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TRENDS, STANDARDS AND RESEARCH DIRECTIONS FOR THE FUTURE OF LAYING HEN WELFARE

T.M. WIDOWSKI[1]

Summary

Whether through changes in animal welfare regulations or corporate commitments for cage-free eggs, most of the egg industry in the Western World is transitioning to cage-free housing for laying hens. The current genetics of commercial hybrids, as well as feeding and management practices, were developed for life in conventional cages, and it is not surprising that new welfare problems have emerged. Multi-tiered aviary systems are commonly used for cage-free production to increase stocking capacity in barns. However, behavioural adaptation to these complex housing systems and skeletal health problems, such as keel fractures, are both significant challenges in aviaries. Recent research indicates that pullets' early-life experiences with environmental complexity, specific structural elements (e.g., perches, ramps), and load-bearing exercise are crucial for their success in adapting to aviaries and for their long-term health and welfare. Feather pecking behaviour, a problem that can result in plumage damage, injury and cannibalism, occurs in all types of housing systems. However, the consequences can be worse in cage-free systems. New areas of research include investigations into the role of the gut-brain axis, the effects of early-life experiences, and effective edible enrichments to prevent feather pecking. Overall, new research is focusing on optimizing hen genetics and pullet management to match the birds to cage-free housing and reduce skeletal and behavioural problems. Finally, research into the development of automated methods for real-time assessment of hen welfare will help prevent problems in the barn and improve animal welfare audits for consumer-driven animal welfare assurances.

I. INTRODUCTION

The last 80 years have witnessed tremendous changes in the way eggs are produced and in the laying hens that produce them. In the decades following World War II, egg production underwent industrialization, transitioning from small, cage-free flocks to larger, cage-based operations as the use of battery cages began in the USA and later spread globally. At the same time, techniques in quantitative genetics were applied in earnest, and selection for production traits produced commercial hybrids with substantially earlier sexual maturity and higher egg production rates. The field of poultry science also flourished, with advances in nutrition and health management. These changes in genetics, housing, and feeding resulted in the production of one of the most nutritious animal proteins with one of the lowest environmental footprints. However, the use of conventional cages also came at a cost. Public perception of battery cages is largely negative and supported by scientific evidence that housing hens in barren cages compromises some important aspects of hen welfare.

Today, whether driven by legislation or corporate commitments, the entire Western world is experiencing another 360 ° shift in housing systems for laying hens. Societal concerns about hen welfare demand the elimination of conventional cages and a return to cage-free, or at least enriched, systems. However, this time, cage-free housing is being implemented on an industrial scale, with flocks of thousands or tens of thousands of hens housed in complex aviary systems. Additionally, we are using a knowledge base developed for the feeding and management of laying hens that were genetically selected for life in cages. Across countries,

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this shift is occurring through different means, at varying rates, and often with differing standards. The welfare trade-offs between conventional cages versus cage-free housing for laying hens are well established in the scientific literature (Hemsworth, 2021). Therefore, it is no surprise that the shift to cage-free housing presents challenges for producers, new welfare issues for laying hens, and a need for new areas of research to address them.

In this paper, I aim to provide an overview of the changing status of egg production in Europe, USA and Canada, current requirements for laying hen standards and assurances, and some of the animal welfare challenges associated with the change to cage-free housing. I will also discuss new and future areas of research needed to improve the welfare of laying hens.

II. STATUS OF THE EGG INDUSTRIES IN EUROPE AND NORTH AMERICA

a) Current Legislation and Corporate Commitments

In Europe, the 1999/74/EC Directive on the keeping of laying hens came into effect in January of 2012, allowing cage-free systems and enriched cages with specific requirements for space, nests, perches, foraging, and dustbathing substrate. Some EU member countries have independently banned all types of caging systems. In 2018, a European Citizens Initiative (ECI) "End the Cage Age," calling for an EU-wide ban on the confinement of poultry, pigs, rabbits, and calves, as well as regulations for imported products derived from these systems, was registered with the EU Commission (European Commission, Animal Welfare). The "End the Cage Age" ECI registered 1.4 million signatures by 2019, with many signatures coming from the Netherlands and Germany (where cages are already prohibited). The EC responded by commissioning an updated Scientific Opinion on the welfare of laying hens (EFSA, 2023) and by initiating public consultation, an impact assessment, and a policy initiative to phase out cages in the EU completely. To date, no changes have been made, although the 2025 "Vision for Agriculture," strategic planning document indicates consideration for updating the EU animal welfare regulations. In 2021, around 47% of eggs in the EU were produced in furnished cags, 36% in cage-free barns, 11% in free range, and close to 6% in organic, with significant regional differences across EU countries in the housing systems used (Majewski et al, 2024).

Following the adoption of Brexit in 2020, the United Kingdom maintained the minimum standards set by the EU Directive, which had been formalized into UK legislation in 2006 and 2007 (The Welfare of Farmed Animals (England) Regulations, 2007). Although a large segment of the British egg industry had already moved to enriched cages by 2012, several major supermarket chains made commitments in 2016 to sell only cage-free eggs with a 2025 deadline. According to the Department for Environment, Food and Rural Affairs (DEFRA, 2025), 71% of egg production is free range, and 17% is enriched cage, with the balance in barn and organic systems.

As of 2024, 10 states in the United States have enacted laws on the production or sale of cage eggs. The impact of these laws was projected to surpass 16% of the hen inventory in the USA by 2026 (USDA Economic Research Service). However, a much greater impact on the US egg industry has been the 2015/2016 corporate commitments by retailers and food service to only sell eggs produced in cage-free systems by 2025. Although not all commitments have been met, the inventory of cage-free laying hens in the US as of October 2025 was estimated at >136M laying hens, or around 46% of the national hen inventory (USDA Egg Markets Overview). Of the cage-free hens in the US, the vast majority are in barns, with a small percentage (~16%) in organic systems and even fewer in free range or pasture-based systems. The other 54% of US hens are in conventional cages.

The Canadian egg industry is also transitioning away from conventional cages, but on a very different pathway. In 2016, the Egg Farmers of Canada (EFC) announced that they would voluntarily stop using conventional cages by 2036. The Code of Practice for the Care and

Handling of Pullets and Laying Hens was published the following year, requiring that all hens be provided with amenities for nesting, perching, and foraging by 2036, and all enriched cage systems installed after 2032 to include amenities to provide opportunities for dustbathing (NFACC, 2017). As a supply-managed industry, the EFC embarked on a steady, organized transition to prevent market disruptions. Although the global wave of corporate commitments also hit Canada in 2016, the Retail Council of Canada, representing the major grocery store chains, retracted their cage-free pledge in 2021, instead indicating support for the National Farm Animal Care Council, the multi-stakeholder body that develops the Codes of Practice for Canada (Edmiston, 2025). When the 2017 Code was published, close to 90% of hens in Canada were housed in conventional cages. As of 2024, just over 43% of the flock was in conventional cages, with close to 37% in enriched and 13.5%, 4.9%, and 1.4% in cage-free barn, organic and free-range systems, respectively (EFC Annual Report, 2024). To date, most Canadian egg producers have opted to transition to enriched cage systems.

b) Welfare Standards, Labeling and Assurance Schemes

In the EU, the table egg industry is the only food animal industry with a harmonized compulsory animal welfare labelling scheme (European Commission, Animal Welfare Labelling). Eggs are stamped with the production method based on EU legislation for laying hens, which defines minimum standards of care for housing in enriched cages and cage-free systems. Across EU countries and the UK, there are also a variety of voluntary and private animal welfare assurance schemes developed to demonstrate higher welfare standards to consumers, such as the Dutch SPCA 3-star Beter Leven system in the Netherlands and RSPCA Assured in the UK.

In the USA, state legislation and corporate commitments for cage-free production generally do not specify any housing or husbandry standards. The United Egg Producers (UEP) developed their own national, industry-led Animal Welfare Guidelines for Cage-free Housing in 2017. Since 1999, the UEP has been working with an independent scientific advisory committee to establish and regularly update animal care guidelines for hens in cages. The scientific committee comprises poultry welfare scientists, veterinarians and an ethicist. The UEP Certified Program involves annual third-party audits of farms to ensure compliance with its guidelines and represents 90% of the eggs produced in the USA (UEP Certified, 2024).

In Canada, the Code of Practice has specific requirements for floor, feeder, nest, perch, and litter or scratch mat space allowances, as well as husbandry and euthanasia practices for pullets and hens across all types of housing systems. The Codes for all farm animals are developed by multi-stakeholder committees (including farmers, processors, government, veterinarians and representation from animal advocacy and retailers) and informed by a Scientific Committee Report on priority welfare issues. Egg farmer compliance with all requirements in the Codes is assured through the national EFC Animal Care Program. Annual on-farm audits are conducted on 2/3 of farms by EFC or provincial field inspectors, with the remaining farms receiving third-party audits. The Animal Care program is bundled with the Start Clean-Stay Clean® food safety program for Egg Quality Assurance TM certification (available at: https://eggquality.ca).

III. ANIMAL WELFARE CHALLENGES AND RESEARCH NEEDS

a) The Importance of Early Life Experience

Most cage-free housing today comprises multi-tier aviary systems that allow for increased bird density within the barn. In aviaries, resources such as feeders, drinkers, perches and nests are located on stacked tiers elevated well above the litter (ground floor). While an aviary may seem like a natural fit for a bird, laying hens are Galliformes, a heavy-bodied, terrestrial species better

suited to life on the ground. Although laying hens do still prefer to roost in high places at night, the numerous aerial transitions required to navigate between different tiers and structures in an aviary often prove challenging for them.

When aviaries were first developed in Europe, it soon became apparent that pullets destined for these complex housing systems had to be reared in similarly complex systems (Janczak and Riber, 2015). When not well-prepared for aviary housing, hens are less able to navigate the system and find (or reach) food, water, and nests, resulting in higher mortality and substantially more eggs laid outside the nest and on the floor. Providing perches early in life was found to be particularly important (Gunnarsson et al., 1999), as was providing resources, such as food and water, at multiple levels (Colson et al., 2008), so that pullets can learn to access vertical space. Up until very recently, there was a lack of any husbandry standards or welfare guidelines for pullets, and little research on pullet behavioural and physical development (Giersburg and Rodenburg, 2023). Now that the success and welfare of laying hens in cage-free housing depends on a bird that is calm, experienced and physically fit, research in this area has been rapidly growing.

In terms of behaviour, rearing experience affects cognitive development, spatial navigation, use of structures, and fearfulness (Campbell et al., 2019). Pullets reared in aviaries versus conventional cages have been shown to perform better on tests of cognition. For example, young hens reared in commercial aviaries were faster to find rewards and had better working memory in a spatial task (Tahamtani et al., 2015) and better success in a T-maze learning task (Rentsch et al., 2023a) than birds reared in conventional cages. Experience with structures such as perches, ramps, and elevated platforms also results in birds performing better on tests of navigation, such as ascending a series of offset platforms to receive a food reward (Gunnarsson et al., 2020; Rentsch et al., 2023b). Early experience with specific structural elements is also important. For example, exposure to ramps in the first weeks of life increases hens' ramp use in the layer house and reduces hesitancy when moving between levels (Norman et al., 2021). Finally, rearing in more complex environments, such as aviaries, has been shown to reduce fear of novelty (Braenstater et al., 2016; Rentch et al., 2024a) and improve overall use of 3-dimensional space (Braenstater et al., 2016).

As one might expect, musculoskeletal development is also affected by the rearing environment, as the amount of load-bearing exercise performed increases with the complexity of the rearing system (Pufall et al., 2021; Anderson et al., 2024; Rentsch et al., 2023c). Pullets reared in aviaries versus conventional cages have increased muscle mass (Casey-Trott et al., 2017a), larger keels (Casey-Trott et al., 2017a; Rentsch et al., 2024) and greater cortical cross-sectional area and breaking strength of the long bones (Regmi et al., 2015; Casey-Trott et al., 2017a). The addition of multi-tiered perching structures alone improved load-bearing activity, muscle mass, bone strength and biomarkers of bone formation in growing pullets housed in floor pens (Anderson et al., 2024). Specific designs of rearing systems can also affect pullet development, as there are considerable differences in the amount of vertical space, the number of structures requiring jumping, and the amount of horizontal space available for running, especially in the brooding sections where chicks are kept for the first few weeks (Pufall et al., 2021).

Interestingly, some of the effects on behavioural and musculoskeletal development mentioned above depend on the genetic strain of the bird. White-feathered strains tend to use more and higher structures in the environment and perform more aerial transitions between tiers (Pufall et al., 2021; Rentsch et al., 2023a); they also reap more of the benefits of growing up in a complex aviary than brown-feathered strains (Rentsch et al., 2023b,c).

b) Skeletal Health

While the load-bearing exercise inherent to cage-free systems does improve bone strength, fragile bones, and in particular, keel bone fractures, are still a significant problem in laying hens. The prevalence of keel fractures is high; estimates from commercial farms range from 20 to 96%, and the condition can impair mobility and is likely painful for hens, at least in the early stages (see Toscano et al., 2020).

Egg production rate and its associated calcium demand is assumed to be a contributing factor for fragile bones, including the keel, but the etiology of keel fractures is complex. Fractures on the medial section of the keel apparently result from collisions with environmental structures. In contrast, fractures located in the caudal area of the keel are non-traumatic in nature, possibly related to the early onset of lay and the repeated strain from laying eggs prior to complete ossification of the keel (Thøfner et al., 2020). Now that egg production rates are close to their biological limit with hens laying nearly an egg per day, breeding goals for laying hens are focusing on laying persistency and maintaining egg quality to support extended production to over 100 weeks of age. One of the significant challenges of extended laying cycles will be maintaining the skeletal health of laying hens in non-cage systems, and research in this area is ongoing (Gautron et al., 2021). Bone traits are moderately heritable, and one study of genetic correlations between production and bone traits indicated that early onset of puberty, rather than egg production persistency, was associated with a loss of bone quality in laying hens (Dunn et al., 2021). Research is also targeting the identification of behavioural phenotypes associated with stronger bones and fewer keel fractures (Toscano et al., 2020).

More research is needed to optimize rearing for lifelong musculoskeletal health and behaviour. Enhanced bone characteristics, such as increased cortical (structural) bone area resulting from prepubertal load-bearing exercise, are maintained throughout adulthood (Casey-Trott et al., 2017b). Lower prevalence and severity of keel fractures have been observed at the end of lay when pullets were reared in high complexity aviaries versus conventional cages (Casey-Trott et al., 2017c) or less complex aviaries (Rentsch et al., 2024b). Fewer fractures could be due to better navigation skills, differences in keel bone properties or both. Hens reared in aviaries versus cages experienced fewer and less forceful collisions when subsequently housed in enriched systems (Pullin et al., 2020) and laying hens reared with ramps not only used ramps more but also had fewer keel fractures (Norman et al., 2021).

c) Feather Pecking

Despite decades of research into its causes and prevention, feather pecking remains one of the major welfare issues in laying hen production. Severe feather pecking, which is the forceful pecking and pulling out of group-mates' feathers (sometimes followed by feather eating), causes damage to plumage and integument and can lead to cannibalism (van Staaveren and Harlander, 2020). Feather pecking has economic as well as welfare implications, as plumage damage results in increased heat loss and thus, increased energy requirement. Although feather pecking occurs in every type of housing system, it can be worse in non-cage systems as it can spread quickly throughout a large flock through behavioural contagion. In countries that prohibit both beak trimming and cages (e.g., the Netherlands, Germany, Austria), feather pecking outbreaks can be devastating for flock welfare.

One hypothesis for feather pecking is that it is a form of redirected foraging behaviour. However, the problem is much more complex with underlying differences in brain chemistry in birds that perform feather pecking (van Staaveren and Harlander, 2020). Factors influencing feather pecking are numerous and multi-factorial, including lack of foraging substrates, genetics, nutritional factors such as feed composition and form, changes in diet, environmental stressors such as stocking density, air quality, social disruption, and the list goes on. One

emerging area of research is the microbiota-gut-brain axis, which is known to influence behaviour (van Staaaveren and Harlander, 2021). Populations of laying hens selected for feather pecking have been found to have distinct microbiota profiles, although the relationship between gut microbes and feather pecking is still unclear.

Another area of more applied research on feather pecking is the identification of effective environmental enrichments. Pecking blocks, which are edible enrichments, have been demonstrated to improve plumage condition in laying hens. Commercially available pecking blocks differ in nutrient composition, with some primarily mineral-based and others containing more grains, molasses or fibre (Ehigbor et al., 2025). Interestingly, preferences for different pecking blocks vary across genetic strain, and their use varies across individual birds and time of day, with high calcium mineral-based blocks consumed more at the end of the day, corresponding with calcium appetite. Pecking blocks offer opportunities to reduce feather pecking and give individual birds some choice over the nutrients in their diet.

The rearing experience of pullets can contribute to the development of feather pecking, even when the behaviour does not emerge until later in the bird's life (Giersberg and Rodenburg 2023). Research is needed to determine how experience with different foraging materials, interruptions in foraging availability, and dietary changes during rearing predict feather pecking in the layer barn. Additionally, research is needed into how nutritional interventions during rearing, especially during critical periods, might influence feather pecking by altering the microbiota, gut function, or feeding/foraging motivation (Mens et al., 2020).

d) Animal Welfare Assessment

Animal welfare audits are a reality for many producers worldwide, and there is a sub-discipline within animal welfare science that focuses on practical animal welfare assessments. Since both farmer livelihoods and assurance of animal welfare depend on sound assessment instruments, measures of animal welfare need to be valid (accurate measures of animal welfare), reliable (good inter- and intra-observer repeatability), and feasible (practical and cost-efficient in the field). Although it has long been acknowledged that animal-based measures (ABMs) (e.g. real-time measures of health, mortality and behaviour) are the best animal welfare indicators, most practical assessments still rely on resource- or management-based measures (e.g. checking compliance with housing or husbandry standards), particularly in poultry. One area of research with considerable effort in Europe is the development of animal welfare surveillance systems through tracking of ABMs, for example, at slaughter plants (EFSA, 2021). In some slaughter plants, these measures are already in place for internal food safety monitoring. For laying hens, potential ante- and post-mortem animal welfare measures include feather condition, DOA, lesions, broken bones, broken keels, and condemnations, which can be tracked at the farm level. There is also considerable research effort to deploy intelligent technologies based on environmental sensors and computer vision in barns to provide real-time monitoring of laying health and welfare (Ma et al., 2025). Potential measures include auditory analysis of hen vocalizations and the identification of changes in flock behaviour to enable early detection of illness or outbreaks of feather pecking. A multidisciplinary effort will be critical for validating automated behaviour and welfare measures.

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FROM NOISE TO INSIGHT: LISTENING TO CONSUMERS ON POULTRY WELFARE

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Summary

Applying social listening, this study conducts a case study of online discussions about welfare in poultry production, demonstrating the methodological and strategic value of this approach for the industry. Using Talkwalker software, researchers monitored Australian news and social media over a one-month period (August-September 2025), gathering 292 relevant news and social media posts that generated a total of 2,900 engagements. A thematic analysis of social media comments revealed strong consumer reactions toward news about welfare in the poultry industry. The findings highlight how social listening enables timely detection of consumer sentiment, misinformation, and reputational risks. For the poultry industry, it offers an essential tool to strengthen transparency, trust, and proactive engagement.

I. INTRODUCTION

Animal welfare is a major issue in contemporary food systems. Growing recognition of farmed animals' sentience has raised expectations, with consumers increasingly demanding higher welfare standards in livestock production, including poultry (Verbeke & Viaene, 2000; Rawles, 2017; Gunderson, 2013). Formerly standard practices, such as caging, beak trimming, or restricted outdoor access, are now facing criticism, even when intended to protect birds' health. Consequently, animal welfare is seen not only as an ethical obligation but also as integral to food quality, trust, and willingness to pay (Alonso et al., 2020). This trend has been reflected by legal recognition of animal sentience in several jurisdictions (Kotzmann, 2023).

One of the major challenges for producers is polarised public opinion. With limited direct access to supply chains, consumers rely on the media for information, which strongly shapes their perceptions of food quality and ethics (Hoban & Kendall, 1994; Tonsor & Olynk, 2011). The impact of media is well-documented. For instance, coordinated campaigns by animal rights groups have driven changes in consumer behaviours, retailer strategies and regulations, such as the 2011 live cattle export ban (National Farmers Federation, 2013) and the planned phase-out of sheep exports by 2028 (Petrie, 2019).

Negative media foster polarised positions, baseless opinions, and emotionally charged debates, especially with the amplification effect of social media (Holt et al., 2018). Research shows that negative content attracts disproportionate attention, with critical or sensational posts shared more widely and discussed in greater detail, as evidenced by higher engagement levels for negative tweets (Hou et al., 2021). Negative online conversations, if left alone, can escalate quickly, generating backlash against producers and threatening their social licence to operate (Buddle & Bray, 2019). For producers, this dynamic highlights the importance of continually monitoring public forums and social media platforms, where information, whether accurate or misleading, can spread rapidly and shape consumer perceptions.

In light of this, social listening, originally a marketing tool for brand promotion and customer engagement (Perakakis et al., 2019), is now widely used in areas such as public health to track misinformation and sentiment (White et al., 2024). This evolution highlights its potential value for the livestock industry beyond commercial implications, where monitoring online conversations around animal welfare can provide timely insights into consumer concerns, misinformation, and shifting expectations. The present study, therefore, presents a case study

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analysis from a larger social listening project, focusing on online conversations related to welfare in poultry. The aim of this research is to provide a thematic analysis of social media users' comments in response to negative news about animal welfare. From this qualitative assessment, we demonstrate the value of social listening as a tool for capturing online sentiment on relevant issues rapidly and detecting potential reputational threats facing poultry producers.

II. METHOD

A mixed-methods study was conducted by applying social listening activities related to welfare in poultry production. To identify and extract relevant news articles and social media posts, we used Talkwalker, a web-based and AI-powered social listening platform that combines deep learning, neural networks, and natural language processing to read and detect basic valence (positive vs. negative). To acquire relevant news articles and social media posts, we formulated a search syntax using Boolean operators. Refer to Table 1, the results are directly related to the poultry industry, including both broilers and egg production. Additionally, all results must directly mention terms related to animal welfare, such as humane, organic, and free-range. The analysis includes Facebook, Instagram, blogs, online news, X (formerly Twitter), YouTube, Reddit, and Bluesky.

Table 1 - Search strategy developed for talkwalker social listening.

Search terms	(chicken OR chook* OR poultry OR egg*) AND ("animal welfare" OR humane OR "cruelty free" OR organic OR "free range" OR "free-range" OR freerange OR "barn laid" OR "barn-laid" OR pasture OR "pasture raised" OR "enriched cage" OR "enriched colony" OR "outdoor access" OR "access to outdoors" OR RSPCA OR "RSPCA Approved" OR ACO OR "ACO Certified" OR "large space" OR "no antibiotics" OR "antibiotic free" OR "free from antibiotics" OR "no added hormones" OR "hormone free" OR "no growth promotant*" OR "vegetarian feed" OR "slow growth" OR "slow grow" OR "beak trim" OR debeak*)
Language	English
Region	Australia
Duration	One month (August-September 2025)
Platforms	Facebook, Instagram, blogs, online news, X (formerly Twitter), YouTube, Reddit, Bluesky.

After identifying the relevant news media and associated social media responses (i.e., public posts and comments), we conducted a thematic analysis of the comments to identify meaningful themes. This is a case study involving an analysis of prominent news and social media posts that attracted strong audience engagement, such as likes, comments, and shares.

III. RESULTS

Within one month (August-September 2025), a total of 292 results (news and social media posts) were obtained, with a total engagement of 2,900 (i.e., likes, shares, and comments). Results indicated that 51.4% of online discussions reflected a positive sentiment, suggesting a generally positive public stance toward animal welfare in the poultry industry. However, with 48.6% of online discussions being neutral or negative, skepticism and criticism remain. Based on the number of engagements and sentiments, we identified several of the most engaging stories and posts related to animal welfare in poultry (Table 2).

Table 2 - Snapshot of top performing stories based on engagement.

Source	Title and Theme	Type	Sentiment	Engagement (Total of likes, shares and comments)	Reach (Total of views)
PR News Wire	European poultry - Quality and trust for consumers in South Korea **Theme**: European poultry's position in the South Korean market	News	Positive	N/A	204
ABC News	Caged eggs to stay on supermarket shelves until 2030 as Retailer (de-identified) abandons pledge **Theme**: Retailers' postponement of animal-welfare reforms for eggs in Australia	News	Neutral	~1,100	176.7K
Animal Justice Party AU	Caged eggs to stay on supermarket shelves until 2030 as Retailer (de-identified) abandons pledge **Theme**: Criticism of retailers' decision to postpone animal-welfare reforms for eggs in Australia	Social media	Neutral	37	276
Crikey AU	Chicken or the chip: How can meat suddenly cost less than potatoes? **Theme**: Analysis of food-pricing dynamics in Australia	Blog	Neutral	51	4.8K

Our case study sought to demonstrate the usefulness of social listening. We conducted a thematic analysis of all comments in response to the ABC's top-performing Facebook post concerning the decision of one of the larger Australian supermarkets to postpone its commitment to ban caged-egg sales (September 2025). The post received 465 reactions, with more than half of them being negative (242 angry and 68 sad), 141 comments (including spam) and 14 shares. We conducted a qualitative thematic analysis of 84 valid user comments (spam or hidden comments were excluded) posted in response to ABC's story. Some major themes emerged as follows:

a. <u>*Commitments to animal welfare (21% of comments)*</u> - Twenty-one percent of comments highlight consumers' consumption habits and commitments to avoiding caged eggs. Many consumers signal ethical consumption choices as part of their identity, reflected by their comments: *"Haven't bought caged eggs for 20 years"; "Free range only"; "I only buy from farm gates".*
b. <u>*Polarised and emotionally charged opinions (17% of comments)*</u> - Seventeen percent of comments expressed a clear disdain against caged eggs and described the practices as *"barbaric," "cruel," "pathetic,"* and *"pure evil."* Many commenters viewed the continuation of battery cages as unacceptable, reinforcing the growing societal rejection of intensive husbandry practices. This sentiment is often accompanied by calls for boycotts and collective action against the practice or the retailer involved.
c. *Distrust of the food supply chain (20% of comments)* - The decision to continue selling caged eggs in supermarkets damages consumer trust, showing that one bad actor could undermine the whole industry. Many consumers saw this decision as self-serving and claimed that the supermarket *"manipulate markets"* and is *"just after dollars.*
d. *Consumer dilemma (12% of comments)* - The story also sparked a debate around the cost of living and affordability, highlighting the tension between consumer financial capacity

and commitment to animal welfare. Several comments noted the prohibitively expensive price of free-range eggs: *"$10-12 for a dozen is ridiculous"*. Meanwhile, others raised an issue of accessibility for low-income consumers: *"Think about low-income families"*.

e. *Widespread debate (30% of comments)* - In the midst of polarised opinions, several debates occurred. These mostly focused on the biosecurity and disease risks associated with the free-range system, providing support for caged eggs. Many commenters said: *"Caged chickens are safer"; "Free range exposed to migratory birds"*.

IV. DISCUSSION

Our analysis of social listening data reveals an extensive, emotionally charged discourse on poultry welfare across social media, with many consumers advocating for stronger welfare standards. Although this analysis focuses on a single case, the findings underscore the amplification effect of social media in shaping public opinion on social issues such as animal welfare. More importantly, these conversations, far from trivial, offer insights into societal attitudes and highlight areas where improvements and proactive measures are expected. Given that negative content tends to spread rapidly and attract heightened engagement, it is increasingly critical for the poultry supply chain to monitor and understand consumer sentiment online.

While the research design and analytical approach may invite criticism for focusing on extreme views, this limitation can be mitigated through intelligent research design and robust analysis. Leveraging unsolicited dialogue offers unique benefits, particularly for emotive issues like animal welfare, which are best understood through observation of natural human behaviours rather than self-reported data. Traditional methods such as surveys, interviews, and focus groups often suffer from cognitive biases related to self-actualization or self-efficacy, leading to an intention–behaviour gap where stated preferences differ from actual behaviours.

By systematically tracking online discussions, the industry can identify and address misinformation, clarify misconceptions about animal welfare practices, and respond proactively before issues escalate into public outrage.

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REDUCING FEAR AND RISK OF PECKING IN POULTRY: WHAT ROLE CAN SENSORY PLANT EXTRACTS PLAY?

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Summary

The stress caused by our modern farming methods sometimes leads to fearful or aggressive behaviour. A plant extract based on a specific Citrus sinensis essential oil, known for its anti-stress effects, was tested in 2 different situations: a) on layers that had not been debeaked; b) during broiler removal. The behavior of the laying hens was modified and redirected towards more exploration and less mortality due to pecking. Broilers showed significantly less fear of humans.

I. INTRODUCTION

During the various phases of poultry farming, stressful situations are not uncommon. Some of these are critical, posing both economic and animal welfare problems. For example, picking up broiler birds can lead to aggressive reaction and unpredictable movement due to fear and losses in carcass quality. Egg-layers need to be beak trimmed to limit pecking, but this trimming is also a source of stress. On the other hand, there could be anti-stress solutions based on sensory plant extracts. A specific essential oil of Citrus sinensis has been described as having positive effects on stress in mice and pigs (Coutens et al., 2020; Menenson et al., 2020). The aim of these trials is to measure the effect of such a plant extract on the behaviour of non-debeaked layers and on the behaviour of broiler chickens when picking up.

II. METHOD

Trial 1: Three hundred non-debeaked Novogen hens have been placed in 12 different pens. All pens offered water, feed, litter and perches, but also one pecking block each. After 14 days of testing, only 6 pens received a 14-day drinking water treatment (OEO) with 100 ppm of a specific sensory additive mainly based on a Citrus sinensis essential oil (Phodé laboratory, France). The other 6 control flocks (CtrL) received nothing in their drinking water. Feed intake and egg production was monitored daily. The number of pecks on the pecking blocks was measured by webcam (18 video of 2 min, twice a week) and monitored as a cumulative number of peack observed during the video periods. The quantity of pecking blocks consumed was measured weekly by weight loss of the blocks. The number of hens that died during the experiment (28 days) and the number injured was monitored daily. The injured hens were immediately removed from the experiment. Feed intake and egg production were analysed using ANOVA GLM, other indicators were analysed using Mann-Whitney U test. $P<0.05$ was considered as significant.

Trial 2: Broilers flocks (Hubbardy 757) were followed from one day old to 8 weeks of age. The study was conducted across 8 Belgian broiler farms, each containing two barns of 13000 birds per barn: a control and a treatment barn. Both groups were fed a standard feed program following Hubbardy recommendation through a starter, grower and finisher feed program. The treatment group (OEO) received the water supplemented with 100 ppm of a specific sensory additive mainly based on a Citrus sinensis essential oil (Phodé laboratory,

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France) for at least 12 days before the test (giving the product time to work). The test was conducted between 32 and 34 days of age.

In each barn, broiler behaviour was evaluated once using the Human Fear Test (HFT), derived from the Welfare Quality Protocol and designed to measure the level of fear and stress experienced by broilers when exposed to human presence (10 footsteps inside the barn). The test involves 3 stages: 1) the time (seconds) it takes for the animals to stop running away from the experimenter; 2) the time (seconds) for 1 or more animals to start moving towards the experimenter; 3) the time (minutes) for the first chicken to pick experimenter's shoe. Data were analysed using a paired Student's t-test. Live weight were recorded and the Average Daily Gain (ADG) calculated at the end of production cycle.

III. RESULTS

Trial 1: Before treatment, the pecking blocks consumption did not differ significantly between the two groups (1.80±0.24 vs. 1.71±0.26g/d). Similarly, the number of pecks given to the pecking blocks did not differ before treatment either (662 vs. 657 pecks). Morover, the number of pecks decreased significantly ($p<0.05$) over time (Figure 1) in both groups. After treatment, consumption of the pecking block tended to increase in the EOE group (2.41±0.15 vs. 1.81±0.22 g/d, $p = 0.080$). After treatment, the number of pecks increase and became significantly higher in the EOE group compared to control (1036 pecks observed per pen vs. 542).
The number of hens dead or discarded due to severe pecking was 9 for the OEO group vs. 35 for the Ctrl one (Figure 2).

Trial 2: Results showed (Table 1) that treated broilers exhibited fewer fear indicators. In the first two stages of the HFT, times to hit each of the targets were reduced by 57% and 46% respectively in treated animals compared with control ($p<0.0001$; $p=0.059$). This suggested a reduction in fear of humans. During the third stage, time was reduced by 8% ($p=0.052$) and suggested greater confidence towards humans and a higher tendency to explore their environment. Although no effect was observed on scratches and pododermatitis, treated broilers showed a 36% reduction in the number of downgraded chickens compared to the control group (data not shown, $p=0.060$). No significant differences were observed between control and treated groups for ADG. This could be related to the short period of treatment.

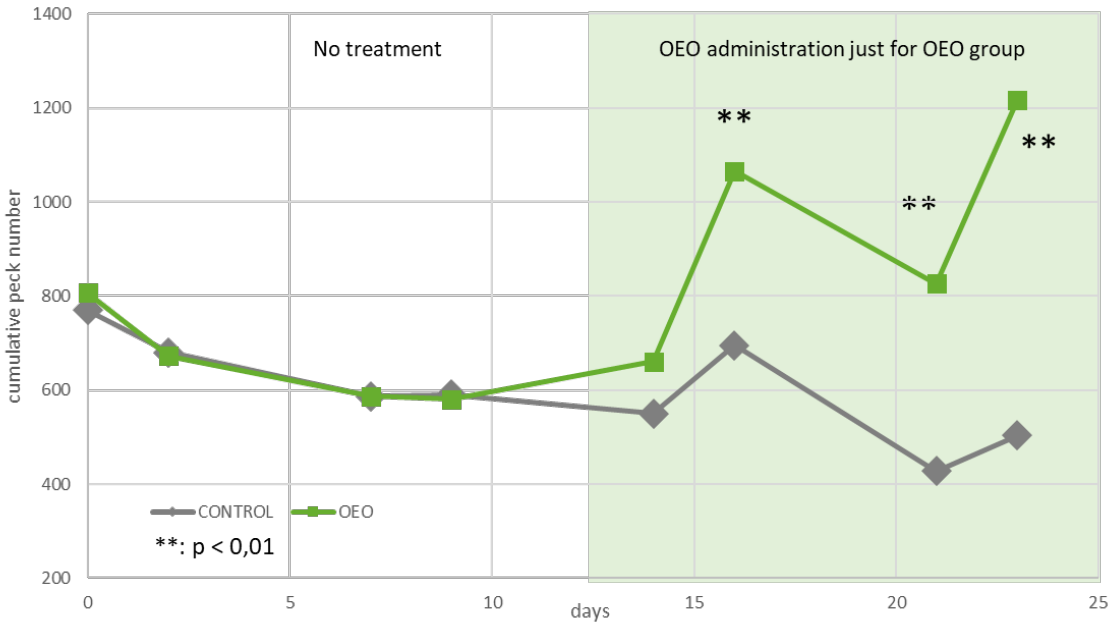

Figure 1 - Total number of pecks on pecking blocks per treatment per observation day.

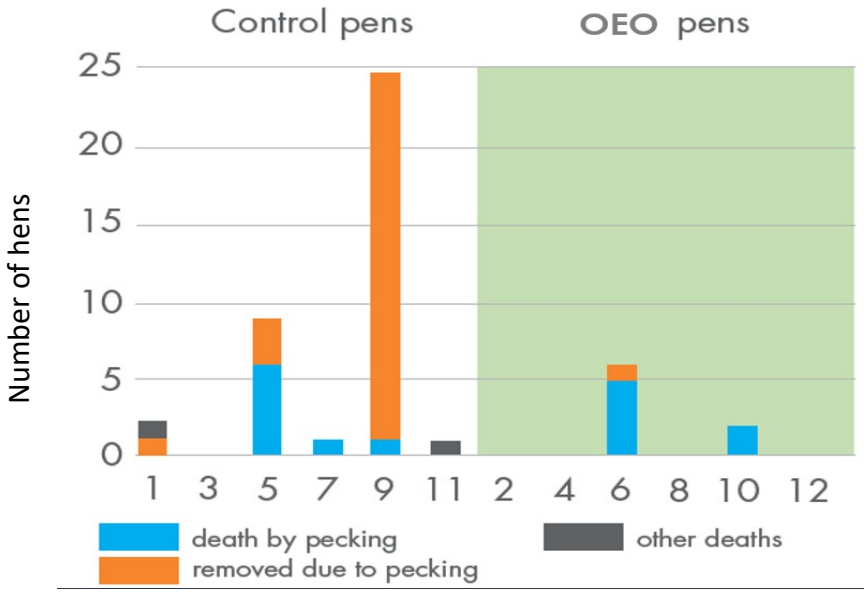

Figure 2 - Number of hens eliminated during the trial and causes for each pen (Trial 1).

Table 1 - Human fear results: latency time after 10 footsteps inside the barn (Trial 2).

Treatments	Stage 1* (s)	Stage 2* (s)	Stage 3* (mn)
Control	7.108	38.531	3.985
OEO	3.372	20.781	3.676
Relative effect	-52%	-46%	-8%
p-value	P < 0.0001	0.059	0.052

*Stage 1: the time (seconds) it takes for the animals to stop running away from the experimenter; Stage 2: the time (seconds) for 1 or more animals to start moving towards the experimenter; Stage 3: the time (minutes) for the first chicken to pick experimenter's shoe.

IV. DISCUSSION

Regarding the usage of pecking blocks by the hens' study, the consistency of the results between the use of pecking blocks and mortality suggests that OEO can play a part in the management of deviant behavior on laying hens' farm. One hypothesis could be that exploring behavior was redirected to picking blocks. Another hypothesis could be that thanks to the treatment, birds were less stressed and more aware of the environment. In that case, this could explain the change of interest for enrichment usually observed in the field. Enrichment is good for redirecting behavior, but stress is still here. With less stress, we can expect more exploring behavior. In that case, the use of enrichment is as much a tool for redirection as it is a tool for measuring animals' cognitive abilities and, therefore, their level of accumulated stress.

Regarding the Human Fear Test with broilers studies, we have here a very clear response on the level of stress of these broilers. This reduction of human fear could be useful to prepare the broilers catching; It could be nice also to reduce human fear for pullets' vaccination. For such an expectation, 12 days of treatment are too long. Further studies need to determine the best time of treatment for this application. On the other hand, the reduction of stress observed here was not connected with better performance. This could be explained by a too short time of administration to get a performance effect. Further investigations need to be conducted to determine if such a stress management could be profitable for animals and industry in terms of performances and meat quality.

All together, these results suggest that a plant extract like this specific Orange Essential Oil could alleviate the fear of birds and improve their perception of environment with a lower

risk of injury and mortality. More studies are needed to better understand the relationship between odors, behavior and welfare.

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RESILIENCE BEFORE RESTRICTION: INTEGRATING EARLY-LIFE EXPERIENCE, NUTRITION AND INDIVIDUAL VARIATION IN BROILER BREEDERS

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Summary

As the genetic potential for broiler chicken growth rapidly increases over time, so too does the level of feed restriction for breeding stock. Paradoxically, feed restriction to mitigate the negative implications for welfare and production from overconsumption and obesity results in other welfare impacts such as chronic hunger, frustration and stress. These impacts also make it increasingly difficult to meet production targets. Decades of research into qualitative feed formulations, optimal delivery, including precision livestock feeding systems, and investigations into various appetite suppressants have shown some promise; however, there are few solutions used in commercial conditions. Chronic stress has detrimental impacts on production and welfare, often resulting in maladaptive behaviours, such as stereotypies, which present further challenges to the birds themselves, the people managing them and meeting production goals. As such, minimising stress is critical for breeder flocks. We argue that a more holistic approach to managing breeder stress requires preparing birds for the inevitable stressors in addition to reducing stress. Stress resilience can be enhanced during key periods of brain development, such as between day 19 of incubation and up to 35 days post-hatch, highlighting the importance of maternal and rearing environments. However, there is a delicate balance between promoting resilience through early life challenges and creating stress vulnerability. This paper explores the evidence and suggests that stress resilience in poultry should be considered through a broad lens, considering not only the frequency and magnitude of the challenge but also the valence of the stressor, role of agency, and physical activity. While developing resilient animals does not negate the need to reduce stress in animals in our care, nor does it reflect overall welfare (i.e., less stress does not mean the animal has positive welfare), understanding what makes chickens more resilient in production systems may help breed and raise chickens that can cope with the allostatic load inherent in some production systems. This paper proposes that future improvements in poultry welfare and production require both reducing stressors *and* fostering resilience at the individual-bird level. With little known about stress resilience in broiler breeders, we provide evidence of rearing environments that build stress resilience in growing broilers, demonstrate individual differences in broiler breeder feeding phenotypes and how such phenotypes may moderate the effects of a qualitative diet intervention. Finally, the two concepts are merged to propose a clear direction for future broiler breeder research. Specifically, that building bird resilience will result in more uniform treatment responses or have additive effects resulting in optimal interventions to improve production and welfare.

I. INTRODUCTION

A major welfare concern in chicken meat production is the feed restriction of broiler breeders (Taylor *et al.* 2024a). Broiler breeders have the same fast-growing genetics as their progeny; however, if they are provided with ad libitum feed access, breeders overconsume, resulting in obesity, lameness, metabolic diseases, high mortality and disrupted reproduction (Mench 2002; Decuypere *et al.* 2010). To combat this, broiler breeders are feed-restricted, sometimes provided with only 20-30% of their ad libitum feed intake (Mench 2002; Decuypere *et al.* 2010).

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Paradoxically, safeguarding the health of these birds, through feed restriction, leads to negative implications for their welfare, such as hunger, frustration, and stress (Taylor *et al.* 2024a). Of the limited amount of broiler breeder research, the majority has focused on qualitative diet formulation, feed presentation and feed additives to reduce voluntary feed intake. The findings of breeder nutrition research have been comprehensively reported recently in a 2025 APSS keynote paper (Chrystal 2025). Demonstratable improvements to feed formulation, feed delivery and presentation and flock management were highlighted, and knowledge gaps were identified. We build on the principles presented by Dr Chrystal by considering the role of individual bird phenotypes and draw on evidence from decades of literature regarding stress resilience from both human and non-human animals, and data from a recent pilot trial that provides some evidence that stress-resilient birds may not only cope better with stress but may also engage with nutritional interventions more effectively.

Stress is a general 'catch-all' term and can be subjective. Here I refer to 'stress' as a challenge that results in a physiological stress response; through nervous and endocrine systems, the Sympathetic-Adrenergic (SA) and Hypothalamus-Pituitary-Adrenal (HPA) axis, respectively. Stress comes in various forms, including psychological stress, such as inescapable negative social interactions or thwarted access to resources and physiological stressors, including disease challenge, and thermal discomfort (Mellor *et al.* 2020). However, stress can also include metabolic digestion, mating and exercise. As such, not all stress is equal in intensity, duration or valence. However, all stress requires energy and various synchronised biological mechanisms to reinstate homeostasis; these efforts to cope with stress and return to homeostasis are sometimes referred to as 'strain' (Wu *et al.* 2025). The impact of the strain depends not only on the characteristics of the stress, but also on the magnitude and duration of the strain and the stage of development of the individual animal.

Stress resilient individuals recover quickly from physical and social stressors and disease challenges, which result from cognitive adaptations (learning and anticipation), behavioural flexibility and shifts in HPA axis reactivity (Colditz and Hine 2016). Individual differences in resilience are often stable, reflecting the animal's genetic profile and early life experience; however, they can be dynamic depending on the animal's ongoing social and physical conditions (Colditz and Hine 2016). It has been well documented that manageable stress during early life builds stress resilience (for example, Levine (1957); Lyons *et al.* (2001); Parker *et al.* (2007)). 'Manageable' stress often includes some level of agency and has positive implications for managing future stress (Colditz and Hine 2016); that is, the animal becomes more competent (Meehan and Mench 2007). There is not one path to build resilience; however, all mechanisms include interactions and modifications of the HPA axis. The age of exposure, intensity and duration of stress influences whether an animal will build resilience (i.e., stress inoculation) or will become stress sensitive, reducing its likelihood of coping in the future (Campbell 2024). One of the mechanisms for building stress resilience includes increased expression of Brain Derived Neurotropic Factor (BDNF) in the hippocampus, frontal cortex, amygdala and nucleus acumbens, and subsequently an impact on cognitive and behavioural flexibility and regulation of the HPA axis (Weinstock 2008). Broadly speaking, the impact on the brain shows that mild stress at critical development periods builds resilience. Conversely, chronic stress suppresses plasticity and animals fail to cope, which can result in the expression of maladaptive behaviours, such as stereotypies (Keeling and Jensen 2002). In poultry, critical periods of brain development (myelogenesis) predominantly occur during the last three days of incubation until 35 days post-hatch (Ehrlich and Mills 1985). During this time, the brain is susceptible to environmental impacts, mostly due to two features: stem cells that line the subventricular zone that produce glial or neural progenitor cells, and the plasticity of dendrites and spines after exposure to specific environmental stimuli. Perhaps then it is not surprising that applying stress during

incubation or early life has been shown to have a stronger impact on development and stress resilience than exposure later in life.

Stress resilience is beyond simply 'returning' to pre-stress productivity and must be understood through a multidimensional lens, including health, physiological stress response and affective states. As such, I argue that resilience extends beyond good health and production; however, they are both important outcomes of better stress resilience. The impact of stress on the biology of chickens is varied, and the mechanisms that modulate the effects appear to be linked to the age at which the animal experiences stress (Ericsson *et al.* 2016). However, evidence suggests that both in-ovo and post-hatch eustress shape resilience in chickens. There have been some efforts to increase stress resilience in poultry, but few interventions focus on stress resilience in broiler breeders. Observations of oral stereotypies in research and commercial broiler breeders suggest that chronic stress is persistent. However, the intra- and inter-flock variation in expression of some abnormal behaviours, such as feather licking (sucking), suggests that some flocks cope better than others (Taylor *et al.* 2024b). Although the nature of such abnormal behaviour is unknown, i.e., whether they are adaptive or maladaptive, the presence of the behaviour and variation across and within flocks suggests that the ability to cope with stress is a necessity for broiler breeders in commercial chicken meat production. Strategies to improve an animal's ability to cope with stress recognise that some stressors are inevitable for commercial chickens (Taylor *et al.* 2024a) and methods that build stress resilience will directly improve welfare and production; in addition, they may also increase the efficacy and uniformity of strategies to mitigate stress.

In this paper, I build on recent nutritional advances in broiler breeders and expand the consideration of strategies that not only reduce hunger stress but build stress resilience to prepare broiler breeders with the biological capacity to recover and adapt from inevitable stressors during rearing and production. Specific environmental factors can enhance resilience, but I argue that resilience may also impact how birds respond to feed and environmental interventions. This may be particularly important for poultry that are expected to experience inevitable cumulative stressors, such as broiler breeders, but also for commercial poultry that are housed in more complex environments, such as free-range housing systems (Armstrong *et al.* 2020). Priming birds to enhance resilience through experience may be an effective method to improve the sustainability of chicken production.

II. SHAPING RESILIENCE IN THE EGG: THE ROLE OF THE MATERNAL ENVIRONMENT

Maternal environments, both behavioural and physiological, act as early-life signals for progeny, calibrating the HPA axis and offspring's behavioural style. For example, maternal licking and grooming result in epigenetic changes of the glucocorticoid receptors in the hippocampus of rodents, which improve pup stress reactivity (Meaney *et al.* 2007). The outcome of this positive maternal interaction is progeny with lower HPA-axis reactivity and better stress recovery; that is, they are more resilient (Meaney *et al.* 2007). Maternal adrenalectomy in rodents reverses the impacts of maternal stress on brain and behaviour changes in her progeny, which can be reinstated with an injection of corticosterone (Weinstock 2008). Commercial poultry are not raised with mother hens; however, the maternal influence on incubation environments has clear impacts on stress resilience. Evidence to support this theoretical framework in broiler breeders is in its infancy, and there is much to learn. However, supraphysiological corticosterone injections into hatching laying hens compromised growth, egg production and quality, behaviour and stress reactivity (Ahmed *et al.* 2014). Although controlled feeding is necessary to prevent excessive body weight and reproductive disorders in broiler breeders, chronic feed restriction is associated with heightened stress and potential intergenerational effects. Bowling et al. (2018) found that increasing feed allowance by 4 % or

14 % (145 g and 160 g /hen/day) to achieve target body weights of 3.6 kg and 4.0 kg, respectively, reduced restriction severity and improved progeny immune function, increased the proportion of male progeny and their growth rate between 35-42 days of age (Bowling *et al.* 2018). Indicators of maternal stress (H:L ratio and expression of stereotypies) were lower in hens fed more, which suggests that the positive effects on the progeny resulted from reduced maternal stress. These results highlight that small adjustments to feed allocation can substantially influence maternal stress physiology and offspring development. Maternal stress has clear implications for progeny; however, completely removing stress is not possible. Building environments that allow hens to cope better may be key to balancing maternal effects. Whilst the potential for environmental shed design to better balance the transgenerational stress effects in broiler breeders is in its infancy, preliminary data suggest there is clear potential. Increasing the complexity of the broiler breeder housing environment reduced HPA reactivity and behaviour of progeny; the effect was stronger as breeder hens aged (Peixoto *et al.* 2021). This effect was not reproducible in the follow-up experiment, which highlights the complexity and how little we understand about the impact of environmental factors on stress and individual stress resilience. However, this work demonstrates the importance of breeder housing environments for progeny stress resilience and warrants further investigation. Focusing on the parent (and grandparent) environment may be key to mitigating stress (i.e., boredom and oral stereotypies directed to conspecifics), impacting stress reactivity and reducing the implications of maternal stress on progeny. Importantly, epigenetic maternal stress affects not only progeny stress resilience but also improves their gut health, metabolism, growth and carcass characteristics (Angove and Forder 2020). As such, building stress resilience is likely to have positive implications for broiler welfare and production.

III. SHAPING RESILIENCE POSTHATCH: THE ROLE OF ENVIRONMENTAL ENRICHMENT AND ACTIVITY

Housing environments that impact early life experience are particularly critical for resilience (Roelofs *et al.* 2016). Regular moderate physical activity and small-moderate novel experiences in the rearing environment that have some form of agency (i.e., the animal has choice and control) are positive stressors that have been shown to increase resilience. Physical activity increases BDNF and hippocampus neurogenesis, enhances antioxidant defences and normalises glucocorticoid receptor sensitivity. That is, *voluntary* wheel running in rodents, blunts corticosterone responses in rodents, and even low intensity shifts in activity, such as free-range access and provision of perches, have been shown to increase behavioural flexibility and reduce stress reactivity (Campeau *et al.* 2010). Scientific literature focused on human resilience has shown that physical activity predicts resilience to trauma, occupational stress, and depression (Szuhany *et al.* 2023). Increasing physical activity of broiler breeders may have cumulative beneficial effects, regarding stress resilience and reducing body weight, and thus the level of feed restriction (Campderrich *et al.* 2019). External factors can enhance resilience, such as social support, engaging in pleasurable activities and positive mood (Fredrickson 1998). For example, broilers housed in complex environments with access to resources that promote the expression of comfort, exploration and resting behaviours were more likely to interpret external ambiguous stimuli as ‘positive’ in a judgement bias test compared to broilers reared in standard environments without complexity. These results suggest that the housing environment can result in a more optimistic outlook, such that affective states (emotions and mood) impact perception of external stimuli.

Evidence from work in our group shows that increasing complexity during early life can enhance resilience of growing broilers, specifically increasing cognitive and behavioural plasticity and modification to the physiological stress response. For example, broiler chickens reared with visual contact to an outdoor range were more cognitively flexible than broilers

reared in less complex, stable environments (Taylor 2021). At 18 days of age, broilers from complex environments learned a Y-maze task (colour associated with mealworm reward) in the same amount of time (attempts) as birds reared in standard conditions. However, once the birds had learned the task (evidenced by 5 consecutive correct choices in the Y-maze), the correct arm (colour and location) was reversed, and the birds from the complex rearing environment learned the reversal task faster. Such results indicate that complex rearing environments altered brain plasticity and behavioural flexibility, which are both important characteristics of resilient animals. Although the mechanism remains unclear, this may be the impact of eustress, or increased activity from higher light intensity, or implications of positive mood. Irrespective, the simple act of providing visual access to an outdoor environment increased stress resilience and could be a practical tool for breeders early in life (i.e., that is, before approaching sexual maturity, where control of photoperiod is critical for development and production). In a subsequent study, we showed that chicks that were played audio recordings of maternal feed calls during the first five days of life were less anxious when challenged at 5 days post hatch, and expression of Corticotropin-Releasing Hormone Receptor 1 (CRHR1) in the hippocampus was greater at slaughter age, relative to control birds that received audio playbacks of white noise (Taylor 2024). CRHR1 has been associated with stress resilience in mice (Dedic et al. 2019); as such, suggested that audio recordings of maternal calls have positive implications for stress resilience in broilers.

We show that increasing the complexity of the rearing environment improved chick resilience through increased cognitive flexibility and altered physiological stress activity. Whilst other literature supports our findings in growing broilers, for example, Altan et al. (2013), there is a deficit of research into the effect of rearing environments on broiler breeder stress resilience. Further research is needed into the impact of novelty, age of exposure, choice, and biological relevance of the environment on stress resilience.

IV. FEEDING RESILIENT BIRDS: A CASE STUDY WITH BREEDER HENS

Whether through early mild stress, maternal cues, or exercise, each of these experiences acts as a *priming event*: a controlled challenge that tunes the stress system to respond efficiently. The outcome depends on context; too much or too little stimulation both impair adaptive calibration. True resilience emerges when early experiences foster flexibility rather than rigidity. Recent work from our collaborative broiler breeder research team provides some evidence of individual variation in nutrition trials. We hypothesise that these feeding and coping phenotypes may be altered through interventions before feeding, using developing stress resilience as a primer for nutrition interventions.

A recent pilot trial, funded by Poultry Hub Australia and conducted at the University of Melbourne (AEC # 2024-31165-60939-4; 4 replicates; 8 pens; 32 birds) assessed the impact of feeding breeder hens' diets with increased water swelling capacity. Hens that were fed a wheat-based diet with an additional 1.0 ml/g DM water swelling capacity through formulating 2% cellulose (Arbocel ® JRS, USA) into the diet displayed fewer indicators of hunger; including behavioural indicators from validated behaviour tests (cumulative feed intake test (CFIT), novel feed test (NFT) and frustration tests (FT),), behaviour in the home pen during feeding (aggression and competition during feeding) and post feeding (resting and food searching behaviour) and post mortem assessments. These results show the potential of qualitative diets to improve breeder welfare and warrant larger trials and long-term assessments to better understand the implications for hen welfare and production.

Although there were clear differences between the treatment groups, we were able to further understand how the treatment diet impacted individual birds using a principal component analysis (PCA) to summarise among-individual differences in feeding-related behaviour and physiology under restriction. Principal component analysis (PCA) transforms correlated

variables into new, independent components that capture the greatest sources of variation in the dataset. This helps visualise structure, detect relationships among traits, and interpret complex biological patterns more easily. From 20 variables measured on each individual, the PCA produced four components that explained 59% of the overall variation in behaviour; identifying four individual feeding phenotypes (Table 1): Hungry and socially reactive (PC1, 18.8%), Satiated and calm (PC2, 16.1%), Persistent feeders (PC3, 13.2%) and Heavy gorgers (PC4, 11%).

Table 1 - Summary of four principal components (PC1–PC4) derived from 20 feeding-related variables measured across a pilot trial. Each component represents a distinct feeding phenotype based on groups of variables that covaried strongly. Numbers are loadings (strength and direction of the relationship); grey values indicate weak loadings (<0.40). For each variable, h^2 shows the proportion of its variance explained by the four components. Component names (in bold) reflect the main behavioural or physiological theme of the variables with high loadings (> 0.4).

	PC1	PC2	PC3	PC4	h^2
Hungry and socially reactive					
Latency to peck feeder (CFIT)	-0.78				0.64
Faecal moisture	0.75				0.64
Empty crop weight (/kg bw)	0.69				0.51
Feed consumption rate (g/s; CFIT)	0.68		0.38		0.62
Number of vocalisations during habituation	0.58				0.42
Latency to peck perspex over feeder (FT)	-0.49				0.36
Number of interactions with enrichment	0.44			0.43	0.46
Satiated and calm					
Time spent resting		-0.32		0.75	0.68
Time spent food searching (foraging & ground pecking)		-0.31		-0.75	0.69
Empty gizzard weight (kg/bw)	0.32			-0.60	0.51
Amount of feed in the gizzard (4 hours after feeding)			0.36	0.58	0.53
Competition during feeding (aggression and displacement)				-0.52	0.36
Number of beak wipes after feeding		0.44	-0.45	0.47	0.67
Persistent feeders					
Time spent feeding (% observation, 48 hours after last feed)		0.90			0.86
Length of first feeding bout during feeding in the home pen		0.88			0.77
Time spent feeding (% observation, 24 hours after last feed)		0.80			0.68
Heavy gorgers					
Amount of feed in the crop (4 hours after feeding)			0.87		0.85
Experimental growth rate (% BW change)			0.71		0.6
Time spent drinking (% of observation)			0.68		0.49
Pre-Treatment weight			0.63		0.47

Following the PCA analysis, each individual was assigned a score for each component (PC1-4) and the effect of treatment on component scores was compared with GLMMs. The only feeding phenotype impacted by diet was ‘satiated and calm’ ($ß_{(29)}$ 0.73, t = 2.24, p = 0.033), with birds being more ‘satiated and calm’ when they were fed Arbocel. There was a trend for Arbocel to reduce ‘hunger and socially reactive’ (p = 0.101) and heavy gorgers (p = 0.119), but no impact on persistent feeders (p = 0.534).

Although these relationships require further investigation with larger sample sizes to clarify relationships, what this data does show is that the impact of diet was greater for some individual birds. From this preliminary investigation, we hypothesise that the impact of individual phenotype (i.e., reactivity and sociality) interacts with diet treatments, which may

benefit from methods that 'prime' birds through the environment and positive interactions to ensure each individual best responds to treatment. We observe that some birds remained calm and flexible despite restrictions, while others were dominated by hunger and frustration. Diet explains a significant proportion, but not all, of these responses. While many efforts have been made to develop and utilise technology to feed individuals, particularly in broiler breeder flocks (for example, Zuidhof *et al.* (2017), these tools are not yet commercially viable. Understanding how individual phenotype impacts the success of diet treatments applied at the flock level may be a practical method to combine strategies that increase the efficacy of interventions and improve flock uniformity. The next step for our research team is to replicate this study and include assessments of production and implications for progeny.

V. CONCLUSIONS: FROM REDUCING STRESS TO CULTIVATING RESILIENCE

Environmental complexity can shift behavioural flexibility and stress-axis function; it is therefore plausible that such 'priming' moves more birds into a resilient calm state. So, whilst management that reduces chronic stressors remains essential, combining stress reduction strategies with complex early-life environments and reducing maternal stress may result in more uniform, resilient parent and growing broilers. A focus on the individual and their stress response reminds us that we are managing and designing interventions for biological systems that are complex with many interrelated parts. Barren environments or stressful interactions with birds may disrupt the very biological systems we are aiming to improve. Priming birds to reduce reactivity and promote recovery from inevitable commercial stressors may, at best, have additive effects on innovative industry solutions or, at the very least, improve the uniformity of treatment responses. Increased uniformity reduces management strain and has known benefits for parent and growing broilers. Stress resilience, therefore, is a win for bird welfare, but also stockpersons, production and economics. Monitoring research interventions and management decisions on the farm at the individual level, through behavioural phenotyping, and technologies such as radio frequency identification technologies, can help identify vulnerable birds and tailor interventions for precision nutrition innovation. As technology rapidly progresses, we can increase the amount of data collected and analysed from individuals to better understand how our treatments and intentions are affecting the whole flock. There are clear benefits for building resilience in commercial poultry production, particularly for animals housed in more complex rearing environments such as breeders and free-range systems, and it should also be noted that early life (manageable) stress not only builds competence and resilience but also curiosity (novelty seeking) (Parker *et al.* 2007) further improving welfare.

Our research team proposes a dual strategy for managing broiler breeder stress: mitigate unavoidable stressors while cultivating resilience in the birds themselves. There is still much to learn about defining, measuring and promoting resilience in broiler breeders. However, including individual assessments and holistic interventions will be key to discovery. We promote a multidisciplinary approach to improving broiler welfare and production that includes understanding and targeting individual phenotypes by integrating nutrition with enrichment and considering stress resilience as a critical production trait.

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THREE DECADES LATER: FEED FORMULATION FLEXIBILITY REALIZED

M.T. KIDD[1] and K.B. NELSON[1]

Summary

In US scenarios, feed formulation flexibility with protein contributing ingredients stems from price-dependent formulation management, but in Australia another important piece represents protein-contributing ingredient imports. In an effort to highlight feed formulation flexibility, these proceedings outline three decades of amino acid nutrition outcomes and reduced protein diet flexibility tools with least cost formulation minimum knowledge for some less limiting amino acids (e.g., histidine) and some non-essential amino acids (e.g., glycine and alanine). Post threonine research, studies discussed herein are primarily from the main authors graduate students. Moreover, studies presented on histidine, glycine, and alanine are from the second author: K. B. Nelson, a current Ph.D. student.

I. INTRODUCTION

In 1999 I presented threonine research at the Australian Poultry Science Symposium (Kidd, 1999), my first introduction to Australia, the Sydney campus, and this venue. The research team I worked for (BioKyowa, Inc. a St. Louis, Missouri, a subsidiary of Kyowa Hakko of Tokyo, Japan) was a decade into research focusing on increasing the inclusion of L-lysine HCl and the practical advantage of using L-threonine in broiler diets. The former may seem straight forward, but at the time the NRC (1994) committee published a reduced lysine need in starting chicks, making the sale of increased lysine and the importance of threonine at a ratio to lysine tough. These proceedings will not consist of a review, but a brief walk down memory lane regarding feed grade amino acid failures, in addition to current success from essential (EAA) and nonessential (NEAA) amino acid ratios. The focus of these proceedings primarily consists of diet formulation with threonine, the branched-chain amino acids, and histidine, followed by NEAA.

II. THREONINE

The 1990s and early 2000s were critical times of change for dietary formulation practices in the United States. In the 1990s many commercial nutritionists had or were considering removing the dietary L-lysine HCl ingredient “cap” restraint in formulation. At this time primary breeder companies were also developing broiler strains that were late maturing and yielded more breast yield breast meat than the feed efficient multi-purpose broiler strains. In addition to research that established higher lysine needs for breast meat than feed conversion (Bilgili et al., 1992), it was also shown that the broiler responsiveness to lysine for breast yield was strain dependent (Acar et al., 1991). A key learning curve in the 1990s is that lysine efficiency required adequate dietary threonine. Although ideal protein requires optimization of both EAA and NEAA, threonine was unique as it is limiting after lysine in most cases, making the threonine to lysine ratio the key ratio for lysine efficiency. Hence, Kidd and Kerr (1997) demonstrated that the threonine minimum for breast meat yield was 8 to 11% higher than that for feed conversion. The first commercial use of L-threonine with a broiler integrator in the United States occurred in 1997 with a customer account managed by BioKyowa, Incorporated. In addition to research on threonine needs and its ratio to lysine, research spurred

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by the Ajinomoto group by Dave Burnham on L-threonine efficacy as an ingredient and digestible amino acid concepts increased the United States threonine market share (Kidd et al., 2002).

III. THE BRANCHED-CHAIN AMINO ACIDS

Similar to that of L-threonine, the Ajinomoto group supported research on the use of L-valine as an ingredient. It was demonstrated that levels around 0.5 kg/ton could support performance without setting minimums for arginine, isoleucine, or tryptophan (Corzo et al., 2011). Because the industry had become acclimated to the use of L-threonine, the sequential use of L-valine was relatively rapid (Kidd et al., 2013).

In regions where corn is the predominant cereal, a consequence of allowing the dietary inclusion of 4 to 5 feed grade amino acids is an increase in the nutrient leucine, especially in diets containing corn distillers grains. Maynard et al. (2022) assessed valine needs in diets with leucine to lysine ratios of 115 or 145 in Cobb 500 males and found limited interactions, although higher leucine tended to increase valine needs for body weight gain, but not breast meat yield (Figure 1). Surface plot responses (Figure 2) in Ross 308 type broilers (e.g., Indian River) demonstrated worsening effects of leucine and valine on feed conversion and breast meat yield, respectively, but responses on the former traits were improved as isoleucine increased (Kidd et al., 2021). Indeed, more work on practical dietary interactions of the branched-chain amino acids, especially in corn-based diets, should be assessed.

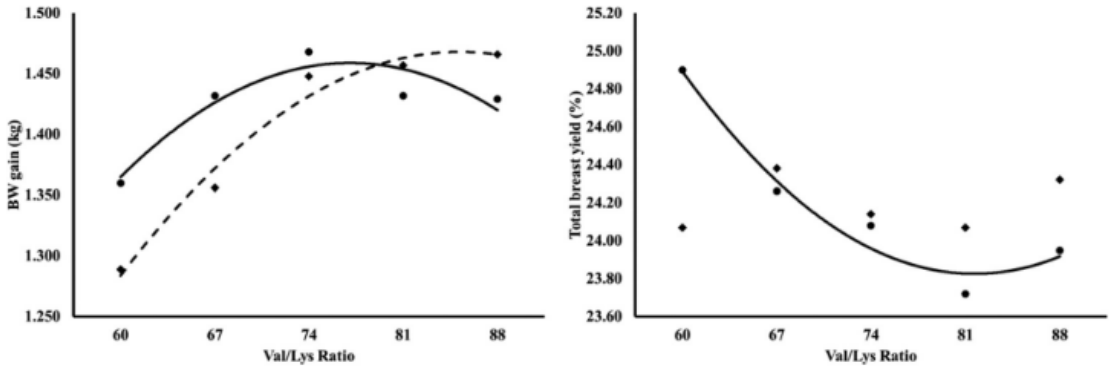

Figure 1 - Cobb 500 male responses to increasing dietary valine with low leucine (115 leucine to lysine solid line) or high leucine (145 leucine to lysine dashed line) for body weight gain (28 to 42 days of age) and breast meat yield (44 days of age) (adapted from Maynard et al., 2022).

IV. HISTIDINE

Recent work in our laboratory has focused on the dietary need of histidine in male Cobb broilers during the growing period. The first work was done to determine if the histidine need in growing broilers is affected by starter histidine levels (Nelson et al., 2024a). Histidine to lysine needs to support performance and processing traits in growing Cobb broilers was between 33 and 38, and starter by grower histidine levels did not interact (Nelson et al., 2024a). The second work on histidine represented a dose titration of histidine, but only breast yield responses occurred at histidine to lysine ratios of 37 to 39 (Nelson et al., 2024b). Furthermore, histidine to lysine ratios were titrated from 30.5 to 43.0 and live performance and carcass yields responded in a positive linear manner to increasing histidine (Nelson et al., 2024b). In the first work, test diets were balanced to EAA; but in the second work, test diets also included glycine. Could NEAA or glycine deficiencies have limited the histidine response in the first work?

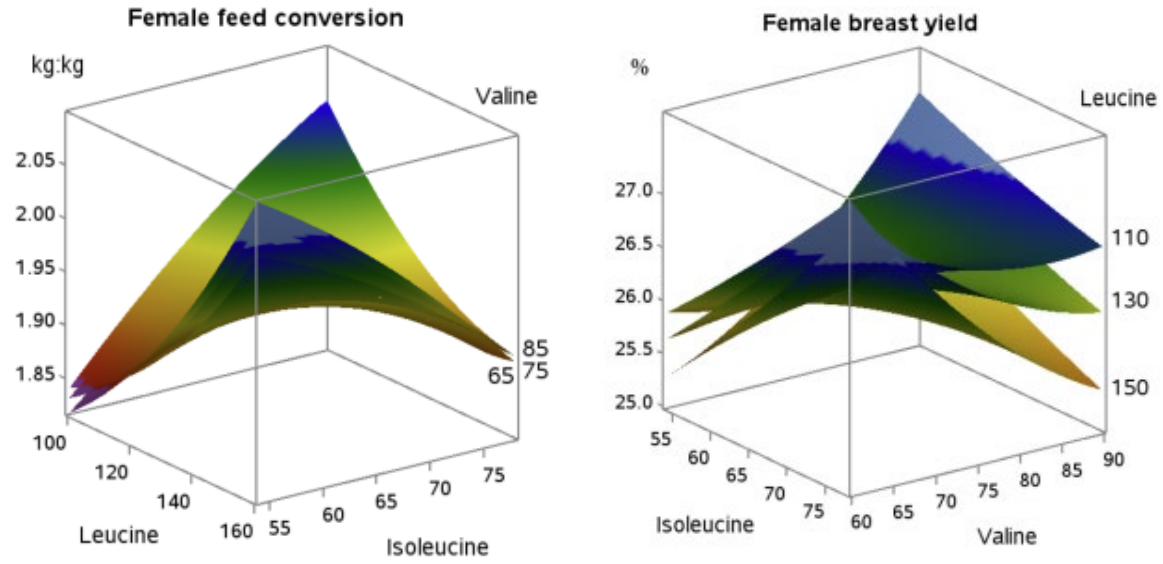

Figure 2 - Response surface plots from 22 to 35 day of age in Indian River female broilers (adapted from Kidd et al., 2021).

V. NONESSENTIAL AMINO ACIDS

A recent investigation into reduced crude protein diets (Table 1) demonstrates the need to better understand both NEAA requirements and interactions. A dietary formulated 50 g/kg crude protein reduction compromised both live performance and carcass yields, regardless that minimum constraints were set on all EAA ratios. When expressed relatively, the reduced crude protein diet supported only 96% of the weight gain achieved by the standard protein diet, despite a feed intake of 107%. Poor feed utilization was also reflected by the 6% reduction in breast fillet yield and a 72% increase in peritoneal fat yield. However, incrementally replenishing levels of alanine, glycine + serine, glutamine + glutamate, and proline resulted in linear improvements ($P \leq 0.05$) in live performance. Furthermore, these NEAA levels at 25 and 75% of the standard protein diet resulted in statistically similar weight gains and feed conversion ratios ($P > 0.05$), respectively. Thus, NEAA supplementation permitted a net crude protein reduction ≥20 g/kg while still maintaining these performance parameters. While these results are promising, it should be noted that NEAA supplementation was unable to restore peritoneal fat yield, owing to the challenges of optimizing reduced crude protein diets.

VI. CONCLUSIONS

The aim of these proceedings was to increase feed formulation flexibility with protein contributing ingredients for Australian nutritionists. Thus, the implementation of reduced protein diets warrants NEAA. Indeed, more work is needed on histidine, and although phenylalanine was not discussed in these proceedings, recent work in our laboratory has shown that it is harder to research than histidine. Finally, it is recommended that the continued use of additional feed grade amino acids be done with a keen eye on NEAA levels and ratios in the nutrient matrix.

Table 1 - Relative live performance in Cobb broilers fed reduced protein diets replenished with portions of nonessential amino acids (NEAA)[1].

