1.480^{a}
1% Ox	No Sup	42	3,039^{d}	2,997^{d}	4,313^{d}	1.438^{d}
1% Ox	1% Ala	41	2,771^{e}	2,730^{e}	4,030^{e}	1.478^{ab}
1% Ox	1% Pyr	43	3,172^{c}	3,126^{c}	4,517^{c}	1.446^{cd}
SEM		0.18	29.47	29.31	43.54	0.004
P-value						
Oxalate		0.685	<0.001	<0.001	<0.001	0.163
Supplement		0.220	<0.001	<0.001	<0.001	0.028
Ox×Sup		0.294	<0.001	<0.001	0.143	0.018

$^{a-e, AB}$means in the columns with different small or capital letters differ significantly ($P < 0.05$). Ala, alanine; Pyr, pyruvate; Ox, oxalate; Sup. Supplement; IBW, initial body weight; FBW, final body weight; BWG, body weight gain; FI, feed intake; FCR, feed conversion ratio.

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RAISING THE ESSENTIAL AMINO ACIDS-TO-TRUE PROTEIN RATIO BEYOND 0.6 IMPAIRS THE GROWTH RATE OF UNCHALLENGED BROILERS FED REDUCED CRUDE PROTEIN DIETS DURING THE GROWER PERIOD

S. MUSIGWA[1], P. COZANNET[2], C.A. ASIAMAH[1] and S. WU[1]

Most essential amino acids (EAA) are usually considered when formulating reduced protein (RP) diets. This results in an imbalanced EAA-to-non-essential amino acids (NEAA) ratio, to which broilers are sensitive (Pesti, 2009). Moreover, the conventional way of measuring dietary protein content using the crude protein (CP) concept is flawed, as it assumes that all protein contains 16% nitrogen (N) and ignores non-protein N sources. True protein (TP), which considers only amino acids, provides a more accurate measurement (Alhotan & Pesti, 2016). Maintaining a ratio of EAA:TP at 0.60 has been shown to restore the performance of broilers fed RP-diets, while going below this ratio impairs the performance (Musigwa et al, 2023). This study evaluated the impact of increasing the EAA:TP ratio above 0.60 on the performance of unchallenged or necrotic enteritis challenged broilers fed RP-diets from d 8 to 19.

A 4 × 2 factorial design was used, with the factors being: diet and challenge. Diet: 3 RP-diets (17% CP) with 3 levels of EAA:TP ratio (0.60; 0.62; and 0.64) and 1 normal CP (NP) diet (19%). Challenge: yes or no. This resulted in 8 treatments replicated 7 times, with 18 mixed-sex birds/replicate. Birds were fed a common starter diet from d 0 to 8 and the grower diet treatments from d 8 to 19. Four iso-energetic grower diets were formulated based on the Cobb 500, 2022 nutrient specifications. The TP contribution of feed ingredients was estimated using a specific N-to-protein conversion factor, K_A, according to Pesti (2009). On d 9, the challenged birds were orally inoculated with *Eimeria* strains, and on d 14 and 15 with *C. perfringens* (NE18). All data were analysed using JMP software, with gender as a covariate.

There was a significant ($P < 0.05$) diet × challenge interaction for weight gain (WG), feed intake (FI) and feed conversion ratio (FCR). Increasing the EAA:TP ratio from 0.60 to 0.64 in RP-diets reduced ($P < 0.05$) WG and FI only in unchallenged birds. Furthermore, birds fed RP-diets with an EAA:TP ratio of 0.60 showed no difference ($P > 0.05$) in WG and FI compared with those fed NP-diets only in the unchallenged group. These improvements were only numerical in the challenged group due to impaired nutrient utilisation. NP-diets only reduced ($P < 0.05$) FCR compared to the RP-diets both in unchallenged and challenged groups. A weak negative correlation was found between EAA:TP ratio and WG ($P < 0.05$, $R = -0.314$). These results show that maintaining an EAA:TP ratio of 0.60 in RP diets can restore growth rate. Further study is needed to find a solution for the observed poor FCR.

ACKNOWLEDGEMENTS: This study was funded by Adisseo France in partnership with AgriFutures Australia and Poultry Hub Australia.

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IMPACT OF A MULTI-ENZYME PREPARATION ON IN VITRO DIGESTIBILITY OF ALTERNATIVE INGREDIENTS FOR BROILERS

B. GUO[1], Y.X. YANG[2], R. SYAHRIADI[1], HG. MASILUNGAN[1] and A. BERTHO[1]

Because of the price increase of major feed raw materials (RMs) in recent years, increasing the utilization of alternative ingredients (AIs) has become popular in broiler industry. However, AIs application in broiler feed is always a challenge, mainly due to their variability of supply, quality, nutritional profiles, and digestibility (Babatunde et al., 2021). Feed enzymes are commonly used to minimize the negative impact from antinutritional factors of these AIs. In addition, using in vitro digestibility assessment facility can provide robust evidence to increase the AIs inclusion rate in broiler feed. In the recent years, a simulated broiler digestive system namely SDS III (Zhongben Intelligent Technology Development Co. Ltd., China) was deployed in many poultry integrators (Liu et al., 2021). Briefly, in this research the system was used to evaluate the impact of feed enzymes on the dry matter digestibility (DMD) of RMs mimicking broiler digestive conditions. It was hypothesized that using the SDS III system could estimate the impact of a multi-enzyme preparation (MEP) on the DMD of AIs.

This trial was carried out at the Research and Innovation Center in China Adisseo (RICA). The MEP used in this trial contained xylanase at 1250 vu/g, and beta-glucanase at 860 vu/g (Rovabio® Advance L2, Adisseo SAS, France). The in vitro digestion procedures followed the previous report (Liu et al., 2021). In total, DMD of seven AIs were tested by SDS III system with (MEP group) or without (Control group) MEP treatment (Figure 1). The results showed that DMD of wheat bran, rice bran and broken rice were significantly improved by MEP ($P<0.01$), whereas no improvement was achieved for the rest AIs. AIs enriched in arabinoxylan such as wheat bran and rice bran are the favorable substances for MEP (Cozannet et al., 2017), resulting in the significant DM improvement. In contrast, high levels of mannan in both palm kernel and copra meals, and low fiber contents in both cassava and rice bran extract are not the ideal substrates for the MEP, leading to the same DMD with or without MEP treatment.

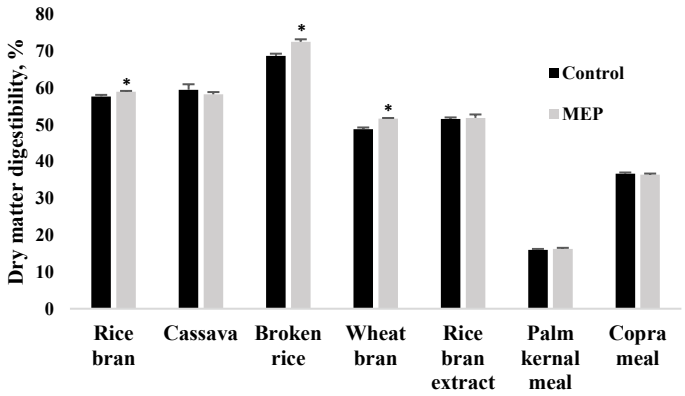

Figure 1 - Effect of MEP on AIs DMD using SDS III. "*" indicates significant differences ($P<0.05$).

It was concluded that the improvement of the MEP on AIs' DMD was correlated with the fiber content and profile, consistent with the previous findings from the in vivo animal trials. In addition, our study provides a reliable in vitro approach to evaluate the impact of feed enzymes on DMD of AIs, hence the reference to utilize AIs in broiler feed more efficiently.

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IMPROVING BROILER-BREEDER PERFORMANCE WITH 25-HYDROXYVITAMIN D_3 OF FERMENTATIVE ORIGIN

W. VAN DER VEKEN[1] and K. BIERMAN[1]

Summary

25-hydroxyvitamin D_3 from a fermentative source was supplemented to broiler-breeders in two separate trials, compared to standard basal diets without a 25-hydroxyvitamin D_3 source. Benefits in terms of reduced day-old chick (DOC) and early embryo mortality were recorded in a dose-response manner for animals supplemented with 25-OH D_3, as well as an overall improved eggshell quality.

I. INTRODUCTION

Poultry diets have developed over the years, including an increased interest in nutrients such as vitamins and minerals. A good example of the latter is vitamin D, mainly known for its involvement in the calcium metabolism and bone strength (Khan et al., 2021).

However, there is more to vitamin D than this. Vitamin D (sub-)deficiencies have been clearly associated with increased mortality and reduced immunological responses, highlighting the broad importance of the vitamin beyond skeletal integrity (Khan et al., 2021; Hashim et al., 2023). This has led to more recent research focusing on the use of different vitamin D metabolites to improve other parameters in poultry, including its use in broiler-breeder diets.

Of these metabolites, 25-hydroxy vitamin D_3 is of major interest: it has a long half-life, acts as the major reserve form of vitamin D within the body and does not rely on the liver in the remainder of the metabolic vitamin D pathway (Sakkas et al., 2018). Within the group of 25-OH D_3 metabolites, the main difference comes down to the production process: either via a synthetic pathway, or via fermentation. In the trials at hand, the first commercially available 25-OH D_3 of fermentative origin was put to the test (Bio D®, Huvepharma).

II. METHOD

Two trials were performed at a commercial research centre in France with the treatments listed in Table 1. For the first trial, a total of 1080 female broiler breeders of the Hubbard D line were used. These were divided at random into 3 batches of 360 female broiler breeders each, divided over 45 repeats of 24 females each (4 hens/cage x 6 cages/repeat). The trial ran for 70 days, from week of age 52 to 62. Egg quality and embryo mortality were evaluated at two different time points (57 and 62 weeks of age).

For the second trial, a total of 720 female broiler breeders of the Hubbard D line were used. These were divided at random in 3 batches of 240 female broiler breeders each, divided over 10 repeats of 24 females each. The trial ran for 105 days, from week of age 48 to 62. In this trial, hatching performance as well as DOC and embryo mortality were evaluated.

Data was analysed using SAS software with the correct statistical models (GLM procedure, alpha = 5%). Models had the following constraints:
- data from each population had to be normally distributed with an even variance
- data was obtained independently

Normality was tested with a Shapiro test. Evenness of variances was tested with Bartlett test. If one of the constraints above was not respected, a Kruskal-Wallis test was used.

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Table 1 - Vitamin D supplementation in both trials (expressed per kg of feed).

Trial 1	Vit D_3 (IU)	25-OH D_3 (µg*)	Total IU
Control	3000	0	3000
25OH A	3000	17.4	4392
25OH B	3000	34.8	5784
Trial 2			
Control	3000	0	3000
25OH B	3000	34.8	5784

One µg of 25-OH D_3 in Bio D® equals 80 IU.

III. RESULTS

In the first trial, the inclusion of 25-OH D_3 in the feed significantly improved mortality parameters, in a dose-response fashion (Figure 1). Broiler breeders in the 25-OH D_3 groups also noted an improved egg quality (Table 2).

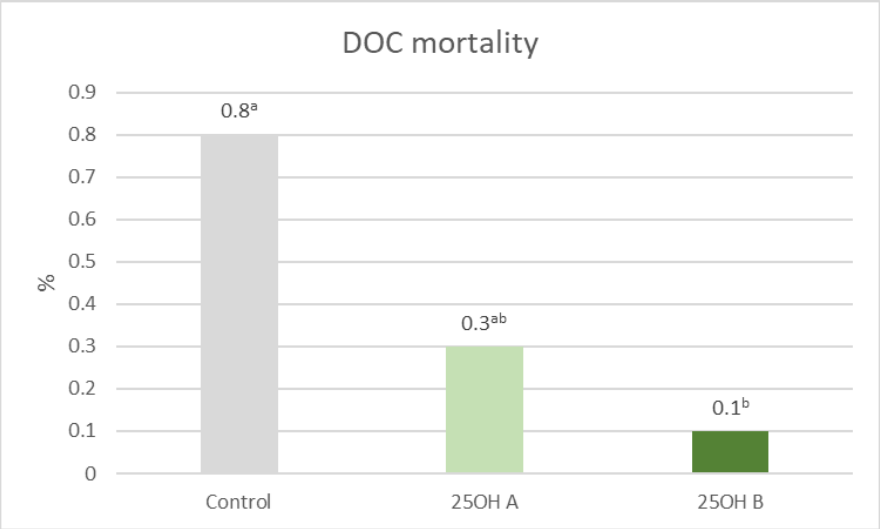

Figure 1 - DOC mortality (%) of the three treatment groups over the full trial. Different superscripts indicate significant differences ($P < 0.05$).

Table 2 - Egg quality parameters for the three treatments in the first trial.

	Control	25OH A	25OH B
Static stiffness (N/mm)	$125^{x} \pm 20.4$	$126.4^{xy} \pm 21.0$	$129.1^{y} \pm 21.5$
Fracture force (N) at 62 weeks	27.7 ± 5.4	28.1 ± 5.2	28.2 ± 5.0
Haugh units	79.6 ± 5.7	79.9 ± 5.6	80.3 ± 5.7

Different superscripts indicate statistical differences ($P < 0.05$)

Table 3 - Mortality and hatching performance parameters of the two groups in the second trial.

%	Control	25OH B	P-value
Early mortality	8.9	7.7	< 0.001
Total embryo mortality	18.8	18.2	0.018
DOC mortality	4.4	3.1	0.086
Uncleared eggs rate*	85.4	86.9	0.001
Hatching rate	75.3	75.9	0.037

Uncleared eggs rate = uncleared eggs / hatching eggs set.
An uncleared egg developed an embryo, so a higher rate is preferred.

In the second trial, supplementing 25-OH D_3 significantly decreased early embryo mortality (<7 days) and total embryo mortality, whilst DOC mortality improved numerically.

Similarly, broiler-breeders in this group noted a significantly better hatching performance compared to the control (Table 3).

IV. DISCUSSION & CONCLUSION

In both trials, the addition of 25-OH D_3 had clear benefits related to the performance of broiler-breeders. The observed effects occurred in a dose-response manner in trial 1 and were most pronounced in mortality-related parameters.

The current hypothesis to explain these results would be a maternal transfer from 25-OH D_3 to the developing eggs, where the increased levels of vitamin D support the early development of the embryo. This transfer-hypothesis is supported by the established use of dietary vitamin D to increase final vitamin D levels in eggs for human consumption, known as "functional food". Regarding improved eggshell quality, the role of calcium in the formation of eggshell is well-established. As vitamin D is tightly linked with the calcium metabolism, improving the vitamin D status of the animal impacts its calcium metabolism as well.

The results underline the importance of adding an effective 25-OH D_3 metabolite in broiler-breeder operations, even if animals are already supplemented with standard levels of regular vitamin D_3.

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BROILER CHICKS SHOW ROBUST INNATE PREFERENCES FOR A BALANCED DIET IMMEDIATELY AFTER HATCHING

R. TUNISA[1], A. KUMAR[1], M. NAVARRO[1], M. BLANCH[2], G. TEDO[2] and E. ROURA[1]

A critical challenge in chicken meat production is to reach a high feed intake of a nutritionally balanced diet as soon as possible in the post-hatching hours. However, feed appetence in chicks has often been ignored and dietary essential nutrient levels have been developed in principle to optimise later stages in the chicken's life. One of the strategies to improve our understanding of both appetence and nutrient balance early in life is to offer dietary choices that allow the expression of innate foraging behaviours (Iqbal et al., 2017; Niknafs & Roura, 2018). This research aimed to develop a double-choice (DC) model to study dietary preferences immediately after hatching. It was hypothesised that broiler chicks develop an accurate sense of taste and smell during embryonic development.

Ninety-six, day-old, male broiler chicks (Ross 308) were individually penned and randomly assigned to 4 DC treatments based on the 4 diets: diet 1 was a control diet (wheat-soybean meal; 2,990 kcal/kg ME; 21.6% CP; 1.28% SID Lys); diet 2 was an iso-caloric but amino acid (AA) deficient diet (0.75%) compared to control by substituting synthetic AA with dextrose; diet 3 was an iso-AA but lower calorie (2821 kcal/kg ME) diet compared to control by substituting fat with Celite; and diet 4 an isocaloric and iso-AA diet based on maize-hydrolysed soy protein. The 4 DC treatments consisted of diet 1 vs diet 1 (T1), diet 1 vs diet 2 (T2), diet 1 vs diet 3 (T3), and diet 1 vs diet 4 (T4). Performance parameters were measured weekly, and dietary choices were recorded daily. The UQ Animal Ethics Committee approved with certificate 2023/AE000084. Diet preferences were presented in Figure 1 as the percentage of test diet intake to total feed intake.

The results showed a significant preference for the control diet (diet 1) over diets 2, 3, or 4. The avoidance of diets 2 and 4 was highly significant ($P < 0.001$) on day 1 post-hatch. Avoidance of diet 3 was significant ($P < 0.05$) at day 3 post-hatch. No significant differences ($P > 0.05$) were observed in T1 (diet 1 vs diet 1) which validated the test.

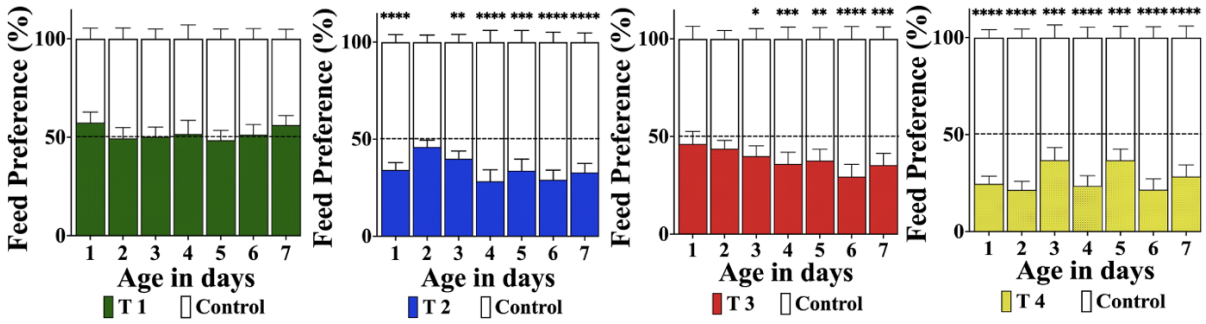

Figure 1 - Dietary preferences between control (diet 1) vs diet 1 (T1); diet 1 vs amino acid deficient diet 2 (T2); diet 1 vs low calorie diet 3 (T3); diet 1 vs alternative formulation diet 4 (T4).

In conclusion, broiler chickens have acute senses of taste and smell, allowing them to make dietary choices based on nutritional and organoleptic principles as early as day 1 post-hatch.

ACKNOWLEDGEMENTS: This research was supported by the AgriFutures Australia Chicken Meat Consortium for Nutrition, Gut Health, and Environment.

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IN OVO DELIVERY OF GRAPEFRUIT ESSENTIAL OIL IMPROVES GUT DEVELOPMENT IN BROILER HATCHLINGS

J.N.K. DISANAYAKA[1], S. NIKNAFS[1], A.A. KHASKHELI[1], M.M.Y. MEIJER[1] and E. ROURA[1]

Essential oils (EO) are widely known for their capabilities as potential alternatives to antibiotic growth promoters. EO have been reported to improve gut functionality by altering intestinal permeability and gut microbiota, inhibiting unwanted bacterial proliferation, and improving digestive secretions and nutrient absorption in broilers (Brenes and Roura, 2010). However, little is known about potential applications of EO on embryonic development. The objective of the present study was to investigate the impact of in ovo delivery of EO on embryonic development and hatchability in broilers. It was hypothesized that selected EO have the potential to improve embryonic development when applied in ovo.

Seven EO treatments including coriander, bergamot, clary sage, lemon myrtle, lemongrass, grapefruit, and cinnamon bark were individually delivered to the amniotic fluid of Ross 308 fertile eggs (n=24 eggs per treatment) at day 17.5 of incubation. One ml of EO solution comprised of 5 µl EO, 5 µl emulsifier polysorbate 80, and 990 µl of 0.9% (v/v) saline were used as the delivery volume. The control group consisted of 1 mL saline injection. Hatchability, chick quality (navel score and deformity) and bodyweight at hatch were measured (n=24). Residual yolk sac, heart, intestine, and gizzard and proventriculus weights were also recorded at hatch (n=6). The relative organ weight and the ratio of chick birthweight to initial egg weight were analysed with one way ANOVA and Tukey's multiple comparison using R statistical software version 4.3.1.

Table 1 summarises the main results obtained in the experiment. Hatchability, chick quality, body weight, yolk residue, heart and, gizzard and proventriculus weights were not significantly ($P > 0.05$) affected by the EO injections compared to the control. In contrast, grapefruit EO and clary sage EO increased ($P < 0.05$) intestine and liver weights, respectively. The increment of the liver weight could be a sign of an increase in pro-inflammatory responses (i.e., toxicity) due to the EO concentration. In conclusion, there was no negative impact of the in ovo delivery of the selected EO, on either hatchability, or day-old chick quality. Grapefruit EO showed promising results on improving gut development that warrant further studies.

Table 1 - Effect of in ovo EO on hatchability, body and organ weights in broiler hatchlings.

Treatment	Hatchability (%)	BW (g)	Intestine W%	Liver W %
Saline (control)	100	47.64±2.17	3.17±0.51^{b}	2.04±0.24^{b}
Coriander	100	47.09±2.35	4.32±0.92^{ab}	2.14±0.29^{ab}
Bergamot	91.6	47.83±1.95	4.37±0.92^{ab}	2.19±0.14^{ab}
Clary sage	87.5	47.47±2.26	4.41±0.52^{ab}	2.48±0.29^{a}
Lemon myrtle	95.8	47.06±2.60	4.40±0.68^{ab}	2.47±0.24^{ab}
Lemongrass	83.3	47.19±5.15	4.03±0.81^{ab}	2.21±0.22^{ab}
Grapefruit	95.8	48.74±2.26	4.55±0.69^{a}	2.39±0.29^{ab}
Cinnamon bark	100	47.09±1.93	3.82±0.52^{ab}	2.31±0.18^{ab}
P value	0.8743	0.3610	0.0385	0.0333

The values are expressed as mean±standard deviation. ^{a,b}Different superscripts within a column are significantly different ($P < 0.05$).

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EFFECTS OF PHYTOMOLECULES ON REPRODUCTIVE PERFORMANCE OF BROILER BREEDERS AND DAY-OLD CHICK QUALITY

M. CABALLERO[1], K. PALANISAMI[1], S. KADARI[1] and D. SHERWOOD[2]

Summary

The effects of a combination of phytomolecule-based compounds, thymol-based in-feed, and carvacrol and cineol-based in water for drinking, were investigated regarding the reproductive performance of broiler breeders and the quality of their progeny. These phytomolecules have been previously investigated for their antimicrobial, antioxidant, and anti-inflammatory properties regarding gut health, and thus the combination was used as a gut health improvement program (GHI) using the in-feed product continuously at 100 g/ton of feed and in-water at 250 ml/1000 liters from week 22 to 28. To evaluate GHI, 2160 female and 216 male Hubbard JA57 broiler breeders were divided in two groups at 22 weeks (w) of age, with 12 replicate pens/cages per group. All birds received the same management and feed allowance. Both groups achieved productivity close to the breed standard, supplemented hens yielded significantly ($p < 0.05$) more total eggs than control, with a significant difference in laying%. The day-old chick (DOC) production per hen housed (DOC/HH) was significantly higher ($P < 0.01$) for the GHI group, yielding 12,5 chicks more than the control. The DOC/HH was influenced by laying rate ($P < 0.05$), hen mortality (NS), and fertility % (NS). Additionally, DOC quality parameters such as yolk free body mass (YFBM), Pasgar score, and serum antioxidant capacity were significantly improved. The use of in-feed thymol combined with carvacrol and cineol in water for drinking, can be regarded as an effective tool to help breeders to deal with various commercial stressors affecting gut health.

I. INTRODUCTION

Following the ban of antibiotic growth promoters in some countries, phytogenic substances have been used in animal production to improve gut health. Feeding phytogenic components in laying hens (Mousavi et al., 2018), and breeder hens (Mustafa, 2019) improved feed efficiency and intestinal morphology through antimicrobial, antioxidant, immunomodulating, and nutrient digestibility actions (Botsoglou et al., 2005; Mousavi et al., 2018; Abdel-Wareth & Lohakare, 2020). Also improving egg production and weight in laying hens (Abdel-Wareth & Lohakare, 2020) and in breeder hens (Chilante et al., 2012; Mustafa, 2019). Among these phytogenics, thymol, a phenolic compound possessing antioxidant, anti-inflammatory, and antibacterial activities (Attia et al., 2018), has been evaluated in poultry animals with positive results, including in reproductive performance and effects in the offspring of the supplemented animals (Videla et al., 2020).

The objective of this study was to evaluate the performance of broiler breeders under a gut health improvement program consisting of an in-feed phytogenic product based on thymol applied at 100g/ton of feed during the laying period, and an in-water phytogenic based on carvacrol and cineol applied during the onset of laying, the critical period of high stress, at 250ml/1000 liters of water. The evaluated parameters were egg production, settable egg rate, hatchability, and quality of day-old chicks regarding their Pasgar Score and total antioxidant capacity (TAC).

II. MATERIALS AND METHODS

All procedures associated with the preparation of the experimental protocol and executions of this study were in agreement with the Central Commission for Animal Protection of the Czech Ministry of Agriculture. A total of 2160 female and 216 male Hubbard JA57 broiler breeders were divided in two groups at 22 w of age. Each group was divided to have 12 replicates, placing 90 female and 9 males in each pen. Both groups received the same management and feed allowance through the trial

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period which ended when the animals reached 70 w of age. One of the groups was provided with a gut-heath improvement program (GHI), consisting of supplementation with an in-feed phytmolecules (Ventar D, containing thymol, EW Nutrition GmbH at 100g per metric ton during the whole experimental period, and in-water phytogenic (Activo Liquid, composed of carvacrol and cineol, EW Nutrition GmbH) at 250 ml/1000 liters of water for drinking during 4 days per week from week 22 to 28.