Diet	Gain	Intake	FCR	NH_3	L. gut	Fat	Fillet
1 PC	100^{ab}	100^{b}	100^{de}	100^{ab}	100	100^{c}	100^{ab}
2 NC	96^{b}	107^{a}	114^{a}	91^{b}	143	172^{a}	94^{b}
3 as 2 + 25% NEAA	98^{ab}	106^{ab}	110^{b}	114^{ab}	155	169^{a}	99^{ab}
4 as 2 + 50% NEAA	102^{ab}	105^{ab}	105^{c}	114^{ab}	120	146^{ab}	98^{ab}
5 as 2 + 75% NEAA	101^{ab}	103^{ab}	103^{cd}	125^{ab}	118	147^{ab}	105^{a}
6 as 2 + 100% NEAA	103^{a}	100^{b}	100^{de}	158^{a}	150	135^{b}	104^{a}
7 as 6 – glycine	101^{ab}	104^{ab}	105^{c}	126^{ab}	143	142^{ab}	99^{ab}
8 as 6 – L-glutamine	104^{a}	102^{ab}	98^{e}	155^{ab}	150	135^{b}	104^{a}
P values							
Diet	0.01	0.01	<0.01	0.02	0.06	<0.01	<0.01
NEAA linear	<0.01	<0.01	<0.01	<0.01	0.18	0.29	0.86
NEAA quadratic	0.36	0.42	0.14	0.47	0.64	0.97	0.72

[1]Data represent Cobb broiler performance from 15 to 35 days of age fed a positive control with the first four limiting amino acids, a negative control with the addition of six more limiting amino acids and potassium, and sequential diets with the addition of NEAA (i.e., L-alanine, L-glutamic acid, L-glutamine, glycine, and L-proline) for variables: Gain: live weight gain; Intake: feed intake; FCR: feed conversion ratio; NH_3: litter ammonia; L. gut: leaky gut; Fat: carcass peritoneal fat; Fillet: Pectoralis major. Adapted from Nelson et al. (unpublished).

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INTRINSIC AMMONIA: AN UNACKNOWLEDGED CHALLENGE

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Summary

Intrinsic (NH_3) ammonia is the source of atmospheric NH_3 in poultry houses and is arguably an unacknowledged challenge to the growth performance of broiler chickens and sustainable chicken-meat production. Therefore, intrinsic NH_3 merits attention.

I. INTRODUCTION

The deleterious impacts of atmospheric ammonia (NH_3) to poultry and the environment are well recognised; nevertheless, albeit indirectly, intrinsic NH_3 is the source of atmospheric NH_3. Uricotelic species, including birds and reptiles, excrete nitrogenous wastes as uric acid (Raidal and Echols, 2007) and uricolysis, or the volatilisation of excreted uric acid in litter, converts uric acid to NH_3 and carbon dioxide (De Sousa et al., 2017). Uric acid of renal origin is the dominant nitrogenous compound in poultry excreta representing 56.2% of total-N in excreta (Krogdahl and Dalsgard, 1981). The uric acid concentration in excreta from broilers offered 222 g/kg protein, maize-based diets was 69.4 mg/g, which represented 40.2% of total excreta-N in Selle et al. (2021). The excreta output was 56 g/bird from 32 to 34 days post-hatch, or a daily output of 169 g/bird, which corresponds to a uric acid output of 11.7 g/bird/day. This excreted uric acid in litter is the prime source of atmospheric NH_3 in poultry houses. Thus, the generation of intrinsic NH_3 in broiler chickens is critical and originates from microbial fermentation along the digestive tract, including the microbial breakdown of urinary uric acid in the caeca (Gilbert et al., 2018), coupled with amino acid catabolism in the gut mucosa plus hepatic deamination of amino acids that are surplus to requirements for protein accretion (Brosnan, 2003) or redundant amino acids arising from protein turnover (Macelline et al., 2025). However, NH_3 is inherently toxic and the intravenous LD_{50} of ammonium acetate in broilers (2.72 mmol/kg) is noticeably less than in mice (5.64 mmol/kg), as reported by Wilson et al. (1968), which may apply to other mammalian species. Although potentially toxic, paradoxically, NH_3 plays a pivotal role in acid-base homeostasis (Weiner and Verlander, 2017).

II. SOURCES OF ENDOGENOUS NH_3 AND NH_4^+

As stated, endogenous NH_3 and NH_4^+ are derived from microbial fermentation along the digestive tract and catabolism of amino acids. Fifteen years ago, Ravindran (2011) contended that the microbial ecology of the gut in broiler chickens was a neglected niche; however, this is no longer the case as reflected in the Bindari and Gerber (2022) review of the gastrointestinal microbial composition of chickens. That microbial fermentation generates NH_3 is evident in the Khempaka et al. (2011) broiler study, which was based on nine dietary treatments. Concentrations of NH_3 in the small intestine ranged from 0.13 to 0.16/100 g digesta as opposed to 0.54 to 1.14/100 g digesta in the caeca, which represents a 5.8-fold increase in NH_3 concentrations following the transition from the small to large intestine. These and similar findings prompted Qaisrani et al. (2015) to conclude that the extent and importance of hindgut protein fermentation on the performance and gut health in broilers needs to be determined. These researchers argued that high NH_3 concentrations in the large intestine of pigs is indicative of undigested protein entering the hindgut and undergoing microbial fermentation, which would apply to poultry. However, in poultry, additional NH_3 levels stem from the entrance of urinary

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uric acid into the coprodeum of the large intestine being propelled by reverse peristalsis into the caecum to be degraded into NH_3 (Gilbert et al., 2018).

The hepatic deamination of unneeded amino acids results from either dietary amino acid imbalances and/or protein turnover. The likelihood is that amino acid imbalances are increasing given the interest in the development and adoption of reduced-crude protein diets. Interestingly, the protein turnover rate in broiler chickens is more than double the rate of humans (30.2 versus 14.6 g/kg body weight$^{0.75}$/day), as tabulated by Muramatsu (1990). Proteins are continuously synthesised and degraded to maintain protein homeostasis and, collectively, these processes are referred to as protein turnover. Thus, protein degradation is an ongoing process to ensure that misfolded, aged or damaged proteins are removed when necessary (Ross et al., 2021).

III. THE TOXICITY OF ENDOGENOUS NH_3

The toxic effects of NH_3 to the brain receives the most attention in human medicine; however, Dasarathy et al. (2017) argued that hyperammonaemia, or 'NH_3 overload', generates NH_3 increases in all tissues leading to the dysfunction of many organs stemming from changes in pH, membrane potential and metabolism. Several broiler studies have linked elevated plasma NH_3 concentrations or NH_3 overload to compromised growth performance (Namroud et al., 2008; Ospina-Rojas et al., 2013, 2014; Aguihe et al., 2022). Because of NH_3's inherently toxicity this metabolite demands detoxification. This is achieved, firstly, by the condensation of NH_3 with glutamic acid to generate glutamine in a reaction catalysed by glutamine synthetase (Hakvoort et al., 2017). Secondly, glutamine enters the Krebs uric acid cycle where it is converted to uric acid, which requires an input of one mole of glycine for every mole of uric acid excreted (Salway, 2018). According to Mapes and Krebs (1978), the conversion of four NH_3 molecules into uric acid in the avian liver requires one molecule of glycine, two molecules of glutamine, one molecule of aspartate and two molecules of formyltetrahydrofolate. Also, NH_3 detoxification attracts metabolic costs as the synthesis and excretion of uric acid to void NH_3-N in urine generates a minimal energy cost of 64.7 kJ/g N excreted as uric acid in poultry (van Milgen, 2021).

Systemic plasma NH_3 concentrations will increase resulting in compromised broiler growth performance in the event of inadequate NH_3 detoxification. For example, the CP content of wheat-based diets was reduced from 210 to 190 and 170 g/kg in Macelline et al. (2023). This triggered a linear increase ($r = -0.607$; $P = 0.010$) in plasma NH_3 concentrations from 1.87 to 2.04 and 2.19 μg/ml, which were in turn linearly related to compromised weight gains ($r = -0.565$; $P = 0.018$) from 15 to 36-days post-hatch. In a another PRF study, the CP content of a sorghum-based diet from 220 to 190 g/kg, which depressed weight gain by 7.51% from 7 to 33 days post-hatch, but a further reduction to 160 g/kg CP compromised weight gain by 38.2% in Greenhalgh et al. (2022). The quaternary amino compound, L-carnitine, was added to these diets at 0, 75 and 150 mg/kg. L-carnitine did not have any impacts on growth performance in birds offered the 220 and 190 g/kg CP diets. In contrast, the 75 mg/kg L-carnitine inclusion in 160 g/kg CP diets improved weight gains by 15.0%, (1580 versus 1374 g/bird), feed intakes by 8.34% (2403 versus 2218 g/bird), and FCR by 5.82% (1.521 versus 1.615). Similarly, 150 mg/kg L-carnitine significantly improved weight gain by 15.9%, feed intake by 12.4% and FCR by 3.16%. However, the pertinent point is that L-carnitine is protective against acute hyperammonemia or NH_3 overload (Kloiber et al., 1988). This raises the real possibility that the significant responses generated by L-carnitine in broilers offered 160 g/kg CP diets were at least partially due to the counteraction of the negative impacts of NH_3 overload.

IV. ENDOGENOUS NH_3 AND AGP MODES OF ACTION

The inclusions of antibiotic growth promotants (AGP) in diets for poultry and livestock was banned in Europe in 2006 and the global AGP usage is in decline. Nevertheless, the question "how did AGP increase growth and feed efficiency in poultry?" was recently posed by Miyakawa et al. (2024). The premise of this question was that, despite the decades during which AGP were used a consensus as to their modes of action, based on scientific evidence, was not reached. Nebulous statements like "the favourable manipulation of the gut microflora to the benefit of the host" seemingly sufficed. The mode of growth promotion by antibiotics was considered in Visek (1978) and this researcher argued that the suppression of NH_3 concentrations along the gut lumen by AGP could be a factor involved in their mode of action. However, it appears that Willard Visek was not aware of an experiment completed a decade earlier by Fujita (1968). In this experiment, three antibiotics (chlortetracycline, penicillin, oxytetracycline) were evaluated in broiler chickens to 28 days post-hatch experiment. As shown in Table 1, penicillin improved weight gain by 19.8% and FCR by 6.51% relative to untreated controls, which clearly exceeded the respective improvements in gain and FCR of chlortetracycline (9.91% and 2.33%) and oxytetracycline (5.17% and 0.93%). Instructively, NH_3 concentrations in digesta along the gastrointestinal tract were determined where concentrations ranged from 687 µg/g in the crop, to 876 µg/g in the small intestine and 3061 µg/g in the caecum in birds offered the control diet. The dietary inclusion of penicillin noticeably reduced NH_3 concentrations in these three segments by 43.7%, 30.6% and 30.0%, respectively. Moreover, based on the reported mean values, small intestinal NH_3 concentrations were negatively related to weight gain ($r = -0.974$; $P = 0.026$) and positively related to FCR ($r = 0.973$; $P = 0.027$). Similarly, caecal NH_3 concentrations were linearly related to weight gain ($r = -0.955$; $P = 0.045$) and FCR ($r = 0.962$; $P = 0.038$).

Table 1 - The impact of antibiotics on broiler growth performance to 28 days post-hatch and NH_3 concentrations along the digestive tract. Adapted from Fujita (1968).

Treatment	Growth performance		Ammonia (NH_3) concentrations (µg/g)		
	Weight gain (g)	FCR	Crop	Small intestine	Caecum
Control	232	2.15	687	876	3061
Chlortetracycline	255	2.10	637	738	2779
Penicillin	278	2.01	387	608	2143
Oxytetracycline	244	2.13	619	808	2728

Thus, the Fujita (1968) experiment provides tangible, retrospective support for the Visek (1978) hypothesis that AGP-induced suppression of NH_3 concentrations in the gut lumen is one of their modes of action in performance enhancement. Also, given that NH_3 is readily absorbed from the gut in broiler chickens (Karasawa and Nakita, 1986), the Fujita (1968) data indicate that the generation of NH_3 by the gut microbiota in chickens is detrimental to avian growth performance. In order to treat hyperammonaemia in humans, Liu et al., (2018) suggested that certain, specific probiotics could be a viable means to reduce NH_3 production by the gut microflora and this approach may hold relevance in poultry. Finally, it would be instructive to identify the sources of endogenous NH_3 in broiler chickens and quantify their relative contributions to the total pool. The mitigation or prevention of NH_3 overload would be facilitated if the more important sources of the problem were established and, if achieved, would certainly benefit chicken-meat production. L-carnitine and relevant probiotics may prove useful in counteracting the negative impacts of endogenous NH_3, which may be one of the impediments to the adoption of reduced-CP broiler diets.

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L-ALANINE, L-CYSTEINE, L-HISTIDINE AND L-TYROSINE INCREASE *LACTOBACILLUS SALIVARIUS, L. RETEURI* AND *L PARACASEI* GROWTH

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Lactobacillus is a bacterial genus that plays beneficial roles in the microbiota, and many of its species are autochthonous to the intestinal tract of chickens (Sirisopapong et al., 2023). Previous data suggest that L-alanine (Ala), L-cysteine (Cys), L-histidine (His) and L-tyrosine (Tyr) suppress the growth of certain chicken pathogens (unpublished). Although the essentiality of these four amino acids has been reported for some *Lactobacillus* species (Kwoji et al., 2022), there is limited knowledge regarding their effects at different concentrations. This study evaluated the effects of these amino acids on the growth of *L. salivarius, L. reuteri* and *L. paracasei*. It was hypothesised that increasing the concentration of these amino acids would alter the growth pattern of these beneficial bacteria.

Each *Lactobacillus* species was cultured for 48 hours under 24 conditions using chemically defined media with each amino acid (Ala, Cys, His and Tyr) adjusted to concentrations of 0, 0.2, 0.4, 0.6, 0.8, or 1 g/L. Growth was measured by optical density at 600 nm (OD_{600}) using the FLUOstar OPTIMA microplate reader. Growth curves were generated and analysed in R using the package gcplyr. One-way ANOVA was used to evaluate the effects of these amino acids at different concentrations. The impact of Ala, Cys, His and Tyr on the *Lactobacillus* species tested was classified as essential (E:no growth at 0 g/L), conditionally essential (cE growth observed at 0 g/L but significantly increased with higher amino acid concentrations, $p < 0.05$), suppressive (S: growth significantly decreased as amino acid concentration increased, $p < 0.05$) or no effects (N) (Table 1). In the order of *L. salivarius*, *L. paracasei* and *L. reuteri*: Ala was cE, N, and E; Cys was N, E, cE; His was E, N, E; and Tyr was E for all three species. Additionally, increasing the concentration of Ala was found to be suppressive to *L. reuteri* growth.

Table 1 - The nutritional roles of L-alanine, L-cysteine, L-histidine and L-tyrosine on Lactobacillus salivarius, Lactobacillus paracasei and Lactobacillus reuteri.

Amino acids	*L. salivarius*	*L. paracasei*	*L. reuteri*
L-alanine	cE	N	E/S
L-cysteine	N	E	cE
L-histidine	E	N	E
L-tyrosine	E	E	E

*E: essential; cE: conditionally essential; S: suppressive; N: no effects.

It is concluded that specific concentrations of amino acids can enhance or suppress the growth of beneficial *Lactobacillus* species. This offers a nutritional strategy to modulate gut microbiota for improved gut health.

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NON-BOUND AMINO ACIDS AND ALTERNATIVE INGREDIENTS TO REPLACE SOYBEAN MEAL IN WHEAT, SORGHUM OR BARLEY-BASED BROILER DIETS

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The Australian meat chicken industry aims to reduce its dependency on imported soybean meal (SBM) by incorporating local alternative high protein feed ingredients. Wheat and sorghum are the primary cereal grains in Australian broiler diets, while barley is used intermittently when it is cost-effective. However, how grain source interacts with SBM reduction and protein-rich alternatives has not been systematically investigated. This study aimed to evaluate whether SBM can be partially or fully replaced by a combination of non-bound amino acids (NBAA) and local protein-rich feed ingredients in broiler diets based on wheat, sorghum, or barley.

This experiment followed a 3 × 3 factorial arrangement of treatments with three sources of grains and three levels of SBM inclusion. Normal SBM inclusion levels were 250, 210, and 170 g/kg the starter (d 0-14), grower (d 15-28), and finisher (d 29-42) phases. Medium and low/nil SBM inclusion levels represented reductions of 25 and 50%, 50 and 75%, 75 and 100% of SBM in three phases, respectively. SBM reduction was achieved by various inclusion levels of NBAA, canola products, and field peas, without controlling dietary crude protein levels, to maintain industry-relevant diet formulations. Each of the 9 treatments was replicated 8 times in floor pens with 20 off-sex male Ross 308 chicks per replicate (n = 1440). The average growth rate (g/b/day) was used to calculate the age to 2.5 kg of live BW. Data were analyzed by two-way ANOVA, and significant effects were followed by LSD tests for mean separation.

Table 1 - Growth performance over the overall period (0-42 d).

Main effects	BW g/b	FI g/b	FCR g/g	Age to 2.5 kg BW d
Grain				
Wheat	3254[a]	4781	1.487[ab]	32.7[b]
Sorghum	3164[b]	4695	1.503[a]	33.6[a]
Barley	3246[a]	4707	1.467[b]	32.8[b]
SBM inclusion				
Normal	3283[a]	4774[a]	1.472[b]	32.4[b]
Medium	3235[a]	4745[ab]	1.485[ab]	32.9[b]
Low/nil	3146[b]	4664[b]	1.501[a]	33.8[a]
P value				
Grain	<0.001	0.110	0.003	<0.001
SBM inclusion	<0.001	0.039	0.018	<0.001
Grain×SBM inclusion	0.713	0.230	0.413	0.711

[ab] Means (n=8, 20 birds per replicate) within columns not sharing the same superscript are significantly different at the 5% level of probability.

Overall (d 0-42), there was no interaction between grain type and SBM inclusion level for BW, FI, FCR and age to 2.5 kg BW (Table 1). Birds fed sorghum-based diets had a lower BW at d 42 compared to those fed wheat or barley-based diets. Low/nil SBM inclusion diets reduced BW and FI, and increased FCR compared to normal or medium SBM inclusion diets. The reduced growth performance in sorghum-based and low/nil SBM diets may result from anti-nutritional factors in sorghum, and asynchrony between amino acid and energy (glucose) supply. In conclusion, partial SBM replacement offers a sustainable approach in meat chicken production, particularly for wheat or barley-based diets.

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WHEAT CRUDE PROTEIN LEVELS IMPACT GROWTH PERFORMANCE AND GUT ENVIRONMENT IN BROILERS

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Wheat-based, reduced-crude protein (RCP) diets often result in poorer growth performance in broilers compared with maize- or sorghum-based diets. This is largely attributed to a higher dietary starch-to-protein ratio, which may impair the synchronised absorption of glucose and amino acids in the small intestine (Selle and Liu, 2017). Moreover, the inherently higher crude protein (CP) content of wheat reduces the inclusion of intact protein sources when formulating RCP diets, potentially worsening the imbalance in nutrient absorption. This study aimed to investigate how different inherent CP contents of wheat influence broiler performance and intestinal environment.

The study included four dietary treatments: treatment 1A (110 g/kg CP wheat with 217 and 195 g/kg dietary CP in grower and finisher phases, respectively); 2B (150 g/kg CP wheat with 215 and 195 g/kg dietary CP); 3C (150 g/kg CP wheat with 205 and 185 g/kg dietary CP); 4D (150 g/kg CP wheat with 195 and 175 g/kg dietary CP). Each dietary treatment was replicated seven times with six birds per replicate, totalling 168 off-sex male Ross 308 broilers, over a feeding period of 9-23 d (grower) and 23-35 d (finisher). Wheat with 110 g/kg and 150 g/kg CP were considered standard and high CP wheats, respectively.

During the grower phase, birds fed diets 1A and 2B showed statistically comparable growth performance, whereas those fed diets 3C and 4D had higher feed intake (FI: $P = 0.005$) and feed conversion ratio (FCR: $P < 0.001$). During the finisher phase, diet 4D had the most compromised performance ($P < 0.001$) compared to the other diets. Over the entire feeding period, WG for diets 1A, 2B and 3C were comparable, but higher ($P < 0.001$) than 4D; however, birds fed 1A had better FCR compared to 2B, 3C and 4D ($P < 0.001$). While birds fed diets 3C and 4D yielded similar FI, diet 1A had lower FI compared to 2B (3527 vs 3763 g; $P = 0.045$) but similar to 3C and 4D. Diet 1A yielded lower gizzard pH compared to 2B (3.01 vs 3.54; $P = 0.037$) and higher ileal pH compared to 4D (7.06 vs 5.65; $P = 0.007$). The correlation analysis indicated that WG increased with ileal pH (r = 0.407, P = 0.031), whereas higher FCR was associated with lower ileal (r = -0.506, $P = 0.006$) and increased gizzard pH (r = 0.534, $P = 0.003$).

In conclusion, broilers fed standard CP diets (1A and 2B) achieved similar WG across wheat types throughout all feeding phases. However, reducing CP in diets containing high CP wheat impaired feed efficiency compared to other treatments, indicating that the inherent cereal grain CP may be one of the reasons for the poorer performance usually observed with wheat-based RCP diets. The high CP wheat also modified gizzard and ileal pH, potentially disrupting gut ecology, metabolic balance and protein solubility. Collectively, these findings highlight that the inherent CP content of wheat is not a trivial factor and should be carefully considered when formulating wheat-based RCP diets for broilers.

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THE EFFECT OF USING AN OPTIMALLY PRESSED CANOLA MEAL ON GROWTH PERFORMANCE, VISCERAL ORGAN WEIGHTS AND GUT MORPHOLOGY OF BROILER CHICKENS

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Summary

In this study, we compared using an optimally pressed canola meal (OP-CM) for the replacement of soybean meal in a traditional corn-soy diet at 5, 10 and 20% inclusions in a 35-d broiler feeding trial. Growth performance, carcass traits, visceral organ weights, faecal scores, and intestinal histo-morphology were evaluated. Broiler performance was maintained across treatments, with no significant differences in final body weight (FBW), cumulative body weight gain (CBWG), feed conversion ratio (FCR), or mortality compared with the control. Birds fed with 20% OP-CM consumed less feed but achieved equivalent growth and recorded the highest European Broiler Index (EBI), indicating efficient nutrient utilization. Carcass yields were comparable to the control. Visceral organ weights, especially thyroid, did not differ significantly among treatments, suggesting there was no major metabolic burden. Morphometric analysis demonstrated slight decreases in duodenal villus height (VH) and villus height to crypt depth ratios (VH:CD) at higher OP-CM inclusions. However, these morphological responses were not associated with poorer growth performance or faecal scores, suggesting that the broiler's intestinal tract is capable for morphological adjustment when CM is included in the diet. In conclusion, an OP-CM can be incorporated into broiler diets at inclusion levels up to 20% without compromising overall growth performance or gut and organ health; on the contrary it seems to enhance production efficiency.

I. INTRODUCTION

Soybean meal (SBM) remains the predominant protein source in poultry nutrition, but its price volatility and reliance on imports have prompted interest in alternative plant-based proteins. Among these, CM has been consistently identified as the most favourable alternative due to its relatively high protein content, balanced amino acid profile being rich in methionine and cysteine, and the presence of functional fibres with prebiotic potential (Newkirk, 2009; Niu et al., 2022). Despite these advantages, studies evaluating CM at high inclusion levels (≥15–20%) have produced inconsistent results. Some trials reported growth performance was maintained (Gopinger et al., 2014; Elbaz et al., 2023), while others observed depressed feed intake or carcass yield (Payvastegan et al., 2017; Ahmed et al., 2015). These discrepancies have been attributed largely to lack of consensus in formulation and/or the quality of CM which depends on processing conditions. The Global Council for Innovation in Rapeseed and Canola (GCIRC) have emphasized the effects of processing on amino acid digestibility, particularly lysine reactivity, as well as glucosinolate retention. In our previous study on the effects of different processing methods on the quality of canola meal (Ng and Neoh, 2025), we demonstrated that OP-CMs and direct solvent extracted canola meals contained significantly higher level of reactive lysine while maintaining low glucosinolate levels when compared with other processing methods. This present trial was designed to verify the performance of OP-CM in replacing SBM of broiler diets at inclusion levels of 5, 10, and 20%. The effects on growth performance, carcass traits, visceral organ weights, faecal score, and intestinal histomorphology were assessed to determine the suitability of OP-CM as a sustainable high-inclusion protein ingredient in broiler feeding.

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II. METHOD

This study was conducted in the Faculty of Agriculture, Universiti Putra Malaysia. A total of 660 Ross 308 broiler chicks were allocated to four dietary treatments with 11 replicates of 15 birds and reared for 35-d. The four treatment diets were as follow: T1: control (0% OP-CM), T2: 5% OP-CM, T3: 10% OP-CM, T4: 20% OP-CM. The OP-CM used in this study was tested with high KOHPS (~85%) and low glucosinolate at 7.30 µmol/g. All the diets (Table 1) were formulated on isocaloric and isoaminoacid basis following Ross 308 nutrient requirement guide. The birds were provided with ad libitum feed and water throughout the entire trial. The room temperature was set at 33 ± 1°C on day 1 and gradually reduced to 28 ± 1°C by day 15. The birds were vaccinated against Newcastle disease and Infectious Bronchitis at 8 and 22-d and against Infectious Bursal disease at 15-d. Body weight (BW) and feed intake (FI) and faecal score were measured weekly while mortality was recorded daily for each group. At 35-d, 11 chickens were slaughtered from each treatment for carcass traits and visceral organ weights. Intestinal duodenum, jejunum, and ileum samples were also collected for VH, CD and VH:CD measurements.

Table 1 - Ingredients and chemical composition of diets used in this feeding trial.

	Starter (1-14 days)				Grower finisher (15-35 days)			
Ingredients (g/kg)	T1	T2	T3	T4	T1	T2	T3	T5
Corn	535.0	514.8	494.6	436.5	587.3	561.7	535.7	484.0
SBM (46%)	340.8	315.4	290.3	238.0	286.9	262.7	238.8	191.0
OP-CM	-	50.0	100.0	200.0	-	50.0	100.0	200.0
Full fat SBM	50.0	50.0	50.0	50.0	60.0	60.0	60.0	60.0
Crude palm oil	-	-	-	-	8.6	9.8	11.1	13.5
Wheat by-product	26.3	23.3	20.0	32.9	20.0	20.0	20.0	20.0
L-Lysine	3.1	3.2	3.2	3.3	2.7	2.7	2.7	2.6
DL-Methionine	4.1	3.8	3.6	3.1	3.6	3.4	3.1	2.6
L-Threonine	1.3	1.2	1.1	0.9	1.1	1.0	0.9	0.6
L-Valine	0.3	0.2	0.1	-	0.3	0.1	-	-
Choline chloride 60	0.7	0.5	0.4	0.2	0.6	0.5	0.4	0.1
Limestone	12.5	12.3	12.1	11.5	9.1	8.9	8.7	8.2
Dicalcium phosphate	7.8	7.3	6.8	5.9	4.7	4.2	3.6	2.6
Sodium bicarbonate	0.7	0.6	0.5	0.4	0.1	-	-	-
Salt	2.2	2.2	2.1	2.1	2.8	2.8	2.8	2.6
Multi-enzymes*	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2
Premixes**	15.0	15.0	15.0	15.0	12.0	12.0	12.0	12.0
Chemical composition (g/kg)								
ME (MJ/kg)	12.3	12.3	12.3	12.3	13.0	13.0	13.0	13.0
Crude protein	230	230	230	230	210	210	210	210
Crude fibre	30.3	33.6	36.8	43.2	28.4	31.8	35.3	42.2
Dig Lys	13.2	13.2	13.2	13.2	11.8	11.8	11.8	11.8

*Multi-enzymes: phytase, xylanase, amylase and protease.

All data was analyzed using one-way ANOVA (SPSS 29.0); Treatment differences were compared using Tukey's HSD test ($P < 0.05$).

III. RESULTS

The growth performance, carcass traits, visceral organ weights and faecal scores are summarised in Table 2. In general, all treatment groups achieved similar FBW ranging from 2606.39 g to 2669.60 g with FCR 1.29 to 1.33. There were no significant differences ($P > 0.05$) among the dietary groups for FBW, CBWG, FCR, EBI and mortality at 35-d. However, cumulative feed intake (CFI) T4 was significantly lower at 3308.27 g ($P = 0.01$) than T2 at 3443.31 g but was similar to other treatment groups. For carcass traits, T3 group's carcass yield was found to be significantly lower at 88.53 % ($P = 0.01$) compared with T1 (93.08 %)

and T2 (93.18 %) groups but not to T4 (92.70 %). Overall, there was no significant difference found in all visceral organ weights. The percentage of abdominal fat was not significantly different (P = 0.22) across all diets. Faecal scores were normal and maintained consistently throughout the experimental period with no significant differences among the groups (P > 0.05) suggesting that high OP-CM inclusion rates did not affect faecal quality.

Table 2 - Growth performance, carcass traits, organs' weight and faecal score results of broilers fed different dietary treatments at 35-d.

Parameters (g)	T1	T2	T3	T4	SEM[1]	p-Value	CV[2]
FBW	2606.39	2642.16	2669.60	2614.61	*38.29*	*0.64*	*4.75*
CBWG	2558.93	2595.42	2621.31	2567.62	*38.25*	*0.65*	*4.83*
CFI	3403.22^{ab}	3443.31^{b}	3413.69^{ab}	3308.27^{a}	*28.72*	*0.01*	*3.10*
FCR	1.33	1.33	1.31	1.29	*0.02*	*0.40*	*5.29*
Mortality (n)	5/165	3/165	6/165	2/165		*0.37*	
EBI	533.45	549.73	553.93	562.70	*13.75*	*0.37*	*8.23*
Carcass traits							
Carcass yield (%)	93.08^{b}	93.18^{b}	88.53^{a}	92.70^{ab}	*0.93*	*0.01*	*3.67*
Breast (%)	30.56	29.86	29.07	29.49	*0.90*	*0.69*	*7.14*
Drumsticks (%)	8.90	9.32	8.72	9.10	*0.21*	*0.26*	*6.02*
Thigh (%)	11.24	10.38	9.84	10.44	*0.50*	*0.29*	*11.91*
Wings (%)	7.20	7.59	7.10	7.95	*0.28*	*0.20*	*9.77*
Abdominal fat (%)	1.15	0.85	0.92	0.93	*0.11*	*0.22*	*27.98*
Gizzard (%)	1.09	1.28	1.10	1.20	*0.10*	*0.52*	*21.41*
Liver (%)	2.07	1.99	2.00	1.87	*0.12*	*0.75*	*14.13*
Spleen (%)	0.14	0.12	0.10	0.13	*0.01*	*0.09*	*24.36*
Thyroid (%)	0.0002	0.0002	0.0001	0.0002	*0.0000*	*0.40*	*43.10*
Faecal score							
7 day	1.91	1.73	2.00	2.27	*0.20*	*0.28*	*33.60*
14 day	1.73	2.18	1.82	2.00	*0.16*	*0.21*	*28.24*
21 day	1.73	2.00	2.00	2.18	*0.13*	*0.13*	*23.11*
28 day	1.82	2.09	1.82	2.18	*0.11*	*0.06*	*20.37*
34 day	1.82	2.00	1.91	1.91	*0.09*	*0.56*	*15.23*

Faecal Scoring (1-5): Score 1: Dry and firmed feces with characteristic white uric acid cover; Score 2: Mostly dry feces with white uric acid cover; Score 3: Moist feces with white uric acid cover; Score 4: Wet feces with less white uric acid cover and droppings lose their shape and Score 5: Extremely wet feces with little to no white uric acid cover.
[1]SEM: Standard error of means. [2]CV: Coefficient of Variation. a-b: values with different subscripts are significantly different (P < 0.05)

Table 3. Histomorphology analysis of duodenum, jejunum and ileum in broiler chicken fed with different levels of canola meal.

Parameters	T1	T2	T3	T4	SEM	p-value	CV
Villi height , µm							
Duodenum	961.77^{c}	726.92^{a}	808.14^{b}	735.11^{ab}	*20.63*	*<0.01*	*13.69*
Jejunum	821.05	814.89	760.51	696.74	*41.42*	*0.14*	*15.83*
Ileum	868.29^{b}	760.81^{ab}	673.60^{a}	788.90^{b}	*27.99*	*<0.01*	*13.36*
Crypt depth, µm							
Duodenum	127.55^{ab}	113.11^{a}	150.43^{b}	117.04^{a}	*7.83*	*0.01*	*20.22*
Jejunum	117.06	99.49	101.34	107.56	*8.29*	*0.45*	*21.96*
Ileum	121.17^{b}	124.14^{b}	86.59^{a}	114.02^{ab}	*8.52*	*0.02*	*24.59*
Villi height: Crypt depth							
Duodenum	7.69^{b}	6.63^{ab}	5.55^{a}	6.46^{ab}	*0.43*	*0.02*	*21.25*
Jejunum	7.12	8.36	7.67	6.90	*0.58*	*0.31*	*22.16*
Ileum	7.53	6.32	8.02	7.01	*0.52*	*0.14*	*21.22*

a-c: values with different subscripts are significantly different (P < 0.05)

Table 3 shows that dietary treatments had a significant impact on the morphology of the duodenum and ileum (P < 0.05) but not in the jejunum. In the duodenum, the T3 group had

significant shorter VH at 808.14 μm ($P < 0.01$) and lower VH:CD at 5.55 ($P = 0.02$) when compared to the T1 group at 961.77 μm and 7.69 respectively. The T3 group also had significant deeper duodenum CD as compared to the T2 and T4 groups ($P = 0.01$). While for the ileum, the T3 group exhibited shorter VH ($P < 0.01$) and shallower CD ($P = 0.02$) when compared to the T1 group, but there were no significant differences in ileum VH:CD observed across all diets. The T2 and T4 groups have morphometric results similar to the T1 group in all parameters except for duodenum VH.

IV. DISCUSSION

This present study demonstrates that OP-CM can be incorporated in broiler diets up to 20% without compromising growth, carcass yield, organ development, or gut health. Performance variables, including BWG, FCR, and mortality, were unaffected, and birds receiving 20% OP-CM achieved the highest EBI despite lower FI. This observation suggests that the higher reactive lysine availability in OP-CM may contribute to better protein utilization. Previously Payvastegan et al. (2017) observed reduced weight gains at 20–30% inclusion of CM. In contrast, Elbaz et al. (2023) and Gopinger et al. (2014) showed that birds tolerated 15–20% CM inclusion when the quality of CM was optimized and is consistent with the outcomes of the present study. Together, these trials suggest that it is the nutrient quality of the CM rather than the absolute inclusion level that determines broiler performances. Carcass traits in the current study were largely unaffected, except for a reduction in yield at 10% inclusion rate but this was not observed at 20% inclusion, indicating the effect was not dose-dependent. Importantly, abdominal fat was consistently reduced in CM-fed birds, in line with reports that CM's fibre and energy profiles can improve carcass composition by shifting energy utilization away from fat deposition (Woyengo et al., 2010). Intestinal histo-morphology showed modest reductions in duodenal VH and VH:CD at higher OP-CM inclusion levels. However, these changes did not impair growth performance, supporting the view that the broiler intestine can undergo structural adaptation without compromising nutrient absorption. Similar findings were reported by Elbaz et al. (2023), where high CM diets induced morphological adjustments but maintained gut function and performance. Overall, this study highlights the value of optimized processing in unlocking the full potential of CM for broilers. By preserving reactive lysine and with lower glucosinolate content, OP-CM achieved stable performance at high inclusion levels where conventionally processed canola meals have often shown limitations. These results provide strong support for expanding CM utilization in poultry diets, contributing both to feed cost flexibility and to more diversity in protein sourcing.

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ALTERNATIVES TO ANTIBIOTICS: STATE OF THE ART AND FUTURE PERSPECTIVES

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Summary

As antibiotic growth promoters (AGPs) are being phased out or completely banned in many parts of the world, there is growing concern about performance losses due to gut health issues, while at the same time there is increasing interest in alternatives to AGPs as feed additives. In the search for alternatives, understanding the fundamental mechanisms governing intestinal health in fast growing, high producing animals has become the number one priority for the feed industry. Currently, a large number of alternatives to AGPs are on the market, to the point that, for the feed formulators, making the right choices seems to be the big question. Hereafter, a brief overview is given of the state of the art scientific knowledge on mechanisms of intestinal health and the recent developments in feed additives that interact with these different mechanisms. The idea is to help decision makers in the feed industry to make the right choices. In the last paragraph, perspectives on future new developments in this field are drawn.

I. INTRODUCTION

Growth promoting antibiotics (AGPs) have been used in poultry feed since the nineteen fifties, following the approval of the use of antibiotics in animal feeds without veterinary prescription by the Food and Drug Administration in the USA in 1951 (Jones and Ricke, 2003). Since then, the beneficial effects of these AGPs on the performance of most production animals have been well documented. Surprisingly, however, after 70 years of intensive worldwide use of these feed additives, the mechanisms by which AGPs function are still not fully understood (Hodak et al., 2023).

The growing concern about farm animals possibly playing a role in the expanding problem of multi resistant bacteria causing life threatening infections in humans has urged the European Commission to completely ban the use of AGPs in animal feed in the E.U. from Januay1, 2006. Immediately thereafter, animal producers in general- and poultry producers in particular- saw losses in performance associated with gut health issues, which ranged from mild dysbiosis to more severe wet litter and even massive outbreaks of necrotic enteritis with high mortality. Following this decision in the E.U., bans on AGPs have been introduced in other parts of the world as well, with similar ensuing effects on the health and performance of the animals (Smith, 2019). These economically rather devastating issues have prompted the industry and the scientific community to search for alternatives to the AGPs. Apparently, this was successful, since in the last two decades a wealth of novel feed additives have been launched, all of which claim to offer a valid alternative to the AGPs. However, while the AGPs seemed to constantly and under all circumstances induce a positive response of the animals in terms of performance, the response to many of the alternatives seems to be somewhat less predictable. Therefore, it turns out to be critically important to go back and try to answer two basic questions: 1. What is the true mode of action of AGPs? and 2. What are the fundamental principles of gut health?

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II. MODE OF ACTION OF ANTIMICROBIAL GROWTH PROMOTERS

The true mode of action of AGPs is still a matter of controversy, which is probably the main reason why new papers on this topic continue to be published today (Perera & Ravindran, 2025). The controversy arises primarily because the concentrations of these antibiotics used in feed are mostly below the MIC values (minimal inhibitory concentrations), meaning that a simple explanation based on antibacterial activity against (certain potentially harmful members of) the intestinal microbiome would not hold true. Even more confusingly, certain studies have reported modulations of the chicken cecal microbiome in response to AGPs (Danzeisen et al., 2011), while others have reported only a change in function but not in structure of that same microbiome (Hodak et al., 2023). There are two major flaws in most of the studies investigating the effects of AGPs on the gut microbiome of poultry. First of all, the focus is almost exclusively on the microbiome of the ceca, ignoring possible effects on the microbiome of other segments of the gastro-intestinal tract. Secondly, MIC values of the antibiotics can only be determined against culturable bacteria, whilst the vast majority of the bacteria residing in the gastro-intestinal tract have not been cultured so far. Moreover, dilution by the gastro-intestinal secretions, and absorption by the intestinal mucosa will, in most cases, result in very low, biologically insignificant concentrations of these feed additives actually reaching the caeca.

Few studies have investigated the effects of AGPs on the microbiota of the small intestine, most of them reporting significant reductions in the population of *Lactobacillus* spp. (for review see Lin, 2014). Lactobacillaceae indeed dominate the microbiome of the small intestine, with the highest densities being found in the ileum. These lactobacilli are known producers of bile salt hydrolase (Begley et al., 2006). Deconjugation of bile salts by bile salt hydrolases is known to reduce the uptake of lipids and cholesterol. Thus improving the digestibility of lipids through reduction of the Lactobacilli population would at least partly explain the mode of action of AGPs (Guban et al., 2006). A broader control of the bacterial populations in the small intestine is also conceivable, thereby protecting against SIBO (small intestinal bacterial overgrowth), a well-known pathological entity in human medicine (Bamba et al., 2023), but not recognized as such in production animals (see below). Such a protection against harmful expansion of the microbiota in the small intestine might explain the reported improvement in small intestinal health parameters such as duodenal villus length and mucosal alkaline phosphatase activity (see below) when birds are fed AGPs (Teirlynck et al., 2009; Yang et al., 2007). Along these same lines, one could argue that the reported effects on the caecal microbiota may be indirect, due to better digestion and absorption of the digestible fraction of the diet in the small intestine, thereby providing a more suitable substrate for the beneficial microorganisms in the caeca (see below). Taken together, these data seem to suggest that optimizing conditions in the small intestine may be equally if not more important than controlling the microbiota in the caeca.

II. ALTERNATIVES TO ANTIBIOTICS TARGETING THE CAECA

In the early days of the search for alternatives to AGPs, newly launched compounds were mostly claimed to have antibacterial activity, when tested in vitro. Another claim was to reduce the pH in the caeca. Considering the more recent concepts about the mode of action of AGPs, trying to suppress microbial fermentation in the caeca or trying to reduce the pH in the caecal lumen might not be advisable. Conversely, providing beneficial microbes (so-called probiotics or direct fed microbials) is one of the widely explored strategies. The first efforts to find alternatives to AGPs were often looking for Lactobacillaceae as candidate probiotics. Excessive expansion of Lactate producers and the associated accumulation of lactate in the colon of mammals and the caeca of birds, however, appears to be undesirable and even harmful

(Wang et al., 2020). Besides, most of supplemented Lactobacilli will not reach the caeca and there is plentiful of spontaneous lactobacilli colonization.

It is well established now that the majority of beneficial microbes in the caeca are butyrate producing Ruminococcaceae and Lachnospiraceae which, in collaboration and cross-feeding with members of the phylum Bacteroidota, break down and thrive on the undigestible fraction of the feed, or, in the absence of feed residues, live on the mucus secreted by the caecal mucosa. Supplementing these butyrate producers in feed is not an option today, as these strictly anaerobic organisms are unculturable or difficult to culture in the lab, let alone to produce in large fermenters for wide scale application. This may, however, change in the years to come. The undigestible fraction of the feed (dietary fiber), which these bacteria live on, consists of macromolecular complexes of non-starch polysaccharides (NSPs) that make up the plant cell walls (Bach Knudsen, 1997) . Providing sufficient amounts of non-starch polysaccharides thus should support caecal fermentation and thereby be beneficial to intestinal health. Broilers indeed respond favorably to supplementation of moderate amounts (2 to 3%) of insoluble dietary fiber on top of their typically low fiber diets (Mateos et al., 2012). Plant cell walls are composed of complexes of macromolecules of different molecular patterns. These different molecular families are fermented by different groups of bacteria in the caeca, thereby differentially influencing intestinal health. As an example, we have looked at the effect of cellulose. It turns out that, in the chicken caeca, cellulose is specifically used by the *Alistipes* genus, belonging to the family of Rikenellaceae. *Alistipes* spp. convert cellulose into succinate, which is absorbed by the epithelial cells (De Maesschalck et al., 2019). The succinate is not used by the cells as an energy source but converted into glucose, which is released in the circulation, thereby contributing to the energetically beneficial intestinal gluconeogenesis (De Vadder et al., 2016).

In very young birds, especially in newly hatched chicks which have not yet acquired a fully mature microbiome in their caeca, providing partly degraded NSPs or synthetic oligomers, commonly known as prebiotics, is another successful strategy for improving intestinal health and performance. As an example, we showed that partly degraded arabinoxylans from wheat or synthetic arabinoxylo-oligosaccharides have the potential to improve intestinal health, even when included at low concentrations (Yacoubi et al., 2018; De Maesschalck et al., 2015).

Currently the most widely used and most successful strategy targeting the caecal microbiota is to include NSP degrading enzymes (so-called NSPases) in the feed. They allow the birds to make better use of the low amounts of NSPs from the ingredients of the feed. Different enzymes target different molecular patterns of the NSPs and therefore should be adapted to the types of ingredients in the feed, e.g. corn vs wheat (Ravn et al., 2018). Including xylanases in broiler feed is now standard practice in regions where wheat based feed formulas are used and AGPs have been phased out.

III. ALTERNATIVES TO ANTIBIOTICS TARGETING THE SMALL INTESTINE

The small intestine is where the absorption of the nutrients takes place. More specifically, the uptake of the nutrients is a receptor-mediated transcellular process carried out by the epithelial cells covering the small intestinal villi, with the cells at the tips of the villi being most actively involved in this. By far the longest villi are found in the duodenum, highlighting the critical role of this upper part of the small intestine. Microbial density and diversity in the upper part of the small intestine is low (10^3 cfu / ml in the duodenum and jejunum vs 10^{11} / ml in the caeca). As mentioned above, the small intestinal microbiome is dominated by lactobacilli. These lactobacilli are involved in bile salt hydrolysis. At the level of the ileum, the lactobacilli are more numerous (10^8 cfu / ml), generating lactate which is used as substrate by the

Lachnospiraceae for butyrate production in the caeca (Wang et al., 2020). In contrast to the conditions in the caeca, where microbial multiplication is fostered by the secretions of the mucosa, in the small intestine numerous physiological mechanisms are in place to keep the microbes in check. It seems plausible that at least part of the beneficial effects of AGPs may be through supporting these antibacterial defences. In the absence of AGPs and considering the ferocious appetite of modern fast growing broilers, it may be that these small intestinal antibacterial defences are insufficient, leading to small intestinal bacterial overgrowth (SIBO). While not fully recognized as such in the veterinary field, SIBO nowadays is recognized as a proper disease entity in humans, with clear diagnostic criteria (Bohm et al., 2013). There are strong indications that SIBO is an issue associated with dysbiosis in broilers, as indicated in the gross dysbiosis scoring (Teirlynck et al., 2011). Indeed, this macroscopic scoring includes changes observed in the small intestine such as large volumes of foamy content, which points to bacterial fermentation.

Antibacterial defence mechanisms in the small intestine are extremely elaborate. Starting from the acid production in the proventriculus, which triggers a temporary viable but non-culturable state of bacteria while passing through the small intestine (Geirnaert et al., 2014). Furthermore, the small intestinal mucosa produces alkaline phosphatase. The secreted isotype of this enzyme can destroy the LPS from gram-negative bacteria in the lumen. Following the attack of this outer layer of bacterial cell wall, secreted antibacterial peptides can kill bacteria by creating pores in the peptidoglycan layer. Finally, the peptidoglycans are further broken down by lysozyme into the smallest biologically active fragments, which are muramyl dipeptides (MDP). The latter are substrates for a receptor in the intestinal mucosa called NOD-1, which, when activated continuously, dampens inflammation. Thus MDP is a signal perceived by the host indicating complete breakdown of bacterial cells. Additional antibacterial defence mechanisms include the breakdown of the nucleic acid degradation product hypoxanthine into xanthine and further into uric acid by the mucosal enzyme xanthine oxidoreductase, generating the release of the powerful antibacterial hydrogen peroxide (Crane et al., 2013). Only few alternatives to AGPs specifically target the small intestine. Most of them support and strengthen the natural host antibacterial defence mechanisms. The best documented such feed additive is probably lysozyme (Sindaye et al., 2023). Lysozyme has direct antibacterial activity by cleaving the peptidoglycan between the N-glucosamine and the N-muramic acid moiety. Enzymes with a similar mode of action but only attacking fragments of peptidoglycan and not intact bacterial cells have no bactericidal activity. Nevertheless, the latter muramidases may also exert beneficial effects on intestinal health (Wang et al., 2021). We recently reported on the mode of action of a probiotic *Bacillus* strain releasing large amounts of hypoxanthine in the small intestine, thereby providing more substrate for the xanthine oxidoreductase, leading to a suppression of the microbial density as well as their associated metabolic activity throughout the small intestine (Choi et al., 2021).

IV. FUTURE PERSPECTIVES

Currently a wealth of alternatives to AGPs is available. These novel nutraceuticals, however, are not as universally applicable as the AGPs. They require a thorough knowledge of the conditions where and when each of the different alternatives should be implemented. Generating more fundamental knowledge of the basic mechanisms ruling optimal function of the digestive system is the key for further developments in this field. Furthermore, one should never forget the old foes, the most important one being coccidiosis. Indeed, improving coccidiosis control remains a cornerstone in the strategy to protect intestinal health. Other than that, however, new feed additives, some of which possibly based on a completely different mode of action, can be expected in the future. In the end, it all comes down to identifying the

weak points in the digestive system and trying to compensate for these. One typical example is the energy requirements of the intestinal epithelial cells. It is well known that the most essential energy source of the epithelial cells in the lower intestinal tract is butyrate, which is one of the reasons why supporting butyrate production by the caecal microbiota is beneficial. Less attention has been paid, however, to the main energy source of the epithelial cells of the small intestine, which is not glucose or butyrate, but glutamine. Part of this glutamine is a spillover from the glutathione cycle in the proventriculus. Especially under conditions of poor development of the proventriculus (and gizzard), as is often the case with pelleted feed, and when the feed proteins are low in glutamine, supplementation of this amino acid could be beneficial. Other amino acids that might become valuable feed additives under specific conditions are arginine, threonine, tryptophan and tyrosine. All of these amino acids have additional functions on top of their roles as building blocks of proteins. Arginine is used by immune cells to generate nitric oxide, an important metabolite in innate host defence, not only in the gastrointestinal tract. Threonine plays a key role in mucin synthesis. Tryptophan is a precursor to serotonine, which has an effect on behaviour.

The most revolutionary changes in the feed additive landscape can probably be expected from nutritional protection against intestinal pathogens. Currently, most control tools are based on medication. These interventions usually occur at a relative advanced stage of the disease process. Even in coccidiosis control, coccidiostats block the pathogen during the replication cycle, when at least part of the damage has already been done. The question is: what makes a (micro)organism a pathogen, and how do the host defences recognize a pathogen as such? For sure, all pathogens arrive in the intestinal tract after oral uptake. In order to cause serious damage, almost all of these organisms must invade. During this process, the first hurdle is the mucus layer. Mucus in the small intestine forms a continuous protective layer and is permeable only to nutrients that are ready to be absorbed through a receptor-mediated transcellular process.

No foreign organisms- viruses, bacteria or protozoa- can pass through this layer, unless they make the mucus more permeable. The impermeability of mucus seems to depend largely on an intact macromolecular complex of the mucin. This mucin is composed of a protein backbone with long, branched polysaccharide side chains that hold a lot of water. At the ends of the side chains, the terminal sugar almost always is sialic acid (synonym: neuraminic acid). These sialic acid molecules determine the impermeability. This is probably the reason why almost all mucosal pathogens produce a powerful enzyme to cleave off these terminal sialic acid molecules- enzymes which, in bacteria and protozoa, are called sialidases, while in viruses they are called neuraminidases.

The neuraminidases of viruses, such as Influenza virus or Newcastle disease virus, are well known and have been studied in great detail, while the sialidases of, for instance, *Eimeria* spp. have hardly been investigated at all. Remarkably, even the *Clostridium perfringens* strains that can cause necrotic enteritis in broilers express a sialidase. The expression of this enzyme by *C. perfringens* in turn triggers the expression of a cascade of additional virulence factors, thus illustrating a coordinated response to the environmental conditions in the intestine (Van Damme et al., 2022). If these neuraminidase / sialidase enzymes could be continuously inhibited, it might, ideally, be possible to prevent the very first step in the virulence mechanisms of almost all intestinal pathogens. Massive research efforts into human influenza have resulted in a large collection of neuraminidase inhibitors being currently available in the pharma industry. These synthetic drugs, however, are probably not the way to go for the feed industry, but for sure, there are numerous natural compounds out there in the plant kingdom, waiting to be discovered as new feed additives to prevent the first step in the pathogenesis of intestinal infections.

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AN INNOVATIVE, NON-INVASIVE METHOD TO ASSESS GUT HEALTH IN BROILERS

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Summary

In this study, a qPCR-based method was applied to quantify host-to-bacterial DNA ratios as a biomarker of gut damage in broilers with different (gut) health status. Another qPCR assay was implemented to quantify *Lactobacillales*, *Lachnospiraceae*, and *Ruminococcaceae* in cloacal swabs of healthy broilers at 21 days of age. The ratios of these subpopulations to total bacterial DNA were used to generate a best fitting model to predict the slaughter weight between 41 and 45 days of age. The gut damage ratios differed significantly between broilers with or without gut health problems. Furthermore, a combination of the ratios from both assays at 21 days of age had a significant correlation with the slaughter weight.

I. INTRODUCTION

Intestinal health is essential for digestion and absorption of nutrients and plays a major role in the general health and optimal performance in poultry. Particularly high performing broilers that are characterized by very high growth and feed intake are susceptible to intestinal health problems due to the considerable stress on the digestive system. Unabsorbed nutrients in the small intestine may trigger dysbiosis which in turn can lead to a compromised intestinal barrier integrity and inflammatory responses (Ducatelle et al., 2023). Gut health issues in broilers are mostly subclinical, meaning that they silently impair growth and feed efficiency, while causing significant economic losses. Intestinal health is normally evaluated by measuring the villi height and crypt depth of different sections of the small intestine, or by scoring lesions associated with specific pathogens. However, these methods require the sacrifice of multiple birds and histologic evaluation often takes a few days. Currently, reliable, practical, and non-invasive biomarkers to evaluate gut health are limited. Therefore, the aim of this study was to evaluate a new, non-invasive qPCR-based method as a tool to objectively evaluate gut health in commercial broilers.

II. METHOD

Cloacal samples using swabs (Enat Swabs, Copan, Brescia, Italy) were collected from commercial broilers with health issues as well as healthy broilers. For broilers with health issues, the swabs were taken from live birds sent to a Dutch veterinary laboratory (Royal GD, Deventer, the Netherlands) for diagnostic purposes (a minimum of three birds and maximum of six birds per submission). After sampling, the birds were euthanized, and complete necropsy and additional laboratory testing were carried out to formulate a main diagnosis. The birds were divided into one of the following health status groups: “gut health problems” or “non-gut health problems”. For healthy broilers, samples were collected as follows: From each of 14 commercial broiler flocks located in the Netherlands, cloacal swabs were obtained from 10 birds randomly selected by a veterinarian at 21 days of age. From the same flocks, the average slaughter weight (SW) and total mortality data were also recorded.

All cloacal samples were subjected to an assay (Gut Damage Assay, Florates B.V., Wageningen, the Netherlands) with two biomarkers for gut damage: total bacterial DNA and host DNA (considered to be derived from desquamated intestinal epithelial cells). The samples from the healthy broilers were subjected to an additional assay (Productivity Assay, Florates

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B.V., Wageningen, the Netherlands) with markers for specific subpopulations of the microbiota: 'Lactate' marker for all *Lactobacillales* and 'Butyrate' marker for *Ruminococcaceae* and *Lachnospiraceae* species. Both the Gut Damage Assay and the Productivity Assay use ligation-based multiplex quantitative RT PCR adapted from methods described by Wattiau et al. (2008) and Naas et al. (2010). The complete assay procedures and marker design are described by Van Hees et al. (2025). In total, 227 broiler samples (129 healthy, 45 health problems that were not gut-related, and 43 gut health problems) from 36 flocks (14 healthy, 10 health problems that were not gut-related, and 12 gut health problems) were included in the analyses (after meeting DNA concentration criteria).

The Ct values for each marker were used to calculate the ratios described in Table 1. Numerical factors were added to generate only positive values for ease of interpretation in the field. Statistical analyses were performed using Microsoft Excel and R Studio Software. The Gut Damage Ratio (GD^{Ratio}) results were analysed using one-way ANOVA with the significance level set at 0.05. If a significant effect was found, Tukey's HSD test was used for post-hoc pairwise comparisons.

To investigate the relationship between different ratios (average, minimum, and maximum values) described in Table 1 and the SW of the 14 healthy broiler flocks, the best subset regression procedure was applied to obtain a best-fitting regression model. A maximum of five variables were selected to prevent inflation of the coefficient of determination (R^2) which was used to compare results and determine which independent variables to include in a multiple linear regression model using Python.

Table 1 - Ratios calculated from the Ct values of each marker of the gut damage and productivity assays.

Ratio	Formula
Gut Damage Ratio (GD^{Ratio})	15 – (Ct Host DNA – Ct Total Bacterial DNA)
Butyrate Ratio (BUT^{Ratio})	10 – (Ct Butyrate – Ct Total Bacterial DNA)
Lactate Ratio (LAC^{Ratio})	12 – (Ct Lactate – Ct Total Bacterial DNA)
Butyrate-Lactate Ratio ($ButLac^{Ratio}$)	8 + (Ct Lactate – Ct Butyrate)

III. RESULTS

Gut Damage Assay - Healthy broilers had a significantly lower GD^{Ratio} (average 12.2) than broilers with gut health problems (average 16.6) ($P < 0.001$). Broilers from the "non-gut health problems" group had a significantly lower GD^{Ratio} than the broilers with gut health problems ($P < 0.001$) and a similar GD^{Ratio} to the healthy broilers ($P > 0.05$) (Figure 1).

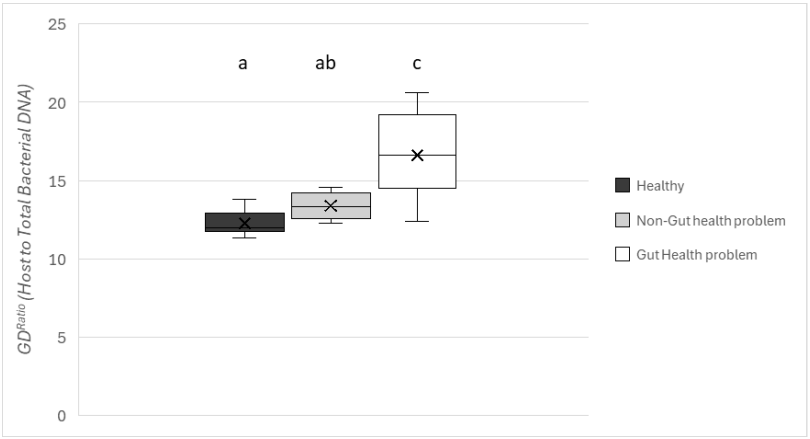

Figure 1 - The Gut damage ratio (GD^{Ratio}) (ratio of host DNA to total bacterial DNA) of healthy broilers, broilers with gut health problems and broilers with health problems unrelated to the gut. $^{a-c}$Treatments with different superscripts differ significantly at $P < 0.05$.

Productivity Assay – The results of the BUT^{Ratio}, LAC^{Ratio}, $ButLac^{Ratio}$ and GD^{Ratio} of each of the 14 healthy broiler flocks are presented in Figure 2. Age at slaughter of these 14 healthy flocks varied between 41 and 45 days, while the SW varied between 2547 g and 2929 g. The total mortality of these flocks ranged from 2.70% to 4.81% (data not shown).

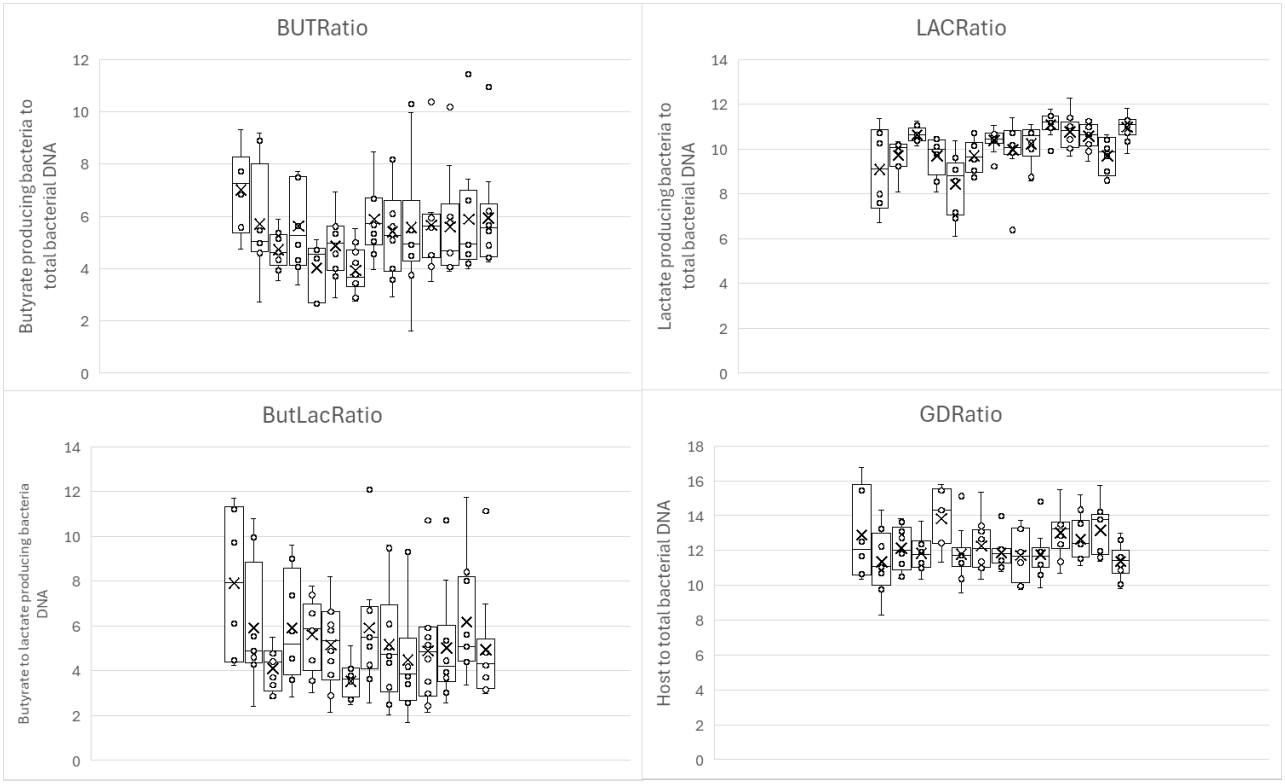

Figure 2 - Average, minimum, and maximum ratios of healthy broilers per flock at 21 days of age.

Following the best subset regression procedure, the final multiple linear regression model included the minimum BUT^{Ratio}, average BUT^{Ratio}, maximum GD^{Ratio}, average GD^{Ratio}, and average $ButLac^{Ratio}$ as independent variables and showed a significant correlation between the actual SW and the SW as predicted by the model (P = 0.002) (Figure 3). The percentage contribution of each of the different factors were 76.7% baseline, 15.3% minimum BUT^{Ratio} and average BUT^{Ratio}, 6.7% maximum GD^{Ratio} and average GD^{Ratio}, and 1.3% average $ButLac^{Ratio}$.

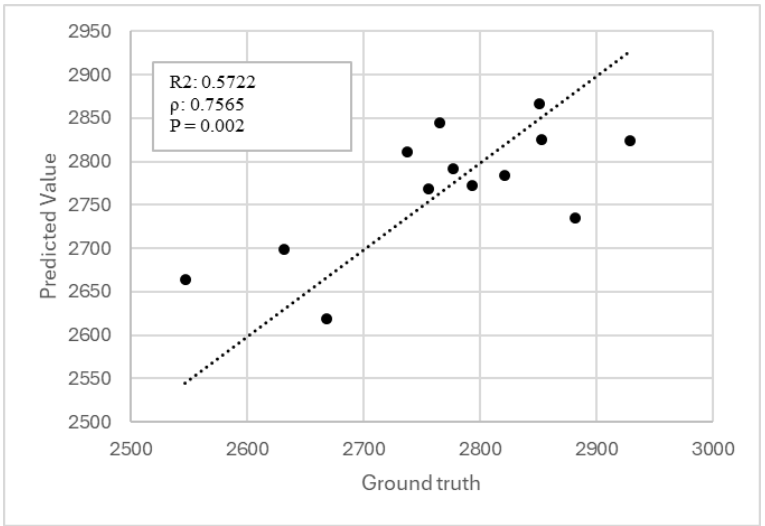

Figure 3 - Predicted slaughter weight (y-axis) and measured slaughter weight (x-axis) per flock.

IV. DISCUSSION

In this study, a novel, non-invasive method to evaluate intestinal health was validated on cloacal swabs from commercial broilers. Using the Gut Damage Assay (analyzing host DNA and total bacterial DNA), it was found that broilers diagnosed with intestinal health problems had a significantly higher GD^{Ratio} than both healthy broilers and broilers health problems unrelated to the gut. A higher GD^{Ratio} indicates a higher level of host cell DNA relative to total bacterial DNA in the cloacal samples. The level of host cell DNA in the samples is expected to increase in response to an increased loss of enterocytes. Enterocytes are continuously renewed, but stressors such as enteric infections that damage the villi can cause an increased cell loss (Ducatelle et al., 2023). The amount of bacterial DNA on the cloacal swabs can vary according to changes in the gut microbiota of the broiler. The intestinal microbiota of healthy animals is rich and highly complex, but various factors such as changes in diet, management practices, stress or disease can disrupt this balance, leading to dysbiosis (Aruwa et al., 2021).

From the Productivity assay, a combination of ratios involving quantities of butyrate and lactic acid producing bacteria in cloacal samples of broilers at 21 days of age had a significant correlation to slaughter weight of the respective flocks. The poultry caeca are dominated by butyrate-producing bacteria of the families *Lachnospiraceae* and *Ruminococcaceae*. It is well known that butyrate is the main energy source of epithelial cells in the lower intestinal tract and plays a role in pathogen control, intestinal barrier function and integrity. It can modulate intestinal microbiota, has anti-inflammatory properties, and can ultimately benefit growth performance in broilers (Guilloteau et al., 2010). Lactate is produced by many bacterial species and is also an important intermediate metabolite for intestinal health. Lactate can also be used in cross-feeding reactions where lactate is converted to butyrate by members of the *Lachnospiraceae*, thereby increasing butyrate concentration and improving gut health (De Maesschalck et al., 2015).

The results of the present study support the potential of the two assays to evaluate the gut health of broilers. This method enables proactive flock management strategies which can help to support more sustainable poultry production.

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A COMBINATION OF POSTBIOTICS AND PHYTOGENICS DRIVES BROILER PERFORMANCE GAINS

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Summary

Modern poultry production demands integrated strategies to meet rising consumer expectations around food safety, animal welfare, and sustainable production practices. These consumer demands drive the need for practical solutions that support gut function, reduce pathogen pressure, and improve efficiency. The research described in this paper highlights the benefits of combining *Saccharomyces cerevisiae* fermentation-based postbiotics (SCFP) and essential oil compounds (EOC) on gut health and performance in broilers. For performance parameters six independent studies were conducted using a randomized block design with pens as experimental units with 12-to-48 replicates/treatment with 216-to-960 birds/treatment. Each study was set up with a completely randomized design with a control group (CON) receiving a basal diet and treatment group (TRT) receiving the combination of SCFP and EOC. For the microbiome analysis, a set of 11 trials were conducted using a microbiome surveillance platform. Birds fed diets supplemented with SCFP + EOC showed improved ($P < 0.05$) body weight and cFCR (adjusted cumulative feed conversion ratio) at key growth stages (14, 28, and 35 days). The analysis revealed the earlier establishment ($P < 0.05$) of beneficial butyrate-producing bacteria (such as *Lachnospiraceae* spp.) and reduced ($P < 0.05$) levels of opportunistic pathogens (such as *Clostridium perfringens*) in birds fed TRT diets compared to CON fed birds. These findings suggest that targeted feed additive combinations can effectively modulate the gut microbiome to drive better productivity. Based on the findings of this study, there is strong evidence for integrating SCFP and EOC as a strategic solution in modern poultry nutrition.

I. INTRODUCTION

Evolving consumer expectations around food safety, animal welfare, and production methods continue to add complexity to modern poultry systems. These multifaceted demands cannot be met by a single solution. As scientific understanding deepens, it becomes increasingly evident that individual feed additive technology might not be addressing the challenges of modern poultry production (Gadde et al., 2017). Instead, an integrated, multi-pronged approach has proven most effective in optimizing performance while aligning with market expectations (Bist et al., 2024). Among the tools available, feed additives have emerged as a cornerstone strategy-delivering measurable improvements in animal health, feed efficiency, and overall production outcomes.

This study aims to evaluate the performance benefits of a combination of *Saccharomyces cerevisiae* fermentation-based postbiotics (SCFP) and a proprietary blend of essential oil compounds (EOC). Postbiotics—comprising inactivated microbial cells, their components, and metabolites—are highly stable and act primarily in the hindgut, where they support beneficial microbial populations and enhance gut health (Perricone et al., 2022). In contrast, EOCs are nature-identical phytogenic compounds that function predominantly in the upper gastrointestinal tract, promoting digestive enzyme activity and suppressing harmful microbes (Abd El-Hack et al., 2022; Gholami-Ahangaran et al., 2022). Together, these technologies offer complementary modes of action across different regions of the gut, presenting a targeted and

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consistent strategy to improve animal performance through microbiome modulation and enhanced digestive efficiency.

II. METHOD

The effect of a combination of SCFP and EOC in broiler birds were evaluated. Six independent studies were conducted under different settings to evaluate the combination treatment. Each study was set up with a completely randomized design with a control group (CON) receiving a basal diet and treatment group (TRT) receiving the combination of SCFP and EOC (Biostrong™ Dual, Diamond V Mills, Cedar Rapids, IA) on-top inclusion of 0.4kg/MT feed starting from 0d till the end of the trials. Body weight adjusted cumulative feed conversion ratio (cFCR), and cumulative feed intake (cFI) were evaluated at days 14, 28, and 35. All trials used a randomized block design with pens as experimental units with 12-to-48 replicates/treatment with 216-to-960 birds/treatment. The broader range in the total number of treatment replicates, as well as the number of birds in each treatment group, was primarily determined by historic data from similar experiments and by the design of the facilities. Performance data across the trials were analyzed using PROC MIXED model of SAS 9.4 (SAS Institute, Cart, NC, USA) with block nested in study as random effect, significant difference was determined at $P < 0.05$.

Microbiome analysis was performed using GALLEON™, which is a non-invasive microbiota platform that combines advanced microarray technology based on 16SrDNA sequences with artificial intelligence. For the present study, cloaca swabs were collected from 11 trials at 21d of age and were analyzed using it to assess differences in gut microbiome using this technology. Microbiota of cloaca samples were analyzed using fluorescence signal from pre-selected bacteria DNA probes. Relative fluorescence data were standardized and submitted to ANOVA in a factorial arrangement with fixed effect of treatment (CON or TEST). Differences between LS-means were considered significant by passing FDR (False Discovery Rate) test with $P = 0.05$.

III. RESULTS

Results from the study showed that inclusion of the combination of SCFP and EOC resulted in a significant ($P<0.05$) improvement in bird body weight at 14, 28, and 35d of age. The increased bodyweights at each time point represented improvements of 1.4, 2.6, and 2.4% in TRT fed birds compared to CON birds. In the case of cFCR, there were significant improvements in birds fed SCFP and EOC diets, resulting in approximately 4, 4, and 5 points improvement at 14, 28, and 35d of age. There was no significant difference in cFI between treatment groups at any time points.

Table 1 - Pooled analysis of body weight, adjusted cumulative feed conversion ratio (cFCR), and cumulative feed intake (cFI) from eight experiments.

Age in days	Body weight (g)				cFCR				cFI (g)			
	CON	TRT	SEM	P value	CON	TRT	SEM	P value	CON	TRT	SEM	P value
0	54.1	53.7	0.2929	0.3181	-	-	-	-	-	-	-	-
14	610.4	618.9	2.9755	0.0383	1.27	1.23	0.0079	0.008	719	714	4.3659	0.4539
28	1621.5	1664.5	9.1260	0.0010	1.44	1.40	0.0080	0.009	2295	2306	11.9239	0.5138
35	2208.2	2261.1	11.5534	0.0014	1.52	1.47	0.0065	0.008	3279	3292	16.1277	0.5854

Microbiome analysis revealed the reduced ($P < 0.05$) abundance of lactate producers and earlier establishment ($P < 0.05$) of butyrate producing bacteria population in TRT fed birds

compared to the CON group at 21d. In addition, there was a reduced ($P < 0.05$) abundance of opportunistic pathogens in TRT birds compared to CON birds (Figure 1).

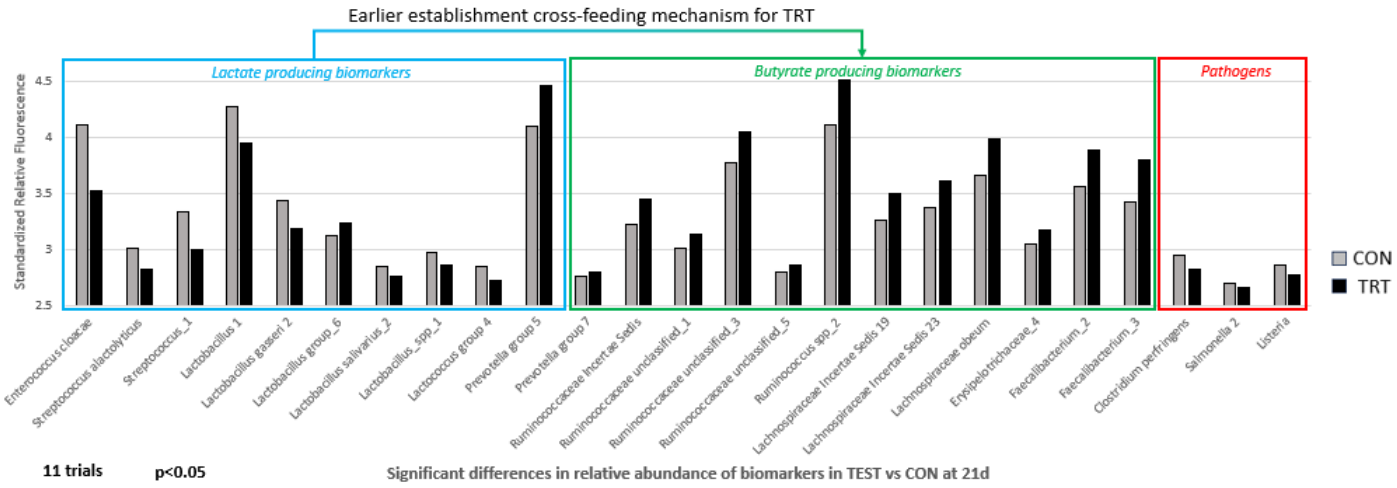

Figure 1 - Microbiome profile showing the reduced abundance of lactate producers, earlier establishment of butyrate producing bacteria population and reduced abundance of opportunities pathogens in TRT compared to CON (all the comparison were significantly different).

IV. DISCUSSION

Previous research has consistently demonstrated the benefits of SCFP in enhancing body weight, feed efficiency, and meat yield in broilers (Fathi et al., 2012; Roto et al., 2017). Building on this foundation, the present study is the first to highlight the benefit of combining SCFP with EO, showing significant improvements in body weight and corrected feed conversion ratio (cFCR) across various production phases. Notably, the absence of changes in corrected feed intake (cFI) suggests that the combination of SCFP and EOC may enhance nutrient digestibility and absorption, leading to more efficient utilization of feed.

SCFP plays a pivotal role in shaping microbial populations in the distal gut—particularly the cecum—by fostering the growth of beneficial bacteria (Feye et al., 2021; Gong et al., 2025). In contrast, EOC primarily exert their effects in the upper gastrointestinal tract (Michiels et al., 2008), where they enhance digestive enzyme activity (Abd El-Hack et al., 2022) and suppress pathogenic bacteria such as *E. coli* and *Salmonella* (Mahfuz et al., 2021; Gholami-Ahangaran et al., 2022). Microbiome analysis from the current study revealed an early establishment of butyrate-producing bacteria (short-chain fatty acid producers) and a marked reduction in opportunistic pathogens in the treatment group (TRT) compared to the control (CON). These short-chain fatty acid producers are key indicators of a mature, resilient gut microbiome (Yue et al., 2024) and are closely linked to improved feed efficiency in broilers (Liu et al., 2021). Collectively, the data suggest that the complementary spatial distribution and functional roles of SCFP and EOC along the gastrointestinal tract offer an advantage in modulating the gut microbiome and enhancing broiler performance.

In conclusion, the present study demonstrated the benefits of combining SCFP and EOC to support improved performance in broilers at different phases. Plausibly, the combination has a beneficial effect in modulating the gut microbiome resulting in improved gut health supporting the improved performance.

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PHYTOGENIC SUPPLEMENTATION ENHANCES PERFORMANCE, INTESTINAL HISTOMORPHOLOGY, AND INFLAMMATORY RESPONSE IN NECROTIC ENTERITIS CHALLENGED BROILERS

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The global restriction on antibiotic growth promoters has led to a rise of necrotic enteritis (NE) in poultry, driving interest in alternative strategies to maintain gut health and productivity. Phytogenic feed additives are among the promising options to support intestinal health and immune function (Abdelli et al., 2021). This study assessed the effects of phytogenic blend (PHB) containing thymol, oleic acid, capric acid, and refined sunflower fat on growth performance, liveability, histomorphology, and excreta biomarkers of inflammation in broilers challenged with NE. A total of 450 d-old Cobb 500 chicks were randomly allocated to three treatments: NC (non-challenged control), CC (NE challenged control), and PHB (NE challenge birds supplemented with 0.01% phytogenic blend). Each group had 10 replicates of 15 birds. To induce NE, birds in the CC and PHB groups were orally inoculated with *Eimeria* spp. on d9 and *Clostridium perfringens* at d14. Birds were fed starter (d0-10), grower (10-24), and finisher diet (d24-35). Feed intake (FI) and body weight (BW) were taken at days 10, 24, and 35, and excreta biomarkers of inflammatory, histomorphology measurements, and lesion scoring were performed at d16. Normally distributed data were analyzed using one-way ANOVA (female % was used as a covariate for performance data if significant), and nonparametric analysis was used for data not following normal distribution.

In the starter phase, birds fed PHB had significantly lower body weight gain (BWG) and higher feed conversion ratio (FCR) ($P < 0.05$) compared to the NC and CC groups. During d10-35, the NE challenge significantly reduced BWG, FI, and increased FCR in CC group compared to the NC group ($P < 0.001$). The CC group also showed reduced villus height (VH) ($P < 0.05$), VH/crypt depth (CD) ($P < 0.001$), and d10-35 liveability ($P < 0.05$), along with increased duodenal lesion scores ($P < 0.05$) compared to NC group. In addition, levels of excreta inflammatory biomarkers, including calprotectin ($P < 0.01$), fibronectin ($P < 0.05$), and lipocalin-2 ($P < 0.001$), were elevated in the CC group.

Dietary supplementation with PHB improved BWG, FI, and reduced FCR ($P < 0.05$) compared to CC during d10-35. Additionally, dietary supplementation of PHB reduced duodenal lesions ($P < 0.05$) on d16 and increased liveability by 10.2% (d10-35, $P > 0.05$, PHB 88.1% vs CC 77.9%) compared to CC, showing no difference from NC (91.9%) birds. PHB supplementation also increased VH/CD ($P < 0.05$) and shifted VH, calprotectin, and fibronectin levels towards values observed in NC birds ($P > 0.05$).

These findings suggest that PHB can potentially mitigate NE-related performance losses by improving growth, intestinal morphology, liveability, and reducing inflammation under NE challenges. However, the reduced performance observed during the starter phase (d0-10) suggests that further studies are needed to determine the optimal inclusion rate of this PHB for routine application in broilers' diets.

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INVESTIGATING THE COMPLEX PATHOBIOME OF ENTEROCOCCAL SPONDYLITIS: EVIDENCE FOR COINFECTION AND DIAGNOSTIC CHALLENGES IN AUSTRALIAN POULTRY

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Summary

Enterococcus cecorum is a significant emerging pathogen globally, causing substantial economic losses in broiler chickens due to septicemia and locomotor disorders such as enterococcal spondylitis. *E. cecorum* pathogenesis is far more complex than previously understood, largely due to coinfection with other pathogens, which can exacerbate disease severity. This study applied high-throughput sequencing (16S rRNA amplicon and shotgun metagenomics) to tissues and organs collected from Australian poultry farms experiencing *E. cecorum* outbreaks, testing the hypothesis that *E. cecorum* disease involves a pathobiome rather than a single pathogen. 16S rRNA sequencing revealed the widespread co-occurrence of the *Enterococcus* genus alongside the profound dominance of the *Escherichia-Shigella* genus across multiple systemic infection sites, such as thigh bone marrow, liver, lung, spleen, and yolk sac. Shotgun metagenomics confirmed *Escherichia coli* as the most dominant species (13.55% abundance) in the cecum, significantly surpassing *E. cecorum* abundance (1.97%). These findings provide compelling molecular evidence for a complex pathobiome structure in enterococcal spondylitis pathogenesis, necessitating a shift towards diagnostics and management strategies that recognise the significant contribution of co-infecting agents.

I. INTRODUCTION

Enterococcal spondylitis (ES) and related osteomyelitis pose a major and increasing global challenge to the commercial broiler chicken industry (Borst, 2012 #1). *E. cecorum* was initially regarded as a commensal until its association with septicaemia and lameness was reported in 2002 (Devriese, 2002 #2). Pathogenesis is typically hypothesised to involve *E. cecorum* translocation from the gut to systemic organs, such as bones and joints, which is often facilitated by compromised intestinal pathology (Schreier, 2022 #3;Borst, 2019 #4).

The traditional diagnostic paradigm of "one-pathogen-one disease" is outdated. Evidence suggests that coinfection, where two or more pathogens infect a single host, has a significant influence on disease progression and clinical outcomes (Hoque, 2021 #5). Cooperative interactions between pathogens can enhance host damage, and *E. cecorum* is known to utilise the effects of other pathogens opportunistically (Borst, 2019 #4). This complex pathogenesis requires adoption of the pathobiome concept, which accounts for the interaction between the host, the environment, and the entire microbial community in determining disease management outcomes. *E. cecorum* infections are highly correlated with other pathogens, including *Enterococcus faecalis* and *Enterococcus faecium* (Szymanski, 2024 #6). Advanced molecular tools, such as high-throughput sequencing, are essential to accurately profile this complex microbial community and support the coinfection hypothesis.

II. METHODS

365 samples from 10 different farms were collected from Australian commercial poultry farms experiencing *E. cecorum* outbreaks, including intestinal contents and systemic organ tissues

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(liver, lungs, spleen, oviduct, yolk sac), and bone marrow. Genomic DNA was extracted directly from tissues. A broad investigation was conducted using 16S rRNA amplicon gene sequencing (Illumina MiSeq, V3-V4 region) to characterise the microbial communities. Shotgun metagenomic sequencing (Illumina NovaSeq) was performed on selected high-priority samples (cecum, oviduct, liver, lungs, bone marrow) to enable species-level identification and functional characterisation. Taxonomic profiling was performed using MetaPhlAn2, which accurately identifies and quantifies species using clade-specific marker sequences bundled in ChocoPhlAn

III. RESULTS & DISCUSSION

16S rRNA sequencing revealed the widespread presence of the *Enterococcus* genus across various systemic sites, including bone marrow, oviduct, yolk sac, liver, lungs, and spleen (Figure 1). Significantly, the sequencing confirmed a profound dominance of the *Escherichia-Shigella* genus in multiple organs associated with ES pathology, specifically the thigh bone marrow, liver, lung, oviduct, spleen, and yolk sac. This robust co-occurrence data (Figure 1) strongly supports the possibility of a complex pathobiome structure in ES pathogenesis.

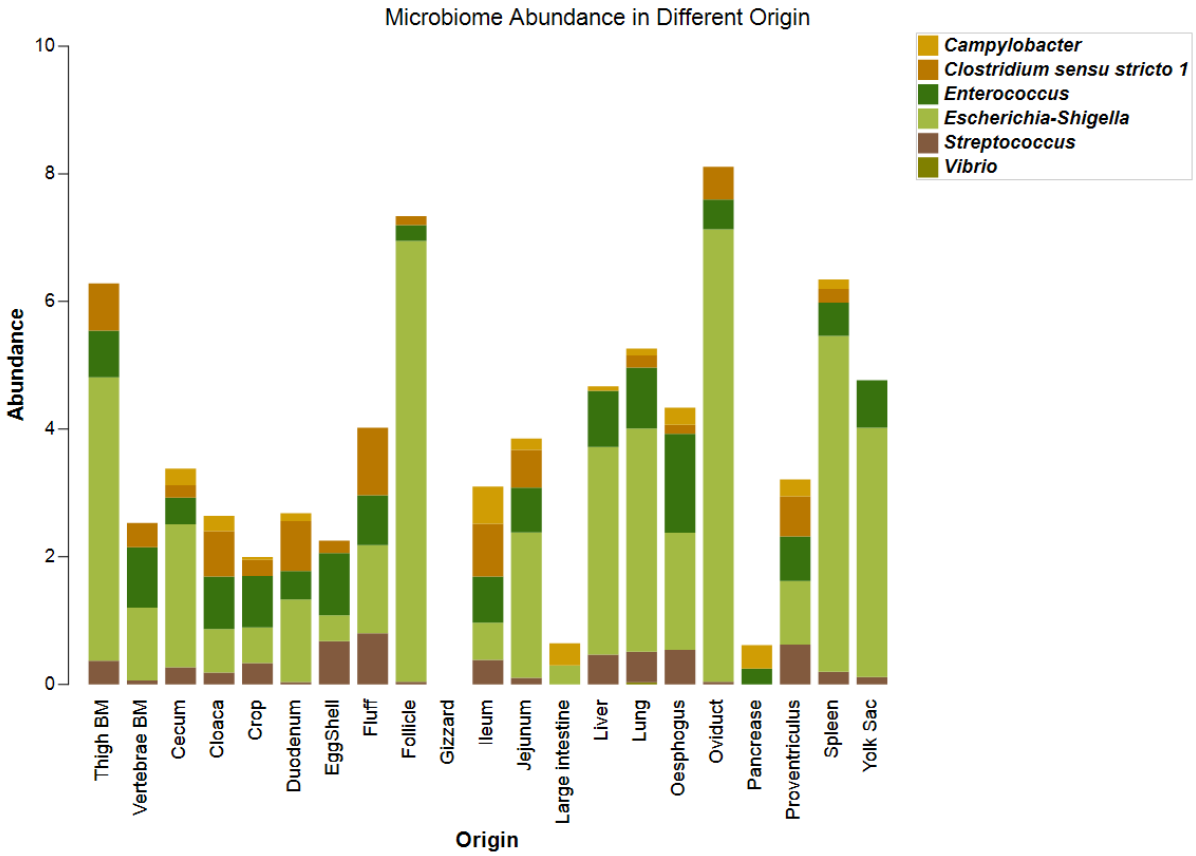

Figure 1 - Relative abundance of pathogenic microbiome taxa across different sample origins.

Shotgun metagenomic sequencing was conducted on a representative cecum sample to provide species-level confirmation of the dominant taxa identified by 16S rRNA sequencing. This analysis confirmed *E. coli* was the most dominant species in the cecum sample, registering an abundance of 13.55%. *E. cecorum* was also confirmed present, with an abundance of 1.97% (Table 1). Partial genome assembly of the detected *E. coli* strongly suggests it is an APEC strain. This is highly relevant as *E. coli* is a known cause of osteomyelitis and is documented to co-infect with *Enterococcus* species during avian colibacillosis (Walker, 2020 #10). The molecular detection of pathogenic *E. cecorum* alongside dominant co-pathogens, such as APEC, across

systemic sites is consistent with published evidence that intestinal pathology facilitates the translocation of bacteria from the gut to other organs (Borst, 2017 #8;Jung, 2014 #9).

Table 1 - Species-level composition of the cecum.

Species	Abundance %
Escherichia coli	13.55176
Campylobacter jejuni	4.82609
Vibrio cholerae	4.76756
Enterococcus cecorum	1.96698

The high-throughput sequencing data provide compelling evidence that ES pathogenesis likely involves coinfection with multiple pathogens. The pervasive co-occurrence and dominance of pathogenic species like APEC alongside *E. cecorum* in systemic tissues point out the complex pathobiome structure in ES, where co-infectors may act as primary drivers of disease or enhance *E. cecorum* virulence. Effective management of *E. cecorum*-associated disease requires a necessary shift to a pathobiome perspective, employing broad diagnostics that recognise the contribution of all significant microbial agents to ensure appropriate mitigation and biosecurity strategies.

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CLOSTRIDIUM PERFRINGENS ENZYMES SIALIDASE, CHITINASE AND COLLAGENASE EXERT A CONCERTED ACTION IN INITIATING NECROTIC ENTERITIS

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Although the *Clostridium perfringens* NetB toxin is essential to cause necrotic enteritis, the pathogen also produces enzymes that can be important. It is hypothesized that these enzymes are involved in the initial steps of the pathogenesis, such as colonization, mucus and extracellular matrix breakdown. We studied *C. perfringens* sialidase, collagenase and chitinase to explore their roles in the initial stages of the pathogenesis.

Using PCR, we showed that the chitinase genes *chiA* and *chiB* were present in pathogenic *netB*-positive type G strains but absent in non-netB containing isolates. Using dot blot assays with recombinant ChiA and ChiB enzymes, it was shown that ChiA specifically interacted with chicken mucus. After Clostron-mediated ChiA mutant generation, we used growth assays in nutrient poor medium to show that the ChiA chitinase is important in *C. perfringens* growth in mucus-supplemented medium. In broiler infection trials, both an *in vivo* colonization trial and a necrotic enteritis trial, it was shown that a ChiA chitinase mutant was less capable to colonize the intestine and was hampered in its disease-causing ability as compared to the wildtype strain.

Sialidases cleave sialic acid from biological molecules, often cell surface components. We show that *C. perfringens* is able to use sialic acid as a carbon source for bacterial growth. We showed that sialic acid, supplemented to culture medium, increases the production of NetB toxin, by measuring the haemolytic activity against chicken erythrocytes. Also, pre-treating avian LMH cells with the recombinant NanI sialidase increases the adherence of *C. perfringens* type G strain CP56 to these cells.

We sequenced the *colA* collagenase gene sequence in 48 *C. perfringens* strains and deduced the amino acid sequences bioinformatically using the ExPASy Translate tool. We discovered that NetB-toxin positive pathogenic strains carry truncated colA collagenase genes, thus encoding small variant collagenases. The Molecular Probes EnzChek® Gelatinase/Collagenase Assay Kit was used to evaluate the activity of the recombinant collagenase fragments using various substrates. It was shown that both recombinant full-length and variant collagenases were capable of degrading type IV collagen, although variant collagenases present in NetB positive strains showed a higher relative activity as compared to full-length collagenase. Detailed protocols of all studies can be found in Van Damme et al. (2021, 2022) and Dierick et al. (2024).

In summary, *C. perfringens* enzymes are contributing to necrotic enteritis. Chitinases are involved in mucus binding and breakdown, while sialic acid releases sialic acid as a nutrient to enhance proliferation and toxin production, and enhance cell adhesion. Collagenases can cleave type IV collagen and thus damage the basal membrane.

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PATHOGENIC *ENTEROCOCCUS CECORUM* CAUSING BACTERIAL CHONDRONECROSIS WITH OSTEOMYELITIS DIFFER FROM COMMENSAL STRAIN IN GENETIC AND PHENOTYPIC CHARACTERISTICS

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Bacterial chondronecrosis with osteomyelitis (BCO) is an emerging disease in broilers, causing lameness, that is currently mainly under control using antimicrobials. The most obvious clinical signs of BCO are related to locomotory problems associated with necrosis in rapidly growing bones, most typically proximal parts of femur and tibia and flexible thoracic vertebrae. Bacteria that are found in BCO lesions are intestinal bacteria that are proposed to have translocated through the intestinal epithelium and have spread systemically, including *Escherichia coli* and *Enterococcus cecorum*.

We isolated commensal strains from healthy flocks and strains from BCO outbreaks, derived from caeca, colon, pericardium, caudal thoracic vertebrae, coxo-femoral, knee and intertarsal joints. Pulsed field gel electrophoresis (PFGE) analysis showed that in outbreaks, clonal *E. cecorum* populations were isolated from different bones/joints and pericardium from animals within the same flock, with intestinal strains carrying the same pulsotype, pointing to the intestinal origin of the systemically present bacteria. Commensal strains were genetically distinct from the outbreak strains. Interestingly, outbreak strains were more sensitive to specific antimicrobials that are used to prevent/treat BCO, such as lincospectin and amoxicillin, as compared to commensal strains, that contained specific antimicrobial resistance genes.

In an oral inoculation model in which 10^8 cfu of a pathogenic *E. cecorum* strain was given on the day of hatch, we were able to induce femoral head necrosis and hock and knee joint lesions. Also, a third of all animals were positive for *E. cecorum* in internal organs, and re-isolation of the inoculated strain was performed. This shows that experimental oral infection causes *E. cecorum* translocation to the blood stream and internal organs, including bones, confirming the intestinal source of the pathogen.

Whole genome sequencing of strains using nanopore sequencing showed that isolates from the gut, bones/joints and internal organs of affected animals contained a set of genes, potentially involved in pathogenesis, that were absent in isolates from the gut of healthy animals. Some of these genes encode cell wall structural components such as genes encoding for enterococcal polysaccharide antigens (epa genes) and capsule formation. These structures are known for supporting environmental survival, resisting phagocytosis and enhancing intracellular survival.

Isolates derived from the affected birds (n=15) induced a significant higher mortality in an embryo mortality model as compared to the isolates from the gut of healthy birds (n=5) (39% vs. 5%), pointing to an increased virulence.

In summary, pathogenic *E. cecorum* isolates form a distinct group that carries a specific genetic background with hypothetical genes encoding virulence traits. Among these, cell wall-related structures are of interest, as they confer survival in hostile environments, including intramacrophage.

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DATA-DRIVEN POULTRY PRODUCTION: FIELD VALIDATION OF MACHINE-LEARNING GROWTH MODELS PREDICTION WITHIN INTEGRATED DIGITAL MANAGEMENT SYSTEMS

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Summary

The poultry industry faces growing biological and operational pressures driven by persistent and rapidly mutating infectious diseases, inconsistent management practices, fragmented data systems, and limited skilled labor. This makes managing a poultry operation like trying to hit a moving target bringing the need for solutions that provide rapid insights and support the making of quick but well-informed decisions and corrective actions. Cloud-based data environments supported by artificial intelligence (AI) and machine learning (ML) offer a scientific framework for addressing these challenges by integrating breeder, hatchery, broiler, feed mill, laboratory, and processing information into a unified system. Such integration enables earlier deviation detection, multi-stage traceability, and more reliable forecasting to support proactive rather than reactive management.

Using anonymized field evidence from commercial implementation such as PoultrySync, this paper illustrates how integrated digital ecosystems and predictive models can support decision-making in modern poultry production. A broiler weight-prediction model trained on 3,500 cycles and validated across 686 cycles achieved a mean absolute percentage error of 2.8%, weekly errors within ±3%, and final-cycle accuracy exceeding 95% despite natural operational variability ($R^2 = 0.61$). Additional field examples highlight how unified digital records accelerate root-cause analysis and improve the consistency and timeliness of production and health reporting. Together, these findings demonstrate the potential of AI-enabled, integrated digital systems to enhance operational control, planning accuracy, and long-term sustainability in poultry production.

I. INTRODUCTION

Global poultry production is a cornerstone of global food security, projected to account for nearly half of the world's meat consumption growth by 2030. This expansion is particularly critical in Africa and the Middle East, where population growth and rising incomes are driving unprecedented demand for affordable protein. However, the industry operates on razor-thin margins, where a mere one-percent gain in efficiency can translate into millions of dollars in savings or losses. Producers in these regions face a confluence of challenges, including the persistent threat of emerging and re-emerging infectious diseases like Newcastle Disease (ND), Infectious Bursal Disease (IBD), and Avian Influenza (AI), which can lead to devastating economic losses. A study in Nigeria, Africa's largest poultry producer, revealed that despite vaccination efforts, a staggering 81.8% of farms had flocks positive for Infectious Bronchitis Virus (IBV) RNA, and less than 4% were adequately protected against it, highlighting significant gaps in conventional disease management protocols.

Traditional farm management, often reliant on manual record-keeping, fragmented spreadsheets, and delayed data consolidation, severely restricts a manager's ability to respond proactively to deviations in flock health or performance. This reactive approach frequently leads to the late recognition of disease outbreaks, suboptimal feed conversion, and compromised animal welfare, directly impacting profitability and sustainability. The digital transformation of

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agriculture offers a new paradigm. The convergence of cloud computing, and artificial intelligence (AI) has enabled the rise of Precision Livestock Farming (PLF), a management concept centered on real-time, continuous monitoring and control of production, health, and welfare of individual animals.

Despite the scale and economic importance of modern poultry systems, producers continue to operate within fragmented information environments where health data, environmental records, production metrics, and laboratory findings are stored in separate systems or paper-based logs. This fragmentation limits the industry's ability to detect emerging problems early, quantify the impact of diseases such as infectious bronchitis (IBV), Newcastle disease, or IBD, or understand how management practices influence performance across the production cycle. In addition to disease pressure, labor shortages and variability in on-farm expertise create further challenges in maintaining consistent data capture and timely decision-making. Cloud-based digital ecosystems supported by real-time sensing, machine-learning forecasting, and AI-driven interpretation offer a scientific framework capable of addressing these gaps. By integrating data from breeder farms, hatcheries, broiler houses, feed mills, laboratories, and processing plants into a unified architecture, these systems enable continuous monitoring, early anomaly detection, and multi-stage traceability that support better disease investigation, benchmarking, and predictive management. This paper examines these emerging capabilities and uses anonymized field implementations as illustrative examples to evaluate how integrated, AI-enabled digital systems can help address the core operational and health challenges faced by poultry producers.

II. THE DIGITAL ECOSYSTEM

Modern poultry enterprises increasingly rely on digital ecosystems capable of unifying production, health, environmental, and laboratory data across the entire value chain. While the specific implementation of such systems varies across commercial providers, the underlying architectural principles share common scientific foundations. These include cloud-native data structures, modular design, real-time sensor integration, standardized data ontologies, and analytics layers that support predictive modelling and cross-stage traceability. The following section describes these general architectural components and functional capabilities as they are understood in current precision livestock farming research. An anonymized commercial implementation—used here solely as an illustrative example—demonstrates how these principles operate in a real-world production environment, without attributing uniqueness or exclusivity to any specific platform.

a) Integrated Value Chain Architecture

A persistent limitation in traditional poultry production is the presence of disconnected data silos, where each stage—grandparents farms, breeder farms, hatcheries, broiler operations, feed mills, laboratories, and processing plants—maintains its own records and information systems as illustrated in Figure 1. This fragmentation restricts the ability to conduct effective root-cause analysis, delays the identification of emerging issues, and complicates responses to food-safety or quality events. Modern cloud-based poultry management ecosystems address this limitation by unifying operational modules into a single digital environment as illustrated in Figure 2. While specific implementations vary among commercial platforms, the underlying objective is consistent: creating a unified relational dataset capable of supporting cross-stage linkage and high-resolution traceability across the entire value chain.

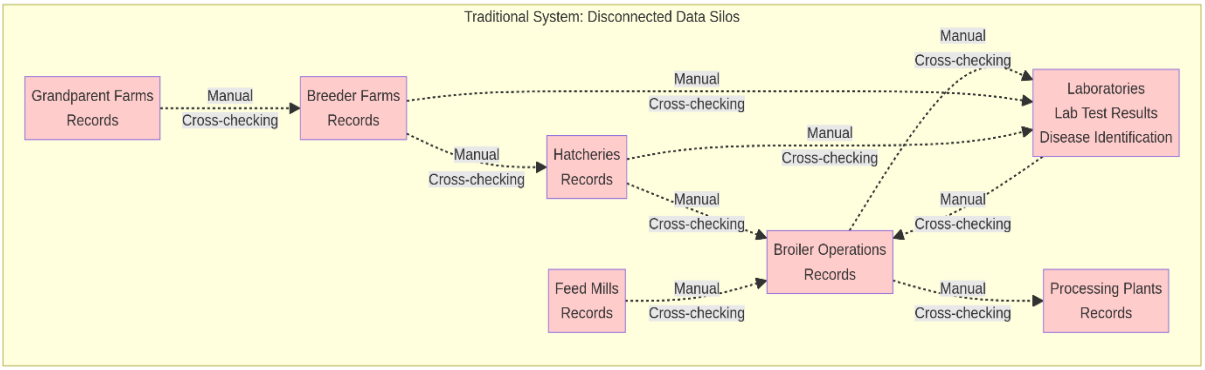

Figure 1 - Traditional Disconnected Stages.

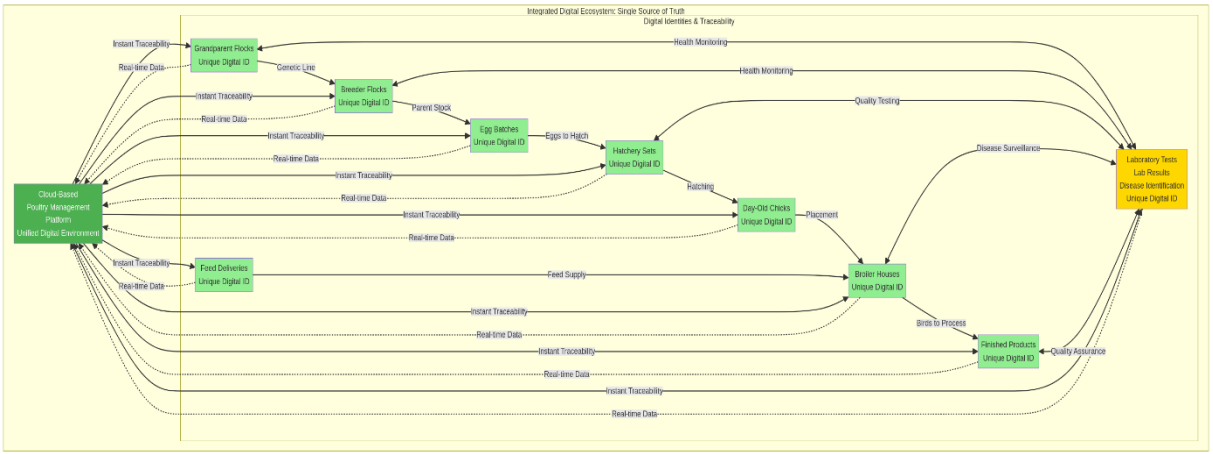

Figure 2 - Integrated Value Chain Architecture.

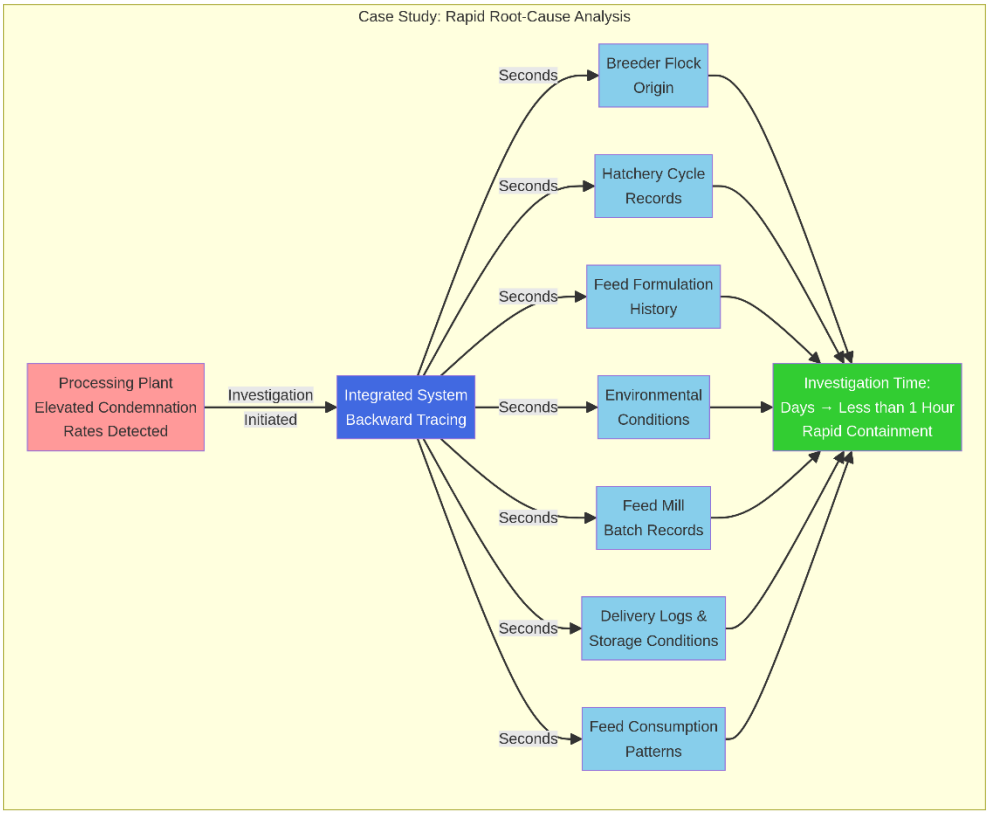

Figure 3 - Internal Investigation for Root-cause Analysis.

Field experience from anonymized commercial implementations demonstrates how integrated traceability can reduce the time required to investigate potential product-quality issues or recalls. In one example, a processing facility flagged abnormalities in a batch of carcasses linked to suspected feed contamination. Using a fully integrated digital system, investigators were able to trace the issue backward through the feed-mill batch records, delivery logs, storage conditions, and on-farm feed-consumption patterns within minutes. The farm-level environmental data and health observations further helped isolate the root cause as displayed in Figure 3. Historically, such investigations would require manual retrieval of records from multiple departments and could take several days. In this field case, the investigation window was reduced to less than one hour, allowing rapid containment measures and preventing the issue from affecting additional production cycles. This represented an approximate 95% reduction compared with typical investigation durations of 24–48 hours under traditional, non-integrated record systems.

b) AI-Powered Predictive and Diagnostic Models

The platform's core analytical power resides in its suite of proprietary AI and machine learning models.

- *Broiler Weight Prediction Model* - To evaluate the performance of the broiler weight prediction framework, a structured field-validation procedure was conducted using anonymized datasets collected from several commercial production cycles. The model generated daily predicted body weights based on historical growth patterns and real-time flock data, and these predictions were compared against actual recorded weights gathered through automated in-house or platform-scale measurements. Table 1 contains a summary of the dataset structure and variable categories for model validation. Table 2 contains detailed variable specifications categorized by either inputs.
- *Dataset Composition* -
    - Total Cycles: 3500 broiler production cycles for training.
    - Variation Factors: Genetics, management style, feed formulation, housing conditions, regional differences.
    - Training data period: July 2022 – July 2025.
    - Prediction Approach: Day-by-day prediction using cumulative daily input data up to each specific age
    - *Model Accuracy Validation* - To assess accuracy, predictions were compared with actual measured weights using standard model-evaluation metrics, the Mean Absolute Percentage Error (MAPE), presented in Equation 1, and Root Mean Square Error (RMSE), presented in Equation 2 and R^2 as the coefficient of determination. Figure 4 illustrates the validation and accuracy assessment process. The predictive model was validated across 686 broiler production cycles, demonstrating robust performance across diverse production conditions. The aggregate performance metrics are summarized in Table 3, indicating high accuracy and reliability in weight prediction throughout the growth period.

Equation 1: (Mean Absolute Percentage Error)

$$MAPE = \frac{1}{n}\sum_{i=1}^{n} \left| \frac{Actual_i - Predicted_i}{Actual_i} \right| \times 100$$

Equation 2:
(Root Mean Square Error)

$$RMSE = \sqrt{\frac{\sum(Actual_i - Predicted_i)^2}{n}}$$

Table 1 - Summary of Dataset Structure and Variable Categories for Model Validation.

Variable Category	Specific Variables	Data Type	Temporal Resolution	Purpose in Model
Dataset Overview	• Total broiler cycles • Regional coverage • Genetic lines • Management styles • Feed formulations • Housing conditions	Categorical/ Numerical	Per cycle	Provide variation and diversity for robust validation across production systems
Production Inputs	• Genetic line • Initial chick weight • Feed type and nutrient specifications • Lighting programs • Ventilation programs	Categorical/ Numerical	Static (per cycle)	Define baseline production parameters and environmental setup
Daily Operational Inputs	• Feed consumption • Water consumption • Mortality/culling counts • Temperature readings • Humidity readings • Stocking density • Growth interruptions (feed outage, heat stress)	Numerical/ Categorical	Daily	Capture real-time operational dynamics and environmental conditions affecting growth
Measured Weight Data	• Daily/weekly sample weights • Automatic weight-scale distributions • Final pre-harvest weights	Numerical	Daily/Weekly	Provide ground truth for model validation and performance assessment
Model Prediction Output	• Day-by-day predicted weight curve	Numerical	Daily	Generate expected weight predictions using all available input data up to each specific age

Table 2 - Detailed Variable Specifications by Category.

Category	Variable	Description	Unit/Format
Production Inputs	Genetic line	Broiler breed or genetic strain	Categorical
	Initial chick weight	Average weight of day-old chicks at placement	grams (g)
	Feed type	Feed formulation identifier and composition	Categorical
	Nutrient specifications	Protein and energy	g/bird and kcal/bird
	Lighting program	Photoperiod schedule and intensity	Hours and lux
	Ventilation program	Air exchange rate and control strategy	m^3/h
Daily Operational Inputs	Feed consumption	Total feed intake per day	g/bird/day
	Water consumption	Total water intake per day	L/day or mL/bird/day
	Mortality/culling	Number of birds removed or died	count/day
	Temperature	Ambient temperature in house	°C or °F
	Humidity	Relative humidity in house	%
	Stocking density	Birds per unit area	birds/m^2
	Growth interruptions	Events disrupting normal growth	Categorical/binary
Measured Weight Data	Sample weights	Periodic weight measurements from sample birds	grams (g)
	Weight-scale distributions	Automated weight data from floor scales (if available)	grams (g)
	Pre-harvest weights	Final weight before processing	grams (g) or kg

Table 3 - Summary of Model Performance Metrics Across All Validation Cycles (n=686).

Performance Metric	Range/Value
MAPE	2.8%
RMSE	0.0815
R^2	0.61
Final-Cycle Prediction Accuracy	> 95%
Prediction Accuracy (Ages 7–35 days)	Within ±3% of observed weights

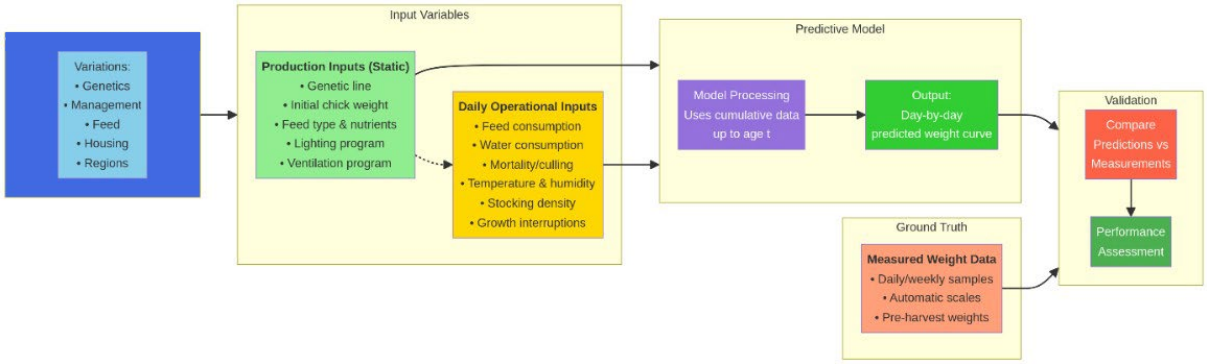

Figure 4 - Validation and Accuracy Assessment.

- *Performance Interpretation* - The validation results demonstrate that the predictive model achieved high accuracy across all production cycles, with MAPE values consistently below 5%, indicating minimal systematic bias. The RMSE value remained below ±75 g, which represents a small absolute error relative to typical broiler weights during the growth period. The R^2 values as 61% was expected as a variance in the target as the dataset contains natural operational noise and all missing values and outliers were preserved to keep this natural noise.

  Notably, the final-cycle prediction accuracy exceeded 95%, with the majority of prediction points for ages 7–35 days falling within ±3% of observed weights. This level of precision is particularly valuable for operational decision-making, as it enables producers to anticipate weight outcomes with confidence and adjust management practices proactively. These early-growth deviations are biologically meaningful because they coincide with periods of rapid metabolic development, heightened feed-conversion sensitivity, and increased vulnerability to subclinical disease expression

  To illustrate the model's day-to-day performance, Table 4 presents a representative example from one production cycle, showing actual versus predicted weights at weekly intervals from day 7 to day 35. The prediction errors remained within a narrow range of −3.0% to +2.7%, demonstrating consistent accuracy throughout the critical growth phase.

Table 4 - Comparison of Actual and Predicted Weights for a Representative Production Cycle.

Age (days)	Actual Average Weight (g)	Predicted Weight (g)	Absolute Error (g)	Relative Error (%)
7	165	160	−5	−3.0%
14	420	415	−5	−1.2%
21	915	940	+25	+2.7%
28	1,530	1,505	−25	−1.6%
35	2,180	2,230	+50	+2.3%

The validation results support the following key findings:

- Robust Generalization: The model performed consistently well across 686 diverse production cycles, encompassing variations in genetics, management practices, feed formulations, housing conditions, and regional factors.
- High Precision in Early Growth: Prediction accuracy was particularly strong during the early growth phase (days 7–35), which is critical for early intervention and management adjustments.
- Minimal Bias: The bidirectional nature of prediction errors (both positive and negative) across different ages suggests that the model does not exhibit systematic bias toward over- or underprediction.
- Operational Relevance: The accuracy levels achieved (MAPE < 5%, errors within ±3%) are well within acceptable thresholds for practical production management applications, enabling data-driven decision-making with confidence.

These results demonstrate that the integrated predictive model, when supplied with comprehensive production and operational data, can deliver accurate, actionable weight predictions that support proactive flock management and optimization of production outcomes.

c) Extending Predictive Frameworks Across the Poultry Value Chain

While broiler weight prediction provides a clear and quantifiable example of the value of AI in poultry production, the same methodological principles can be extended to other stages of the value chain. In practice, modern cloud-based ecosystems increasingly incorporate additional forecasting and optimization models that operate on top of a shared data infrastructure.

At the breeder and grandparent levels, AI/ML models can be used to predict egg production curves, fertility, and hatchability, using data on flock age, body condition, nutritional programs, health status, and environmental conditions. These forecasts allow hatcheries to plan setting capacity, staffing, and downstream chick placements more accurately, while also helping identify suboptimal breeder or GP performance earlier in the cycle.

In hatcheries, predictive models can associate hatchability and chick-quality outcomes with specific combinations of egg-storage time, incubator profiles, turning regimes, and breeder-flock characteristics. This supports data-driven adjustments in incubation profiles and egg-handling protocols rather than relying solely on static guidelines.

At the processing stage, integrated datasets enable models that link live performance to carcass yield, cut-up mix, and condemnation rates, allowing processors to forecast both volume and product mix more reliably and to trace deviations back to upstream causes such as feed formulation shifts, environmental stress, or health events.

The full benefit of these models emerges when they operate within a single, integrated digital ecosystem, rather than as isolated tools. In the anonymized commercial implementation considered in this paper, a platform such as PoultrySync serves as an example of how outputs from broiler, breeder, hatchery, and processing models can be combined in a unified environment to support planning, benchmarking, and root-cause analysis across stages. This aligns model development with real operational challenges—such as synchronizing chick placements with processing capacity or matching expected live weights with contract commitments—rather than building stand-alone algorithms disconnected from day-to-day decision-making.

III. DISCUSSION

The findings of this study demonstrate how AI-enabled predictive modeling, when embedded within an integrated digital ecosystem, can support a meaningful shift from reactive to predictive poultry management. The broiler weight prediction model provides the clearest example: its ability to forecast flock-specific growth trajectories with high accuracy illustrates how machine learning can provide operationally relevant insights that extend well beyond traditional descriptive reporting. This aligns with the broader goals of Precision Livestock Farming (PLF), where continuous sensing and automated interpretation are used to enhance decision-making across production systems.

The value of such forecasting is not limited to weight estimation itself. Accurate growth predictions inform a wide range of planning decisions, processing capacity scheduling, harvesting logistics, feed procurement, and contract fulfillment—allowing enterprises to reduce uncertainty and operate with greater precision. In regions where environmental stressors, disease pressure, and input-cost variability are significant, these predictive tools offer tangible benefits by enabling producers to anticipate deviations earlier and adjust management strategies proactively.

Beyond broilers, the same methodological principles apply to other stages of the poultry value chain. Predictive models for egg production, fertility, hatchability, incubation performance, and carcass yield are increasingly being integrated into cloud-based systems, creating multi-stage forecasting environments that reflect real operational needs. When these models operate on top of a unified data architecture—as in the anonymized commercial example

used in this study—they support a connected decision framework in which breeder, hatchery, broiler, and processing outcomes can be analyzed collectively rather than in isolation. This integrated perspective is critical for understanding root-cause relationships, benchmarking performance, and optimizing long-term planning across the chain.

Traceability represents another core pillar of this ecosystem. Cross-stage data linkage allows deviations detected at the processing plant, such as elevated condemnations or abnormal yields, to be rapidly traced back through broiler-house environments, feed batches, hatchery cycles, or breeder flock histories. This capability is essential not only for food-safety investigations but also for continuous improvement and biosecurity programs, where systematic identification of operational bottlenecks remains a major challenge [14]. The case study presented in this paper illustrates how such a system can reduce investigation time from days to minutes, reinforcing the practical value of integrated traceability.

IV. CONCLUSION

The results of this study highlight that the greatest benefit of AI in poultry production does not come from isolated models, but from their integration inside a single, end-to-end digital environment. Whether forecasting broiler weights, predicting hatchability, interpreting field observations, or tracing quality deviations, these models gain strength from shared data flows, standardized ontologies, and the ability to contextualize outputs across multiple stages of production. In an industry increasingly constrained by labor shortages, disease risk, and market volatility, such systems provide a scalable framework for enhancing managerial effectiveness and operational sustainability.

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EXPLORING RISK FACTORS FOR FLOOR EGG PRODUCTION IN FREE-RANGE LAYER FLOCKS

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Summary

Floor eggs remain a major challenge in cage-free egg production, reducing food safety, egg quality, and farm profitability. This study investigated risk factors for floor egg prevalence in 37 free-range flocks across Australia. Case flocks (≥3% floor eggs) averaged 7.0% prevalence compared with 1.3% in Control flocks (<3% floor eggs) ($p < 0.001$). Univariate analyses identified significant associations with nest box density, flock size, the farmer's perspective of an acceptable level of floor eggs, perch space/bird, lighting colour, and nest box platform type. Multivariate logistic regression revealed that flock size and nest box platform design were significant predictors of floor egg prevalence. Compared with large flocks (>10,000 hens, n=15), medium-sized flocks (3,001–10,000 hens, n=12) had substantially higher odds of being classified as Case flocks (odds ratio (OR) = 29.1, $p = 0.010$). Small flocks (200–3,000 hens, n=10) also had higher odds of being a Case flock (OR = 5.9), which was approaching statistical significance ($p = 0.098$). Flocks without a solid nest box platform were at greater risk of floor eggs, with an odds ratio of 27.9 ($p = 0.022$). The final model explained 52.9% of the variation in floor egg prevalence (Nagelkerke $R^2 = 0.529$).

These findings highlight that medium-sized flocks may face unique management challenges that increase susceptibility, while the presence of solid nest box platforms may have a protective effect. Practical interventions, including the inclusion of a solid nest box platforms, may reduce floor egg numbers. Further controlled research is required to validate these associations and refine management recommendations.

I. INTRODUCTION

Cage-free egg production is rapidly expanding in Australia and globally, driven by consumer demand for higher welfare standards and retailer commitments to phasing out conventional cages (Bray & Ankeny, 2017; Campbell et al., 2020). While cage-free systems offer hens greater behavioural opportunities, they also present management challenges, including the occurrence of floor eggs (FE), that is eggs laid outside nest boxes, on for example, litter, slats or perches. The occurrence of FE is problematic for several reasons. Floor eggs are more likely to be contaminated with faeces or litter, increasing the risk of bacterial spoilage and food safety concerns (e.g., *Salmonella*, *E. coli*) (Parisi et al., 2015). They are also more prone to cracking and downgrading, contributing to economic losses (Bist et al., 2023; Chai et al., 2023). Floor eggs also require manual collection increasing labour demands and diverting staff away from other management tasks (Putt et al., 2025). Further, once established, floor laying behaviour can spread quickly within a flock making early prevention particularly important.

A previous national survey identified FE prevalence range from <1% to 17% across Australian flocks (Putt et al., 2025). However, that study provided only preliminary associations with housing and management variables. Building on that, the present study aimed to investigate FE risk factors in greater detail through a follow-up survey, incorporating both structural and management-related variables with a goal to generate evidence-based insights to support producers in reducing FE prevalence and farm profitability.

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II. METHOD

This Case:Control study was a follow-up to a previous project (Putt et al., 2025) and farmers from that project were invited to complete this more detailed phone-based survey. This study was approved by the University of Sydney Human Ethics Committee (protocol 2024/264). Each interview lasted approximately 30 minutes and was conducted by the same interviewer with one interviewee for each flock. Prior to participation, farmers received a new information statement outlining the extended scope of the research and provided renewed consent. The final survey comprised 62 new questions across key domains: housing and space allocation (e.g., stocking density, nest box density), labour and management inputs (staff full time equivalent /1000 birds, years active), nest box management, lighting regime, feeding management, flock characteristics, and litter practices. Data collected in the initial study were also considered during the analysis. Survey responses were entered into REDCap, a secure web-based application, with each flock assigned a unique identifier. Farms with multiple flocks completed separate surveys, and all data were de-identified and stored in encrypted form. Responses were exported from REDCap for analysis in SPSS (IBM Corp., Armonk, NY). The Case:Control flock criteria were Case flocks ≥3% floor eggs; Control flocks <3% floor eggs. Descriptive statistics were generated for all variables and are presented as mean ± standard error. Associations between risk factors and outcomes were assessed using chi-squared tests for categorical variables and Student's t-tests for continuous variables. Variables showing notable associations were included in a multivariate logistic regression model. Statistical significance was set at $p < 0.05$.

III. RESULTS

This study included 37 flocks across Australia, including New South Wales (n=28), Queensland (n=1), Tasmania (n=4), and Western Australia (n=4). The average flock size was 12,446 birds (median = 9,300). Indoor stocking density averaged 10.15 hens/m² (median = 9.55 hens/m²). Case flocks (n=16) had an average FE prevalence of 7.0% (median = 4.6%), whereas Control flocks (n=21) averaged 1.3% (median = 1.0%) ($p < 0.001$). All flocks were housed in free-range systems with daytime outdoor access. Perches were provided in all flocks, but perch space/hen and their distribution within sheds varied.

Exploratory univariable analysis identified associations between FE prevalence and several factors, i.e. nest box density ($p = 0.048$), flock size ($p = 0.010$), accepted FE ($p = 0.015$), perch space/bird ($p = 0.027$), lighting colour ($p = 0.042$), and nest box platform type ($p = 0.006$) (Table 1). Case flocks generally had higher nest box densities and smaller flock sizes compared with Control flocks. The farmer's acceptance of higher FE levels also showed higher FE prevalence. Use of cool white rather than warm or natural lighting, and solid rather than slated or no nest box platforms, appeared to reduce FE levels. Multivariate logistic regression identified two significant predictors of FE prevalence (≥3%). Compared with large flocks (>10,000 hens), medium-sized flocks (3,001–10,000 hens) had markedly increased odds of being Case flocks (OR = 29.1, $p = 0.010$). Small flocks (200–3,000 hens) also showed elevated odds (OR = 5.9), which was approaching statistical significance ($p = 0.098$), likely due to low sample size. Flocks without a solid nest box platform were at substantially higher risk of FE, with an odds ratio of 27.9 ($p = 0.022$). The final model explained 52.9% of the variation in FE prevalence (Nagelkerke $R^2 = 0.529$) (Table 2).

Table 1 - Mean values of selected continuous and categorical variables for case (≥3% floor eggs) and control (<3% floor eggs) flocks, with associated *p*-values.

Variable		Case ≥3%		Control <3%		
		Mean	N	Mean	N	P
Floor eggs (%)		7	16	1.326	21	<0.001
Nestbox density (hens/m^2)		97.44	9	68.67	15	0.048[a]
Farmer accepted level of floor eggs (%)		3.81	16	2.00	21	0.015[a]
Perch space/bird (cm/bird)		15.01	13	12.54	15	0.027[a]
Lighting colour	(Cool)	3.50	1	1.00	9	
	(Natural)	16.00	2	2.50	1	
	(Warm)	5.90	13	1.49	11	0.042[b]
Solid nestbox Platform	(No)	7.22	15	1.15	11	
	(Yes)	3.76	1	1.52	10	0.006[c]
Flock Size (hens)	(200-3000)	11.17	6	1.13	4	
	(3001-10000)	4.72	8	1.87	4	
	(10001- 33300)	3.63	2	1.22	13	0.010[b]
Tunnel Ventilation	(Yes)	3.93	4	1.15	12	
	(No)	8.03	12	1.56	9	0.093[c]

All variables were tested under Brown-Forsythe test to confirm equal variance. [a] Students t-Test. [c] Fishers exact test. [b] Chi-squared test. N = number of flocks

Table 2 - Multivariate logistic regression model of risk factors associated with floor egg prevalence (≥3%) in cage-free layer flocks.

Parameter	Estimate	S.E.	OR	Z	Wald Test		
					Wald statistic	df	P
Intercept	-1.291	0.797	0.275	-1.619	2.621	1	0.105
Solid nestbox platform (Yes)	-3.336	1.453	0.036	-2.297	5.275	1	0.022
Flock Size (medium)	3.372	1.304	29.133	2.587	6.691	1	0.010
Flock Size (small)	1.772	1.072	5.885	1.654	2.735	1	0.098

Case:Control level '≥3%' coded as class 1. Reference category for 'Presence of solid nestbox platform' = 'No'. Reference category for 'Flock Size' = 'large'. N = 37. Nagelkerke R2 = 0.529. P = 0.006. 'OR' describes Odds ratio.

This analysis included 37 flocks and a Case–Control restriction compared to Putt et al., 2025 which included 43 flocks where means were analysed by ANOVA. Hence in this analysis tunnel ventilation was not statistically significant (p = 0.09) and was excluded from the regression model, likely reflecting the adjusted sample size rather than biological irrelevance. Perch space was significant in t-tests (p = 0.03) but not retained in the regression, though reduced perch space may still be biologically in lowering FE levels.

IV. DISCUSSION

This study provides new insights into risk factors associated with FE levels in Australian free-range systems. By examining flock and farm-level variables, this study has identified two key predictors of FE prevalence: flock size and nest box platform design.

Flock size emerged as a strong predictor, though the effect was not linear. Medium-sized flocks (3,001–10,000 hens) had significantly higher odds of elevated FE prevalence compared with larger flocks (>10,000 hens). Medium-scale flocks may lack the intensive management resources typically available in large-scale operations, while also facing more complex housing and monitoring challenges than very small flocks (Blasch et al., 2022; Oliveria et al., 2019). Additionally, larger flocks may devote greater attention to FE control, consistent with previous findings that farmers managing larger flocks reported stricter acceptability thresholds for FE (Putt et al., 2025). Small flocks (<3,000 hens) also had elevated odds, though this effect was not statistically significant (p > 0.05). It is possible that this reduced effect was partly driven by

outliers in the small-flock category, where a few flocks reported exceptionally low FE levels. These flocks may reflect unique management strategies or highly controlled environments that are not representative of small-scale production more broadly. Future studies should therefore examine small-flock systems more closely to distinguish genuine size effects from outlier-driven variation.

Nest box design also proved highly influential. The presence of a solid nest box platform was strongly protective, reducing the odds of high FE prevalence by nearly 28-fold. This suggests that even relatively simple structural features can play a critical role in directing hens to appropriate laying sites (Weeks & Nicol, 2005). These results align with international studies emphasising the importance of nest accessibility, stability, and comfort in shaping laying behaviour (Riber & Nielson, 2013; Struelens et al., 2005). Importantly, this finding highlights a practical and low-cost intervention for producers, offering a tangible means of reducing FE without requiring major system redesigns.

Taken together, both flock-scale differences and specific housing design features influence the risk of FE in cage-free egg production and a foundation for evidence-based strategies to improve farm profitability and reduce food safety risks. Future research should prioritise controlled experimental trials to validate these associations and investigate the potential behavioural mechanisms influencing FE laying.

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INCREASED HEPATIC FATTY-ACID β-OXIDATION MAY EXPLAIN THE REDUCED FEED INTAKE IN BROILER CHICKENS FED HIGH CANOLA MEAL DIETS

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In Australia, canola meal (CM) is considered a cost-effective, low-carbon-footprint alternative protein source to soybean meal in meat chicken diets. However, inclusion of high level of CM often suppresses feed intake as shown in our recent study (Kandel et al., 2025). To understand the molecular mechanisms underlying this phenotypic response, we performed liver RNA-sequencing analysis (RNA-seq) on 24-day-old Ross 308 broilers fed either a control diet (0% CM + 6% canola seeds) or a high-CM diet (11.8% CM + 8.3% canola seeds) (n = 8 birds per diet). Kyoto Encyclopedia of Genes and Genomes (KEGG) database was used to integrate the function of the differentially expressed genes (DEGs) identified between the 2 treatments.

RNA-seq reads were aligned to transcripts from more than 11,500 genes, and comparison of liver transcriptomes identified 405 DEGs, 209 upregulated and 196 downregulated in the high CM group ($P < 0.002$; FDR < 0.05). The KEGG pathway analysis revealed 12 significantly enriched pathways, consolidated into three major metabolic pathways including lipid catabolism, cell-cycle/proliferation, and amino acid metabolism in response to high CM diets. DEGs involved in lipid metabolic pathways including fatty acid metabolism ($P = 0.029$), fatty acid elongation ($P = 0.047$), and a trend toward fatty acid degradation/β-oxidation ($P = 0.092$) were mainly upregulated, indicative of a shift toward fat utilisation. Lipoic-acid metabolism was also upregulated ($P = 0.017$), supporting enhanced mitochondrial entry of carbon via pyruvate dehydrogenase. In contrast, cell-cycle and motor-protein programs were downregulated ($P = 0.008$ and $P = 0.043$, respectively), while the ribosomal protein synthesis was strongly enriched and upregulated ($P < 0.001$), indicating increased protein synthesis for metabolic adaptation. Amino acid pathways were redistributed rather than uniformly up or down. For lysine, the degradation pathway that converts lysine carbon into energy (to acetyl-CoA) was reduced, while the biosynthetic pathway linked to maintenance functions increased ($P = 0.029$). For tryptophan ($P = 0.031$), hepatic catabolism via the kynurenine arm was reduced indicating lower flux toward acetyl-CoA, succinyl-CoA and NAD^+ synthesis and thus, less tryptophan catabolism, potentially directing more tryptophan for serotonin production.

In conclusion, a high CM diet upregulated hepatic genes linked to fatty acid β-oxidation and altered tryptophan metabolism toward serotonin synthesis, both associated with enhanced satiety and reduced feed intake. These effects may be partly attributed to the high fibre and glucosinolate content of CM, which enhance satiety through gut–brain signalling and reduce thyroid-driven appetite regulation. To mitigate these effects, the use of low fibre or dehulled CM, enzyme supplementation, and low glucosinolate cultivars is recommended.

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IMPACT OF A STIMBIOTIC FEED ADDITIVE ON MICROBIOME MODULATION AND SKIN PIGMENTATION IN BROILER

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Summary

The objective of this study was to evaluate the effect of a Stimbiotic feed additive on broiler from 1 to 42 days of age, focusing on its impact on the gut microbiome at 21 days old, as recommended by Teemu et al. (2020) and skin pigmentation at slaughter. The trial was conducted with Hubbard broilers fed a basal corn–soybean meal diet across two different seasons, but within the same location. Birds were allocated into two groups/barns with two dietary treatments: Control feeding program (including a mannanase-based NSPase), or Stimbiotic replacing mannanase (100 g/ton). Gut microbiome composition, metabolite production (short-chain fatty acids), and skin pigmentation were assessed. The inclusion of the Stimbiotic demonstrated a clear capacity to enhance gut health by increasing SCFA production, promoting beneficial *Bifidobacterium* populations, and reducing levels of Avian Pathogenic *Escherichia coli*. These outcomes suggest improved nutrient absorption in the gut. This optimized intestinal environment also directly contributed to enhanced skin pigmentation in broilers.

I. INTRODUCTION

The global broiler industry is currently facing significant challenges, driven by rising production costs, increasing pressure to reduce antibiotic use, and growing consumer demand for sustainable, welfare-friendly, and high-quality protein. One strategy to address these demands involves optimizing nutrient utilization in feed, particularly fiber. Beneficial fiber fermentation in broiler diets can improve gut health, nutrient digestibility, and overall performance. These effects are strongly influenced by fiber type, particle size, solubility characteristics, as well as bird age and management practices. Blends of insoluble and partially fermentable fibers, combined with prebiotic sources (such as xylooligosaccharides, XOS), have demonstrated the potential to modulate the intestinal microbiota, strengthen the mucosa, and enhance growth — representing a sustainable alternative for modern broiler diets (Rybicka et al., 2024). There is growing interest in poultry nutrition to stimulate fiber-fermenting microbiota in the ceca, aiming to reduce dysbiosis, promote a healthier gut microbiome, and improve energy extraction from the fibrous fraction of the diet — a source historically underestimated as an energy contributor to growth. To this end, a class of additives known as Stimbiotics (STB) has been developed. STB are defined as non-digestible but fermentable additives that enhance fiber fermentation at doses too low for the additive itself to significantly contribute to short-chain fatty acid (SCFA) production. Unlike prebiotics, which are quantitatively fermented by the microbiome, STB function by improving the fermentation of fiber already present in the diet (Bedford, 2019). The objective of this study was to evaluate the effect of a STB feed additive on broiler performance from 1 to 42 days of age, with specific focus on its impact on gut microbiome function at 21 days old and skin pigmentation at slaughter.

II. METHOD

The trial was conducted at the experimental unit of one of the leading companies in the Mexican broiler market (Veracruz State), involving 486,000 Hubbard broilers. Data collection was

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performed in two periods, May 2024 and February 2025. Birds were fed a corn–soybean meal–based diet formulated to meet the nutritional requirements specified by lineage standards. Diets were divided into four phases: Starter (1–10 days; 2,950 kcal AME, 1.28% dLys, 0.96% Ca, 0.58% Av.P), Grower 1 (11–21 days; 3,000 kcal AME, 1.18% dLys, 0.80% Ca, 0.40% Av.P), Grower 2 (22–35 days; 3,100 kcal AME, 1.06% dLys, 0.74% Ca, 0.37% Av.P), and Finisher (36–42 days; 3,200 kcal AME, 0.96% dLys, 0.72% Ca, 0.36% Av.P). The birds were housed in two barns per period (121,500 birds each) within the same poultry farm, both equipped with identical automated negative-pressure facilities, receiving uniform management, water supply, and thermal conditions. This two-period design was implemented to capture and strengthen the representativeness of seasonal variations in the intestinal microbiota. Each barn represented one treatment: a) Control Group: Mannanase-based NSPase at 300 g/ton, providing an energy matrix of 66 kcal/kg of complete feed. b) STB Group: Same feed composition and nutritional levels as the control, but without mannanase and with the inclusion of 100 g/ton of a STB additive (Signis, AB Vista, Marlborough, UK). On day 21, twelve birds from each barn (representing the average weight of each barn) were selected and euthanized. Cecal content samples were collected in Biofreeze® (Alimetrics, Finland) vials for the analysis of short- and branched-chain fatty acids (SCFAs and BCFAs, respectively). These samples were also subjected to 16S ribosomal RNA gene sequencing by qPCR to determine the relative abundance of *Bifidobacterium* and Avian Pathogenic *Escherichia coli* (APEC). Gene copy numbers per gram of digesta were log-transformed (log_{10}) following the methodology described by Teemu et al. (2020), and all values were analyzed in a factorial design (2 treatments × 2 periods) using Minitab 18® with two-way ANOVA. Results with $P < 0.05$ were reported as statistically significant. At 42 days of age, an additional 44 birds (representing the average body weight of each barn) were evaluated at the slaughterhouse for skin pigmentation scoring (Δb), following the method described by Castañeda et al. (2005), using a Minolta CR-200 Chroma Meter (Minolta Corp., Ramsey, NJ). The Minolta device was calibrated with a standard white plate. Measurements were taken on the fat vein (lateral apterylum region) of each bird, an area selected because it contains no feathers or major blood vessels.

III. RESULTS AND DISCUSSION

Cecal SCFA and BCFA contents from 21-day-old broilers, as well as Δb skin pigmentation values measured at 42 days of age, are presented in Table 1. SCFA levels were significantly higher in Period 1 (a result typically associated with improved performance in the region where the trial was conducted) compared with Period 2 ($P < 0.05$). In contrast, BCFA levels were not influenced by either the dietary treatments or the experimental period ($P > 0.05$). Consequently, the SCFA:BCFA ratio, a recognized marker of beneficial hindgut fermentation, was only influenced by the experimental period, with Period 2 showing a higher ratio compared to Period 1 ($P < 0.05$). BCFAs are primarily end-products of protein fermentation, whereas SCFAs are indicative of carbohydrate (especial fiber) fermentation. This finding suggests that higher levels of protein fermentation occurred during Period 2, potentially reflecting a less favorable gut health status. Indeed, the intestinal tract of broilers harbors complex microbial communities that are critical for both growth and gut integrity, producing SCFAs, modulating intestinal morphology, and supporting nutrient digestion and absorption. These microbial communities ferment indigestible carbohydrates (particularly fibrous components) into SCFAs, which serve as a primary energy source for epithelial cells, promote villus development, and establish a microbial profile generally associated with enhanced performance. Conversely, elevated undigested protein levels in the small intestine—commonly observed under stress conditions such as heat or excessive humidity—can increase BCFA production. BCFAs are derived from protein fermentation metabolites, including ammonia, hydrogen sulfide, nitric oxide, and biogenic amines (such as tyramine, histamine, cadaverine, putrescine, and spermine), which

may negatively impact broiler health and performance. Therefore, nutritional strategies or seasonal conditions that maximize SCFA production relative to BCFA generation should be regarded as valuable approaches to fostering a healthier intestinal environment (Elling-Staats et al., 2021).

Table 1 - Short and branched-chain fatty acids (SCFAs and BCFAs, respectively) contents in the ceca of 21 days of age broilers and skin pigmentation values obtained from broilers 42 days of age broilers.

Treatment	SCFA, mmol/kg	BCFA, mmol/kg	BCFA:SCFA, %	Skin Pigmentation, Δb
CTRL *x* P1	115.85	0.80	0.72	20.14[b]
STB *x* P1	117.50	0.98	0.77	24.15[a]
CTRL *x* P2	70.14	1.02	1.28	22.53[a]
STB *x* P2	106.04	1.51	1.42	22.37[ab]
Main Effects				
Feed Additives				
CTRL	99.23	0.88	0.92	21.51
STB	113.68	1.16	0.99	23.38
Period				
P1	116.73	0.90	0.75	22.51
P2	88.09	1.26	1.35	22.46
P-value				
Feed Additives	0.127	0.285	0.668	0.001
Period	0.025	0.236	0.012	0.591
Feed Ad. x Period	0.162	0.621	0.845	0.001

CRTL (Mannanase-300 g/t). STB (Stimbiotic-100 g/t). P1 (May 2024). P2 (Feb 2025). SCFA (Short-chain fatty acids in mmol/kg of sample); BCFA (Branched-chain fatty acids in mmol/kg of sample); BCFA:SCFA (percentage ratio between the fatty acid contents). Δb Skin Pigmentation (measured on a color intensity scale by the Minolta CR-200 Chroma meter - Minolta Corp., Ramsay, NJ). [ab]P<0,05.

Broiler carcass skin color is a key quality attribute in the United States and Mexico. Modern commercial broiler strains raised worldwide possess the genetic capacity to deposit pigments in the skin; however, pigmentation outcomes are influenced by feed ingredient quality, the type and dosage of exogenous additives (such as pigment sources), and the overall health status of the birds (Bilgili et al., 1998). Birds with compromised health typically show reduced nutrient absorption, including the uptake of key pigments. As shown in Table 1, enhanced fiber fermentation achieved through the use of a STB additive—specifically formulated to stimulate the fermentation of arabinose- and xylose-based substrates, which are typically abundant in corn–soybean diets—led to improved gut health compared with mannanase supplementation. In this study, this effect translated into significantly greater skin pigmentation intensity in the STB group relative to the control group ($P < 0.05$). Pigmentation results from the combination of xanthophylls and red pigments, whose absorption depends directly on intestinal integrity. Several factors influence this process, including pigment concentration and type, interactions with dietary fats, the presence of mycotoxins, genetics, sex, environmental conditions, and, in particular, diseases that compromise the intestinal mucosa, such as enteritis and coccidiosis. Studies have shown that improvements in fermentative processes and reductions in dysbiosis can enhance carcass pigmentation in broiler chickens (Vendrell et al., 2001; Williams, 2005; Ronsmans et al., 2015).

The $\log_{10}$-transformed results of *Bifidobacterium* and APEC counts in the ceca of broilers at 21 days of age are presented in Table 2. Seasonal variations in fiber fermentation profiles were associated with an increase in *Bifidobacterium* populations (a bacterial genus generally regarded as beneficial in poultry gastrointestinal health) in Period 1 ($P < 0.05$). The inclusion of the STB additive proved to be an effective strategy to reduce APEC prevalence (P

< 0.05), particularly during challenging seasonal conditions (high humidity and poor litter quality).

In conclusion, the inclusion of an STB additive in broiler diets demonstrated potential to support gut health by reducing APEC levels. STB show strong potential as microbiome modulators, offering an innovative nutritional strategy to maintain microbial balance and ensure optimal gut functionality in broiler production systems.

Table 2 - *Bifidobacterium* sp. and Avian Pathogenic *E. coli* (APEC) contents in the ceca of broilers slaughtered at 21 days of age.

Treatment	*Bifidobacterium* sp., gene copies/g of sample	APEC, gene copies/g of sample
CTRL *x* P1	6.10	8.11[a]
STB *x* P1	5.89	8.25[a]
CTRL *x* P2	5.10	8.79[a]
STB *x* P2	5.10	7.51[b]
Main Effects		
Feed Additives		
CTRL	5.74	8.36
STB	5.63	8.00
Period		
P1	5.99	8.18
P2	5.10	8.15
P-value		
Feed Additives	0.680	0.042
Period	0.001	0.924
Feed Ad. x Period	0.666	0.014

CRTL (Mannanase-300 g/t). STB (Stimbiotic-100 g/t). P1 (May 2024). P2 (Feb 2025). APEC (Avian Pathogenic E. coli). [ab]P<0,05.