Production performance parameters, such as mortality, feed intake, egg production, settable eggs, egg weight and feed efficiency were recorded. To calculate feed efficiency, the combined feed allowances for both hens and roosters were divided by hatched chicks per week for each pen. Nine Incubation trials were conducted, starting at w 30 with an average frequency of four weeks, until the end of the period, setting 90 eggs per pen. Individual egg candling was performed on incubation-day 10 and clear eggs were broken to count unfertile eggs. Day-old chick quality, of the hatched chicks, was evaluated after each incubation trial taking 240-day-old chicks (DOC): 20 random chicks hatched from each of the 90 eggs per hen-pen that were incubated. These DOC were individually weighted and evaluated through the Pasgar score (Boerjan, 2002). The Pasgar score evaluates alertness (reflex), the appearance of the navel, legs, beak, and the thickness of the belly. Ten DOC were randomly picked and sacrificed to record yolk-free body mass, the serum from these chicks was collected to test TAC using total antioxidant status (TAS) kits (Randox Laboratories, UK), following the instructions of the supplier. The data were subjected to a completely randomized design using one-way ANOVA. The pen was used as the experimental unit for analyses. All statements of significance were based on a $P < 0.05$.

III. RESULTS

In general, both groups had good productivity, close to the breed standard; supplemented hens laid 3% more eggs than control birds (Figure 1), with a marked difference in production persistence starting at 35 weeks of age (Figure 2), where a significant interaction was found between laying rate and age ($P < 0.05$). The settable eggs% was similar for both groups, with a slight numerical difference in favor of the GHI group (91.94 vs 91.20 in the control group), thus the difference in hatching eggs per hen housed (HE/HH) is driven by the higher egg production in the GHI group.

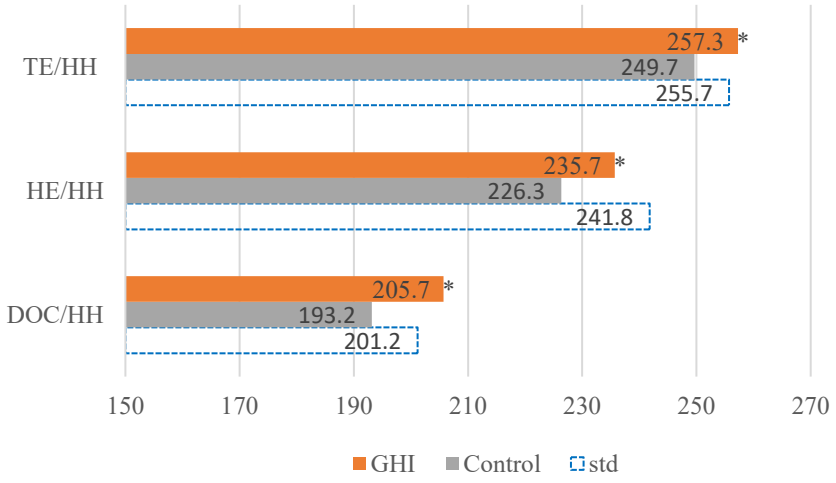

Figure 1 - Total eggs (TE), Hatching Eggs (HE) and ay-old chicks (DOC) per hen housed (HH) for Hubbard JA57 broiler breeders under GHI vs a control group. For all parameters a significant different was found between the groups ($P < 0.05$), indicated by (*). Breed standard (std) included for reference.

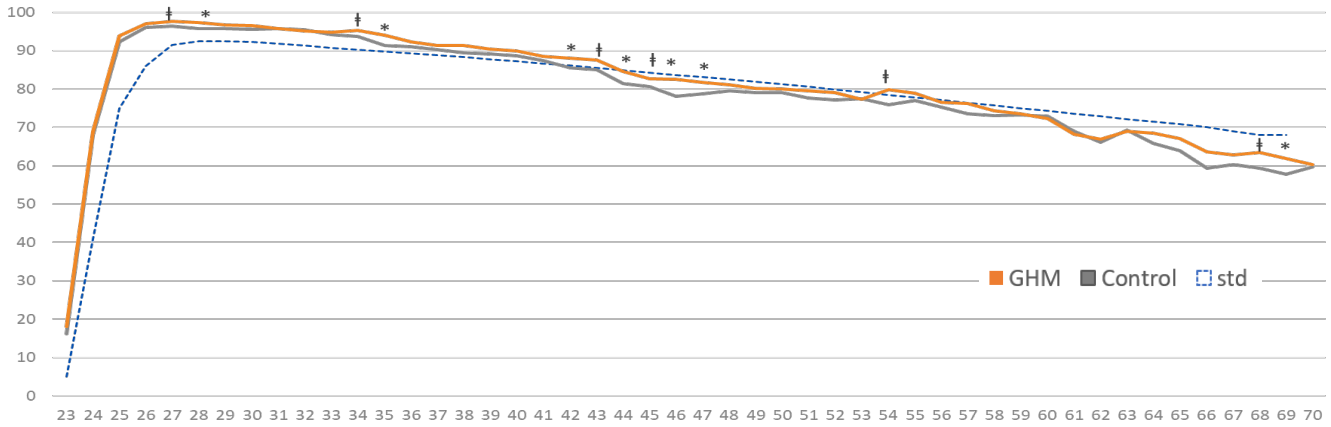

Figure 2 - Laying rate from onset of laying until 70 weeks of age for Hubbard JA57 broiler breeders under GHI compared with the control group. Significant differences (P < 0.05) are indicated by (*), and trends towards differences (P < 0.1) by (ǂ).

Day-old chick production was significantly higher (P < 0.01) for the GHI group, yielding 12.5 more DOC than the control group (Figure 1). Besides higher egg production, the DOC/HH was influenced by hen mortality (6.48% in GHI, and 8.83% in the control group), and hatchability %, which was 2 percentual points higher for the GHI group (Table 1).

Table 1 - Fertility, hatchery performance and, DOC quality parameters of Hubbard JA57 broiler breeders under GHI vs a control group.

	Results			P value		
Variable	Control	GHI	SEM	age	trt	trt*age
Fertility (%)	91.88	93.93	0.799	NS	NS	NS
Hatchability (%)	87.27	89.82	0.749	NS	NS	NS
Hatch of Fertile (%)	95.01	95.65	0.595	NS	NS	NS
Egg weight (g)	64.46	64.71	0.569	*	NS	NS
DOC weight (g)	42.03	42.17	0.353	*	NS	NS
Pasgar Score (units)	8.94	9.10	0.087	*	*	*
YFBM (%)	87.47	88.00	0.340	*	*	NS

Significant differences (p < 0.05) are indicated by (*);
Hatchery performance is indicated by hatchability and hatch of fertile;
DOC quality is indicated by DOC weight, pasgar score and yolk free body mass (YFBM)

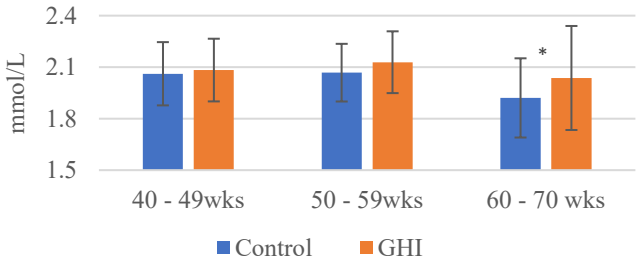

Figure 3 - Total antioxidant capacity (TAC) in serum from DOC from Hubbard JA57 broiler breeders under GHI program compared with a control group. Each breed age range is the average of 3 sampling points spread through the different weeks. Significant differences (P<0,05) are indicated by (*).

Feed efficiency in terms of feed consumed per egg produced was 2% better for the GHI program, needing 4 grams less of feed that the control group to produce each egg. This non-significant increase is mainly due to the higher egg production. No differences were found neither in egg or in DOC weight, however other indicators of DOC quality were significantly improved (p<0.05) by the GHI. The Pasgar score was increased in DOC from relatively young breeders (40 to

49 wks) and mid age in a minor proportion (from 50 to 59 wks), after this point no differences were found. A similar result was found for YFBM, which was higher than the control group only in hens aged less than 49 weeks. The total antioxidant capacity (TAC) evaluates the antioxidant response against the free radicals in a given sample. From weeks 60 to 70, this variable was significantly higher ($P < 0.05$) by 5.7% in DOC's serum from GHI program chicks compared with the control group (Fig. 3).

IV. DISCUSSION

This study showed that phytogenic gut-health additives can improve DOC production and quality. This effect is likely related with gut health enhancing effects such as antimicrobial, reducing pathogens; anti-inflammatory, driving the use of most nutritional resources towards production; and anti-oxidant, maintaining the gut barrier integrity and function (Botsoglou et al., 2005; Mousavi et al., 2018; Abdel-Wareth and Lohakare, 2020). The higher DOC production was due to persistency after 35 weeks of age, fertility, and hatchability, in partial agreement with the results from Chilante et al. (2012) showing increased laying rate in breeder hens fed a mixture of essential oils containing carvacrol and pepper extract from 25 to 34 w of age with similar hatchability and better cumulative livability, however Videla and collaborators (2020) did not find this effect using thymol in quails.

Besides egg storage and incubation (Narinc & Aydemir, 2021), breeder nutrition can also influence DOC quality, as it has the potential to improve parameters as bacterial contamination of eggs and transfer of antioxidants and immunity to the progeny (Johnson-Dahl et al., 2017; Bonagurio et al., 2020; Surai, 2020), which influence the Pasgar score (Vieira, 2007). The DOC from the hens in the program had higher scores, indicating better egg quality and transfer of nutrients. Studies have shown a positive relation between YFBM and subsequent performance of the bird after placement (van der Wagt et al., 2020). A small yolk sac in relation with body weight at hatch is preferred, which agrees with the findings of this study in the GHI group. The GHI supplementation in breeders resulted in increased TAC in chick serum during the late phase of laying (after week 60), similar to findings by Johnson-Dahl et al. (2017) supplementing canthaxanthin. In conclusion, the GHI, positively impacted breeder productivity and DOC quality, and can be regarded as an effective nutritional tool to deal with various commercial stressors.

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THE INFLUENCE OF A BLEND OF PROTECTED ORGANIC ACIDS AND ESSENTIAL OILS ON THE INTESTINAL AND OVERALL HEALTH STATUS OF COMMERCIAL PULLETS

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Organic acids (OA) and essential oils (EO) are among the most studied natural feed additives as they are shown to exhibit antimicrobial properties. Additionally, organic acids also influence diverse regulatory functions on host physiology, metabolic regulation, inflammation, and immunity (Khan and Iqbal, 2016). Essential oils, on the other hand, has broad spectrum antimicrobial, immunomodulatory, and antioxidant properties (Gopi et al., 2014). When combined, OA and EO can have a synergistic effect, being more efficacious in improving intestinal health and in modulating the microbiota (Stefanello et al., 2020). However, in their free form, these compounds may be lost during feed processing, lose their activity, or be absorbed early in the gastrointestinal tract (Michiels et al., 2008). The microencapsulation of OA and EO using a lipid matrix offers the potential to not only protect them, but also to control their subsequent release as they pass through the GIT, maximizing their effectiveness (Hassan et al., 2018). A study was conducted to test the hypothesis that a blend of protected organic acids and essential oils P(OA+EO) will promote better intestinal and overall health status of pullets raised under commercial conditions.

A total of 208,000 2-week-old pullets were raised in the same shed and divided into two groups: T1, the control group which received the standard diet in mash form, and T2, the group which received the same diet plus the P(OA+EO) at 300 g/t. The P(OA+EO) consists of fumaric, sorbic, malic, and citric acids and thymol, vanillin, and eugenol. It was supplemented from 2 to 18 weeks and the trial lasted until 21 weeks. At weeks 6, 12 and 21, 12 birds/treatment were used for blood sampling and necropsy for *I See Inside* (ISI) methodology, described by Kraieski et al. (2017). Fluorescein isothiocyanate-dextran (FITC-d) was inoculated to the birds and blood samples were collected after 1.5 h to evaluate intestinal integrity. The macroscopic ISI score was used to rate overall health status, while the macro-intestinal ISI score evaluated the condition of specific segments of the intestine. The histologic intestinal ISI score assessed the health of the ileum. A low ISI score indicated a better health status. Data were subjected to ANOVA to analyze parametric data (FITC-d data) and Kruskal Wallis to analyze non-parametric data (ISI data) using the XLSTAT software. A $P \leq 0.05$ was used to indicate statistical significance and a $P < 0.10$ was used to indicate tendency. Pullets fed with P(OA+EO) had lower ($P < 0.001$) levels of FITC-d, indicating reduced leaky gut. They also presented lower overall ISI scores at weeks 6 ($P = 0.002$) and 12 ($P = 0.003$), lower macro intestinal ISI scores at weeks 6 ($P = 0.0001$) and 21 ($P = 0.004$), and lower histologic intestinal ISI score at weeks 6 ($P = 0.09$), 12 ($P = 0.0006$), and 21 ($P < 0.0001$). Furthermore, at week 21, pullets on P(OA+EO) did not present *Eimeria* oocysts but were present in the control treatment ($P < 0.0001$). Both treatments reached 21 weeks with body weights and uniformity in accordance to the genetic guidelines. In conclusion, the blend of protected organic acids and essential oils evaluated in this study has potential applications to promote better intestinal health and overall health status in commercial pullets.

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SHELF-LIFE ENHANCEMENT OF GROUND CHICKEN MEAT USING COMBINATIONS OF NATURAL ANTIOXIDANT AND VINEGAR

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Summary

Recent consumer concerns over the use of synthetic preservatives have stimulated an interest in the development of preservatives based on rosemary extract to extend the shelf-life of fresh chicken meat. The purpose of this study was to confirm the efficacy of rosemary extract in suppressing spoilage microorganisms and increasing the shelf-life of fresh ground chicken thighs while maintaining quality and sensory attributes. Rosemary extract was evaluated on its own and in combination with natural vinegar-based acetic acid salts such as dried vinegar. At refrigerated storage temperatures, dried vinegar alone or in combination with minimal use of rosemary extract was effective in controlling the growth of aerobic plate count and *Enterobacteriaceae* in ground chicken. The combination treatment (0.0033% rosemary extract + 0.75% dried vinegar) significantly enhanced the shelf-life of ground chicken to 14 days as compared to the control (no preservative), which reached spoilage threshold (6 logs CFU/g) for aerobic plate count by 6 days. Therefore, the studies conducted using dried vinegar in combination with rosemary extract showed suppression of the growth of spoilage microorganisms and extended the shelf-life of freshly ground poultry meat by approximately 100%.

I. INTRODUCTION

Natural vinegar-based solutions have been known to possess antimicrobial properties and can create an antimicrobial-antioxidant system when combined with plant extracts, which could potentially extend the shelf-life of poultry meat (Heintz et al., 2018 and Heintz et al., 2019). Plant extracts which contain phenolic compounds, have therapeutic effects against viruses, bacteria, and fungi. Rosemary belongs to the mint family and has antinociceptive, antioxidant, antimicrobial, and anti-inflammatory effects (Zaouali et al., 2010 and Sternisa et al., 2020). It contains phenolic compounds (such as carnosol, rosmarinic acid, and caffeic acid), flavonoids (such as diosmin, luteolin, and gencuanine) and monoterpene (such as camphor, cineole, and borneol) (Sternisa et al., 2020). The present study was conducted to evaluate the effect of adding different combinations of dried vinegar and rosemary extract on the shelf-life of freshly ground chicken meat.

II. METHOD

Different treatments of ground chicken thighs (97% poultry and 3% water adjusted with dried vinegar or rosemary extracts as per CFR 319.140) were prepared. For uniform distribution of powdered rosemary extract (Sigma-Aldrich), 1.5% water was added across all the treatments. Treatments included control (no preservative), rosemary extract at a concentration of 0.0033% 0.0067%, 0.0102%, 0.0132% and 0.0170% alone or in combination with 0.75% dried vinegar (IsoAge DV100). For each treatment, 454 g of fresh ground chicken was weighed, and the appropriate antimicrobial treatment solutions were mixed. The prepared ground chicken was

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divided into 20 g samples, with duplicates allocated to each treatment. Samples were aerobically packaged and appropriately labeled and stored in a refrigerator at 4 °C. On each sampling day i.e., day 0, 2, 4, 6, 8, 10, 12, and 25, two samples from each treatment were removed and placed into a filter whirl-pack bag containing 180 mL buffered peptone water. Samples were stomached for 2 minutes followed by serial dilution. The sample filtrate was diluted in buffered peptone water and spread plated for enumeration of aerobic plate count on Plate Count and for enumeration of *Enterobacteriaceae* on Violet Red Bile (VRB) agar, respectively. Plates were incubated at 37 °C for 24-48 hours. Spoilage threshold and maximum population density at end of stationary phase was considered as 6 and 8 log CFU/g for aerobic plate count and *Enterobacteriaceae*, respectively. Data generated were used for primary modelling using modified Gompertz equation to calculate maximum growth rate (log/day) and days to reach spoilage threshold for each treatment. Differences among the treatments were determined using one-way ANOVA. Statistical analysis and model building was performed in JMP Pro version 15.1.0 (SAS Institute Inc., NC, US), with significance set at $P \leq 0.05$.

III. RESULTS

Overall, rosemary extract (0.0102% or greater) in combination with 0.75% dried vinegar slowed the growth of aerobic plate count and *Enterobacteriaceae* in ground chicken meat (Table 1). Specifically for aerobic plate count and *Enterobacteriaceae*, the combined treatment (≥ 0.0102% rosemary extract and 0.75% dried vinegar) significantly increased the shelf life of ground chicken by 8 and 11 days as compared to the control (no preservative), which reached spoilage the threshold by 6 and 8 days, respectively. Furthermore, sensory review conducted showed that with rosemary was more acceptable than the control treatment on day 8.

Table 1 - Time (days) to end of shelf life at 4 ˚C for ground chicken meat.

Treatment	Days to end of shelf life (spoilage threshold)	
	Aerobic Plate Count (6 logs CFU/g)	*Enterobacteriaceae* (8 logs CFU/g)
Control	6.46 ± 0.079^{A}	8.17 ± 0.006^{D}
0.0033% Rosemary extract	6.28 ± 0.026^{A}	8.05 ± 0.063^{D}
0.0067% Rosemary extract	5.93 ± 0.093^{A}	7.71 ± 0.148^{D}
0.0102% Rosemary extract	6.56 ± 0.039^{A}	8.33 ± 0.079^{D}
0.0132% Rosemary extract	6.90 ± 0.196^{AB}	8.09 ± 0.064^{D}
0.0170% Rosemary extract	5.85 ± 0.509^{A}	7.92 ± 0.148^{D}
0.75% Dried vinegar	9.52 ± 1.05^{BC}	18.26 ± 0.711^{E}
0.0033% Rosemary extract + 0.75% dried vinegar	11.66 ± 0.075^{CDE}	18.43 ± 0.291^{E}
0.0067% Rosemary extract + 0.75% dried vinegar	12.76 ± 0.902^{DE}	17.51 ± 3.057^{E}
0.0102% Rosemary extract + 0.75% dried vinegar	14.16 ± 0.224^{E}	18.51 ± 0.298^{E}
0.0132% Rosemary extract + 0.75% dried vinegar	12.07 ± 0.550^{CDE}	18.71 ± 0.112^{E}
0.0170% Rosemary extract + 0.75% dried vinegar	11.40 ± 0.461^{CD}	19.01 ± 0.305^{E}

*Dried vinegar was IsoAge DV100

Data represented as mean ±standard deviation (days). Different letters in the same column depict significant differences between treatments ($p \leq 0.05$).

IV. DISCUSSION

Dried vinegar has previously been shown to have has effective antimicrobial properties (Heintz et al., 2018), which occur through interactions with bacterial cell membrane processes such as electron transfer and ionic gradient. Similarly, other studies the have shown the antimicrobial effect of rosemary extract on spoilage organisms (Jawad et al., 2018; Tural et al., 2019). This study has shown that combining dried vinegar and rosemary extract can greatly increase the shelf life of ground chicken meat by 100% compared to untreated chicken meat. This agreed

with previous studies reported by Sternia et al., 2020 that showed a combination of rosemary extract and buffered vinegar inhibited the growth of various species of *Pseudomonas*. The combined antimicrobial action is not completely understood but is believed to be due to the addition of secondary plant metabolites that react with a wide range of cellular components causing protein translocation, phosphorylation, and other enzyme-dependent reactions (Wu et. al., 2020). Therefore, additional research may be required to completely understand the synergistic level by challenging the solutions in a controlled inoculated study with spoilage organisms at varying levels of temperatures and simultaneously explore the mechanism of actions of dried vinegar and rosemary combined.

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POSITIVE EFFECT OF *QUILLAJA SAPONARIA* AND *YUCCA SCHIDIGERA* BLEND IN COCCIDIOSIS CHALLENGE MODEL AND POTENTIAL MODE OF ACTION

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Summary

Numerous scientific publications and commercial use data indicate the positive effect of *Quillaja saponaria* and *Yucca schidigera* combination product (QY) on broiler performance when exposed to an *Eimeria* spp. challenge. This has created the perception of an anticoccidial effect of such a combination. To assess this hypothetical effect and identify the specific mode of action of QY in coccidia challenged broilers a series of *in vivo* and *in vitro* trials have been carried out. In the *in vitro* study QY did not exhibit a direct anticoccidial effect, assessed by the reduction of sporozoite viability during *in vitro* incubation at physiologically relevant concentrations in comparison to registered anticoccidial products such as salinomycin and toltrazuril. However, QY demonstrated numerous beneficial effects when used alone or in combination with either a coccidiosis vaccine or an in-feed anticoccidial when birds were exposed to coccidiosis challenge. QY had a positive effect on performance prior to the challenge (d 0-14) on oocyst shedding expressed as oocyst per gram feces (OPG) and performance during the recovery phase (d 28-35), but not during the acute phase (d 14-28). This suggests that the positive effect of QY under coccidiosis challenge is due to improved immunity development, reduced inflammation and tissue damage and faster recovery, rather than direct anticoccidial effect.

I. INTRODUCTION

Natural triterpenoid saponins from *Quillaja saponaria* such as QS 21, QS 17, QS 18 and QS7 are known to support specific immune response towards different pathogens (Lacaille- Dubois, 2019; Marciani et al., 2000). As well, natural polyphenols from both *Q. saponaria* and *Yucca schidigera* such as piscidic acid, vanillic acid, ferulic acid, p-coumaric acid, resveratrol and yuccaols are known to have antioxidant and anti-inflammatory effects (Maier et al., 2015; Piacente et al., 2005). Furthermore, numerous scientific publications (Bafundo et al., 2020, Bafundo et al., 2022) and commercial field experience indicate positive effects of the *Q. saponaria* and *Y. schidigera* combination product (QY) (Magni-Phi®), containing a minimum of 3.5% triterpenoid (Quillaja) saponins and typically 0.8-1.0% of total polyphenols expressed as gallic acid equivalent on broiler performance when exposed to an *Eimeria* spp. challenge. This has created a perception of an anticoccidial effect of this combination. The current study aims to assess this effect and helps to identify the specific mode of action of QY in coccidia challenged broilers.

II. METHOD

a) In vivo study. A total of 1 848 day old male Ross 308 broilers were allocated into 7 treatments: T1 - uninfected untreated control (UUC); T2 - infected untreated control (IUC); and five infected treatments: T3 - supplemented with QY; T4 - supplemented with in-feed anticoccidial: narasin+nicarbazin (100 ppm 0-21 d), followed by salinomycin (60 ppm 22-35d)

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(ACC); T5 - same coccidiostat treatment supplemented with QY (ACC+QY); T6 - birds vaccinated for coccidiosis at day 0 with a commercial attenuated vaccine Evant® (VAC); and T7 - the same vaccine treatment supplemented with QY (VAC+QY). In treatments 3, 5 and 7 QY (Magni-Phi®) was used at 250 g/t from 0 to 35 d. Each treatment had 8 replicates, 35 birds each, in floor pens on fresh wood shavings. All treatments, except UUC, were challenged on day 14 by spray on feed and litter with a mix of *E. acervulina*, *E. maxima*, *E. mitis* and *E. tenella* isolates at a total of 199,000 oocysts/bird, to mimic natural field infection causing subclinical infection.

Body weight (BW) and daily weight gain (DWG) were measured at day 14, 28 and 35 and feed conversion ratio (FCR) was calculated respectively. Fecal samples for oocyst count per gram (OPG) were collected per pen and pooled per treatment at day 6, 7, 8 and 14 to confirm the status of the birds prior to the challenge. In addition, fecal samples were collected and counted per pen after the challenge for OPG at day 21, 22, 28 and 35. Total mean lesion score (TMLS), as a sum of individual *E. acervulina*, *E. maxima* and *E. tenella* lesion scores, were recorded according to Johnson and Reid (1970) at day 21, 22 and 28 based on four birds per pen each time. Statistical analysis using Fisher LSD test was applied; paired QY (+) and (–) treatments were compared with paired T test at $P \leq 0.05$.

b) In vitro study. In the *in-vitro* study *E. tenella* sporozoites were incubated for 72 h in 20% *Quillaja* extract (QE) and QY solutions with concentration respectively 8.75; 17.5 and 35.0 µl/l; 50, 100 and 200 mg/l mimicking *Quillaja* saponin intestinal concentration corresponding to 250 g/t, 500 g/t, and 1000 g/t in-feed application of QY, taking into account respective dry matter of feed and intestinal content. The effect of the QE and QY on sporozoites was assessed based on sporozoites counts at 24, 48 and 72 hours of incubation and compared to sporozoites count in negative controls (solvent only – PBS and dimethyl sulfoxide – DMS) and positive controls – salinomycin at 9 and 12 mg/l mimicking intestinal concentration respective to in-feed application of 45 and 60 ppm and toltrazuril at the in-water therapeutic dose of 25.0 mg/l.