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SUBSTRATE TYPE INFLUENCES IN VITRO CECAL FERMENTATION IN 7-DAY-OLD CHICKS: GUIDANCE FOR DIET ADJUSTMENT DURING THE EARLY-LIFE STAGE

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The cecum is the primary site of microbial fermentation in chicken. In early-age broilers, the cecal microbiota is still developing and therefore more susceptible to dietary influences. In addition to short-chain fatty acids (SCFA), branched-chain fatty acids (BCFA) can also serve as indicators of protein fermentation and can reveal the early directional tendencies of cecal metabolism (Elling-Staats et al., 2023). However, the composition and particle size of substrates that reach the caeca remain uncertain, highlighting the need for standardized *in vitro* digestion and fermentation approaches.

This study applied an *in vitro* fermentation model (Yao et al., 2023) with 7-day-old cecal inocula to evaluate *in vitro* digested cereals (wheat, barley, maize), protein meals (soybean, canola, lupin), and a complete starter feed, compared to ex-vivo ileal digesta (EID). All substrates were digested using a unified protocol (Jing et al, 2025) to standardize the procedure. In addition, a particle-size limit (<400 µm) was applied by sieving to mimic the selective barrier of the cecal villous network at the ileo-cecal junction(Vanderghinste et al., 2025). Gas kinetics, pH, SCFA and BCFA (i-butyrate, valerate, i-valerate) were measured. Indices including total SCFA and BCFA/SCFA proportion (BCP) were calculated.

Digested maize produced the highest cumulative gas (151.5 ± 5.7 mL/g DM), followed by wheat (140.3 ± 4.9) and barley (124.0 ± 11.2), while canola was the lowest (18.1 ± 9.4). Total SCFA showed a similar trend, with values ranging from 8.41 ± 0.58 mmol/g DM in maize to 4.01 ± 0.4 in canola. The BCP clearly distinguished protein sources with values all exceeding 5.0%, compared with ~2.3–2.5% for cereals. Notably, EID (7.51 ± 0.74) yielded similar total SCFA to digested starter feed (7.86 ± 0.29) but much less gas, suggesting that the *in vitro* digestion process may lead to a different fermentable substrate composition. In addition, cereals produced measurable ethanol (up to 0.57 ± 0.05), indicating potential activation of alternative metabolic pathways. Ongoing 16S rRNA gene sequencing analysis after fermentation aims to investigate whether these distinctive fermentation product fingerprints are associated with shifts in microbial community composition and diversity metrics. Their metabolomic functions will also be predicted using PICRUSt (gene function) analysis.

In conclusion, substrate type and particle size strongly influenced *in vitro* fermentation at this early-life stage. The higher BCP in protein substrates may promote fermentation with low energetic yield but higher metabolic risk *in vivo* unless balanced by low BCP carbohydrate-rich cereal substrates. These findings provide a reference for optimizing feed strategies, including enzyme and fiber uses, to reduce harmful by-products and support healthy microbial development. This *in vitro* model can provide a practical approach to predict and differentiate the fermentative and nutritive values of feed ingredients.

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ASSESSING THE IMPACT OF PROBIOTIC INTERVENTION ON BACTERIAL CHONDRONECROSIS WITH OSTEOMYELITIS (BCO) LAMENESS INCIDENCE IN BROILER CHICKENS

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Summary

Bacterial chondronecrosis with osteomyelitis (BCO) is a leading cause of lameness in modern broilers, with implications for welfare and production. This study evaluated the efficacy of two probiotic interventions: a hatch spray of *Enterococcus faecium* (2×10^9 CFU/bird) and a triple-strain *Bacillus* feed additive (*B. subtilis* 597, *B. subtilis* 600, and *B. amyloliquefaciens* 516 at 500 g/t from day 1 to 56). Using a wire-floor aerosol challenge model with one thousand three hundred, Cobb 500 broilers, lameness incidence was monitored from day 21–56 across five groups: positive control, negative control (no probiotics), *E. faecium* probiotic only, *Bacillus*-based probiotic only, and the probiotic combination.

All probiotic treatments significantly ($p<0.05$) reduced lameness compared with the negative control 49% incidence at the end of the experiment. The final rates were 31.7% (*E. faecium*), 31.0% (*Bacillus*), and 25.7% (combination). The combined probiotic applications (spray at hatch and feed supplementation) significantly ($p<0.05$) yielded the greatest benefit (~23% points reduction), with tendencies for lower lesion scores and reduced mortality (<5%) and without any adverse effect on body weight. These findings highlight probiotics as sustainable, antibiotic-free strategies to mitigate BCO lameness and enhance skeletal health in modern broiler production.

I. INTRODUCTION

Rapid growth rates in modern broilers have improved meat yield but also increased susceptibility to skeletal disorders, including bacterial chondronecrosis with osteomyelitis (BCO).Bacterial chondronecrosis with osteomyelitis (BCO) lameness is one of the major health & welfare challenges faced by the poultry industry, affecting 3 to 15% of commercial broilers in United States of America (Wideman, 2016; Hartcher and Lum, 2019). This condition develops when gut bacteria, such as *Enterococcus spp.* or *Escherichia coli*, translocate across the gut barrier and establish infection in compromised bone tissue, resulting in necrosis, osteomyelitis, and impaired locomotor function (Wideman, 2016; Asnayanti et al., 2024). Bacterial chondronecrosis with osteomyelitis (BCO) is both a welfare concern and an economic burden due to mortality,reduced performance and culling. Bacterial chondronecrosis with osteomyelitis (BCO) is responsible for 1–2% of bird condemnation at market age in the United States of America and can result in annual losses of tens of millions of dollars for producers (Wideman, 2016; Alharbi et al., 2024).

As antibiotic use faces increasing restrictions, alternative solutions are critical. Probiotics including *E. faecium* 669 and triple-strain *Bacillus* based feed additive (*B. subtilis* DSM 32325, *B. subtilis* DSM 32324, and *B. amyloliquefaciens* DSM 25840) have demonstrated benefits in gut health, immune modulation, and pathogen exclusion (Castaneda et al., 2020). This study assessed whether hatch-day administration of E. faecium 669 (GalliPro® Hatch. Novonesis), continuous dietary supplementation with a triple-strain Bacillus additive (GalliPro® Fit, Novonesis, B. subtilis DSM 32325, B. subtilis DSM 32324, and B.

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amyloliquefaciens DSM 25840), or their combination could reduce bacterial chondronecrosis with osteomyelitis incidence and severity in broilers exposed to a bacterial chondronecrosis with osteomyelitis (BCO) challenge.

II. METHOD

To evaluate the dietary treatments' effects on BCO lameness, an aerosol transmission challenge model was implemented, using a modified version of the approach reported by Asnayanti et al. (2024). A total of one thousand five hundred and sixty day-old Cobb 500 broiler chicks were housed in 26 floor pens (1.5 m × 3.0 m each), with an initial stocking density of approximately 750 cm² per chick. On day 14, bird numbers were adjusted to 50 chicks per pen to maintain a final density of 900 cm² per chick (one thousand three hundred birds in total).Two wire-floor pens (2 x 50 birds) were designated as infection source pens (T1 Positive control "seeder birds" group) and were strategically positioned near the evaporative cooling pads at the front of the facility with the other groups (T2, T3, T4, T5) were housed on wood-shaving litter flooring and replicated across six pens (6 x 50 birds), as follows:-

- T1 Positive Control: Wire-floor, infection source.
- T2 Negative Control: Litter floor, no probiotics.
- T3: Litter floor, *E. faecium* probiotic sprayed at on newly hatched chicks at 2.0×10^9 CFU/bird.
- T4: Litter floor, triple-strain *Bacillus* probiotic in feed (500 g/t, 1.60×10^6 CFU/g feed) from day 1-56.
- T5 Combination: Litter floor, T3 + T4 probioticss.

Clinical lameness was monitored daily from day 22–56. Suspect birds were euthanized and necropsied for tibial and femoral lesions. Lesions were scored per Wideman's classification (Wideman 2016). Incidence was analyzed via T-test and logistic regression ($p < 0.05$).

III. RESULTS & DISCUSSIONS

Cumulative lameness incidence showed clear differences among treatment groups over the course of the trial (Figure 1). Birds in the positive control group (T1, PC), reared on wire flooring, exhibited the earliest onset of lameness (beginning around day 33) and the highest cumulative incidence, reaching approximately 77% by day 56. Lameness also developed progressively in the negative control group (T2, NC), which was housed on litter flooring but exposed to aerosol transmission, culminating in a final incidence of nearly 50%. In contrast, birds in the probiotic-treated groups—T3, T4, and T5 demonstrated a delayed onset and substantially reduced cumulative lameness. By day 56, final lameness incidence was approximately 31.7% in T3, 31% in T4, and 25.7% in T5, ($p < 0.05$). The T5 group consistently maintained the lowest lameness trajectory throughout the observation period, suggesting a potential synergistic benefit from combining both probiotic application types (spray at hatch and feed supplementation). All probiotic interventions significantly reduced lameness compared to the NC group.

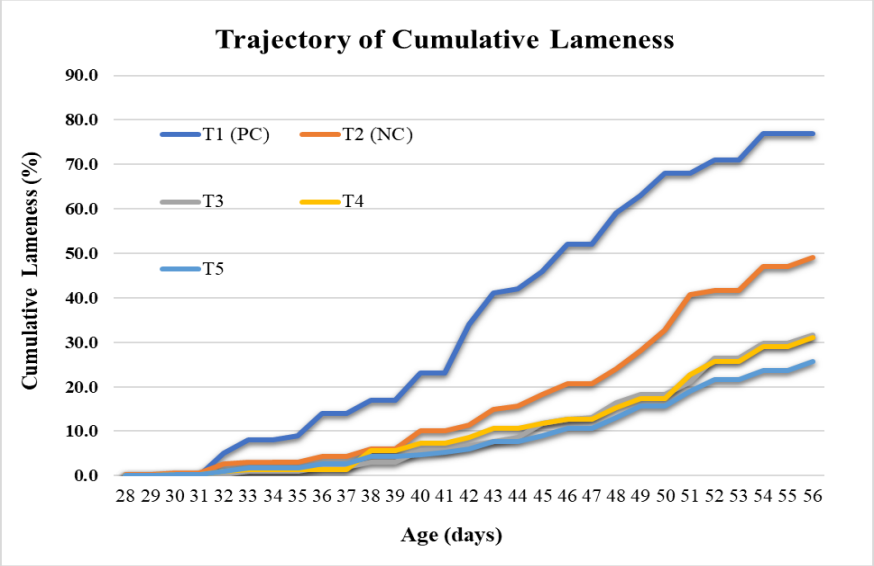

Figure 1 - Percentage cumulative lameness by treatment groups from d28-56 of this study.

Administering probiotics to broilers significantly reduced cumulative lameness compared to the negative control (T2, NC) group at day 56 (Figure 2.). Lameness incidence was reduced by 17.3 % points compared to T3 (31.7% vs. 49.0%), 18 % points in T4 (31.0% vs. 49.0%), and 23.3 % points in T5 (25.7% vs. 49.0%) compared to T2 (NC), with T5 which combined both type of probiotic applications showing the greatest improvement in comparison with the negative control group ($p < 0.05$).

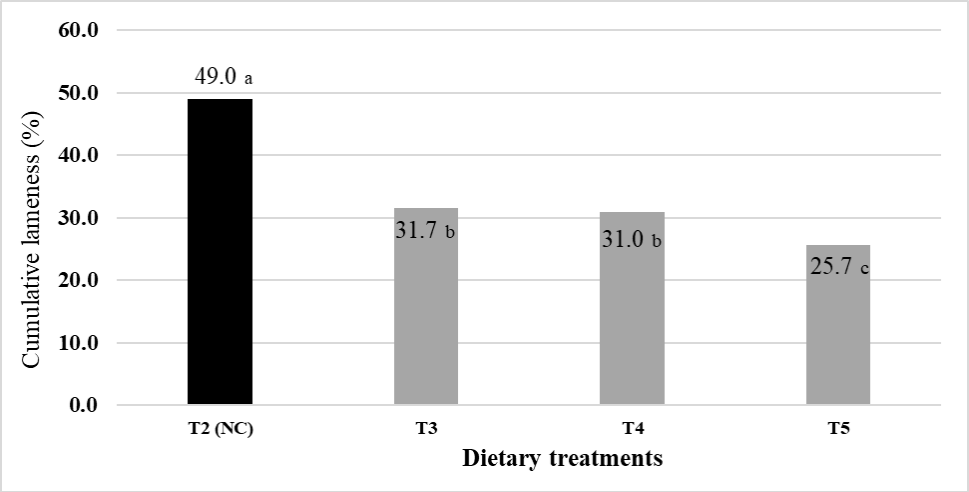

Figure 2 - Comparing the effectiveness of probiotic treatments (T3, T4, and T5) for lameness reduction by comparing them with the negative control group (NC). [a,b,c] Values with distinct superscripts in the same category are significantly different at $p < 0.05$.

Regarding the assessment of femoral head lesion types among lame birds across all treatment groups, normal bone structure in the right femur was observed in 27.5% of PC birds and 22.5% of NC birds, compared to higher rates in treated groups—T3 (17.5%), T4 (20.0%), and T5 (22.5%). Transitional degeneration remained under 10% across all treatments, while femoral head necrosis, a critical indicator of advanced BCO, reached 12.5% in PC birds but dropped to 2.5% in T5. Notably, femoral head necrosis (a hallmark of advanced BCO) was 12.5% in positive controls but only 2.5% in the combination group. These tendencies may indicate that probiotics not only delay onset but also mitigate lesion severity. However, the differences were not significant.

In addition, mortality was monitored throughout the trial, with a focus on cumulative deaths from day 29 to day 56. Mortality rates followed the same trend: 11% in positive controls, ~6.5% in negative controls, and <5% in probiotic groups, with the lowest in T5. Importantly, probiotic supplementation did not compromise growth performance. Mean body weights at day 56 (4.70–4.76 kg) were statistically similar among treatments.

IV. CONCLUSION

This study demonstrated the effectiveness of a probiotic intervention, which included an *Enterococcus faecium* spray administered at hatch and a triple-strain *Bacillus*-based supplement incorporated into the feed, in mitigating BCO-related lameness in broiler chickens. When administered individually, both the *E. faecium* spray and the *Bacillus* feed supplement reduced lameness by approximately 35–37%. The combination of both probiotics resulted in the greatest reduction, approaching 48%, suggesting a synergistic effect. The lameness reduction obtained with the probiotic association leads to a difference of 23 % points compared to the group of challenged birds not fed with any probiotic supplementation. These findings highlight the potential of targeted probiotic strategies as sustainable, antibiotic-free tools to enhance broiler welfare and productivity. Validation under field conditions will determine their broader application in commercial production systems.

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BACTERIAL XYLANASE SUPPLEMENTATION ENHANCES GROWTH PERFORMANCE, NUTRIENT DIGESTIBILITY, INTESTINAL BARRIER FUNCTION AND MICROBIOMES COMPOSITION IN BROILERS

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Summary

Poultry diets based on corn, wheat, and soybean meal provide energy and protein but also contain indigestible non-starch polysaccharides (NSPs) that can impair nutrient utilization. Two experiments evaluated bacterial xylanase supplementation effects on growth performance, nutrient digestibility, and gut health in broilers. In Experiment 1, a total of 1,050 birds were used for feeding study using three dietary treatments: control (corn–soybean meal basal, CON), negative control with reduced metabolizable energy (-85 kcal/kg ME, NC), and NC supplemented with 100 g/ton bacterial xylanase (NCX). In Experiment 2, a total of 192 birds were housed in metabolic cages for nutrient digestibility study using the same diets. Bacterial xylanase supplementation improved feed efficiency of the young birds fed a reduced-energy diet. The growth performance during the other period and overall (0–35 days) phases did not differ among treatments ($P > 0.05$). On day 35, birds in the NCX group had significantly lower serum fluorescein isothiocyanate-dextran (FITC-d) levels ($P < 0.01$), indicating improved intestinal integrity. Ceca microbiota analysis revealed that NCX groups increased abundances of Bifidobacterium pseudolongum and Dietzia aerofata. Xylanase supplementation significantly improved fiber, NDF, ADF digestibility, and apparent metabolizable energy (AME) ($P < 0.05$). Overall, bacterial xylanase supplementation in reduced-energy broiler diets enhanced early feed efficiency, supported intestinal health, promoted a beneficial cecal microbiome, and improved nutrient utilization, with indications of enhanced overall production efficiency.

I. INTRODUCTION

Supplementing corn-wheat-soybean meal diets with bacterial xylanase reduces the anti-nutritive effects of AX by lowering digesta viscosity and breaking down cell wall structures that otherwise trap essential nutrients..In addition, xylanase generates arabinoxylo oligosaccharides AXOS), which serves as fermentable substrates in the ceca, stimulating microbial fermentation, enhancing short- chain fatty acid (SCFA) production like butyrate and supporting a more favorable microbial profile (Craig et al., 2020; Bautil et al., 2021). Together, these mechanisms enhance nutrient utilization and maintain gut health, critical for poultry productivity and welfare, especially under nutritional stress. Reduced- energy diets are often employed as a cost- saving strategy but can impair gut function and performance. Xylanase supplementation helps counter these effects by supporting nutrient absorption, barrier integrity, and microbial balance. Despite extensive growth performance studies, research on xylanase's effects on intestinal permeability

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and microbiota under dietary energy restriction remains limited. This study investigates xylanase impact on growth performance, nutrient digestibility, gut permeability, and microbiota composition and function in broilers fed reduced-energy diets, elucidating its role in enhancing health and productivity.

II. METHOD

Two experiments evaluated bacterial xylanase supplementation effects on growth performance, nutrient digestibility, and gut health in broilers. In Experiment 1, a total of 1,050 birds were randomly assigned in a completely randomized design to three dietary treatments: control (corn–soybean meal basal, CON), negative control with reduced metabolizable energy (-85 kcal/kg ME, NC), and NC supplemented with 100 g/ton bacterial xylanase (NCX). The ME of all dietary treatments are shown in Table 1. The 35-day feeding trial included starter (1–10 days), grower (11–28 days), and finisher (29–35 days) phases, with *ad libitum* access to pelleted feed and water. On day 35, blood and cecal samples were collected (one bird/replicate) for serum fluorescein isothiocyanate-dextran (FITC-d) to determine intestinal permeability and cecal content (one bird/replicated) was collected for microbiome analyses. In Experiment 2, a total of 192 birds were housed in metabolic cages and assigned to the same dietary treatments with eight replicates of eight birds. Titanium dioxide (0.3%) was included as an indigestible marker from days 19 to 28. Birds were euthanized on day 28 for ileal digesta collection to assess nutrient digestibility. Growth performance and nutrient digestibility data were analyzed by ANOVA, with means separated using Duncan's multiple range test. Microbiota diversity was assessed using Kruskal–Wallis and PERMANOVA tests. Statistical significance was set at $P < 0.05$ and trends at $0.05 \leq P < 0.10$. GraphPad Prism v10.4.0 was used for data visualization.

Table 1 - Main ingredient composition and calculated metabolizable energy of experimental diets[1].

Ingredient (%)	Starter (day 0-10)			Grower (day 11-28)			Finisher (day 29-35)		
	CON	NC	NCX	CON	NC	NCX	CON	NC	NCX
Corn	41.89	43.69	43.69	36.23	38.13	38.13	32.43	34.33	34.33
Soybean meal (48%CP)	31.64	31.30	31.30	27.13	26.80	26.80	20.89	20.56	20.56
Wheat	10.00	10.00	10.00	15.00	15.00	15.00	20.00	20.00	20.00
Full fat soybean	6.00	6.00	6.00	8.00	8.00	8.00	10.00	10.00	10.00
Cassava chip	5.00	5.00	5.00	7.50	7.50	7.50	10.00	10.00	10.00
Soybean oil	1.55	-	-	2.84	1.26	1.26	3.85	2.27	2.27
Bacterial Xylanase	-	-	0.01	-	-	0.01	-	-	0.01
Calculated ME (kcal/kg)	3,000	2,915	3,000	3,100	3,015	3,100	3,200	3,115	3,200

[1]CON = basal diet; NC = reduced energy diet 85 Kcal/kg; NCX = NC + 100 g/ton Belfeed XylanaseTM.

III. RESULTS

During the starter phase (0–10 days), broilers fed the NC diet had significantly higher feed intake and poorer FCR compared to the CON and NCX groups ($P < 0.05$) indicating the effect of bacterial xylanase in enhancing feed efficiency of young birds fed reduced energy diet (Table 2). However, there was no significan difference in growth performance among dietary treatment during grower (11–28 days), finisher (29–35 days), and overall (0–35 days) phases ($P > 0.05$). Although not statistically significant, the NC + xylanase group exhibited numerically higher European Production Efficiency Factor (EPEF) values compared to the other groups.

Tabel 2. Effects of xylanase supplementation on growth performance of broilers during the starter phase (0 -10 days) and European Productivity Efficiency Factor at day 35.

Items	Dietary treatment			Pooled SE	*P*-value
	CON	NC	NCX		
Initial BW (g)	43.96	43.82	44.12	0.115	0.568
BW (g)	338.12	336.74	334.78	1.168	0.514
Feed intake (g/bird/day)	322.42 [b]	329.27 [a]	322.76 [b]	1.146	0.019
FCR	1.097 [b]	1.124 [a]	1.110 [b]	0.004	0.014
Mortality, (%)	0.286	0.000	0.000	0.095	0.377
EPEF at 35 day	547.32	544.67	558.43	4.273	0.382

[a,b] Means within the same row with different superscripts differ significantly (P < 0.05).
CON = basal diet; NC = reduced energy diet (-85 kcal/kg); NCX = NC + 100 g/ton xylanase.
FCR = feed conversion ratio; BW = body weight; SE = standard error.
EPEF = European production efficiency factor = [% Livability × BW (kg) × 100] / [FCR × Trial duration (days)].

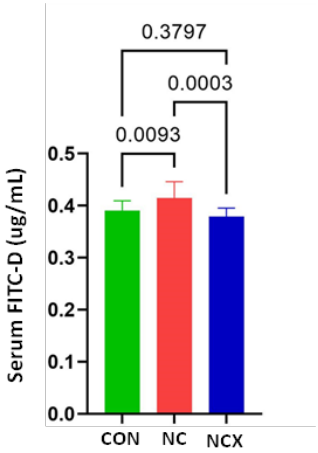

Figure 1 - Serum fluorescein isothiocyanate-dextran *(*FITC-d*)* levels in broilers at 35 days of age *(*P < 0.05*).*

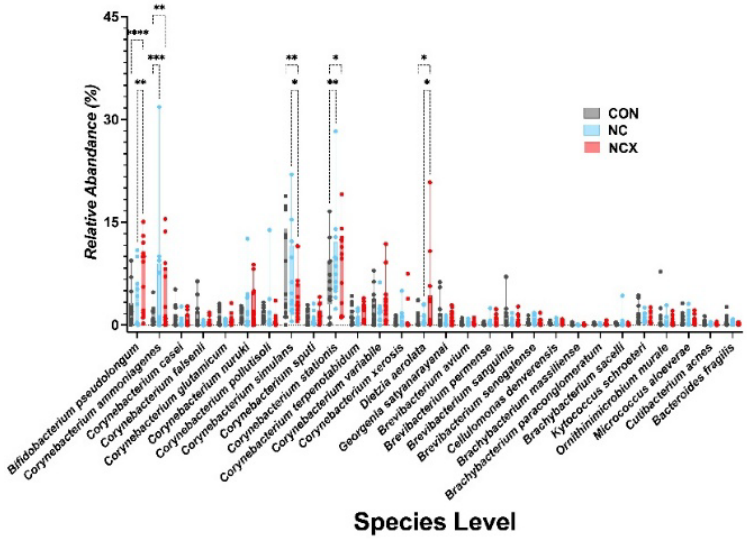

Figure 2 - Relative abundance *(%)* of cecal microbiota at species levels in broilers at day 35. * *(*P < 0.05*)*, ** *(*P < 0.01*)*, *** *(*P < 0.001*)*, **** *(*P < 0.0001*)*

Tabel 3 - Effect of xylanase supplementation on ileal nutrient digestibility in broiler chickens.

Items	Dietary treatment[1]			Pooled SE	*P*-value
	CON	NC	NCX		
Dry matter (%)	91.27	91.38	94.41	0.72	0.130
Protein (%)	79.82	79.60	80.96	0.33	0.200
Fat (%)	93.85	94.87	94.51	0.34	0.497
Fiber (%)	28.22[b]	30.50[ab]	39.02[a]	1.901	0.044
NDF (%)	24.40[b]	21.25[b]	34.51[a]	2.08	0.017
ADF (%)	17.71[b]	20.71[b]	31.57[a]	1.68	<0.001
AME (kcal/kg)	3,003[a]	2,928[b]	2,995[a]	12.26	0.015

[1] CON = basal diet; NC = reduced energy diet (-85 kcal/kg); NCX = NC + 100 g/ton xylanase
[ab] Means within the same column with different superscripts significantly difference (P<0.05)
NDF = Neutral Detergent Fiber, ADF = Acid Detergent Fiber, AME = Apparent Metabolizable Energy
AME = $GE_{feed} – [GE_{excreta} × (Ti_{feed} / Ti_{excreta})]$ (Khalil et al., 2020)
Nutrient Digestibility (%) = 100 – [100 × (Ti-feed × nutrient ileal) / (Ti-ileal × nutrient feed)] (Short et al., 1996)

Broilers fed the NCX diet had significantly lower serum FITC-d levels at day 35 when compared to the CON and NC groups (P < 0.01, Figure 1), suggesting improved gut integrity and reduced intestinal permeability. For relative abundance (%) of cecal microbiota at species

levels in broilers at day 35, birds fed NCX diet had significantly higher abundances of *Bifidobacterium pseudolongum* and *Dietzia aerofata* and lower abandance of *Corynebacterium simulans* abundance when compared to those of CON and NC groups ($P < 0.05$, Figure 2).

Broilers fed NCX diet had significantly higher ($P < 0.05$) digestibility values for crude fiber, neutral detergent fiber (NDF), acid detergent fiber (ADF), and apparent metabolizable energy (AME) than those of CON and NC groups.

IV. DISCUSSION

Feeding bacterial xylanase improved feed efficiency in NC birds during the first 10 days of age, likely due to enhanced fiber digestibility and AME (Nusairat and Wang, 2020; Gorenz et al., 2022). Xylanase supplementation also significantly reduced serum FITC-d levels at day 35 ($P < 0.01$), indicating improved intestinal barrier integrity, probably resulting from xylanase-mediated degradation of NSPs, which reduces intestinal viscosity, supports better mucosal function, and improves nutrient absorption (Nguyen et al., 2021). Birds in the NCX group exhibited higher abundance of *Bifidobacterium pseudolongum*, *Corynebacterium ammonianenes*, *Corynebacterium stationis*, and *Dietzia aerofata*, while *Corynebacterium simulans* abundance was reduced. Consistent with Pourabedin et al., (2015), xylanase hydrolyzes complex arabinoxylan fibers, releasing smaller, fermentable XOS, which serve as a preferred substrate for *Bifidobacterium* species. These bacteria produce SCFAs, primarily acetate and lactate, which lower gut lumen pH and inhibit acid-sensitive pathogens, supporting gut health. In summary, dietary supplementation of xylanase in reduced-energy broiler diets improved early feed efficiency, enhanced fiber digestibility, preserved intestinal integrity, and promoted a beneficial cecal microbiome. These effects highlight bacterial xylanase as a functional feed additive that mitigates the negative impacts of low-energy diets while supporting overall gut health and performance in broiler chickens.

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EVALUATION OF COPPER SOURCE AND DOSE ON PHYTASE EFFICACY IN YOUNG BROILERS

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Summary

This study assessed the effects of Cu source and dose on growth performance and bone mineralization in broilers, dosed with graded levels of phytase. The trial utilized 2,016 4-day old male Ross 308 broilers which were allocated to 18 dietary treatments (trt) in a factorial arrangement, incorporating three Cu trt, copper sulfate (CuS) at 10 and 200 ppm, and 200 ppm from monovalent copper oxide (Cu_2O). Diets contained a novel phytase product at the same six dose levels per Cu trt (0, 500, 1,000, 1800, 2,500 and 3,500 FTU/kg). The BW was determined for the trial period (4-10 days). On day 10, birds were sampled, tibias were collected and pooled per pen to determine tibia bone ash (%). Data were analyzed in JMP using the fit model platform to evaluate interactions, subsequently polynomial regression was conducted. All Cu trt resulted in a diminishing curvilinear increase in BW with increasing phytase dose at day 10. All Cu trt resulted in a diminishing curvilinear increase in tibia ash weight (%) with increasing phytase dose at day10. For tibia mineralization, a significant Cu source x phytase interaction was observed ($P < 0.05$). For tibia mineralization, a significant nonlinear regression analysis indicated that tibia ash percentage plateaued at 2000 FTU/kg phytase for CuS and 1,250 FTU/kg for Cu_2O, suggesting that Cu_2O is less susceptible to phytic acid chelation. Results from this *in vivo* study suggest that the Cu source and dose influence bone mineralization, in doing so influencing phytase efficacy in young broilers.

I. INTRODUCTION

Phytate (inositol hexaphosphate) is a major component of plant-based feeds, accounting for 60–80% of total phosphorus in plants (Pires et al., 2023). Beyond being a phosphorus storage molecule, phytate exerts anti-nutritional effects by chelating divalent minerals such as Zn^{2+}, Ca^{2+}, and Cu^{2+}, thereby rendering them largely insoluble and poorly available for absorption (Osunbami & Adeola, 2025).

In monogastric animals, high dietary levels of certain trace minerals, particularly copper (Cu) and zinc (Zn), can further impair phosphorus digestibility through several mechanisms: (1) direct inhibition of phytase activity, (2) formation of insoluble mineral–phytate complexes resistant to enzymatic hydrolysis. Copper is required in very low amounts in broilers, with recommended levels ranging from 3 to 10 ppm (NRC, 1994). However, in many regions outside Europe, copper is supplemented at pharmacological levels (80–200 ppm) for growth promotion. Such elevated supplementation can exacerbate the formation of insoluble complexes between copper and phytic acid, reducing phosphorus bioavailability. The use of exogenous phytase has been consistent proven to enhance phytate degradation, releasing phosphorus and other minerals (Adeola & Cowieson, 2011). Nevertheless, its effectiveness is influenced by pH, dietary factors, and the interaction between both.

Indeed, Banks et al. (2004) reported that supplementation with 250 ppm Cu from divalent $CuSO_4$ markedly reduced phosphorus digestibility in broilers, by 19% when 600 FTU of phytase were included in the diet, and by 29% in the absence of phytase. The impact of Cu on phosphorus released was also reported by Hamdi et al (2018), where divalent Cu sulfate reduced the phytic phosphorus released by phytase, much more than monovalent Cu oxide, with a difference of 10% between sources. With this background, we hypothesize that both phytase dose and copper dose would significantly affect body weight and bone mineralization in young broilers, and that the

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valence of copper source would also influence these parameters. The objective of the present study was to evaluate the effects of two different copper sources (a monovalent and a divalent Cu) and supplementation levels, combined with varying phytase doses, on performance and bone mineralization in phosphorus-deficient diets fed to young broilers.

II. METHOD

a) Animals and housing

A total of 2,016 4-day-old male broiler chicks (Ross 308) were used in this study. The trial lasted for 10 days and was divided into two stages: Stage 1 (day 0–3), for an adaptation phase; Stage 2 (day 4-10), for the test phase. Birds were housed in 126 metabolic cages, with 16 broilers per cage, and 7 replicate cages allocated to each dietary treatment.

b) Experimental design and diets

In Stage 1 (day 0–3), all broilers received a commercial-type pre-starter rearing diet, that met all nutrient requirements of the bird (11.2 MJ/kg, 23% CP, 1.35% dLys, 0.94% tCa, 0.53% oP). From day 4 to 10 (Stage 2), a basal diet with same nutritional values as in Stage 1, except by being free of supplemental copper was prepared (based on maize, 52%; soybean oilcake 35%, sunflower oilcake 1% and gluten 4%). The products were then added on this diet.

Eighteen dietary treatments were tested (Table 1), arranged in a 3 × 6 factorial design. They consisted in: a control with the incorporation of 10 ppm of divalent Cu sulfate (CuS); the incorporation of 200 ppm of divalent CuS; or the incorporation of 200 ppm of monovalent copper oxide (Cu_2O). Diets contained a novel phytase product at the same six dose levels per Cu treatment (0, 500, 1,000, 1800, 2,500 and 3,500 FTU/kg). During Stage 2, the treatment diets were applied, and all feed and water were provided *ad libitum*.

Table 1 - Factorial arrangement (3 × 6) of the 18 dietary treatments tested in Ross 308 broilers.

Treatment	Copper source	Copper dose, ppm	Phytase dose, FTU/kg diet	Analyzed Cu[1]	Phytase activity[2]
T1	$CuSO_4$	10	0	10.8	54
T2	$CuSO_4$	10	500		560
T3	$CuSO_4$	10	1000		930
T4	$CuSO_4$	10	1800		1811
T5	$CuSO_4$	10	2500		2028
T6	$CuSO_4$	10	3500		3557
T7	$CuSO_4$	200	0	224	7
T8	$CuSO_4$	200	500		424
T9	$CuSO_4$	200	1000		1029
T10	$CuSO_4$	200	1800		1973
T11	$CuSO_4$	200	2500		2911
T12	$CuSO_4$	200	3500		3567
T13	Cu_2O	200	0	210	0
T14	Cu_2O	200	500		380
T15	Cu_2O	200	1000		731
T16	Cu_2O	200	1800		1899
T17	Cu_2O	200	2500		2334
T18	Cu_2O	200	3500		3252

[1]Analysed using inductively coupled plasma atomic emission spectroscopy (ICP) based on AOAC method 935.13.
[2]Defined as the amount of enzyme that catalyzes the release of 1 µmol of iP per minute from 5.1 mM sodium phytate in pH 5.5 buffer at 37°C based on AOAC method 3002.

c) Body weight measurements and sample collection

At the end of day 3 every bird was weighed individually. On day 10, all pens were weighed to determine pen weight. Feed intake per pen and mortality were monitored throughout the study but are not reported here, as the 10-day trial duration was insufficient for a reliable evaluation of performance parameters.

Birds were euthanized at d10 via cervical dislocation. Both the right and left tibias were excised from each bird, kept separate, and pooled per cage to form the experimental unit. A total of 3,024 tibia samples were collected (12 birds × 2 tibias per metabolic cage). Tibias were immediately frozen, later defleshed, and ashed in a muffle furnace following AOAC method 920.39. Bone ash percentage on a dry matter (DM) basis were determined.

d) Statistical analysis

Data were analyzed using JMP (SAS Institute, Cary, NC). The fit model platform was employed to evaluate linear and quadratic responses to analyzed phytase dose and its interactions with copper (Cu). Nonlinear regression analysis was further conducted for each Cu treatment according to the following equation: $y=a(1-b\cdot Exp(-c\cdot x))$, where y = dependent variable, a = asymptote value, b = scale parameter, c = growth rate, and x = independent variable (analyzed phytase value).

III. RESULTS

All Cu treatments resulted in a diminishing curvilinear increase in BW with increasing phytase dose at day 10. However, there was no Cu treatment effect and all Cu treatments exhibited similar asymptote, scale and growth rate for BW.

Cu treatments resulted in a diminishing curvilinear increase in tibia ash weight (%) with increasing phytase dose at day10. For tibia mineralization, a significant Cu source x phytase interaction was observed ($P < 0.05$). The supplementation of diets with 200 ppm from Cu_2O was able to achieve an asymptote at a lower phytase dose compared to 200 ppm and 10 ppm of CuS due to its higher ($P<0.05$) growth rate (0.0031 vs. 0.0019 and 0.0025, respectively). For tibia mineralization, a significant nonlinear regression analysis indicated that tibia ash percentage plateaued at 2000 FTU/kg phytase for CuS and 1,250 FTU/kg for Cu_2O, suggesting that Cu_2O is less susceptible to phytic acid chelation (Figure 1).

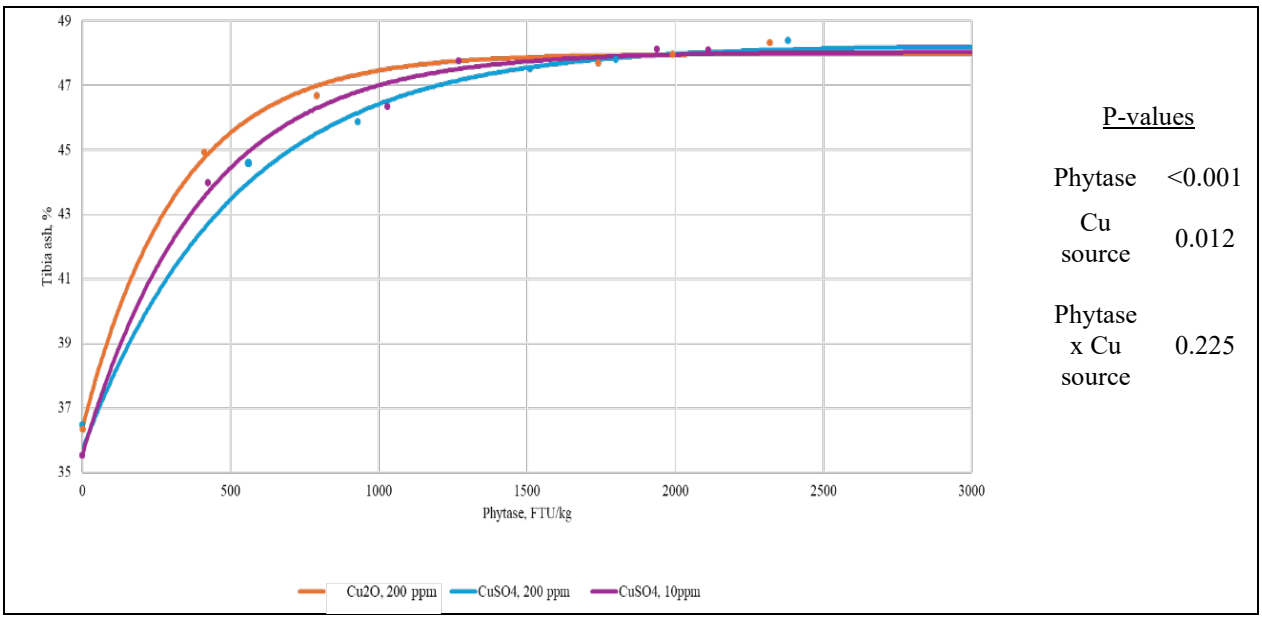

Figure 1 - Effects of copper source and phytase dose on tibia bone ash (%) in Ross 308 broilers.

IV. DISCUSSION

At low doses of phytase, the reduced solubility of Cu_2O likely limited the availability of free soluble Cu, resulting in fewer interactions with phytate, and thereby minimizing the inhibitory effect of copper on phytase efficacy. The same results were reported by Hamdi et al. (2018), in an *in vitro* study testing two sources of Cu (divalent Cu sulfate and monovalent Cu oxide) tested at three Cu levels (15, 150 or 300 mg Cu/Cu). Authors observed that divalent Cu sulfate reduced the phytic phosphorus released by phytase, much more than monovalent Cu oxide, and the impact became even more pronounced when Cu supplementation exceeds nutritional levels (150–300 mg Cu/kg). Besides, literature highlights that monovalent cations are less prone to interact with phytate than divalent and trivalent cations (Crea et al., 2008). Further research is needed to explore if the effect of Cu on phytase is related to the valency status.

At high doses of phytase supplementation (2000 FTU), there was no impact of Cu source on phytase efficacy. Previous findings were observed by Dersjant Li et al. (2022), where the ileal phytate disappearance increased with the increase in phytase dose.

Nutritionists using lower doses of phytase should be cautious of the impact of divalent CuSO4 on phytase efficacy and the reliability of associated matrix values in diet formulation.

In conclusion, at low doses of phytase, monovalent copper oxide can preserve phytase efficacy and reducing the impact of Cu on the matrix values that can be applied to phytase. Future research is planned to quantify the impact of Cu source on phytase matrix values.

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IMPROVING RED SORGHUM UTILISATION IN BROILER CHICKENS USING EXOGENOUS ENZYMES OR A REDUCING AGENT

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Sorghum is a drought-tolerant cereal widely grown in Australia, offering a more sustainable and locally sourced alternative to imported feedstuffs. While white sorghum generally supports better broiler performance, red sorghum often contains higher polyphenols that interact with kafirin, limiting nutrient availability. This study evaluated strategies to improve red sorghum utilisation with exogenous enzymes or a reducing agent, sodium metabisulphite (SMBS). A total of 280 Ross 308 off-sex males were allocated to seven treatments for 35 d (8 replicates of 5 birds). Diets included a white sorghum control and a red sorghum control (both with 100 g/kg wheat, xylanase at 4000 U/kg and phytase at 1,500 FTU/kg). The red sorghum control was further supplemented with amylase (80 KNU/kg), double phytase (3,000 FTU/kg), protease (with or without matrix values; 30,000 NFP/kg) or SMBS (up to 3.28 g/kg). Excreta were collected on d 27-29 for nitrogen-corrected apparent metabolisable energy (AMEn), and distal jejunal digesta were sampled on d 35 for starch digestibility. Over 35 d, both white and red sorghum controls supported growth above Ross 308 objectives (2022). Broilers fed red sorghum with SMBS achieved the greatest weight gain (WG; 2976 g/b; P = 0.014), comparable to the white sorghum control (2870 g/b) but higher than the red sorghum control and amylase groups (2812 and 2811 g/b). Feed intake (FI) tended to differ (P = 0.059), with SMBS promoting higher intake, while feed conversion ratio (FCR) was unaffected across all treatments. AMEn was influenced by treatment (P < 0.001), with the white sorghum control highest (11.12 MJ/kg). The SMBS-supplemented red sorghum diet maintained AMEn comparable to the white sorghum control, whereas amylase and protease additions resulted in lower AMEn. Distal jejunal starch digestibility coefficient tended to improve with SMBS (0.822 vs. 0.799; P = 0.061) compared to the red sorghum control. Collectively, exogenous enzymes did not influence birds' growth nor nutrient utilisation, highlighting the strong binding effect of sorghum kafirin on starch and protein. On the other hand, SMBS was able to directly disrupt disulphide cross-links within kafirin-starch complexes, thereby improving growth performance and enhancing starch digestion in the jejunum of broilers fed red sorghum, maintaining energy utilisation comparable to white sorghum. These findings highlight SMBS as a practical strategy to increase the feeding value of red sorghum and support its broader use in sustainable poultry production.

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Table 1 - Effects of dietary treatments on growth performance, energy and nutrient utilisation in broiler chickens.

	d 0-35			d 27-29	d 35
Item	WG[1] (g/b)	FI[2] (g/b)	FCR[3] (g/g)	AMEn[4] (MJ/kg)	Jejunum starch DC[5]
White sorghum control (PC)	2870[ab]	4004	1.395	11.12[a]	0.827
Red sorghum control (NC)	2812[b]	3974	1.413	10.72[ab]	0.799
NC + double-dosing phytase	2851[ab]	4058	1.424	10.74[ab]	0.782
NC + amylase	2811[b]	3967	1.413	10.40[b]	0.783
NC + sodium metabisulfite	2976[a]	4126	1.387	10.76[ab]	0.822
NC + protease	2841[ab]	3989	1.405	10.54[b]	0.803
NC + protease with matrix	2909[ab]	4092	1.408	10.42[b]	0.786
SEM[6]	14.2	17.8	0.0052	0.030	0.0374
P-value	0.014	0.059	0.224	<0.001	0.061

[ab]Means (n = 8) within columns not sharing a common superscript are significantly different (P < 0.05).[1]WG = weight gain; [2]FI = feed intake; [3]FCR = feed conversion ratio; [4]AMEn = nitrogen-corrected apparent metabolizable energy; [5]DC = digestibility coefficient; [6]SEM = standard error of the mean.

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KINETICS AND LOCALIZATION OF SPORE GERMINATION AND OUTGROWTH OF A *BACILLUS*-BASED PROBIOTIC IN AN IN-VITRO MODEL OF THE POULTRY GASTROINTESTINAL TRACT

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Summary

This study explores the critical factors governing the efficacy of spore-forming *Bacillus*- based probiotics in poultry, focusing on the transition from dormant spores to metabolically active vegetative cells within the gastrointestinal tract (GIT). Using the Dynamic Avian Intestine in-vitro System (DAISy), the research identifies the duodenum as the pivotal site for spore germination and outgrowth—a process triggered by pH level, nutrient availability, among other factors. The experimental design simulated the digestive tract of a 21-day-old broiler, incorporating physiologically relevant pH, enzyme concentrations, and retention times across the crop, proventriculus, gizzard, duodenum, and ileum.

Quantitative analysis of spore and vegetative cell counts, alongside measurements of amino acids and sugars, revealed that spore activation and germination commence in the duodenum, with vegetative cell proliferation becoming prominent at the onset of the ileum. The findings demonstrate that rapid outgrowth is essential for maximizing the number of active probiotic cells during the limited retention time in the gut, thereby supporting improved animal health and productivity under diverse dietary conditions. The study proposes that outgrowth kinetics should be a primary selection criterion for spore-forming probiotics in poultry production.

I. INTRODUCTION

Bacillus-based probiotics are increasingly used and recognized in poultry for their capacity to enhance gut health, bolster immunity, and improve overall animal performance. *Bacillus* species are distinguished among probiotics by their ability to exist as both vegetative cells and as robust spores (Tan & Ramamurthi, 2014). The spore form confers stability during feed processing, ensures a prolonged shelf life, and enables survival through the harsh conditions of the gizzard (Bernardeau *et al.,* 2017). In contrast, vegetative cells, once activated from spores, are metabolically active and execute key probiotic functions such as pathogen inhibition (Vieco-Saiz *et al.*, 2024). Therefore, reliable and rapid transition from spore to vegetative cell in the animals intestine—encompassing activation, germination, and outgrowth—might be critical for the efficacy of *Bacillus*-based probiotic products (Koopmann *et al*., 2022). Nevertheless, significant variability observed in published findings—often attributed to differences in study methodology and the probiotic strains used—impedes the capacity to formulate overarching conclusions about their ability to reliably transition into active cells (Bernardeau *et al.*, 2017). This study aimed to investigate the outgrowth process of a spore-forming probiotic in greater detail and to identify the site in the gastrointestinal tract (GIT) and time required for spore-to-vegetative cell transition.

II. MATERIALS AND METHODS

To investigate the germination and outgrowth of spores to vegetative cells within the chicken GIT, the DAISy model was used in combination with a standard European grower diet

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comprised primarily of corn, soybean meal and soy oil (Hüser and Nerenz, 2024). The spore-forming probiotic *Bacillus* CECT 5940 (Evonik Operations GmbH, Hanau, Germany) was incorporated into the GIT in-vitro model at a concentration of 2 E+7 spores, together with the feed to obtain analytically reliable data. The simulation mimicked the digestive tract of a 21-day-old broiler, including the crop, proventriculus, gizzard, duodenum, and ileum, and featured dynamic retention times representative of physiological conditions. Each segment was individually adjusted for pH to accurately reflect the in-vivo environment, and all relevant enzymes in appropriate amounts were added—such as Pepsin- HCl, and Pancreatin combined with bile salts and carbonate to replicate key digestive processes. Intestinal reflux was not considered to make calculation more consistent. Samples were collected from separate DAISy compartments representing distinct sections of the intestine during the experiment. Duodenum samples were obtained at 30-minute intervals.

Each sample was analyzed for spore counts and total colony-forming units using four technical replicates to assess spore germination and microbial growth in the simulated GIT. Additionally, amino acid concentrations released from the feed were separated using ion-exchange chromatography and quantitatively analyzed photometrically following ninhydrin derivatization. Mono- and oligosaccharides were assessed via ligand-exchange chromatography coupled with ELSD detection. These analyses were performed for each small intestinal segment and served as indicators of efficient feed digestion by intestinal enzymes, thereby confirming the effective functioning of the in-vitro model.

III. RESULTS

The essential initial step was to determine where and how spores are activated to begin the germination process. The general mechanisms have been thoroughly studied and reviewed by Koopman *et al*. (2022). For most spores, amino acids and sugars in the presence of water serve as primary activators. Within the GIT, concentrations of amino acids and sugars were expected to be elevated in the duodenum due to enzymatic breakdown of feed components. In the crop condition, no free sugar was detected and only the substituted amino acids from the diet methionine (Met), lysine (Lys), and threonine (Thr) were present. The duodenum samples contained supplemental free amino acids and amino acids from enzymatic breakdown of feed with a total concentration of 4.5 g/L which was equivalent to total analysed dietary amino acid level, while the combined glucose and maltose concentration was 12 g/L.

By collecting in-vitro digesta samples from various compartments of the DAISy model spanning from the duodenum to the ileum the hypothesis on spore germination and outgrowth was validated. A distinct kinetic pattern emerged, demonstrating both a decrease in spore numbers and an increase in vegetative cells. Spores were activated and underwent germination, with each spore producing one vegetative cell as shown by the constant CFU numbers (incl. spore + vegetative cells) in the compartments duodenum and jejunum in Figure 1. Upon reaching the onset of the ileum, vegetative cells began to proliferate, resulting in detection of more vegetative cells than the number spores initially introduced.

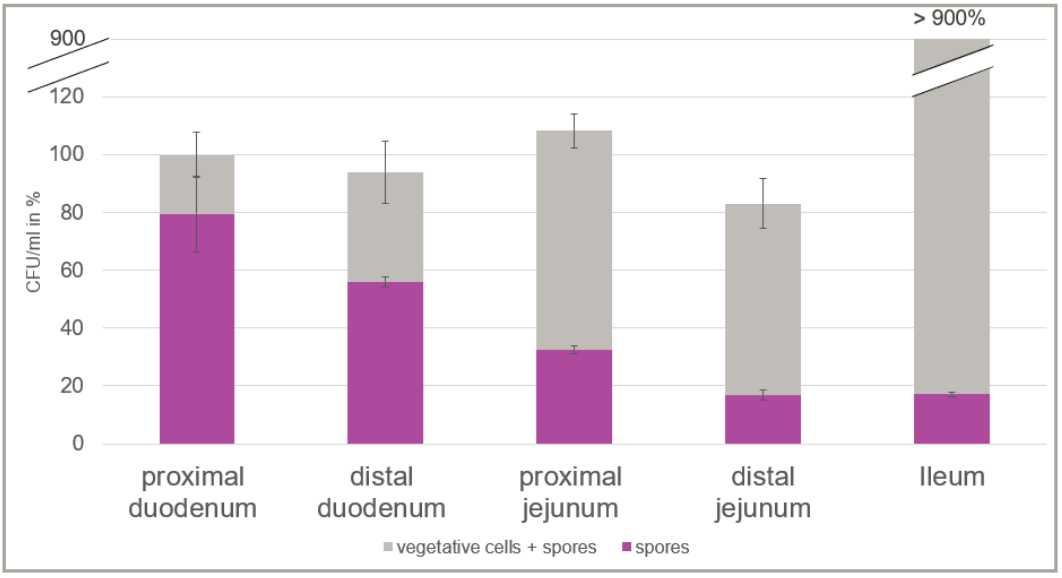

Figure 1 - Total aerobic microbes (including spores and vegetative cells) did not show any significant differences from proximal duodenum to distal jejunum comparing each segment with each other ($P > 0.32$). In contrast, total aerobic microbe counts in the ileum were significantly higher compared to all other segments ($P < 0.0001$). Spore numbers significantly decreased with each segment from proximal duodenum to distal jejunum ($P < 0.048$), but there was no significant difference in spore counts between distal jejunum and ileum samples ($P = 0.99$).

IV. DISCUSSION

The use of *Bacillus* spore products in the poultry industry is predicated on their ability to form stable spores, which confer resilience during feed processing and storage, and enable survival through the harsh conditions of the upper GIT. Upon reaching the small intestine, specifically the duodenum, these spores encounter a favorable environment characterized by a pH upshift and the presence of germinants such as amino acids and sugars released from feed by pancreatic enzymes. This triggers the activation, germination, and subsequent outgrowth of spores into metabolically active vegetative cells, as supported by both previous literature and the data presented in this study (Bernadeau *et al*., 2017).

Our results confirm that duodenum and jejunum are the critical sites for spore germination of *Bacillus* CECT 5940, with the process occurring within approximately 60 minutes under simulated physiological conditions. The subsequent proliferation of vegetative cells in the ileum underscores the importance of rapid outgrowth, as the retention time in the poultry gut is limited. This temporal constraint necessitates that probiotic strains selected for poultry applications exhibit not only robust spore stability but also efficient and consistent outgrowth kinetics.

Furthermore, the study highlights the need for a paradigm shift in probiotic selection criteria. Traditionally, emphasis has been placed on spore numbers, viability and stability, and species; however, our data indicate that the ability to rapidly transition to an active vegetative state within the gut is equally important for achieving the desired health outcomes. This is particularly relevant in poultry species, where feed transit times are short and the window for probiotic action is narrow.

In conclusion, the DAISy in-vitro model provides a robust platform for evaluating the kinetics and localisation of spore germination and outgrowth. The identification of the duodenum and jejunum as the primary site for activation, coupled with the demonstration of rapid outgrowth, supports the adoption of outgrowth speed as a critical selection criterion for *Bacillus*-based probiotics in poultry.

Further studies could investigate the molecular mechanisms involved in germination triggers, as well as the interaction with the feed and nutrient composition and spore properties to improve probiotic effectiveness.

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BUTYRIC AND VALERIC GLYCERIDES BLEND AND ESSENTIAL OILS IMPROVED BROILER PERFORMANCE AND INTESTINAL MORPHOLOGY

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Summary

Owing to their antimicrobial properties and growth-promoting effects, organic acids (OAs) and essential oils (EOs) are reported to be promising alternatives to antibiotic growth promotors (AGPs). However, investigation into simultaneous supplementation or direct comparison of different OAs and EOs is still scarce. Two experiments were conducted to determine the effectiveness of a butyric and valeric glyceride blend (BVg), tributyrin (TB), and a blend of essential oil compounds with benzoic acid (EOB) as AGP replacements in broilers. It was hypothesized that BVg and EOB would improve growth performance by modulating intestinal morphology. In experiment 1, a total of 792 male broiler chickens (Ross 308) were allocated to three dietary treatments: 1) control group; 2) control group + TB; 3) control group + BVg. Results showed that final body weight (BW) was significantly improved in the BVg group by 7.1% compared with the control group. In addition, the supplementation of BVg significantly improved FCR by 8 and 6 points when compared with the control group and the TB group, respectively. In experiment 2, 900 male Cobb 500 broiler chickens were allocated to five dietary treatments: 1) control group; 2) control group + enramycin (ENR); 3) control group + bacitracin methylene disalicylate (BMD); 4) control group + BVg; 5) control group + EOB. The supplemented groups all significantly improved the final BW compared with the control group. The BVg supplementation significantly improved FCR by 20 points over the control and showed numerical improvements of 3.6, 2.7, and 0.6 points over groups of enramycin, BMD, and EOB, respectively. Irrespective of the additives used, a significantly positive impact on villus height (VH) of jejunum could be observed. Villus height/crypt depth (CD) ratio was significantly improved in broilers fed with either BVg or EOB. In summary, the use of OAs and EOs, especially the supplementation of BVg and EOB, allowed significant growth improvement in broiler and delivered intestinal morphology benefits.

I. INTRODUCTION

It is essential to find novel alternatives following the total ban or restricted use of AGPs to support broiler gut health. Many feed additives have been explored for this purpose, including probiotics, prebiotics, OAs, EOs, enzymes, and among which both OAs and EOs are often favored because of their strong antimicrobial traits. Organic acids are well established as antibacterial and antifungal agents. Specifically, butyric acid has been shown to enhance gut development, control enteric pathogens and reduce inflammation (Bedford and Gong, 2018). In contrast, valeric acid is a relatively new OA and has been studied far less. Nevertheless, a recent study demonstrated that the supplementation of glycerol esters of valeric acid enhanced broiler BW and intestinal morphology while reducing the incidence of necrotic enteritis (Onrust et al., 2018). As volatile compounds extracted from plants, EOs are also of particular interest especially considering their variety of beneficial properties, such as flavoring, stimulation of enzyme secretion, and antioxidant or antimicrobial activities (Weber et al., 2012; Sumon et al., 2025). To our knowledge, the comparison of different combinations of OAs including BVg, TB, and EOs, has not yet been investigated. Given these circumstances, this study aims to evaluate

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the potential effect of BVg on growth performance and gut morphology, as well as compare its effectiveness against TB and EOB as potential AGP replacement.

II. METHOD

In experiment 1, a total of 792 male broiler chickens (Ross 308) were allocated to three dietary treatments with 12 replicates each. Treatments include: 1) control group; 2) control group + TB at 500, 500, and 250 g/ton in the starter, grower and finisher phases; 3) control group + BVg (Gastrivix™ Avi, Perstorp Waspik BV, the Netherlands) at 500, 500, and 250 g/ton in the starter, grower and finisher phases. The corn-wheat-soybean meal based diets were formulated to meet the nutrient requirements recommended by NRC (1994) for broilers. The diets were provided in three phases: starter (d 0-14), grower (d 15-28), and finisher (d 29-42). Feed intake, BW, and mortality were recorded, and average daily gain (ADG), FCR, European Production Efficiency Factor (EPEF) were calculated accordingly.

In experiment 2, a total of 900 male broiler chickens (Cobb 500) were allocated to five dietary treatments with 12 replicates each. Treatments include: 1) control group; 2) control group + ENR (10 ppm); 3) control group + BMD (55ppm); 4) control group + BVg at 500, 500, and 250 g/ton in the starter, grower and finisher phases; 5) control group + a blend of essential oils (thymol, eugenol, piperine) with benzoic acid (EOB) at 300g/ton throughout all feeding phases. The experimental diets were corn and soybean meal based and formulated according to the nutritional levels recommended by the Cobb 500 supplement manual (2018). Diets were delivered in three phases: starter (d 1-21), grower (d 22-35) and finisher (d 36-42). At d 21, one bird per replicate was euthanized for collection of histology samples, which were evaluated for VH, CD, as well as the VH/CD ratio in the middle portion of the jejunum. The data were analyzed by one-way ANOVA, with the pen as experimental unit. Tukey's test was used at the level of 5% probability to compare the means of the experimental treatments.

III. RESULTS

The effects of different OAs on broiler growth performance are presented in Table 1. Final BW during the overall period of the trial was significantly improved in the BVg group by 7.1% compared with the control group ($P < 0.05$). Moreover, the supplementation of BVg lowered FCR by 8 points compared with the control group ($P < 0.05$) and 6 points compared with the TB group ($P < 0.05$).

Table 1 - Effect of BVg[1] and tributyrin on growth performance of broilers (d 0-42).

Treatment	BW, g	ADG, g/d	ADFI, g/d	FCR	Mortality, %	EPEF
Control	2732^{b}	63.8^{b}	103.1	1.62^{b}	5.7	374^{b}
Control + tributyrin	2820^{ab}	65.9^{ab}	105.6	1.60^{b}	6.2	385^{b}
Control + BVg	2927^{a}	68.5^{a}	105.7	1.54^{a}	2.7	433^{a}
SEM[2]	35.2	0.84	1.36	0.016	1.29	9.9
P-value	0.006	0.005	0.561	0.044	0.481	0.003

$^{a-b}$Values within a column with different letters differ significantly ($P < 0.05$).
[1]BVg: butyric and valeric glycerides.
[2]SEM: standard error of means.

In experiment 2, the final BW was significantly improved compared with the control in all treatment groups (Table 2). The BVg group performed significantly better than the control group: BW was improved by 14.3% ($P < 0.05$) while FCR was reduced by 20.4 points ($P < 0.05$). Broilers fed diets added with BVg performed numerically better compared with birds of the other treatment groups. Body weight was improved by 1.7%, 1.4% and 0.7% compared with ENR, BMD and EOB groups respectively ($P > 0.05$). The supplementation of BVg numerically

reduced FCR by 3.6, 2.7 and 0.6 points compared with ENR, BMD and EOB groups respectively ($P > 0.05$). The intestinal morphology analysis showed that, regardless of the treatment, a significantly positive impact on VH could be observed (Table 3). Enramycin, BMD and BVg also showed significantly lower CD compared with the control and EOB groups ($P < 0.05$). Villus height/crypt depth ratio was significantly improved regardless of the OA or EO used ($P < 0.05$).

Table 2 - Effect of AGPs, BVg[1] and EOB[2] on growth performance of broilers (d 0-42).

Treatment	FI, kg	BW, kg	FCR
Control	4.913	2.835[b]	1.737[b]
Control + ENR[3]	4.997	3.186[a]	1.569[a]
Control + BMD[4]	4.973	3.193[a]	1.560[a]
Control + BVg	4.965	3.239[a]	1.533[a]
Control + EOB	4.946	3.216[a]	1.539[a]
SEM[5]	0.034	0.036	0.017
P-value	0.649	<0.001	<0.001

[a-b]values within a column with different letters differ significantly ($P < 0.05$).
[1]BVg: butyric and valeric glycerides.
[2]EOB: a blend of essential oils (thymol, eugenol, piperine) with benzoic acid
[3]ENR: enramycin
[4]BMD: bacitracin methylene disalicylate
[5]SEM: standard error of means.

Table 3 - Effect of AGPs, BVg[1] and EOB[2] on gut morphology (jejunum) of broilers.

Treatment	Villus height (μm)	Crypt depth (μm)	VH/CD ratio
Control	455.25[b]	135.25[a]	3.37[c]
Control + ENR[3]	566.25[a]	105.22[ab]	5.33[b]
Control + BMD[4]	677.25[a]	94.41[b]	7.17[a]
Control +BVg	647.32[a]	96.35[b]	6.72[b]
Control + EOB	648.12[a]	128.84[a]	5.31[b]
SEM[5]	11.21	2.24	0.061
P-value	0.049	0.050	0.048

[a-c]values within a column with different letters differ significantly ($P < 0.05$).
[1]BVg: butyric and valeric glycerides.
[2]EOB: a blend of essential oils (thymol, eugenol, piperine) with benzoic acid
[3]ENR: enramycin
[4]BMD: bacitracin methylene disalicylate
[5]SEM: standard error of means.

IV. DISCUSSION

In addition to being the primary energy source for colonocytes, butyrate is also known to provide other health benefits such as reducing inflammation and preventing enteric dysbiosis (Bedford and Gong, 2018). In contrast, research related to valerate is less abundant, but recent research suggested valerate and butyrate can contribute to disease resistance through the induction of host defense peptides gene expression (Sunkara et al., 2012). Therefore, testing the combined effect of butyrate and valerate as an alternative to AGPs is worthwhile. As demonstrated in experiment 1, BVg supplementation increased final BW by 7.1% over the control group and 3.8% over the TB group. Indeed, while both esterified butyric and valeric acids have shown benefits individually, their combination may yield synergistic effects. Butyrate supports gut barrier integrity, while valerate may further enhance pathogen resistance and intestinal morphology (Skrzypecki et al., 2020; Hodgkinson et al., 2023). This was also supported by the finding that only the BVg supplementation significantly improved FCR compared with the

control group, while no improvement was observed with the TB supplementation. The addition of BVg also further increased EPEF compared with the TB group (+12.5%).

The effects of BVg and EOs on broiler growth performance and gut morphology were studied in experiment 2. The absence of AGPs or other additives resulted in lower BW and poorer FCR. The results of BW and FCR were similar among all treatments where additives were added, with the BVg group showing the heaviest BW and lowest FCR numerically. Research on valeric acid is scarce, making the comparison between low-dose butyrate–valerate esters and EOs a novel approach. A recent meta-analysis (Gracia et al., 2024) reported that broilers fed BVg were 3% heavier, consumed 1.5% more feed, were 1.25% more efficient, and had a 5.7% higher EPEF than controls. These benefits may be attributed to the combined histone deacetylase (HDAC) inhibition potential of butyrate and valerate, since HDAC overexpression is associated with a variety of disease pathologies ranging from colitis to cancer (Yuille et al., 2018). The EOs used in the present study are known to have strong antimicrobial effects and ability to increase intestinal amylase activity in broilers (Weber et al., 2012), which may support nutrient utilization in young birds. Therefore, this may explain why the EOB supplementation maintained similar broiler BW and FCR as the two AGP groups. The morphology analyses showed that all additives significantly improved VH compared with the control. The supplementation of BVg significantly improved VH/CD ratio against the control group, and numerically enhanced it compared with ENR and EOB groups. Previous studies have shown that both butyric acid and valeric acid stimulate the growth of intestinal villi and VH/CD and showed morphological changes in the jejunum are more responsive to dietary factors than those in the ileum. (Xu et al., 2025). The underlying mechanisms are likely multifaceted, including their role as energy sources for intestinal epithelial cells and their capability of reducing bacterial invasion and colonization to intestinal epithelial cells. Thus, improved intestinal structure, shown by a positive impact on villus and crypt development, may ensure efficient nutrient absorption and contribute to the observed benefits in growth performance.

In conclusion, these trials demonstrated that the combined use of butyrate and valerate glycerides significantly improved growth performance compared with TB alone, as evidenced by increased ADG and enhanced FCR. Additionally, BVg and EOB supplementation positively affected broiler performance and enhanced intestinal morphology, indicating their potential as AGP alternatives.

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MYCOTOXINS AND CONCURRENT STRESSORS: A COMBINATION OF CONCERNS IN COMMERCIAL BROILER PRODUCTION AND THE POTENTIAL EFFECTIVENESS OF A DETOXIFIER

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Summary

This study demonstrated that feeding a naturally contaminated diet with multiple mycotoxins, and in particular, with aflatoxin B1 (AFB1, ~22 µg/kg), close to the EU limit (20 µg/kg), significantly reduces the performance of broilers. Notably, these adverse effects were more profound (~10% BW loss) when broilers were raised under a production farm setup, encountering additional stressors. Our study showed that the environment can affect the impact of mycotoxins in broilers. Namely, the cumulative pressure under production conditions is what dictates the severity of mycotoxins related to performance losses. Dietary supplementation with a mycotoxin detoxifier consisting of binder components, preservatives, yeast cell wall, and a blend of plant extracts effectively reduced the detrimental effects of mycotoxins on performance (Return On Investment (ROI = 1:3.5), supported by improvement of blood biochemistry markers and related immune responses.

I. INTRODUCTION

The contamination of feed with mycotoxins poses various challenges to broiler chickens, negatively affecting their health and productivity. Additionally, commercial broilers face many other stressors in intensive production systems, such as endotoxins, pathogens, and pro-inflammatory substances, which may interact with mycotoxin exposure and worsen their harmful effects. To the best of our knowledge, there is limited research on the impact of environmental stressors on the toxicity of mycotoxins. Therefore, this study used novel experimental setups with two different environmental conditions to examine (1) the effects of naturally contaminated feed with AFB1 on the growth performance, antibody titre, and blood biochemistry of broilers, (2) whether a common environmental stressor amplifies the effects of aflatoxins on these parameters, and (3) how a detoxifying technology can reduce these impacts.

II. MATERIALS AND METHODS

This experiment received prior approval from the local ethics committee for animal experiments at Poulpharm Belgium (EC: P24329). A total of 2,160 one-day-old male Ross 308 birds were randomly allocated to five treatments, and each treatment consisted of twelve replicate pens with 36 broilers per pen (60 pens). The birds received a control (clean) or naturally contaminated (AFB1) corn-based diet. The pens were divided over two separate houses: 1) 24 pens in a controlled Research Facility, with T1RF (clean feed) and T2RF (contaminated feed), and 2) 36 pens placed in a Commercial Broiler Facility between the other 55000 broilers with T1CF (clean feed), T2CF (contaminated feed), and T3CF (contaminated feed + Detoxifier (Escent® Innovad), to replicate the cumulative environmental stressors present in industrial production. The trials in both houses started simultaneously, maintaining the same stock density of broilers in the pens and ensuring a consistent environmental temperature, lighting, and management program across both conditions, as well as feeding identical diets. The diets for the two sites were produced in a single batch from both clean and naturally contaminated corn, then divided and distributed to

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the research farm (RF) and the commercial farm (CF). On days 21, 30, and 35, BW and FI were recorded per pen, and FCR was calculated. Blood samples were collected aseptically from the wing vein of two birds per pen on days 21, 30, and 35 and the antibody titers against Newcastle Disease Virus (NCDV) were determined. At the end of days 21 and 35, blood samples were also collected for plasma chemistry analysis. Plasma levels of total uric acid (UA), creatinine (CREA), blood urea nitrogen (BUN), urea (UREA), and aspartate aminotransferase (AST) activity were measured. Return on investment (ROI) was calculated by considering the feed cost, FI, FCR, final BW, and the cost of the Detoxifier (Escent®) over 35 days. The European Production Efficiency Factor (EPEF) over the 35-day phase was also determined using the formula: EPEF = [(liveability (%) × body weight) / (FCR × age)] × 100.

Statistical analysis - A 2 × 2 factorial design for treatments T1RF, T2RF, T1CF, and T2CF was used to assess the impacts of mycotoxin, environmental stress, and their interactions. The data were analysed using a two-way ANOVA, with mycotoxin and environmental stress (housing system) as fixed factors. When interaction effects were significant, the Tukey multiple comparisons test was used to separate the means. The impact of the detoxifier was analysed using a one-way ANOVA to compare T1CF, T2CF, and T3CF in the commercial setup.

I.RESULTS

The performance results of broiler chickens at different growth periods are summarized in Table 1. AFB1 exposure negatively impacted the BW, FI, and FCR of the broilers on days 21, 30, and 35 (Overall $P < 0.05$). Considering the levels of mycotoxins in the contaminated diets, this result indicates that AFB1, at levels around the legal limit (approximately 22 µg/kg on day 21), negatively impacted the performance of the broilers. This adverse effect became more noticeable as AFB1 levels increased towards the end of the trial (around 45 µg/kg on day 35), resulting in a 12% reduction in final body weight. Including the detoxifier technology in contaminated feed (T3CF) improved BW, FCR, and FI to levels close to those of clean feed; therefore, no statistical differences were observed between the clean feed (T1CF) and contaminated feed with Detoxifier (T3CF) for all growth parameters in the commercial setup. On days 21, 30, and 35, BW and FI of broilers in the commercial setup were lower than those in the research facility ($P < 0.01$). The FCR was also negatively affected by environmental stressors in all dietary phases ($P < 0.01$). Concerning the effect of mycotoxins in the presence of stressors, a significant interaction was found between the mycotoxins and environmental stress on days 30 and 35, indicating that the adverse effects of mycotoxins (AFB1) became more pronounced and noticeable under production stress. In the present study, economic profits increased with the addition of detoxifier technology to the diet. It reduced FCR, improved the BW of broiler chickens, leading to lower feed costs per kg of weight gain, and increased ROI (1:3.5) compared to the contaminated feed without a detoxifier. The EPEF in the contaminated treatment with detoxifier (T3CF) (377) was higher compared to the contaminated treatment without detoxifier T2CF (349) in the commercial setup. The detection of IBV antibody showed a clear difference between the two environments, with lower antibody levels in the research facility compared to the commercial setting (Figure 1). In the commercial setting, birds showed a stronger response to the vaccines, with a higher number of positive birds, confirming higher infectious pressure in that environment. Interestingly, towards the end of the trial (D30 and D35), only in the commercial setting, the birds fed contaminated feed (T2CF) showed higher ($P < 0.05$) antibody titers against IBV vaccination compared to those on the clean feed diet (T1CF). Interestingly, birds fed the detoxifier (T3CF) showed the same antibody titer levels against IBV as those on the clean feed diet (T1CF), indicating the positive effect of the detoxifier on humoral immune responses. On day 35, broilers fed the contaminated diets showed a significant increase in plasma uric acid (UA) levels (mg/dL) ($P = 0.001$), as shown in Table 2. However, no

difference was observed between clean feed (T1CF) and contaminated feed + Detoxifier (T3CF) ($P < 0.05$), indicating that the addition of the detoxifier to the contaminated feed prevented this elevation. The plasma concentrations of BUN, UREA, and CREA (mg/dL) were not significantly altered by any of the treatments (data not shown).