III. RESULTS

a) In vivo study. Overview of parasitological results is provided in Table 1. Prior to the challenge at day 14 oocysts shedding was identified only in VAC and VAC+QY confirming the vaccine cycling and the coccidia-free status of all other treatments. A successful natural *Eimeria* challenge was obtained, evidenced by significantly higher TMLS and OPG at both 21-22 and 28 days in IUC compared to UUC. At day 21 and 22 only ACC+QY provided OPGs significantly lower than IUC. At day 35, QY and VAC+QY provided OPG significantly lower compared to IUC. Only ACC and ACC+QY provided significant reduction of macroscopic coccidia lesions at day 21-22 in comparison to IUC. There was no significant difference in any of the parasitological parameters between VAC and VAC+QY indicating no QY interference with coccidiosis vaccination.

Oocysts shedding in the UUC appeared at day 28, indicating contamination of the UUC group from neighboring pens. It caused coccidiosis cycling manifested by increased OPGs at day 35, at a level significantly higher than IUC and all treatments.

Mortality was not significantly different from the UUC for any of the infected groups including IUC. Overview of zootechnical performance is provided in Table 2. The highest BW and lowest FCR for the overall 0-35 day period were achieved in the ACC and ACC+QY treatments and were significantly better compared to all other groups. VAC had significantly higher FCR than all other infected treatments. QY significantly improved FCR compared pairwise for IUC vs QY and VAC vs VAC+QY with a P value of 0.024 and 0.048 respectively.

Table 1 - Overview of parasitological parameters during the different study periods per treatment group.

Treatment	Total OPG d 14	Total OPG d 21	Total OPG d 22	Total OPG d 28	Total OPG d 35	TMLS d 21-22	TMLS d 28
UUC	0.0	67^{a}	47^{a}	32022^{a}	91503^{d}	0.9^{e}	1.5^{c}
IUC	0.0	54451^{b}	56809^{c}	213835^{bc}	10381^{c}	1.9^{ab}	2.1^{bc}
QY	0.0	57203^{b}	87653^{c}	228213^{bc}	1916^{a}	2.2^{a}	3.2^{a}
ACC	0.0	19347^{b}	14649^{bc}	475283^{c}	6935^{bc}	1.0^{de}	2.7^{ab}
ACC+QY	0.0	6082^{b}	3921^{b}	183419^{b}	6588^{bc}	1.4^{cd}	2.9^{a}
VAC	3600	13100^{b}	14686^{bc}	336172^{bc}	3538^{abc}	1.6^{bc}	2.8^{ab}
VAC+QY	16000	31513^{b}	25519^{bc}	163397^{b}	2865^{ab}	1.7^{bc}	2.6^{ab}

OPG – total oocyct count per gram feces; TMLS – total mean lesion score as a sum of individual *E. acervulina*, *E. maxima* and *E. tenella* lesion scores, according to Johnson and Reid (1970). Means with different superscripts are significantly different at $p < 0.05$ (LSD Fisher test)

In the period before the challenge (0-14 d) ACC and ACC+QY had significantly highest BW and lowest FCR. QY improved FCR significantly when used alone compared to non-treated groups and provided statistically the same FCR as the coccidiostat groups. VAC had significantly highest FCR and lowest BW. QY helped to partly alleviate the negative effects of the coccidiosis vaccination: VAC+QY had significantly higher BW compared to VAC.
In the acute period after the challenge (14-28 d) the IUC had significantly lower DWG and higher FCR compared to UUC, demonstrating the success of the challenge model and the impact of subclinical coccidiosis on performance. Among different infected treatments, significant improvement over IUC was recorded only in ACC and ACC+QY, showing significantly highest BW and lowest FCR. All other treatments were not different from IUC and QY did not provide significant improvement when compared pairwise to the respective non QY group.

In the recovery period (28-35 d), a deterioration of performance was noticed in the UUC caused by late coccidia cycling in this treatment evident also in the OPG counts mentioned above. The only treatment that outperformed the IUC was ACC+QY having significantly higher BWG and lower FCR. QY when added on top of the vaccine or the anticoccidial brought positive effect, statistically significant or tendency, with a P value of 0.05 and 0.09 respectively.

Table 2 - Overview of zootechnical performance during the different study periods per treatment group.

Treatment	BW 14d	BW 28d	BW 35d	DWG 0-14d *	DWG 14-28d	DWG 28-35d **	DWG 0-35d	FCR 0-14d	FCR 14-28d	FCR 28-35d ***	FCR 0-35d ****
UUC	436^{cd}	1494^{b}	2202^{bc}	27.8^{cd}	75.3^{a}	99.2^{b}	55.3^{bc}	1.26^{a}	1.49^{c}	1.63^{ab}	1.47^{c}
IUC	450^{bc}	1470^{b}	2191^{bc}	28.8^{bc}	61.4^{cd}	101.6^{b}	55.0^{bc}	1.27^{a}	1.84^{a}	1.68^{a}	1.52^{b}
QY	453^{b}	1499^{b}	2216^{b}	29.0^{b}	62.6^{c}	103.2^{ab}	55.6^{b}	1.18^{b}	1.80^{a}	1.67^{ab}	1.48^{bc}
ACC	471^{a}	1616^{a}	2334^{a}	30.3^{a}	68.5^{b}	103.1^{ab}	58.2^{a}	1.17^{b}	1.67^{b}	1.68^{ab}	1.43^{d}
ACC+QY	470^{a}	1585^{a}	2362^{a}	30.3^{a}	67.0^{b}	109.8^{a}	58.8^{a}	1.18^{b}	1.69^{b}	1.58^{b}	1.42^{d}
VAC	417^{e}	1391^{d}	2098^{c}	26.6^{e}	58.9^{cd}	97.7^{b}	51.4^{d}	1.29^{a}	1.87^{a}	1.65^{ab}	1.56^{a}
VAC+QY	433^{d}	1404^{cd}	2133^{bc}	27.6d^{e}	58.3^{d}	103.0^{ab}	52.8^{cd}	1.24^{ab}	1.87^{a}	1.60^{ab}	1.51^{b}

BW – body weight in grams; DWG – daily weight gain in grams; FCR – feed conversion ratio
Means with different letters are significantly different at $p < 0.05$ (LSD Fisher test)
*Difference between VAC and VAC+QY is close to significant showing tendency P = 0.083 (paired T test)
**Difference between VAC and VAC+QY and ACC and ACC+QY is close to significant showing tendency P = 0.165 and P = 0.170 respectively (paired T test)
***Difference between VAC and VAC+QY and ACC and ACC+QY is close to significant showing tendency P = 0.052 and P = 0.091 respectively (paired T test)
**** Differences between QY and IUC and VAC and VAC+QY is significant P = 0.024 and P = 0.048 respectively (paired T test)

b) In vitro study: Toltrazuril significantly reduced sporozoites counts in comparison to the negative control at 24, 48 and 72 hours of incubation. Salinomycin, at concentrations mimicking 45 and 60 ppm in-feed application, significantly reduced the sporozoites counts at

48 and 72 hours of incubation while neither QE nor QY provided significant reduction at any of the tested timepoints or concentrations (Fig. 1).

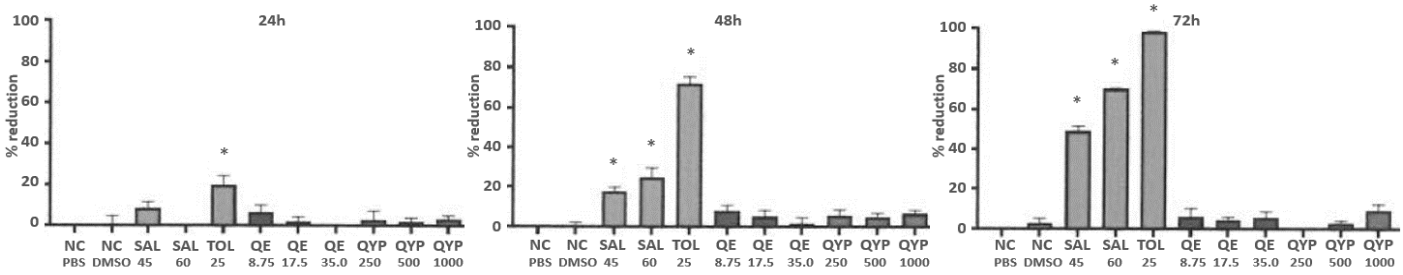

Figure 1 – Reduction (%) of sporozoites counts of different treatments compared to non-treated control after 24, 48 and 72 hours of incubation. Significant difference ($P < 0.05$) based on Kruskal-Wallis test with Dunn's numerous comparison with control is indicated with *.

NC PBS – negative control phosphate buffered saline; NC DMSO – negative control dimethyl sulfoxide; SAL 45 and SAL 60 – positive control salinomycin mimicking intestinal concentration respective to in-feed application of 45 and 60 ppm; TOL 25 toltrazuril 25.0 mg/l; QE 8.75; QE 17.5 and QE 35.0 Quillaja extract solution with saponin concentration of 8.75, 17.5 and 35.0 µl/l; QYP 250; QYP 500 and QYP 1000 Quillaja and Yucca product mimicking intestinal concentration respective to in-feed application of 250, 500 and 1000 g/t.

IV. DISCUSSION

The *in vitro* study did not show a direct anticoccidial effect of the QE or QY on sporozoite viability at physiologically relevant concentrations. Furthermore, the *in vivo* study demonstrated that QY did not affect vaccine cycling before the challenge, but improved performance of the coccidia vaccinated group, demonstrating compatibility with the coccidiosis vaccine. Although QY did not exhibit direct anticoccidial effect on sporozoites the *in vivo* coccidiosis challenge model confirmed the positive effect of QY on birds infected with coccidia as demonstrated in previous studies with *Eimeria spp.* challenged birds. Thus, the QY positive effect on performance prior to the challenge (d 0-14), on OPG and performance during the recovery phase (d 28-35), but not during the acute phase (d 14-28), suggest that the positive effect of Quillaja and Yucca under coccidiosis challenge is due to improved immunity development, reduced inflammation and tissue damage, and faster recovery, rather than direct anticoccidial effect. This is in line with previously reported data of improved cell mediated immunity related to *Q. saponaria* saponins and reduced inflammation and anti-oxidative effect related to *Y. schidigera* and *Q. saponaria* polyphenol fractions.

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EFFECTS OF BUTYRINS AND VALERINS ON GROWTH AND SLAUGHTER PERFORMANCE OF YELLOW FEATHER BROILERS

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Short-chain fatty acids (SCFAs) have multiple functions on gut health, including supporting intestinal epithelial cells as energy sources, strengthening gut barrier function, regulating immune response and developing skeletal muscle (Martin-Gallausiaux et al., 2021; Liu et al., 2021). Among the different SCFAs, butyric acid is the most studied. Previous research has demonstrated that butyric acid can provide approximately 70% of the total energy consumption of the colonocyte (Roediger, 1982). Valeric acid is naturally present in the lower intestinal tract. Compared with butyric and valeric acid or salt, butyrin and valerin have less odor problem and are more and more common used in animal feed. However, there is limited knowledge about the synergic effect of butyrins and valerins on yellow feather broiler growth and slaughter performance.

This study was conducted to evaluate the effects of a synergic combination of butyrin and valerin (Gastrivix™ Avi) on yellow feather broilers. A total of 1,680 one-day-old Datu yellow feather broilers were randomly assigned into two dietary treatment groups (each treatment had six replicates with 140 birds) and fed either a non-supplemented diet or a diet supplemented with Gastrivix™ Avi (500, 500 and 250 g/MT in starter, grower and finisher phases as manufacturer recommended). Feed consumption and body weight of the birds were recorded at 63 days of age. On day 63, 12 birds (2 birds/replicate) were randomly selected from each treatment and euthanized by cervical dislocation for slaughter performance analysis.

Table 1 - d1 to d63 growth and slaughter performance of yellow feather broiler fed with or without Gastrivix™ Avi (data expressed as mean ± standard deviation).

Treatment	ADFI (g/bird/d)	ADG (g/bird/d)	FCR	Dressing yield, %	Abdominal fat (g/bird)
Control	68.23±1.71	28.65±0.82	2.38±0.02[a]	90.83±0.86	40.72±15.10
Gastrivix™ Avi	68.80±1.76	29.30±0.81	2.35±0.00[b]	91.43±1.31	32.85±8.95

Means within a column not bearing a common superscript are significantly different ($P < 0.05$).

A significant improvement in overall FCR was observed comparing the Gastrivix™ Avi supplemented group with the control group. The FCR was 3 points lower for the broilers that received the butyrin/valerin combination in the diet ($P = 0.007$) compared to non-supplemented broilers. Numerically higher dressing yield at day 63 was observed with supplementation of the butyrin/valerin combination compared with the control group. It was also noticed that abdominal fat weight at day 63 was numerically lower by 7.87 g/bird with the supplementation of the combination of butyrin and valerin in comparison to the non-supplemented group. In conclusion, butyrin/valerin combination at the recommendation dosage could improve the yellow feather broiler growth performance. Results suggest that butyrin/valerin combination could have an impact on the lipid metabolism in the animal which would explain the tendency to decrease the abdominal fat ($P=0.07$), however further studies would be needed to confirm this theory.

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USING GASTROINTESTINAL FLUIDS AS AN EX VIVO METHOD TO ASSESS ANTIBACTERIAL PROPERTIES OF GLYCERIDES OF LAURIC ACID

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Summary

This study aims to evaluate the antimicrobial activity of glycerides of lauric acid (GLA) in the gastrointestinal tract (GIT) using an *ex vivo* method. Digestive fluids were collected from broiler chickens that were fed a standard diet or a diet supplemented with GLA. The samples were then tested for antimicrobial activity against *Enterococcus faecalis* and *Escherichia coli*. The results showed that the GIT fluids from animals receiving GLA had antibacterial action against both Gram-positive and Gram-negative microorganisms. The study suggests that GIT fluids can be a useful *ex vivo* approach for assessing the antibacterial activity of feed supplements, and it highlights the mode of action of GLA in suppressing pathogenic bacteria. Further research is needed to identify the specific metabolites responsible for the antibacterial action against Gram-negative bacteria.

I. INTRODUCTION

In vitro experiments can provide insight in the antibacterial potential of molecules, but do not always represent their activity *in vivo* because some factors that impact their function are not considered. This explains why phenomena observed in *in vitro* and *in vivo* models can differ. Therefore, we suggest an *ex vivo* method to detect and quantify antimicrobial activity to define more precisely the behavior of molecules in the gastrointestinal tract (GIT). For this purpose, we assessed glycerides of lauric acid (GLA). GLA have been described *in vitro* to have strong antibacterial activity against Gram-positive (G+) and limited activity against Gram-negative (G-) microorganisms (Lieberman et al., 2006). GLA alter the bacterial phospholipid membrane. The G+ are more sensitive because their characteristic single lipid bilayer cell membrane (Jackman et al., 2020). The use of GLA as a feed supplement to prevent intestinal bacterial infections helps animals to be more resilient to pathogen threats and to perform better (Fortuoso et al., 2019). To validate GIT fluids as an *ex vivo* test, they were extracted from animals receiving a GLA-supplemented feed.

II. METHOD

One hundred Ross 308 broiler chickens were used. Fifty broilers were allocated to the control group, receiving a standard corn-wheat based diet. The other fifty broilers received the same diet supplemented with GLA at a dose commercially relevant of 3 kg/ton. At day 28, samples of crop, ileum, and caecum contents were taken for antimicrobial assessment. Samples from the caeca were also collected for 16S rRNA sequencing. Samples for antibacterial activity were centrifugated and filter-sterilized. Subsequently, GIT fluids were evaluated by utilizing Minimal Inhibitory Concentration (MIC) assays following the Clinical and Laboratory Standards Institute (CLSI) standard protocol against *Enterococcus faecalis* ATCC 29212 and *Escherichia coli* DSM 103262, an avian pathogenic *E. coli* (APEC). The MICs were performed

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in triplicate and the results are expressed as average dilution of digestive fluids. This means that the digestive fluids with a higher dilution have a stronger antibacterial effect.

III. RESULTS

GIT fluids from animals receiving GLA were shown through MIC analysis to have antibacterial action against G+ and G- microorganisms. Specifically, the GIT fluids from the crop, ileum, and caeca strongly inhibited *E. faecalis* growth ($P = 0.0029$; $P < 0.0001$ and $P = 0.0353$; respectively) (*Figure 1*). This *ex vivo* antimicrobial activity demonstrates that added GLA maintains its antimicrobial action throughout the GIT, ensuring animal protection from pathogen assaults.

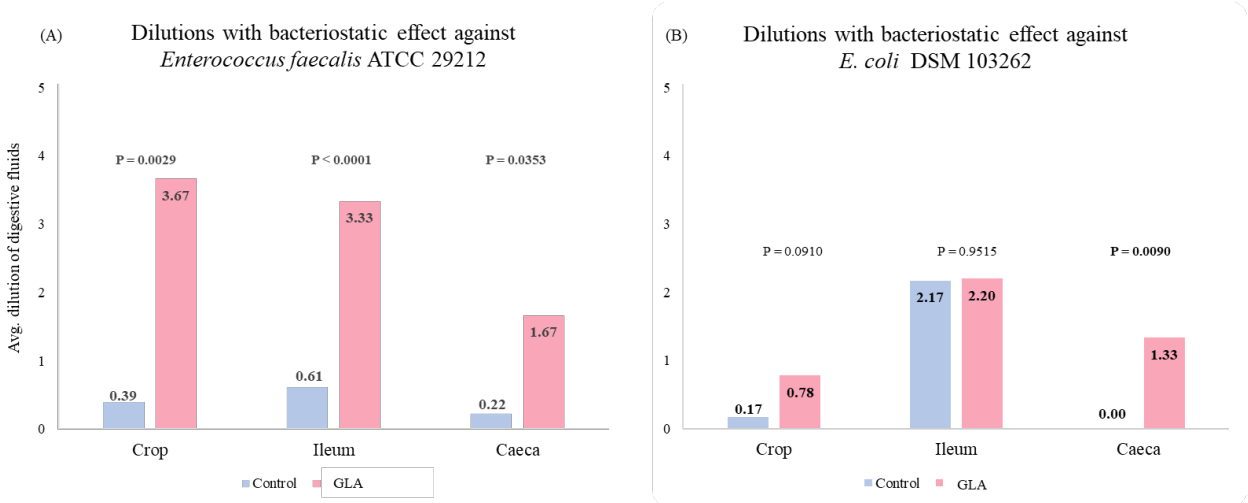

Figure 1 - Activity against *Enterococcus faecalis* ATCC 29212 (A) and *E. coli* DSM 103262 (B) of digestive fluids from broilers receiving a standard diet (control) or standard diet plus GLA at 3kg/Ton.

Interestingly, antibacterial action against the APEC strain was only shown for cecal fluids ($P = 0.0090$). We hypothesized that this antibacterial activity could be explained by a microbial shift on cecal microbiota exerted by GLA towards a microbial profile of metabolite producers that may inhibit G- bacteria. Microbiota analysis showed indeed a shift on G+ positive bacteria favoring *Lactobacillaceae* presence and decreasing *Streptococcaceae* populations.

IV. DISCUSSION

Our investigation demonstrated that GIT fluids are a promising *ex vivo* approach for determining the antibacterial activity of molecules supplemented to the feed, and it also reveals that GLA has a dual mode of action to suppress pathogenic bacteria. First, they can directly restrict growth of G+ bacteria throughout the GIT. Second, they cause a change in the microbiota towards a population producing antimicrobial compounds, indirectly inhibiting G- bacteria in the ceca. More research will be required to describe and pinpoint the precise metabolites produced by the microbiota that are responsible for this G- antibacterial action.

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ESCHERCHIA COLI LOAD IN THE GUT AND WEIGHT GAIN IN BROILERS: INSIGHTS FROM A SYSTEMATIC REVIEW AND META-ANALYSIS

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Escherichia coli, a gram-negative bacterium is one of the dominant members of the commensal microbiota in young broiler chickens (Ocejo et al., 2019). However, under certain circumstances, it can also cause colibacillosis, which is an economically important disease. In studies aimed at improving broiler chicken production, the increase in the load of the phylum *Proteobacteria* that includes *E. coli,* is regarded as an indicator of dysbiosis and poor gut health (Diaz Carrasco et al., 2019; Shin et al., 2015), contradicting the role of the organism as a commensal bacterium (Rychlik, 2020) Therefore, the precise link between *E. coli* load and broiler growth performance has remained elusive. This systematic review was conducted to investigate whether there is an association between *E. coli* load in different gut sections of broiler chickens and body weight gain at different stages of growth. We conducted a comprehensive search following Preferred Reporting Items for Systematic Reviews and Meta-analysis (PRISMA) guidelines to collect eligible peer-reviewed published articles from PubMed and the Web of Science. Among the initial pool of 2,108 articles screened, 60 were deemed suitable for inclusion in the final meta-analysis. Criteria for inclusion were peer-reviewed articles in English, experimental studies with broiler chickens as subjects, inclusion of body weight and *E. coli* in counts presented as averages with SD or SEM, inclusion of a negative control group, and maximum chicken age of 42 days. To address the inherent variability among studies, standardized procedures were adopted during data extraction. Ultimately, we focused on records from the ileum and caecum at 21, 35, and 42 days of age in untreated negative control groups of broilers from thae selected studies. Random effects meta-regression models were constructed for each gut section and age combination, using a significance level of 0.05. The residuals of each final model were visually checked for normality, and bubble plots were generated to show the relationship between *E. coli* count and weight gain at the study level. The results unveiled a positive association between *E. coli* count in both ileum and caecum at 21 days of age and the weight gain in broiler chickens. However, no significant associations were observed at 35 and 42 days of age. In summary, the association between *E. coli* load and body weight gain in broiler chickens appears to be age dependent. This dependency could be attributed to the relative dominance of *E. coli* during the early stages of the birds' development when microbial diversity is limited. The dynamic association between the *E. coli* load and weight gain underscores the importance of carefully assessing commensal *E. coli* to support bird health during the critical early growth phases, while considering its potential pathogenicity.

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MULTI STUDY ANALSYSIS COMPARING SALINOMYCIN WITH NARASIN

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A multi study analysis was performed to compare the efficacy of salinomycin with narasin. The analysis was performed using data of 18 different anticoccidial sensitivity tests (ASTs) conducted over a 7-year period (2013-2019). The *Eimeria* isolates were collected in commercial farms from 13 different countries all using different coccidiosis control programmes. Sensitivity of *Eimeria* parasites to anticoccidial products can be assessed by performing AST trials (Cervantes & McDougald, 2022). Faecal samples were collected in commercial farms from 13 different countries. In all farms coccidiosis prevention was done by inclusion of anticoccidial products in the feed. The historical use and nature of the products at the time of sample collection was different for each sample. Sensitivity trials were conducted by 2 different research institutes, using comparable, standardised AST protocol. Groups of birds, reared in cages, were supplemented with salinomycin or narasin starting 2 days before oral inoculation with the different *Eimeria* field strains. The inclusion rate of both ionophores was always identical per trial and varied between 60 and 70 ppm. Feed samples were collected, and the target concentration of the coccidiostats were confirmed. From day of allocation until the end of the trial, average daily gain (ADG), average daily intake (ADI) and feed conversion ratio (FCR) were evaluated. All variables were analysed using a linear mixed effects model with the treatment, research institute and an interaction between them included as fixed effects, and a random effect for the specific study. The estimated marginal means were used to evaluate the differences. On average the salinomycin group had a higher ADG (45.1 g/day vs. 44.0 g/day, $p = 0.14$) and ADI compared with the narasin group (84 g vs 82.2 g, $p = 0.04$). The FCR was lower (better) in the salinomycin group (2.03 vs 2, $p = 0.36$) even without applying a correction for the higher weights. When calculating the averages per treatment the salinomycin group had a higher ADG and ADI compared with the narasin group. The FCR also improved in the salinomycin group even without applying a correction for the higher weights. When evaluating performance of different ionophores there will always be a difference between results of samples collected on different farms. Main drivers for the efficacy are the intrinsic efficacy of the product and the historical use of the different products on the farm. The best possible way to evaluate the difference between products is to do a multi study analysis using samples with different background and evaluate their average performance. This study indicated that birds treated with salinomycin had a higher ADG and ADI compared with birds treated with narasin. The FCR was lower (better) in the salinomycin group even without applying a correction for the higher weights.

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EFFICACY OF DIFFERENT IONOPHORE-NICARBAZIN COMBINATION PRODUCTS IN COCCIDIOSIS CHALLENGE MODEL

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Summary

This study investigated the efficacy of different ionophore-nicarbazin combination products using 400 male, day old, Ross 308 birds divided into wired floor battery cages (5 birds each) in 8 groups: 1) Uninfected Untreated Control (UUC); 2) Infected Untreated Control (IUC); 3) Monensin 50 ppm + nicarbazin 50 ppm; 4) Salinomycin 50 ppm + nicarbazin 50 ppm (SAL+NIC); 5) Narasin 50 ppm + nicarbazin 50 ppm (NAR+NIC); 6) Maduramicin 3.75 ppm + nicarbazin 40 ppm (MAD+NIC); 7) Semduramicin 15 ppm + nicarbazin 40 ppm (SEM+NIC low) and 8) Semduramicin 18 ppm + nicarbazin 48 ppm (SEM+NIC high), with 10 replicates per treatment. Birds were individually weighed at day 14 and provided with feed supplemented according to the allocation above. At d16 all birds, except for the UUC group, were individually gavaged with 1 ml of a mixed coccidia inoculum containing 130 000 *Eimeria acervulina*, 22 000 *E. maxima* and 8 000 *E. tenella* sporulated oocysts, all of them, from a recent field isolate originating from the Netherlands. All remaining feed was weighed at day 23. Half of the birds from each group were individually weighed, sacrificed and lesion scored at day 22, and the remaining birds followed the same protocol at day 23. Oocyst shedding (OPG), intestinal mean lesion scores for the three major *Eimeria* spp. used for the challenge and total mean lesion score (TMLS) as a sum of the above, body weight (BW), average daily weight gain (DWG), daily feed intake (DFI) and feed conversion ratio (FCR) were recorded and compared between treatments.

All treatments provided an improvement of DWG compared to IUC. Highest improvement was recorded in the SEM+NIC high group, which was the only group not differing from UUC ($P > 0.05$), completely alleviating the effect of the challenge. According to Chapman (1980) this indicates sensitivity for the respective treatment. All other treatments were significantly different ($P < 0.05$) to both UUC and IUC indicating partial resistance (reduced sensitivity). However, none of the treatments demonstrated complete resistance. The least effect was recorded in the NAR+NIC treatment, where DWG was significantly lower than all other combination treatments ($P < 0.05$). Feed intake was impaired in all treatments, except for both SEM+NIC low and high groups where it was not different from the UUC ($P > 0.05$). All treatments improved FCR compared to IUC ($P < 0.05$), with highest improvement in the SAL+NIC group. Lesion scores in all treatments except NAR+NIC were significantly reduced compared to IUC ($P < 0.05$). The highest reduction of TMLS was recorded in SEM+NIC high which was significantly lower than all other treatments ($P < 0.05$), except for SAL+NIC. None of the treatments significantly reduced *E. acervulina* lesions compared to IUC ($P > 0.05$). *E. maxima* lesions were significantly reduced ($P < 0.05$) in SEM+NIC high, SAL+NIC and MON+NIC compared to IUC and only NAR+NIC did not significantly reduce the lesions of *E. tenella* in comparison to IUC. None of the ionophore-nicarbazin combination treatments reduced oocyst shedding in comparison to IUC ($P > 0.05$).

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I. INTRODUCTION

Coccidiosis caused by unicellular protozoa from the genus *Eimeria* is one of the major veterinary challenges in the poultry industry, causing annual losses of more than $14 billion worldwide (Blake et al., 2020). *Eimeria* spp. need to invade intestinal epithelial cells to replicate. This leads to destruction of the intestinal epithelia, inflammation, reduced nutrient absorption and secondary infections such as nonspecific enteritis (also referred to as dysbacteriosis), and necrotic enteritis. In some cases, coccidiosis leads to intestinal hemorrhages and increased mortality. Although coccidiosis major detrimental effect remains impaired performance – increased feed conversion ratio (FCR) and reduced body weight gain (BWG). *Eimeria* parasites are well adapted to the host and the industrial production settings and quite robust in the environment. Thus, eradication strategies are not feasible, and coccidiosis requires continuous management programs based on in-feed anticoccidial use or vaccination. The latter can provide good coccidiosis control when properly applied, but also might increase the risk of intestinal inflammation and respective drop in performance (Stanev et al., 2021). Therefore, the majority of poultry producers nowadays can only rely on in-feed anticoccidials to prevent the negative effect that *Eimeria* spp. exert on broilers health, welfare, and performance (Noack et al., 2019). However, the capability of *Eimeria* to develop resistance towards different anticoccidial drugs might compromise their effect on the field. Due to the synergistic and complementary effect of ionophores and nicarbazin, combination products are the backbone of the in-feed anticoccidial programs worldwide, providing enhanced efficacy, at reduced dose of the individual components, thus increased safety margins and reduced risk of resistance development (Chapman and Rathinam, 2022). This study aims to investigate the efficacy of different ionophore-nicarbazin combination products to a contemporary *Eimeria spp*. isolate, containing *E. acervulina*, *E. maxima* and *E. tenella*, all of them originating from the Netherlands and respectively the extend of resistance development.

II. METHOD

Conway and McKensey (2007) have described a methodology for estimation of the efficacy of different anticoccidial drugs in poultry. Based on that methodology, the current study aimed to assess the efficacy of different ionophore-nicarbazin combination products using 400 male, day old, Ross 308 birds in wired floor battery cages (5 birds each) distributed in 8 treatment groups: 1) Uninfected Untreated Control (UUC); 2) Infected Untreated Control (IUC); 3) Monensin 50 ppm + nicarbazin 50 ppm (MON+NIC); 4) Salinomycin 50 ppm + nicarbazin 50 ppm (SAL+NIC); 5) Narasin 50 ppm + nicarbazin 50 ppm (NAR+NIC); 6) Maduramicin 3.75 ppm + nicarbazin 40 ppm (MAD+NIC); 7) Semduramicin 15 ppm + nicarbazin 40 ppm (SEM+NIC L) and 8) Semduramicin 18 ppm + nicarbazin 48 ppm (SEM+NIC H), with 10 replicates per treatment. Birds were reared in coccidia free environment during the first two weeks of life to avoid immunity development against coccidiosis. No anticoccidial drug was provided during this period. At day 14 all birds were individually weighed and provided with feed supplemented according to the allocation listed above. At day 16 all birds, except for the UUC group, were individually gavaged with 1 ml of a mixed coccidia inoculum containing 130 000 *E. acervulina*, 22 000 *E. maxima* and 8 000 *E. tenella* sporulated oocysts, all from a recent field isolate originating from the Netherlands. Half of the birds from each group were individually weighed, sacrificed and lesion scored at day 22. The remaining birds followed the same protocol at day 23 and all remaining feed was weighed. Efficacy of the treatments was assessed based on oocyst shedding (oocyst per gram of feces or OPG), species specific mean coccidiosis intestinal lesion scores (MLSs) for the three *Eimeria* spp. used for the inoculation according to Johnson and Reid (1970) and total mean lesion score (TMLS) being the sum of the three individual species specific MLSs, body weight (BW) at day 14 and day 23, daily weight gain

(DWG) day 14-23, daily feed intake (DFI) day 14-23 and feed conversion ratio (FCR) day 14-23. Data were analyzed using ANOVA and Fisher's LSD test was used for mean comparison ($P < 0.05$). Test animals were humanely handled. The study was conducted according to Good Clinical Practice and approved by the Poulpharm Ethical Committee.

III. RESULTS

There were no significant differences in mortality between the treatments (2 birds in UUC and one bird in MON+NIC and NAR+NIC each) and was not related to the challenge. Overview of the parasitological results is provided in Table 1. Mean lesion scores for the three major *Eimeria* species in broilers, TMLS and OPG were significantly higher in the IUC compared to UUC, demonstrating effective infection model.

TMLS on day 22-23 in all treatments, except NAR+NIC, were significantly reduced compared to IUC. Highest reduction was recorded in SEM+NIC H which was significantly lower compared to all other treatments, except SAL+NIC. None of the products significantly reduced *E. acervulina* lesions at day 22-23 compared to IUC. *E. maxima* lesions at day 22-23 were significantly reduced in SEM+NIC H, SAL+NIC and MON+NIC compared to IUC. Only NAR+NIC did not significantly reduce the lesions of *E. tenella* compared to IUC.

None of the combination products reduced oocyst shedding (total OPG) at day 23 in comparison to IUC.

Table 1 - Overview of parasitological. parameters per treatment group.

Treatment	MLS *E. acervulina* d 22-23	MLS *E. maxima* d 22-23	MLS *E. tenella* d 22-23	TMLS d 22-23	OPG d 23
UUC	0.4^{b}	0.29^{e}	0.16^{de}	0.85^{e}	$4\,600^{c}$
IUC	1.98^{a}	1.20^{ab}	1.20^{a}	4.38^{a}	$903\,200^{b}$
MON 50 NIC 50	2.29^{a}	0.78^{cd}	0.71^{bc}	3.70^{b}	$549\,320^{bc}$
SAL 50 NIC 50	2.04^{a}	0.71^{d}	0.38^{de}	3.00^{cd}	$575\,800^{bc}$
NAR 50 NIC 50	2.33^{a}	1.39^{a}	1.00^{ab}	4.62^{a}	$1\,547\,800^{a}$
MAD 3.75 NIC 40	2.18^{a}	1.08^{b}	0.44^{cd}	3.70^{b}	$753\,500^{b}$
SEM 15 NIC 40	2.30^{a}	0.94^{bcd}	0.38^{de}	3.62^{bc}	$615\,800^{bc}$
SEM 18 NIC 48	2.08^{a}	0.68^{d}	0.10^{e}	2.86^{d}	$524\,360^{bc}$

Means with different superscripts are significantly different at $P < 0.05$ (LSD Fisher test).
MLS – mean lesion score for the respective *Eimmeria* spp. According to Johnson and Reid (1970); TMLS – total mean lesion score as a sum of the MLS for *E. acervulina, E. maxima* and *E. tenella*; OPG – oocyst per gram feces

An overview of the performance results is provided in Table 2. BW at day 23, DWG and DFI were significantly lower and FCR significantly higher in IUC compared to UUC demonstrating effective infection model.

All products provided DWG improvement compared to IUC. Highest improvement was recorded in the SEM+NIC H group, which was the only group not differing from UUC, completely alleviating the effect of the challenge. Thus, according to Chapman (1980) the isolate can be considered sensitive to SEM+NIC H only. All other treatments were significantly different to both UUC and IUC indicating partial resistance. The least effect was recorded in the NAR+NIC treatment, with DWG significantly lower than all other combination treatments. FI was impaired in all treatments, except SEM+NIC L and H groups, which were not different from the UUC. All treatments improved FCR compared to IUC and were not significantly different from UUC except for NAR+NIC group which was significantly higher than UUC.

Table 2 - Overview of zootechnical performance per treatment group.

Treatment	BW d 14	BW d 23	DWG d 14-23	DFI d 14-23	FCR d 14-23
UUC	494^{ab}	1222^{a}	81.70^{a}	120^{a}	1.46^{cde}
IUC	494^{a}	1072^{c}	63.60^{d}	109^{b}	1.71^{a}
MON 50 NIC 50	480^{b}	1161^{ab}	74.50^{b}	105^{b}	1.43^{de}
SAL 50 NIC 50	484^{ab}	1174^{ab}	76.60^{b}	106^{b}	1.40^{e}
NAR 50 NIC 50	484^{ab}	1139^{bc}	71.10^{c}	107^{b}	1.57^{b}
MAD 3.75 NIC 40	491^{ab}	1174^{ab}	75.20^{b}	107^{b}	1.47^{cde}
SEM 15 NIC 40	483^{ab}	1152^{ab}	73.50^{b}	113^{ab}	1.52^{bcd}
SEM 18 NIC 48	487^{ab}	1221^{a}	80.20^{ab}	118^{a}	1.53^{bc}

Means with different superscripts are significantly different at $P < 0.05$ (LSD Fisher test).
BW – body weight in grams; DWG – daily weight gain in grams; DFI – daily feed intake in grams; FCR – feed conversion ratio

IV. DISCUSSION

When assessing overall efficacy of anticoccidials to different strains, it is important to consider both parasitological and zootechnical parameters. The results of the study indicate that all ionophore-nicarbazin combinations are still effective in controlling coccidiosis, as none of the treatments demonstrated complete resistance. The above is evidenced by their wide use in commercial poultry production.

Resistance development is inevitable and well correlated with previous exposure of the field strains to specific anticoccidial products (Stanev et al., 2021). In line with this, the recent Dutch field isolate showed certain reduced sensitivity especially for products widely used for long time such as NAR+NIC. On the other hand, products that the isolate was naive to like SEM+NIC provided highest improvement compared to IUC and was not different from UUC indicating complete sensitivity.

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HOW XYLANASE ALTERS ARABINOXYLAN UTILISATION IN BROILERS; CHARACTERIZATION OF THE IN VIVO FORMATION OF ARABINOXYLAN-OLIGOSACCHARIDES

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The objective of the presented study was to extract and identify the arabinoxylan-oligosaccharides (AXOS) released *in vivo* in the different segments of the gastro-intestinal tract (GIT) of broilers and demonstrate the influence of xylanase on arabinoxylan (AX) utilization. The study used samples from digesta collected from the gizzard, ileum, ceca and excreta of broilers, fed wheat-soybean meal diet without (CTL diet) or with xylanase supplementation (ENZ diet).

Molecular weight (Mw) distribution analysis was performed with purified (A)XOS by HPSEC-RI and oligomers present were profiled by HPAEC-PAD. Different HPSEC profile for ENZ compared to CTL were shown. CTL diets mainly presented molecules between 10-100 kDa Mw, while smaller molecules between 10-1 kDa Mw were more abundant for ENZ. This shift in size distribution was aligned with AXOS presence for ENZ.

The *in vivo* AXOS profile matched the one obtained during *in vitro* hydrolysis of soluble wheat AX by the same xylanase. This supports that AXOS detected for ENZ were formed by the supplemented xylanase. AXOS were also detected in excreta for ENZ, and presented similar profile to the ileum. The *in vivo* formation of AXOS is in line with the AX depolymerization and with AX solubilization from the water-extractable cell wall matrix. Additionally, AXOS formation in the upper GIT may further explain the positive influence of xylanase supplementation on ceca fermentation processes (Singh et al., 2021). As previously documented (Kouzounis et al., 2021; Singh et al., 2021), the release of (A)XOS in the upper GIT was correlated with pronounced cecal fermentation. In the current study, soluble compounds with Mw < 10 kDa were present in the ceca, however, AXOS were not present, suggesting that extensive fermentation, of AX species, has occurred in the ceca.

Using a recently developed HILIC-MS^n methodology, formed AXOS were identified; AXOS with degree of polymerization (DP) 4-10 were present in ileum and excreta samples of birds fed ENZ diet, which aligned with improved AX utilization in the hindgut.

In this study, xylanase supplementation of broiler diets resulted in AXOS formation *in vivo*. The detection and characterization of released oligosaccharides further delineated the impact of dietary xylanase on hindgut fermentation in broilers. In particular, it is proposed that low-substituted AXOS and XOS, released *in vivo* by the xylanase, were extensively fermented by the ceca microbiota. Our work highlights the contribution of dietary xylanase to animal health and provides valuable insight on the utilization of AX and AXOS along the GIT of broilers.

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PLANT-DERIVED ISOQUINOLINE ALKALOIDS MODULATED CECAL MICROBIOME AND AMINO ACID METABOLISM IN BROILERS REARED IN TROPICAL CONDITIONS

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Summary

Broilers reared under tropical conditions will likely be subjected to heat stress. The study aimed to evaluate the effect of a standardized blend of plant-derived isoquinoline alkaloids (IQ) on the cecal microbiome of broilers and amino acid metabolic functions in heat-stressed broilers reared a tropical climate (Thailand). Inclusion of IQ significantly improved body weight, average daily weight gain, feed intake and mortality. IQ significantly modulated the cecal microbiome (days 14 and 35) by improving microbiome diversity, increasing abundances of phylum *Bacteriodetes* and *Cyanobacteria,* and decreasing *Firmicutes* and *Proteobacteria*. At the species level, IQ supplemented diets significantly increased abundances of *Turicibactor sanguinis*, *Lactobacillus salivarious*, *Lactobacillus sacidophilus,* and *Akkermansia municiphila* (day 14), and suppressed the levels of *Enteroccucus cecorum*, *Enteroccuus villorum*, *Escherichia fergusonii,* and *Helicobacter pullorum.* In addition, it also suppressed levels of *Enterococcus decorum*, *Campylobacter jejuni*, and *Methanobrevibacter woesei* in the ceca of broilers at day 35 of age. IQ supplemented diets significantly upregulated L-tryptophan degradation VI, XII, IX, L-phenylalanine biosynthesis, L-lysin degradation VIII, L-methionine salvage cycle III, L-isoleucine degradation, and L-serine biosynthesis I of cecum microbiome at day 14 of age. At day 35, IQ supplementation upregulated L-methionine biosynthesis and L-tryptophan biosynthesis (Kruskal-Wallis p-value < 0.05). It was concluded that using a standardized blend of plant-derived IQ positively impacted the gut microbiome of broilers and essential amino acid metabolism pathways in the ceca of heat-stress broilers. Improvements were related to control of the stress level via microbiome-gut-brain axis and L-methionine biosynthesis essential amino acid metabolism pathways and contributed to more economical and sustainable broiler production.

I. INTRODUCTION

The microbiota acts as a “metabolic organ” and plays an essential part in regulating host physiology. The microbiota exerts various regulatory functions, including host development and nutrition, digestive performance, intestinal physiology, and intestinal immune homeostasis (Dominguez-Bello et al., 2015). Various physiological functions of the host have been shown to be mediated by the composition of microbiota or their metabolites, and these structural components will also be widely affected by dietary nutrients (Vades et al., 2018). The gut microbiome's amino acid metabolism (AAM) is widely effective in gut health. Heat stress (HS) negatively affects the growth and gut health of broilers by reducing feed intake and body weight and compromising gut integrity. In this regard, HS affects the composition of the microbiota and its metabolization (Khongthong et al., 2022). Plant-derived feed additive *Macleaya cordata* extract contains the active ingredient isoquinoline alkaloids (IQs). This ingredient positively influences growth performance and has an anti-inflammatory effect (Khadem et al., 2014). Supplementation with IQ in the diet could improve gut health under HS conditions (Khongthong et al., 2022). Therefore, elucidating the composition and amino acid metabolic functions of the microbiota changed by heat stress and the interaction between the

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microbiota and nutrient metabolic process will provide new insights into optimizing poultry's intestinal macroecological health and nutritional efficiency. This study aimed to evaluate the effect of plant-derived isoquinoline alkaloids (IQ) on the modulation of the cecal microbiome and amino acid metabolism functions in relation to the control of heat stress levels in broilers reared in summer in a tropical climate area.

II. MATERIALS AND METHODS

This experiment was approved at the Prince of Songkla University with ethics approval: MHESI 68014/1571. 720 1-day-old male Ross 308 broiler chicks were randomly allocated to three treatments: 1) negative control (fed on a basal diet), 2) IQ60 (supplemented with 60 mg/kg feed Sangrovit® Extra, Phytobiotics Futterzusatzstoffe GmbH, Germany), 3) IQ100 (supplemented with 100 mg/kg feed). Each treatment was divided into eight replicates with 30 chickens per replicate. Birds were housed in an open production house in the Southern area of Thailand (April-May). House temperatures were 33.0-35.0 °C, and relative humidity (RH) was 70-75%, equating to heat stress index (HI) of 160-200. Each replicate was assigned to a wire-floored cage (2.5 x 2.5 m) and equipped with a self-feeder and waterer. All birds were provided *ad libitum* access to feed and water. Nutrient contents in diets were matched to the requirements of broilers reared in tropical climates (Aviagen, 2019). Birds were fed pelleted diets throughout the trial (42 days), split up into a starter (days 1 – 21), grower (day 21 -35), and finisher period (day 35 – 42). Live body weight (BW), body weight gain (BWG), feed intake (FI), feed conversion ratio (FCR), and survival rate were recorded.

On days 14 and 35, one chicken was randomly selected from each replicate and sacrificed, resulting in 8 samples per treatment per time interval. All chickens were euthanized, and then cecal contents were collected and kept at -80 °C until 16S RNA Sequencing Analysis. The V3-V4 region of the 16S rRNA genes were PCR amplified from the genomic DNA in Illumina MiSeq cartridge V3. The amplicons were sequenced on the Illumina MiSeq sequencer (2x250 bp paired-end run). Operational Taxonomic Units (OTU) abundance was quantified as alpha and beta diversity. The raw data obtained by sequencing was processed by the DADA2 pipeline of Quantitative Insights into Microbial Ecology 2 (QIIME2) version 2020.11 for data cleaning, denoising, and cluster analysis. PKSSU 4.0 database (Yoon et al., 2017) was used as a reference. Alpha diversity and Beta diversity were calculated using R version 4.2.2 (http://www.r-project.org/) with vegan package version 2.5-7. The linear discriminant analysis effect size (LEfSe) algorithm in R was used to identify significant differences in the relative abundances of microbial taxa between broilers with different groups (Segata et al., 2011). Metabolic potential based on 16S rRNA sequences of microbial communities were processed on plugin q2-picrust2 in QIIME2 (version 2019.10), and the Kyoto Encyclopedia of Genes and Genomes (KEGG) database was used to predict the functional gene content of the various microbial communities. KEGG Ortholog (KO) gene was counted and then further annotated by Enzyme Commission (EC) Number. The reaction-annotated gene count data were summed and regrouped by EC number and then internally normalized per each sample. The metabolic pathway was built based on the MetaCyc Metabolic Pathway Database. In addition, we used STAMP v2.1.3 software package to analyse the differences in amino acid metabolism pathways among treatments.

III. RESULTS AND DISCUSSION

a) Performance parameters - The average FI, BW, BWG, FCR, and mortality of broilers fed treatment diets are presented in Table 1. Supplementation with IQ at 60 and 100 mg/kg feed increased FI, final BW, and BWG compared to the control ($p < 0.05$). The FCR was significantly lower for birds fed IQ at 14 days but was not significantly different at 35 days. The current results are similar to those reported by Khongthong et al. (2023), who fed IQ to birds before inducing artificial heat stress.

Table 1 - Effect of isoquinoline alkaloids (IQ) on growth performance parameters of broiler chickens from 14 to 35 days of age.

Parameters	Control	IQ60	IQ100	SEM	*P*-value
Day 0					
BW (g)	43.56	43.45	43.55	0.00	0.913
Days 14					
BW (g)	435.28[b]	465.71[a]	470.20[a]	7.67	0.002
BWG (g)	263.04[b]	293.33[a]	297.71[a]	7.12	<0.0001
FI (g)	380.55[b]	406.23[a]	414.82[a]	10.03	0.0056
FCR	1.45[b]	1.38[a]	1.39[a]	0.016	0.0001
Das 35					
BW (g)	2,482.38[b]	2,538.13[a]	2,553.30[a]	22.28	0.009
BWG (g)	830.02	839.23	855.78	14.47	0.215
FI (g)	1,350.19[b]	1,362.99[b]	1,374.73[a]	5.29	0.0004
FCR	1.63	1.62	1.61	0.02	0.757
Mortality (%), 1-42 days	3.40 [b]	1.40 [a]	1.40 [a]	0.20	<0.0001

b) Modulated cecal microbiome - Cecal microbiota diversity was significantly improved with the treatment diets (Table 2). In terms of alpha-diversity, the number of observed nOTUs, the estimated OTU richness (Chao1), and the estimated community diversity (Shannon index) were higher on days days 14 and 35 in broilers fed the treatment diets (Kruskal-Wallis p-value < 0.05). In addition, principal coordinates analysis (PCoA) of Bray-Curtis distance matrices also found that the microbial community (Beta-diversity) in the treatments was markedly different from the control at day 14 of age (Permannova, p<0.001). The taxa composition in ceca at days 14 and 35 had a lower proportion of *Firmicutes* and *Proteobacteria* with the IQ100 treatment compared to the control group (p<0.05). In contrast, *Bacteriodetes* and *Verrrucomicobia* were higher in the treatments (p<0.05). In addition, at day 35 of age, the proportion of phyla *Euryarchaeita* in the ceca of broilers fed the treatment diets were lower than the control (p<0.05). It was previously reported that the composition of gut microbiota in broilers was changed by heat stress, in which *Firmicutes*, *Proteobacteria,* and *Tenericutes* increased, and *Bacteroides* and *Facecalibacterium* decreased (Shi et al., 2019). The multivariate regression analysis in our study showed that the total abundance of *Bacteriodetes* in the ceca of broilers under heat stress conditions has positively correlated with FI ($R^2 = 0.79$) at day 14 of age. Whereas abundances of *Firmicutes* and *Proteobacteria* were negatively correlated with FI ($R^2 < -0.5$) at days 14 and 35 of age.

Table 2. Taxonomic alpha diversity of cecal microbial communities from natural heat stress broiler fed either a control or supplementation with plant-based isoquinoline alkaloids with 60 and 100 mg/kg feed.

Taxonomic alpha diversity	Control	IQ60	IQ100	*p*-Value
Day 14				
nOTUs	513.28[b](8.46)	576.60[a](16.24)	596.79[a](17.93)	0.042
Chao1	515.37[b](48.64)	583.13[a](49.79)	599.82[a](43.32)	0.042
Shannon	4.72[b](0.59)	5.92[a](0.43)	6.10[a](0.39)	0.002
Day 35				
nOTUs	708.37[b](50.04)	805.75[a](53.27)	829.37[a](37.04)	0.024
Chao1	1063.16[b](62.18)	1363.69[a](87.16)	1424.17[a](143.08)	0.001
Shannon	5.63[b](0.33)	6.33[a](0.30)	6.42[a](0.26)	0.008

At day 14 of age, abundances of *Turicibactor sanguinis*, *Lactobacillus salivarious*, *Lactobacillus sacidophilus,* and *Akkermansia municiphila* were higher in the ceca of broiler feed the IQ60 treatment diet compared to the control (In Fig 1, using linear discriminant analysis score >2 in the LEfSe analysis). Also, abundances of *Enteroccucus cecorum*, *Enteroccuus villorum*, *Escherichia fergusoni*i and *Helicobacter pullorum* in ceca of broilers in control group were higher than broilers fed the treatment diets. At day 35 of age, *Enteroccucus cecorum*, *Campylobacter jejuni* and *Methanobrevibacter woesei* in ceca of broilers in control group were higher in total abundance than broiler feed the treatment diets.