Table 1 - Effect of treatments on BW, FCR, and FI of broilers.

		Research farm (RF)		Commercial farm (CF)			Two-way ANOVA		
	Day	Clean Corn	Contam-inated Corn	Clean Corn	Contami nated Corn	Contam-inated Corn+ Escent	Myco-toxin	Env. Stress	Myco-toxin ×Env. Stress
		T1RF	T2RF	T1CF	T2CF	T3CF	P	P	P
BW	21	1.087	1.065	1.072 a	1.039 b	1.063 ab	0.002	0.017	0.527
	30	1.865	1.835	1.813 a	1.625 b	1.748 ab	0.001	< 0.001	0.01
	35	2.493	2.449	2.317 a	2.030 b	2.168 ab	0.001	< 0.001	< 0.001
FCR	21	1.227	1.242	1.253 a	1.282 b	1.262 ab	0.008	0.042	0.412
	30	1.333	1.330	1.503 a	1.582 b	1.561 b	< 0.001	< 0.001	< 0.001
	35	1.453	1.454	1.503 a	1.582 b	1.561 ab	0.001	0.001	< 0.001
FI	21	1.350	1.317	1.290	1.280	1.288	0.031	< 0.001	0.425
	30	2.717	2.677	1.771 a	1.583 b	1.683 ab	0.001	< 0.001	0.017
	35	3.622	3.561	3.414 a	3.150 b	3.279 ab	< 0.001	< 0.001	0.002

Mean values with SD are shown (n = 12 replicates per treatment. Each replicate (pen) consisted of 36 birds).
Two way ANOVA; Comparing T1RF, T2RF, T1CF, T2CF
Means followed by different superscript letters (a,b) within the same line are different by one-way ANOVA and post hoc Tukey's test ($P < 0.05$ only comparing T1CF, T2CF, T3CF; P = P value

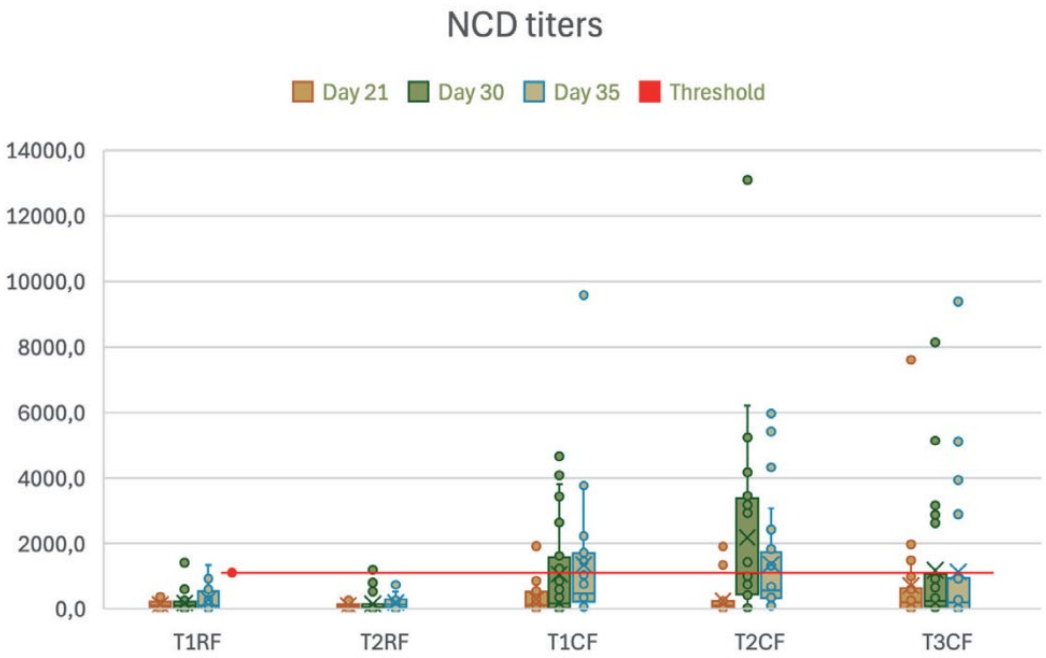

Figure 1 - Newcastle disease (NCD) antibody titers over study days and treatment groups. The red line indicates the threshold above which birds are considered positive. T1RF: (Clean feed in research farm), T2RF: (Contaminated feed in research farm), T1CF: (Clean feed in commercial farm), T2CF: (Contaminated feed in commercial farm), T3CF: (Contaminated feed + Escent (1 kg per ton) in commercial farm).

Table 2 - Effect of treatments on blood uric acid.

		Research farm (RF)		Commercial farm (CF)			Two-way ANOVA		
	Day	Clean Corn	Contam-inated Corn	Clean Corn	Contam-inated Corn	Contam-inated Corn+ Escent	Myco-toxin	Env.	Myco-toxin × Env
		T1RF	T2RF	T1CF	T2CF	T3CF	P value	P value	P value
UA (mg/ dL)	21	4,67	4,46	5,44	6,96	6,24	0.243	0.001	0.095
	35	5,42	6,47	4,08ab	4,76 b	4,06 a	0,001	<0,01	0,162

Mean values with SD are shown (n = 12 replicates per treatment. Each replicate (pen) consisted of 36 birds).
Two way ANOVA; Comparing T1RF, T2RF vs. T1CF, T2CF
Means followed by different superscript letters (a,b) within the same line are different by one-way ANOVA and post hoc Tukey's test ($P < 0.05$ only comparing T1CF, T2CF, and T3CF

II.DISCUSSION

The results of this study confirmed previous findings on the effects of AFB1 on broiler performance (Yunus et al., 2011), showing that low to moderate levels significantly impair the growth performance of broilers. In our study, the impact of the same mycotoxin exposure was profoundly higher (~10 times higher BW loss) in the commercial setting compared to the research facility, highlighting the importance of the cumulative pressure under real farm production. In this regard, most scientific reports do not accurately reflect practical conditions, as they are unfortunately conducted in 'well-controlled' research settings. To this, such unrealistic studies often mislead, as they indicate that only extremely high levels of mycotoxins, often 25 times higher than EU guidelines (e.g., ~500 µg/kg AFB1), can impact broiler performance. Increased levels of UA in the mycotoxin-contaminated groups in both environments indicate cell damage caused by mycotoxins, leading to protein breakdown. Aflatoxin-induced subtoxicities have been linked to kidney weight loss, thickening of the glomerular basal membrane, reduced glomerular filtration rate, decreased urine flow, and apoptosis in other animal species (Owumi et al., 2020). Regarding antibody titers, our findings align with previous work, which demonstrated that similar dietary exposure to AFB1 (75 mg/kg) resulted in a temporal effect on humoral serological responses in broilers, with increased responses observed at later growth stages (Yunus and Bohm, 2013). A possible explanation for this increase could be immunoreactivity overstimulation after an initial disruption phase of immunity balance caused by mycotoxins, which plateaued afterward (at D35). Interestingly, Detoxifier managed to approximately halve the relevant humoral responses. In conclusion, these results highlight the need for continuous monitoring of mycotoxins and application of effective in-feed mitigation strategies to prevent significant economic losses related to performance drop and/or sub-clinical disease in broilers.

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BENEFICIAL IMPACT OF ESSENTIAL OIL SUPPLEMENTATION ON GROWTH PERFORMANCE, MEAT QUALITY, AND GUT MICROBIOTA IN COMMERCIAL BROILER CHICKENS

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Summary

The aim of this study was to evaluate the impact of feed or drinking water supplementation of an essential oil blend (EO) derived from lavender, eucalyptus, and pine on the growth performance, meat quality and gut microbiota of broiler chickens. The experimental treatments were: (1) Control (CON), (2) EO blend in feed (EO-Feed) at 200 g/ton or 200 ppm, (3) EO blend in water (EO-Water) at 200 ppm, and (4) nano-emulsified EO in water (Nano-EO) at 200 ppm. The supplementation of EO had a marked improvement on the performance of broiler chickens. Additionally, EO supplementation improved the meat quality of the breast, reflected in a greater water-holding capacity (WHC) in all EO supplemented groups compared to the control. Water supplementation of EO and Nano-EO modulated the cecal microbiota, increasing the relative abundance of beneficial *Lactobacillus johnsonii*, a key probiotic associated with improved gut health and immunity, while reducing *Romboutsia ilealis* which is also a beneficial bacterium associated with improved immune response and *Streptococcus alactolyticus*, indicating a shift toward a more favorable microbial profile. Overall, both water-soluble and nano-emulsified EO treatments significantly improved growth performance compared with the control, with Nano-EO yielding the greatest effect.

I. INTRODUCTION

Maintaining gut health is vital for optimal growth and immunity in chickens and can be supported through green feed additives such as essential oils (EO), organic acids, and probiotics that enhance microbiota balance and intestinal integrity. Essential oils, rich in bioactive compounds such as terpenes and terpenoids, provide antimicrobial, antioxidant, and digestive benefits that can enhance gut health, nutrient utilization, and overall performance in broilers (Abd El-Hack et al., 2022). Yet, their use in commercial systems, including optimal dosage, formula, delivery methods, and effects on meat quality, remains underexplored. This study investigated the optimum dosage, formulation, and administration route of an EO blend containing lavender, eucalyptus, and pine oils on growth performance, meat quality, and gut microbiota of broiler chickens. It was hypothesized that Nano-EO will have the greatest impact on the production performance of broilers due to the smaller particle size and greater bioavailability.

II. METHOD

A 42-day trial was conducted with 256 Ross 308 broilers allocated to 32 pens (4 treatments × 8 replicate pens × 8 birds/pen) at the Queensland Animal Science Precinct facility, The university

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of Queensland, Gatton Campus (Queensland, Australia). Four treatments were tested including control (CON), EO blend in feed (200 g/ton or 200 ppm; EO-Feed), EO blend in water (200 ppm; EO-Water), and nano-emulsified EO in water (200 ppm; Nano-EO). Birds were fed wheat–soybean meal basal diet meeting nutrient requirements for all growth phases following Aviagen nutrient recommendations, with *ad libitum* access to feed and water throughout the trial. Growth performance was measured bi-weekly. On day 42, after recording all birds' body weight and all pens residual feed, one bird per pen was sampled for meat quality (pH, colour, water holding capacity (WHC), cooking loss, shear force, proximate composition) and gut microbial profile. Microbial DNA was extracted using the Qiagen DNeasy® PowerSoil® Pro Kit and used to generate full-length 16S rRNA amplicons via PacBio Kinnex™ library preparation and sequencing; data were processed through the PacBio HiFi-16S workflow and analyzed in MicrobiomeAnalyst for microbial profiles.

Data were analysed by two-way ANOVA using the General Linear Models procedure in SAS (2016), with dietary treatment as a fixed factor and block as a random effect. Pen was the experimental unit for performance data, and individual birds for meat quality and microbiota. Mean differences were considered significant at $P < 0.05$ and separated using Tukey's test.

III. RESULTS

By day 28 and 42, birds supplemented with Nano-EO or EO-Water at 200 ppm had significantly higher body weight, with Nano-EO consistently performing best (Table 1). Nano-EO had a greater average daily gain (P=0.01), and both EO-Water and Nano-EO, increased average daily feed intake compared to EO-Feed and CON. Overall, Nano-EO and EO-Water improved growth performance, with Nano-EO showing the strongest effect. Although Nano-EO showed a numerically lower feed conversion ratio, the effect was not statistically significant.

Table 1 - The effects of EO supplementation on production performance in broiler chickens.

Parameters*	Experimental diets**				SEM	P value
	Control	EO-Feed	EO-Water	NANO-EO		
BW g, D10	265.37^{b}	269.97^{bc}	278.21^{a}	274.51^{ac}	2.55	0.007
BW g, D28	1594.72^{b}	1608.85^{bc}	1655.15^{ac}	1688.80^{a}	19.20	0.006
BW g, D42	3125.17^{b}	3205.96^{bc}	3258.90^{ac}	3308.71^{a}	37.43	0.011
ADG g, (D1 – 42)	75.33^{b}	77.30^{bc}	78.59^{bc}	79.81^{a}	0.91	0.011
ADFI g, (D1 – 42)	118.11^{b}	118.46^{b}	122.48^{a}	123.89^{a}	1.55	0.019
FCR, (D1 – 42)	1.56	1.54	1.55	1.51	0.02	0.349

*BW: body weight; ADG: Average daily gain; ADFI: Average daily feed intake; FCR: Feed conversion ratio

** EO-Feed: Essential oil blend in feed (2 kg/ton); EO-Water: Essential oil blend in water (200 ppm); -EWO nano-emulsified essential oil blend in water (200 ppm)

$^{a-b}$: values with different superscripts within the same row, differ (P<0.05). SEM: Standard Error of Mean.

As presented in Table 2, no significant differences were observed for most meat quality traits and chemical composition except for cold carcass weight improved in EO-Water (no different to EO-Feed) and Nano-EO groups ($p = 0.004$) and water-holding capacity (WHC; $p = 0.012$), which was improved with EO-Water (no different to EO-Feed and Nano-EO). The relative abundance of *Lactobacillus johnsonii* was higher in EO-Water and Nano-EO compared with the CON. In contrast, both *Romboutsia ilealis* and *Streptococcus alactolyticus* exhibited the opposite pattern, being more abundant in the CON than the EO-Water and Nano-EO treatments. The CON and EO-Feed showed similar bacterial abundances (Figure 2).

Table 2 - The effects of EO supplementation on breast meat quality parameters and chemical composition of broiler chickens.

Item*	Experimental diets**				SEM	P Value
	Control	EOB-Feed	EOB-Water	NANO-EO		
Cold Carcass Weight D42	2302[b]	2363[ab]	2509[a]	2506[a]	49.3	0.004
Dress out %	70.4	72.5	73.8	72.6	1.09	0.196
Breast pH	5.89	5.77	5.88	5.96	0.05	0.138
Breast Colour L*	53.1	53.8	54.3	54.2	0.72	0.572
Breast Colour a*	1.95	1.80	1.95	1.56	0.25	0.597
Breast Colour b*	2.18	2.35	2.28	2.16	0.24	0.936
Hue (◦)	0.86	0.90	0.87	0.94	0.07	0.864
Chroma	2.98	3.02	3.04	2.71	0.29	0.768
Cooking loss%	26.4	26.3	27.1	27.5	1.12	0.714
Shear force	23.0	19.5	23.2	20.9	1.91	0.484
WHC	66.9[b]	68.5[ab]	69.6[a]	68.0[ab]	0.61	0.012
Meat Approximate Analyses						
Moisture%	75.1	75.4	75.2	75.8	0.41	0.361
Nitrogen% (DM)	3.3	3.2	3.2	3.2	0.16	0.708
Protein% (as is)	20.7	19.8	20.3	20.3	0.97	0.708
Fat% (as is)	2.19	1.94	2.23	1.96	0.21	0.424
Ash% (as is)	1.24	1.40	1.26	1.21	0.08	0.411

WHC: Water holding capacity; L: Lightness; a*: Redness; b*: Yellowness.
**EO-Feed: Essential oil blend in feed (2 kg/ton); EO-Water: Essential oil blend in water (200 ppm); -EWO nano-emulsified essential oil blend in water (200 ppm)
[a-b]: values with different superscripts within a row differ (P<0.05). SEM: Standard Error of Mean.

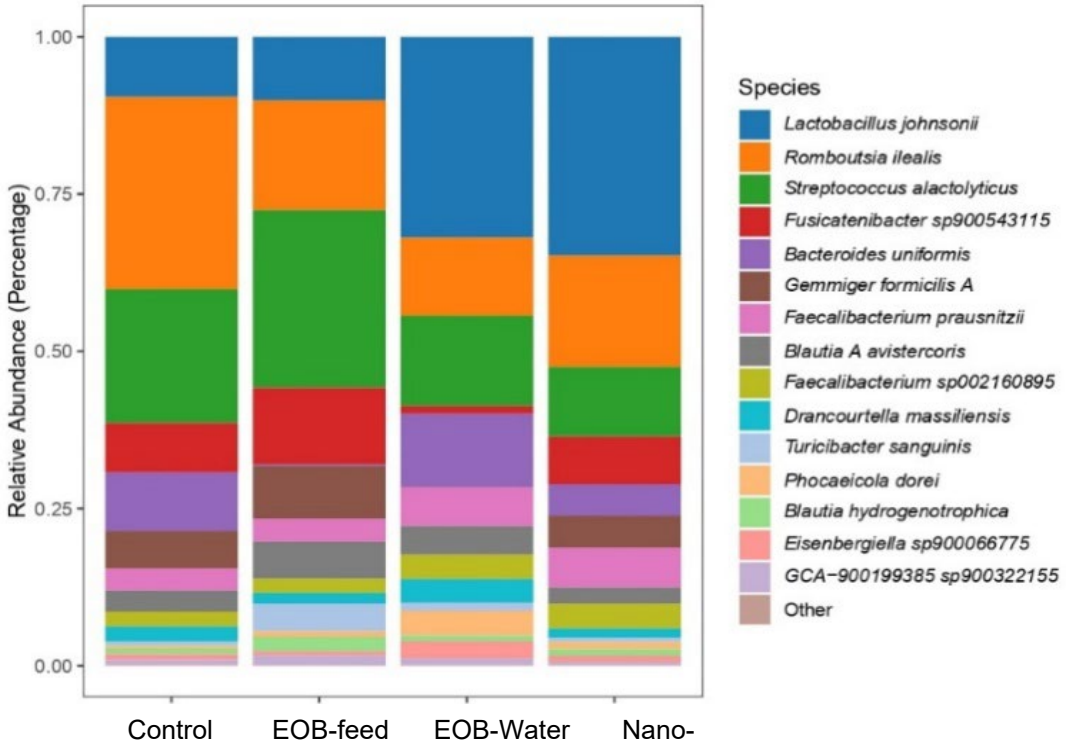

Figure 2 - Relative abundance (%) of the top 15 bacterial species across four treatments: Control, 2 Kg of essential oil-blend (EOB) per ton, 200 ppm EOB in water, and Nano 200 ppm EOB in water.

IV. DISCUSSION

Supplementation with EO-Water and Nano-EO significantly improved body weight and carcass yield on day 42, consistent with Merdana et al. (2024), who reported higher broiler weights with commercial EOs. These effects are closely linked to shifts in gut microbiota, as both treatments increased *Lactobacillus johnsonii* abundance while reducing *Romboutsia ilealis* and *Streptococcus alactolyticus*. Although EOs reduced the abundance of *Romboutsia ilealis* in our trial, it has been reported to be linked to improved feed efficiency and immune function in broiler chicken (Song et al. 2022), while *Streptococcus alactolyticus* can be a component of the normal microbiota but may also be associated with infection if its population increases to pathogenic levels. The bioactive constituents of lavender (*Lavandula angustifolia*; linalool, linalyl acetate), eucalyptus (*Eucalyptus globulus*; 1,8-cineole), pine (*Pinus spp.*; α-pinene, α-terpineol), Gamma-Terpinene, Thymol, Carvacrol and Menthol contribute antimicrobial, anti-inflammatory, antioxidant, and digestive-stimulatory properties that further support growth and muscle development (Guo et al., 2022). These mechanisms likely explain the improved carcass yield and meat quality observed, including greater WHC, a finding consistent with the antioxidant protection of cell membranes reported by Popović et al. (2019) and Liu et al. (2019).

V. CONCLUSION

This study shows that EOs can effectively improve growth performance, meat quality and microbial balance in broiler chickens. The findings support maintaining broiler chickens gut health using green additives in an antibiotic-free production cycle.

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XYLANASE SUPPLEMENTATION IN REDUCED ENERGY BROILER DIETS WITH VARYING WHEAT- CORN RATIOS: EFFECTS ON GROWTH PERFORMANCE

T.T.H. NGUYEN[1,2], J.C. KIM[3] and N.K. MORGAN[4]

Wheat and corn, the primary grains in broiler diets, contain arabinoxylans (AX), a non-starch polysaccharide that can impair nutrient absorption by increasing digesta viscosity, reducing nutrient access to enzymes, and altering gut microbiota. Xylanase application is ubiquitous in poultry diets to hydrolyse AX, but its efficacy varies between wheat- and corn- based diets due to their differences in AX structure and quantity (Cowieson, 2010). In reduced-energy diets, AX may further depress bird's performance by restricting nutrient availability. This study evaluated the potential of xylanase supplementation to enhance growth performance in broilers fed reduced energy diets with varying wheat-to-corn ratios. It was hypothesised that supplementing xylanase into reduced energy diets would mitigate the negative impacts of energy reduction and improve broiler growth in wheat-rich diets.

A total of 792 mixed-sex Cobb 500 broilers were assigned to 11 dietary treatments, eight replicate pens per treatment, nine birds per pen. Diets were fed as three phases (Starter: d0–12; Grower: d12–24; Finisher: d24–35). The positive control (PC) wheat- and corn- based diet was formulated to optimal AME (2930, 3000, 3070 kcal/kg), while negative control (NC) diets were formulated to reduced AME (2780, 2850, 2920 kcal/kg). Nutrient composition was maintained consistently across all treatments. The control diet contained 50:50 mixture of PC wheat-based diet and PC corn-based diet. The test diets contained NC wheat-based diet and NC corn-based diet at ratios of 0:100, 25:75, 50:50, 75:25 or 100:0 supplemented with either 0 or 4,500 U/kg xylanase. Body weight (BW), feed intake (FI), and feed conversion ratio (FCR) were measured for each phase. On d35, ileal viscosity and foot pad dermatitis were assessed. Data were analysed as a 2×5 factorial arrangement (two enzyme levels and five grain ratios) and comparisons between the control group and xylanase-supplemented NC treatments.

At d0-35, birds fed diets containing 75% corn and 25% wheat showed greater BW gain than those fed the 100% corn-based diet (2514g vs. 2408g; $P = 0.022$). A significant grain ratio and enzyme level interaction was observed for FCR ($P = 0.013$); in the presence of xylanase, FCR was lower in birds fed the 75%, 50% or 25% corn and the 100% wheat diets compared to those fed the 100% corn-based diet (1.51, 1.50, 1.50, and 1.51 vs. 1.57, respectively), but no significant differences were observed in the absence of xylanase. The dietary treatments had no impact on FI ($P > 0.05$). Xylanase significantly reduced ileal viscosity in birds fed the 100% wheat-based diet, but no effects were seen in birds fed the treatments containing corn (2.54 vs. 2.62 to 2.75cP; $P = 0.016$). Compared to the control group, the d0-35 FCR value observed in birds fed the 100% corn-based diet supplemented with xylanase was significantly higher (1.50 vs. 1.57; $P < 0.001$). Higher incidences of footpad score 0 ($P = 0.039$) and lower incidences of score 2 ($P = 0.049$) were observed in birds fed the 100% wheat-based with xylanase compared to the control group. A footpad score of 0 was predominant across all treatments.

In conclusion, the xylanase supplementation improved feed efficiency in broilers fed reduced-energy wheat-rich diets by reducing digesta viscosity. The xylanase had no impact in birds fed the 100% corn-based diet, but improved feed efficiency in birds fed the 75%, 50% or 25% corn-based diets.

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OPTIMISING ENERGY AND AMINO ACID DENSITY IN MODERN BROILER DIETS: IMPLICATIONS FOR PERFORMANCE, NUTRIENT DIGESTIBILITY, NUTRIENT EXCRETION, AND FEED COST EFFICIENCY

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Summary

Feed cost volatility and rapid genetic progress have intensified the need for evidence-based nutrient density strategies that maximise profitability while maintaining birds' performance and reducing nutrient wastage in broiler chicken diets. Energy and amino acids continue to be the most expensive components of broiler diets and the main drivers of both biological performance and feed cost per kg of body weight (BW). Yet, commercial nutrient specifications are often adopted with limited testing under local ingredient prices and supply conditions. This paper summarises outcomes from a coordinated series of four large-scale feeding trials conducted under commercially relevant Australian conditions (wheat-based diets with contemporary ingredient choices and feed enzymes), designed to quantify the independent and interactive effects of dietary metabolisable energy (ME) and amino acid (AA) density across growth phases, ingredient matrices (grain and supplemental fat sources), and breeds. The main objective was to determine optimal ME and AA densities to improve production efficiency and reduce nutrient excretion, assess whether grain and oil/fat sources modify these optima, compare Ross 308 and Cobb 500 responses to altered nutrient densities, and develop predictive equations linking dietary inputs and intakes to growth performance. Four feeding trials were conducted using floor pens with industry relevant replication and bird numbers (min 8 replicates of 25 birds each), generating a robust dataset for performance, carcass traits, breast meat quality indicators, nutrient digestibility, nutrient excretion, and feed cost per kg of body weight. Key outcomes showed that reducing dietary ME by 100 kcal/kg in the starter phase and 75 kcal/kg in the grower phase did not impair BWG or FCR but consistently improved feed cost efficiency. Higher AA density improved BWG and FCR and increased breast meat yield but was not always cost-effective and increased the incidence of woody breast and white striping. Wheat-based diets generally supported superior performance compared with sorghum- and barley-based diets. Breed-specific responses were modest but economically relevant, with Cobb birds achieving higher BW and Ross birds tending towards better FCR when corrected to a common BW. These data support a re-balancing of nutrient density specifications towards slightly lower ME in the starter and grower phases, coupled with breeder-recommended AA densities, as a robust and commercially applicable strategy to improve feed cost efficiency without compromising growth performance. The predictive equations generated from this dataset provide a practical tool for nutritionists to explore tradeoffs between nutrient density, biological responses and feed cost under different ingredient price scenarios.

I. INTRODUCTION

Energy and protein (and the AA supply used to support lean tissue accretion) remain the most expensive components of broiler diets, and recent market volatility has reinforced the importance of flexible, least-cost formulation strategies that not only maintain performance and carcass yield but also optimize cost efficiency. Under modern production systems, feed typically represents at least ~70% of operational costs, making dietary nutrient density a primary lever

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for profitability. Over the last decade, global demand for starch, oil and high-protein feedstuffs has intensified competition between animal agriculture, human food and biofuel sectors. For example, canola oil prices surged by nearly 75% in 2021, driving up the cost of animal fats and other vegetable oils, while protein meals such as soybean meal and meat and bone meal increased by more than 25% relative to 2020 levels. Although prices have partially stabilised, volatility in global commodity markets remains a structural challenge.

Genetic progress has markedly improved growth rate and feed efficiency, but this progress has also increased appetite and the capacity for rapid tissue deposition, altering the response surface to dietary ME and AA density (Aftab, 2019). Conceptually, at constant (adequate) intakes of protein and other nutrients, increasing energy intake supports protein accretion to a point where birds reach their genetic potential, beyond which additional energy is preferentially stored as fat, reducing feed efficiency (De Lange and Swanson, 2006). Consequently, optimal dietary ME is not simply "higher is better": the question is where ME should sit relative to AA supply (and particularly digestible lysine as a reference AA within ideal protein concept) to maximise lean gain and FCR at least cost.

A substantial body of research has explored broiler chickens' responses to dietary energy and AA density. Increasing AA density, particularly when balanced relative to lysine, consistently improves growth performance and carcass yield (Liu et al., 2019; Barekatain et al., 2021), and modern high-yielding lines appear especially responsive to higher AA density (Xie et al., 2026). Conversely, modest reductions in ME often stimulate feed intake and help maintain energy intake, although effects on FCR are variable and depend on bird age, environment, diet structure, and AA balance (Classen, 2017; Barekatain et al., 2021; Maharjan et al., 2021a). More recent work has further highlighted that nutrient density interacts with grain source, fat quality, and overall diet digestive dynamics (Noblet et al., 2022). Consistent with these findings, major breeder companies have progressively increased recommended digestible AA specifications while reducing recommended ME density (Aviagen, 2014, 2022; Cobb-Vantress, 2022). In practice, however, these specifications are often implemented with limited validation under local ingredient matrices. Consequently, diets are commonly formulated to least cost at a fixed nutrient density, rather than actively optimising nutrient density to maximise margin over feed cost.

To address this gap, the project summarised here executed four coordinated feeding studies to quantify: (1) phase-specific ME and AA effects and the optimal digestible lysine to ME (DLys:ME) ratio; (2) the effect of grain and fat sources on responses to reduced ME and the implications for digestibility and carcass traits; (3) breed and specification interactions (Ross 308 vs Cobb 500; breeder vs adjusted specifications) for performance and cost; and (4) predictive modelling approaches linking dietary ME and digestible lysine densities and intakes to performance outcomes. These data aim to strengthen precision nutrition decision-making for Australian broiler production amid ongoing ingredient price volatility and sustainability expectations.

II. PROJECT OVERVIEW AND EXPERIMENTAL DESIGN

The project comprised four large-scale floor-pen trials conducted at the University of Sydney Poultry Research Unit floor pen facilities, using Ross 308 and Cobb 500 broilers and diet structures representative of Australian commercial practice. Experimental diets were wheat–soybean meal or mixed-grain based, and included canola meal and canola seed, with meat and bone meal used as an organic phosphorus source. All diets contained a commercial xylanase at 400 FXU/kg feed (Ronozyme WX 2000 CT) and phytase at 2,000 FYT/kg (Ronozyme HiPhorius™ 10); the corresponding phytase matrix values for ME, AA, Ca, available phosphorus (AvP), and Na were applied in formulation according to the manufacturers'

recommendations. Diets were steam pelleted under commercial-style conditions using a pellet mill set to 85°C conditioning temperature.

The first feeding trial evaluated the interactive effects of four dietary ME levels (standard, −50, −100 and −150 kcal/kg) and three AA densities (standard, +3% and +6% relative to breeder recommendations) on growth performance, nutrient intake, digestibility, nutrient excretion and carcass traits in male Ross 308 broilers. A 4 × 3 factorial arrangement was implemented across four feeding phases (starter 0–10 d, grower 10–24 d, finisher 24–35 d and withdrawal 35–42 d), with 12 treatments, eight replicate pens and 25 birds per pen.

The second study focused on commercially realistic phased adjustments in ME and AA densities across the four phases, generating seven practical diets around a standard (breeder recommended) control. These treatments were designed to test combinations of lower ME in starter and grower phases, modest increases in AA density and further adjustments in ME in finisher and withdrawal diets.

Study 3 examined the interactive effects of three grain sources (wheat, sorghum and barley) and three supplemental fats (canola oil, poultry tallow and beef tallow) on performance, carcass yield and abdominal fat deposition, using a 3 × 3 factorial design (nine treatments; eight replicates; 25 birds per pen). Diets were formulated to common nutrient specifications but differed in the contribution of starch and fat from the grain and oil sources.

The last feeding study evaluated the interactive effects of nutrient density and breed in straight-run birds. A total of 1080 day-old Ross 308 and 1080 Cobb 500 chicks were allocated to three diet specifications (Ross 308, Cobb 500 and an adjusted AGF specification with lower ME in starter and grower and slightly higher ME in withdrawal). The design was a 3 × 2 factorial with eight replicate pens per treatment and 45 birds per pen. Key outputs included body weight, FCR, age to reach 2.5 kg, carcass yield and the incidence of woody breast and white striping.

III. GROWTH PERFORMANCE

Data from these studies indicated that the response to dietary ME density was age dependent. In terms of BWG, younger birds (up to 24 d) responded positively to reduced ME density and, at the control AA density, achieved higher BWG than birds fed the higher ME diets. In contrast, increasing AA density improved BWG and FCR regardless of age. Because maintenance energy can represent a substantial proportion of total ME intake in young birds, this may partly explain the limited response of younger birds to higher dietary ME (Toghyani et al., 2024). Feed intake regulation in poultry is complex (Denbow, 1999). Body weight is maintained across the lifecycle through adjustments in feed intake and energy expenditure, both governed by interconnected neuronal and endocrine networks that maintain energy homeostasis (Richards and Proszkowiec-Weglarz, 2007). Although compensation may be incomplete, depending on the severity of nutrient restriction, birds can increase feed intake to partially offset deficiencies in ME or AA and support growth. These findings were supported by subsequent studies showing that reducing dietary ME by 100 kcal/kg in starter diets and 75 kcal/kg in grower diets did not compromise growth performance, regardless of dietary grain or supplemental fat source (Wang et al., 2025) or broiler genotype (Ross vs Cobb) (Xie et al., 2026).

Interestingly, ME × AA interactions for BWG were more evident in older, heavier birds. In these birds, the response to increased AA density was clearer when ME density was at the control (higher) level, whereas the effect of AA density was less distinct at the lower ME densities. This supports the hypothesis that, at higher AA intake, an adequate supply of ME is required to efficiently utilise the additional AAs and convert them into muscle mass. Partitioning of ME and AAs between maintenance and lean tissue growth is influenced by their dietary balance (Classen, 2017) and it also changes with bird age (Aftab, 2019). Importantly, all experimental diets, regardless of ME or AA density, were formulated to meet ideal digestible AA ratios. Therefore, under high AA intake and adequate ME supply (control ME diets),

particularly in older birds with greater body mass, less AA would be expected to be catabolised to meet energy demands, allowing a greater proportion to be directed toward protein deposition and muscle accretion, thereby improving feed efficiency. Overall, accumulative reduction in ME did not affect BWG but each 50-kcal reduction in ME increased FCR by around 2.0 points. Similarly, Maharjan et al. (2021a) reported a significant reduction in feed intake (−61 g/bird) and a 7.3-point improvement in FCR when dietary ME was increased by 125 kcal/kg. In our study, increasing AA density accelerated growth rate by almost half a day and improved FCR by approximately 3 points for each 3.0% increase in AA density.

Despite these contrasting performance responses to lower dietary ME and higher AA density, feed cost per kg BW decreased linearly as ME density decreased, whereas it increased with higher AA density. This suggests that, under our conditions, the gains in growth rate and feed efficiency associated with higher AA density were not sufficient to fully offset the additional ingredient cost required to achieve that higher nutrient density.

IV. BREAST MEAT YIELD AND QUALITY

Decreasing dietary ME density linearly increased breast meat yield (R^2 = 0.78) and reduced abdominal fat pad weight (R^2 = 0.96), irrespective of AA density. Similarly, increasing AA density at each incremental level improved breast meat yield (R^2 = 0.97) and decreased abdominal fat pad weight (R^2 = 0.98). Dietary ME density had no significant effect on woody breast mean scores; however, increasing AA density, particularly at the highest level, significantly increased woody breast mean scores (R^2 = 0.99). Reducing ME density also resulted in a significant linear decrease in white striping mean scores (R^2 = 0.87) compared with the standard ME diets. In contrast, increasing AA density was associated with higher white striping mean scores (R^2 = 0.97) (Toghyani et al., 2025).

Dietary AA density, particularly when formulated to an appropriate, balanced AA profile, has long been recognised as a key physiological driver of protein synthesis and, consequently, breast meat yield. Accordingly, numerous studies have reported improvements in carcass and breast yield when digestible AA density is increased (Maharjan et al., 2020a; Vieira and Angel, 2012). Increased dietary AA levels have also been associated with reduced abdominal fat pad yield (Maharjan et al., 2020b). Vieira and Angel (2012) further noted that modern, high-yielding broilers are particularly responsive to AA density, especially lysine. The effect of dietary ME density on abdominal fat deposition is also broadly consistent across the literature and aligns with our observations; several studies have reported that reducing dietary ME lowers fat pad weight (Dozier III and Gehring, 2014; Maharjan et al., 2020b; Majdolhosseini et al., 2019). However, in contrast to the present findings, some reports have shown either no effect (Jespersen et al., 2024; Maharjan et al., 2021a; Majdolhosseini et al., 2019) or even an increase (Zahi et al., 2014) in breast meat yield when higher ME diets are fed.

The higher breast meat yield observed in birds fed lower ME diets in the present study may be an indirect consequence of ME density altering feed intake and, therefore, AA intake. Across 0 - 42 d, the average calculated digestible Lys intake was 14.4 and 14.5 g/bird/day in birds fed diets with 100 and 150 kcal/kg lower ME, respectively, which was significantly higher than 13.9 g/bird/day in birds fed the standard-ME diets. In addition, digestible Lys intake was linearly associated with both breast meat yield (R^2 = 0.69) and abdominal fat pad weight (R^2 = 0.81). Brito et al. (2017) suggested that higher digestible Lys intake, within the “ideal protein” concept, may upregulate mRNA expression of genes involved in the mitochondrial electron transport chain, potentially increasing mitochondrial energy production and thereby promoting protein deposition and breast muscle yield.

Over the last decade, broiler producers have increasingly reported woody breast (WB) and white striping (WS) in the pectoralis major (Meloche et al., 2018a). While their pathology is well described, the causes remain multifactorial. Genetics contributes, but both conditions

show low heritability and strong non-genetic (environmental/nutritional) effects (Bailey et al., 2015). WS has been associated with rapid growth and high energy diets, particularly those high in fat and relatively low in protein (Kuttappan et al., 2012). In our studies, lower ME (lower fat) diets linearly reduced WS scores, with the lowest WS observed at −150 kcal/kg ME, whereas the higher growth rate under high AA density (especially +6%) likely contributed to the increased WB and WS incidence. Mechanistically, WS-affected breast has been linked to altered lipid composition (Kuttappan et al., 2012), and recent evidence implicates disrupted energy metabolism, oxidative stress, and impaired vascularisation/hypoxia pathways in these myopathies (Li et al., 2024; Marchesi et al., 2019). Increasing digestible lysine has been reported to improve breast yield but also increase WB/WS severity (Cruz et al., 2017), while a moderate lysine reduction during mid growth can lower WB/WS without compromising performance (Meloche et al., 2018b).

V. DIGESTIBLE LYSINE TO ME RATIOS

In practical feed formulation, optimising the utilisation of dietary ME and AAs is critical for both economic and environmental outcomes. ME and AA concentrations should be adjusted proportionally; otherwise, lean tissue deposition may become limiting and surplus energy is diverted to fat deposition (Barekatain et al., 2021). Conversely, when AAs are increased independently, they may be increasingly catabolised to meet energy demands (Gous et al., 2018; Richards and Proszkowiec-Weglarz, 2007). For this reason, the digestible lysine-to-ME ratio (DLys:ME) is widely used to align AA supply with dietary energy. In the present study, quadratic broken-line (QBL) models were used to estimate DLys:ME requirements for optimal BWG and FCR. In the starter and grower phases, the predicted DLys:ME ratio was higher for optimising FCR than BWG (Figures 1 and 2), and both exceeded breeder recommendations (Aviagen, 2022). Although QBL models could not be fitted for BWG in the finisher phase or FCR in the withdrawal phase, the estimated optimum ratios for finisher FCR and withdrawal BWG were also higher than breeder guidelines. These results align with previous work showing performance benefits from higher DLys:ME ratios (Barekatain et al., 2021; Mansilla et al., 2022) and with studies reporting that AA requirements to optimise FCR are higher than those needed to maximise BWG (Maharjan et al., 2021b; Liu et al., 2019).

VI. NUTRIENT DIGESTIBILITY AND EXCRETION

Decreasing ME density or increasing AA density did not affect gross energy or nitrogen digestibility, but it markedly influenced starch and fat digestibility and nutrient excretion (Toghyani et al., 2025). Starch digestibility increased with AA density, with the +6% AA diet showing the highest value. In contrast, crude fat digestibility declined linearly as ME density decreased ($R^2 = 0.99$). Increasing AA density (medium and high) increased nitrogen excretion by >14% regardless of ME ($R^2 = 0.88$), whereas lowering ME reduced fat excretion ($R^2 = 0.80$), with the −150 kcal/kg diets decreasing fat excretion by ~50% compared with standard ME. Reducing ME also decreased Ca ($R^2 = 0.96$), Mn ($R^2 = 0.91$), and Cu ($R^2 = 0.81$) excretion. Increasing AA density reduced Ca ($R^2 = 0.76$) and Fe ($R^2 = 0.83$) excretion but increased K excretion ($R^2 = 0.99$).

Digestibility and nutrient excretion are influenced by feed intake and dietary nutrient concentration (Svihus, 2011). In the present study, although feed intake was higher with lower ME and higher AA density (Toghyani et al., 2024), crude protein and gross energy digestibility were unchanged. Synthetic AAs are rapidly absorbed and do not require digestion (Wu, 2009), and their total inclusion in the grower diets was similar across treatments (7.7–8.3 g/kg). Nevertheless, high AA diets increased nitrogen excretion, suggesting that nitrogen utilisation efficiency was broadly similar, but higher nitrogen intake increased nitrogen output. This

contrasts with Khoddami et al. (2018), who reported higher protein digestibility in high density diets, attributed to greater synthetic AA inclusion.

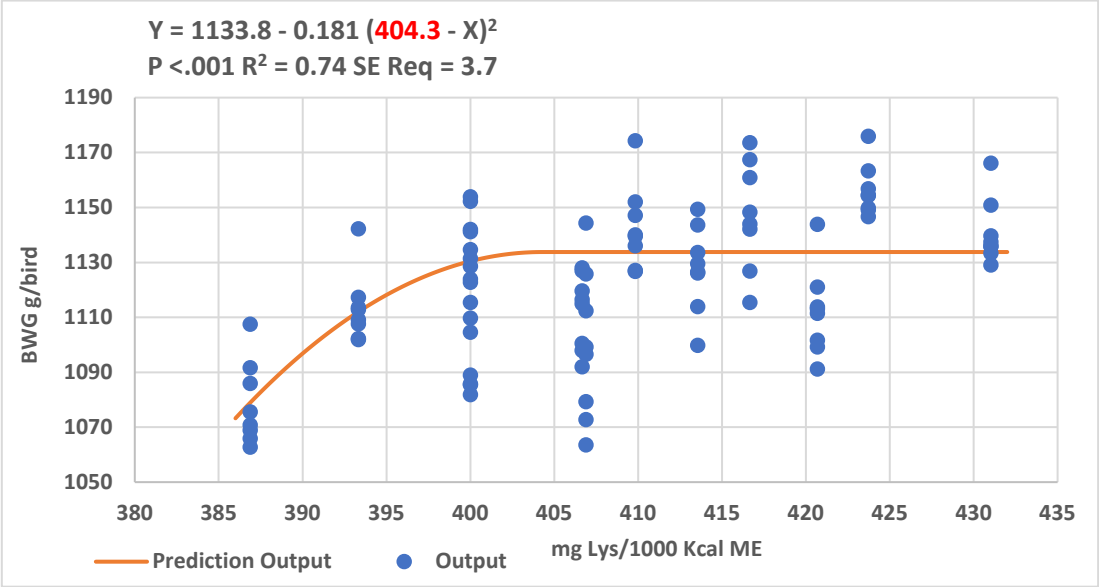

Figure 1 - Quadratic broken-line models fitted to describe the relationship between digestible lysine to ME ratio and BWG during the grower period.

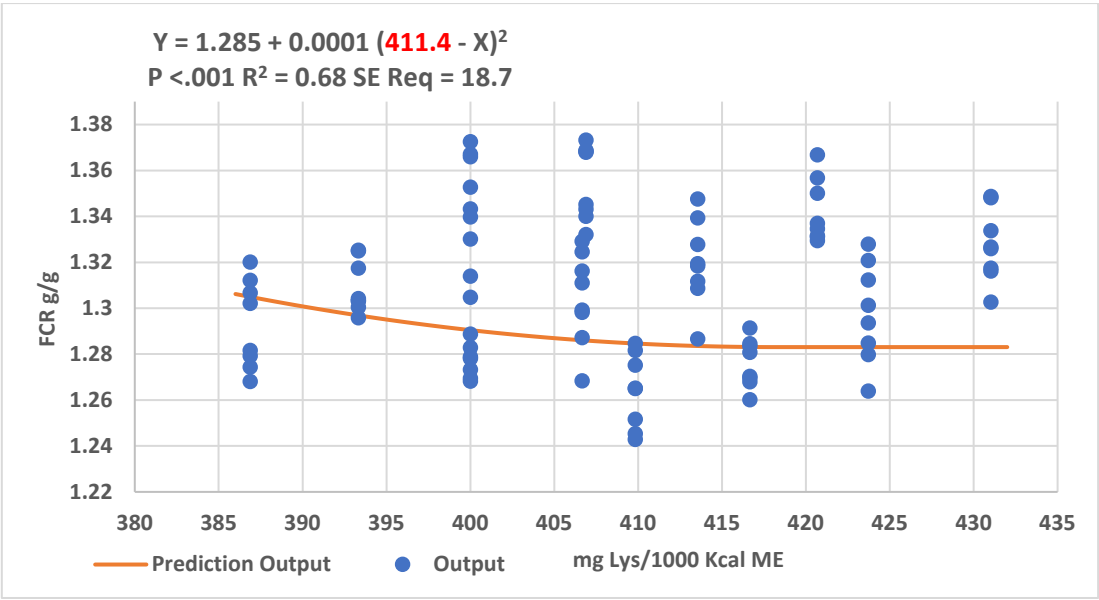

Figure 2 - Quadratic broken-line models fitted to describe the relationship between digestible lysine to ME ratio and birds FCR during the grower period.

Fat responses differed from nitrogen: the low ME (low added-fat) diets reduced apparent fat digestibility yet also reduced fat excretion. This pattern likely reflects both dietary fat level and physiological adaptation. Fat and oil digestibility in poultry can decline at higher inclusion rates (Ravindran et al., 2016), and in our grower diets fat content differed markedly (5.6% at standard ME/AA vs 2.6% at −150 kcal ME; >110% difference). Digestive enzyme activity adapts to substrate supply (Corring, 1980), so birds fed low fat diets from hatch may secrete less lipase and have reduced capacity to digest fat. Diet-induced upregulation of lipid absorption pathways has also been reported in animal models (Petit et al., 2007). In addition, apparent fat digestibility is influenced by endogenous fat losses. Ileal endogenous fat loss in broilers has been estimated at ~1,714 mg/kg DM intake (Tancharoenrat et al., 2014), and higher dietary fat

can "dilute" endogenous losses, improving apparent digestibility (Kil et al., 2010). Therefore, the lower fat, reduced ME diets likely contained a greater proportion of endogenous fat in ileal digesta, contributing to the lower measured fat digestibility.

The reduction in Ca excretion with lower ME diets likely reflects their lower fat inclusion rather than ME density *per se*. Dietary fat can modify Ca utilisation by altering intestinal solubility and absorption, and in some cases forming insoluble Ca–fat "soaps" that reduce absorption (Bandali et al., 2018). Feeding high fat diets may also increase acid load and renal Ca losses (Dawson-Hughes, 2020), potentially increasing urinary Ca excretion (Leeson and Summers, 2005). The lower Ca and Fe excretion, and higher starch digestibility, observed with higher AA density may reflect greater growth rate and body mass, increasing tissue demand for these minerals. Manganese and copper are enzyme cofactors involved in bone formation and energy/protein metabolism (e.g., superoxide dismutase, pyruvate carboxylase) (Suttle, 2010). The linear reduction in Mn and Cu excretion with lower ME diets may therefore indicate shifts in metabolism associated with diet structure and the energy source (fat vs carbohydrate), although this area remains poorly studied beyond trace-mineral source effects.

VII. PREDICTIVE MODELS

Regression models were developed using comprehensive datasets from three feeding studies in which male broilers were fed wheat-based diets with canola oil as the primary supplemental fat source, standardising the energy supply across experiments. The results confirm that nutrient intake, particularly ME and lysine, is a stronger predictor of BWG than dietary concentrations alone. Multivariable models incorporating both ME and lysine intake achieved the highest accuracy for BWG, explaining up to ~60% of the variation across the starter, grower, and withdrawal phases (Table 1). In contrast, FCR was better predicted by dietary nutrient density rather than intake.

Overall, these regression models reinforce the need to consider both ME and lysine when formulating diets: intake is most informative for predicting growth (BWG), whereas nutrient density appears more influential for feed efficiency (FCR), particularly early in life. These models provide practical tools for precision nutrition and could be further improved by incorporating factors such as environment, gut health, and alternative ingredients.

Table 1 - Regression models to predict body weight gain in response to dietary ME and Lys concentration and intake for different phases of growth.

ME (kcal/kg) and Lys (g/kg) concentration	Regression model (multivariable)	R square	RMSE[1]	P-value
Starter	BWG = 284.98 – 0.0389 * ME + 0.0901*Dig Lys	0.11	8.54	0.001
Grower	BWG = 766.55 + 0.0314 * ME + 0.2280*Dig Lys	0.03	30.6	0.023
Finisher	BWG = 270.46 + 0.1320 * ME + 0.5985*Dig Lys	0.12	34.4	0.001
Withdrawal	BWG = - 213.52 + 0.096 * ME + 0.6937*Dig Lys	0.09	41.9	0.091
ME (kcal/b/d) and Lys (g/b/d) intake				
Starter	BWG = 82.67 + 0.6439* ME intake + 3.794* Lys intake	0.56	6.01	0.001
Grower	BWG = 367.99 + 1.499* ME intake + 2.283* Lys intake	0.62	19.1	0.001
Finisher	BWG = 600.25 + 0.753* ME intake + 1.527* Lys intake	0.32	30.2	0.001
Withdrawal	BWG = 105.57 + 0.823* ME intake + 0.789* Lys intake	0.62	27.3	0.001

[1]RMSE: Root Mean Square Error

VIII. CONCLUSION

This project delivers phase-specific ME and AA targets for modern broilers under Australian conditions to support cost-effective, high performing diets. Reducing ME by 100 kcal/kg

(starter) and 75 kcal/kg (grower) lowered feed cost without compromising growth, intake, or carcass traits, whereas in older birds (>24 d) each 50 kcal/kg ME reduction increased feed intake and increased FCR by ~2 points (0.02) (independent of AA density), and increasing ME above breeder recommendations did not improve performance. Thus, when intake is not limiting, moderate ME reductions can reduce $/kg BW, improve leanness, and reduce white striping, with little effect on woody breast. Higher AA density and increased DLys:ME can improve growth rate and FCR, but profitability depends on ingredient prices and requires clear input–output assessment. Across the full cycle, wheat-based diets (vs sorghum and barley) and canola oil in wheat diets supported better BWG and FCR. The optimised ME targets appear broadly applicable to both Ross 308 and Cobb 500 despite breed differences. Precision ME/AA strategies also improved sustainability by lowering excretion of fat, Ca, Mn, and Cu. Predictive modelling showed BWG is best explained by ME and lysine intake, whereas FCR is better predicted by dietary concentrations; intake-based FCR models may benefit from correcting FCR to a standard body weight.

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INTERACTIVE EFFECT OF LYSINE TO ENERGY RATIO AND FEED PHYSICAL CHARACTERISTICS IN STARTER DIETS ON BROILER GROWTH

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Early life nutrition can effect growth, health and welfare across the broiler production cycle, and one strategy involves increasing amino acid density such as heightening the digestible lysine to metabolizable energy(dLys:ME) ratio to support early lean muscle accretion (Barekatain et al., 2021). Hence, feed physical properties also affect gut development and nutrient release, formulation needs to consider both nutritional density and physical characteristics. This study examined the interactive effect of dLys to ME ratio post pellet oil inclusion on starter growth and pellet durability and assessed any carry over effect at market age.

A total of 504 day-old off-sex male Ross 308 broilers were used in a 2 × 4 factorial arrangement of treatments with seven replicates of nine birds per cage. Birds received eight experimental starter diets from day 0 to 10, differing in two dLys:ME ratios (standard and high) and four levels of post pelleted oil inclusion (0%, 20%, 60% and 80%), followed by common grower (d 11-24) and finisher diets (d 25-35) that were formulated to meet Ross 308 nutrients specification (Aviagen, 2022). All starter diets were wheat, soybean meal, and canola based with fixed ME of 2975 kcal/kg. dLys was set at 1.32% for standard dLys:ME diets and 1.40% for high dLys:ME ratio diets. Total oil inclusion was 2.1% for the standard and 2.6% for the high ratios. Oil was split between pre- and post-pelleting to create differences in pellet physical characteristics and pellet durability (PDI), which was tested in triplicate using the NHP 200 New Holman Automatic Pellet Tester.

A significant interaction ($P < 0.001$) between dLys:ME ratio and post-pellet oil inclusion was observed for PDI. In high dLys:ME diets, PDI was significantly reduced at 80% of oil inclusion (85.0%) compared to 0%, 20% and 60% inclusions (89.6, 90.1 and 90.0%, respectively), while PDI was similar across the post-pelleting oil inclusions in the standard dLys:ME diets. No dLys:ME ratio × post-pellet oil inclusion level interaction was found for growth performance. As main effect, birds offered the high dLys:ME ratio diet had higher body weight (BW; $P < 0.05$), body weight gain (BWG; $P < 0.05$) and lower feed conversion ratio (FCR; $P < 0.001$) during the starter phase compared to those fed the standard ratio diet, but no carry-over effect was observed on day 35. Quadratic regression analysis indicated that PDI was positively correlated with FCR during the first week (d 0-7; $R^2 = 0.34$; $P < 0.001$) and the overall starter phase (d 0-10; $R^2 = 0.46$; $P < 0.001$).

In conclusion, increasing digestible lysine level during the starter phase improved growth performance; however, the improvements did not persist beyond this period, suggesting that nutritional interventions may need to continue into subsequent growth phases past starter period. Based on the quadratic regression, the optimal feed efficiency was observed at around 90% PDI during starter phase. An 80%, post-pellet oil inclusion negatively influenced PDI of high dLys:ME diets. This reduction in pellet quality was likely attributable to the lower inherent binding capacity of the high dLys:ME diet matrix because of less wheat inclusion (4%) compared to the standard diet. Despite this, FCR was not compromised, indicating that nutrient supply may outweigh physical feed characteristics in supporting early growth performance.

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OPTIMISING ENERGY DENSITY IN BROILER DIETS USING A CENTRAL COMPOSITE DESIGN

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Dietary energy is the most expensive component associated with broiler production. Previous research has suggested that the optimum level of dietary energy is below breeder recommendations (Aftab, 2019). A study was conducted using a central composite design (CCD) to optimise dietary energy levels across the broiler growth phases.

A total of 940 one-day-old male Ross 308 broilers were allocated to 94 pens (10 birds per pen) and randomly assigned to one of the 18 dietary treatments. To estimate random error, four treatments were set at the centre point with six replicates each, while the remaining treatments were replicated five times each. The experiment consisted of three growth phases (starter, grower, and finisher), each tested at five levels of nitrogen corrected apparent metabolizable energy (AMEn). AMEn ranged between 10.67 and 12.76 MJ/kg and was changed by varying the amount of added oil. Diets in each phase differed only in AMEn with all other essential nutrients being constant. Digestible lysine was reduced from 1.3% in the starter to 1.2% in the grower and finisher phases. The starter, grower, and finisher diets were offered from days 0-14, 15-28, and 29-35, respectively. Pellet size was increased at each growth phase, with pellet diameter ranging from 3 to 4.7 mm and length from 3 to 6 mm. The day 14 growth performance was analysed by regression, while a two-factor and a three-factor CCD was used to evaluate the day 28 and 35 growth performance, respectively.

AMEn had a significant linear effect on the day 0-14 feed intake (FI) ($P < 0.01$) and feed conversion ratio (FCR) ($P < 0.01$), with both parameters decreasing as dietary AMEn increased. Body weight (BW) at day 14 was not affected by AMEn ($P > 0.05$). Similarly, AMEn did not affect BW at day 28 but resulted in a significant effect on FI and FCR from day 0-28. FI from day 0-28 decreased linearly as AMEn in the grower phase increased ($P < 0.01$), while FCR linearly decreased as AMEn in both the starter ($P < 0.01$), and grower ($P < 0.01$) phases increased, with AMEn in grower phase having a more pronounced effect. These results are consistent with previous studies where birds regulate their FI in response to dietary energy, resulting in minimal changes in BW but a linear response in FCR (Leeson et al., 1996).

The final (day 35) BW showed a significant quadratic response ($P < 0.05$) to AMEn in the finisher phase. BW achieved the maximum response at AMEn levels of 12.28, 11.67, and 11.53 MJ/kg in the starter, grower, and finisher phases, respectively. Total FI was affected only by AMEn in the finisher phase, with FI decreasing linearly ($P < 0.05$) as energy increased. Interestingly, the overall FCR (day 0-35) responded linearly to AMEn in the starter phase ($P < 0.05$) but was not affected by AMEn in the subsequent phases.

In conclusion, dietary energy influenced growth performance across all phases. Final BW was optimized at intermediate AMEn level in the finisher phase. AMEn in the starter phase significantly affected FCR from days 0-14, 0-28, and 0-35. Energy dense starter diets can improve overall feed efficiency.

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INFLUENCE OF DIETARY ENERGY AND PARTICLE SIZE ON PERFORMANCE, NUTRIENT DIGESTIBILITY, AND GUT DEVELOPMENT IN BROILER STARTERS

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Modern broilers selected for feed conversion utilise energy differently (Aftab, 2019). Chang et al. (2015) fed a lower energy diet (92.5% of the recommended level) and found no detrimental effect on birds' body weight. Coarse particles benefit birds' nutrient digestibility by improving gizzard function (Rueda et al., 2024; Svihus, 2011) but the influence of coarse particles combined with lower energy diets is not known. This study determined the influence of particle size on broilers fed different dietary energy levels. It is hypothesised that coarse particles benefit birds' performance and nutrient digestibility, especially when combined with low energy (95% of recommended) by enhancing gizzard function.

Day-old broiler chicks (288) were used in a 2 × 3 factorial arrangement of treatments with energy levels of 2,850 and 3,000 kcal/kg and particle sizes (ground through screens with 2.5, 5.0, and 8.0 mm openings). Chicks were randomly allocated to 36 floor pens (8 birds per pen). On d-17, birds were transferred to enriched cages for 4 days for excreta collection. Diets contained 0.5% titanium dioxide as indigestible marker. All birds were euthanised with sodium pentobarbitone intravenous injection (1 ml per 2 kg BW) to collect digesta from the lower ileum. Nutrient analysis was determined as per AOAC (2016). Two-way ANOVA analysis determined the effect of energy level, particle size and their interaction. Individual birds were considered the experimental units for measurements of the digestive tract.

Birds fed low-energy had greater feed intake (1,501 vs. 1,438 g, $P = 0.001$) and weight gain (1,265 vs. 1,228 g, $P = 0.009$). The influence of particle size on feed conversion ratio (FCR) was dependent on energy level, where coarse particles had the lowest FCR when the diet contained high energy, and fine particles were most efficient in the low-energy groups ($P < 0.05$). A similar interaction was observed for the coefficient of apparent ileal digestibility (CAID) of DM, GE, and P, where coarse particles resulted in the greatest CAID when fed the high-energy diet. However, CAID of starch and N were greater in birds fed the low-energy diets ($P < 0.05$), and AMEn was greater in birds fed high-energy diets (13.98 vs. 12.53, $P = 0.001$). Birds fed diets with coarse particles had gizzard weights 14.2% greater than those fed fine particles ($P = 0.011$) and lower gizzard pH (2.95 vs. 3.39; coarse vs. fine, $P = 0.010$), but small intestine length and weight were similar among treatments. Reducing dietary energy to 2,850 kcal does not compromise growth performance in broiler starters. However, improvements in FCR and nutrient digestibility are observed only when coarse particles are used in conjunction with high-energy, likely a result of improved gizzard function.

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OPTIMISING THE CALCIUM AND PHOSPHORUS NUTRITION OF BROILERS USING BLOOD METABOLOMICS

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Summary

Calcium (Ca) and phosphorus (P) are essential macro minerals for poultry, making critical contributions to skeletal architecture, metabolic processes, immunity, nerve function and more. However, optimisation of the Ca and P status of birds is challenging due to the complex interactions that exist between diet features such as limestone quality, vitamin D, phytase, phytate, digestible phosphorus, Ca and P ratios etc (Salisbury et al., 2021). The emergence of novel analytical methods such as advanced metabolomics, combined with digital tools such as supervised machine learning, offers new insight into bird health and welfare. This short article describes some of the adjacencies and explores the usefulness of these technologies to enhance the Ca and P status of a bird.

I. INTRODUCTION

In 1984, during the Florida Nutrition Conference, Dr. T.S. Nelson discussed the merits of formulating poultry diets on an available Ca basis. These recommendations were somewhat ahead of their time due to a lack of practical information on some key prerequisites such as available Ca requirements of poultry at different production phases, absence of commercially viable exogenous phytases, an incomplete understanding of the chemical and mechanical heterogeneity of limestone, and a lack of a standardised methodology to measure Ca availability. Since 1984, considerable advances have been made in many of these domains and these have been recently summarised (Walk et al., 2021). These include standardised methods for the measurement of both Ca and P, data on endogenous Ca (and P) losses, digestible Ca requirements at different broiler ages, interactions between dietary phytate and Ca digestibility, phytase super-dosing, limestone quality and the prediction of Ca digestibility of limestone using *in vitro tests*, the role of vitamin D and associated metabolites, and the publication of the standardised digestible Ca content of common poultry feed ingredients. Despite the considerable progress that has been made, there is a persistent reluctance by poultry nutritionists to adopt a digestible Ca formulation system. This is in part due to the critical role that Ca plays in bird health and welfare and an associated lack of confidence that the evidence presented in the published literature will adequately transfer to commercial production systems, and partly due to uncertainty of the net economic value of such a move. As Ca (and P) metabolism is complex and involves many interactive components, additional tools that can bring confidence to nutritionists play a vital role in the adoption of the digestible Ca formulation system. One such tool is a novel blood metabolomics platform (Verax^{TM}, dsm-firmenich, Animal Nutrition and Health, Kaiseraugst, Switzerland) that pairs point-of-care metabolomics with supervised machine learning to rapidly establish the Ca and P status of a bird. It is the purpose of this article to explore the contributing haematological parameters that define the Ca and P status of a broiler chicken and how nutritionists can use this novel precision nutrition platform to create learning loops that will bring confidence when formulating using digestible nutrient systems.

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II. CALCIUM, PHOSPHORUS AND HAEMATOLOGY

Plasma Ca and P are regulated via the parathyroid hormone (PTH) and calcitriol (1,25-dihydroxyvitamin D_3; Wideman, 1987). These mechanisms are largely automatic and respond to falling or rising blood Ca and P concentrations by adjusting intestinal absorption, renal excretion and bone mobilisation. Additionally, birds have a Ca and P-specific appetite (Wilkinson et al., 2011) which offers additional opportunity for birds to self-regulate their Ca and P intake. However, none of these regulatory mechanisms are foolproof and derangement in blood Ca and P concentrations are common. For example, Cowieson et al. (2024) noted that dilution of either diet Ca or P via changes to limestone and inorganic phosphate concentrations resulted in rapid perturbation in blood Ca and P levels. Similar plasticity in blood Ca and P concentrations have been observed to be associated with dietary protein level (Dao et al., 2022), brooding conditions (Crespo et al., 2024), genetics and bird age (Livingston et al., 2020; Ruiz-Jimenez et al., 2021; Ruiz-Jimenez et al., 2022), coccidiosis and coccidiosis vaccination (Cowieson et al., 2020), limestone and dietary Ca features (Fallah et al., 2018; Manangi et al., 2018; Marjina Akter, 2018), dietary calcium concentration *per se* (Mansilla et al., 2020) and poultry species (Livingston et al., 2020; Sauer et al., 2020; Ripplinger et al., 2023).

An additional complication in blood Ca regulation is that there are several forms of Ca in blood, some of which are not regulated via PTH and calcitriol. The three major forms of Ca in blood include protein-bound (typically to albumin), anion-bound (typically to phosphate, lactate or citrate) and so-called 'ionised' or free Ca (Fig. 1). In a healthy broiler chicken, total blood Ca should be in the 11.5-12.5 mg/dL range, whilst blood P should be approximately 6.5 mg/dL (Fig. 2; Livingston et al., 2020) and around 47-48% will be 'ionised' or metabolically active. It is the ionised Ca that the PTH attempts to regulate and in the case of a healthy broiler chicken this is usually in the range of 1.3-1.4 mmol/L (5-6 mg/dL). The problem with ionized Ca in blood is that it is influenced not only by ingestion of bioavailable Ca and P (and the various factors involved in Ca digestion such as vitamin D, phytase etc), but it is also influenced by blood pH and the acid/base status of the bird in general.

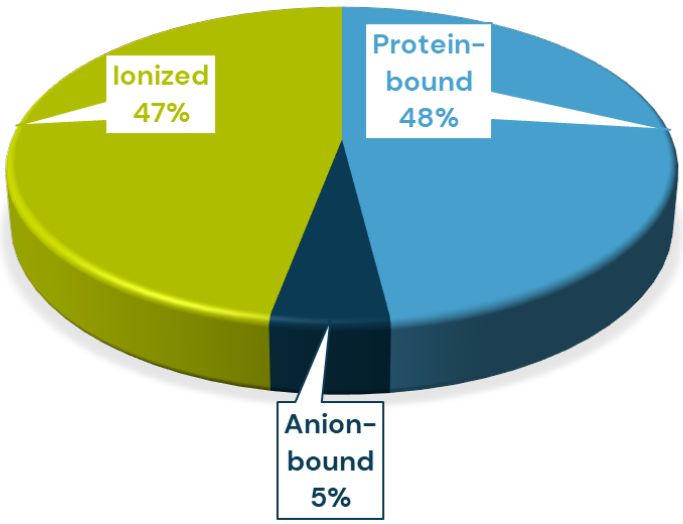

Figure 1 - Relative proportions of the three major blood calcium fractions in broilers.

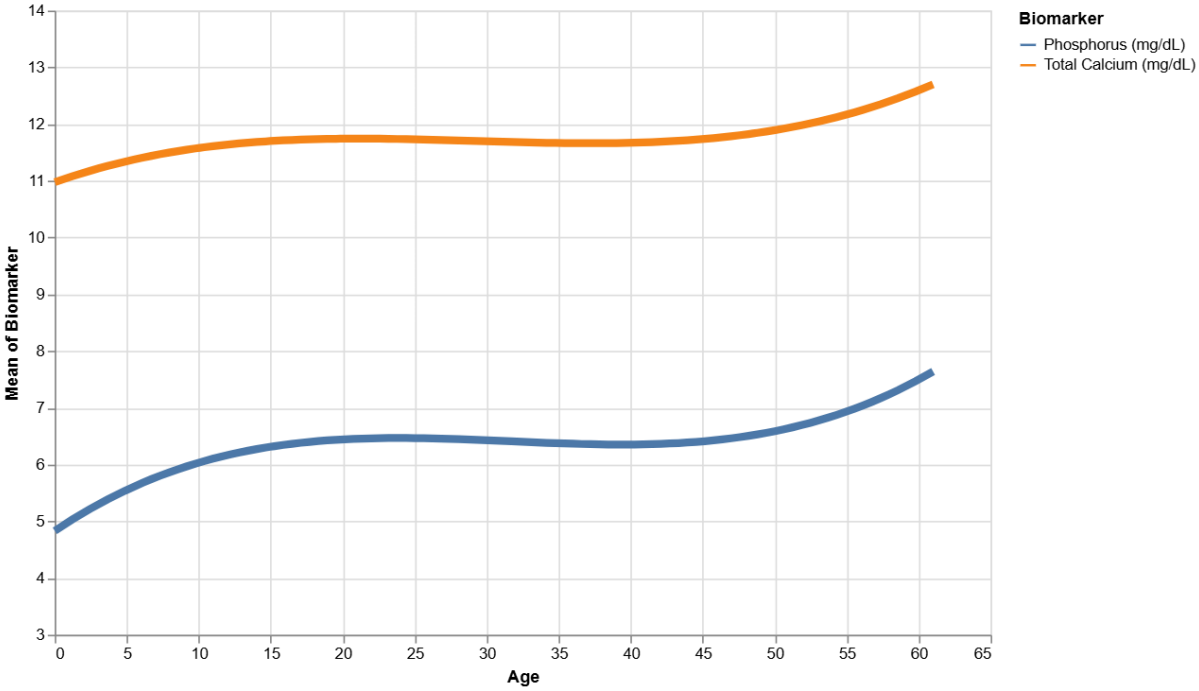

Figure 2 - Reference ranges for total calcium and phosphorus in broiler blood (mg/dL).

III. BLOOD ACID BASE BALANCE

Blood pH in broilers is regulated via the respiratory and renal systems, much like in mammals. The optimal pH for broiler blood is around 7.4 (Fig 3.; Livingston et al., 2020) although this is age dependent. However, it is common for derangement in blood pH to occur via either, or both, respiratory or metabolic routes. For example, excessive ingestion of chloride (Cl) raises blood Cl concentration (Livingston et al., 2022), forcing renal compensatory loss of bicarbonate (HCO_3) and a concomitant drop in blood pH i.e. a classical hyperchloremic metabolic acidosis. Similar perturbation can occur via changes in respiratory rate e.g. respiratory alkalosis associated with tachypnoea. There are a variety of common causes of unusually high or low blood pH including respiratory tract infections, ventilation issues, ambient temperature, ambient CO and CO_2 concentrations, altitude, diet and water chemistry (especially Na, Cl, K and HCO_3 load), the enteric microbiome (especially molar ratio of lactate and butyrate production), heavy metal toxicity, renal pathologies or toxicological status and more. These features can either raise or lower blood pH and in addition to the *per se* consequences on the bird, there are important associative changes to the binding of Ca to blood proteins.

Albumin is the dominant plasma protein and is responsible for regulation of blood oncotic pressure, nutrient transport, plasma osmolality and a variety of other functions. Importantly, albumin is anionic at blood pH and will readily form covalent bonds with Ca and other cations. The degree to which this occurs is dependent on the concentration of albumin in blood and, critically, blood pH. Under alkalotic conditions the binding of Ca to albumin increases (to >50%) whereas in acidotic conditions the opposite occurs and the ionised Ca concentration of blood, relative to total Ca, rises. This pH-dependent change in binding of Ca by albumin can influence blood ionised Ca by around +/- 15% i.e. from a normal value of 1.35 mmol/L to deranged values from <1.2 mmol/L to >1.5 mmol/L. This is essentially pH-mediated hypo- or hypercalcaemia. Importantly, chronic hypocalcaemia (ionised Ca <1.2 mmol/L or thereabouts) can lead to a significant rise in various skeletal pathologies such as rickets and femoral head necrosis whereas chronic hypercalcaemia causes renal damage and can lead to uraemia, gout and wet litter. Thus, it is critically important that optimisation of

blood Ca and P status in birds includes measurement of pH and cation and anion concentrations in general, as well as plasma proteins.