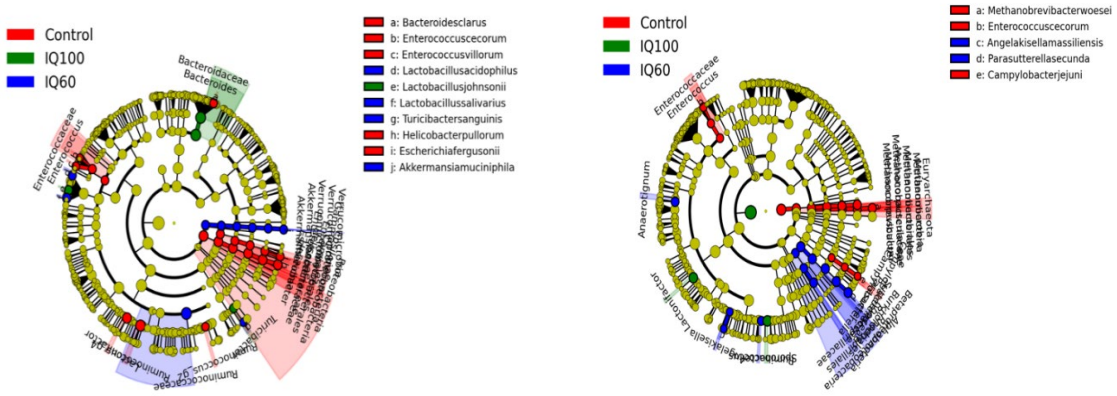

Figure 1 - LEfSe analysis in cecal microbiome of heat stressed broilers fed control or IQ-supplemented diets with 60 (IQ60) and 100 mg/kg (IQ100) at days 14 (A) and 35 (B) of age. Cladogram of microbiome in different groups compared at different evolutionary levels with LDA scores in a different group and LDA effect size of greater than 2 as a threshold.

c) Metabolic Profiles from Cecal Microbiome - Based on the functional annotation and abundance information of KOs in the KEGG gene database, we created the metabolic pathway based on the MetaCyc Metabolic Pathway Database and then selected the amino acid metabolism pathway. Principal coordinates analysis (PCoA) of Bray-Curtis distance matrices of amino acid metabolic functions (Beta-diversity) showed that the treatment diets were markedly different from the control ($p < 0.01$). Our finding revealed enrichment of amino acid metabolism activity in the ceca at days 14 and 35 in broilers fed the treatment diets. L-phenylalanine biosynthesis, L-serine biosynthesis I, L-tryptophan degradations, L-phenylalanine biosynthesis, L-lysin degradation VIII, L-methionine salvage cycle III, and L-isoleucine degradation of cecal microbiome at day 14 were higher in broiler feed supplemented IQ60 diet than that control group. Superpathway of L-methionine biosynthesis, L-tryptophan degradation VI, phosphatidyl serine and phosphoethanolamine biosynthesis, and arginine dependent acid resistance of cecal microbiome were more highly regulated in broilers fed the IQ100 treatment diet than that control group. At day 35, L-tryptophan biosynthesis was higher in the broilers fed the IQ60 treatment diet than the control group. L-methionine biosynthesis, L-tryptophan biosynthesis, and L-methionine salvage cycle in broilers fed the IQ60 treatment diet were higher than the control group (Kruskal-Wallis p-value < 0.05) (results not shown but available from the author). In summary, isoquinoline alkaloid supplementation in the broiler diet modulated beneficial microbiota diversity, improved *Bacteroides,* and reduced *Firmicutes* and *Proteobacteria* in the ceca of broilers reared in tropical conditions. IQs upregulated the tryptophan metabolism pathway, which is related to control the stress level via the microbiome-gut-brain axis and L-methionine biosynthesis beneficial amino acid metabolism pathways in the ceca of heat-stress broilers was also improved, therefore contributing to more economical and sustainable broiler production.

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GROWTH INHIBITORY EFFECT OF A TRIPLE-STRAIN *BACILLUS*-BASED PROBIOTIC ON ENTEROCOCCUS CECORUM ISOLATED FROM BROILERS: AN IN VITRO STUDY

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Summary

Twenty-two *E. cecorum* isolates were collected from broiler chickens, 12 isolates from West European farms and 10 isolates from US farms, to evaluate the *in vitro* inhibitory capability of a triple-strain *Bacillus*-based probiotic, consisting of *B. subtilis* (DSM 32325), *B. subtilis* (DSM 32324), and *B. amyloliquefaciens* (DSM 25840). The triple-strain *Bacillus*-based probiotic was hypothesized to exhibit a significant *in vitro* inhibitory effect against *E. cecorum*. This study assessed this hypothesis through a pathogen inhibition assay of the probiotic supernatant against twenty-two different *E. cecorum* isolates. The inhibitory effect of the supernatant against bacterial growth was evaluated using optical density (OD 600 nm) by measuring the turbidity of the broth. All experiments were conducted in triplicate. Results revealed that the triple-strain probiotic had a growth inhibitory effect against *E. cecorum* that ranged from 80% and 96% for the 12 France and 10 US isolates. The *in vitro* capability for direct inhibition of clinical *E. cecorum* isolates may result from the release of specific inhibitory lipopeptides like surfactins and fengycins that inhibit bacteria through the formation of pores in cell membranes as biosurfactants.

I. INTRODUCTION

Enterococcus cecorum, a gram-positive coccus, plays an integral role in the composition of the gastrointestinal microbiota across a diverse array of mammalian and avian species. It is worth noting that this range encompasses not only a variety of avifauna, such as chickens, ducks, and pigeons, but also several mammalian species, including pigs, calves, horses, cats, and dogs (Baele et al., 2022). *E. cecorum* has been implicated sporadically in a range of human illnesses, including peritonitis, septicemia, and empyema, typically presenting in individuals with pre-existing conditions or vulnerabilities such as alcohol abuse, liver cirrhosis, malnutrition, or undergoing continuous ambulatory peritoneal dialysis (De Baere et al., 2000). Human disease associated with *E. cecorum* has been documented in only a small number of publications (De Baere et al., 2000) (Greub et al., 1997) (Hsueh et al., 2000) (Woo et al., 2004). In the last 15 years, *E. cecorum* has surfaced as a noteworthy opportunistic pathogen affecting poultry. The infection attributed to *E. cecorum* was detected in 2002 in broilers aged 4-5 weeks, originating from Scotland (Wood et al., 2002). *E. cecorum* is usually recognized as a harmless commensal within the gut of broiler chickens. However, the occurrence of dysbiosis, an imbalance in the gut microbiome has the potential to compromise the gut barrier integrity resulting in a "leaky gut". This condition can facilitate intestinal bacterial overgrowth, providing an opportunity for *E. cecorum* to exploit weakened tight junctions with potential to translocate into the blood stream followed by transport to specific sites (e.g., joints) or organs (e.g., heart). *E. cecorum* has been implicated in causing bone lesions, notably chondronecrosis and osteomyelitis in

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broiler chickens. Disease outbreaks associated with *E. cecorum* were characterized by a range of symptoms including sepsis, pericarditis, local myositis, and particularly bone and joint lesions, which encompass purulent arthritis of the hock, femoral head necrosis, and T6 vertebral osteomyelitis. *E. cecorum* presently poses a significant threat to the global profitability of broiler operations with increased mortality rates, poor production, and increased condemnation (Dolka et al.,2017). Therefore, for poultry production, evaluation of probiotics has been undertaken to determine the potential effect of growth inhibition of *E. cecorum*.

II. METHOD

From USA and West European farms, twenty-two isolates of *E. cecorum* were collected from broiler chickens displaying clinical signs of lameness. The goal was to evaluate the *in vitro* inhibitory capability of a triple-strain Bacillus-based probiotic, consisting of *B. subtilis* (DSM 32325), *B. subtilis* (DSM 32324), and *B. amyloliquefaciens* (DSM 25840). Out of the 22 isolates, 10 were provided by the University of Arkansas (Prof. Douglas Rhoads and Dr. Adnan Ali Khalaf Alrubaye), while the remaining 12 West-European isolates were obtained from a laboratory in France. These *E. cecorum* strains were isolated from various body sites including the heart, liver and joints or via cloacal swabs.

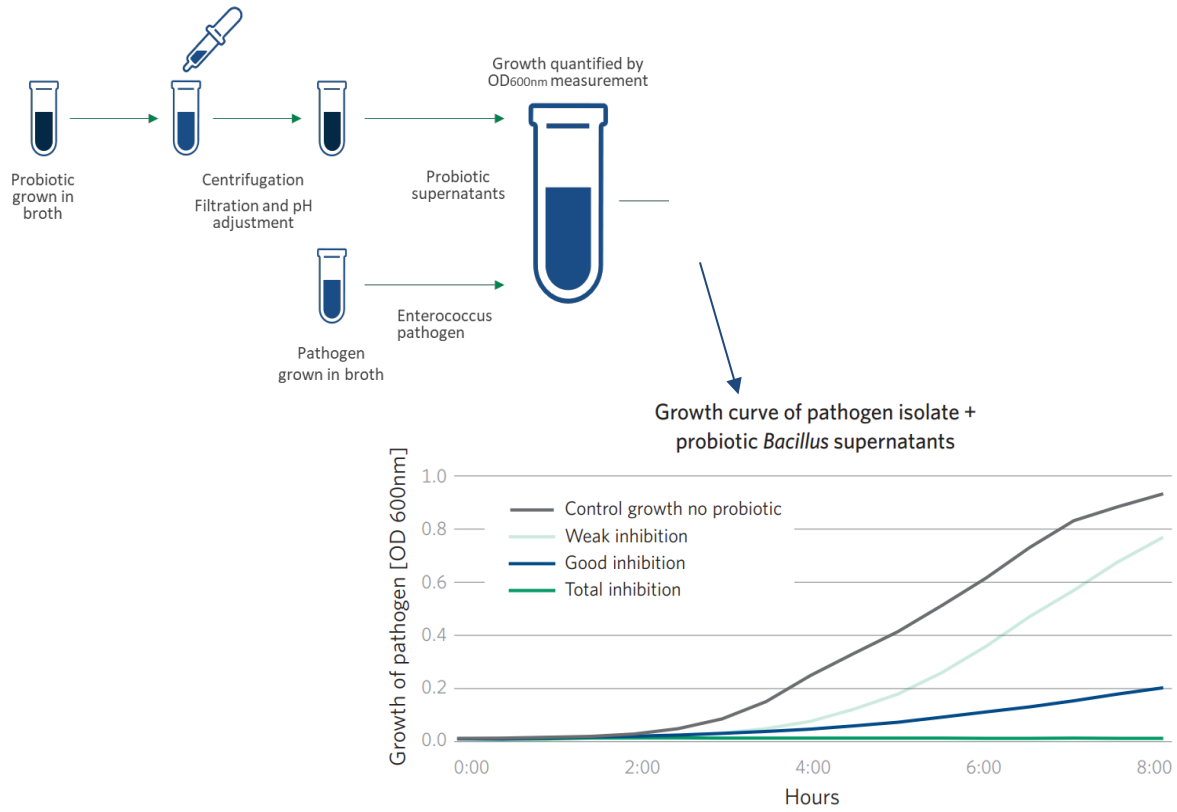

Figure 1 - Supernatant Assay Method and example of outcome for E. cecorum isolates.

A pathogen inhibition assay was conducted to determine the ability of the probiotic supernatant to inhibit the growth of the different *E. cecorum* isolates. The probiotic was cultured in BHI broth and the *E. cecorum* isolates were cultured in TSA-sheep blood agar at 37˚C for 24 hours. The probiotic broths were centrifugated for bacterial cell sedimentation, separating the supernatant from the precipitates through filtration. A 100 µl aliquot of this cell-free probiotic supernatant, which has previously been shown to contain active substances including bacteriocins, was subsequently combined with 100 µl of new BHI broth and 20 µl of *E. cecorum* in saline diluent inoculated in wells. Post-inoculum incubation to assess the

inhibitory effect of the supernatant against bacterial growth was evaluated using the Optical/OD plate reader for 96 well plates by measuring the turbidity (Figure 1). All experiments were conducted in triplicate. The average OD of triplicates are calculated for both probiotic and control growth. This is the inhibition percentage for each timepoint. To calculate the percentage decrease in growth, an average of these percentage inhibition were performed to get one number per strain.

III. RESULTS AND DISCUSSION

As presented in Figure 2, results revealed that the triple-strain probiotic had a very high inhibition of the growth of *E. cecorum* ranging from 80% and 96% for the 12 West European and 10 US isolates.

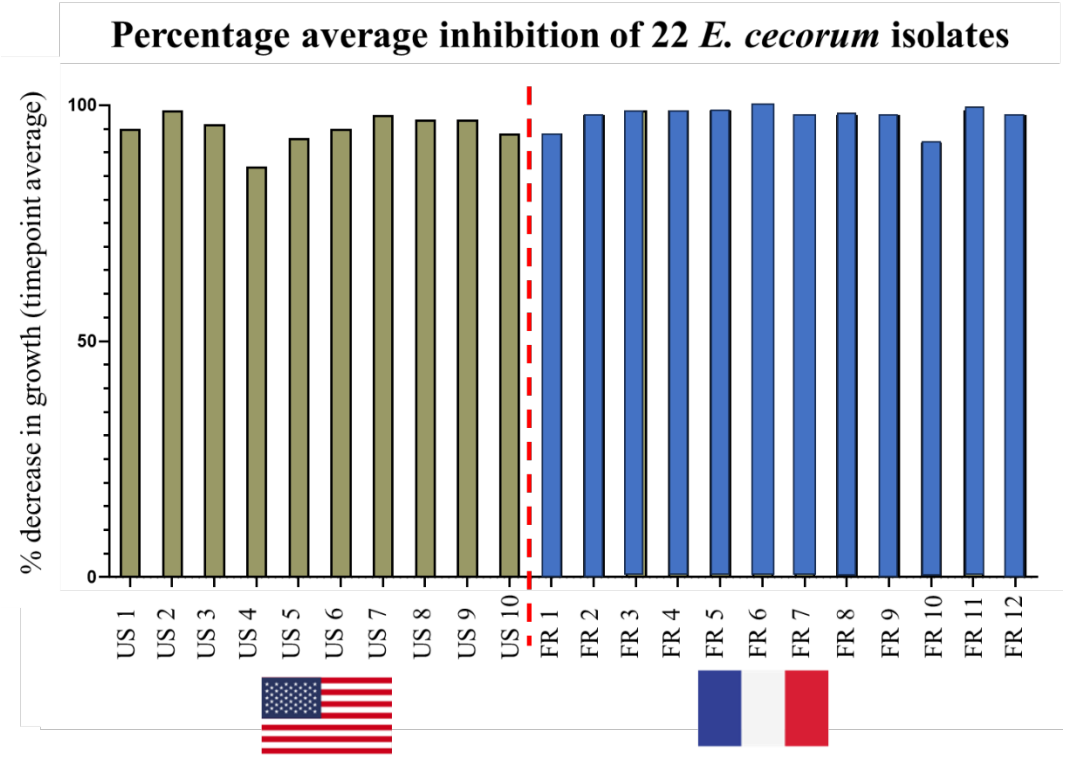

Figure 2 - Average inhibition of triple-strain probiotic on all E. cecorum isolates.

This strong *in vitro* capability for direct inhibition of clinical *E. cecorum* isolates can be explained by the ability of the different *Bacillus* strains in the probiotic to release specific inhibitory substances (such as lipopeptides like surfactins and fengycins) to its surrounding that inhibit bacteria through the formation of pores in cell membranes. The *in vitro* activity shown in this study suggests that the probiotic should have *in vivo* efficacy.

In conclusion, the observed direct pathogen inhibitory effect *in vitro* as demonstrated through supernatant assay is just one facet of the multifaceted modes of action attributable to *Bacillus*-based probiotics. The action of triple-strain probiotic has been shown to reduce the *in vitro* growth of *E. cecorum*.

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IN OVO CARVACROL DELIVERY INFLUENCES YOLK SAC MEMBRANE IMMUNE FUNCTION IN BROILER CHICKS

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During broiler embryonic development, the yolk sac membrane (YSM) is known to develop and maintain immune function (Wong & Uni, 2021). After in ovo delivery, the bioactive compound carvacrol primarily migrates to the yolk (Meijer et al., 2023). However, it is unknown whether this could impact immune function in the YSM. The aim of this experiment was to study the impact of in ovo carvacrol on the YSM with emphasis on indicators of immune development. It was hypothesised that in ovo carvacrol delivery would enhance YSM immune function by influencing cytokine and immunoglobulin mRNA expression.

Two treatments were injected into the amniotic fluid of fertile Ross 308 eggs (38-week-old breeder flock) at embryonic day (E)17.5: either 1mL of (1) 0.9% saline or (2) carvacrol (0.5% v/v) with polysorbate 80 (1:1 v/v) in 0.9% saline. Yolk sac membranes were collected on E19.5 and hatch (d0) (n = 10/treatment/day) to study the impact of the treatments. Relative mRNA expressions of IL1β, IL2, IL4, IL8, IFNγ, TNFα, TGFβ, NFκB and IgM were measured by qRT-PCR. Data were analysed using PROC MIXED (SAS 9.4).

Table 1 - Relative mRNA expression of immune related genes (fold change, FC) in the yolk sac membrane, showing the main effects in ovo treatment (saline or carvacrol) and age (E19.5 and d0).

Treatment	Age	IL1β	IL2	IL4	IL8	IFNγ	TNFα	TGFβ	NFκB	IgM
Control		1.19	0.87	0.94	0.84	1.35[a]	1.12	0.77	0.85	1.70
Carvacrol		1.09	0.79	1.60	0.57	0.35[b]	1.18	0.80	1.00	1.03
SEM		0.16	0.27	0.17	0.16	0.38	0.12	0.06	0.06	0.76
	E19.5	1.49[a]	1.30[a]	0.18	1.09	-[1]	1.57[a]	1.11[a]	1.21[a]	2.14[a]
	D0	0.77[b]	0.36[b]	0.17	0.32	0.85	0.74[b]	0.46[b]	0.63[b]	1.01[b]
	SEM	0.16	0.27	0.17	0.31	-	0.12	0.08	0.06	0.58
P-value										
Treatment		0.59	0.92	0.12	0.14	<.001	0.88	0.77	0.12	0.51
Age		<.001	<.001	0.23	<.001	-	<.001	<.001	<.001	0.02
Treatment x Age		0.98	0.66	0.71	0.05	-	0.35	0.11	0.07	0.29

[1]No amplification of IFN-γ was found at E19.5; [a,b,c]. Means within a factor lacking a common superscript differ (P ≤ 0.05).

A main effect was observed for in ovo treatment, with carvacrol decreasing IFNγ expression at d0. Main effects of age showed declined expressions from E19.5 to d0 for all measured genes. An interaction between treatment and age on IL8 expression (Δ FC = 0.41), showed that in ovo carvacrol led to a more pronounced decrease at d0, reflecting potential anti-inflammatory effects of carvacrol in the YSM. Over time, immune-related gene expression in the YSM decreased regardless of in ovo treatment, indicating a decline in YSM immune function.

It was concluded that in ovo delivery of carvacrol reduced some inflammatory cytokine expression in the YSM of broiler chicks at hatch.

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THE BINDING CAPACITY OF A BROAD SPECTRUM MYCOTOXIN ADSORBENT FOR THE EMERGING MYCOTOXINS FUSARIC ACID, BEAUVERICIN AND STERIGMATOCYSTIN

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Mycotoxins are secondary metabolites that are produced by certain filamentous fungi. They are found to be common contaminants of food and feed sources (CAST, 2003). Poultry are sensitive to the presence of mycotoxins in feed and the effects of different mycotoxins on individual birds depend on many factors including type of production, age, and production stage. The presence of mycotoxins in poultry feed can cause clinical or subclinical symptoms, and consequently result in reduced production performance (Resanovic et al. 2009). These concerns highlight the need for solutions to reduce harmful effects of mycotoxins in poultry. Current solutions are available in the form of commercial mycotoxin adsorbents. However, for a specific, less well-known group of mycotoxins, the emerging mycotoxins, little information is available about the effects of mycotoxin adsorbents. Besides, emerging mycotoxins are not controlled by legislation, and they are often not measured during feed analysis (Vaclavikova et al. 2013). The objective of this trial was to study the binding capacity of a broad spectrum mycotoxin binder to the emerging mycotoxins fusaric acid, beauvericin, and sterigmatocystin.

The binding efficacy (%) of a broad spectrum mycotoxin adsorbent (Excential Toxin Plus, Orffa Additives BV) was determined towards fusaric acid (15 ng/ml), beauvericin (0.75 ng/ml) and sterigmatocystin (25 ng/ml). Three conditions were tested at two pH levels, one replicate per treatment (pH 3; resembling stomach, pH7; resembling intestinal tract) for each mycotoxin sample: 1 = with buffer and mycotoxin standard mixture (*in duplo*); 2 = with buffer, mycotoxin standard mixture and adsorbent product; 3 = with buffer and adsorbent product. The solutions were first incubated at pH 3 at 37°C for one hour after which a sample was collected for mycotoxin extraction. The remaining buffer was then adjusted to pH 7, incubated for another three hours and sampled for mycotoxin extraction. All samples were analyzed by means of liquid chromatography-tandem mass spectrometry (LC-MS/MS) after which the binding efficacies (%) were calculated, no statistics were included (one replicate per treatment).

For fusaric acid there was complete binding by the mycotoxin adsorbent at pH 3 (> 91%) and partial binding (71%) at pH 3-7. For beauvericin, there was partial binding at pH 3 (77%) and complete binding (> 91%) at pH 3-7. Sterigmatocystin was completely bound (> 91%) by the mycotoxin adsorbent at both pH 3 and pH 3-7.

Overall, it can be concluded that the tested mycotoxin adsorbent partially binds fusaric acid and can completely bind the emerging toxins beauvericin and sterigmatocystin in the pH range of the gastrointestinal tract (pH 3-7).

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SALMONELLA CHALLENGE EXPERIMENTS: A PARADOX

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Summary

Non-typhoidal serovars of *Salmonella enterica* subsp *enterica* are able to colonize the intestinal tracts of chickens, creating risks of contamination of meat and egg food products. These serovars seldom cause disease in chickens over 3 weeks of age. Colonization is generally transient but can continue to circulate in a flock for many months. Vaccination of breeders and layers is the most effective method of control of infections with serovars Enteritidis and Typhimurium. Throughout the process of vaccine development, challenge studies are required to evaluate the vaccines' efficacy. However, establishing a successful challenge model where the control birds are colonized to a sufficient extent to be able to demonstrate a statistically significant reduction in pathogen load in infected birds that have been vaccinated is problematic. A meta-analysis of published *S.* Enteritidis challenge studies was performed to identify challenge model conditions that will provide consistent colonization in challenged but unvaccinated control birds. Challenge with the pathogen close to sexual maturity was significantly more effective in achieving at least 80% colonization of control hens compared with earlier or later age groups.

I. INTRODUCTION

Non-typhoidal serovars of *Salmonella* seldom cause disease in adult chickens but colonization of the gastrointestinal tract does occur. This colonization is variable and usually of short duration in birds over 1 week of age (Berndt et al., 2007). Typically, the organisms are expelled over a few weeks (Lister & Barrow, 2008). Despite this, some of these serovars, particularly Enteritidis and Typhimurium, remain consistent concerns for zoonotic transfer inside eggs or on eggshells. Development of immunity in the chicken to *Salmonella* is regarded as due to cell mediated responses (CMI) (Beal et al., 2006), yet historically inactivated vaccines, which provide only humoral responses have proven to be highly effective (Berghaus et al., 2011; Crouch et al., 2020).

The evaluation of vaccine efficacy against non-typhoidal serovars of *Salmonella* requires a challenge model that will provide a high level of caecal colonization or faecal shedding in the unvaccinated control birds. This is necessary to give any chance of detecting a statistically significant reduction of colonization in the vaccinates. Given the transitory colonization in chickens, this is often difficult to achieve. As a result, researchers have developed unnatural challenge methods, such as extremely high oral doses, intravenous administration of the organism or antibiotic modification of the normal microflora to allow an oral dose of the challenge organism to colonize. In attempting to better understand these paradoxes, a meta-analysis of *Salmonella* challenge studies in the published literature was conducted.

II. METHOD

A literature search was conducted, seeking articles covering vaccine experiments in layer hens against S. Enteritidis or S. Typhimurium, preferably covering a span of bird ages (4 to over 50 weeks of age) using oral challenge and faecal or caecal detection. PubMed, CAB and SCOPE databases were searched for articles between 1990 and 2022 using the terms: layer* OR hens

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OR chicken* AND Vaccine OR inoculat* OR challenge OR dose OR infection AND oral AND *Salmonella* AND caecum OR caeca OR cecal OR cecum OR faecal OR fecal OR faeces OR feces. The *Salmonella* colonization rate in the non-vaccinated control groups in these studies and the actual oral challenge dose rate used, were retrieved from the publications, and used for comparative meta-analyses. The colonization rates in the control birds were compared across age groups and challenge dose rate categories.

III. RESULTS

Publications meeting the search criteria yielded 34 articles (the full list may be requested from author). Most publications dealt with *S*. Enteritidis, so only this serovar was considered in the final analyses. Challenge dose rates varied between 10^5 to 10^9 colony forming units (CFU) per bird. Outcomes were grouped into bird age categories of "early rearing" (4-6 weeks), "mid-late rearing" (9-14 weeks), "point of lay" (16-20 weeks), "prime lay" (24-29 weeks) and "older" (>=30 weeks).

Assessing colonization levels by bird age, point of lay control birds had significantly higher faecal or caecal colonization (mean 80.1% positive compared to 48.3% at 4-6 weeks, 47.7% at 9-14 weeks, 24.0% at 24-29 weeks and 66.8% at 30 weeks or older) than any other age group. Older flocks (>=30 weeks) produced a mean colonization of 66.8% which was higher than early or mid-late rearing group.

At 9-14 weeks and 24-29 weeks, oral doses between 10^5 and $<10^9$ CFU/ bird did not produce colonization rates higher than 45%, which would be inadequate to deliver a significant difference between control and vaccine groups unless the vaccine treated groups had a colonization rate of zero. For the point of lay age (16-20 weeks of age) any dose above 10^5 CFU/bird produced an average of 90% successful colonization, whereas the older age group (>=30 weeks) required challenge doses of $>10^7$ CFU/ bird to achieve even a minimal colonization rate.

IV. DISCUSSION

Considering typical vaccine challenge studies included in treatment groups of between 5 to 16 birds, a control colonization rate of at least 80% would be necessary to detect a statistically significant reduction ($P<0.05$) in the vaccinated compared to control group (Dhand & Khatkar, 2014).

The age of greatest success with vaccine efficacy studies appears to be sexual maturity (point of lay - which varies between 16-20 weeks depending on breed). Variance in challenge dose rate at this age did not affect the outcome and increases in the challenge dose rate above 10^5 CFU/ bird did not reflect the level of caecal or faecal enumeration outcomes. Birds older than 30 weeks required much higher challenge doses, for example $>=10^8$ CFU/ bird, to achieve colonization in unvaccinated control groups. Several studies have shown that hens experience a temporary suppression of their CMI at sexual maturity (Wigley et al., 2005; Johnston et al., 2012) and we hypothesize that this phenomenon is responsible for the more consistent success with experimental challenge at this age. This phenomenon also may explain the reason for paradoxical success of inactivated vaccines against *Salmonella* in the field. Suppression of CMI may allow latent organisms in the intestine to multiply and then spread through the layer flock, thus explaining the persistence of the organism throughout the laying cycle. To the contrary, a flock vaccinated with, for example, an inactivated *S.* Enteritidis vaccine, would have high serum antibody (Pavic et al., 2010), as the humoral system is not suppressed, which may limit intestinal replication and inhibit the spread of infection.

V. CONCLUSION

Bird age plays a major role in achieving *Salmonella* colonization in hens. The most reliable age for evaluating the efficacy of vaccine using a challenge model is as the hen reaches sexual maturity.

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DEVELOPMENT OF THE BURSA OF FABRICIUS IS AFFECTED BY IN OVO CARVACROL DELIVERY IN BROILER CHICKS

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The compound carvacrol has the potential to enhance immune function, thereby reducing disease impact (Liu et al., 2019). The bursa of Fabricius serves as primary immune organ responsible for B-cell development in chicks which may represent a promising target for in ovo delivery of carvacrol to modulate early bursal development. This study aimed to explore how carvacrol affects immune function in the bursa of broiler chicks around hatching. It was hypothesised that in ovo carvacrol delivery would improve bursal development.

Bursal samples were collected on embryonic day (E)19.5, d0 and d14 (n = 10/treatment/day), to study the impact of two in ovo treatments: either 1 mL of (1) 0.9% saline or (2) carvacrol (0.5% v/v) with polysorbate 80 (1:1 v/v) in 0.9% saline. Treatments were administered into the amniotic fluid of fertile eggs at E17.5. Histomorphology was evaluated at d14. Relative mRNA expressions of IL1β, IL6, IL8, IL10, IFNγ, TNFα, NFκB, IgM, IgY and IgA were measured by qRT-PCR. Data were analysed using PROC MIXED (SAS 9.4).

Table 1 - Relative (compared to E19.5 control) mRNA expression (shown as fold change) of immune-related genes in the bursa, showing main effects in ovo treatment (saline or carvacrol) and age (E19.5, d0, d14).

Treatmen	Age	IL1β	IL6	IL8	IL10	IFNγ	TNF	NFκ	IgM	IgY	IgA
Control		2.29	0.74	1.92	4.23	0.90	0.93	0.97	1.23	22.65	2.65
Carvacrol		3.26	0.64	1.46	2.80	0.76	0.93	1.05	1.28	17.89	2.81
SEM		0.26	0.31	0.31	0.60	0.11	0.06	0.03	0.05	2.25	0.29
	E19.	1.20^{c}	1.23	1.31^{b}	1.71^{b}	1.36^{a}	1.12^{a}	1.09	1.09^{b}	1.17^{b}	1.33^{b}
	d0	1.85^{b}	0.49	0.70^{c}	0.74^{c}	0.22^{b}	1.08^{a}	0.95	0.91^{b}	1.47^{b}	1.74^{b}
	d14	6.24^{a}	0.35	3.15^{a}	8.09^{a}	0.91^{a}	0.59^{b}	0.99	1.76^{a}	58.17	5.11^{a}
	SEM	0.32	0.16	0.19	0.73	0.13	0.07	0.04	0.07	2.60	1.10
P-value											
Treatment		0.23	0.14	0.38	0.44	0.11	0.67	0.17	0.44	0.889	0.69
Age		<.00	<.00	<.00	<.00	<.00	<.001	0.06	<.00	<.001	<.00
Treatment x Age		0.50	0.002	0.51	0.59	0.26	0.25	0.32	0.77	0.43	0.55

[a,b,c] Means within a factor lacking a common superscript differ (P ≤ 0.05).

Histological analysis of bursal follicles at d14 showed that in ovo carvacrol increased cortex size (P = 0.03, Δ = 6663.06 μM²) and elevated cortex/medulla ratio (P = 0.04, Δ = 0.19), potentially indicating increased B-cell maturation. No treatment effects (P > 0.05) were observed for the genes studied, while the age variable resulted in different mRNA expression patterns. Expression of IL1β, IL8, IL10, IgM, IgY and IgA was highest in older chicks (d14). In contrast, TNFα decreased over time, while IFNγ decreased at d0, but rebounded at d14. An interaction between treatment and age on IL6 expression showed that in ovo carvacrol led to a more pronounced decrease at d14 (Δ FC = 1.10), reflecting anti-inflammatory effects.

It is concluded that in ovo injection of carvacrol decreased bursal inflammatory cytokine expression at d14, while promoting bursal cortex development.

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EFFICACY EVALUATION OF NOVEL ORGANIC IRON COMPLEXES IN LAYING HENS: EFFECTS ON LAYING PERFORMANCE, EGG IRON CONTENT AND BLOOD BIOCHEMICAL PARAMETERS.

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Summary

A study was conducted to determine the optimal dose of novel iron (Fe) amino acid complexes (Fe-Lys-Glu) by measuring laying performance, egg Fe concentrations, and blood biochemical parameters in laying hens. A total of 1260 18-wk-old Beijing White laying hens were randomly divided into 7 groups with 12 replicates of 15 birds each. After a 2-wk acclimation to the basal diet, hens were fed diets supplemented with 0 (basal negative control - NC - with innate 75.1 ppm Fe), 15, 30, 45, 60, and 75 ppm Fe as Fe-Lys-Glu or 45 ppm Fe from $FeSO_4$ (positive control, PC) for 24 wks. Results showed that compared with the NC and PC, dietary supplementation with 30 to 75 ppm Fe from Fe-Lys-Glu significantly (linear and quadratic, $P < 0.05$) increased the laying rate and average daily egg weight; hens administered 45 to 75 ppm Fe as Fe-Lys-Glu showed a remarkable (linear, $P < 0.05$) decrease in FCR. The Fe concentrations in egg yolk and serum were elevated by increasing Fe-Lys-Glu levels, and the highest Fe content was found in 75 ppm Fe group. In addition, hens fed 45 ppm Fe from Fe-Lys-Glu had (linear and quadratic, $P < 0.05$) higher yolk Fe contents than PC. The red blood cell count (RBC) and haemoglobin content (linear and quadratic, $P < 0.05$) increased in the groups fed with 30 to 75 ppm Fe as Fe-Lys-Glu in comparison with NC and PC groups. In conclusion, supplementation Fe-Lys-Glu in laying hens could substitute for $FeSO_4$ and the optimal level of Fe-Lys-Glu is 45 ppm Fe in layers diets based on the quadratic regression analysis of performance.

I. INTRODUCTION

Iron (Fe) is an indispensable element for organisms that plays crucial roles in several fundamental metabolic processes, including erythropoiesis, oxygen transport, DNA synthesis, energy metabolism, mitochondrial electron transport, immune-protection, and cognitive function (Abbaspour et al., 2014; Zhou et al., 2018; Drygalski and Adamson, 2013). Iron should be maintained at an appropriate level which is required to satisfy the metabolic needs and specialized functions for animals (Wang and Pantopoulos, 2013), while Fe deficiency causes anaemia, abnormal development, and severe deficiency could lead to death (Sarlak et al., 2021; Baker and Greer, 2010). Traditionally, hens are fed with a form of inorganic Fe salts in the diets, such as Fe sulfate ($FeSO_4$) (Bess et al., 2012). However, the inorganic Fe sources have several issues, like low bioavailability, high oxidation, and excretion, which can pollute the environment (Ma et al., 2014). As a result, chelates or complexes of Fe with amino acid or protein, organic Fe compounds has been developed as substitute for inorganic Fe. Meanwhile, it was reported that organic trace elements have higher bioavailability compared with inorganic microelements (Xie et al., 2019). The relative bioavailability of organic mineral elements was determined by the chelation strength and greater chelation strength indicates higher biological value and beneficial effects to laying hens (Zhang et al., 2016). Studies are mainly focused on ferric glycine (Fe-Gly). Compared with the same dosage of $FeSO_4$, dietary supplementation of

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60 ppm from Fe-Gly improved egg weight and increased Fe in egg albumen and yolk (Xie et al., 2019). However, a new organic form of Fe active compounds, Fe amino acid complexes (1:1 complex of Fe lysine and Fe glutamic acid, Fe-Lys-Glu), in laying hens has not been studied. The purpose of this study was to investigate the effects of dietary Fe-Lys-Glu level on laying performance, egg Fe content, and blood biochemical parameters in laying hens.

II. MATERIALS AND METHODS

A total of 1260 18-week-old Beijing White laying hens with similar body weights were randomly allocated into 7 groups with 12 replicates (15 birds/rep). After 2-week adaptation to the environment and to the basal corn-soybean meal diet (formulated nutrient requirements based on NRC, 1994), hens were fed with 0 (negative control, NC), 15, 30, 45, 60, and 75 ppm Fe as Fe-Lys-Glu or 45 ppm Fe as $FeSO_4$ (positive control, PC) for 24 weeks. Analyzed Fe content on basal diet was 75.1 ppm. Every three birds were placed in a cage (0.50m length×0.45m width ×0.40m height) with a 16L:8D photoperiod with 15 to 20 lux light intensity during experimental period. Feed and water were provided *ad libitum*. The housing temperature and relative humidity were maintained at 24°C±3°C and 50% to 60%, respectively.

a) Laying performance

Egg numbers and total egg weight of each replicate were recorded daily, and the feed consumption was weighed once a week. Based on the collected data, the laying rate, average daily egg mass, average daily feed intake, and feed conversion ratio were calculated.

b) Iron content in egg yolk and blood biochemical parameters

For the Fe content in egg yolk at the end of 12 and 24 wk, 6 eggs per replicate were randomly selected to measure the Fe concentrations in egg yolk using AAS according to the method described by Revy et al. (2004). The results were expressed as mg/kg fresh weight. At the end of the trial, one bird was chosen in random from each replicate (n=12). After fasting for 12 h, blood samples from the wing vein were collected to determine blood indexes as red blood cells (RBC), hemoglobin (HGB), and hematocrit (HCT) by a Sysmex microcell counter CL- 180 automated hematology analyzer (Sysmex Company, Lincolnshire, IL, USA). Part of the blood was centrifuged at 3500 r/min for 10 min at 4°C to obtain samples for serum Fe content determination measured by AAS (Revy et al., 2004).

c) Statistical Analysis

All data were analyzed with general linear model procedure of SPSS software (SPSS 21.0, SPSS Inc., Chicago, IL, USA) and multiple comparisons between the groups were performed by least significant difference test. Besides, the first six groups of hens fed with 0, 15, 30, 45, 60, and 75 ppm Fe from Fe-Lys-Glu in diet of data were analyzed by linear and quadratic regression analysis. $P < 0.05$ was considered statistically significant.

III. RESULTS

The results are summarized in Table 1 and the regression analysis of sensitive indexes are presented in Table 2.

Table 1 - Effects of dietary Fe-Lys-Glu supplementation on performance, Fe concentration in yolk and serum, and blood biochemical parameters of laying hens.

	Supplementary level of Fe-Lys-Glu (ppm of Fe)						$FeSO_4$	SEM	p-value		
	0	15	30	45	60	75	45		Trt	Linear	Quadratic
LR (%)	88.35^{d}	91.14^{bc}	93.18^{ab}	93.39^{ab}	94.05^{a}	93.21^{ab}	90.58^{cd}	0.840	<0.001	<0.001	0.006
ADEW (g/h/d)	47.24^{c}	48.21^{bc}	49.11^{ab}	49.70^{ab}	50.06^{a}	49.13^{ab}	48.15^{bc}	0.639	0.038	0.004	0.044
ADFI (g/h/d)	106.7	108.2	108.8	105.8	107.1	106.5	106.8	0.861	0.190	0.275	0.347
FCR	2.27^{a}	2.25^{a}	2.22^{ab}	2.14^{c}	2.13^{c}	2.16^{bc}	2.21^{ab}	0.025	<0.001	<0.001	0.105
Fe in yolk (ppm fresh weight)											
12 wk	51.98^{e}	54.17^{cd}	54.83^{c}	57.37^{b}	57.43^{b}	63.41^{a}	53.50^{d}	0.396	<0.001	<0.001	<0.001
24 wk	56.39^{d}	61.50^{bc}	61.93^{b}	63.42^{a}	63.31^{a}	63.19^{a}	61.02^{c}	0.317	<0.001	<0.001	0.080
Fe in serum (mg/L)											
12 wk	1.92^{d}	2.13^{c}	2.11^{c}	2.32^{b}	2.48^{a}	2.55^{a}	2.18^{c}	0.027	<0.001	<0.001	0.981
24 wk	2.21^{e}	2.41^{d}	2.60^{c}	2.78^{b}	2.97^{a}	3.11^{a}	2.47^{cd}	0.052	<0.001	<0.001	0.600
RBC (10^{12}/L)	2.73^{d}	2.88^{cd}	3.00^{abc}	3.16^{ab}	3.09^{ab}	3.17^{a}	2.70^{d}	0.066	<0.001	<0.001	0.004
HGB (g/L)	208.8^{c}	214.5^{bc}	221.5^{b}	233.6^{a}	241.6^{a}	238.9^{a}	207.2^{c}	3.400	<0.001	<0.001	0.179
HCT (%)	38.44	37.89	38.28	38.14	37.06	37.29	38.87	0.683	0.517	0.092	0.710

^{abcde}The means in the same row with distinct superscripts show significant differences ($P < 0.05$). LR, laying rate; ADEW, average daily egg weight; ADFI, average daily feed intake; FCR, feed conversion ratio; RBC, red blood cell; HGB, hemoglobin; HCT, hematocrit.

Table 2 - Estimated change of sensitive indexes in laying hens with Fe-Lys-Glu supplementation.

Variation	Equation of Regression	Estimated Fe supplementation	Estimated maximum response	P-values	R^2
LR	$y=88.436+0.204x-0.0021x^2$	48.57	93.39	0.006	0.294
ADEW	$y=47.103+0.099x-0.001x^2$	49.50	49.55	0.044	0.167
RBC	$y=2.722+0.012x-8.847e^{-05}x^2$	67.82	3.13	0.004	0.556
Fe in yolk-12wk	$y=52.551+0.034-0.001x^2$	17.00	52.84	<0.001	0.817

LR, laying rate; ADEW, average daily egg weight; RBC, red blood cell.

IV. DISCUSSION

The present study found a remarkable increase in LR of layers fed with 15 to 75 ppm Fe and ADEW when fed 30 to 75 ppm Fe as Fe-Lys-Glu compared with diet without adding exogenous Fe. Additionally, FCR of hens fed 45, 60, 75 ppm Fe from Fe-Lys-Glu was decreased markedly in comparison with NC group. It is because Fe organic source elevates the absorption of trace mineral elements in hens by reducing the ability to bind with nutrients or antinutritional factors, thereby improving the laying performance (Kwiecień et al., 2015). However, when the dosage of Fe-Lys-Glu reached 75 ppm Fe, the laying performance showed a downward trend compared to 60 ppm. This suggests that optimal dosage of Fe-Lys- Glu should not exceeding 60 ppm. Additionally, even though the addition level of Fe-Lys-Glu was lower than that of $FeSO_4$, it still showed better laying performance. It indicates that Fe-Lys-Glu has greater bioavailability than inorganic ferrous. Through the quadratic regression analysis of LR and ADEW, we can see that when the supplemental dose of Fe-Lys-Glu reached 48.6 and 49.5 ppm Fe, respectively, the laying performance of laying hens reached the best.

Several studies reported that Fe contents in egg yolk were affected by different sources of Fe and experiment period (Sarlak et al., 2021; Bess et al., 2012). They found that adding Fe-amino acids (Fe-AA) to diets distinctly increased the enrichment of Fe in egg yolk, compared with adding $FeSO_4$. We found that additive level of Fe-Lys-Glu and experimental period effectively affected the deposition of Fe in egg yolk, which was consistent with previous studies.

Iron is a necessary element for RBC production and synthesis of HGB (Trivedi and Bardi, 2021). It was reported that adding Fe-Gly in piglet diets increased serum Fe, RBC count, HGB content, and HCT compared with negative control and $FeSO_4$ groups (Li et al., 2018). Similar results were found in pregnant sows (Egeli et al., 1998). The results may demonstrate that organic Fe has higher biological value than inorganic Fe as our study presented an upward linear tendency in Fe content of serum with increasing dosage of Fe-Lys-Glu. The RBC count and HGB content were significantly increased in hens fed 30 to 75 mg Fe/kg from supplemented Fe-Lys-Glu diet.

In conclusion, the present study demonstrated that Fe-Lys-Glu was an effective Fe source that could substitute for $FeSO_4$ in laying hen diets. Dietary Fe-Lys-Glu supplementation is beneficial to improve the laying performance, yolk Fe content and blood biochemical parameters of laying hens in varying degrees. Based on the quadratic model analyses, supplemental 45 ppm Fe via Fe- Lys-Glu could provide the optimum laying performance (LR and ADEW). It is recommended that the dosage of Fe-Lys- Glu should not be higher than 60 mg Fe/kg.

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OREGANO IMPROVES PERFORMANCE, HEALTH AND PRODUCTIVITY OF LAYING HENS IN LATE LAYING PHASE

A. BECKMANN[1] and T. BORCHARDT[1]

Summary

The aim of this study was to investigate the influence of oregano essential oil (OEO) on the biological performance and productivity of laying hens at the end of the laying period (week 57-72). The addition of 22.5 g of OEO per tonne of feed resulted in an improvement in laying performance as well as a significant increase in egg mass. In addition, mortality was lower in the experimental group that received OEO. The improvement in performance and health also had a positive effect on the profitability of the hens that received OEO.

I. INTRODUCTION

The prices of pullets in Germany have risen significantly compared to previous years, largely due to the ban on killing male chicks and the associated more cost-intensive alternatives of rearing male chicks or sexing the egg. High feed prices, as well as significantly higher labour and energy costs, are factored into this. Other European Union countries will probably follow the same approach in the near future. Therefore, it is increasingly in the interest of laying hen farmers to extend the laying period, or to be able to use the hens in a second laying period through an induced moult. Since an extended laying period is also accompanied by a decrease in performance and fitness and thus may seem less economically attractive to the farmer, hens are even more dependent on optimal flock and feeding management at the end of the laying period than before.

OEO is known for its strong antimicrobial, antiparasitic, anti-inflammatory and antioxidant properties (LEYVA-LÓPEZ et al. 2017), and thus can contribute greatly to increasing gut health, immune function and performance of laying hens. The aim of this study was to investigate the influence of OEO on the biological performance and productivity of laying hens at the end of the laying period.

II. METHOD

The influence of 22.5 g OEO per tonne of feed (DOSTO® Powder, Dostofarm GmbH, Germany) on biological performance, egg quality and productivity of laying hens was investigated at a federal Experimental and Educational Center for Poultry in Germany. For the feeding trial, 392 hybrid hens (Lohmann Brown-Classic) were divided into two experimental groups at 21 weeks of life. The laying hens were kept in floor housing (8 hens per m²) in a total of 14 pens with 28 birds each. From 57 to 72 weeks of life, the two groups were fed a laying hen complete feed (17% CP, 11.4 MJ/kg ME and 3.8% calcium) with or without OEO supplementation. The standardized composition of the OEO is shown in Table 1. The number of produced eggs was recorded daily, average egg weights were determined weekly from the two-day production of individual pens. Those eggs were sorted according to weight classes (S, M, L, XL) for marketable eggs and separated from dirty eggs and cracked eggs. The feed consumption of the hens of each group was determined every 28 days by weighing of added feed and feed residuals. Deaths were documented daily per feeding variant. After the feeding period, the income over feed cost (IOFC) was calculated as follows: Egg revenue - feed cost

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(number of eggs of the respective weight class x price of the weight class - feed price (€/kg) x feed consumption per housed hen (kg)).

For data collection and preparation, Microsoft Excel® (version 2011, Microsoft Corporation, Redmond/USA) was used. The collected performance parameters were analyzed by one-factorial variance analysis with the fixed effect feeding variant by using the statistical program SAS. The statistical significance level was set at $P \leq 0.05$.

Table 1 – Standardized composition of the used OEO.

Bioactive ingredient	Content (%)
Carvacrol	60.0 - 65.0
Thymol	1.0 - 3.0
p-Cymene	5.0 - 10.0
γ-Terpinene	4.0 - 9.0
α-Terpinene	0.5 - 2.0
trans-Sabinene hydrate	0.3 - 1.0
β-Caryo-phyllene	2.0 - 5.0
Terpinene-4-ol	0.5 - 2.0
Linalool	0.8 - 5.0
Myrcene	0.5 - 3.0
α-Pinene	0.2 - 2.5
α-Thujene	0.2 - 1.5

III. RESULTS

The addition of OEO to the feed of laying hens during the last 16 weeks of the laying period increased the number of eggs per housed hen (HH) and thus overall laying performance with a trend towards significance ($P = 0.098$). While in the control group the laying rate per HH averaged 84.6% from 57 to 72 weeks of life, the OEO inclusion resulted in an average laying rate of 90% (Fig. 1). Moreover, a significant increase in egg mass ($P < 0.05$) was observed by the addition of OEO in laying hen feed (Fig. 2).

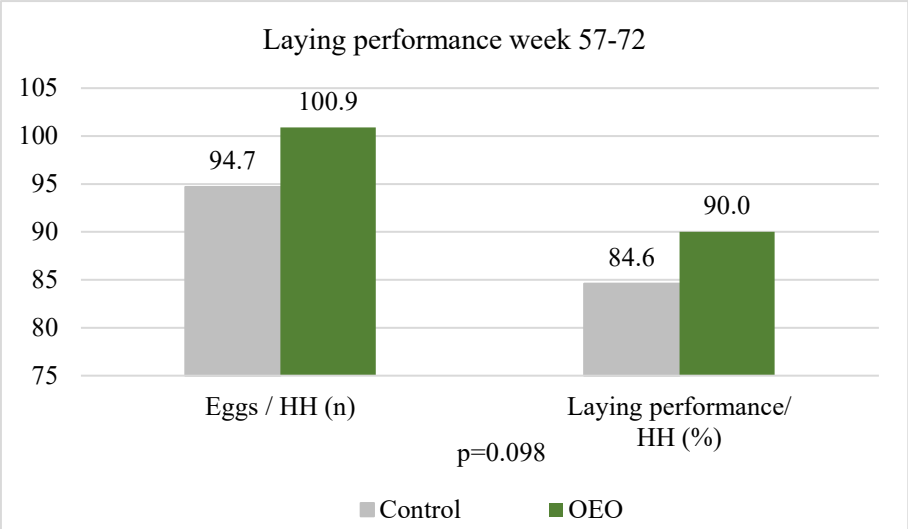

Figure 1 - Laying performance of hens fed OEO in the layer feed was increased by 5.4%.

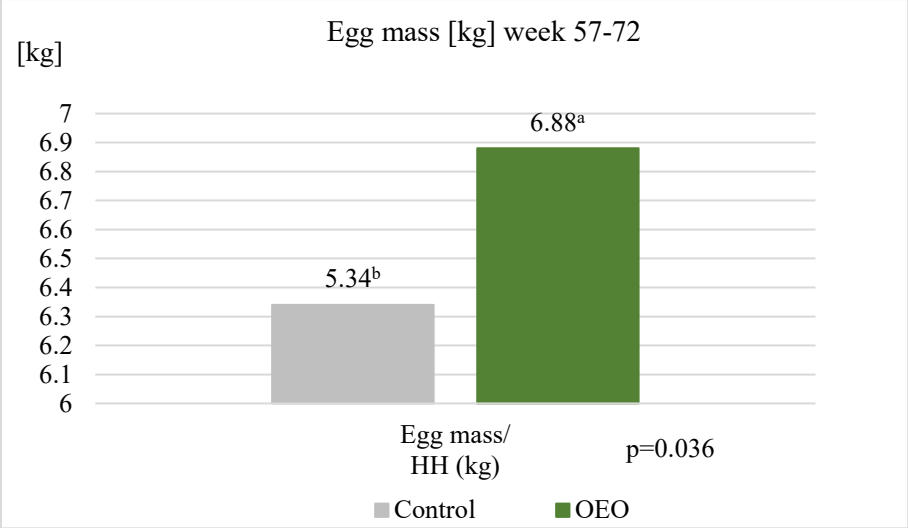

Figure 2 - The use of OEO at the end of the laying period significantly increases egg mass. The different letters show significant differences ($P < 0.05$).

The present study showed that supplementation with OEO increases the performance and egg mass of laying hens, which is associated with economic benefits. The calculation of the "income over feed costs" (IOFC) included feed costs, egg class distribution and their individual producer prices, and feed intake data. The addition of OEO resulted in a 0.23 EURO higher income per HH after deduction of feed costs (Fig. 3). In addition, there was a numerical decrease in the mortality rate from 8.24% in the control to 3.06% in the OEO supplemented group during the period from 57 to 72 weeks of life (Fig. 4); however without statistical significance below $P \leq 0.05$.

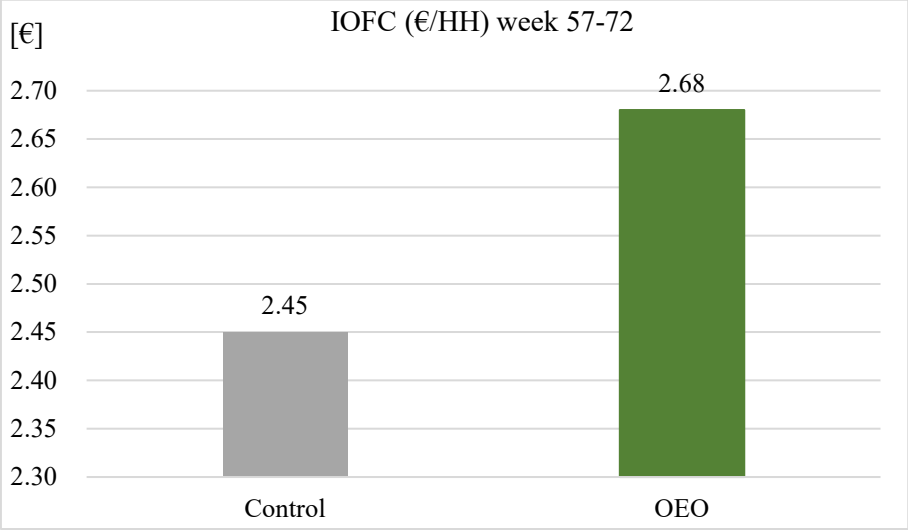

Figure 3 - The use of OEO in laying hen feed at the end of the laying period increases profitability.