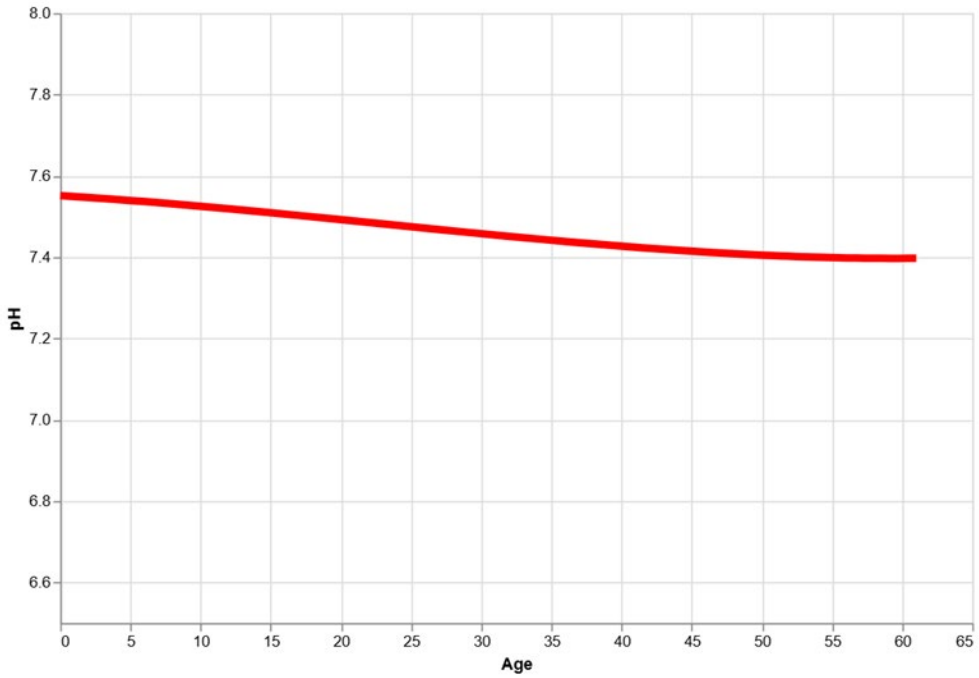

Figure 3 - Effect of broiler age on the reference range for blood pH.

IV. PRACTICAL APPLICATIONS AND CLINICAL RELEVANCE

As the interactions mentioned above are complex, it is useful to integrate these various biomarker signatures into machine learning-based algorithms that allow automation, simple graphical interfacing and to enable forecasting. As part of the ongoing precision service development, dsm-firmenich Animal Nutrition and Health have surveyed thousands of poultry farms across all major geographies to build clear associations between animal productivity, welfare, health, nutritional status and sustainability, with haematology. Dashboards are available, built from both mechanistic and predictive models, that show the nutritionist, veterinarian or live production professional, insights regarding the status of a flock. Specifically for optimisation of Ca and P, these insights integrate the relevant contributions of blood pH, albumin and acid/base status in general, with the direct measurement of blood total Ca, P and ionised Ca. Before making nutritional, husbandry or veterinary interventions, it is critical that the holistic view is taken. Adjusting dietary Ca and/or P supply, without first establishing the acid/base status of a flock, may be counterproductive. For example, addressing symptoms suspected to be linked to hypocalcaemia by increasing limestone inclusion may compound the problems by creating additional pressure on the HCO_3 load in blood, exacerbating metabolic alkalosis and further reducing ionisable Ca in blood.

V. CONCLUSIONS

Optimisation of a birds Ca and P status using conventional metrics such as bone ash and growth rate can be effective strategies but lack precision, with underlying mechanisms that are often obscure. The blood metabolome on the other hand contains a wealth of features that shed light on important biochemical processes, health, welfare, nutritional status and more. Further, counter-intuitive associations between plasma proteins, anions, cations, pH, Ca and P can be elucidated via the use of machine learning algorithms. As digital platforms for

precision animal farming mature, it is likely that these tools will become increasingly relied upon by veterinarians and nutritionists to make proactive decisions.

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EXCESS ARGININE INJECTED IN OVO DISRUPTS NUTRIENT TRANSPORT AND DETOXIFICATION PATHWAYS IN BROILER CHICKS

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The immature digestive and immune system upon hatching is partially associated with the susceptibility of broiler chicks to diseases. Arginine in ovo feeding improved the immunity of day 21 post-hatch broilers (Gao et al., 2017), suggesting a path towards improving broiler chick resilience. However, a study aimed at determining the optimal arginine in ovo feeding dose supporting embryonic development of broilers reported that doses above 60 mg/egg reduced hatchability and day-old chick quality (Disanayaka et al., 2025). The current study investigated the underlying factors disrupting embryonic development after high levels of arginine in ovo. It would help optimise arginine response and clarify nitrogen detoxification mechanisms in broilers, aiding efforts to reduce ammonia excretion in poultry production. It was hypothesized that excess arginine in ovo triggers detoxification mechanisms in broiler embryos.

In ovo injections of 0.9% saline solutions without (control) or with 80 mg of arginine were administered at day 17.5 in the amniotic fluid of fertile eggs. Jejunum samples (n = 6 per group) from day-old chicks were collected for transcriptomic analysis. RNA was extracted using the RNeasy Mini Kit (QIAGEN), quantified with NanoDrop, and sequenced following library preparation. Differential expression analysis was performed using the EdgeR package (RStudio 4.2.1) with a significance threshold of $P < 0.01$. Enrichment and functional analyses were conducted using Database for Annotation, Visualization, and Integrated Discovery (DAVID). The Gene Ontology (GO) database was used to identify biological functions.

A total of 14,119 genes were expressed in the jejunum, and 150 were differentially expressed in the arginine-injected group. Among them, 83 genes were downregulated while 67 genes were upregulated ($P < 0.01$, $\log_2$ fold change < -0.5 and $\log_2$ fold change > 0.5, respectively). The biological processes enriched in the jejunum with differentially expressed genes were mainly transmembrane transport, sodium and potassium ion transport, and xenobiotic metabolic process ($P < 0.01$). Solute carrier (SLC) genes were the most downregulated genes associated with the transmembrane, sodium and potassium transport (SLC5A1, SLC5A11, SLC9A2, SLC9A3, SLC16A13, SLC22A13L and SLC26A9). Notably, the downregulation of SLC5A1 and SLC5A11, both sodium-glucose co-transporters, indicated the reduced glucose uptake by the jejunum, which could impair energy metabolism in chicks. Moreover, the downregulation of sodium and potassium ion transportation could be an indication of osmolarity imbalance occurring with the excess arginine in ovo. In addition, cytochrome P450 (CYP) genes were enriched under xenobiotic metabolic processes. CYP1A2, which was upregulated in this study, is one of the key oxygenase enzymes participating in the detoxification process. However, the remaining CYPs (CYP2D6, CYP2AC1, CYP2AC2) reported in this study were downregulated, suggesting that the embryo's xenobiotic metabolism may not be efficient. In conclusion, the chick quality impairment due to excess arginine in ovo may be related to the downregulation of SLC-mediated glucose uptake and inefficient detoxification pathways.

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PROTEIN SOURCE AND STARCH-PROTEIN DIGESTIVE DYNAMICS SHAPE GUT MICROBIOTA AND GROWTH IN BROILER CHICKENS

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Dietary protein sources modulate starch-protein digestion dynamics and growth performance in broiler chickens. However, the specific dietary components and host digestibility factors that shape gut microbial communities, and thereby influencing growth, remain poorly understood. This knowledge gap limits the precision design of diets that meet the requirements of both the chicken host and its microbiota for optimal growth and health. We hypothesised that diet protein sources affect growth performance directly and indirectly by altering gut microbiota. To test this, we analysed caecal bacterial communities using 16S rRNA gene amplicon sequencing in broilers fed three isoenergetic (12.9 MJ/Kg ME) and isonitrogenous (203 g/kg true protein) pelleted diets differing in protein digestion rates: Rapid: non-bound amino acids (NBAA); Medium: whey protein concentrate (WPC); and Slow: soybean meal (SBM). Caecal samples collected from the same birds used in a previous study (Macelline *et al.*, 2022) were processed to generate new microbiome data. While the earlier work focused on diet effects on digestion and growth, this study examined host-microbiome-diet ineractions.

Male, off-sex (parent line) Ross 308 chicks were sampled at day 35 post-hatch receiving NBAA, WPC, or SBM diets from day 14 to 35 (Macelline *et al.*, 2022). For each treatment, 11 birds were sampled from the six replicate cages (1-2 birds per cage). Diet was treated as a fixed factor and cage as a random factor in statistical models. Birds fed SBM exhibited significantly higher weight gain (WG) and lower feed conversion ratio (FCR) than those fed WPC or NBAA (pairwise Wilcoxon following Kruskal-Wallis tests, $P_{adjusted} < 0.05$), with no significant difference between WPC and NBAA. WPC-fed birds had significantly lower feed intake than the other treatments, which did not differ from each other. SBM-fed birds showed lower starch levels in digesta along the small intestine, particularly in the jejunum, and proximal ileum, compared to the other diets (pairwise Wilcoxon rank sum test, $P_{adjusted} < 0.05$).

Clostridia (relative read abundance: 63-88%) and Bacilli (1-26%) were the dominant bacterial classes across all diets. Diet significantly altered caecal bacterial diversity and composition. WPC-fed birds had the significantly lowest bacterial richness and a distinct community structure, while SBM and NBAA groups were more similar. Redundancy analysis on community composition (Bray-Curtis dissimilarity) revealed that shifts in microbiota were associated with distal jejunal starch and protein levels. Multivariate generalised linear model analysis identified bacterial guilds significantly enriched in SBM (*Lachnospiraceae*, *Lactobacillaceae*, *Butyricicoccaceae*, and *Acutalibacteraceae*) and WPC (*Enterococcaceae* and *Bifidobacteriaceae*) diets, with their relative abundances correlating with WG, apparent metabolizable energy, and FCR (Spearman's correlation, $P_{adjusted} < 0.05$). Structural equation modelling (SEM) revealed that SBM-guilds had a direct positive effect on WG, and an indirect effect through increasing feed intake. SEM also identified a negative influence of distal jejunal starch-to-protein ratio on SBM-guilds, indicating a microbiota-mediated pathway that the SBM diet supported optimal growth in chickens. Overall, the findings demonstrate that dietary protein source influenced starch digestion and reshaped caecal microbiome composition and ultimately affected broiler growth. Therefore, protein formulation should consider carbohydrate digestion dynamics and their downstream effects on gut microbiota.

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EVALUATION OF A MODEL FOR PREDICTION OF RAW MATERIALS METABOLISABLE ENERGY FOR BROILERS

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Summary

Accurate energy evaluation of raw materials is essential for formulating broiler diets that meet birds' requirements. A precise estimation of apparent metabolizable energy corrected to zero nitrogen balance (AMEn) represents a key prerequisite for the successful adoption of more precise energy evaluation systems, including the net energy system. This study aimed to assess the performance of a prediction model for estimating the AMEn content that was measured on corn (n = 68 samples), wheat (n = 44), soybean meal (n = 49), soybean meal expeller (n = 18), and corn DDGS (n = 76). These 255 AMEn measurements were conducted over the past decade at Adisseo for NIRS calibration programs. Nutrient composition of feedstuffs was determined using standard laboratory methods, and their AMEn content was measured by the difference method in 19 to 21 days of age broilers fed a reference diet and test diets containing one of the 5 ingredients. The prediction model was based on 1/measured chemical indicators and adjustments coefficients of gross energy (GE) and AMEn/GE obtained from a compilation of literature measurements on 80 diets and 2/ reference AMEn values corresponding to the average chemical composition and AMEn values from 4 recent feeding tables. Measured AMEn values averaged 3729, 3287, 2538, 2891 and 2497 kcal/kg DM for corn, wheat, soybean meal, soybean meal expeller and corn DDGS, respectively. The variability of these values was markedly higher for corn DDGS (CV: 12%) than for the other ingredients (3 to 9%). The predicted AMEn values of the ingredients according to our model were close to the measured AME values with the highest difference for soybean meal (193 kcal) In addition, the comparison of measured and predicted AMEn values according to a covariance model showed a strong agreement, R = 0.93; common slope = 0.93. Raw material effect was significant, intercept was 41, 59, -171, -22 and 0 kcal/kg DM for corn, corn DDGS, soybean meal, soybean meal expeller and wheat, respectively. These results indicate that nutrient composition can reliably predict AMEn values for poultry while highlighting the intrinsic variability of coproducts such as DDGS.

I. INTRODUCTION

Accurate evaluation of raw materials is crucial in broiler feed formulation, as it impacts nutrient use, growth of birds and economic efficiency. Since feed accounts for the largest part of production costs, precise assessment of energy and digestible nutrients is essential for cost-effective balanced diets. Apparent metabolizable energy for zero nitrogen balance (AMEn) is the currently most used energy system for feed production. Beyond AMEn, the Net Energy (NE) system is considered the most accurate approach for evaluating feed energy, as it accounts for the actual energy available for maintenance and production after losses due to digestion, metabolism, and heat increment. Precise AMEn is mandatory for precise calculation of NE (Noblet et al., 2024). Errors in evaluation can lead to over-formulation (waste, high costs) or under-formulation (poor growth, feed efficiency, and bird health). AMEn can be sourced from in vivo trials, near-infrared spectroscopy (NIRS), or feed tables. In vivo methods are accurate but costly, slow and sensitive to factors like bird age and technology. Alternatively, NIRS is a fast, non-destructive tool but it needs important initial investments for model calibration. The

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objective of the present study is to evaluate the quality of equations based on wet chemistry for prediction of broiler raw materials energy values.

II. MATERIALS AND METHODS

a. Chemical analyses and AMEn measurement on raw materials

Average AMEn and chemical composition values from four recent feeding tables were used as reference values for each ingredient (Table 1). Data for NIRS calibration were obtained from Adisseo's raw material database, which included samples of corn (n = 68), wheat (n = 44), soybean meal (n = 49), soybean meal expeller (n = 18), and corn DDGS (n = 76). Proximate analyses were performed following AOAC (1990) methods for moisture, ash, nitrogen (Dumas), crude fiber, and crude fat; GE was determined using an adiabatic bomb calorimeter, and starch by the Ewers polarimetric method (EEC, 1972). NDF, ADF, and ADL were analyzed using the sequential Van Soest and Wine (1967) method with amylolytic and proteolytic pre-treatments. Broiler balance studies were conducted as described by Cozannet et al. (2010) and AMEn values (n = 255) were calculated using the difference method (Table 2).

Table 1 - Composition of reference raw materials (% DM and kcal/kg DM)[1].

	Corn	Wheat	Soybean meal	Soybean meal expeller	Corn DDGS
Chemical composition, % DM					
Dry matter	86.4	87.8	88.5	94.9	90.1
Ash	1.3	1.8	7.1	6.5	4.6
Crude protein	8.7	13.8	52.7	44.8	31.2
Crude fat	4.1	1.7	1.8	8.9	11.8
Crude fiber	2.5	2.6	5.2	6.4	8.0
NDF	12.8	13.3	11.5	11.0	42.2
Starch	73.4	67.2	4.7	5.2	4.2
Energy utilization					
GE	4434	4358	4727	5039	5147
AMEn/GE	83.1	76.8	52.1	55.9	53.5
AMEn	3684	3349	2459	2817	2759

[1] Average values of Brazilian Tables for Poultry and Swine 5th edition 2024, CVB Feed Table 2021, Fundación Española para el Desarrollo de la Nutrición Animal 2021 and Feed Table INRAE-CIRAD-AFZ 2022

b. Model description and calculations

Gross energy (GE) and metabolizability (AMEn/GE) of energy in poultry differ between nutrients and are then closely related to nutrient composition (Wu et al., 2019). Four major studies conducted in broilers over the last decade (described by Noblet et al.., 2024) and involving 80 chemically diverse diets (18.0–29.9% CP, 1.3–12.3% fat, 27.5–56.9% starch, 2734–3750 kcal/kg AME, DM basis) were then used in the development of prediction equations of GE and AMEn/GE in broilers. A covariance model with "study" as a fixed intercept and nutrients content as covariates was used (J. Noblet et al., unpublished data). The results indicate that the GE value would change by -50, 15, 50 kcal/kg per % increase of ash, CP and crude fat (relative to DM), respectively. Similarly, coefficients for AMEn/GE ratio were 0.20, 0.73, 0.47 and -0.93 % unit per % CP, crude fat, starch and crude fiber change or 0.24, 0.61, 0.39 and -0.72 % unit per % CP, crude fat, starch and NDF, respectively. These coefficients are then used for adjusting the GE and AMEn/GE values of an ingredient according to the difference between its actual chemical composition and the corresponding values for the reference raw material according to the following model:

$$Ynew = Yref + A1\,x\,(X1new - X1ref) + A2\,x\,(X2new - X2ref) + Ai\,x\,(\ldots.)\ldots..$$

where Ynew and Yref correspond to GE or AMEn/GE of the new and the reference ingredients, Xinew and Xiref to the chemical indicators for each model and Ai to the coefficients of the chemical indicators of each model. Finally, the AMEn content is equal to adjusted GE multiplied by the adjusted AMEn/GE. As mentioned above, the so-called reference values correspond to the mean of 4 tabulated values (Brazilian Tables, 2024; CVB, 2021; FEDNA, 2021; INRAE-CIRAD-AFZ, 2022; Table 1). Inversely, the same method can be used for adjusting the AMEn measured on ingredients for a common chemical composition.

III. RESULTS

Average measured AMEn values were in close agreement with average tabulated reference values (+45, -62, 79 and 74 kcal/kg DM for corn, wheat, soybean meal and soybean meal expeller), except for corn DDGS (-262 kcal/kg DM) (Tables 1 and 2). Considerable within-ingredient variability was observed in chemical composition and AMEn contents, with coefficients of variation of 2.7, 6.5, 10.2, 3.6, and 11.1% AMEn for corn, wheat, soybean meal, soybean meal expeller, and DDGS, respectively. Predicted GE and AMEn values are presented in Table 2. The differences between measured and predicted averaged 58, 117, 60, 10 and -151 kcal GE /kg DM and 70, 16, 193, 107 and -28 kcal AMEn per kg DM for corn, wheat, soybean meal, soybean meal expeller, and DDGS, respectively. A covariance analysis using raw material as a fixed effect (n = 5) and measured AMEn as covariate also indicates a rather good agreement between measured and predicted AMEn values. Model presented significant effect of measured AMEn, significant raw material effect ($P < 0.001$), interaction was not significant ($P = 0.66$), $R = 0.93$ and Residual standard deviation = 174 kcal/kg DM. The common slope was 0.91 and allowed us to evaluate model quality. Intercept was 41, 59, -171, -22 and 0 kcal/kg DM for corn, corn DDGS, soybean meal, soybean meal expeller and wheat, respectively. It confirmed the strong agreement between observed and predicted values.

Table 2 - Raw materials nutrient content (% DM), gross energy (GE) and apparent metabolizable energy values corrected for zero N balance (AMEn ; kcal/kg DM) in broilers.

	Corn (n=68)	Wheat (n=44)	Soybean meal (n=49)	Soybean meal expeller (n=18)	Corn DDGS (n=76)
Chemical composition, % DM					
Dry matter	86.5 (1.4)[1]	87.5 (1.3)	88.9 (1.0)	94.9 (1.2)	88.8 (1.4)
Ash	1.5 (12.4)	1.7 (13.3)	7.6 (6.6)	6.4 (5.2)	5.2 (17.1)
Crude protein	8.5 (9.0)	13.0 (12.0)	53.0 (2.2)	45.8 (4.5)	31.4 (12.0)
Crude fat	4.4 (19.6)	2.4 (27.3)	2.3 (35.5)	8.6 (21.7)	11.2 (18.0)
Crude fiber	2.8 (16.7)	2.9 (16.1)	5.4 (31.3)	5.6 (11.0)	7.9 (11.0)
NDF	12.1 (15.2)	12.7 (10.2)	9.8 (27.1)	11.0 (11.8)	36.0 (7.2)
Starch	74.0 (3.4)	69.7 (3.3)	7.0 (20.7)	5.7 (12.2)	4.4 (45.2)
Energy utilization					
GE measured	4488 (1.0)	4450 (1.1)	4785 (1.1)	5043 (1.5)	5002 (2.8)
GE predicted[2]	4430 (1.0)	4332 (1.1)	4724 (1.1)	5033 (1.5)	5204 (2.7)
AMEn measured	3729 (2.7)	3287 (6.5)	2538 (10.2)	2891 (3.6)	2497 (11.1)
AMEn predicted[2]	3659 (3.1)	3271 (2.5)	2345 (3.9)	2784 (3.3)	2517 (8.7)

[1] mean and coefficient of variation between brackets
[2] Prediction using reference average tabulated values of Table 1 and differences in nutrient composition between measured ingredients and reference ingredients.

Inversely, the model based on nutrients content can be used for adjusting within each ingredient the 255 AMEn values for a common chemical composition that can be the average chemical composition of each reference ingredient (Table 2). These predicted AMEn values were 3719, 3371, 2504, 2849, and 2569 kcal/kg DM for corn, wheat, soybean meal, soybean meal expeller and corn DDGS with a reduced variability (2.4, 2.5, 8.3, 3.4 and 7.5% CV), which confirms the impact of variability in chemical composition on AMEn content of feedstuffs.

IV. DISCUSSION AND CONCLUSIONS

The results confirm that the nutrient-based prediction model provides accurate estimates of AMEn for five major broiler feed ingredients. But it requires robust reference values issued from feeding tables and/or in vivo measurements that can also be compared according to this model. Preliminary calculations on a larger number of ingredients would suggest that only averaging such tabulated values could be simplistic. In our conditions, measured AMEn values were consistent with published data, validating both the analytical methods and representativeness of ingredients dataset. However, a substantial variability was observed within ingredients, particularly for DDGS, reflecting the influence of grain source and processing conditions. Such variability generates challenges for precision nutrition, as discrepancies between expected and actual energy values can impair performance and efficiency. When NIRS calibration is not available, the nutrient composition-based model represents a practical tool for estimating ingredient AMEn. It enables rapid and standardized estimation while reducing reliance on labor- and cost-intensive in vivo assays.

In conclusion, composition-based prediction models can enhance the accuracy and efficiency of feed formulation. It provides also a way to safely consider changes for more precise energy system such as NE. Further refinement of model coefficients, particularly for highly variable by-products, would improve prediction robustness across raw materials.

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AN EVOLUTION IN MYCOTOXIN MITIGATION: TOWARD A HOLISTIC AND ROBUST MULTI-MODAL STRATEGY

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Summary

Building on over three decades of mycotoxin research, recent advances in blends of carefully selected ingredients and mechanistic understanding have enabled significant expansion of the mitigation capacity across a broader and more diverse spectrum of mycotoxins, yielding enhanced efficacy in practical applications. A comprehensive panel of mycotoxins—encompassing major classes produced by *Aspergillus*, *Fusarium*, and *Penicillium* genera, as well as key contaminants such as ergot alkaloids and other emerging *Alternaria*-derived toxins, or enniatins, have been systematically evaluated for interactive potential with various novel formulations. A specific and novel combination of functionalized yeast-based ingredients in combination with a bacterial biomass rich in proteins exhibited superior mycotoxin-binding affinities relative to individual components or previously tested mixtures. The enhanced interaction profile suggests a potential synergistic action that potentiates the sequestration capacity for high-prevalent and field-relevant mycotoxins commonly encountered in feed matrices, such as deoxynivalenol. Complementary biochemical analyses coupled with in vitro and in vivo biological assays have substantiated the functional benefits of this discovery, offering mechanistic insights into binding specificity, stability under gastrointestinal conditions, and potential downstream effects on animal health and performance.

I. INTRODUCTION

Mycotoxins are a diverse group of naturally occurring toxic secondary metabolites produced by various filamentous fungi, primarily belonging to the genera *Fusarium*, *Aspergillus*, *Penicillium*, and *Alternaria* (CAST, 2003). These compounds frequently contaminate a wide range of agricultural commodities, including cereal grains, forages, and stored feed ingredients, both pre- and post-harvest, under conditions conducive to fungal proliferation and secondary metabolism. The ubiquitous distribution of fungal spores in the environment, combined with climatic and agronomic conditions favorable to fungal growth and mycotoxin biosynthesis, has markedly increased the prevalence and complexity of mycotoxin contamination in the global food and feed supply chains. Advancements in high-throughput and multiplex analytical platforms—such as liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS)—have significantly expanded the capability to detect and quantify tens to hundreds of mycotoxin metabolites in any given sample (Jackson et al., 2012), revealing a far more pervasive contamination landscape than previously recognized.

II. PREVALENCE OF MYCOTOXINS

Historically, the Food and Agriculture Organization (FAO) estimated in the 1980s that approximately 25% of global crops were contaminated with mycotoxins (Park et al., 1999). However, modern surveillance utilizing refined sampling strategies and enhanced analytical sensitivity has revealed positive contamination frequencies between 60% and 80% in contemporary crop production systems (Eskola et al., 2019). These findings have also uncovered the frequent occurrence of "masked" or conjugated mycotoxins—metabolically modified forms not detectable by conventional methods—as well as a growing number of emerging and modified fungal metabolites whose toxicological significance is still under active investigation (Berthiller et al., 2013). Extensive global mycotoxin monitoring programs have demonstrated

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that over 92% of feed ingredients tested contain detectable levels of mycotoxins, with the average number of co-occurring mycotoxins per sample increasing from 5 to 8 over time (Weaver et al., 2021). The mycotoxin profiles vary significantly by geographical region, crop type, storage conditions, and climate. However, trichothecenes—notably deoxynivalenol (DON)—remain among the most prevalent, particularly those produced by *Fusarium* spp., followed by fumonisins (FB1, FB2), zearalenone (ZEA), and variable presence of *Aspergillus* and *Penicillium* toxins (e.g., aflatoxins, ochratoxin A, penicillic acid, and citrinin) which tend to appear in specific contamination "hot spots" (Ni et al., 2012; Kerry et al., 2017).

In addition to DON, increasing attention has been directed toward lesser-studied or emerging mycotoxins such as fusaric acid (FA), beauvericin, moniliformin, and enniatins, which are frequently detected but not yet routinely regulated. Despite the well-established toxicity of major mycotoxins, regulatory frameworks remain limited: in regions such as the EU and the U.S., only aflatoxin B1 (AFB1) in feed and its metabolite aflatoxin M1 (AFM1) in milk are subject to strict legal limits (Directive 2002/32/EC; FDA Guidance for Industry, 2000). Other compounds, including DON, ZEA, OTA, patulin, and fumonisins, are currently governed by guidance levels or non-binding recommendations (Commission Regulation (EC) No 1881/2006; Guidance for the Industry and FDA, 2010). One of the critical limitations in current regulatory policies is the inability to account yet for the co-occurrence and potential interactive effects of multiple mycotoxins within a single feed matrix, because of the complexity of mixtures encountered in the field and complexity of the dose-dependent relationship of toxins and impact on animals. Numerous in vitro and in vivo studies have demonstrated that mixtures of mycotoxins could exhibit additive, synergistic, or antagonistic toxicological interactions, thereby amplifying their adverse effects on animal health and productivity beyond what would be predicted from individual toxin levels (Alassane-Kpembi et al., 2015; Oh et al., 2017; Smith et al. 1997). While acute mycotoxicosis is well documented, chronic, low-dose exposure has now been characterized in the scientific literature as a major concern in livestock production and economics (Bryden, 2012; Grenier et al., 2013). Such exposure has been linked to subclinical effects including immunosuppression, reproductive dysfunction, impaired gut integrity, and reduced nutrient absorption, ultimately compromising animal performance and economic returns.

III. MITIGATION STRATEGIES

Given the inherent resilience of mycotoxins to conventional feed processing and storage practices, and the impracticality of achieving their complete elimination while maintaining optimal crop yields and ensuring feed safety and availability, there is a growing demand for efficacious in-feed solutions capable of neutralizing or deactivating mycotoxins post-ingestion when given to animals (Jouany, 2007). This mitigation strategy aims not only to reduce the toxicological burden on animals consuming contaminated feeds—often unknowingly—but also to safeguard the integrity of animal-derived food products. To be effective within commercial production systems, such solutions must undergo rigorous scientific evaluation to characterize their mode of action, spectrum of activity across multiple toxin classes, compatibility with various feed types, and amenability to the gastrointestinal environment of target species.

Research efforts have increasingly shifted toward the identification and development of biological or bioactive compounds—including microbial components, enzymatic systems, and microbial consortia—that can interact with a broad spectrum of structurally diverse mycotoxins, particularly those commonly co-occurring in complex feed matrices. Key development targets include achieving effective sequestration for relevant mycotoxins, multi-functional mitigation, using low inclusion levels in the diet and ensuring biological and nutritional compatibility, and environmentally responsibility, which allows for flexible formulations while minimizing environmental impact.

IV. NOVEL COMPONENTS TO BIND MYCOTOXINS

The foundational mechanisms underlying mycotoxin sequestration have been extensively studied, with numerous investigations demonstrating that yeast-derived parietal components can establish effective chemical interactions with mycotoxins via physicochemical and stereochemical complementarity between mycotoxins and β-glucans (Yiannikouris et al., 2004). These interactions are primarily governed by non-covalent binding mechanisms such as hydrogen bonding, van der Waals forces, and hydrophobic interactions, allowing selective adsorption of structurally diverse mycotoxins (Yiannikouris et al., 2006).

A novel composition integrating a functionalized yeast cell wall, including a protein-rich bacterial biomass (YCWB), with or without the inclusion of algal-derived material, has demonstrated, when used together, a marked enhancement in mycotoxin sequestration efficacy evaluated through specific biochemical assays. Comparative in vitro analyses revealed a twofold improvement in deoxynivalenol (DON) binding and an almost threefold increase in fusaric acid (FA) adsorption, relative to conventional yeast-based technologies (Figure 1).

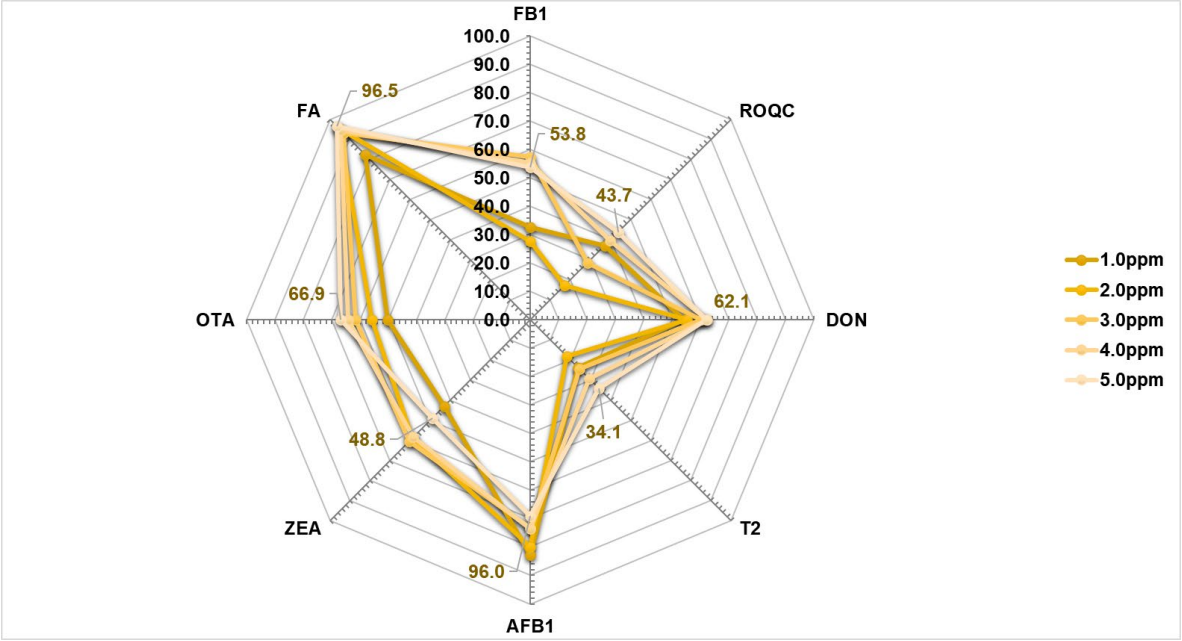

Figure 1 - Spider-web representation of the percent of mycotoxin adsorption by a functionalized yeast cell wall with a protein-rich bacterial biomass by-product (YCWB), tested against 5 titers of mycotoxins ranging from 1.0 to 5.0 µg/mL (or ppm) with an iso-mixture of 8 mycotoxins, comprising aflatoxin B1 (AFB1), zearalenone (ZEA), ochratoxin A (OTA), fusaric acid (FA), fumonisin B1 (FB1), Roquefortin C (ROQC), deoxynivalenol (DON), T-2 toxin (T2), and evaluated in a reaction media maintained at pH3.0, 37°C under orbital agitation.

To further evaluate the biological relevance of the formulations, in vitro studies were conducted using swine jejunal epithelial cells—a physiologically pertinent model, given that the small intestine is the primary site of exposure to ingested mycotoxins. The impact of the novel composition on cellular viability was assessed under controlled exposure to deoxynivalenol (DON) alone, and in combination with fusaric acid (FA), both of which are commonly co-occurring *Fusarium* toxins, as confirmed by global feed surveillance data (Weaver et al., 2021). When cells were exposed to YCWB and DON alone at concentrations ranging from 0.5 to 10 ppm, a dose-dependent improvement in cell viability, achieving an average viability recovery of 33%, was found with the tested material. With a combined DON and FA, the YCWB formulation demonstrated a relative viability restoration reaching up to 58% (Figure 2). These findings highlight the capacity of YCWB to mitigate the cytotoxic effects of individual and co-occurring mycotoxins at biologically relevant concentrations, further supporting its application as an effective in-feed strategy for intestinal protection and mycotoxin risk reduction.

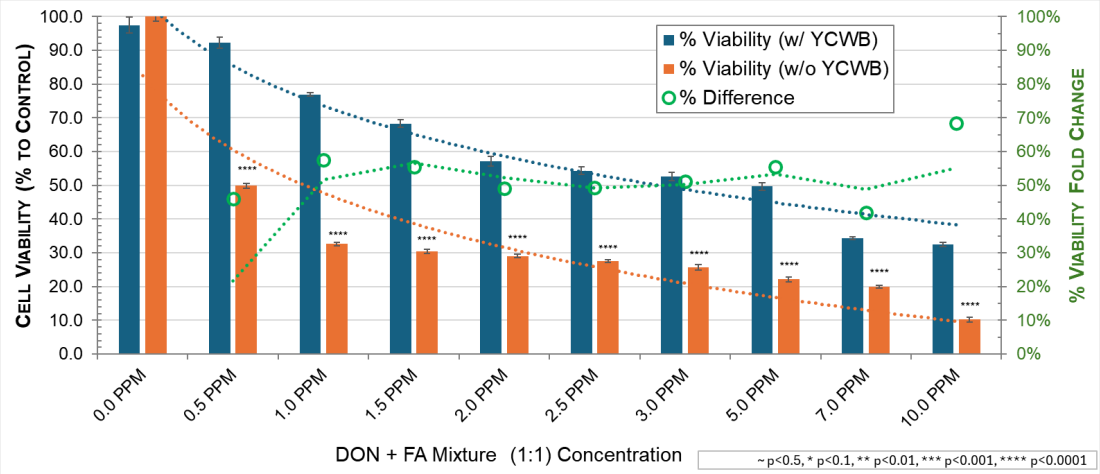

Figure 2 - Histogram of the percent cell viability relative to the control of IPEC-J2 cells in the presence of 9 different titers of deoxynivalenol (DON) and fusaric acid (FA) iso-mixture (0.5 to 10.0 µg/mL or ppm) with (blue bars) or without (orange bars) the use of the composition, comprising a functionalized yeast cell wall with a protein-rich bacterial biomass by-product (YCWB), at an inclusion rate of 0.2% (w/v). The differential viability percentage is displayed on the green curve. Student t test was used to evaluate statistical significance.

V. CONCLUSION

Overall, this next-generation composition represents a significant advancement in in-feed mycotoxin mitigation. It offers broad-spectrum efficacy, enhanced binding for both regulated and emerging mycotoxins, and flexibility for integration into diverse feed systems, positioning it as the next evolution for improving feed safety and protecting animal performance in mycotoxin-challenged production environments.

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STRATEGIES TO OPTIMIZE ENERGY UTILIZATION IN MODERN LAYING HENS TO EXTEND EGG PRODUCTION

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Summary

Egg production from a laying hen is a reproductive event, not direct growth. This nuance results in increased attention needed to interpret responses to dietary energy. Reliance on performance and production data tells an incomplete story, especially in laying hens selected for reduced body weight and increased feed efficiency where ability to alter feed intake with changes in dietary energy can be limited. A holistic approach is needed to fully understand laying hen responses to dietary energy. Hen body condition (lipid storage) is critical for egg production with a lack of body fat resulting in reduced egg production and excess body fat also resulting in reduced egg production. There are proven methods to maximize energy extraction from the diet including carbohydrate enzymes and at least some supplemental fat in the diet. Additional feed additives (lipase and emulsifiers) are being developed, but these have either been inconsistent or newly commercialized so less certainty around their responses in laying hens is warranted. Alternative feed additives that have not traditionally been associated with energy responses, need to be reevaluated in the context of reducing maintenance energy requirements and thereby increasing energy available for productive (reproductive) support.

I. INTRODUCTION

Dietary energy has traditionally been one of the top two expensive components of typical laying hen diets along with amino acids and proteins (Xie et al., 2024). In 2005, the Renewable Fuels Standard (RFS) linked feed corn to transportation through the support of ethanol production that was directly utilized as an additive/substitute for gasoline (Condon et al., 2013). The RFS was first expanded in 2007 resulting in ethanol production ramping up and corn prices spiking to above US$7.00/bushel in the short term before returning to approximately US$4.00/bushel as a new normal. This increase in the price of energy was mainly associated with the carbohydrate portion of the diet as starch and ultimately glucose is the feedstock for ethanol production. The RFS resulting in nearly 40% of the US annual corn crop being diverted into ethanol surpassing animal feed as the primary user of corn (USDAa, 2025). In 2019, the RFS was refunded to expand the use of food/feed oil to generate renewable diesel fuel (USDAb, 2025). This new funding will result in the processing of more soybeans to generate the needed oil in addition to other traditional feed oils to help satisfy the RFS goals for renewable diesel. This movement of feed oil to transportation will result in increased price pressure on feed/soybean oil. On positive side, this increased soy processing should result in more soybean meal availability. These additional funds for renewable diesel directly link feed oil to transportation resulting in continued and increased price pressures on dietary energy. As a result, the cost of feed energy will continue to drive the cost of egg production into the future. This requires a review of current and future technologies to better understand and maximize energy utilization in laying hens.

II. ENERGY IN LAYING HENS

The first step in understanding energy within the egg production/laying hen system is to review how best to measure energy utilization within the production system. Traditionally, feed conversion ratio or feed efficiency has been used to document changes in dietary energy within

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poultry models. This is especially true for meat birds that still have an ability to respond to differences in dietary energy with differences in feed intake and weight gain. Laying hens, especially light-framed white egg producing hens, have been selected for egg production and only moderate increases in feed intake. This results in a hen with a limited ability to alter feed intake with dietary energy differences. At least in the short term, less than 30 weeks, egg production is insensitive to differences in dietary energy (Murugesan and Persia, 2013; Lyons et al., 2023). This is especially true when laying hens are fed industry relevant diets (Lyons et al., 2023). Table 1 shows a list of manuscripts that report no differences in egg production with various concentrations of dietary energy.

Table 1 - White laying hen responses in egg production when fed reduced dietary energy over short (less than 30 wk) feeding periods.

Experiment	Dietary Energy or Reduction (kcal/kg)	Egg Production (%)	Experiment Duration (Wk)
Harms and Burns, 2004	3,067	81.6	8
	-130	82.4 ND↑	
	-220	81.7 ND↑	
Jalal et al, 2006	2,903	78.8	15
	-51	80.9 ND↑	
	-99	80.0 ND↑	
Jalal et al, 2007	2,903	83.9	28
	-90	83.2 ND↓	
Murugesan and Persia, 2013	2,884	92.6	12
	-90	93.7 ND↑	
Bobeck et al., 2014	2,903	88.3	24
	-77	89.2 ND↑	
	-154	88.6 ND↑	
Persia, Unpublished	2,855	95.3	16
	-66	93.7 ND↓	

As direct performance measurements are not always reliable indicators of energy status, alternative measurements should be utilized. In this case, measurements of body composition or abdominal fat pad weights have been shown to be more sensitive to changes in dietary energy in comparison to egg production or feed efficiency (Murugesan and Persia, 2013; Bobeck et al., 2014; Lyons et al., 2023). When examining the model closer, the relationship between reproductive events and minimum adipose reserves support the use of body composition or abdominal fat pad weights as a more sensitive response as the laying hen will cannibalize her own energy reserves to produce eggs before falling out of egg production. Egg production will continue until a minimum body fat composition is reached at which time egg production is reduced. This response is under studied in poultry species but well established across mammalian models (Herd and Sprott, 1996). It is noteworthy that once egg production is reduced and productive energy needs are reduced, body fat composition can recover with consistent energy intake. The response of laying hens to dietary energy is complex and varies

over time but requires consideration of all uses of energy within the body, not just egg production or feed conversion, but should also include some estimate of productive energy use, maintenance energy use and storage of energy.

III. DIETERY ENERGY MODULATION IN LAYING HENS

Several feed additives have been developed, researched and implemented commercially that improve the energy metabolism or net energy from common poultry diets. These feed additives commonly facilitate the utilization of one of the major carbon-containing nutrient classes including carbohydrate, lipids and protein. In this case, the focus will not be on extracting energy from the protein or amino acid fraction of the diet as deamination for energy utilization of amino acids results in increased N waste and is not a long-term solution (Latshaw and Zhao, 2011). Both carbohydrate and lipids are the major energy providing nutrients that can be increased with feed additive use or dietary manipulation.

Supplemental exogenous enzymes have been utilized to increase energy utilization in research dating back almost 100 years and in commercial use upwards of 20 years (Hervey, 1925). The two major carbohydrate enzymes in use and used in combination with other enzymes include xylanase and glucanase and have been shown to release 50 to 150 kcal/kg diet in poultry and swine depending on the substrate available in the diet (Bedford et al., 2024). The responses in an adult laying hen model have been inconsistent with most reports suggesting either positive FCR responses or positive digestibility/metabolic energy responses, but not within the same experiment. (Pirgozliev et al., 2010; Mirzale et al., 2012; Bobeck et al., 2014; Abdollahi et al., 2021; Nguyen et al., 2022). Although not as clear as in meat producing poultry, the evidence suggests that carbohydrate enzyme supplementation for laying hens does provide an alternative method to provide energy into the laying hen model.

Although fats and lipids are a smaller component of poultry diet than either carbohydrates or proteins, they are energy dense and positively contribute to energy metabolism and utilization both directly and indirectly. Directly, supplemental crude fat contents of 2 to 4% of the diet have been shown to reduce the passage rate of feed within poultry models resulting in increased overall dietary digestibility including dietary energy. This response is referred to as the extra caloric effect of fat within poultry diets (Miller et al., 1983). This response is interesting to consider as one of our methods to use energy enzymes is to reduce the amount of supplemental dietary fat within the diets in exchange for the energy released from the diet by the enzyme. This response is important to monitor as the removal of the supplemental oil might reduce the extra caloric effect of supplemental fat, especially in diets higher in corn or other high energy grains or in diets formulated to lower dietary energy.

Enzymes have also been evaluated to increase the energy digestibility of lipids and fats in poultry diets with mixed results. In growing broilers, Hu and others (2018) showed decreased FCR, and increased either extract digestibility and nitrogen corrected apparent metabolizable energy with the feeding of a lipase enzyme. In contrast, dietary lipase had no positive effects on broiler chickens in other experiments (Meng et al, 2004; Movagharnejad et al., 2020). In a long-term laying hen experiment, lipase had no effect on egg production or feed efficiency but did increase yolk weight and polyunsaturated fatty acid content (Lichovnikova et al., 2002). The mixed results in young growing birds and the lack of performance differences in laying hens suggests that lipase use may have limited value in laying hen diets.

Alternative feed additives have also been investigated to increase the fat or lipid content of poultry diets. Dietary emulsifiers have been shown to increase broiler performance and ileal fat digestibility (Movagharnejad et al., 2020; Haetinger et al., 2021). Laying hen models have been evaluated, but not as thoroughly. Two experiments have shown positive responses when emulsifiers have been added to reduced energy diets in laying hens, but other supporting response criteria such as digestibility or body composition have been limited to missing (de

Oliveira et al., 2021; Ferreira et al., 2022). This lack of supporting data for either lipid digestibility or body composition allow for questions to be asked about the performance responses as hens will reduce body weight and body fat before they reduce egg production (Lyons et al., 2022).

IV. REIMAGINING ENERGY MODULATION IN LAYING HENS

From a nutrition standpoint, energy is usually considered from a dietary standpoint, but one consideration that is not usually accounted for is alterations of maintenance energy requirements and is an opportunity to rethink and expand thought processes around energy use in laying hens. Direct fed microbials (DFM) have typically been used in laying hens for multiple purposes, but review of the literature reveals that egg production can be increased with DFM supplementation, especially later in the productive cycle (Panda et al., 2003; Yoruk et al, 2004; Khan et al., 2011; Abdelqader et al., 2013a,b; DeLeon et al., 2023). This increased egg production is associated with increased energy needs, but only a few DFM have been developed to produce dietary enzymes during their normal metabolism in the bird digestive track that might lead to increased dietary energy utilization. Most DFM have been developed to modify immune responses or to interact more directly with the intestinal microbial population. Even with this focus on non-digestive processes with DFM development, it has been shown that DFM can increase egg production energy in laying hens (Lyons et al., 2025). In this case, egg production energy is defined as the amount of energy captured and produced daily during egg production and is measured as the gross energy content of the internal component of the egg times the amount of yolk and albumin produced daily. This measurement suggests that DFM can increase productive energy in a laying hen model. But the question remains is where is that energy coming from? In the Lyons and coworkers (2025) data, feed intake was not altered so it did not contribute to the increased energy. Body weights and body composition were not altered, or in one case was increased. It cannot be said with certainty as energy digestibility measurements were not completed, but the 2 of the 3 DFM evaluated were not designed to produce excess dietary enzymes within the digestive tract and were generated to modulate the immune system and microbial responses, so it is unlikely that the DFM directly increased dietary energy utilization. Nonetheless, this excess productive energy must come from some source. We suspect that this energy in fact was spared by alterations to the immune system resulting in reduced maintenance energy and freeing up excess productive energy. Additional research and verification is needed to truly demonstrate this response, but this line of thought does expand our ability to further classify energy responses within the complex laying hen model.

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DEVELOPMENTS TOWARDS PRECISION NUTRITION FOR POULTRY

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As precision agriculture technologies become cheaper, smart feed blending systems are now becoming affordable and offer a promising return on investment for poultry production facilities. Broiler chickens grow rapidly with nutrient requirements changing daily. However, broilers are fed 3-5 diet stages throughout their growth, meaning nutrients are under- and over-supplied throughout production (Kleyn, 2013). Thus, blending rations daily utilising feed blending technology to meet the daily energy and lysine requirements was demonstrated to improve feed efficiency and reduce the coefficient of variation in broiler flocks (Nawab et al., 2025). Importantly, it demonstrated a good return on investment for the installation of the feed blending systems required. However, it is sensible that nutrient requirements do not only change gradually over time as an animal age, but there are also diurnal variations present. The most obvious example is a laying hens' metabolism which undergoes a cyclic process to produce eggs, requiring relatively higher dietary protein and energy in the morning (AM) and higher calcium (Ca) in the afternoon/evening (PM) than the rest of the day (De Los Mozos et al., 2012). Therefore, a feeding strategy termed AM/PM or split feeding, which offers high protein and energy diets during the AM and high Ca diets during the PM has been optimised for feed efficiency and nutrient digestibility (Akter et al., 2025). In a follow-up study, the optimised diet then demonstrated significant improvement in feed efficiency, tibia ah content, tibia breaking strength, range use and reduce feather pecking compared to hens offered a standard commercial feeding program in free-range laying hens (Jahan et al., 2025). Further improvements were made to this diet in optimising the fine:coarse limestone ratio of AM/PM diets providing further advantage (Moss et al., 2024). Recently, there is increased attention as to the importance of circadian rhythms in optimising the growth of animals. For example, recent research in fish demonstrates that circadian rhythms control muscle development, and that it is cyclic throughout the day/night and not continuous (Kelu 2020). Thus, consideration of the circadian rhythm may be important in optimising muscle deposition and broiler production. Recent research in this field and practicalities for production will be discussed.

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REDUCING DIETARY CRUDE PROTEIN ENHANCES ILEAL AMINO ACID DIGESTIBILITY AND DECREASES CECAL MICROBIAL DIVERSITY IN BROILER BREEDERS

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Reliance on imported soybean meal may present considerations for the long-term sustainability of chicken meat production in Australia. Reducing dietary crude protein (CP) is a potential strategy to moderate this dependency. However, excessively low CP diets are associated with negative impacts on productivity due to potential amino acid deficiencies. In addition, low CP diets may shift bacterial fermentation profiles (Yuan et al., 2024) and less is explored in broiler breeders. The current study investigated the effects of a moderate decrease in dietary CP on amino acids digestibility and cecal microbiota composition. It was hypothesised that a moderate decrease in CP diet would improve production efficiency by increasing amino acids digestibility and changing microbiota profiles.

A total of 103 Ross 308 broiler breeders (91 hens and 12 roosters) were allocated to three dietary treatments with CP levels of 16% (high), 14% (standard), and 12% (low), each having four replications (seven or eight hens per replication), over a three-week period (36–38 weeks of age). Diets were iso-caloric and essential amino acids were maintained or exceeding the requirements (Aviagen, 2021). At the end of the trial, ileal digesta was collected from ileum of four birds from each pen, pooled and freeze dried for further analysis. Amino acids and digestible marker were analysed both in feed and digesta samples. Acid insoluble ash was used as a digestible marker, and the digestibility coefficient was calculated based on the ratio of marker in feed and digesta. In addition, cecal content was collected from eight birds per treatment for microbiota analysis. DNA was extracted using the QIAamp® PowerFecal® Pro DNA Kit (Catalogue No. 51804; QIAGEN) and full-length sequencing of the 16S rRNA gene was conducted by the Australian Genome Research Facility using the PacBio platform (AGRF Ltd., Melbourne, VIC, Australia). The digestibility data were analysed using PROC GLM in SAS software (SAS 9.4). The HiFi reads were processed using the Nextflow workflow (version 23.04.5). Taxonomic classification was performed with the VSEARCH classifier against the Silva 138.1 database. Read counts across samples were analysed with MicrobiomeAnalyst.

The results showed that the apparent ileal digestibility of lysine, leucine, valine, isoleucine, phenylalanine, proline, glycine, and glutamic acid were significantly higher in the 12% CP group compared to the 14% CP group ($P < 0.05$). This might be a consequence of the higher levels of crystalline amino acids in the low compared to the standard CP diets. Microbiota analysis using 16S rRNA sequencing revealed that the top three classified phyla (representing 70% of the microbial population) across CP levels in a descending order were: Firmicutes > Bacteroidota > Proteobacteria. The top five classified genera independent of CP levels were: Phocaeicola (17.2%) > Ligilactobacillus (11.6%) > Lactobacillus (10.9%) > Blautia (5.7%) > Mediterraneibacter (5.6%). The 12% CP groups showed a significant decrease in alpha diversity indices (Chao1 and ACE; $P < 0.05$). The β-diversity analysis (PERMANOVA, Bray-Curtis) showed that the 12% CP group differed from both 14% (FDR = 0.003) and 16% CP (FDR = 0.0045), while no significant difference was observed between 14% and 16% CP (FDR = 0.208). Linear Discriminant Analysis Effect Size (LEfSe) revealed that the relative abundance of *Butyricicoccaceae* at the family level and *Butyricicoccus_A* at the genus level were significantly higher in the 16% CP. These bacteria are known butyrate producers which contribute to intestinal epithelial health and anti-inflammatory signalling (Kien et al., 2007).

In conclusion, reducing dietary CP to 12% increased ileal amino acid digestibility and altered microbial composition. This microbial shift could affect progeny gut development through microbiota mediated maternal programming and warrants further investigation.

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THE EFFECTS OF ARGININE, GUANIDINOACETIC ACID AND CITRULLINE SUPPLEMENTATION IN REDUCED PROTEIN DIETS IN AGED LAYING HENS

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Arginine is a key AA involved in multiple metabolic pathways, notably protein synthesis, growth hormone stimulation, and nitric oxide production (Kwak et al. 1999). Nitric oxide regulates pituitary nitric oxide synthase activity, and influences the secretion of hormones supporting follicular development and ovulation, potentially increasing egg production, egg weight, and egg mass (Uyanga et al. 2022). Despite the importance for egg production, research on the use of crystalline arginine (Arg), guanidinoacetic acid (GAA), and citrulline (Cit) in older laying hens fed reduced protein diets is lacking. This study evaluated the effects of Arg, GAA, and Cit supplementation in reduced protein diets on the production performance, egg quality, nutrient digestibility, serum uric acid level and bone quality of aged laying hens. A total of eight dietary treatments were allocated to 13 replicate cages of two hens per cage per treatment (n = 208) from 60 to 75 weeks of age. The dietary treatments included a standard protein (SP) diet (CP = 15.8%, AME = 2690 kcal/kg, Ca = 4%, digestible Arg = 876), a reduced protein (RP) diet (CP = 13.8%, AME = 2690 kcal/kg, Ca = 4%, digestible Arg = 0.704), and RP diets supplemented with two levels (0.06% or 0.12%) of either Arg, Cit or GAA at the expense of wheat. A significant Arg source × level interaction was observed for feed intake during 60–67 weeks (P = 0.007) and 60–75 weeks (P = 0.014), where increasing Cit supplementation increased feed intake, while Arg and GAA did not. Additionally, Cit supplementation as a main effect significantly increased egg weight (P = 0.049) compared to GAA from 60–67 weeks. From 68–75 weeks, Cit supplementation improved egg mass (P = 0.014) and feed intake (P = 0.009) compared to Arg and GAA. Over the entire 60–75 week period, Cit increased egg weight (P = 0.048) and egg mass (P = 0.019) compared to GAA. Hens offered the RP diets with Cit supplementation had the lowest feed cost per kilogram of eggs produced from 60 to 75 weeks of age (P = 0.007). Thus, a moderate reduction in dietary protein level combined with Cit supplementation was effective in enhancing egg mass, egg weight and economic returns in aged laying hens. Cit produced a greater Arg sparing effect than Arg itself, likely due to Cit resisting catabolism by intestinal arginase (where Arg may be catabolized) (McCarty 2010), or Arg may be more greatly catabolized in the liver (Allerton et al. 2018).

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EFFECTS OF A COMMERCIAL TRIPLE-STRAIN *BACILLUS*-BASED PROBIOTIC ON EGG QUALITY, PERFORMANCE, WELFARE AND LIVABILITY OF LAYING HENS

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Summary

Sustaining persistency of lay in commercial hens requires continuous attention to egg quality, feed efficiency, and bird welfare throughout the laying cycle. Nutritional interventions that establish a balanced gut microbiota from the first day of life can positively influence long-term productivity and health. This study evaluated the impact of dietary supplementation with a commercial triple-strain Bacillus-based probiotic on production performance, egg quality, livability, and welfare in laying hens. Three hundred sixty Leghorn day-old chicks were assigned to two treatments: one control group (CON) receiving a standard diet, and a probiotic group (PRO, 1.6×10^6 CFU/g of finished feed) from day old to 42 weeks of age. Each treatment comprised 30 replicates, with two cages per replicate and three hens per cage. The group of hens fed with PRO showed better zootechnical performance compared to CON ($p< 0.05$) with higher hen-day egg production (87,3 vs. 85,9 %), higher hen-housed production (85,9 vs. 84,1%) as well as better feed efficiency with a lower feed consumption per dozen eggs (1.242 vs. 1.276 kg). Regarding egg quality parameters, egg weight was also in favor ($p< 0.05$) of PRO (56.4 vs. 55.9g), but there was no significant difference between PRO and CON for Haught unit score (87.751 vs. 86.421 respectively) and eggshell thickness (0.420 vs. 0.413 mm respectively). Furthermore, PRO group did show higher circulating levels of serotonin (+20%) and lower mortality rate (-4% points) compared to CON ($p< 0.05$). In conclusion, continuous supplementation with a triple-strain *Bacillus*-based probiotic from hatch enhanced productivity, egg quality, and welfare without compromising egg integrity, supporting its role as an antibiotic-free tool for sustainable layer production.

I. INTRODUCTION

Modern laying hens are required to maintain high productivity over extended cycles, yet sustaining persistency of lay while preserving egg quality remains a significant challenge (Bain et al., 2016) . As hens age, both egg productivity and quality often decline, this deterioration is frequently accompanied by increased mortality and reduced liveability. These challenges compromise both welfare and economic efficiency.

Early establishment of a healthy gastrointestinal microbiota has been shown to support nutrient utilization, immunity, and resilience against stress. Probiotics, particularly triple-strain *Bacillus* formulations, are highly suitable for layer production because they survive feed processing, germinate in the gastrointestinal tract, and beneficially modulate microbial balance (Khan et al., 2020). Previous studies have demonstrated improvements in productivity, immune function, and welfare in poultry receiving *Bacillus*-based probiotics. (Meuter et al., 2024).

The objective of this study was to evaluate the effects of a commercial triple-strain *Bacillus*-based probiotic, administered continuously from hatch to 42 weeks of age, on production performance, egg quality, livability, and welfare indicators in laying hens under controlled experimental conditions.

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II. METHOD

A total of 360 day-old White Leghorn chicks were randomly allocated to two treatment groups: control (CON), fed a standard layer diet, and probiotic (PRO), fed the same diet supplemented with a commercial triple-strain *Bacillus* probiotic (GalliPro® Fit, 1.6×10^6 CFU/g feed). Each treatment consisted of 30 replicates. Each replicate comprised two adjacent conventional cages, and each cage housed three birds. Treatments were coded and randomly assigned. Mash Feed will be provided throughout the study via one trough feeder per cages the feeding phases will be as follows: Starter 1 (Week 0 – 4), Starter 2 (Week 4 – 8), Grower (Week 8 – 12), Developer (Week 12 – 16), Pre-Lay (Week 16 – 18), Peak (Week 18 – 36), Layer (Week 36 – 42).During the grow up phase (W0-18), birds were housed in an environmentally controlled facility and placed in floor pens cotaining an appropriate depth of clean wood shavings. All birds received were evenly divided and randomized into two pens (1 pen/treatment) and tagged in the neck for identification purposes. Following transfer to cages, birds were randomizedinto replicate cages of the same treatment. Birds receivd additional tags in the wing so that birds within a replicate had consecutive tag numbers for future data collection.. The birds were housed in standard cages providing approximately 632 cm^2 per bird, under environmentally controlled conditions with consistent temperature, humidity, and lighting across treatments. Feed and water were provided ad libitum. Mortality was recorded daily, and necropsies were performed to determine causes of death. Body weghts were measured at 0, 4, 8, 12, 16, and 40 weeks of age. Feed intake and conversion were calculated at defined intervals. Egg production was recorded daily from week 20 through week 40 and expressed as both hen-day (HD) and hen-housed (HH) percentages. Egg weights were measured daily, and egg quality assessments (Haugh unit, shell thickness, shell color) were performed at weeks 20, 24, 28, 32, 36, and 40. Welfare indicators included cumulative mortality and circulating serotonin levels.. The latter was assessed at week 16 and 1.5-mL blood samples were collected from the basilica vein of 30 birds per treatment by using syringes containing 0.1 mL of a 0.9% sodium chloridesolution containing 50 mg/mL of EDTA.The plasma was separated by centrifugation and was stored frozen at −4°C until analysis. Plasma 5-HT levels were measured by using a commercial enzyme immuno-assay kit. Data were analyzed using a general linear model (JMP 18, SAS Institute), and treatment means were compared using Dunnett's post-hoc test. Statistical significance was determined at $p < 0.05$.

III. RESULTS

At 16 weeks of age, there was no significant difference between groups on uniformity (ranging from 89% for PRO to 92% for CON) and average bird weight (ranging from 1.10 Kg for PRO to 1.12 Kg for CON). Furthermore, circulating serotonin levels were 20% higher in the probiotic group at week 16 ($p<0.05$, Figure 5) before their transfer to the cages, suggesting reduced stress and improved overall well-being during the pullet rearing phase. Probiotic supplementation improved key production performance parameters. From weeks 22 to 42, hens receiving the probiotic maintained higher hen-day egg production, averaging 87.3% compared with 85.9% in the CON group ($p<0.05$, Figure 1). Hen-housed egg production was also significantly higher in PRO fed hens at 85.9%, compared with 84.1% in CON fed hens ($p<0.05$, Figure 2). These results indicate that probiotic supplementation supported persistency of lay during mid-lay.

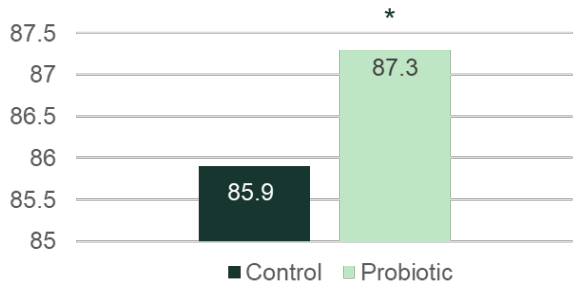

Figure 1 - Hen-Day Egg Production (HD)% W22-42 period (*p<0.05).

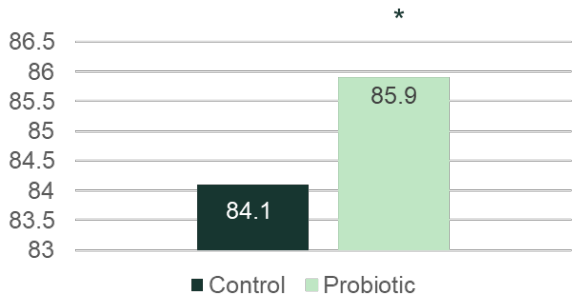

Figure 2 - Hen-Housed Egg Production (HH)% W22-42 period (*p<0.05).

Feed efficiency was also enhanced in the probiotic group. Hens receiving probiotics consumed only 1.242 kg of feed per dozen eggs, compared with 1.276 kg in the control group, demonstrating superior feed-to-egg conversion (p<0.05, Figure 3). Egg weights were significantly higher in PRO hens at 56.4 g compared with 55.9 g in CON hens (p<0.05, Figure 4). However, no significant differences were observed between treatments for Haugh unit scores, which averaged approximately 87 in PRO hens and 86 in CON hens, or for eggshell thickness, which was 0.42 mm in PRO hens and 0.41 mm in CON hens. These findings confirm that the probiotic enhanced egg mass without compromising internal or external egg quality.

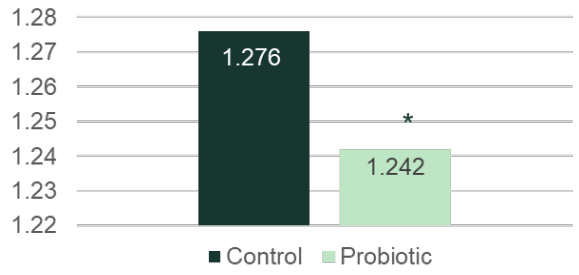

Figure 3 - Feed Consumption per Dozen Eggs Kg W22-42 period (*p<0.05).

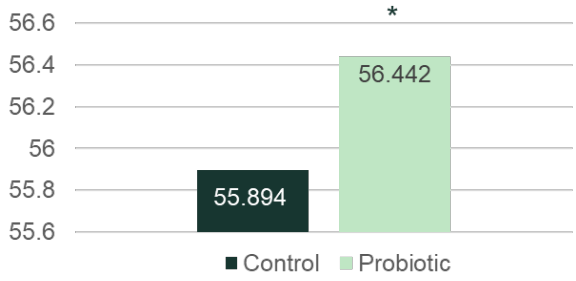

Figure 4 - Egg Weight, g W22-42 period (*p<0.05).

Probiotic supplementation also improved welfare outcomes. Mortality was significantly reduced by approximately 4 percentage points in PRO hens compared with CON hens (p<0.05, Figure 6).

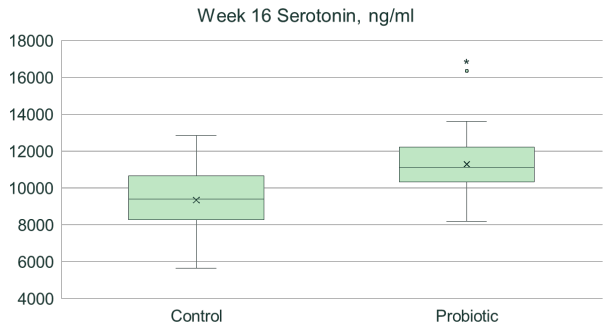

Figure 5 - Circulating Serotonin levels at week 16 (p<0.05)

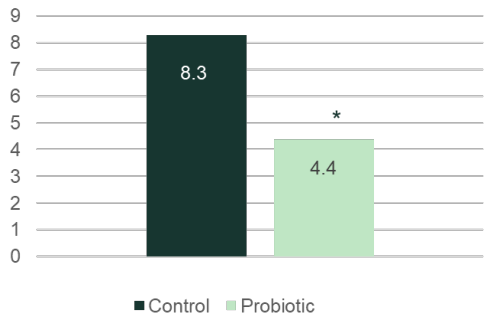

Figure 6 - Mortality % from 18 to 42 w of age (*p<0.05).

IV. DISCUSSION

The findings of this study demonstrate that continuous supplementation with a triple-strain Bacillus probiotic positively influenced both performance and welfare in laying hens. The probiotic contributed to improved persistency of lay, as shown by higher hen-day and hen-housed egg production. The reduction of approximately 40 g of feed per dozen eggs represents a significant improvement of feed efficiency and a meaningful economic saving in large-scale operations. Improvements in laying rate (+1.8 pts), egg weight (+0.5 g), and feed conversion ratio (–3 pts) resulted in a calculated ROI of 37 AUD/t feed, demonstrating the tangible financial value of this intervention. In terms of egg quality, the probiotic improved egg weight, confirming more efficient nutrient assimilation, while maintaining Haugh unit scores and shell thickness. This indicates that the benefits in productivity were achieved without compromising the physical or internal quality of the eggs. Welfare findings were also notably improved with lower mortality rates during the whole trial period, combined with elevated serotonin levels at the end of the rearing phase. This is an important consideration for both producers and consumers, as welfare-friendly production systems are increasingly demanded by the market. Overall, the study confirms that supplementing feed of layers with the commercial triple-strain Bacillus probiotic can serve as an effective antibiotic-free nutritional tool to enhance productivity and welfare in layers.

V. CONCLUSION

Feeding a commercial triple-strain Bacillus probiotic continuously from hatch to 42 weeks of age translated into clear economic and welfare benefits for egg producers. Beyond improved productivity, the consistent gains in performance and livability showed the potential of the probiotic solution to sustainably optimize the output and welfare of hens throughout the production cycle.

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EFFECTS OF ENERGY AND LYSOLECITHIN LEVELS ON EGG PRODUCTION, PRODUCTIVE PERFORMANCE AND FAT DEPOSITION ON POST-PEAK BROILER BREEDERS

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Summary

This study investigated the effects of dietary energy and lysolecithin (LPL) supplementation on reproductive performance and fat deposition in post-peak broiler breeders. A total of 264 Ross 308 breeders (240 hens and 24 roosters) at 50 weeks of age were assigned to a 2 × 2 factorial arrangement with two energy levels (2800 vs. 2760 kcal ME/kg) and two LPL inclusion levels (0 vs. 500 g/t). The trial lasted 12 weeks, during which egg production, body weight, egg mass, feed efficiency, and organ weight were evaluated. Results showed that LPL supplementation significantly improved egg production during 58–61 weeks ($P = 0.03$), leading to a higher overall laying rate ($P = 0.02$). Hatchable egg utilization also increased during 50-53 weeks ($P = 0.03$) with a tendency for improvement on the overall period ($P = 0.08$). Egg weight was consistently increased by both higher energy and LPL ($P < 0.01$), with LPL further enhancing egg mass and feed conversion ratio during late production. Importantly, LPL markedly reduced abdominal fat deposition ($P = 0.001$) without affecting body weight or liver weight. In contrast, dietary energy level alone did not show effects on reproductive or productive performance. In conclusion, LPL supplementation supported the persistency of egg production and reduced excessive fat accumulation in aging breeders. These results highlight LPL as a potential nutritional strategy to maintain reproductive performance and flock fertility during the late laying phase.

I. INTRODUCTION

Fertility management in broiler breeders is a key determinant of hatchery output and flock welfare. However, maintaining persistency of post-peak egg production remains challenging. Between 40 and 60 weeks of age, hens exhibit declines in egg production and hatchability due to physiological and metabolic changes. Excessive fat accumulation in the uterovaginal junction of overweight hens further reduces sperm storage capacity and compromises fertility (Yu et al., 1992; Robinson et al., 1993). In order to mitigate these risks, feed restriction programs are commonly used to control body weight gain of hens and egg mass; however, excessive restriction post-peak may impair laying performance and welfare. Lysolecithin (LPL), a hydrolysed lecithin, has been shown to enhance nutrient digestibility and absorption by upregulating intestinal nutrient transporter expression and improving micelle formation in laying hens (Han et al., 2010; Juntanapum et al., 2020). Several studies further demonstrated that LPL improves nutrient utilization and feed efficiency in poultry (Boontiam et al., 2017; Chen et al., 2019). Improved nutrient efficiency may sustain laying performance and reduce fat deposition, thereby supporting reproductive longevity in breeders. Despite these findings in broilers and laying hens, evidence in broiler breeders during the late laying period is limited. The objective of this study was to evaluate the effects of dietary LPL supplementation and dietary energy level on egg production, productive performance, and fat deposition in broiler breeders between 50 and 61 weeks of age. It was hypothesized that dietary LPL supplementation in a low-energy diet would sustain post-peak laying performance comparable to that of a higher-energy diet by enhancing nutrient utilization and reducing excessive fat deposition.

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II. METHOD

A total of 264 Ross 308 broiler breeders (240 hens and 24 roosters) at 50 weeks of age, with similar physiological status, were selected and assigned to experimental treatments for 12 weeks. The trial was conducted at Isfahan University of Technology (Isfahan, Iran) with the approval of ethical committee in the University. Birds were allocated to a 2 × 2 factorial design consisting of two dietary energy levels (2800 and 2760 kcal ME/kg) and two dietary LPL levels (0 and 500 g/ton of feed). Each treatment consisted of six replicates, with 10 hens and 1 rooster per pen. Experimental diets were corn–soybean meal-based and formulated to contain 13% crude protein and 0.56% digestible lysine. Dietary energy was adjusted by varying the inclusion level of soybean oil, which consequently altered the linoleic acid concentration of the diet (1.35% in the high-energy diet vs. 0.95% in the low-energy diet). LPL was provided using a commercial source (Lipidol®, Pathway Intermediates). Birds were managed under standard breeder conditions. Body weight was measured individually at 50 and at 53, 57, and 61 weeks of age. Eggs were collected four times daily; egg production and hatchable egg utilisation were recorded on a per-hen basis. Hatchable egg utilisation was calculated as the percentage of hatchable eggs, determined by the ratio of total hatchable eggs to total eggs laid in each pen. Feed conversion ratio (FCR), coefficient of variation (CV), and egg mass were calculated in three consecutive laying cycles (50–53, 54–57, and 58–61 weeks of age). At the end of the trial, five hens per replicate were randomly selected, humanely euthanized followed by FASS guideline (FASS, 2010) and liver and abdominal fat were excised and weighed. Data were analyzed using the GLM procedure of SAS (SAS Institute, USA) under a completely randomized design. The fixed effects of dietary energy, LPL and their interaction were included in the model. Tukey's test was applied to determine significant differences among treatments, with significance declared at $P < 0.05$.

III. RESULTS

The effects of dietary energy and LPL levels on egg production are presented in Table 1. LPL supplementation increased egg production during 58-61 weeks (P = 0.03), resulting in a higher overall laying rate across the trial (P = 0.02). In addition, hatchable egg utilization was increased during 50–53 weeks (P = 0.03) and was tended to improve overall (P = 0.08) by LPL supplementation. Dietary energy level alone had no effect on egg production or hatchable egg utilizaton.