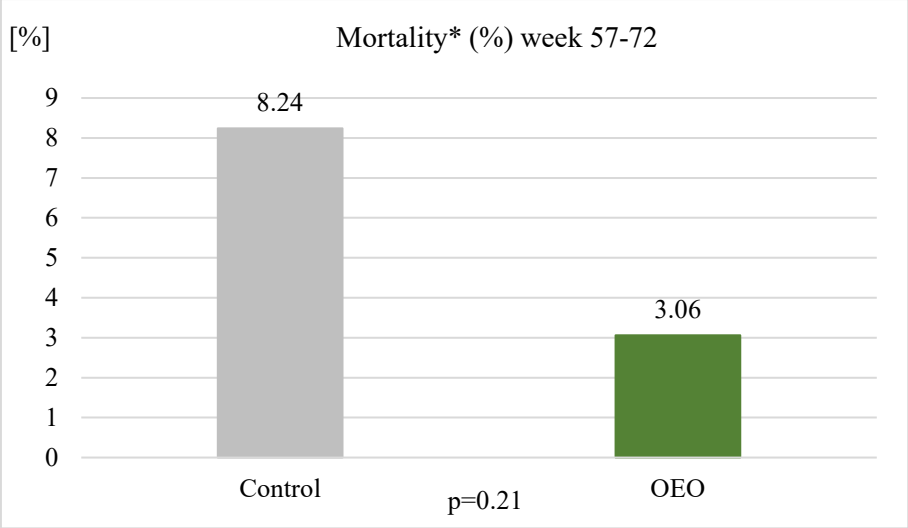

Figure 4 - The use of OEO in laying hen feed at the end of the laying period reduces the mortality rate.
* In a stress situation due to a problem with the lighting program.

IV. DISCUSSION

The findings confirm positive results from a previous study, adding the same natural OEO to laying hens diets. The trial demonstrated significant improvements in feed conversion rate and eggshell thickness when feed was supplemented with OEO from week 60 to 72 of life. These improvements could be attributed to a higher chymotrypsin and lipase activity ($P < 0.05$) in the ileum and consequently an improved fat and protein digestibility (Feng et al, 2021). He et al (2017) showed that laying performance, average egg weight, feed conversion and likewise amylase and trypsin activities were significantly improved ($P < 0.01$) by the addition of 100 g OEO/t laying hen feed (He et al., 2017). Increased enzyme activity in the gut allows fats and proteins from the feed to be used more efficiently for egg mass production, which can result in improved feed efficiency, performance and associated economic benefits for the farmer (Reshadi et al., 2020).

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METHIONINE AND ALPHA-TOCOPHEROL SUPPLEMENTATION IMPROVE LIVER HEALTH OF AGED LAYING HENS

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Summary

Aged laying hens are exposed to oxidative stress which can contribute to liver damage. This study aimed to investigate the effects of methionine (Met) sources (DL-Met and DL-2-hydroxy-4-(methylthio)-butanoic acid (OH-Met)), Met levels (100, 110, and 120% of breeding company recommendation) and alpha-tocopherol (VE) levels (20 and 40 IU) supplementation on liver functioning in 70 wk aged laying hens. Met source and level interaction had significant effect on liver index ($P < 0.01$) along with significant interaction effect of Met source and VE level ($P < 0.01$). Further, OH-Met reduced liver fat percentage compared with DL-Met ($P < 0.01$). Met sources had no effect on liver Triglycerides (TG) and total cholesterol (TC) but Met level significantly affected TG ($P < 0.05$) with Met source and level interaction ($P < 0.01$), however, lower VE level reduced TC in liver ($P < 0.05$). Met source and Met level influenced superoxide dismutase (SOD), while catalase activities (CAT) was influenced by Met level only ($P < 0.05$). It was found that OH-Met at 110% level significantly increased SOD compared with other Met levels. DL-Met at 100% level and OH-Met at 110% exhibited higher CAT activities ($P < 0.05$). DL-Met at 110% level and OH-Met at 100% showed significant reduction of malonaldehyde (MDA; $P < 0.05$). Further, OH-Met significantly increased mRNA gene expression of glutathione synthase (*GSS)* and nuclear factor erythroid 2-related factor (*Nrf2)* compared with the DL-Met group ($P<0.05$). A higher number of normal livers were found at 100 % OH-Met with 20 IU VE supplementation. Taken together, this research demonstrates that the liver health of aged laying hens was slightly improved by OH-Met through enhanced hepatic antioxidative functions.

I. INTRODUCTION

Aged hens with their regressive antioxidant capacity are susceptible to reactive oxygen species (ROS) and reactive nitrogen species (RNS), thereby various liver metabolic disorders may occur (Gu et al., 2021; Ponnampalam et al., 2022). Though supplementation of Met and VE are involved in antioxidant defense system (Agostini et al., 2016; Mazur-Kusnirek et al., 2019), previous studies highlighted oxidative stress may increase the Met and VE demands beyond standard requirement of chickens (Lugata et al., 2022). Therefore, we hypothesized that current industry standards may be insufficient to fulfill the Met and VE dietary requirement of aged laying hens for antioxidation functions. Thus, this study aimed to investigate the effects of Met source, Met levels and VE levels on oxidative potential in aged laying hens.

II. METHODS

A total of 864, Hy-line brown laying hens (70 wk age) were randomly divided into 12 treatment groups with 6 repeats (12 birds in each repeat). The experimental birds were subjected to a factorial array of treatments using two Met sources (DL-Met and OH-Met) with three Met levels (100, 110, and 120% of breeding company recommendation), and two VE levels (20, 40 IU) The experimental birds were fed a corn-soybean meal diet. At the end of 12 wk of experimental period, one bird close to the average body weight of the replicate was selected per each replicate and euthanized by cervical dislocation. The body cavity was opened to collect and weigh the livers. The liver index was calculated by using the equation: Liver index (%) = [liver weight (g) / live weight (g)] × 100%.

The visible evaluation of the livers from different treatments was performed for lesions.

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Tissue samples from the liver were collected in RNA-free centrifuge tube, snap frozen in liquid nitrogen and stored at -80°C for mRNA analysis. Total RNA was extracted from liver tissue. The total RNA was reverse transcribed into cDNA. Quantitative real- time PCR for CDO, GSS, Nrf2, and GST was performed according to instructions of the manufacturer. Gene expression were analyzed using the relative PCR amplification analysis method ($2^{-\Delta\Delta CT}$). The content of fat in liver was detected by ether extraction method. The liver triglycerides (TG), total cholesterol (TC), SOD, MDA, reduced glutathione (GSH), oxidized glutathione (GSSG), CAT, were determined using a commercial kit.

The statistical differences were determined by three-way ANOVA in a 2 × 3 × 2 factorial arrangement with Duncan's test for multiple comparisons. Meanwhile, one-way ANOVA and Duncan's multiple comparisons were used when a significant interaction was observed. A value of $P < 0.05$ was considered significant. $0.05 < P < 0.10$ was viewed as a trend.

III. RESULTS

Met sources and Met levels interaction effect were observed on liver index, DL-Met at 100% level and DL-Met with 20 IU VE level exhibited higher liver index value ($P < 0.05$), as described in Table 1. Furthermore, OH-Met group had lower fat percentage than DL-Met ($P < 0.05$). We observed significant effect of Met level on TG wherein OH-Met had lower TG at 100% compared to DL-Met at the same level ($P < 0.05$). Total cholesterol in liver was significantly decreased by VE at 20 IU ($P < 0.05$).

Table 1- Liver index and lipid at the end of 12 weeks of experimental period.

Met source	Met level	VE level	Liver index	Fat (%)	TG (mmol/gport)	TC (mmol/gprot)
DL-Met			1.98	23.49	0.24	0.063
OH-Met			1.94	21.08	0.23	0.059
	100%		2.03	23.43	0.25	0.062
	110%		1.89	21.76	0.24	0.063
	120%		1.96	21.67	0.21	0.058
		20 IU	1.94	21.96	0.23	0.058
		40 IU	1.98	22.61	0.24	0.065
DL-Met	100%		2.15	24.67	0.28	0.067
	110%		1.77	23.21	0.22	0.066
	120%		2.02	22.58	0.22	0.057
OH-Met	100%		1.91	22.18	0.21	0.057
	110%		2.01	20.31	0.26	0.061
	120%		1.91	20.75	0.21	0.060
DL-Met		20 IU	2.02	24.06	0.24	0.060
		40 IU	1.93	22.91	0.24	0.067
OH-Met		20 IU	1.85	19.86	0.22	0.056
		40 IU	2.03	22.30	0.23	0.063
Pooled SEM			0.030	0.510	0.006	0.001
Probability						
Met source			0.534	0.014	0.346	0.162
Met level			0.138	0.231	0.039	0.297
VE level			0.423	0.495	0.649	0.011
Met source*Met level			0.002	0.899	0.001	0.146
Met source*VE level			0.019	0.062	0.853	0.890
Met level*VE level			0.948	0.260	0.853	0.979
Met source*Met level*VE level			0.572	0.282	0.599	0.887

Note: Met, methionine; VE, Vitamin E; OH-Met, DL-2-hydroxy-4-methylthiobutanoic acid; DL Met, DL methionine; TG, triglyceride; TC, total cholesterol; IU, International Units

The supplementation of OH-Met increased both GSH and GSSG content compared with the DL-Met, while concomitant decrease in SOD activity in this group ($P < 0.05$) (Table 2). SOD activity decreased linearly with increasing level of DL-Met but OH-Met at 110% level had the highest SOD activity compared with other Met levels. In addition, Met at 110% level showed significantly higher SOD and CAT activities ($P < 0.05$). But GSH content linearly increased with the increase of Met level ($P < 0.05$). DL-Met at 110% level and OH-MET at 100% showed significant reduction of MDA content. DL-Met at 100% level and OH-Met at 110% level exhibited higher CAT activities compared to other supplementation level of the groups ($P < 0.05$). As shown in Table 3, *GSS* and *Nrf2* expression in OH-Met was significantly higher than DL-Met ($P < 0.05$). With the increase of Met level, the *GSS*

expression increased significantly, and the *Nrf2* expression increased initially and then decreased significantly (P < 0.05). The *GSS* expression at 20 IU of VE was higher than at 40 IU (P < 0.05). For *GSS* and *Nrf2* expression, a marked interaction between Met source and level was observed (*P*<0.05). Additionally, distinct interaction between Met level and VE level on *Nrf2* was found (P < 0.05). The highest *Nrf2* expression was observed at 110% and 20 IU of Met level and VE level, respectively.

Table 2 - Liver antioxidant indices at the end of 12 weeks of experimental period.

Met source	Met level	VE level	SOD (U/mgprot)	MDA (nmol/mgprot)	GSH (μmol/gprot)	GSSG (μmol/gprot)	CAT (U/mgprot)
DL-Met			106.94	0.66	9.19	8.21	45.98
OH-Met			100.90	0.69	10.75	9.20	46.71
	100%		103.44	0.64	9.08	8.85	48.20
	110%		110.81	0.67	10.00	8.09	48.59
	120%		97.51	0.72	10.82	9.18	42.24
		20 IU	103.01	0.66	10.34	8.32	44.88
		40 IU	104.83	0.70	9.59	9.09	47.81
DL-Met	100%		113.21	0.65	8.35	9.05	53.05
	110%		106.48	0.57	9.04	7.60	42.79
	120%		101.12	0.76	10.17	7.98	42.11
OH-Met	100%		93.66	0.64	9.82	8.65	43.36
	110%		115.14	0.77	10.95	8.58	54.40
	120%		93.90	0.68	11.47	10.37	42.38
Pooled SEM			1.659	0.015	0.220	0.209	0.935
Probability							
Met source			0.041	0.333	<0.001	0.017	0.637
Met level			0.002	0.120	0.003	0.089	0.002
VE level			0.531	0.275	0.068	0.057	0.062
Met source*Met level			0.001	0.001	0.826	0.021	<0.001
Met source*VE level			0.528	0.957	0.654	0.793	0.428
Met level*VE level			0.569	0.929	0.777	0.290	0.956
Met source*Met level*VE level			0.301	0.970	0.966	0.697	0.715

Note: Met, methionine; VE, Vitamin E; OH-Met , DL-2-hydroxy-4-methylthiobutanoic acid; DL Met, DL methionine; TG, triglyceride; TC, total cholesterol; IU, International Units; SOD, superoxide dismutase; MDA, malondialdehyde, GSSG, glutathione disulfide; CAT, catalase activities.

Table 3- mRNA expression of genes in antioxidant related pathway.

Met source	Met level	VE level	*CDO*	*GSS*	*Nrf2*	*GST*
DL-Met			0.993	1.113	1.013	1.061
OH-Met			1.068	1.463	1.127	1.038
	100%		1.021	1.070	0.995	1.020
	110%		1.048	1.325	1.272	1.046
	120%		1.022	1.468	0.943	1.082
		20 IU	1.043	1.344	1.050	1.036
		40 IU	1.018	1.231	1.090	1.063
DL-Met	100%		1.056	0.988	1.180	1.115
	110%		0.960	1.059	0.992	1.059
	120%		0.962	1.291	0.867	1.009
OH-Met	100%		0.987	1.152	0.809	0.926
	110%		1.137	1.591	1.553	1.033
	120%		1.082	1.644	1.019	1.156
DL-Met		20 IU	1.000	1.188	0.986	1.060
		40 IU	0.985	1.038	1.040	1.062
OH-Met		20 IU	1.085	1.501	1.113	1.012
		40IU	1.052	1.425	1.141	1.065
Pooled SEM			0.027	0.040	0.036	0.030
Probability						
Met source			0.166	<0.001	0.006	0.710
Met level			0.897	<0.001	<0.001	0.708
VE level			0.658	0.048	0.311	0.655
Met source*Met level			0.161	0.034	<0.001	0.089
Met source*VE level			0.859	0.512	0.747	0.679
Met level*VE level			0.181	0.793	0.034	0.707
Met source*Met level*VE level			0.538	0.842	0.011	0.489

Note: Met, methionine; VE, Vitamin E; OH-Met, DL-2-hydroxy-4-methylthiobutanoic acid; DL Met, DL methionine; CDO, Cysteine Dioxygenase; GSS, Glutathione Synthetase; Nrf2, Nuclear Factor Erythroid 2-Related Factor 2, GST, Glutathione S-Transferase

We found that OH-Met at 100% level had a higher number of normal livers than the DL-Met group. Further, OH-Met at 100% level with 20 IU VE supplementation had the most normal livers among all the groups, as described in Table 4. In conjunction with VE at 20 IU, OH-Met diets led to more normal livers and fewer livers with severe lesion.

Table 4 - Degree of liver damage

Items	OH-Met						DL-Met					
Met Level	100%	110%	120%	100%	110%	120%	100%	110%	120%	100%	110%	120%
VE Level	20 IU	20 IU	20 IU	40 IU	40 IU	40 IU	20 IU	20 IU	20 IU	40 IU	40 IU	40 IU
Normal	5	4	4	3	3	4	2	4	3	2	4	3
Mild	1	1	2	3	2	1	3	1	2	4	2	3
Severe	-	1	-	-	1	1	1	1	1	-	-	-

Note: Met, methionine; VE, Vitamin E; OH-Met, DL-2-hydroxy-4-methylthiobutanoic acid; DL Met, DL methionine. N= 6 birds per treatment

IV. DISCUSSION

Liver indexes and hepatic lipid accumulation in aged layer hens can be an indication of liver health and metabolic function (Madeira et al., 2018). Antioxidant enzymes such as SOD, CAT, and GSH-Px are vital components of the antioxidant defense system (Cadenas and Davies, 2000). Previous research showed that antioxidant status or concentration can be manipulated through nutritional regulation, and the Met source (Wang et al., 2019). Consistently, our data confirmed that Met sources influenced antioxidant enzymes concentration. In the past, OH-Met was reported to increase GSH compared to DL-Met in broilers regardless of the tissue and level (Jankowski et al., 2017; Zhang et al., 2018). In line with our results, Ruan et al., (2018) also showed that GSH content increased linearly with the increase of Met. Thus, the increase GSH contents due to OH-Met supplementation indicates its antioxidant potential with scavenging free radical mechanism (Surai et al., 2019). The current study confirmed that *Nrf2* expression in OH-Met group was significantly higher compared with DL-Met. In this regard, the Nrf2-Keap1 system signal transduction pathways is one of the quickest responding systems to the changing environment, and it can upregulate the antioxidant defense networks (Pomatto and Davies, 2018). Additionally, OH-Met at 100% level with 20 IU VE had the most normal livers among all the groups. It can be argued that OH-Met supports antioxidant system better, resulting in normal liver functions. In conclusion, the study indicated that the inclusion of OH-Met enhanced liver antioxidant capacity, lowered fat content, and promoted lipid metabolism through the up-regulation of key genes in aged laying hens.

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THE SUPPLEMENTATION OF A SYNERGISTIC BLEND OF ORGANIC ACIDS IN COMBINATION WITH HYDROXY CHLORIDE COPPER IMPROVES PRODUCTIVE TRAITS AND REDUCES SHELL DEFECT DURING EARLY PHASE OF LAYING

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As the poultry industry continues to reduce antibiotic usage, the interest in effective alternatives is increasing. Organic acid (OA) may help to reduce pathogen load, modulate gut microbiota, and maintain gut integrity (Abd El-Hack et al., 2022). Cu on the other hand, may elicit a bacteriostatic effect in the hindgut when fed above the requirement (125–250 ppm) (Nguyen et al., 2022). This study aimed to investigate the efficacy of a synergistic blend of short and medium-chain fatty acids including slow-release lauric acid, target-release butyrate, and a phenolic compound (PFY) (Presan-FY, Selko Feed Additives) in combination with Hydroxychloride Cu (IBC) (IntelliBond, Selko Feed Additives) on the performance of layers and egg quality during the early phase of laying. A total of 400 Dekalb laying hens (19 weeks of age) were allocated in two dietary treatments with 25 replicates of 8 birds each. The treatments included (1) basal diet plus 180 g/t Virginiamycin+15ppm $CuSO_4$ (AGP) and (2) basal diet with 1 kg/t PFY+ 125 ppm IBC (PFY+IB). Diets were fed for a total of 23 weeks including an initial 2-week adaptation period from 19 to 20 weeks of age. Hens were kept in cages in an open-sided type of housing with an ambient temperature ranging from 25-37^{0}C. Hen-day egg production (HD), egg weight, feed consumption (ADFI), and FCR were recorded from 21 to 42 weeks, whereas egg quality parameters were measured at weeks 4, 8, 12, 16, and 24 of the study. Data were analyzed using the MIXED procedure in SAS and Tukey's range test was used to separate treatment means (P <0.05).

The dietary treatments did not influence the feed intake (P >0.05). However, the egg weight (+1.6 g/egg) and egg mass (+2.4 g) were significantly increased with PFY+IBC supplementation (P <0.05, Table 1). In addition, PFY+IB tended to increase HD (+1.63%, P=0.10) and significantly reduced FCR (-9 pts, P =0.0003) compared to AGP. Overall, feeding PFY+IB supplemented diets tended to increase yolk weight compared to AGP (14.36 vs. 14.07%, P =0.07) and significantly decreased the percentage of shell-less eggs (0.01 vs. 0.05, P =0.04). The dietary treatments did not affect mortality rate, albumen height, eggshell thickness, eggshell weight, and yolk weight. In conclusion, the results suggest that the combined supplementation of PFY+IB is an effective replacement for in-feed antibiotics that can enhance the production performance of layers and reduce shell defects during the early stages of laying.

Table 1 - Performance of hens supplemented with AGP and PFY+IB from 21 to 42 weeks.

Treatment	AGP	PFY+IB	SEM	P-value	Δ from AGP
ADFI, g	103.18	103.78	0.739	0.56	0.60
Hen-day, %	93.42	95.05	0.827	0.10	1.63
Egg weight, g	53.32^b	54.86^a	0.264	0.0001	1.53
Egg mass, g	49.80^b	52.13^a	0.518	0.002	2.33
FCR, kg/kg	2.182^a	2.096^b	0.019	0.003	-0.09
Mortality, %	2.5	1.0	0.045	0.51	-1.5

^{a,b} Means in a row with no common superscripts differ significantly (P ≤ 0.05).

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CONSEQUENCES FOR TREES AND SHRUBS GROWING IN SOILS WITH HIGH NUTRIENT LEVELS ON FREE RANGE LAYER FARMS

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Summary

Three free range layer farms with contrasting trees/shrubs (Oldman saltbush, olive trees and grapevines) had their soils sampled at increasing distances from the shed. Samples were taken underneath trees/shrubs and from the adjacent open range areas. Also, the trees/shrubs on these farms had plant tissues taken at increasing distances from the shed. Two farms had developed soil nutrient gradients, whereby nitrate and phosphorus were found at higher concentrations closest to the shed and reduced levels further from the shed. Most of the nutrient accumulation was in the top 10 cm soil, especially under saltbush and olive trees. Plant tissue analyses revealed no toxic levels or luxury uptake of nitrate, nitrogen and phosphorus despite soils being high in these nutrients.

I. INTRODUCTION

Free range layer hens deposit nutrients onto the range via their excreta. Over time, gradients of soil nutrients develop across the range with higher concentrations found closest to the shed and under shelters and trees (Wiedemann et al., 2018). Hens congregate underneath trees and shrubs seeking shade and shelter, but little is known about how well trees/shrubs handle the additional nutrient loads. Nitrogen and phosphorus are the two main plant nutrients whose excessive amount may lead to negative impacts on the environment. We hypothesized that the nutrient concentrations in soil would decrease with increasing distance from the shed, while anticipating higher concentrations under trees. Furthermore, we expected that nutrient concentrations in plant tissues of trees/shrubs would be higher when closer to the shed.

II. METHOD

Three farms were selected with fixed sheds and fixed ranges. Farms 1 and 2 had relatively similar soil textures with silty loam (pH 7.3 in $CaCl_2$) and silty clay loam (pH 6.9 in $CaCl_2$) respectively, and flat topography. Farm 3 had deep sand (pH 6.4 in $CaCl_2$), and a south-east facing slope (5 – 10%). Farm 1 had an outdoor stocking density of 10,000 hens/ha, while farms 2 and 3 were stocked at 1,500 hens/ha. The closest trees/shrubs on farm 1 were Oldman saltbush (*Atriplex nummularia*), planted 10 m from the shed on the south range. On farm 2 the first row of olive trees (*Olea europaea*) was 15 m from the shed on the north range, while the first row of wine grapevines (*Vitis vinifera*) on farm 3 was 23 m on the south-east facing upslope from the shed. Soil was sampled under the first row of trees/shrubs closest to the shed on each farm, and at 50 m and 100 m from the shed. Similarly, soil was taken from open range areas adjacent to the trees/shrubs at the same distances from the shed. All soil samples were taken in triplicate 30 m apart running parallel to the shed. Nitrate and phosphorus were measured at the depths of 0 – 10 cm, 10 – 30 cm and 30 – 60 cm. Due to soil constraints (hard clay layer), farms 1 and 2 were only sampled at two depths (0 – 10 cm and 10 – 30 cm). All soil samples were analysed for nitrate and phosphorus by the Eurofins/APAL laboratory (Adelaide, South Australia).

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Farm 1 saltbush plant tissue samples were collected in early summer when saltbush had new active growth. The first fully expanded leaf on a new growing tip was taken (100 leaves x 3 replicates x 3 distances from the shed). Olive trees on farm 2 were sampled when trees were at the late flowering to early fruit set stage. The first fully expanded olive leaf was sampled on fresh new growing tips without flowers/fruit (100 leaves x 3 replicates x 3 distances from the shed). Grapevines were sampled on farm 3 during veraison (onset of fruit ripening). Only the leaf blade (no petiole) was taken opposite a bunch of grapes (30 leaf blades x 3 replicates x 3 distances from the shed). All plant tissue samples were analyzed by the Eurofins/APAL laboratory (Adelaide, South Australia).

General ANOVA was conducted on soil nitrate and phosphorus data (Genstat v 21.1, VSN International, UK). Each farm was analysed separately. The main effects in the model were open range versus trees/shrubs, distance from the shed, and soil depth. Means and SEM were calculated for plant tissue results.

III. RESULTS

Nitrate levels were significantly higher in soils under saltbush (farm 1) and olive trees (farm 2) (108.6 ± 12.9 mg/kg and 83.9 ± 9.7 mg/kg, respectively) compared to the adjacent open areas of the range (43.1 ± 12.9 mg/kg and 40.0 ± 9.7 mg/kg, P = 0.002 & P = 0.004 respectively). Only farms 1 and 2 had significant nitrate gradients across the range (P = 0.003 and P < 0.001 respectively), whereby levels were highest close to the shed and lowest furthest from the shed (Figure 1 A). Nitrate levels close to the shed exceeded those of the desired level for olives, and vines. There are no levels available for saltbush. On the distant areas of the range, nitrate levels were within the desirable range. Farm 3 had very low nitrate levels. On all farms the top 10 cm of soil had approximately double the nitrate levels of those found at 10 – 30 cm (Figure 2 A). There were significant interactions for nitrate, whereby soil 0 – 10 cm under olives had significantly higher nitrate (128 ± 19.3 mg/kg) than soil at the same depth in the open areas (35 ± 19.3 mg/kg, P = 0.002). Yet, at the soil depth 10 – 30 cm, there were no differences between soils under olives and open areas (range 35 – 45 mg/kg). Similarly, there was higher nitrate under vines in the 0 – 10 cm soil depth (4.29 ± 0.51 mg/ kg, P = 0.012) compared to open areas (1.72 ± 0.51 mg /kg) and no differences at the other two depths (0.91 and 1.56 mg/kg respectively). There were no interactions for nitrate on farm 1.