Table 1 - The effect of energy and LPL levels on laying performance of broiler breeders.

Energy (ME/kg)	2800 kcal		2760 kcal		SEM[1]	*P*-values		
LPL (g/t)	0	500	0	500		ME	LPL	ME x LPL
Egg Production, %								
50-53 week	74.16	77.38	77.20	78.61	1.76	0.31	0.28	0.66
54-57 week	76.48	76.60	75.24	78.01	1.70	0.96	0.47	0.51
58-61 week	71.05	76.07	71.36	76.77	0.50	0.82	0.03	0.93
Overall	73.22	75.83	74.68	78.79	1.20	0.13	0.02	0.60
Hatchable egg utilization, %								
50-53 week	94.37	98.17	95.08	96.04	0.86	0.49	0.03	0.18
54-57 week	93.68	96.33	94.01	94.14	1.10	0.48	0.30	0.34
58-61 week	88.29	89.98	88.39	89.54	1.23	0.90	0.34	0.85
Overall	92.11	94.83	92.81	92.92	0.65	0.44	0.08	0.10

[1] Standard error of the mean.

The productive performance results are summarized in Table 2. Egg weight was consistently increased by both higher energy and LPL supplementation (P < 0.01). Consequently, egg mass was significantly higher in LPL-fed hens during 58–61 weeks (P = 0.01). FCR was also improved by LPL supplementation during the late laying period (P = 0.01). Average body weight and coefficient of variable eggs were unaffected by either energy level or LPL supplement.

Table 2 - The effect of energy and LPL levels on productive performance of broiler breeders.

Energy (ME/kg)	2800 kcal		2760 kcal		SEM[1]	*P*-values		
LPL (g/t)	0	500	0	500		ME	LPL	ME x LPL
Average body weight, g								
50-53 week	4126	4150	4125	4224	69.61	0.97	0.23	0.35
54-57 week	4146	4115	4125	4119	19.80	0.71	0.44	0.59
58-61 week	4282	4282	4260	4266	23.20	0.91	0.50	0.92
Egg weight, g								
50-53 week	63.80	64.89	63.39	64.42	0.13	0.01	0.001	0.84
54-57 week	64.43	65.23	63.76	64.58	0.19	0.009	0.002	0.98
58-61 week	64.93	65.76	64.23	64.97	0.25	0.001	0.001	0.76
Egg mass, g								
50-53 week	47.31	50.21	48.94	50.64	1.08	0.43	0.09	0.65
54-57 week	49.34	49.23	47.97	50.39	1.04	0.92	0.35	0.31
58-61 week	46.11[b]	50.02[a]	45.86[b]	49.83[a]	0.35	0.89	0.01	0.96
Coefficient of variable eggs, %								
50-53 week	7.03	6.06	7.43	6.36	0.45	0.66	0.22	0.95
54-57 week	7.60	5.93	7.00	6.11	0.54	0.80	0.15	0.65
58-61 week	6.05	5.88	5.98	6.56	0.50	0.72	0.81	0.67
FCR[2]								
50-53 week	3.35	3.15	3.23	3.13	0.07	0.42	0.09	0.57
54-57 week	3.19	3.19	3.27	3.14	0.06	0.83	0.42	0.41
58-61 week	3.38[a]	3.10[b]	3.39[a]	3.12[b]	0.08	0.84	0.01	0.97

[ab] Values within a column followed by different superscripts are significantly different. P < 0.05; Tukey's pairwise test.
[1] Standard error of the mean.
[2] Feed conversion ratio

The effects of treatments on organ weights are shown in Table 3**.** Abdominal fat deposition was markedly reduced in LPL fed hens (P = 0.001), while liver weight remained unaffected by either factor.

Table 3 - The effect of energy and LPL levels on organ weights of broiler breeders.

Energy (ME/kg)	2800 kcal		2760 kcal		SEM[1]	*P*-values		
LPL (g/t)	0	500	0	500		ME	LPL	ME x LPL
Liver	1.64	1.63	1.52	1.54	0.01	0.12	1.00	0.78
Abdominal fat	3.23	2.85	3.11	2.70	1.01	0.03	0.001	0.81

[1] Standard error of the mean.

IV. DISCUSSION AND CONCLUSION

The present study demonstrated that dietary lysolecithin (LPL) supplementation improved reproductive and productive performance in broiler breeders during the late laying period. Birds fed LPL showed higher egg production, improved egg mass, and better feed conversion ratio, particularly between 58–61 weeks of age. These findings are consistent with previous reports that LPL enhances nutrient digestibility and utilization in poultry (Han et al., 2010; Juntanapum et al., 2020; Boontiam et al., 2017; Chen et al., 2019). Importantly, the increase in egg weight and mass observed in this study remained within the normal range for Ross 308 breeder standards (Aviagen, 2021). Excessive egg weight is undesirable because it can impair shell quality, reduce hatchability, and negatively affect chick uniformity (Grobas et al., 1999). Therefore, the moderate improvement in egg mass with LPL supplementation suggests enhanced nutrient efficiency without the negative consequences of oversized eggs. Another notable finding was the reduction in abdominal fat deposition in hens fed LPL. Excessive fat accumulation in breeders has been linked to reduced sperm storage capacity and fertility decline (Yu et al., 1992; Robinson et al., 1993). By reducing abdominal fat while maintaining productive performance, LPL may contribute to sustaining fertility and extending the reproductive longevity of aging breeders. Similar reductions in fat deposition with LPL have also been reported in laying hens (Jiang et al., 2021), supporting the hypothesis that improved nutrient absorption reduces excessive lipid storage.

Dietary energy did not affect egg production, egg mass, or fat deposition. It should be noted that the energy reduction between treatments was relatively small (≈1.4%), which may fall within normal analytical or mixing variation. Therefore, the lack of a clear energy effect should be interpreted with caution. However, egg weight was higher in the high-energy diet. Egg weight is directly related to the linoleic acid concentration of the diet rather than the total fat content (Leeson & Summers, 2005). This result may be explained by the higher linoleic acid concentration in the high-energy diet due to the additional soybean oil inclusion.In conclusion, the results suggest that dietary LPL supplementation sustains post-peak egg production and persistency, and reduces abdominal fat deposition in broiler breeders.

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ANAEROBIC AND TWO-STAGE *BACILLUS SUBTILIS* FERMENTATION IMPROVE IN VITRO DIGESTIBILITY OF SUNFLOWER MEAL FOR POTENTIAL USE IN POULTRY FEED

A.M. ABDULRAHMAN[1,2] , K. KLIEM[1] and C. RYMER[1]

Summary

Soybean meal is the main protein source in poultry diets but raises sustainability and supply concerns. Sunflower meal (SFM) is a cheaper alternative, though limited by high fibre. This study evaluated solid-state fermentation (SSF) with *Bacillus subtilis* under different methods and temperatures using an 8 × 2 factorial design. Treatments included aerobic, anaerobic, and two-stage fermentations with respective controls at room temperature or 30 °C. After 14 days, anaerobic and two-stage fermentations significantly ($p < 0.05$) reduced fibre, prevented fungal growth, and improved crude protein and amino acid digestibility. These methods show promise for producing a sustainable cheap alternative to soybean meal in poultry feed.

I. INTRODUCTION

Soybean meal (SBM) remains the primary protein source in poultry feed (Beski et al., 2015). However, its extensive use raises sustainability concerns, including high water requirements, deforestation, and greenhouse gas emissions during production and transport (Ercin et al., 2012; Ferreira et al., 2016). This has driven interest in alternative protein sources that are cost-effective and environmentally sustainable (Sun et al., 2024). Sunflower meal (SFM) is widely available and affordable (Ciurescu et al., 2019), but its high fibre content reduces nutrient utilization in poultry (Lannuzel et al., 2022). Solid-state fermentation (SSF) is a practical strategy to improve the nutritional value of high fibre feedstuffs, as it operates without free-flowing liquid and produces products that can be used directly (Pandey, 2003). *Bacillus subtilis* is a suitable fermenting microbe due to its ability to degrade fibre, reduce anti-nutritional factors, and increase protein availability (Teng et al., 2012). Yet, aerobic fermentation may cause nutrient losses, amino acid depletion, and contamination risks (Heuzé et al., 2025; Liu et al., 2025). This study compares aerobic, anaerobic, and two-stage SSF of SFM with *B. subtilis*, focusing on in vitro amino acid digestibility under room temperature and 30 °C conditions.

II. METHOD

An 8 × 2 factorial design evaluated four *Bacillus subtilis* fermentation methods and their uninoculated controls at room temperature or 30 °C. Treatments included 14-day aerobic, 14-day anaerobic, 2-day aerobic followed by 12-day anaerobic, and 4-day aerobic followed by 10-day anaerobic fermentations. SFM was unsterilized, supplemented with 1% molasses, and each treatment was replicated three times. The inoculum, prepared as described by Choe et al. (2012), reached 5.3×10^7 CFU/mL and was applied at 5% (v/w). After 14 days, samples were freeze-dried and stored at −20 °C for chemical and amino acid analysis.

Crude protein (CP) was determined using the AOAC official method 2001.11 (AOAC, 2005), using a nitrogen-to-protein conversion factor of 5.29, as recommended for SFM by Mariotti et al. (2008). Neutral detergent fibre was measured with an Ankom 200 Fibre Analyser

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using F58 filter bags, following the manufacturer's protocol, and the values were subsequently corrected for ash (aNDF). Amino acids (AAs) concentration was quantified after acid hydrolysis (6 M HCl), in line with AOAC method 994.12 (AOAC, 2005) with minor modifications. Briefly, 200 mg of each sample was hydrolyzed using 10 ml 6M HCl at 110 °C for 24 h in sealed, nitrogen-flushed bottles, then neutralized with 8 ml 7.5M NaOH. Hydrolysates were diluted, adjusted to pH 3.3, filtered (0.2 μm), and stored at −80 °C. Analysis was performed using a Shimadzu LCMS-8050 triple quadrupole mass spectrometer coupled with a Nexera UPLC. Samples were injected without derivatization, with chromatographic separation achieved under a 25 min gradient at 40 °C and 0.25 mL/min flow rate.

The in vitro digestibility of SFM was determined following Witten and Aulrich (2022) with modifications to mimic broiler chicken digestion. In stage one (crop), 1 g of sample was incubated in 15 mL phosphate buffer (pH 6.0) at 41 °C, 200 rpm for 30 min. Stage two (proventriculus/gizzard) involved adding 1.5 mL pepsin (25 mg/mL), adjusting pH to 2.4–2.5 with 6M HCl, and incubating for 135 min under identical conditions. In stage three (small intestine), 1.5 mL pancreatin (100 mg/mL) was added, pH adjusted to 6.4 with 7.5M NaOH, and incubation continued for 120 min. Enzymatic activity was stopped by centrifugation (3321 × g, 10 min, 4 °C). Residues were washed, centrifuged again, freeze-dried (90 h), and finely ground. CP and AAs were measured before and after digestion, and digestibility was calculated using the following formulas:

$$\text{In vitro CP digestibility (\%)} = \left[1 - \frac{\text{Residue weight (g)} \times \text{Residue crude protein}}{\text{Initial sample weight (g)} \times \text{Initial sample crude protein}}\right] * 100$$

$$\text{In vitro AAs digestibility (\%)} = \left[1 - \frac{\text{Residue weight (g)} \times \text{Residue AAs}}{\text{Initial sample weight (g)} \times \text{Initial sample AAs}}\right] * 100$$

Data were analyzed using a two-way analysis of variance (ANOVA) under the General Linear Model (GLM) procedure in Minitab version 22.1. Tukey's test was used for post hoc comparisons. Statistical significance was determined at a threshold of $p < 0.05$.

III. RESULTS AND DISCUSSION

By Day 14, all uninoculated controls exhibited extensive fungal contamination, while minor fungal growth was observed in aerobic *Bacillus subtilis* treatments at both 30 °C and room temperature (RT). In contrast, anaerobic and two-stage fermentations remained free of visible contamination, indicating greater stability under practical conditions. Table 1 presents the interaction effects of fermentation method and incubation temperature on aNDF, CP digestibility, and AAs digestibility. Both two-stage treatments significantly reduced aNDF ($p < 0.05$) compared with controls, with values similar to those of the anaerobic fermentation. These findings align with previous studies reporting substantial fibre degradation in oilseed meals subjected to sequential aerobic–anaerobic processes (Shi et al., 2017; Li et al., 2022; Liu et al., 2025), largely attributed to microbial enzymes such as cellulases, xylanases, and phytases, which disrupt cell wall structures (Wang et al., 2020). Improvements in CP and AAs digestibility were also evident, particularly in anaerobic and two-stage groups, which consistently achieved values exceeding 80%. Significant increases ($p < 0.05$) were observed for CP and several indispensable AAs compared with controls. These results are consistent with Shi et al. (2017), who reported enhanced digestibility of CP and AAs following two-stage fermentation, and with meta-analyses and feeding trials showing that inclusion of fermented SBM improves nutrient utilization in pigs and poultry (Heuzé et al., 2025; Liu et al., 2025). Such improvements are generally linked to the reduction of anti-nutritional factors (Ying, 2020). Furthermore, Figure 1 demonstrates a strong negative correlation between aNDF content and both CP digestibility ($r = −0.739$) and total AAs digestibility ($r = −0.788$),

highlighting the close association between fibre reduction and enhanced nutrient utilization in SFM. Overall, the findings confirm that anaerobic and two-stage *B. subtilis* fermentations effectively reduce fibre, suppress spoilage, and improve protein quality, thereby enhancing the potential of SFM as a sustainable alternative to SBM in poultry nutrition.

Table 1 - Interaction effects (fermentation method × incubation temperature) of crude protein (CP) and indispensable amino acids digestibility (% DM basis), and aNDF content (g/kg DM basis) in sunflower meal at day 14 of fermentation.

Treatments	aNDF	Digestibility %							
		CP	Lys	Arg	Thr	Val	Leu	Ile	His
CA×30°C	480.75^{ab}	66.27^{e}	48.05^{f}	36.12^{k}	40.53^{d}	51.54^{d}	52.06^{g}	53.57^{f}	56.80^{f}
CA×RT	462.91^{abc}	69.53^{d}	52.38^{f}	56.77^{j}	48.34^{cd}	62.14^{c}	62.94^{f}	63.73^{e}	67.18^{e}
CAn×30°C	437.98^{abcd}	65.65^{e}	70.74^{bcd}	70.10^{gh}	65.67^{abc}	69.20^{bc}	70.47^{de}	71.45^{cd}	76.99^{cd}
CAn×RT	409.80^{abcd}	81.84^{bc}	76.91^{abc}	82.59^{bcd}	68.43^{ab}	77.60^{a}	77.12^{abcd}	77.92^{abc}	81.82^{abc}
CTS-2d×30°C	406.48^{bcde}	83.96^{abc}	80.31^{a}	77.54^{def}	68.81^{ab}	81.76^{a}	80.27^{ab}	80.75^{a}	83.28^{abc}
CTS-2d×RT	484.53^{a}	69.87^{d}	56.65^{ef}	60.78^{ij}	53.85^{bcd}	63.31^{c}	63.27^{f}	64.27^{de}	67.43^{e}
CTS-4d×30°C	425.81^{abcd}	81.71^{c}	67.86^{cd}	71.17^{fgh}	69.84^{ab}	74.40^{ab}	73.40^{a}	74.35^{abc}	76.73^{cd}
CTS-4d×RT	469.92^{abc}	70.37^{d}	52.85^{f}	58.25^{j}	54.91^{bcd}	63.26^{c}	62.32^{f}	63.45^{e}	66.54^{e}
BA×30°C	397.29^{cde}	81.25^{c}	70.24^{cd}	74.89^{efg}	69.88^{ab}	74.59^{ab}	73.06^{cd}	72.82^{bc}	79.79^{bc}
BA×RT	406.88^{bcde}	71.54^{d}	63.38^{de}	66.07^{hi}	65.07^{abc}	62.13^{c}	65.08^{ef}	65.24^{de}	72.04^{de}
BAn×30°C	412.50^{abcd}	84.29^{abc}	81.76^{a}	89.25^{a}	77.95^{a}	80.67^{a}	79.74^{abc}	79.84^{ab}	84.99^{ab}
BAn×RT	370.87^{de}	83.21^{abc}	81.01^{a}	88.79^{ab}	75.75^{a}	78.78^{a}	77.78^{abc}	77.84^{abc}	84.87^{ab}
BTS-2d×30°C	334.04^{e}	85.32^{a}	81.08^{a}	84.26^{abc}	78.58^{a}	78.86^{a}	77.36^{abcd}	77.50^{abc}	83.28^{abc}
BTS-2d×RT	374.65^{de}	84.17^{abc}	82.10^{a}	84.51^{abc}	76.98^{a}	79.50^{a}	78.20^{abc}	78.07^{abc}	84.51^{ab}
BTS-4d×30°C	372.14^{de}	85.40^{a}	79.63^{ab}	81.01^{cde}	76.25^{a}	78.28^{a}	77.33^{abcd}	77.34^{abc}	85.51^{ab}
BTS-4d×RT	366.41^{de}	84.96^{ab}	84.72^{a}	84.00^{abcd}	79.98^{a}	82.44^{a}	81.23^{a}	81.20^{a}	87.44^{a}
SEM	14.300	0.615	1.740	1.250	3.810	1.570	1.370	1.400	1.290
p-Values	0.002	<0.001	<0.001	<0.001	0.044	<0.001	<0.001	<0.001	<0.001

Means that do not share a letter within the column are significantly different. CA: control aerobic. CAn: control anaerobic. CTS-2d: control-2 days aerobic followed by 12 days anaerobic. CTS-4d: control-4 days aerobic followed by 10 days anaerobic. BA: *B. subtilis* aerobic. BAn: *B. subtilis* anaerobic. BTS-2d: *B. subtilis*-2 days aerobic followed by 12 days anaerobic. BTS-4d: *B. subtilis*-4 days aerobic followed by 10 days anaerobic. Lysine (Lys), Arginine (Arg), Threonine (Thr), Valine (Val), Leucine (Leu), Isoleucine (Ile), Histidine (His), Phenylalanine (Phe). RT: room temperature. aNDF: ash-corrected neutral detergent fibre.

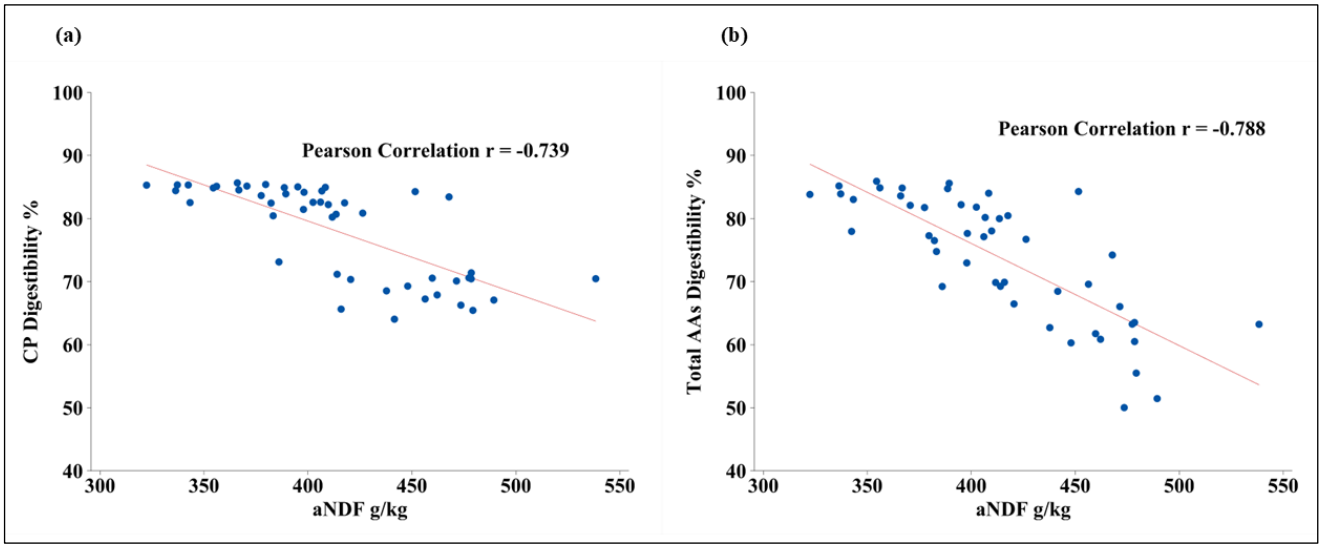

Figure 1 - Correlation between aNDF content (g/kg DM basis) of sunflower meal and crude protein (CP) digestibility (% DM basis) (a) and total amino acids (AAs) digestibility (% DM basis) (b). Total amino acids (AAs) digestibility represents the average digestibility of 17 amino acids, excluding methionine and tryptophan.

IV. CONCLUSION

This study demonstrated that anaerobic and two-stage fermentations with *Bacillus subtilis* enhanced the nutritional value of sunflower meal (SFM). Both approaches reduced fibre (aNDF), suppressed fungal contamination during fermentation process, and enhanced the digestibility of crude protein and amino acids. In contrast to our earlier laboratory trials, where aerobic fermentation caused amino acid losses and low digestibility despite fibre reduction, these methods delivered more reliable outcomes. Overall, the results highlight the potential of anaerobic and two-stage fermentation to generate a balanced, sustainable alternative to soybean meal in poultry feed. However, further in vivo validation is recommended under practical broiler chicken feeding conditions.

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PATHOGENESIS AND REPLICATION OF AVIAN HEPATITIS E IN CHICKENS.

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Summary

This study evaluated avian hepatitis E virus (aHEV) generation via *in ovo* inoculation in commercial brown and SPF layer hens. Embryonated eggs were intravenously inoculated with infectious liver homogenate on day nine of incubation. Embryos and hatched chicks were culled at varying time points to determine virus detection and disease presentation. aHEV RNA was detected by PCR at day 14 of incubation. Gross lesions and discolouration of the liver were observed in infected treatment groups. No differences in body weight or evidence of hepatosplenomegaly was observed between controls and infected groups. Histopathological liver and bone lesions were observed in infected chicks. These findings demonstrate that *in ovo* intravenous inoculation can produce aHEV virus infection in chicks.

I. INTRODUCTION

Big liver and spleen (BLS) disease in chickens presents as an increase in mortality and morbidity with a significant drop in egg production (Handlinger & Williams 1988). BLS was first reported in Australian broiler breeders (Handlinger & Williams 1988) and has since been identified in Asia, Europe and America (Haqshenas et al. 2001; Kwon, Sung & Meng 2012; Marek et al. 2010). Although the disease was characterised in the 1980s, avian hepatitis E virus was not isolated until the 1990s (Haqshenas et al. 2001; Payne et al. 1999). Avian hepatitis E virus (aHEV) is a single stranded RNA virus of the *Hepeviridae* family, genus *Orthohepevirinae*, species *Avihepevirus magniiecur* (Meng 2008). aHEV is transmitted via the oral faecal route and has a predilection for disease presentation in adult birds (Handlinger & Williams 1988; Haqshenas et al. 2001). However, birds as young as 12-18 weeks have been found positive for viral antibodies (Handlinger & Williams 1988; Hewitt et al. 2025).

Aspects of aHEV virus pathogenesis have not been extensively studied due to the lack of culture models. In the present study, the replication of aHEV after *in ovo* inoculation was evaluated using a commercial brown egg laying breed and Specific-Pathogen Free layer hens. Embryos were inoculated with infectious liver homogenate to study virus replication and disease progression.

II. METHOD

An Australian commercial layer flock (8000 hens) presented with increased mortalities and reduced egg production. aHEV infection was confirmed by enzyme linked immunosorbent assay (ELISA, Innovative Diagnostics) and polymerase chain reaction (PCR) (Troxler et al. 2011). Liver and spleen samples were collected post mortem for histology.

a) In ovo Infection Trial

Fertile Specific-Pathogen Free (SPF) (n = 50) and commercial brown egg laying (n = 61) eggs were obtained from Australian SPF services and a commercial layer hatchery respectively. Eggs were incubated at 37.7°C with 55% humidity. On day 9, SPF (n = 36) and eggs from a brown commercial breed (n = 44) were intravenously inoculated with aHEV-infected liver homogenate. Briefly, prominent veins were identified via candling and exposed using a Dremel

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tool. Mineral oil was added to make membrane opaque for visualisation. Control birds (SPF n = 10 and commercial brown n = 10) received phosphate buffer solution. At day 14, half of each group (SPF control = 5, commercial brown = 5, SPF infected = 18, commercial brown infected = 20) were sacrificed. Livers and spleens were collected for PCR and histology. Remaining eggs were kept in incubation till hatching at day 21.

Post-hatch (44% hatch rate), control and infected chicks were provided *ab libitum* water and feed. Body weight was recorded on days 1, 4, 6, 8, 10, and 12. At two weeks of age, blood was collected and serum was used to test for aHEV antibodies using ELISA. Chicks were euthanised. Spleens and livers were collected in RNA-later for PCR to detect viral RNA. Additional liver tissue and a leg from two birds per treatment group were collected in 10% neutral buffer formalin. Samples for histology were cut into sections and stained with hematoxylin and eosin stain.

III. RESULTS

a) Postmortem gross lesions

Gross lesions during post mortem were hepatosplenomegaly and discolouration of the liver. One bird had gross lesions, haemorrhaging and discolouration across both lobes of the liver. All liver samples collected from post mortem examinations were positive for avian hepatitis E viral RNA as detected by PCR.

b) In ovo trial

On day 14, half of the embryos were randomly selected and euthanised. One commercial breed control, eight commercial brown and four SPF infected embryos had died early in incubation, therefore no samples were taken from these specimens. All controls were negative for viral aHEV RNA and confirmed PCR positives were reported in infected treatment groups. Gross lesions and discolouration of the liver were observed in three commercial breed and four SPF infected embryos. On day 21 of incubation, a total of 33 chicks hatched (SPF control = 4, commercial brown, control = 3, SPF infected = 8, commercial brown infected = 8). Three control (SPF = 1, commercial brown =2) and 26 infected (SPF = 10, commercial brown = 16) either died early in incubation or had delayed hatching and were euthanised. One commercial brown and seven SPF infected chicks were euthanised within three days due to splayed legs. Of the chicks culled prior to being two weeks of age, three commercial brown and four SPF infected had visible gross lesions or discolouration on the liver. Chicks had no reported lesions on the liver or spleen at two weeks of age. No aHEV antibodies were detected in serum.

Both control groups and commercial brown infected chicks exhibited normal increases in body weight over the two-week experiment. Post-hatch, SPF infected birds initially exhibited a lower body weight compared to controls over the first week and then a gradual increase to day 12. Significant weight differences were observed between commercial brown and SPF infected chicks, as well as commercial brown control and SPF infected ($P < 0.05$). On day four, SPF control and infected showed a significant difference ($P = 0.04$), however significance was lost from day six. Spleen and liver weights were obtained for each chick culled post hatch and average organ to body weight ratios were obtained. No significant differences were observed between infected and control groups. However, liver to body weight ratio of SPF infected was significantly lower ($P = 0.011$) than commercial brown infected.

c) Histology

Microscopic lesions were observed in livers of infected treatment groups. Lymphocytic infiltration in hepatocytes. (Figure 1.B) and intrahepatic globular leukocytes were observed.

Plasma cells were identified in high lymphocyte activity regions in aHEV infected livers. High glycogen content was observed in controls (Figure 1.C) and infected birds. High fat content (Figure 1.D) in SPF infected chicks culled for leg deformities was due to the immobilisation of fat to the liver. No lesions were found in commercial brown control (Figure 1.A). Mild lymphocytic periphlebitis was reported in SPF controls with no other sign of viral infection.

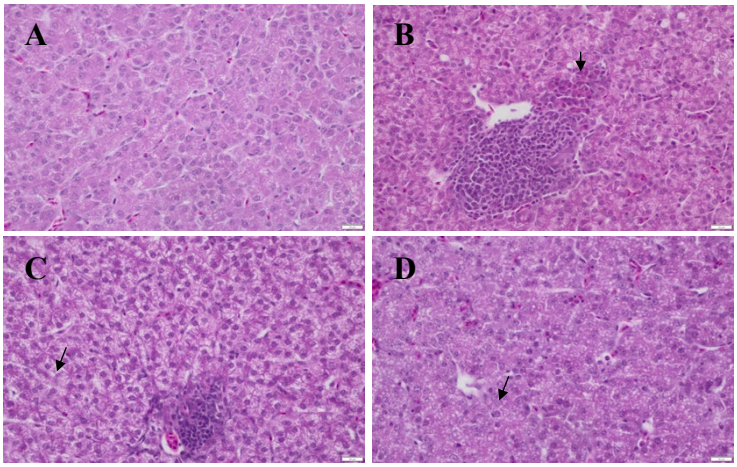

Figure 1 - Microscopic images at 40x magnification of the liver. (A) Commercial brown control: no pathological signs of viral infection. (B) Commercial brown infected: severe lymphocytic periphlebitis in multiple locations across the liver sections and globular leukocytes (arrow). (C) SPF control: high glycogen content (arrow) and mild lymphocytic activity. (D) SPF infected with karyorrhexis (arrow) and high fat content due to the immobilisation of fat to the liver.

Normal bone formation of the metatarsus and tibia was reported in both control groups (Figure 2.A and 2.C). In aHEV infected chicks, darker eosinophilic staining was observed. Bone formation was significantly thinner in regions where the extracellular matrix was denser and the lacunae surrounding osteocytes appears to be smaller in infected bones. In control chicks, the osteoblasts maintain their columnar structure until approximately half way of the intracellular matrix before becoming cuboidal. However, in infected bones the disintegration phase of osteoblasts is observed to occur at a faster rate. Thinner mineralisation bands were reported in commercial brown infected chicks (Figure 2.B), whilst resting and reversal lines in SPF infected were evident (Figure 2.D).

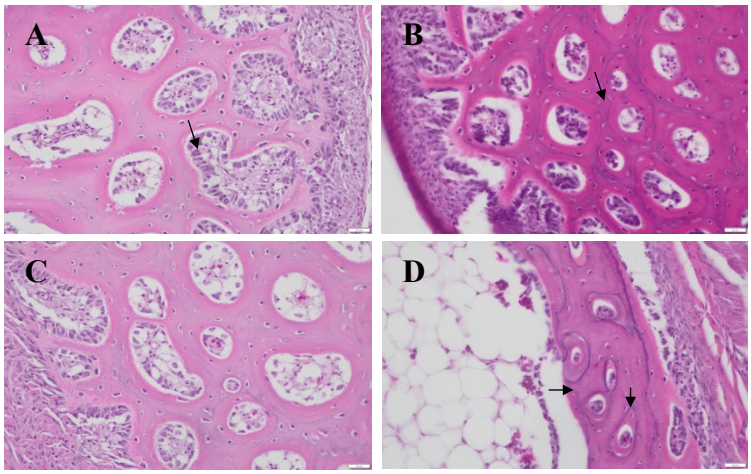

Figure 2 - Microscopic images at 40x magnification of the metatarsus bone. (A) Commercial brown control with normal bone formation. (B) Commercial brown infected showing dark eosinophilic staining and thinner mineralisation bands (arrow). (C) SPF control with normal bone formation. (D) SPF infected with resting lines (vertical arrow) and reversal lines (horizontal arrow).

IV. DISCUSSION

Replication of aHEV is key to developing culturing methods for the investigation of viral pathogenesis and ultimately vaccine production. While *in ovo* inoculation has been used in broiler breeder embryos, this technique has not been established in layer hen embryos (Payne et al. 1999). This study investigated intravenous inoculation in layer hen embryos as well as aspects of aHEV disease progression.

aHEV RNA was detected in infected liver and spleen tissues from day 14 embryos. RNA has previously been detected in the liver, faeces and lymphocytes in SPF infected chickens, inoculated intravenously as embryos, at one day old after hatching (Zhang et al. 2023). Consistent with Zhang et al. (2023), viral RNA was present at hatch but viral specific antibodies were not detected. Gross liver lesions were observed in infected SPF and commercial brown embryos and culled birds. However, no lesions were visible at two weeks of age despite histopathological evidence of infection. No differences in body weight were found between infected and control groups. The breed-specific differences noted between SPF and commercial brown requires further investigation.

Histopathological bone changes linked with aHEV infection have not been previously reported. Viral induced changes to the lacunar system within bones has been noted in humans and similar bone lacunae alternations have been observed in human osteoporosis cases (Buccino et al. 2023). Virus-induced osteoporosis has been reported in chickens experimentally infected with avian leukosis virus at the embryo stage where a cytostatic effect was observed (Smith & Ivanyi 1980). The results in this study demonstrated that intravenous inoculation in embryos was able to generate viral aHEV RNA in tissue as well as stimulate clinical signs of disease in both embryos and chicks. This development will assist in furthering research on the pathogenesis of aHEV in chickens.

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THE SWEET CHALLENGE: *PASTEURELLA MULTOCIDA* LPS VARIATION AND FOWL CHOLERA CONTROL

L. OMALEKI[1],J. HOSMER[1], J. ZAUGG[2,3] and V. MURIGNEUX[3]

Fowl cholera caused by *Pasteurella multocida* is an ongoing problem in Australian poultry production due to the increasing adoption of free-range production system. Currently, autogenous killed whole-cell vaccines are one of the main measures used to prevent the disease. Traditionaly, a scheme known as Heddleston serotyping was used to choose strains to be included in killed autogenous vaccine against fowl cholera. The serotyping was done using polyclonal antibodies raised against the 16 Heddleston reference strains, as the protection offered by killed autogenous vaccines was believed to be serovar specific. However, it is now known that the killed *P. multocida* vaccines protect only against field strains that share the same—or very similar—outer coat, which is mainly made up of lipopolysaccharides or LPS (Harper and Boyce, 2017). The LPS shows considerable structural variations between strains, exceeding that identified by the Heddleston serotyping scheme. So far, nine different genetic loci (L1–L9) (1, 2) have been identified that code for the 16 recognised Heddleston serovars. Most of the genes in the LPS genetic loci code for glycosyl transferases that are responsible for adding a specific sugar to a specific position on the LPS to form the outer structure. Inactivating mutations within any of these genes can result in truncation of the LPS structure. Whole-genome sequencing can be used to identify and compare the variations in the LPS outer structure of the wildtype strain causing an outbreak and the vaccine strains. This was used previously by our group to provide explanations, based on the detected diversity in the genetic loci coding for the LPS coat, for the repeated vaccination failures seen on a number of free-range layer farms (3).

We have compiled a comprehensive database of known inactivating mutations that change the LPS outer coat. We focused on the mutations circulating in the Australian livestock industry (primarily chickens), supplemented with those found in isolates from the NCBI Sequence Read Archive. We used a numbering scheme to assign subtypes to the 9 major LPS types, based on these mutations. For example, we documented 25 recurrent mutations within LPS type L3 (L3-1 to L3-25), the most prevalent LPS type found in the *P. multocida* isolates in Australia and internationally.

In this study, we sequenced 61 *P. multocida* isolates from avian hosts on the Oxford Nanopore Technology (ONT) platform. The isolates were then typed using our newly developed pipeline that accepts ONT reads, assembles the genome and performs quality control, MLST, LPS typing and subtyping. Thirty-six of the isolates were L1, twenty-four L3 and one was L6. The LPS from fifteen of the isolates carried major mutations, e.g. truncating/stop-gain, and could be therefore subtyped using our workflow. This included thirteen L3 isolates (54%) and only two of the L1 carrying isolates. We are currently investigating the diversity in the LPS sequence of those isolates that did not show inactivating mutations in the LPS genetic region and could not be subtyped through the pipeline.

In conclusion, we are working towards establishing a new typing scheme and relevant tools for *P. multocida* isolates. This will be offered as a user-pay service for industry stakeholders, allowing isolates to be submitted to our group for analysis. These tools will also be made available on Galaxy Australia—an open, web-based platform for accessible, reproducible and transparent computational research for use by the wider research community. Ultimately, the new *P. multocida* typing and subtyping platform will improve strain selection for killed autogenous vaccines to protect the poultry industry against fowl cholera. Production of autogenous vaccines requires isolating and inactivating the outbreak strains; sequencing and analysis of the LPS genetic region will allow farm veterinarians to confirm alignment between vaccine strains and circulating fowl cholera strains, identify potential mismatches and make informed decisions.

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USING PLANT-DERIVED SOLUTIONS AS ANTIBACTERIAL AGENTS FOR HATCHING EGGS

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Summary

In commercial procedures for bacterial control of eggshells, synthetic sanitizers are generally used as routine standards. However, the reliance on synthetic sanitizers has increased the range of toxic outcomes in poultry systems, including those associated with the sanitization of hatching eggs using these products. Researchers have been continuously studying green protocols to control bacterial proliferation in post-laying eggshells. In this context, our study demonstrated that applying a sanitization protocol with essential oils from ginger (*Zingiber officinale*) rhizomes or peppermint (*Mentha piperita*) leaves can significantly reduce the bacterial load on eggshells. In terms of antibacterial activity, these essential oils can be incorporated into the repertoire of efficient poultry sanitizers aimed at egg sanitization of hatching eggs.

I. INTRODUCTION

Bacterial contamination can lead to increased complications, losses, and increased costs for poultry producers. Regardless of the production scale, losses due to bacterial contamination, including those related to hatching eggs, can impair productivity and quality indices. The sanitization of hatching eggs is a practice that, when combined with other antibacterial measures, aims to ensure egg safety and improve poultry productivity. Although the use of synthetic products for the sanitization of hatching eggs is sometimes criticized because of their contribution to bacterial resistance and potential toxicity to both poultry and humans, interest in green alternatives has been growing. In organic poultry systems, for example, the use of formaldehyde for sanitization is prohibited. The use of essential oils has become one of the main focuses of scientific research on green alternatives for bacterial control in hatching eggs. These compounds not only help ensure the bacteriological quality of eggs but also promote more sustainable poultry production.

The *Zingiber officinale* plant can be used for food, pharmaceuticals, material production, and ornamental purposes (Inta et al., 2023). Rhizome is the most commonly used part, especially for medicinal purposes (Wahyuni et al., 2023; Inta et al., 2023). The essential oil obtained from the rhizome of *Zingiber officinale is* composed of camphene, bisacurone epoxide, eucalyptol, β-phellandrene, α-zingilbene, α-pinene, and geranial, among other components (Abdullahi et al., 2020). This essential oil showed antibacterial activity in the disk diffusion assay (López et al., 2017), inhibiting the growth of *Staphylococcus aureus*, *Escherichia coli*, *Klebsiella pneumoniae*, and *Salmonella enterica* serovar Typhi. The plant *Mentha piperita*, including its leaves, stems, and flowers, as well as extracts and essential oils derived from it, has been used in both traditional and modern medicine (Souza et al., 2021). Its essential oil contains various compounds, such as menthol, menthone, menthyl acetate, menthofuran, and 1,8-cineole, and exhibit antibacterial activity, as it is able to inhibit both Gram-positive and Gram-negative bacteria, including *Staphylococcus aureus*, *Micrococcus flavus*, *Bacillus subtilis*, *Salmonella* Enteritidis, and *Escherichia coli* (Reddy et al., 2019). The bacteria inhibited *in vitro* by the essential oils of *Zingiber officinale* and *Mentha piperita* may be present on eggshells, posing risks to food safety and poultry production.

In this context, the essential oils of *Zingiber officinale* and *Mentha piperita* have attracted interest for use in sanitizing formulations. This study evaluated the antibacterial potential of these essential oils and tested a sanitizing solution formulated with them to assess its effectiveness in egg sanitization.

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II. METHOD

The essential oils were commercially purchased and extracted via steam distillation, with *Zingiber officinale* from the rhizome containing α-zingiberene as the main component and *Mentha piperita* from the leaves predominantly containing menthol (Table 1).

Table 1 - Components of essential oils[1].

Components	Approximate %
Zingiber officinale	
α-Zingiberene	34.00
β-Sesquiphellandrene	12.00
Ar-Curcumene	8.00
Camphene	7.00
β-Bisabolene	6.00
α-Pinene	2.00
β -Elemene	1.00
Geraniol	0.50
Identified total	70.50
Unidentified	29.50
Mentha piperita	
Menthol	42.00
Menthone	27.00
Eucalyptol	5.00
Menthyl acetate	5.00
Isomenthone	4.00
Menthofuran	4.00
Limonene	3.00
β-caryophyllene	2.00
Neomenthol	2.00
Pulegone	1.00
Isopulegol	0.20
Octanol-3	0.10
Trans-sabinene hydrate	0.10
Carvone	0.10
Identified total	95.50
Unidentified	4.50

[1]BioEssência®, São Paulo, Brazil

The antibacterial activity of the essential oils was evaluated using disk diffusion and broth dilution methods. For the disk diffusion assay, two reference strains were used: *Escherichia coli* and *Staphylococcus aureus* (American Type Culture Collection, Manassas, VA, USA). The bacterial suspensions were adjusted to a 0.5 McFarland standard and 100 µL were inoculated on Mueller–Hinton agar plates. Sterile paper disks, each impregnated with 10 µL of an essential oil, were placed on the agar surface in triplicate and incubated at 36 °C for 24 h. Disks containing 0.5% Tween 80 served as controls under the same conditions. After incubation, the antibacterial effect was measured by the diameter of the inhibition zones (mm). For the broth dilution assay, the same procedure was followed, except that 10 µL of a single essential oil was added to tubes containing Mueller–Hinton broth after 100 µL of bacterial cultures were added. A tube containing undiluted essential oil was used as a negative control. The tubes were then incubated at 36 °C for 24 h, and bacterial growth was assessed visually.

Sanitizing solutions were prepared by diluting *Zingiber officinale* and *Mentha piperita* essential oils, each in a separate solution, to a final concentration of 2.0% in 93.8% grain alcohol. Commercially obtained, brown table eggs were transported and stored under refrigeration in a poultry laboratory. Table eggs were used to simulate the sanitization of hatching eggs because

productive parameters were not evaluated, making the use of actual hatching eggs unnecessary. After 24 h, each egg was manually sprayed with approximately 2.5–3 mL of solution. The total aerobic mesophilic bacteria, Enterobacteriaceae, and total coliforms on the eggshells were subsequently evaluated using the eggshell washing method. All procedures in this study were performed in triplicate. A group of non-sanitized eggs was included as a control for comparison. Grain alcohol was not included as an isolated treatment, as it has been previously shown that its activity is insignificant in the absence of essential oils (Vale et al., 2024). Statistical analyses were conducted using SAS software, version 9.4. The data were analyzed using analysis of variance (PROC GLM), and the means were compared using Tukey's test. Non-parametric data (counts of Enterobacteriaceae and total coliforms) were analyzed using the Kruskal–Wallis test. Differences among treatments were considered statistically significant at $P < 0.05$.

III. RESULTS

The essential oils of *Zingiber officinale* and *Mentha piperita* exhibited *in vitro* activity against *Escherichia coli* and *Staphylococcus aureus*, with inhibition zones exceeding 17 mm (Table 2). There was no difference between the oils against *Escherichia coli* ($P > 0.05$), but *Mentha piperita* was significantly more effective than *Zingiber officinale* against *Staphylococcus aureus* ($P < 0.05$). No bacterial growth was observed in the broth dilution assay, confirming the results obtained by the disk diffusion test.

Table 2 - In vitro antibacterial activity of the essential oils.

Essential oils	Inhibition halo (mean ± standard deviation, mm)	
	Escherichia coli	*Staphylococcus aureus*
Zingiber officinale	17.13 ± 2.28 [a]	20.90 ± 1.72 [b]
Mentha piperita	18.91 ± 1.37 [a]	25.22 ± 1.80 [a]
P value	0.3111	0.0384

a,b Values within a column followed by different letters differ significantly ($P < 0.05$).

The sanitizing solutions containing *Zingiber officinale* and *Mentha piperita* essential oils significantly reduced the bacterial load on the eggshells ($P < 0.05$) (Table 3). Mesophilic bacteria counts were reduced by approximately 40.00% on eggshells after sanitization with *Zingiber officinale* and 50.00% with *Mentha piperita*, whereas both essential oils reduced Enterobacteriaceae and total coliforms to levels below 10 CFU/mL.

Table 3 - Bacterial counts on eggshells after treatment with essential oils.

Essential oils	Mesophilic bacterial count (log_{10} CFU/mL)		*P* value
	Before spraying	After spraying (1 h)	
Zingiber officinale	4.79 ± 0.26 [a]	2.67 ± 0.67 [b]	0.0017
Mentha piperita	4.79 ± 0.26 [a]	2.34 ± 0.44 [b]	
	Enterobacteriaceae		*P* value
	Before spraying (log_{10} CFU/mL)	After spraying (1 h) (CFU/mL)	
Zingiber officinale	1.22 ± 0.43 [a]	<10 [b]	0.0013
Mentha piperita	1.22 ± 0.43 [a]	<10 [b]	
	Total coliform count		*P* value
	Before spraying (log_{10} CFU/mL)	After spraying (1 h) (CFU/mL)	
Zingiber officinale	1.03± 0.25 [a]	<10 [b]	0.0002
Mentha piperita	1.03± 0.25 [a]	<10 [b]	

a,b Values within a row followed by different letters differ significantly ($P < 0.05$).

IV. DISCUSSION

Previously, Sharma et al. (2016) reported that the essential oil of *Zingiber officinale* also inhibited both bacterial strains *in vitro*, with inhibition zones of 10.6 ± 0.3 mm for *Escherichia coli* and 12.0 ± 0.2 mm for *Staphylococcus aureus*. In these bacteria, the essential oil of *Zingiber officinale* can compromise cell membrane permeability and negatively affect the metabolism of energy, respiration, and DNA (Wang et al., 2020). As reported by Piveta et al. (2022), *Mentha piperita* essential oil exhibited inhibition zones of 43 mm against *Escherichia coli* and 35 mm against *Staphylococcus aureus*, which is consistent with the findings of our study. This antibacterial activity can be explained by the findings of Kang et al. (2019), who demonstrated that *Mentha piperita* essential oil increases bacterial membrane permeability; promotes the leakage of nucleic acids, proteins, and ATP; reduces cell viability; and alters bacterial morphology. Consistent with our findings, El-Kashef and El Sabry (2025) and El-Soufi et al. (2025) reported that the application of essential oil-based sanitizers in the sanitization of hatching eggs, including those evaluated in this study, effectively reduces the bacterial load on eggshells. This further reinforces the potential of essential oils as effective antibacterial alternatives to undesirable conventional sanitizers for hatching eggs.

In conclusion, the essential oils of *Zingiber officinale* and *Mentha piperita* not only inhibited the growth of *Escherichia coli* and *Staphylococcus aureus* but also proved effective as post-laying egg sanitizers, reducing bacterial colonization on the eggshell surface.

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DIFFERENTIAL DETECTION OF *ASCARIDIA GALLI* AND *HETERAKIS GALLINARUM* EGGS IN INTESTINAL AND CAECAL DROPPINGS OF FLOOR HOUSED LAYING HENS

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With the increasing use of floor-based egg production systems, helminth infections have become more common in commercial poultry. *Ascaridia galli* and *Heterakis gallinarum*, the two most prevalent nematodes of chickens, inhabit the small intestine and caeca, respectively, and often co-occur in the field. Current excreta egg count (EEC) methods do not differentiate between the eggs of these two related ascarid species (Tarbiat et al., 2021). However, chickens produce two distinct excreta types based on their gastrointestinal origin—intestinal and caecal. Intestinal excreta (IE) is passed 12-15 times a day and in larger quantities than caecal excreta (CE), which is passed only once or twice a day. It is highly likely that the worm eggs in the two types of excreta predominantly contain eggs from *A. galli* and *H. gallinarum,* respectively. We hypothesised this in a study measuring the level of nematode eggs in IE or CE obtained from hens artificially infected with *A. galli* or *H. gallinarum* and kept in a floor-based husbandry system.

Forty Hy-Line Brown laying hens (mean body weight (BW 2.00 kg), 40 weeks of age, were cleared of any prior nematode infection and artificially infected with *A. galli* (n=20) or *H. gallinarum* (n=20), housed in separate floor pens, and monitored for clinical signs, egg shedding in IE, CE, mixed intestinal-caecal excreta (ME), and total worm recovery in the gastrointestinal tract. Birds were monitored daily, with BW, EECs, and worm count recorded at set intervals from 44 weeks until the experiment ended at 66 weeks of age. Both infections remained subclinical, although hens with *A. galli* showed slightly lower body weight gain (0.52 g/week) than those with *H. gallinarum* (2.18 g/week) throughout the infection period. Analysis of EEC data (eggs/gram of excreta) by infecting nematode, excreta type and time post infection, showed little overall difference between infections or excreta type, but a highly significant interaction between these factors ($P<0.0001$). Eggs of *A. galli* were predominantly detected in IE and those of *H. gallinarum* in CE, consistent with the hypothesis. *A. galli* infection resulted in mean EECs of 747 and 91 respectively in the IE and CE. This shows that, *A. galli* egg count in IE was 8-fold higher than that in the CE (per grams of excreta sample). For *H. gallinarum* infection, the corresponding values were 23 and 842 indicating that *H. gallinarum* eggs were shed at a 36-fold higher level in the CE, than in IE. For ME samples, EECs were reduced by 45% for *A. galli* and 60% for *H. gallinarum* relative to levels in their preferred excreta types. Based on postmortem worm counts (including larvae), the detected and quantified nematode eggs in different excreta types appeared to be outputs of stable adult worm populations of individual birds.

In conclusion, these results provide a practical option for non-lethal differentiation between the level of infections with *A. galli* and *H. gallinarum* infection based on conducting EEC on the two distinct types of excreta. ME samples have little practical utility in this regard. Further studies are needed to evaluate this differential egg excretion under a wider range of conditions and to determine the level of variation between individual chickens.

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RECENTLY DETECTED INFECTIOUS BRONCHITIS VIRUS STRAINS SHOW POOR PROTECTION CONFERRED BY VACCINES IN AUSTRALIAN FREE-RANGE LAYER FLOCKS

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Summary

Infectious bronchitis virus continues to threaten commercial poultry production due to ongoing viral evolution and incomplete vaccine protection. This study investigated IBV prevalence, tissue-specific detection performance, and genetic diversity in Australian commercial free-range layer operations to evaluate current control success and inform surveillance strategies. Tracheal, cloacal and caecal tonsil swabs and tissues (n = 127) were collected from multiple farms across three major companies in Queensland and New South Wales. Samples were screened using RT-PCR and positive detections underwent full S1 gene sequencing. IBV was detected on all participating farms, with an estimated flock-level infection rate of 20-30% despite routine vaccination. Caecal tonsil sampling improved detection outside acute infection, outperforming tracheal swabs. Infection status did not correlate with bird age or vaccination timing. Phylogenetic analysis revealed distinct clustering by farms and companies, and divergence between states. Several circulating strains were genetically distant from vaccine lineages, indicating reduced cross-protection potential. Persistent post-vaccination infection and strain drift highlight a surveillance gap with economic significance. A coordinated national genomic monitoring program incorporating caecal tonsil sampling is recommended to support timely vaccine updates and strengthen control outcomes.

I. INTRODUCTION

Infectious bronchitis virus continues to threaten commercial poultry production due to ongoing viral evolution and incomplete vaccine protection (Jordan, 2017). Despite the fact IBV rapidly evolve due to mutations and recombination (McKinley *et al.*, 2011), little is known about the current IBV strains circulating in Australia as a result to the absence of a surveillance program. In the present study we aimed to isolate IBVs circulating in free-range layer farms from Queensland and New South Wales, reporting on having problems with chronic respiratory disease, watery albumens, cystic oviducts and oddly shaped eggs. Once isolated, we aim to study their potential divergence from the previously detected field strains, as well as comparing them with the current vaccine strains available in Australia.

II. METHOD

Samples: Tracheal, cloacal and caecal tonsil swabs and tissues (n = 127) were collected from multiple farms across three major companies in Queensland and New South Wales. Samples were screened using RT-PCR and positive detections underwent full S1 gene sequencing. All samples were placed in RNAlater-containing 5 mL tubes and sent to our laboratory by the company's treating veterinarians. All the farms were included in the study because they exhibited clinical signs that could be attributable to an IBV infection, including drop in egg production and watery egg albumen.

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RNA extraction and RT-PCR: The extractions were performed using the RNeasy Plus Kit (QIAGEN, Australia) following the manufacturer's instruction with slight modifications. For swabs, the tubes containing them were vortexed at maximum speed for at least 30 seconds per sample, and then 250 mL of the RNAlater containing the viral particles subjected to lysis into 600 µL of RLT lysis buffer supplemented with 1% β-mercaptoethanol (BME). For the caecal tonsils, a fraction of the tissue of approximately 20-30 mg was extracted aseptically, mixed with 600 µL of RLT lysis buffer with 1% BME. Then, after adding 2 metal beads, lysed in a TyssueLyser II (QIAGEN, Australia) at 25 Hz for 45 sec., the tubes inverted, and lysed at the same frequency for another 45 sec. Then, they were subjected to the washing steps using the kit's buffers for a final elusion in 30 µL of Nuclease-free water. Then, the complimentary DNA (cDNA) strand was produced using the SuperScript IV reverse transcriptase following the manufacturer's instructions and using 5 µL of RNA extract as template. The cDNA was then subject to polymerase chain reaction (PCR) targeting the N-3'UTR region of the IBV genome. Previous studies have shown that this region can successfully predict the IBV type (Hewson *et al.*, 2009). Then, all amplicons were submitted to the Australian Genome Research Facility (Sydney, Australia) for genome sequencing using Sanger termination dye technology and electrophoresis. The primer sequences (All1F and Del1R) can be found in Hewson *et al.* (2009).

Data analysis: All the amplicon sequences were compared using Geneious Prime software package (Dotmatics), including multiple alignments using Clustal Omega and phylogenetic trees inferences using the Maximum Likelihood algorithm and 1,000 replicates as bootstrap.

III. RESULTS

IBV was detected on every farm tested, and the proportion of positives varied significantly depending on the sample used. Also, the sequence analysis of the filed PCR amplicons, when compared with the sequences of the same segment on the vaccine strains, demonstrated the presence of variants that differ from the vaccines (Figure 1).

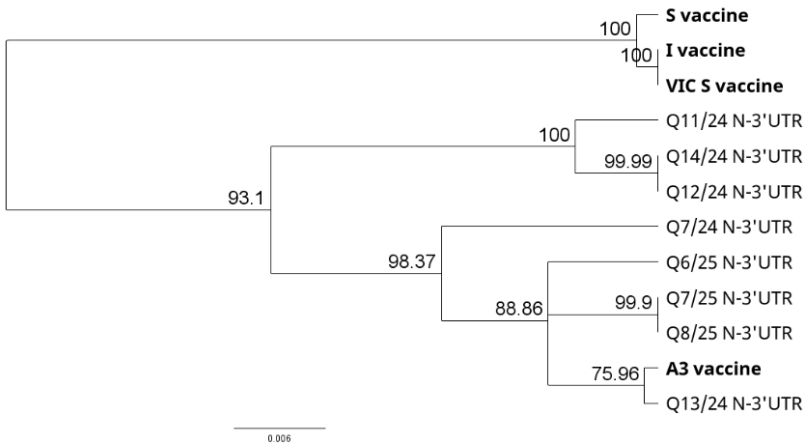

Figure 1 - Phylogenetic tree inferred from the samples obtained from the layer farms located in QLD in 2024 and 2025. In bold are the vaccines available in Australia (same amplicon, N-3'UTR).

The highest proportion of positives were found in the caecal tonsils, swabs or tissues ($P < 0.05$) with a proportion of positives of 80% and 38.9%, respectively. This is compared with the proportion of positive from tracheal (10.9%) and cloacal (12.9%) swabs (Figure 2).

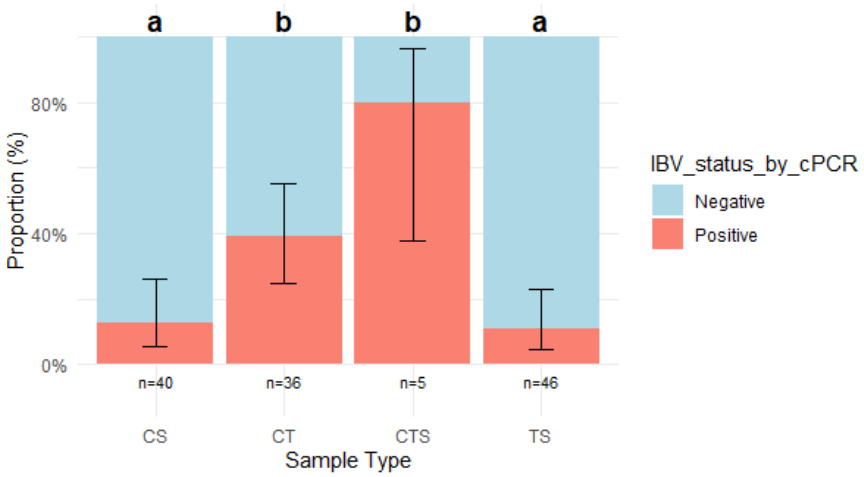

Figure 1 - Proportion of birds testing positive for IBV by PCR, grouped by sample type: CS (cloacal swab), CT (caecal tonsil), CTS (caecal tonsil swab), and TS (tracheal swab). Bars show the proportion of IBV-positive and IBV-negative birds, with stacked colouring. Error bars indicate 95% Wilson confidence intervals for the proportion of positives. Total sample sizes (n) per sample type are shown below each bar. Differences in IBV positivity among sample types were statistically significant (Fisher's exact test p = 0.0002). Pairwise Fisher's exact tests with Holm correction identified significant differences between CS and CTS (padj = 0.0189), TS and CT (padj = 0.0189), TS and CTS (padj = 0.0138), and CS and CT (padj = 0.0468), as indicated by letters above bars.

In terms of the age of the hens (10 days to 27 weeks of age in those form NSW, 19 to 77 weeks of age in those from QLD), those that were found positive appeared younger in comparison with those that were negative (Figure 3). However, the differences between groups were not found to be significant.

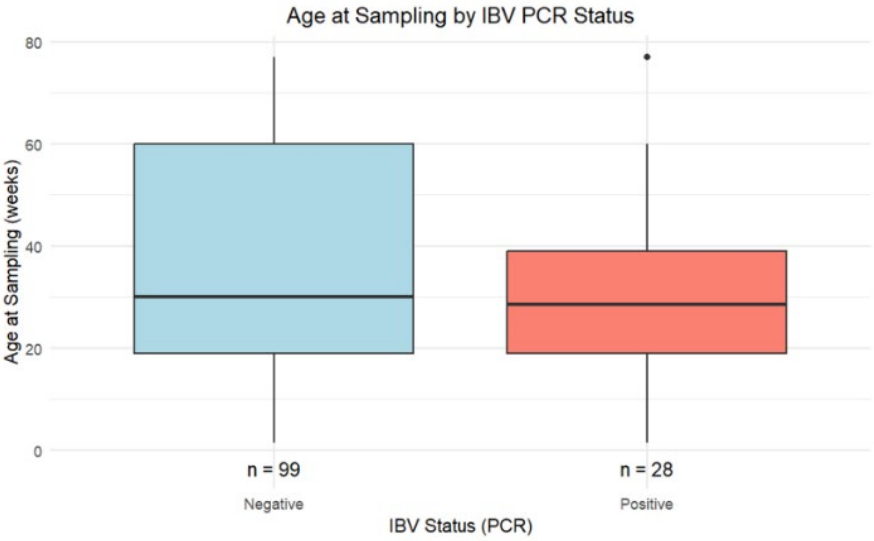

Figure 3 - Boxplot showing age at sampling of birds grouped by IBV status (positive vs negative) determined via PCR, showing the distribution of age (days) with medians, interquartile ranges, and whiskers representing minimum and maximum values. Sample sizes (n) per group are shown below each box. A Wilcoxon rank-sum test indicated no statistically significant difference in age at sampling between IBV-positive and IBV-negative birds (p = 0.2833).

Regardless of their vaccination status, Figure 4 shows that hens vaccinated only with the I vaccine were 31.3% positive to IBV, while 18.3% were positive in those vaccinated at different time points with the I vaccine followed by the A3 vaccine, and 28.6% when vaccinated with I, A3 and VicS. For some samples, the vaccination status was not reported, and those had a positivity proportion of 20.0%.

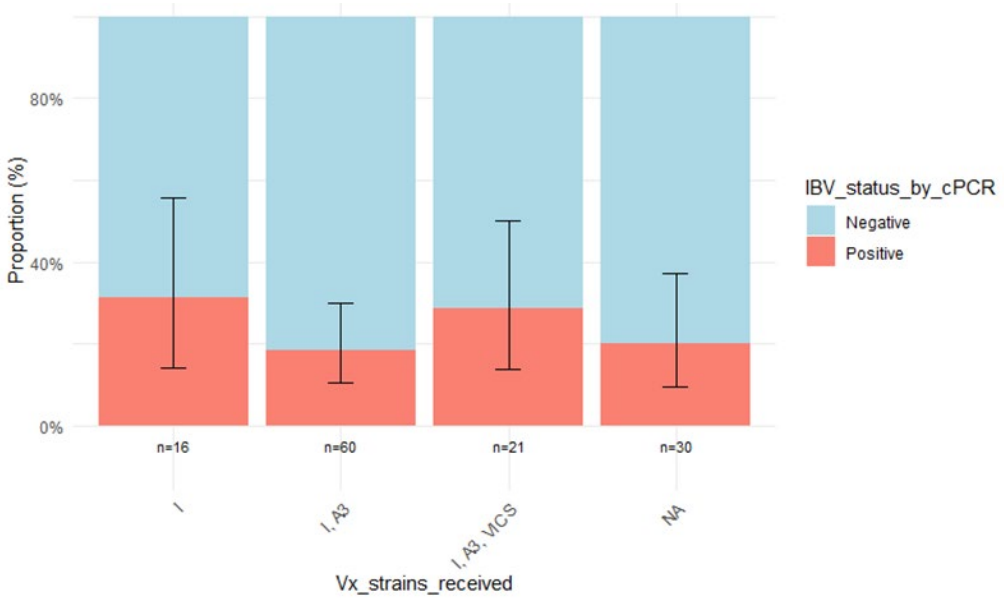

Figure 4 - Proportion of birds testing positive and negative for IBV by PCR, grouped by vaccine strain combinations received. Stacked bars show the relative proportions of positive and negative samples within each vaccine group. Error bars represent 95% Wilson confidence intervals for the proportion of positives, and total sample sizes (n) are shown below each bar. Differences in IBV positivity among vaccine combinations were not statistically significant (Fisher's exact test, p = 0.5683).

IV. DISCUSSION

Our molecular epidemiology study showed that IBV variants strains are still circulating and causing clinical signs in layer farms in Australia. Also, due to genetic drift, the current vaccines seemed to offer little or no protection against the challenge with these variant strains. This is a phenomenon continuously observed with IBV around the globe, where little or no cross protection is observed between the emergent variants and vaccines (Abozeid, 2023; de Wit *et al.*, 2011; Gallardo, 2021). All the variants detected in the present study belong to the GV genotype but appeared to be distant to the other GV isolates already sequenced. In conclusion, it seems evident that new vaccines are needed in Australia, as it has been seen in other countries due to the constant emergence of variants, which appears to be a continuous challenge (Jordan, 2017). A complete sequence of the S1 protein, or even of their complete genome, seems necessary to confirm the identity of these variants.

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FLYING UNDER THE IMMUNE RADAR: WHAT DUCKS REVEAL ABOUT POULTRY RESPONSES TO INFLUENZA A VIRUSES

L.K. CAMPBELL[1]

Mallard ducks *(Anas platyrhynchos*) are the natural reservoir for diverse influenza A virus (IAV) strains and typically remain asymptomatic despite supporting high viral replication (Webster et al, 1992). This contrasts with domestic poultry, where infection with highly pathogenic strains of avian influenza (HPAI) often results in severe disease and high mortality (Alexander et al, 1986). Understanding how ducks control virus replication without pathology provides valuable insight into host factors that influence susceptibility in chickens and other production birds.

Recent transcriptomic analyses of ducks infected with HPAI H5N1 revealed an early antiviral response in lung and spleen tissues, with rapid resolution of inflammation by 72 hours post-infection (Campbell et al, 2021). Ducks down-regulated key pro-inflammatory genes in tissues where the virus was replicating, indicating modulation of tissue-damaging pathways. An evaluation of pattern-recognition receptor expression profiles across avian species highlights the potential roles of retinoic acid-inducible gene-I (RIG-I), melanoma differentiation-associated gene 5 (MDA5), and their downstream regulators in shaping divergent disease outcomes (Campbell and Magor, 2020).

A comparative analysis of data available across species demonstrates that these immune strategies follow distinct trajectories: ducks initiate an early and rapid antiviral response that peaks early in infection and resolves even as viral load remains high, preserving tissue integrity and allowing continued viral shedding without disease. Chickens exhibit delayed sensing of viral RNA and slower induction of antiviral genes, which can lead to excessive inflammation and severe pathology. This difference in regulation likely underpins the balance between tolerance, susceptibility and resistance to infection.

Building on these findings, current work underway aims to establish primary cell cultures from Australian wild ducks to investigate genes involved in resistance to IAV infection. Primary fibroblast cultures have been successfully established from Australian wood duck (*Chenonetta jubata*) and black swan (*Cygnus atratus*) tissues. These cells exhibit species-specific growth requirements distinct from domestic poultry, underscoring the need for tailored media and culture conditions in wild avian systems. By examining the activity of innate immune regulators, including pattern-recognition receptors, this comparative framework seeks to identify molecular signatures that control the outcome of infection, whether a host clears, tolerates or succumbs to the virus.

Understanding how wild birds naturally limit viral damage provides new perspectives for improving disease resilience in domestic poultry. Insights from natural reservoir hosts can inform strategies to reduce the impact of avian influenza outbreaks in chickens through improved breeding, management, and preparedness approaches that strengthen flock immunity without compromising productivity.

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VACCINATION FOR H5N1 PREPAREDNESS?

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Summary

The global spread of HPAIV H5N1 poses a significant threat to wild birds and the poultry industry, with Australia currently at moderate-to-high risk of an incursion. Poultry vaccination, illustrated by France's large-scale duck campaign, can substantially reduce outbreak size but carries trade restrictions and potential evolutionary pressures on the virus, limiting its immediate applicability in Australia. Native Australian species, including black swans, show heightened susceptibility due to unique immune adaptations, highlighting the need for targeted conservation strategies. Selective vaccination of at-risk captive native species offers a feasible, low-risk preparedness approach, exemplified by the planned Ceva–Mandai pilot program in Singapore, and may serve as a model for mitigating ecological and evolutionary impacts of H5N1 incursions.

I. INTRODUCTION

The H5N1 strain of highly pathogenic avian influenza virus (HPAIV) has recently spread globally, reaching South America and Antarctica, leading to significant wild-bird population declines (Airey and Short 2024). Oceania is currently the only region unaffected by HPAIV H5N1, but the risk of the virus becoming established in Australia's is deemed 'Moderate-High' (Airey and Short 2024). Preparedness for the incursion of H5N1 in Australia is a government priority, with Australia recently investigating over $100 million in enhanced national preparedness and biosecurity whilst also boosting the surveillance of wild birds and conducting national simulation exercises (e.g. Exercise Volare). Part of Australia's preparedness planning includes considering the benefits and disadvantages of vaccination in different populations. In this talk, we evaluate the benefits and limitations of vaccinating two key groups—poultry and native species—and introduce a new framework for assessing vaccination's broader ecological and evolutionary impacts.

II. POULTRY VACCINATION

To understand the benefits of poultry vaccination, France's vaccination campaign offers an interesting case study. Beginning October 2023, France rolled out a compulsory vaccination campaign targeted mainly at intensive duck production areas (mallard/force-plucked duck sectors) to control ongoing HPAI H5Nx circulation (Guinat, Fourtune et al. 2025). Coverage reached very high proportions of commercial duck flocks during 2023–2024. Specifically, as of July 1, 2024, >35 million ducks had received 2 doses and 1.5 million had received 3 doses (Guinat, Fourtune et al. 2025). It is estimated that this vaccination campaign adverted 314-756 outbreaks, representing a 96%-99% reduction in epizootic size (Guinat, Fourtune et al. 2025).

However, this success does not come without its caveats. Firstly, whilst the World Organisation for Animal Health (WOAH) states that vaccination against HPAIV does not automatically bar a country from being considered HPAI-free (WOAH 2023) – the reality is that many trade partners impose restrictions or bans when vaccination is used. For example, when France began vaccinating commercial meat ducks against HPAI (in 2023), the United States Department of Agriculture (USDA) / its veterinary services entity placed import restrictions on poultry (especially ducks), duck eggs, and untreated duck products from France and from certain

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European countries, effective 1 October 2023 (Polansek 2023). The rationale for these restrictions is that vaccinated birds may still be infected and that vaccination would result in the infection being asymptomatic and hence missed. In support of this notion, it is important to note of France's 10 HPAI H5 poultry outbreaks that reported in 2023-2024, 2 occurred in vaccinated flocks (Guinat, Fourtune et al. 2025). A further concern is that vaccination can exert selection pressures that accelerate antigenic change in circulating H5 viruses if vaccine antigens and coverage are mismatched or inconsistent. Here, with the support of Australian Eggs, we have developed a novel *in vitro* system that can be applied to understand the effects that vaccination can have on viral evolution in both chickens and ducks. Briefly, primary duck and chicken cells were cultured and infected with LPAIV H7N6. LPAI was used for infection as the potential generation of additional mutations in a HPAI H5N1 strain was deemed inconsistent with biosafety principles. Avian influenza was passaged 6 times on chicken and duck cells, and the viral HA was deep sequenced after each passage. We then used a generalised Pareto fit to assess the greater-than-wildtype fitness of the mutations that emerged in the viral HA after passaging in each species (Fig 1). The distribution of fitness used in conjunction with the mutation rate indicates that, whilst the emergence of mutations is significantly lower in duck cells than in chicken cells when these mutations do emerge, they achieve a greater fitness value. That is, it takes much less time to observe high fitness mutations in duck cells than in chicken cells (e.g. 6 passages compared to 125 passages for fitness above 1.25). These data have potential information for targeting surveillance for novel mutations in different poultry species. More importantly, this system can be adapted to include the presence of vaccinated poultry serum (including those from different H5 vaccines) to understand in a high throughput manner how vaccination is likely to influence viral mutations in both chickens and ducks.

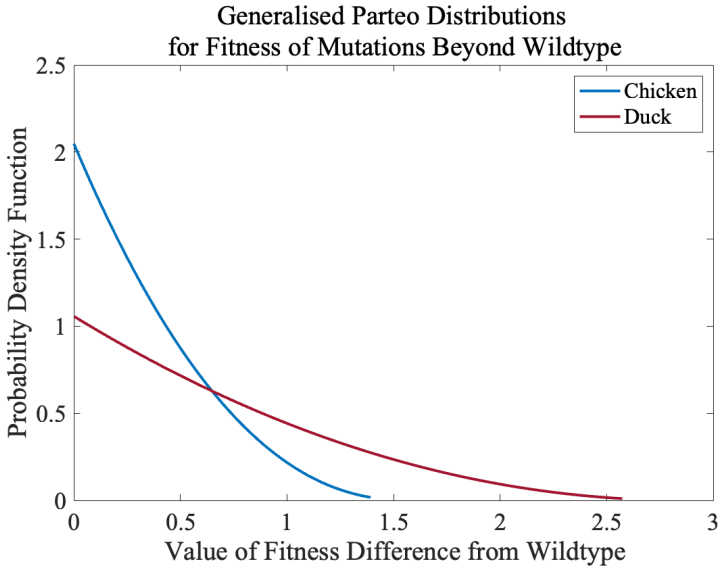

Figure 1 - Fitted generalised pareto in chicken (blue) and duck (red) cells.