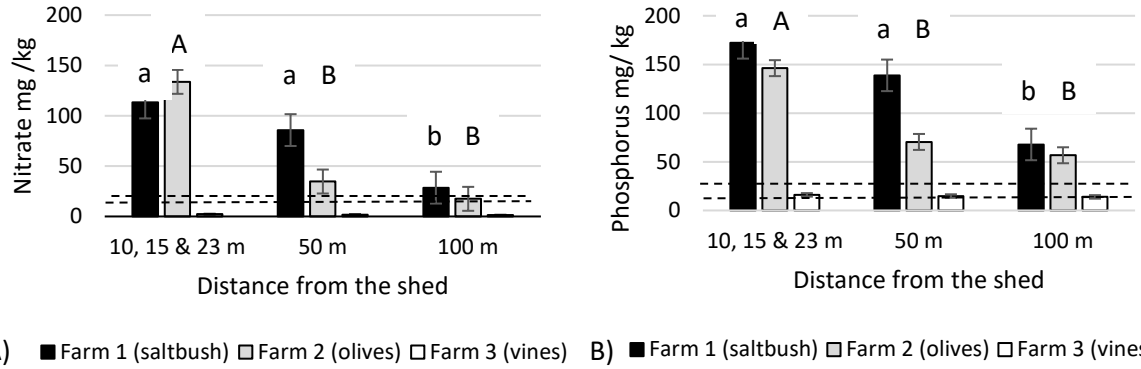

Figure 1 – Main effect of distance from the shed, A) Mean nitrate (mg/kg) and B) phosphorus in soil. Between dashed lines are the desired levels of nitrate and phosphorus for olives and vines. Different lowercase letters are significant (5% level) for farm 1, and different capital letters are significant for farm 2. Farm 3 not significant.

Phosphorus (Colwell) levels were higher in soils under saltbush compared to the adjacent open range areas on farm 1 (148.6 ± 13.3 mg/kg vs. 104.3 ± 13.3 mg/kg respectively,

P = 0.028). No significant differences were found between trees/shrubs and open range areas on farms 2 and 3. Levels of phosphorus were highest closest to the shed on farms 1 and 2 (Figure 1 B). Farm 3 phosphorus levels were very low and there were no significant main effects or interactions. Phosphorus was not leaching into the deeper soil layer (10 – 30 cm) on farms 1 and 2 (Figure 2 B). Levels were high in the top 10 cm and there was significant distance from shed x soil depth interactions on farm 1 and 2 (P = 0.003 and P < 0.001 respectively). Closest to the shed at soil depth 0 – 10 cm, phosphorus was significantly higher on farm 1 and 2 (315 ± 23.0 and 253 ± 11.5 mg/kg respectively). There were no differences at 10 – 30 cm regardless of distance from the shed on both farms 1 and 2 (range 15 – 39 mg/kg).

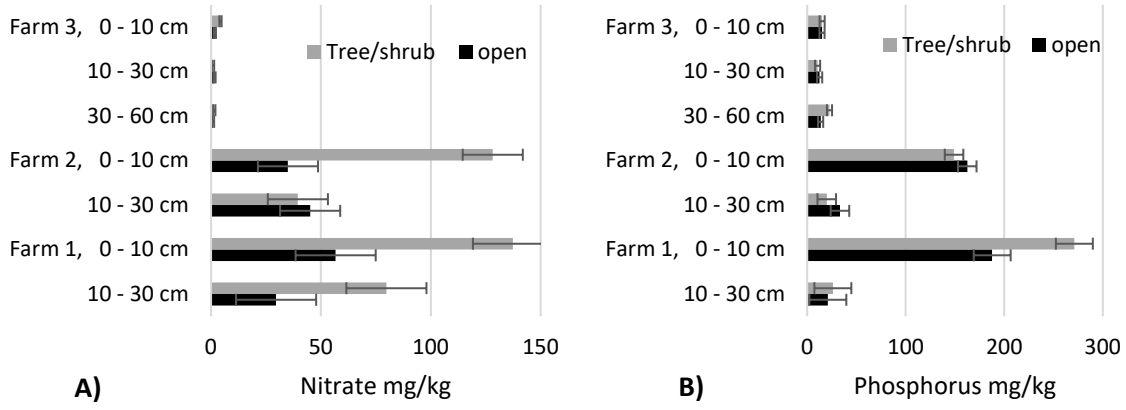

Figure 2 - A) Nitrate and B) phosphorus (Colwell) levels (mg/kg) on three farms at three soil depths under trees/shrubs and adjacent open range areas. Farms 1 and 2 only two soil depths measured.

In saltbush plant tissues, there were no nitrate and nitrogen % changes with distance from the shed on farm 1 (Table 1), this was despite a strong distance from the shed effects for nitrate in the soil on farm 1. However, olive leaf did show higher nitrogen % closest to the shed compared to further away from the shed. At all distances from the shed there was adequate nitrogen in the leaves of olives. Nitrogen % was below the target range for grapevines at the veraison stage on farm 3, except at 100 m from the shed. Both olives and grapevines had below 30 mg/kg nitrate in their plant tissues, well below that of saltbush. Phosphorus in plant tissues showed no trend across the ranges of farms 1, 2 and 3, even though there were strong phosphorus gradients across the range soils on farms 1 and 2. Phosphorus % in plant tissues was within the target range for olives and grapevines. There are no target ranges available for saltbush.

Table 1 - Mean ± SEM plant tissue analysis for nitrate, nitrogen, and phosphorus in the leaves of saltbush farm 1, olives farm 2 and grapevines farm 3 at various distances from the shed. NA – Not available.

Farm	Nutrient	Target range	Distance from shed		
		%	10 - 23 m	50 m	100 m
1	Nitrate	NA	415 ± 83	502 ± 190	350 ± 254
2	(mg/kg)	NA	< 30	< 30	< 30
3		NA	< 30	< 30	< 30
1	Nitrogen	NA	3.72 ± 0.02	3.94 ± 0.13	3.38 ± 0.17
2	(%)	1.5 – 2.0	1.78 ± 0.02	1.66 ± 0.05	1.63 ± 0.06
3		2.2 – 4.0	2.13 ± 0.02	2.03 ± 0.05	2.29 ± 0.09
1	Phosphorus	NA	0.25 ± 0.02	0.25 ± 0.02	0.21 ± 0.01
2	(%)	0.10 – 0.30	0.15 ± 0.01	0.17 ± 0.01	0.16 ± 0.01
3		0.15 – 0.30	0.25 ± 0.03	0.25 ± 0.01	0.32 ± 0.04

IV. DISCUSSION

Hens mostly congregated in large numbers closest to the shed and under nearby trees and shrubs. As a result, nitrate and phosphorus were found at higher levels in soils closest to the shed with a decrease as distance from the shed increased (except farm 3). These findings support those of Zoli et al., (2023), Wiedemann et al., (2018) and a study with free range broilers (Kratz et al., 2004). Even though there were strong nutrient gradients in the soils across the range for nitrate and phosphorus, this was not reflected in plant tissues. There were no toxic levels found in plant tissues and no evidence of luxury uptake. The farms in this study have been operating as free range for nine years (farms 1 & 2) and 11 years (farm 3). At this stage the trees/shrubs on all three farms were healthy and surviving the high nutrient levels. However, long term sampling of soils under trees/ shrubs and plant tissue analyses are needed to monitor the health of trees/ shrubs on free range farms. In addition, a diversity of tree/ shrub species should be analysed to gauge how well they manage high nutrient loads.

The clay-based soils on farms 1 and 2 retained nitrate and phosphorus, notably in the top 10 cm of soil closest to the shed. In contrast, the sand on farm 3 did not retain nutrients (including nitrate and phosphorus) and was mostly deficient, even under the grapevines closest to the shed where the hens would mostly range. Nutrients in the sand had most likely been leached further down the sand profile (deeper than 60 cm), and/or the grapevines and the inter row perennial grasses had intercepted some of the nutrients. Soil texture is a possible mechanism in that can strongly influence nutrient accumulation or leaching properties of soils (Gaines and Gaines 2008).

A practical strategy to help manage nutrients on the range is the use of moveable shelters in areas closest to the shed (0 - 25 m) permanently located trees/shrubs beyond 25 m. Furthermore, it is important to protect the top layer of soil (0 – 10 cm) from water and/or wind erosion as this layer contains the highest levels of nitrate and phosphorus. To minimise erosion, rock rubble can be placed closest to the shed (0 – 10 m) and hay bales spread across any bare areas on the range.

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INCREASED COST OF POULTRY INPUTS: A STIMULANT TO ZAMBIA'S INDIGENOUS CHICKEN SECTOR

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Zambia's poultry industry is comprised of the commercial and indigenous chicken (IC) sectors. Large horizontally integrated private establishments run the commercial sector. In contrast, the IC sector involves small-scale farmers (SSF), producing IC under a free-range system. The IC sector has an important role in complementing the commercial chicken sector while improving rural livelihoods and national food and nutritional security. IC production is characterised by negligible production costs regarding feed, infrastructure and management. In Zambia, unstable production and erratic supply of maize and soybean over the past eight years have contributed to increased feed prices (PAZ, 2023). In this paper, we demonstrate the impact of the increasing costs of inputs on the financial sustainability of the commercial chicken sector, a situation likely to stimulate the IC sector.

Between 1991 and 2012, Zambia's private poultry breeders invested over US$95 million in the commercial sector, resulting in over a 100% increase in the annual production of day-old chicks to 65 million (Bagopi et al., 2014; Bonaglia, 2009; Rakner, 2003). Despite this growth, fewer SSF have fully participated in producing commercial chickens, a case attributed to high production costs and limited access to the formal markets. Over 80% of SSF in the country rear IC, as an essential component of agriculture. This role of IC for SSF is a phenomenon common across Sub-Saharan Africa. In recent years, consumer demand for IC meat and products has significantly increased resulting in major price increases compared to commercial chickens and related products.

We analysed the market data from the Poultry Association of Zambia website (http://www.paoz.org/) for the first quarters from 2016 to 2023. The analysis revealed challenges in the commercial chicken sector attributed to high prices for live inputs, poultry feed and feed ingredients. During the review period, prices for day-old chicks and point-of-lay pullets increased by 57-125%. Prices for broiler and layer feeds increased by 67-96%, while soybean and fishmeal prices rose by 143-229%. Further, analysis showed increase in prices for IC, commercial broilers, and ex-layers by 150%, 79%, and 71%, respectively. Farm gate and national retail egg prices rose by 100% and 124%, respectively.

To demonstrate the impact of price dynamics on profitability for smallholder broiler and IC farming, we computed the gross profit margin for (a) three batches of 100 broilers and (b) one batch of 100 IC in six months. Results showed that the gross profit margin for broilers was 18.2% compared to 95.6% for producing IC. Strong returns for SSF from IC production are likely to support accelerated growth in the IC sector, however, harnessing the potential of the IC sector requires targeted interventions to address notable concerns for SSF including low access to the markets, lack of value addition, poultry diseases and others.

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EGG PRODUCER PERCEPTION OF FLOOR EGG PROBLEMS: CONTRIBUTING FACTORS

C. CIARELLI[1], P.J. GROVES[2] and W.I. MUIR[3]

Summary

A survey was undertaken to garner some initial information about factors that may contribute to floor eggs within Australian cage-free egg production systems. For each of 69 egg-laying flocks, the percentage of floor eggs and the producer's perspective as to whether that level of floor eggs was of concern, were recorded. Categorical and continuous variables about the farm, flock and housing environment were recorded during the laying phase of each flock. The impact of each variable in the context of the producer's perspective of the level of floor eggs being of concern, was analysed. The percentage of floor eggs ranged from 1-28%, with levels of 1-3 % for the producers not concerned with floor eggs and 2-28% for producers concerned about floor eggs. The inclusion of 2-3% floor eggs for producers both concerned and not concerned with floor eggs illustrates the different perspective of this issue amongst producers. No continuous variables were significantly associated with producer's concern with floor eggs; however, the categorical variables of cool white lighting and feather pecking were associated with floor eggs of concern to producers. The findings of this survey were valuable as few studies have explored these production variables with the incidence of floor eggs. They will also contribute to the development of a future survey designed to specifically identify key factors associated with floor eggs in Australian cage-free systems.

I. INTRODUCTION

During recent years, Australian supermarkets have changed from a predominant supply of eggs sourced from hens housed in conventional caged systems to an increasing number of eggs sourced from cage-free housing (Australian Eggs 2022). This move reflects the increasing demand for eggs produced by hens housed in systems other than cages (Edwards and Hemsworth 2021), where they have greater freedom of movement and more opportunity to express natural behaviour including scratching and dust bathing (Lay Jr et al. 2011). With recent updates to the Australian Animal Welfare Standards and Guidelines for Poultry, including a proposed phasing out of conventional layer hen cages (Department of Agriculture, Fisheries & Forestry 2022), and limited local adoption of furnished cages (Edwards and Hemsworth 2021), cage-free egg production is likely to increase. Within cage-free systems, hens may not lay their eggs in the designated nest boxes but in areas outside the nest box (Bécot et al. 2023), referred to as floor eggs, ground eggs, floor laying or mislaid eggs.

Compared to nest laid eggs, floor eggs have a greater risk of contamination due to their contact with faecal matter, moisture, dirt and dust, greater risk of cracks or fractures of the eggshell and requires staff to frequently walk the sheds to collect floor eggs and to move the birds around (O'Flaherty 2019). Floor eggs cannot be sold as first grade eggs, but as second grade eggs destined to produce pasteurised products, generating significantly reduced revenue (Australian Eggs 2023). As an initial investigation into the variables that may contribute to the incidence of floor eggs, some questions about them were included in surveys of producers of

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cage-free systems within an Australian Eggs funded project "Epidemiological risk factors for Spotty Liver Disease". While the larger survey was not specifically designed to evaluate floor eggs, it gathered information about each farm, flock and housing environment. This information was aligned with the producer's concern about their level of floor eggs and provided some initial indicators of factors that may be involved with floor eggs.

II. MATERIALS AND METHODS

A cross-sectional survey of cage-free egg laying systems within Australia was conducted during 2020-2022. Sixty-nine flocks housed in either barn or free-range systems from all Australian states except Tasmania were enrolled in the study. Initial survey interviews were conducted on farm (n = 43), but, with travel restrictions due to Covid-19, later interviews were undertaken by telephone (n = 26). Survey questions included whether the producer was concerned with the flock's level of floor eggs and the percent floor eggs. Categorial variables including the Australian state in which the farm was located, strain of laying hen, farming system and indoor shed structure during lay, whether perches and platforms were present during lay, lighting colour in the shed, and the incidence of feather pecking during lay. Information was collected about continuous variables, including flock age at transfer to the laying shed, number of birds transferred, total shed floor area, total stocking density, total nest space, nest density, age at first access to nest boxes and, for free range systems the age, when hens were first given access to the range. The responses to each question were entered into REDCap® and then downloaded into Excel, cleaned, and prepared for analysis. Categorical variables were analysed using contingency tables and Pearson χ^2 or, when an expected value was < 5, Fisher's exact two tailed test was used. Continuous variables were analysed using a Student's T-test with the dependent variable being whether the producer was concerned about the level of floor eggs.

III. RESULTS AND DISCUSSION

Of the 69 flocks included in the survey, the producers of 17 flocks (25%) had concerns about the level of floor eggs. For these flocks, floor egg production ranged from 2-28% with an average 7% and median 4%. In contrast, 52 flocks (75%) had floor egg levels between 1-3%, which the producer did not perceive to be a concern. Across these flocks, 1% floor eggs was both the average and median. In comparison, a recent qualitative enquiry about floor eggs with Australian cage-free egg producers found 4 of 10 producers (40%) were not concerned with floor eggs and the remaining 60% were concerned, experienced 1-15% floor egg (Campbell 2023).

The statistical assessment of categorical factors in the context of producer concern about the level of floor eggs is presented in Table 1. Most of the flocks were included in the analysis for light colour (n = 63, as 6 flocks housed with mixed lighting were removed from the analysis), layer strain, indoor shed structure and for continuous factors (Table 2), although some were not included due to missing information.

For flocks where the producer perceived a problem with floor eggs, the categorical factors of Australian state in which the flock was located, the type of cage free production system, the style of indoor housing, perch availability, platform availability and strain of bird did not contribute to floor eggs ($P > 0.05$; table 1). However, the use of cool white lighting within the shed increased the perceived problem of floor eggs ($P = 0.017$) relative to farmer perception with flocks with warm white lighting. The role of lighting of nest boxes and light intensity has been explored with floor egg production (Abbleby et al. 1984; Pillan et al. 2023) but lighting colour has not considered. In flocks where feather pecking had occurred, the

producer perceived the level of floor eggs to be concerning (P = 0.008). In comparison, Gunnarsson et al. (1999) found no correlation between floor eggs and feather pecking.

Table 1 - Univariate analysis of categorical variables during lay with producer concerns of floor eggs.

Categorical variables during egg-laying	Variable	No concern about floor eggs (N)	Concerned about floor eggs (N)	P-value [a,b]
Australian state	NSW	31	12	0.854[a]
	VIC	14	4	
	SA	1	0	
	QLD	2	0	
	WA	4	1	
Cage free farm system	Free range	50	16	1.000[b]
	Barn	2	1	
Indoor shed structure	Conventional, (flat deck)	33	13	0.387[b]
	Aviary	13	4	
Perches available	No	5	5	0.105[b]
	Yes	47	12	
Platforms available	No	35	15	0.124[b]
	Yes	17	2	
Light colour in the shed	Cool white	8	8	0.017[b]
	Warm white	39	8	
Brown egg-layer strain	A	8	6	0.206[a]
	B	37	10	
	C	6	1	
Feather pecking observed	No	50	12	0.008[b]
	Yes	2	5	

[a] Pearson χ^2 test used, [b] Fisher's exact 2-tailed test was used when an expected value was < 5, N-number of flocks.

Table 2 - Student T-test analysis of continuous variables during lay with producer concerns of floor eggs.

Continuous variables during egg-laying	No concern about floor eggs Mean	SE	Concerned about floor eggs Mean	SE	t-value	df	P-value
Bird age at transfer (wks)	15.87	0.10	15.59	0.31	1.146	67	0.256
Number birds transferred	19269	1390	17600	1742	0.634	67	0.528
Total floor area (m^2)	1589	82.4	1686	155	-0.575	67	0.567
Stocking density (b/m^2)	11.82	0.41	10.87	1.08	1.008	67	0.317
Total nest space (m^2)	194.03	16.0	180.25	20.52	0.451	64	0.654
Nest density ($birds/m^2$)	104.19	3.52	100.53	5.27	0.519	63	0.606
Bird age at initial access to nests (wks)	16.48	0.11	16.36	0.20	0.551	42	0.585
Bird age at first access to the outdoors* (wks)	21.51	0.31	21.43	0.56	0.129	57	0.898

N-number of flocks. * free range systems only.

None of the continuous variables significantly contributed to the producer's perspective of a concerning level of floor eggs (Table 2). There are very few reports on floor eggs that have addressed these types of continuous variables apart from nest density. In this regard, Williams (2021) recommends 100-120 hens/m^2 nest box. This range includes the nest densities for flocks where floor eggs were both a concern and not a concern for producers in this study.

It should be reiterated that this survey was not specifically designed to explore variables that may be involved in the production of floor eggs but was an opportunistic early investigation into potential risk factors, many of which have not been previously explored for floor eggs. This provides evidence and rationale for more targeted exploration of factors that may predispose flocks to the laying of floor eggs in Australian cage-free systems.

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UTILISING DYNAMIC TIME WARPING IN DATA MINING FOR FREE-RANGE LAYERS

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Traditionally, laying flocks have been compared for performance outcomes and subjected to pre-grouping based on known pre-determined variables (e.g., breed, age, diet, housing or management strategies). However, cluster analysis can use the outcome target parameters (e.g., performance) and identify homogenous groups (clusters) based on similarities in the outcome data (e.g., egg production) (Sibanda et al., 2020). These clusters can then be used for classification or benchmarking purposes.

This study aimed to assess whether the egg laying rate curve can be used to benchmark high and low performing laying flocks. From a statistical perspective, the egg laying rate curve is a temporal sequence of laying rate and each individual curve can be treated as an individual time-series. Dynamic time warping (DTW) based clustering was used to group similarly shaped curves (time-series) while taking into account the short term peaks and drops in egg laying rate. This enabled classification of sequences varying over time such as with peaks and drops in egg production.

Laying rate data from 51 free-range egg laying flocks (as 51 time-series) were subjected to DTW based clustering. The number of clusters was determined by analysing the within-cluster sum of squares, leading to 3 clusters with 15, 28 and 8 flocks assigned to Cluster 1, Cluster 2 and Cluster 3, respectively. To confirm whether the clusters were different in terms of performance, the clusters were compared using one-way ANOVA. Significant differences in laying rate, floor eggs, and egg weight were found among the clusters during the early laying period. Most parameter disparities were found in the late laying period. Also, the clusters differed significantly in total eggs/hen housed (Table 1), having high (Cluster 1), medium (Cluster 3) and low (Cluster 2) egg production.

Table 1 - Comparison of productive performance of laying hen flocks in 3 clusters based on egg laying curve*.

Cluster No. of flocks	Cluster 1 15	Cluster 2 28	Cluster 3 8	Pooled SE	P-value
	Early laying period (week 23-35)				
Laying rate (%)	91.96^{b}	90.37^{a}	92.03^{b}	0.44	0.00325
Floor eggs (%)	1.46^{a}	2.72^{b}	4.80^{c}	0.18	<0.00001
Egg weight (g/egg)	58.56^{a}	58.68^{ab}	59.31^{b}	0.17	0.04088
	Late laying period (week 36-70)				
Laying (%)	92.11^{c}	89.86^{b}	87.31^{a}	0.17	<0.00001
Floor eggs (%)	0.54^{a}	1.06^{b}	1.83^{c}	0.06	<0.00001
Feed (kg) /dozen eggs	1.49^{a}	1.51^{b}	1.52^{b}	0.004	0.00001
Egg weight (g/egg)	61.85^{a}	62.02^{b}	61.65^{a}	0.06	0.00006
Mortality (%)	0.15^{b}	0.14^{b}	0.09^{a}	0.006	<0.00001
	Total production (week 21 to 70)				
Eggs/hen housed at 70 wk	307.47^{b}	299.99^{a}	301.87^{ab}	2.80	0.0521
Floor eggs (%)	0.89^{a}	1.64^{b}	2.68^{c}	0.07	<0.000001

*LS Means and pooled standard error. Where P < 0.05, means with different letters are significantly different.

Thus, DTW can be useful for benchmarking laying hen flocks. Investigating the management and input differences among the clusters could help identify novel factors that improve egg production.

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BROMELAIN CAN ALLEVIATE THE NEGATIVE EFFECTS OF NECROTIC ENTERITIS IN BROILER CHICKENS

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Bromelain is a mixture of proteinases, derived from the stem and fruits of pineapple (*Ananas comosus*), which may have additional anti-bacterial and anti-inflammatory properties (Jančič and Gergieva, 2021). This study assessed the effect of dosage and administration methods of a bromelain-containing product (ANR-pf®, Anatara Lifesciences Ltd, Australia), on bird performance under a subclinical necrotic enteritis (NE) challenge.

A total of 540 Ross 308-day-old male chicks were randomly allocated into 6 treatments of 6 replicates each. The bromelain formulation was delivered to chickens via gavaging or in drinking water, twice on d 8 and 13. The treatments details are included in Table 1. Birds were challenged with *Eimeria* spp. on d 9 and *Clostridium perfringens* on d 14 and 15. On d 14 and 19, fresh faecal contents were collected to determine oocyst counts.

Table 1 - The effect of dietary treatment on growth performance.

	Treatment	oocyst counts (Log10/g)		day 0-35			
		Day14	Day19	FI[3]	WG[4]	FCR[5]	Mortality%
T1	NC[1] (Non-challenged)	0[b]	0[d]	3622[ab]	2398[a]	1.510[d]	2.78
T2	NC + 0.8 mL/kg gavaged	0[b]	0[d]	3641[a]	2385[a]	1.527[cd]	1.39
T3	NE[2] challenged	5.04[a]	4.35[a]	3410[b]	2155[c]	1.583[ab]	5.56
T4	NE challenged + 0.4 mL/kg gavaged	4.99[a]	4.22[ab]	3523[ab]	2198[bc]	1.581[ab]	0.00
T5	NE challenged + 0.8 mL/kg gavaged	5.07[a]	4.12[bc]	3470[ab]	2202[bc]	1.599[a]	4.72
T6	NE challenged + 0.8 mL/kg in water	5.05[a]	3.94[c]	3554[ab]	2291[ab]	1.551[bc]	1.39
	SEM[6]	0.033	0.070	52.3	37.6	0.014	2.08
	P-value	<0.001	<0.001	0.031	<0.001	0.001	0.399

[1]Non-challenged; [2] Necrotic enteritis; [3]Feed intake; [4]Weight gain; [5]Feed conversion ratio. [6]Standard error of means. [-d]values within a column with different letters differ significantly ($P < 0.05$)

Both non-challenged groups had improved WG and FCR compared to NE-challenged birds during d 0-35 ($P < 0.001$). Among NE-challenged-groups, birds administered bromelain in drinking water (T6) showed improved WG ($P < 0.001$) and lower FCR ($P < 0.001$) than the non-supplemented (T3) and gavaged with high dose of bromelain (T5) groups, respectively. Day 19 oocyst counts in NE challenged birds was lower in the water-delivery treatment (T6) compared to the non-supplemented (T3) or the low dose gavage treatment (T4) ($P < 0.001$).

Although the underlying mechanism of the two administration routes remains unclear, the water delivery method may have extended the contact time of the active compounds of the product, mostly bromelain, which may have contributed to the lower oocyst counts. Bromelain extract has shown to soften the shell wall of oocysts, and cause damage to the central cytoplasmic mass (Daiba et al., 2022). This could have partially led to the improved bird performance observed in this group. In conclusion, the current study suggests that administering this additive in the drinking water of broilers, may ameliorate the negative effects of NE and lead to improved performance of birds.

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