In the context of Australia, currently free from H5N1, poultry vaccination would not hold the same benefits as its implementation in France. Moreover, implementation of vaccination programs in Australia would require robust DIVA (Differentiating Infected from Vaccinated Animals) strategies, enhanced active surveillance (targeted sampling and laboratory testing), and increased virological/genetic monitoring to detect breakthroughs—adding logistical and diagnostic burdens. Accordingly, at this point in time, without significant changes to Australia's avian influenza surveillance the disadvantages of poultry vaccination outweigh the advances. However, it is important to note that upon the incursion of H5N1 in Australia this

risk-benefit analysis will change significantly, and the poultry industry must be ready to adapt accordingly.

III. VACCINATION OF NATIVE SPECIES

Incursion of H5N1 into Australia will not just have important implications for the poultry industry but also our native species. Australia's native fauna are unique in terms of their immune response and genomics, and their susceptibility is likely to be markedly distinct from Northern Hemisphere species (Karawita, Cheng et al. 2023). To examine this in more detail we have recently generated a high-quality reference genome and transcriptome for the Australian black swan (*Cygnus atratus)* and compared it to the genome of the Northern Hemisphere mute swan (*Cygnus olor*) (Karawita, Cheng et al. 2023). We show that in the duck and mute swan (relative to their nearest common ancestor) immune genes have expanded over the course of evolution. In contrast, the black swan and chicken have not expanded their immune gene repertoire (Karawita, Cheng et al. 2023). We then infected primary duck, chicken and black swan cells with H5N1 and assessed the transcriptomic response. We showed that black swan cells produce a hyperinflammatory response to infection, akin to that of the chicken (Karawita, Cheng et al. 2023). In contrast, the duck had a more regulated antiviral response to infection. We further show that black swan cells lack the expression of a key sensor of viral infection: TLR7 (Karawita, Cheng et al. 2023). In contrast, TLR7 is expressed in mute swan tissues (Karawita, Cheng et al. 2023). Accordingly, we conclude that the black swan is more susceptible to avian influenza compared to its Northern Hemisphere counterparts due to the unique evolution of its immune response.

The susceptibility of black swans to avian influenza is unlikely to be restricted to this species and rather more likely to be a general feature of Australia's native fauna. Indeed, it is striking to note that in the Northern Hemisphere H5N1 has infected numerous different carnivores species who feed on dead or dying birds (Airey and Short 2024). This is particularly relevant in the Australian context for the Tasmanian Devil, whose population numbers have already been decimated by devil facial tumour disease and is known to have an impaired immune response (Cheng, Sanderson et al. 2012). The question therefore remains as to whether the vaccination of native species is a feasible strategy for H5N1 preparedness. Australia's federal authorities have explicitly included vaccination of priority native spec**ies** in preparedness planning and have issued policy guidance (Policy Decision 25-01) on emergency vaccination of rare, protected or valuable avian species as a response option during an HPAI incursion. New Zealand has moved from planning to small-scale trials and emergency preparedness for vulnerable native birds—conservation agencies have trialled vaccines (proof-of-concept/safety and immunogenicity work) for a handful of threatened species to protect core breeding populations. On a large scale, vaccination of native species is unlikely to be feasible. However, targeted vaccination of at-risk captive native species remains possible. Indeed, on the 20th of November 2025, Ceva, in collaboration with National Parks Board (NParks) and Mandai Wildlife Group, signed an official Memorandum of Understanding to launch a pilot avian influenza vaccination programme for zoological birds in Singapore (Webb 2025). This pilot, planned to start in 2026, will target a selected subset of bird species— especially species considered vulnerable to HPAI (such the white-backed vulture and brahminy kite), or of conservation significance (Webb 2025). The question therefore remains as to whether vaccination of at least captive native species should serve as a prepardness measure or whether such a strategy should only be implemented upon the incursion of H5N1. Unlike poultry vaccination, vaccination of native species does not hold any trade implications. Therefore, assuming that H5N1 vaccination in native species is safe and effective there is strong argument to commence vaccination of select captive populations of native species.

IV. CONCLUSIONS

In conclusion, vaccination remains a nuanced tool in Australia's preparedness for H5N1, with poultry vaccination offering limited benefits under current conditions but potentially becoming critical following an incursion. Targeted vaccination of captive native species, particularly those at high conservation risk, presents a feasible and low-risk strategy to mitigate severe disease impacts without affecting trade, and pilot programs such as the Ceva–Mandai initiative provide an important model for implementation.

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THE RELEVANCE OF STARCH AND PROTEIN DIGESTIVE DYNAMICS ON BROILER PERFORMANCE: A META-ANALYSIS

M.Z. WANG[1], S.P. MACELLINE[1] and S.Y. LIU[1]

The extent of nutrient digestion by the end of the distal ileum is conventionally used to evaluate feed quality, and poultry diets are typically formulated based on ileal digestible amino acids. However, the rate of starch and protein digestion may be more relevant to feed efficiency (Liu and Selle, 2015). Quantifying digestion rates is time-consuming and expensive. Consequently, a meta-analysis was conducted to investigate whether jejunal digestibility coefficients of starch and protein can serve as a reliable predictor of digestion rate and growth performance, and whether starch and protein digestibility should be considered in tandem when formulating.

A total of 260 observations across 32 studies conducted at the Poultry Research Foundation were collected for analysis. A robust random-effects meta-regression (REMA) was conducted using *metafor* package in R version 4.5.0, with studies included as a random factor, and final bird age and duration of the experiment included as covariates. The coefficients of variation (CVs) for starch digestibility were 24.6%, 10.0%, 7.8%, and 7.0% in the proximal jejunum (PJ), distal jejunum (DJ), proximal ileum (PI), and distal ileum (DI), respectively. The CVs for protein digestibility were 33.2%, 17.4%, 9.6%, and 8.7% in the PJ, DJ, PI, and DI, respectively. A low CV represents more consistent digestibility in each section. It was deduced from REMA that the digestible starch to protein ratio in PJ, DJ, PI, and DI was impacted by the dietary starch to protein ratio ($S:P_{diet}$), with coefficients of 2.550, 1.430, 1.590 and 1.262, respectively. BWG could be predicted based on starch and protein digestibility in PJ using the equation presented below. Correlations between starch digestibility in PJ and DI with BWG are shown in Figure 1.

$$BWG = -1032 + 331 \times Starch\ dig\ in\ PJ\ \times Protein\ dig\ in\ PJ\ + 61 \times End\ age + 35\ \times duration\ (P < 0.001)$$

In conclusion, the interaction between starch and protein digestibility confirms the necessity to consider starch and protein in tandem when formulating. Moreover, jejunal digestibilities of starch and protein act as reliable predictors of growth performance, and establishing a comprehensive database on the digestibility of starch, protein, amino acids in jejunum will optimise feed formulations, thus improving feed efficiency.

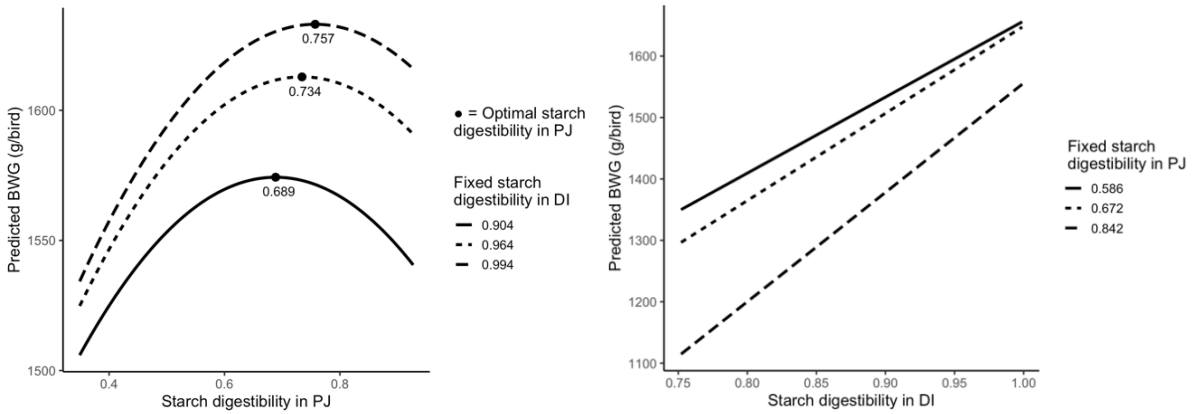

Figure 1 - The correlation between apparent digestibility of starch in PJ and DI and BWG (mean end age = 29.0, duration days = 20.6).

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IN VITRO XYLO-OLIGOSACCHARIDE RELEASE AND *IN VIVO* CAECAL SHORT-CHAIN FATTY ACID LEVELS IN BROILERS FED WHEAT- OR MAIZE-BASED DIETS

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Endo-xylanase hydrolysis of xylan releases xylo-oligosaccharides (XOS), which act as prebiotics in poultry by stimulating fibre-fermenting bacteria and enhancing short-chain fatty acid (SCFA) production in the caeca. This study hypothesized that a carbohydrase preparation would generate XOS in a substrate-dependent manner, and that *in vitro* release and *in vivo* caecal fermentation responses could be linked. Two broiler diets (wheat-soy or maize-soy) were tested ± a mixture of xylanase (560 TXU/kg) and β-glucanase (250 TGU/kg; Natugrain® TS, BASF SE, Germany) in a 2 × 2 factorial arrangement *in vitro* and *in vivo*. *In vitro*, diets were subjected to a two-step digestion (gastric and intestinal) to quantify XOS release (degree of polymerisation 2 – 5). *In vivo*, each treatment had eight replicates of 12 birds. Overall (d 0-35) growth performance was recorded, and caecal SCFA were measured at d 21 from four birds per replicate pen. *In vitro*, a diet × enzyme interaction was observed where enzyme addition increased XOS2 (P = 0.049) and XOS3 (P = 0.010) release in the wheat-soy diet but not in the maize-soy diet. XOS4 and XOS5 were absent in the maize-soy diet, whereas both increased with enzyme addition in the wheat-soy diet (P = 0.042 and 0.001, respectively; independent *t*-test), highlighting that wheat has more enzyme-susceptible xylans and more potential for enzyme-mediated prebiotic XOS generation. *In vivo*, an interaction was observed for d35 body weight (P = 0.013), with a greater enzymes response in birds fed the maize-soy diet than the wheat-soy diet. Enzyme supplementation improved overall FCR (P < 0.001) by 9.3 points in the wheat-soy diet and 6.8 points in the maize-soy diet. The wheat-soy diet led to greater total caecal SCFA level (P = 0.013) than those fed the maize-soy diet. Enzyme increased caecal butyric acid (P = 0.039) and tended to increase total SCFA (P = 0.082). In the wheat-soy diet, *in vitro* XOS2-XOS5 levels positively correlated with *in vivo* caecal SCFA production (P < 0.045), with XOS5 showing the strongest link (*r* = 0.523, P = 0.018). In conclusion, enzyme supplementation improved feed efficiency in both diets. Enhanced XOS release and caecal fermentation were more pronounced with the wheat-soy diet. Further research is needed to fully clarify how enzyme-mediated XOS release consistently translates into improved gut function and bird performance.

ACKNOWLEDGEMENT: This study was funded by BASF SE in partnership with Poultry Hub Australia.

Table 1 - Effects of carbohydrases on *in vitro* XOS release and performance of broilers fed wheat-soy or maize-soy diets.

Diet type	Wheat-soy		Maize-soy			P-value		
Xylanase + β-glucanase	-	+	-	+	SEM	Diet	Enzyme	Interaction
In vitro feed XOS release (mg/kg)								
XOS2	8.3^{b}	13.7^{a}	3.1^{c}	3.9^{c}	1.08	<0.001	0.012	0.049
XOS3	2.0^{b}	5.2^{a}	0.7^{b}	0.7^{b}	0.54	<0.001	0.007	0.010
XOS4	1.2	2.6	ND	ND	-	-	-	-
XOS5	0.9	1.4	ND	ND	-	-	-	-
In vivo feeding								
d 35 body weight (g/b)	2,291	2,335	2,230	2,366	23.5	0.114	<0.001	0.013
d 0 - 35 FCR (g/g)	1.48	1.39	1.47	1.40	0.016	0.979	<0.001	0.488
d21 Caecal butyric acid (µmol/g)	17.6	21.1	16.6	19.0	1.4	0.250	0.039	0.715
d21 Caecal total VFA (µmol/g)	99.7	104.4	73.0	93.3	7.3	0.013	0.082	0.340

[ac]Means (n = 5 for *in vitro*, n = 8 for *in vivo*) in a row followed by a different superscript are significantly different (P < 0.05).

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DOSE RESPONSE EFFECTS OF LYSOLECITHIN IN A LOW ENERGY DIET ON GROWTH PERFORMANCE AND PROFITABILITY OF BROILER PRODUCTION

M. OLDNALL[1] and K. SUNG[1]

Feed nutrition evolves alongside genetic improvements in poultry. Dietary energy plays a crucial role in feed intake and efficiency, directly influencing the profitability of broiler production. Recent broiler standards reflect downward adjustments in dietary energy, driven by rising feed costs, welfare considerations, and improved efficiency (Aviagen, 2022). However, excessive or inadequate energy reduction can negatively affect nutrient balance and growth performance. Functional feed additives may support energy utilization and efficiency, thereby enhancing the value of feed energy. Lysolecithin (LPL) has been reported to improve nutrient absorption and feed efficiency in broilers. (Boontiam et al, 2017; Zhao and Kim, 2017). This study evaluated the dose-response of LPL supplementation in a low-energy diet on growth performance and profitability.

A total of 1056 one-day-old male broilers (Ross 308) were allocated with six dietary treatments in a completely randomized block design based on initial body weight. Each group had 8 replicates with 22 birds per pen. Treatments included: positive control (PC, basal diet with formulated according to the Ross 308 nutrient standard); negative control (NC; basal diet with 200 kcal/kg lower ME); NC + 0.0065% LPL (LPL 6.5); NC + 0.015% (LPL 15); NC + 0.025% (LPL 25); and NC + 0.035% (LPL 35). LPL was supplied from a commercial product (Lipidol® Prime, Pathway Intermediates). Birds were fed experiment diets in 3 feeding program, starter (d 0-14), grower (d 15-28) and finisher (d 29-35) periods. Body weight (BW), feed intake (FI) and feed conversion ratio (FCR) were recorded weekly and production profit was calculated as live bird income minus total feed cost. Data were analysed using the GLM procedure of SAS software. Fixed effects of dietary treatment were tested, including contrasts for positive vs. negative control and orthogonal polynomial contrasts (linear and quadratic trends). Tukey's test was applied for pairwise comparisons, with significance declared at $P < 0.05$.

BW during d 0–7 was unaffected by treatments. At d 14, NC birds were heavier than PC ($P = 0.010$), while a linear response to LPL was observed by d 35 ($P = 0.049$). FI was consistently higher in NC compared with PC during weeks 1–3 ($P < 0.05$). LPL supplementation further increased FI with quadratic responses in weeks 2 and 4 ($P < 0.05$). During week 5, LPL supplementation linearly improved BW gain ($P = 0.012$), resulting in better FCR ($P = 0.006$). Over the entire trial (d 0–35), LPL linearly increased BW gain and improved FCR ($P < 0.05$). Economic evaluation showed that LPL supplementation improved profitability by up to €0.15 per bird compared with NC. Notably, LPL 25 and LPL 35 also exceeded PC profitability by €0.08 and €0.09 per bird, respectively.

In conclusion, LPL supplementation at 0.025–0.035% in a low-energy diet improved growth performance, feed efficiency, and profitability of broilers, demonstrating its value as a nutritional tool to compensate for reduced dietary energy density.

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IMPACT OF WHEAT PARTICLE SIZE IN REDUCED SOYBEAN MEAL DIETS ON BROILER PERFORMANCE

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Feed particle size plays a critical role in broiler growth performance. Coarser particles or whole-grain feeding could stimulate gizzard development, its activity and grinding efficiency, which together may improve nutrient utilization. Therefore, this study was designed to evaluate the interactive effects of hammer-milling screen size (HMS) of wheat and whole-wheat feeding with soybean meal (SBM) inclusion levels on nutrient digestibility and growth performance of broiler chickens offered typical wheat-SBM-canola-based diets and diets containing low or no SBM.

A total of 366, one-day-old, off-sex male Ross 308 broiler chickens were used in a 4 × 2 factorial arrangement, including four HMS of wheat and two levels of SBM inclusion. In the starter phase, all diets used 3.2 mm ground wheat, and 5% wheat was pre-pelleted in whole wheat treatments. In the grower phase, SBM was included at either 210 or 52.5 g/kg with wheat ground to 3.2 or 6.0 mm or partly replaced by 7.5% whole wheat. In the finisher phase, SBM was 170 or 0 g/kg with wheat ground to 3.2 or 6.0 mm, or 15% whole wheat. Peas, canola meals and non-bound amino acids were used to balance the digestible amino acids as per breeder recommendations in low/nil SBM diets. Each diet was offered to 7 replicates of 6 birds per bioassay cage from 0 to 35 days post-hatch.

During the overall period, there was no interaction between the HMS and inclusion level of SBM on any growth performance parameters. As a main effect, low/nil SBM diets decreased body weight gain (2434 versus 2354 g/bird, $P < 0.001$), increased FCR (1.523 versus 1.494, $P = 0.002$), and increased age to 2.5 kg body weight ($P < 0.001$). Wheat HMS did not influence the growth performance. There was an interaction effect between wheat HMS and inclusion level of SBM on relative *Pectoralis* minor weight ($P = 0.021$) and gizzard weight ($P < 0.001$). Whole wheat feeding reduced *Pectoralis* minor weight compared to other HMS levels in standard SBM diets, but no difference was found with low/nil SBM diets. In addition, whole wheat feeding increased gizzard weight by 29.4% and 14.0% in standard and low SBM level, respectively, than other dietary treatments. Low/nil SBM diets resulted in lower relative *Pectoralis* major weight ($P < 0.001$) but higher relative leg quarters weight ($P < 0.001$) and fat pad weight ($P < 0.001$) compared to standard SBM diets. In addition, pH values were lower across the intestines of birds fed low/nil SBM diets, as observed in the gizzard ($P = 0.045$), jejunum ($P = 0.032$), and ileum ($P < 0.001$), regardless of wheat HMS. Over days 31–33, standard SBM (130 g/kg) diets showed higher protein disappearance rates at the distal jejunum and ileum, than diets containing low/no SBM. Ileal pH at day 35 was negatively correlated to FCR ($r = -0.903$; $P = 0.002$).

In conclusion, HMS of wheat or whole grain feeding had no impact on growth performance in broiler chickens offered varying in SBM levels; however, the complete replacement of SBM in diets compromised body weight gain, feed conversion ratio, and breast meat yield.

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IMPROVING AN *IN VITRO* DIGESTION MODEL TO EVALUATE FEED INGREDIENTS IN BROILER CHICKENS

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The assessment of feed ingredient digestibility plays a crucial role in least-cost feed formulation in poultry nutrition. However, *in vivo* measuring digestibility in chickens has animal welfare and economic drawbacks compared with *in vitro* techniques. *In vitro* digestion models are useful for evaluating nutrient digestibility in poultry diets, but some seem to oversimplify digestive conditions (Losada et al., 2010) while others involve complex procedures that hinder reproducibility (Bean et al., 2016). In addition, there has been limited study on how feed particle size influences enzymatic digestibility. The gizzard mechanically grinds feed, reducing particle size and increasing surface area for enzyme access before entering the small intestine. Thus, *in vitro* models must account for ingredient-specific grinding behavior and simulate changes in digesta particle size found in the gastrointestinal tract. The objective of this research was to refine an *in vitro* digestion model based on particle size and use it to evaluate the digestibility of wheat and soybean meal (SBM).

Digesta was collected from three gastrointestinal sites (gizzard, jejunum, and ileum) at three ages (7, 28, and 42 days), using samples from at least 12 birds per age, which were divided into three replicate subsamples for particle size distribution analysis. Soybean meal and wheat were ground using a standard coffee grinder for 15 seconds to mimic the particle size distributions of intestinal digesta. Duplicate samples of ground and unground substrates (duplicates) were compared using a t-test. Duplicates samples of ground substrates were digested with four exogenous enzyme treatments (no enzyme, VP, multigrain, and both combined). Results were analyzed using one-way ANOVA.

The results showed that wheat had a higher starch and protein digestibility after grinding ($P<0.05$), indicating that particle size reduction improved enzymatic access and digestion rate. However, in ground wheat, the addition of different enzymes did not lead to further improvements in digestibility ($P>0.05$). For SBM, protein digestibility was similar across all treatments, regardless of grinding or enzyme addition ($P>0.05$). In conclusion, these results demonstrate the differential role of particle size in shaping ingredient-specific digestion responses and support the inclusion of physiologically relevant grinding in future *in vitro* models to improve their predictive value.

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VALIDATION OF A NEW NIRS APPROACH TO ESTIMATE METABOLIZABLE ENERGY IN BROILER DIETS

V. LARAT[1], M. CECCANTINI[2], P. THIERY[2], G. FONTINHAS-NETTO[2] and B. GUO[3]

Summary

A new Near-Infrared Spectroscopy (NIRS) approach was developed to estimate the actual metabolizable energy (AME) of broiler diets under field conditions. Using data from 47 in-vivo trials and over 1,500 matched feed–excreta samples, a robust NIRS calibration model was created, achieving a cross-validation error of 0.30 MJ/kg DM. Two independent validation trials confirmed that NIRS-predicted AME closely matched in-vivo measurements, both in individually caged birds at 21 days and in commercial pens at 42 days, with prediction errors similar to the calibration error. Results also highlighted that theoretical AME values commonly differ from real AME by about 5% in practice. Overall, this new NIR calibration provides nutritionists with a rapid, cost-effective, and reliable tool to evaluate feed energy value without conducting labor-intensive digestibility trials.

I. INTRODUCTION

Nutritionists worldwide face the common challenge of formulating diets that enable animals to reach their genetic potential while maintaining economic efficiency. Achieving such precision requires an accurate understanding of the nutritional quality of feed ingredients, which can vary substantially with origin, processing conditions, price dynamics, and storage time. The recent introduction of novel raw materials and by-products further increases variability, as their nutrient profiles may be inconsistent or poorly characterized. Although such ingredients may initially appear economically attractive, inadequate nutrient supply or poor digestibility can ultimately compromise animal performance. Consequently, precise feed analysis is essential to avoid over- or under-formulation and the associated economic losses.

Near-infrared (NIR) spectroscopy has become a widely adopted analytical tool within the feed industry due to its speed, accuracy, versatility, and suitability for routine assessment of both raw materials and complete diets. However, even when two diets present identical nutrient specifications at the feed mill, their performance on-farm may differ markedly. Variations in health status, environmental conditions, housing, management practices, and farm-specific factors all influence the animal's capacity to utilize the diet. Among the parameters determining the nutritional value of a feed, Apparent Metabolizable Energy (AME) remains central, as it reflects the usable energy available to the bird after accounting for losses in excreta. Yet in practice, AME is often treated as a static value within formulation software, with limited consideration of how real production conditions affect digestibility.

To bridge this gap between formulation and field performance, an NIRS-based calibration (Feed Digestibility Check, Adisseo) was designed to accurately predict the true AME of broiler diets under commercial farming conditions. This approach enables nutritionists to better align formulated energy values with the actual digestible energy available to birds in production environments, thereby improving both precision nutrition and economic outcomes.

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II. METHOD

Using broiler AME data from 47 in vivo trials, an AME calibration equation was established based on the Near-infrared spectra (NIRS) of 1,548 dried excreta collected during the in-vivo trials and the corresponding feed samples. These samples were scanned over 1,100–2,500 nm on a NIRS instrument and analyzed by wet chemistry for gross energy and moisture to calculate AME. The feed and excreta spectra were concatenated and paired with the in vivo AME values to build the calibration model. The AME of the samples ranged from 10.96 to 16.54 MJ/kg dry matter (DM) (mean 14.42 MJ/kg DM; SD = 0.77 MJ/kg DM), and the model's cross-validation error (SECV) was 0.30 MJ/kg DM. In a similar approach of predicting Energy from combined NIRS spectra of feces and feed, Coulibaly et al. (2013) obtained a SECV of 0.24 MJ/kg DM on AME of broiler feed.

To check the validity of the NIRS model, two independent validation trials were conducted in different facilities using the same eight treatments (four diets at theoretical AME values of 13.39, 13.75, 14.10 and 14.57 MJ/kg DM, each with and without enzyme supplementation).

III. RESULTS AND DISCUSSION

In Trial 1, 90 excreta samples were collected from individually caged birds at 21 days of age and in vivo AME values were determined for each of the 90 individual birds for comparison with the NIRS predictions of the model.

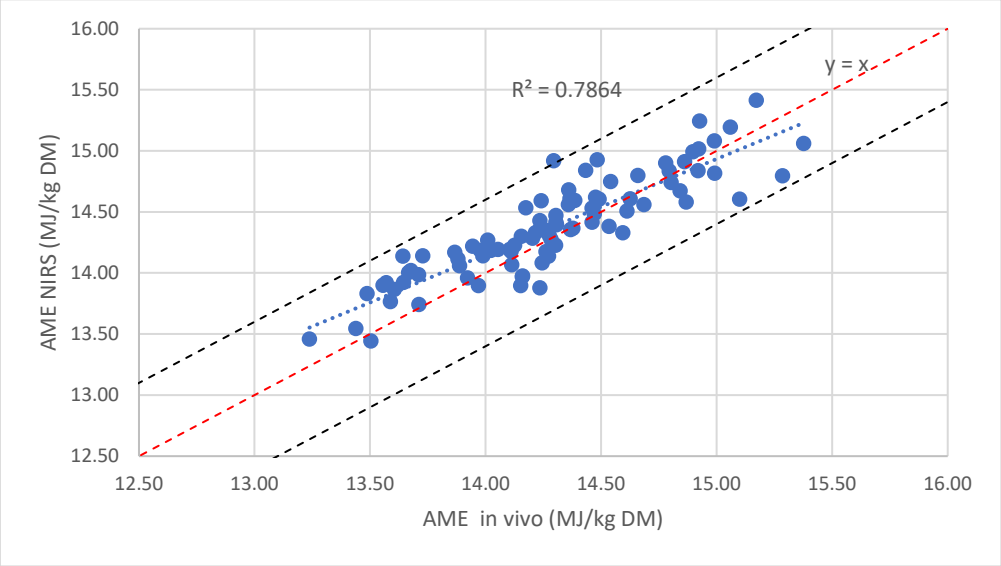

Figure 1 - Relationship between NIRS predicted and in vivo AME content (MJ/kg DM) measured on individual birds. The red dotted line is the line of equivalence, and the black dotted lines indicate the limits to the 95% probability precision of the calibration (± SECV x 2).

The prediction error (SEP) for the 90 samples in trial 1 was found to be 0.26 MJ/kg DM, in the same range of order than the model error, confirming the robustness of the NIRS calibration as discussed in Knudsen et al. (2023).

In Trial 2, 40 excreta samples (5 samples per treatment) were collected from 40 pens of 15 birds each at 42 days of age to verify the NIRS AME predictions under commercial conditions.

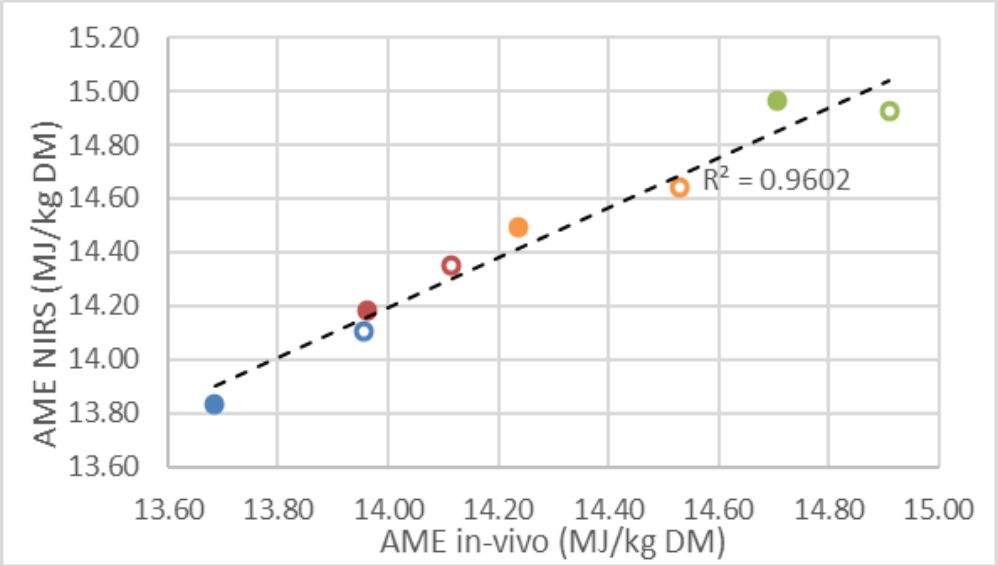

Figure 2 - Relationship between averaged of 5 NIRS predicted and measured in-vivo AME content (MJ/kg DM) for each treatment under commercial conditions farming. The dotted line is the prediction relationship. Each color represents a different diet: the colored disc being the diet without enzyme and the empty one being the diet with enzyme supplementation.

In Trial 2, the average AME values of the eight treatment regimens predicted by the NIRS model were strongly correlated with the in vivo AME values measured on these treatments in trial 1 (Fig. 2), further demonstrating its validity under field conditions. On average, the difference between theoretical and in vivo AME values was 5% (0.61 MJ/kg DM), illustrating the typical discrepancy faced by nutritionists in practice.

IV. CONCLUSION

A new NIR calibration is therefore a rapid and reliable tool for assessing feed AME under real-world production conditions, providing nutritionists with vivo-aligned digestibility information without the time and cost of traditional trials.

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CRYSTALLINE ARGININE IMPROVES FEED EFFICIENCY AND STRESS RESILIENCE COMPARED TO INGREDIENT-BASED SOURCES IN HEAT-EXPOSED BROILERS

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Summary

This study compared crystalline and ingredient-based arginine supplementation in broilers under heat stress. Crystalline arginine improved early-phase feed efficiency and reduced stress markers such as ASS/ASL activity, total antioxidant capacity (T-AOC) and nitric oxide (NO) content in the serum attributable to its higher bioavailability. Increasing arginine level through crystalline arginine improved FCR until first 28 days, however, treatment effect diminished in the final finishing phase as the ambient temperatures decreased. The findings suggest that crystalline arginine holds potential for stress mitigation during thermal stress.

I. INTRODUCTION

Poultry production in tropical regions continues to struggle with significant performance losses due to persistent heat stress. Specifically, exposure to hyperthermic conditions negatively impacts feed intake, and growth, while concurrently compromising both carcass quality and immunocompetency in broiler chickens (Lara & Rostagno, 2013). These effects represent a critical threat to both economic viability and animal welfare.

To address these challenges, nutritional strategies have gained significant attention. Among them, the use of functional amino acids, especially L-arginine, has shown promising potential (Khajali & Wideman, 2010). L-arginine plays a key role in thermoregulation as a precursor to nitric oxide (NO), a molecule that promotes vasodilation and helps the body release excess heat. It also supports immune function and helps manage oxidative stress, which is particularly important when birds are exposed to high temperatures (Tan et al., 2021).

Although L-arginine has known benefits, it is rarely supplemented directly in practical diets. Instead, nutritionists typically rely on arginine-rich ingredients, such as soybean meal, peanut meal, meat and bone meal, or fishmeal to increase the overall arginine intake (Ravindran, 2013). However, this indirect method has potential drawbacks. It can lead to an excess inclusion of non-essential amino acids and total nitrogen, which in turn may increase metabolic heat production—counteracting the very benefits arginine is meant to provide under heat stress conditions (Temim et al., 2000; Siegert et al., 2016).

To date, few studies have directly compared the effects of crystalline L-arginine versus ingredient-based arginine supplementation in heat-stressed broilers. To address this, the effects of crystalline and ingredient-based arginine on broiler performance were compared under tropical summer conditions. The hypotheses tested in this study were (1) increasing arginine supplementation and (2) the crystalline arginine supplementation compared with ingredient-bound arginine supplementation would improve performance indices and markers of heat stress in broilers reared under tropical summer conditions.

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II. METHOD

A total of 1,000 one-day-old male broiler chicks (Vencobb strain) were randomly assigned to four dietary treatments using a 2 × 2 factorial arrangement of treatments. The tested factors were arginine source (crystalline vs. ingredient-based) and supplementation level (standard 107% vs. elevated 120% digestible Arg:Lys ratio). Each treatment had 10 replicate pens with 25 birds per pen. Birds were housed on deep litter flooring and provided ad libitum access to feed and water for 42 days.

Diets were based on a corn–soybean meal formulation and provided in three phases: starter (1–14 days), grower (15–28 days), and finisher (29–42 days). Arginine levels were adjusted by supplementing either crystalline L-arginine or arginine-rich peanut meal, while all other indispensable amino acids, including threonine, were balanced across treatments using crystalline amino acids to maintain identical amino acid specifications. The diets were formulated to provide metabolizable energy (AME) levels of 2950, 3050, and 3150 kcal/kg and standardized ileal digestible lysine (SID Lys) levels of 1.250%, 1.152%, and 1.000% for the starter, grower, and finisher phases, respectively.

The trial was conducted during the summer season in an open house system, relying on natural fluctuation of ambient temperature. Morning temperatures ranged from 27.4°C to 36.6°C, and evening temperatures from 25.1°C to 30.9°C. Relative humidity ranged from 20.7% to 54.9% in the morning and 42.2% to 68.5% in the evening.

At the end of the experimental period, blood samples were collected from selected birds, and serum was separated by centrifugation for biochemical analyses. Nitric oxide (NO) concentration was determined indirectly by measuring nitrite (NO_2^-), a stable metabolite of NO, using a Griess-based colorimetric assay kit (Elabscience, China; E-BC-K035-M). Absorbance was measured at 550 nm using a microplate reader.

Total antioxidant capacity (T-AOC) was assessed using a ferric reducing antioxidant power (FRAP) colorimetric assay kit (Elabscience, China; E-BC-K225-M), and absorbance was measured at 593 nm.

Serum ASS and ASL protein levels were quantified using a microplate-based colorimetric method. Absorbance was measured at 450 nm using a microplate reader, and concentrations were calculated from a standard curve and expressed as ng/mL.

III. RESULTS

This study was conducted during the summer season, under naturally occurring elevated ambient temperatures that can impose physiological and metabolic stress on poultry. However, unfortunately, the temperature decreased in the finishing phase and the ambient temperature reached below 27 °C. Under these conditions, birds fed crystalline arginine significantly reduced argininosuccinate lyase (ASL) activity (1665 vs. 2606 U/L; $P = 0.042$) compared to the ingredient-bound arginine group. Argininosuccinate synthetase (ASS) activity was not affected by treatment. In addition, total antioxidant capacity (T-AOC) was significantly higher in the crystalline group (24.02 vs. 14.7 U/mL; $P < 0.001$), and serum nitric oxide (NO) levels were also markedly increased (114.43 vs. 59.74 μmol/L; $P < 0.001$), suggesting potential enhancements in oxidative balance and vascular signaling. A minor but significant increase in NO was also observed at the higher arginine level (93.16 vs. 84.05 μmol/L; $P = 0.034$).

Table 1 - Effects of dietary arginine level (107% vs. 120%) and arginine source (crystalline vs. ingredient-bound) on, urea cycle enzyme activities (ASS, ASL), total antioxidant capacity (T-AOC), and nitric oxide (NO) concentrations in broiler chickens at day 28.

		ASS (ng/mL)	ASL (ng/mL)	T-AOC (mmol/L)	NO (μmol/L)
Main factors					
Level	107	4.42	2137	19.73	84.05^{X}
	120	5.179	2098	18.62	93.16^{Y}
Source	Crystalline	5.262	1665^{X}	24.02^{Y}	114.43^{Y}
	Ingredient	4.33	2606^{Y}	14.7^{X}	59.74^{X}
P-value					
Interaction		0.881	0.448	0.056	0.14
Level		0.397	0.972	0.106	0.034
Source		0.292	0.042	<0.001	<0.001

A, B or X, Y: Values within a column with different superscripts differ significantly at $P < 0.05$ or tend to differ at $0.05 \leq P < 0.10$.
ASS: Argininosuccinate synthetase; ASL: Argininosuccinate lyase.

In terms of growth performance, feed conversion ratio (FCR) was significantly improved in the crystalline group at both 1–2 and 1–4 weeks ($P < 0.001$). Higher arginine level (120%) also led to better FCR during 1–4 weeks ($P = 0.0039$). However, body weight gain (BWG) and feed intake (FI) were not significantly affected at any time point ($P > 0.05$), and no differences in BWG were observed in the overall period from 1 to 6 weeks.

Table 2 - Growth performance by treatment (T1–T4) and two-way factorial effects (Level [107 vs 120] × Source [Crystalline vs Ingredient]) at 1–2, 1–4, and 1–6 weeks.

		1-2wk			1-4wk			1-6wk		
Level	Source	BWG (g)	FI (g)	FCR	BWG (g)	FI (g)	FCR	BWG (g)	FI (g)	FCR
107	Crystalline	453.1	531.5	1.173	1354.3	1852.3	1.363	2482.4	3913.9	1.577
107	Ingredient	445.8	531.3	1.192	1332.1	1860.4	1.386	2459.9	3908.4	1.589
120	Crystalline	459.4	532.3	1.159	1355.7	1839.4	1.357	2426.8	3881.8	1.600
120	Ingredient	453.0	532.8	1.176	1349.7	1855.9	1.375^{b}	2437.5	3884.0	1.594
SEM		7.6	8.1	0.0031	12.9	15.6	0.0053	42	59.7	0.0054
P-value		0.662	0.999	<0.001	0.552	0.799	<0.001	0.793	0.972	0.041
Main factors										
Level	107	449.4	531.4	1.183^{A}	1343.2	1856.3	1.382^{A}	2471.2	3911.1	1.583^{B}
	120	456.2	532.6	1.168^{B}	1352.7	1847.6	1.366^{B}	2432.2	3882.9	1.597^{A}
Source	Crystalline	456.3	531.9	1.166^{y}	1355.0	1845.9	1.363^{y}	2454.6	3897.9	1.589
	Ingredient	449.4	532.0	1.184^{x}	1340.9	1858.1	1.386^{x}	2448.7	3896.2	1.592
P-value										
Interaction		0.954	0.967	0.772	0.534	0.789	0.328	0.696	0.949	0.116
Level		0.381	0.888	<0.001	0.466	0.578	0.004	0.360	0.638	0.018
Source		0.374	0.983	<0.001	0.282	0.436	<0.001	0.889	0.978	0.555

A, B: Main effect of arginine level significantly different at $P < 0.05$.
x, y: Main effect of arginine source significantly different at $P < 0.05$.
BWG: Body weight gain; FI: Feed intake; FCR: Feed conversion ratio.

IV. DISCUSSION

These findings suggest that crystalline arginine may help birds cope better with heat stress, possibly due to higher bioavailability and lower metabolic burden associated with non-essential amino acid catabolism, which otherwise contributes to increased endogenous heat production for uric acid synthesis. Similar effects of L-arginine on stress biomarkers and nitrogen metabolism have been reported in poultry and waterfowl under thermal stress (Lee et al., 2024; El-Hadad et al., 2024).

Elevating the arginine level, specifically through crystalline arginine supplementation, improved performance and attenuated the stress response in broilers, particularly during the early growing phase. Although crystalline arginine effectively mitigated the stress response, as indicated by improved biomarkers, its effect on overall growth was limited, especially throughout the final finishing phase. This lack of sustained growth difference is most likely attributable to a progressive decrease in ambient temperature, which reduced the relative thermal stress burden. As previously observed by Brugaletta et al. (2023), the benefits of nutritional intervention aimed at combating heat stress may diminish concurrently with an improvement in environmental conditions. Furthermore, the marked enhancement of T-AOC and NO levels suggests that crystalline arginine may exert additional protective effects by reinforcing antioxidant defense and supporting nitric oxide-mediated vasodilation under thermal stress.

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PROTEASE SUPPLEMENTATION CAN AMELIORATE NEGATIVE EFFECTS OF HIGH TRYPSIN INHIBITOR IN BROILER DIETS

D SANCHEZ TORRES[1] G. RUEDA [1], J. PRADA [1], R. QUISIEH [1] and E CASTRO[2].

Summary

The presence of protease inhibitors—primarily the Kunitz trypsin inhibitor (KTI) and the Bowman–Birk inhibitor (BBI)—limits SBM nutritional value compromising performance in broilers. The aim of this study was to evaluate the effect of protease supplementation on growth performance of broilers fed diets containing high TI content and raised to 35 d of age. A total of 1632 Ross 308 male broiler chicks were randomly allocated to one of four treatments with 12 replicate pens per treatment and 34 birds per replicate: all dietary treatments contained soybean meal (SBM, TI: 3.8 mg/g), full fat soy (FFS, TI: 4.7 mg/g), and 3% raw soybeans added in the grower and finisher phases (TI: 34.0 mg/g). The dietary treatments were: positive control (PC) that was formulated according to the breed's feed specifications; negative control (NC) that was formulated with a 5% reduction of digestible amino acids compared to PC; NC+exogenous protease at 250 g/MT (P250); and NC+exogenous protease at 500 g/MT (P500). For the last two treatments, the composition was same as the NC with the addition of either 250 or 500 g/MT exogenous protease, respectively. No differences in BWG at 35 days were observed among treatments (P=0.207), while significant difference was observed for feed intake (P=0.0495) and FCR (P=0.015) between the NC and T4 P500 with P500 having a better FCR at a lower FI, the PC and P250 were intermediate. Supplementing protease at 500 g/MT decreased feed intake by 3.14% and FCR by 5 points compared to NC, and 2.35% and 1.5 points beyond the PC for feed intake and FCR, respectively. Based on the results, supplementation of exogenous protease can improve growth performance in broilers through mitigating the negative effect of high levels of TI as well as compensating for 5% of amino acid reduction.

I. INTRODUCTION

Trypsin inhibitors are naturally occurring proteins found predominantly in soybeans and other legumes. These inhibitors bind to trypsin—an essential enzyme responsible for breaking down dietary proteins into absorbable amino acids. When trypsin activity is compromised, protein utilization decreases, leading to reduced growth rates and impaired feed conversion efficiency. The Bowman–Birk inhibitor is a small, disulfide-rich protein featuring dual reactive loops capable of inhibiting both trypsin and chymotrypsin (Birk, 1985). Its structure underlies its stability during processing, where achieving adequate inactivation without amino acid degradation remains a challenge (Avilés-Gaxiola et al., 2018). Despite advances in breeding and CRISPR-mediated reduction of inhibitor expression (Kim et al., 2024), residual inhibitor activity persists in commercial SBM (Chen et al., 2014).

Recent literature highlights that not all proteases are equally affected by BBI inhibition (Pérez-Calahorra et al., 2023). Some exogenous enzymes, including feed-grade serine endoproteases, may evade or hydrolyze BBI, maintaining activity in the gastrointestinal tract. The concept of using feed proteases to mitigate BBI has been supported by studies showing improved amino acid digestibility and performance in broilers fed high-TIA SBM supplemented with exogenous protease (Wang et al., 2006; Wedekind et al., 2020). These proteases have been consistently associated with enhanced feed conversion efficiency, growth rate, and nutrient utilization in poultry, particularly under conditions of elevated trypsin-inhibitor activity.

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II. METHOD

A total of 1632 Ross 308 male broiler chicks were randomly allocated to one of four treatments with 12 replicate pens per treatment and 34 birds per replicate: All treatments were formulated using soybean meal (SBM), full fat soy (FFS) with low TI levels (3.8 and 4.7 mg/g, respectively) and 3% of raw soy with 34,0 mg/g of TI; A positive control (PC) diet was formulated according to the breed's feed specifications. The negative control (NC) was formulated with 5% lower levels of digestible amino acids compared to PC. The remaining two treatment were compositionally same as NC with the addition of exogenous protease at 250 g/MT (P250) and 500 g/MT (P500), respectively.

The calculated level of TI for grower diet was 2,33 mg/g and 2,19 mg/g for the finisher. Mash diets were provided over 3 dietary phases: starter (0-14d), grower (15-21d), and finisher (22-35d). Body weight and feed intake were determined at 35 d of age. Data was analyzed as one-way ANOVA, and means were separated using Tukey HSD test ($P \leq 0.05$).

Table 1 - Broilers performance at 35d.

	PC	NC	P250	P500	P Value	CV
BW	1937.46	1907.49	1919.89	1910.75	0.2070	5.56%
Feed Intake	2839.75^{ab}	2862.58^{a}	2817.62^{ab}	2772.66^{b}	0.0495	9.78%
FCR	1.466^{ab}	1.501^{a}	1.468^{ab}	1.451^{b}	0.0150	7.72%
FCR Adj.	1.475^{ab}	1.515^{a}	1.480^{ab}	1.464^{b}	0.0176	7.73%
EEI	357.2	333.76	347.76	351.39	0.1276	8.32%

III. RESULTS AND DISCUSSION

No differences in BWG at 35 days were observed among treatments (P=0.207), while significant difference was observed for feed intake (P=0.0495) and FCR (P=0.015) between the NC and P500 . PC and P250 being intermediate. Supplementing protease at 500 g/MT decreased feed intake by 3.14% and FCR by 5 points compared to the negative control, and 2.35% and 1.5 points beyond the PC for feed intake and FCR, respectively.

IV. CONCLUSION

Based on the results, supplementation of exogenous protease can improve growth performance in broilers through mitigating the negative effect of high levels of TI as well as compensating for 5% of amino acid reduction.

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PEAS AND LENTILS OR CANOLA MEAL USED AS SOYBEAN MEAL ALTERNATIVES IN BROILER BREEDER DIETS IMPROVED YOLK PIGMENTATION

B. XU[1], B.C. RAY[1], A. KUMAR[1] and E. ROURA[1]

The Australian poultry industry relies on imported soybean meal (SBM) as the primary protein source in feed formulations. This reliance on SBM raises concerns about the long-term sustainability of meat and egg production in Australia. Local protein sources, such as canola meal (CNM), field peas and lentils (PL), have been tested as partial substitutes of SBM with positive results in broiler chickens. However, much less is known on the impact of these protein sources in broiler breeder diets. Therefore, the aim of this study was the assessment of alternative protein sources in broiler breeders. It was hypothesised that local protein sources (i.e., CNM, PL) can be used in broiler breeder diets with no negative impact on egg quality parameters compared to SBM.

A total of 261 Ross 308 broiler breeders (237 hens and 24 roosters) were fed four feeding treatments differing in the cereal (corn or wheat) or the protein source (SBM, CNM, or a PL mix): corn-soybean (CS) with 15% SBM, wheat-soybean (WS) with 8.5% SBM, wheat-canola (WC) with 10.8% CNM, and wheat–pea-lentils (WPL) with11.5% peas 11% Lentils for a period of six weeks (30 to 35 weeks of age). Each dietary treatment was distributed following a randomised block design into 24 pens with ten hens and one rooster in each pen. Feed intake was restricted following the breeder guidelines. Egg quality parameters were assessed at week 35 when four eggs were collected from each pen and analysed for Haugh unit and albumen height (Egg Multi Tester, Robotmation Co., ltd. JP), yolk colour score (Roche Yolk Colour Fan), eggshell thickness (Digital Caliper, 0-150mm) and eggshell breaking strength (Egg Force Reader, Orka Food Technology Ltd. USA). Data were analysed using PROC GLM (SAS 9.4).

The results showed that both the cereals and the protein sources significantly ($P<0.0001$) affected yolk colour whereas the CS produced the darkest yolks compared to any of the wheat diets (Table 1). Within the wheat diets, colour intensity was the darkest for the WPL which is significantly higher than WC and WS. No significant effects were observed for any of the other parameters studied. This is consistent with the amount of carotenoid content (µg/g DM, DM-dry matter), which is the highest in corn (~35µg/g DM) compared with wheat (~3.2µg/g DM) (Trono, 2019). Lentils and peas contain (~15µg/g DM) whereas very little present in soybean meal and canola meal (Langyan et al., 2022).

Table 1 - Egg quality indices of broiler breeders at 35 weeks of age.

Parameter	CS	WS	WC	WPL	SEM	P-value
Average egg weight (g)	58.14	60.29	59.20	59.54	0.753	0.279
Albumen height (mm)	8.15	8.24	8.15	8.22	0.082	0.777
Haugh unit	90.89	90.83	90.46	90.88	0.549	0.935
Yolk colour score	9.06[a]	2.92[c]	3.54[c]	5.12[b]	0.214	0.000
Shell thickness (mm)	0.362	0.364	0.369	0.357	0.003	0.073
Egg shell breaking strength (kgf)	4.249	4.135	4.093	4.003	0.163	0.770

CS-corn soybean, WS-wheat soybean, WC-wheat canola, WPL-Wheat-pea-lentils, n=6 per treatment (each replication is the average of four eggs)

In conclusion, SBM can be successfully replaced with CNM or PL in broiler breeder diets resulting in improvement of egg yolk pigmentation. This could have an added benefit to the antioxidative stress management and viability of the developing embryo.

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COMPARISON OF TWO COMMERCIAL PROTEASES ON THEIR EFFICACY FOR INCREASING AMINO ACID DIGESTIBILITY IN BROILERS FED A MIXED-CEREAL DIET

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Efficient protein digestion in broiler chickens is essential for optimizing growth and health as well as minimizing undigested protein excretion and reducing the environmental impact of nitrogen pollution. Exogenous proteases added to feed are used to improve protein and amino acids (AA) digestibility and utilization but with variable effects reported across studies (Wijayanti et al., 2025). Direct comparison studies of commercially available proteases are scarce.

This study evaluated the efficacy of two commercial proteases for improving the apparent ileal digestibility (AID) of AA in broilers fed a mixed-cereal diet. The two proteases comparatively evaluated were protease L, produced in *Bacillus licheniformis*, and protease S, produced in *Bacillus subtilis*. A total of 336 straight run Ross 308 hatchlings were assigned to 3 treatments (8 birds/cage, 14 cages/treatment) in a randomized complete block design and fed standard diets until 16 days (d) of age. Between 17 and 21 d of age, birds were fed one of three experimental diets, *ad libitum*, in pelleted form. Diet 1 of the three was a basal control (Control) based on corn, wheat and soybean meal with added sunflower and rapeseed meals, and supplemented with 1,000 FTU/kg phytase with the phytase full matrix application (down-specification in Ca, P, digestible AA, Na and metabolizable energy (ME)), and an additional protease S matrix application (down-specification of up to 0.02% points in digestible AAs and -20 kcal/kg in ME), vs. commercial broiler nutrient and energy adequate specification (CVB, 2018). Diet 2 was the Control supplemented with protease L at 50 g/ton, expected to supply 30,000 NFP/kg and diet 3 was the Control supplemented with protease S at 50 g/ton, expected to supply 4,000 U/kg. Both proteases were dosed according to their manufacturers recommended dosage. The analyzed dry matter, fat, CP, Ca, P, Lys, Met, Cys, Thr levels were 88.2, 4.8, 21.2, 0.65, 0.49, 1.26, 0.97, 0.68, and 0.93 % (basal starter diet); 90.1, 5.9, 19.5, 0.63, 0.49, 1.17, 0.77, 0.68, and 0.81 % (basal grower diets); and 89.8, 7.7, 18.7, 0.52, 0.40, 1.17, 0.77, 0.68, and 0.71 (basal finisher diet), respectively. Ileal digesta sampled from all birds on d 21 was used to determine the AID of 17 AAs, expressed on a per cage basis. Both proteases increased ($P < 0.05$) the AID of Cys compared to the Control (+4.25 and +4.64 % points for L and S, respectively); protease S additionally increased the AID of Met (+1.66 % points; $P < 0.05$) and tended to increase AID of Arg, Ile and Val ($P < 0.1$).

While usage of protease and phytase together can have an additive effect, these results demonstrate that both proteases are effectively increased AA digestibility in birds fed phytase-supplemented diets with modest AA content reduction; however, protease S showed a comparatively greater benefit than protease L under the test conditions.

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LOW-SOYBEAN MEAL DIETS HAVE MODIFIED AMINO ACID LIMITATIONS IN BROILER CHICKENS

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Summary

This paper explores the possibility that current breeder recommendations for ideal amino acid ratios do not suit low-soybean meal (SBM) broiler diets. The altered amino acid metabolism in such diets suggests that the traditional distinction between amino acids as essential and non-essential is less applicable, because the metabolic demand for glycine, serine, glutamine, and glutamate is increased.

I. INTRODUCTION

In conventional broiler diets, a primary focus is balancing essential amino acids in accordance with the breeders' recommendations. This approach originates from the ideal amino acid ratio concept proposed by Dean and Scott (1965), which defines a set of proportions across essential amino acids, expressed relative to lysine, to meet physiological requirements for protein synthesis and growth without creating imbalances. Conventional maize-SBM or wheat–SBM based diets are typically deficient in methionine, lysine, threonine, isoleucine, valine, arginine and tryptophan, which are therefore supplemented as non-bound amino acids (NBAAs) to meet breeders' recommendations. Since SBM provides a relatively well-balanced amino acid profile for broiler chickens (Heuzé et al., 2020), the inclusion of alternative plant protein sources (e.g., canola meal, sunflower meal, palm kernel meal) or animal protein sources (e.g., meat and bone meal, fishmeal) may increase the risk of deficiencies in certain essential amino acids when these ingredients partially replace SBM in the diet. For instance, in starter diets, reducing SBM from 262 to 167 g/kg and replacing it with 90 g/kg canola meal increased the supplementation of NBAAs such as threonine by 19%, lysine by 29%, valine by 58%, isoleucine by 133%, and arginine by 163% at comparable dietary crude protein (CP) concentrations (Kandal et al., 2025). In wheat–SBM based broiler diets, reducing dietary CP from 230 to 204 g/kg resulted in valine and arginine becoming limiting amino acids (Maynard et al., 2022). Similarly, reducing dietary CP from 203 to 187 g/kg in maize-based broiler diets resulted in valine alone becoming limiting (Maynard et al., 2020). Furthermore, Macelline et al. (2023) reported that broiler responses to two ideal amino acid ratios shifted when dietary CP concentrations were reduced from conventional to low levels. Dietary-CP reductions by reducing the inclusions of SBM prominently reduces the concentrations of non-essential amino acids in diets. For instance, reductions in dietary-CP by around 4 percentage points led to a 23.4% decrease in dietary glutamate plus glutamine concentrations, as reported by Macelline et al. (2024). Findings from Wang et al. (2024) indicate that achieving broiler performance objectives with low-CP diets requires precise adjustment of the dietary amino acid profile. Emerging evidence also indicates that non-essential amino acids play a pivotal role in reduced-CP diets, where their availability may become limiting (Siegert et al., 2025). Therefore, this paper consolidates the concept that broiler chickens fed diets formulated with low SBM inclusions may have modified amino acid requirements in comparison to those fed standard broiler diets.

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II. AMINO ACID PROFILES IN COMMON FEEDSTUFFS: HOW BALANCED ARE THEY?

Amino acid profiles of feedstuffs used in broiler diet formulations determine which amino acids become limiting in relation to the targeted amino acid recommendations for broiler chickens. It is important to note that broiler diets are formulated not only to achieve the desired amino acid balance but also to meet specific CP and metabolizable energy levels. The identity of the fourth limiting amino acid depends on the dietary composition; valine has been identified as the fourth limiting amino acid in maize–SBM diets (Maynard et al., 2020), whereas valine and isoleucine may become co-limiting when animal protein meals are included (Dozier et al., 2011). Furthermore, Edmonds et al. (1985) demonstrated that methionine, lysine, threonine, arginine, and valine are the most critically limiting amino acids in low-protein, corn–SBM diets. In Table 1, the standardized ileal digestible (SID) amino acid profiles of commonly used feedstuffs were compared with the amino acid recommendations of Maharjan et al. (2021), using chi-square distance values as described by Guilloteau et al. (1983). A lower chi-square distance indicates a closer match between the amino acid profile of a given feedstuff and the amino acid requirements of broiler chickens. Among the evaluated feedstuffs, SBM exhibited the amino acid profile most closely aligned with broiler requirements, whereas meat and bone meal showed the greatest deviation. Among cereal grains, maize had a more favourable amino acid profile than wheat or sorghum. Therefore, replacing SBM with feedstuffs that have higher chi-square distance values increases the likelihood of amino acid imbalances in broiler diets; thereby, necessitating greater supplementation with NBAAs to meet the amino acid requirements. Wheat-based, reduced-CP diets required 49 g/kg NBAA inclusions whereas maize-based, reduced-CP diets formulated with 39 g/kg NBAA inclusions in Chrystal et al. (2021).

III. ALTERED AMINO ACID METABOLISM IN LOW-SOYBEAN MEAL DIETS FOR BROILER CHICKENS

Reducing SBM inclusion in broiler diets necessitates greater NBAA supplementation, as discussed in Section 2. NBAA are absorbed more rapidly than protein-bound amino acids (PBAAs), resulting in higher systemic plasma amino acid concentrations (Wu, 2009). Liu et al. (2013) reported that the digestion rate constant of NBAAs is nearly four times greater than that of PBAAs. Chrystal et al. (2021) formulated conventional and low–SBM diets to contain equivalent concentrations of essential digestible amino acids, yet the ileal digestibility coefficients differed markedly between treatments. Moreover, the reduction of SBM in broiler diets necessitates a greater inclusion of feed grains, which typically contain higher concentrations of non-starch polysaccharides (NSP); however, the extent of NSP varies depending on the grain source. The observed differences in apparent nutrient digestibility are likely attributable to alterations in endogenous secretions, as elevated dietary NSP levels in low-SBM formulations have been shown to stimulate mucin secretion (Tanabe et al., 2005) and impair bile acid reabsorption (Choct, 1999). Consequently, the requirement for amino acids abundant in endogenous secretions such as threonine, serine, glutamic acid, and aspartic acid may increase. The cost of amino acid catabolism in broiler chickens was discussed by Macelline et al. (2025). Approximately 18% of dietary amino acids are oxidized for energy in pig enterocytes (Stoll et al., 1998), and glutamate and glutamine are likely the primary energy substrates in broiler enterocytes too (He et al., 2018). Reducing dietary CP by four percentage points can lower dietary glutamate plus glutamine concentrations by 23.4% (Macelline et al., 2024), this is while Yin et al. (2019) observed higher glutamate catabolism in low-CP diets. At deficiency of glutamate, other amino acids such as serine, glycine, arginine, proline, and branched-chain amino acids may be catabolized as alternative energy sources (Wu, 1998).

Table 1 - Chi-square distance values between amino acid profiles in feed stuffs and amino acid recommendation for broiler chickens.

Amino acid, %	AA recom.[1]	Standardized ileal digestible (SID) amino acid concentrations						
		SBM[2]	CM[3]	FM[4]	MBM[5]	Maize	Wheat	Sorghum
Arginine	1.24	3.23	2.03	2.90	3.23	0.34	0.53	0.31
Histidine	0.49	1.13	0.88	0.85	0.71	0.22	0.26	0.19
Isoleucine	0.90	1.99	1.09	1.91	1.25	0.24	0.39	0.33
Leucine	1.59	3.36	1.09	3.06	2.48	0.91	0.75	1.12
Lysine	1.26	2.69	1.63	2.89	2.21	0.19	0.29	0.18
Methionine	0.58	1.21	0.70	1.15	0.58	0.15	0.18	0.13
Phenylalanine	0.95	2.33	1.23	1.79	1.29	0.33	0.52	0.45
Threonine	0.85	1.64	1.26	1.86	1.32	0.29	0.30	0.25
Tryptophan	0.21	0.61	0.39	0.38	0.23	0.06	0.12	0.09
Valine	0.99	2.00	1.38	2.22	1.88	0.31	0.46	0.41
Aspartic acid	0.93	1.74	1.31	3.12	3.28	0.49	0.33	0.76
Cysteine	1.89	4.90	2.47	3.57	2.12	0.45	0.53	0.50
Glutamic acid	0.32	0.62	0.72	0.77	0.35	0.14	0.25	0.14
Glycine	3.40	9.32	5.38	5.58	4.66	1.44	3.36	1.94
Proline	0.77	1.73	1.47	4.19	5.04	0.24	0.44	0.24
Serine	1.13	2.12	1.60	3.42	3.88	0.76	1.23	0.71
CP, %	-	48.1	36.2	54.0	52.0	7.86	11.5	8.75
X^2 distance	-	1.79	5.16	11.8	30.1	12.5	21.2	18.2

[1]Amino acid recommendations (Maharjan et al., 2021)
[2]Soybean meal, [3]canola meal, [4]fish meal, [5]meat and bone meal, maize, wheat, sorghum SID digestible amino acid concentrations for broilers from Brazilian tables (Rostagno et al., 2017); X^2 = Chi-square distance

The lack of bioequivalence between NBAA and PBAA can promote post-enteral amino acid catabolism due to amino acid imbalances at sites of protein synthesis (Selle et al., 2022), resulting in deamination and increased ammonia production. Macelline et al. (2023) reported higher plasma ammonia concentrations in birds offered low–SBM diets. Ammonia is toxic and associated with poor growth (Aguihe et al., 2022; Namroud et al., 2008). Although uric acid synthesis detoxifies ammonia, this process is energetically costly and requires additional aspartate, glutamate and glycine. Overall, these findings indicate that broilers fed low–SBM diets exhibit altered amino acid metabolism compared with those fed conventional diets.

IV. AMINO ACIDS IN THE SPOTLIGHT OF LOW-SOYBEAN MEAL DIETS

Modified amino acid metabolism in low-SBM diets suggests that breeders' amino acid recommendations may not meet actual requirements. As these guidelines largely exclude so-called non-essential amino acids, limitations are most likely among this group. Glycine often becomes limiting in low-SBM diets due to its role in ammonia detoxification. The importance of balancing dietary glycine equivalence (glycine + 0.7143 × serine) has been highlighted by Siegert and Rodehutscord (2019) and Selle et al. (2021), with further evidence from Aguihe et al. (2021), Siegert et al. (2025), and Maynard et al. (2022) showing that dietary glycine is critical in low-SBM diets. Glutamic acid and glutamine may also become limiting because of their high utilization as energy sources in enterocytes. Bezerra et al. (2015) estimated glutamic acid requirements between 43.8 and 53.2 g/kg, which exceed typical levels in low-SBM diets. The anabolic role of glutamine in such diets has also been emphasized (Selle et al., 2024). Given the potential increase in endogenous amino acid secretions associated with low–SBM diets, revised recommendations for aspartate, asparagine, and threonine may further enhance broiler performance by ensuring adequate availability of these amino acids for body protein synthesis.

V. CONCLUSION

The amino acid profile of feed ingredients defines the need for NBAA supplementation. Replacing SBM with other feed stuffs increasing reliance on NBAAs to meet requirements as SBM has most balanced amino acid profile for broiler chickens. Broilers fed low-SBM diets exhibit distinct amino acid metabolism, indicating that present breeder recommendations of amino acids may not reflect their true needs. These differences likely involve non-essential amino acids such as glycine, glutamic acid, and glutamine, which are critical for ammonia detoxification, enterocyte energy metabolism and production of endogenous secretions. Accordingly, future research studies on low-SBM diets should re-evaluate amino acid recommendations, with particular emphasis on non-essential amino acids, to improve nutrient utilization and growth performance in broilers fed low-soybean meal diets.

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IN FEED MATRIX VALUES ESTIMATION OF A HEAT STABLE E. COLI PHYTASE IN BROILERS

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The objective of this trial was to evaluate the potency of an *E. coli* derived phytase (OptiPhos Plus) at two inclusion levels on growth performance and bone characteristics in broilers. Broilers were randomly assigned to three treatment groups with eight pens and 35 male birds per pen per treatment. Broilers were fed starter (day 0-10), grower (day 11-21) and finisher (day 22-35) diets. The diets were based on corn/wheat/soy based and provided as pellet (crumble in starter). The positive control composition was (a) starter: 22 % crude protein, 1.26 % dig. Lys, AME of 2925 kCal/kg (12.23 MJ/kg), 0.90 % Ca and 0.485 % available P (AvaP), (b) grower: 21.5 % crude protein, 1.14 % dig. Lys, AME of 3000 kCal/kg (12.54 MJ/kg) 0.75 % Ca, 0.38 % AvaP and (c) finisher: 20.2 % crude protein, 1.04 % dig. Lys, AME of 3040 kCal/kg (12.71 MJ/kg), 0.55 % Ca, 0.34 % AvaP. A negative control feed was produced by reducing Ca and AvaP levels in the positive control feed by 0.10 % and 0.22 % in feed respectively in all feeds replacing MCP and limestone. The phytase was added to the negative control diet at levels of 1000 or 2000 FTU/kg. Phytase analysis according to ISO 30024 revealed average values over the 2 feeds (corrected for background endogenous phytase level of the negative control feed) of 1305±94 FTU and 2448±122 FTU/kg which indicates values close to the targeted value of 1000 and 2000 FTU/kg respectively. Growth, feed intake and feed conversion were monitored per feeding phase. At day 21 of age, four birds per pen were euthanised, the tibia bones were collected, pooled per pen and analysed for bone ash. Statistical analysis on the experimental data was performed by one-way ANOVA using GenStat.

At a targeted inclusion of 1000 FTU/kg, performance was already brought back to the level of the control [2732 g/bird body weight (BW)] and a feed conversion ratio (FCR) of 1.379 at 1000 FTU/kg versus 2730 kg and 1.389 for the control. However, dosing a targeted value of 2000 FTU/kg resulted in similar body weight and FCR compared with 1000 FTU/kg. Adding 1000 FTU/kg of phytase to the negative control feed brought back bone ash to the positive control (511 g/kg fat-free bone vs 509 g/kg respectively) while at 2000 FTU/kg a value of 515g/kg fat free bone was obtained. However, none of the effects were statistically different (P> 0.05).

Table 1 - Growth performance of broilers (day 1 to 35).

	Body Weight	Feed Conversion Ratio
Positive Control	2730	1.389
Negative control + phytase at 1000 FTU/kg	2732	1.379
Negative control + phytase at 2000 FTU/kg	2781	1.376
SEM	20.6	0.06

It can be concluded from this trial that this phytase at a targeted inclusion level of 1000 FTU/kg can compensate for the 0.22 % AvaP and 0.1 % Ca reduction in the feed, while at a targeted inclusion level of 2000 FTU/kg no significant additional improvement in bones ash was noted. This is higher compared to the expected effect of 0.176 % and 0.2 % AvaP in feed for this phytase at 1000 and 2000 FTU/kg respectively which might be linked to the fact that the real analysed values in the feed of this trial were higher than targeted.

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GUANIDINOACETIC ACID UNLOCKS FEED ENERGY REDUCTION WITHOUT COMPROMISING PERFORMANCE AND CARCASS QUALITY IN BROILERS

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Feed energy is the costliest component of broiler diets; therefore, safe reductions in metabolizable energy (ME) have strong economic value. Prior studies have shown that guanidinoacetic acid (GAA) can maintain broiler performance under reduced dietary protein (Barekatain et al., 2024). However, whether GAA enables reductions in dietary ME without compromising growth performance and carcass quality remains unclear. To address this, a controlled trial was conducted at Kasetsart University, Thailand.

A total of 1,200 Ross 308 broilers were randomly assigned to six dietary treatments in a 2 × 3 factorial arrangement of treatments with main factors as diet and GAA supplementation. T1 diet (Conventional ME) was a maize-soybean meal basal diet formulated as follows: grower feed: 12.97 MJ/kg, 21.5% crude protein (CP), and 1.15% dig lysine; finisher feed: 13.60 MJ/kg, 19.5% CP, and 1.05% dig lysine. T1- 0.42 MJ/kg (T2); T1- 0.84 MJ/kg (T3), each fed without or with 0.06% GAA (T4 = T1 + GAA; T5 = T2 + GAA; T6 = T3 + GAA). Each treatment had four replicate pens (50 birds/pen). Body weight (BW), average daily gain (ADG), feed intake (FI), and feed conversion ratio (FCR) were recorded through day 35, and carcass traits were evaluated on day 36 (6 birds/pen). Data were analyzed as a 2 × 3 factorial by two-way ANOVA (SAS University Edition); pens were the experimental unit. When effects were significant ($P < 0.05$), Duncan's test was used for mean separation.

The results showed that supplementation with 0.06% GAA significantly increased final BW and improved FCR over the overall period ($P < 0.05$). No significant interaction between diet ME level and 0.06% GAA supplementation was detected for growth performance and carcass traits ($P > 0.05$). T5 (T1-0.42 MJ/kg + GAA) matched the conventional ME control (T1) for BW, ADG, FI, and FCR. In addition, T5 treatment significantly improved carcass composition, resulting in higher outer-breast yield and lower abdominal fat compared with both T1 and T2 ($P < 0.05$). A greater energy reduction (-0.84 MJ/kg) without GAA (T3) impaired performance ($P < 0.05$), while adding GAA at this energy level (T6) partially mitigated the negative effects but did not fully restore performance to T1 levels. These findings demonstrate that supplementing 0.06% GAA allows a reduction of 0.42 MJ/kg ME in broiler diets without loss of performance, delivering immediate feed-cost savings, particularly when corn or oil prices are elevated. Moreover, carcass value is improved (higher breast yield and lower abdominal fat), supporting higher processing returns. Thus, a 0.42 MJ/kg ME reduction with 0.06% activated GAA supplementation represents a practical and low-risk strategy for broiler feed formulation.

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EFFECTS OF LYSOLECITHINS SUPPLEMENTATION ON NUTRIENT DIGESTIBILITY AND GROWTH PERFORMANCE OF BROILER CHICKENS FED DIETS WITH COCONUT OIL

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Summary

The study was conducted to determine the effects of lysolecithins on nutrient digestibility and growth performance of broiler chickens fed diets with coconut oil (CCO). The treatments were a standard corn-soybean-CCO-based diet (NC), a positive control with 0.2092 MJ less metabolizable energy (PC), PC with emulsifier A (EMU), PC with a concentrated lysolecithins-based emulsifier (LEC) at 0.25 kg/ton (LECld), and PC with LEC at 0.50 kg/ton (LEChd). Dietary emulsifiers improved the energy digestibility compared to PC in the finisher phase ($P < 0.0001$). Additionally, the LECld and LEChd showed greater AME values than NC and PC (15.22 and 15.34 vs 14.15 and 12.77 MJ/kg, respectively; $P < 0.0001$). On the growth performance, LECld had the lowest overall average daily feed intake (72.09 g; $P = 0.0333$) while maintaining the feed efficiency (1.545; $P = 0.9543$), resulting in the most favorable feed cost efficiency. The results showed that lysolecithins supplementation improved the nutrient digestibility, growth performance, and cost efficiency of broilers fed diets with CCO.

I. INTRODUCTION

Lysolecithins are natural emulsifiers with higher hydrophilic/lipophilic balance (HLB), lower critical micelle concentration, and better oil-in-water emulsifying properties compared to lecithin (Wealleans *et al*., 2020). They improve fat emulsification by emulsifying large droplets into smaller fat droplets, increasing the surface area for lipase activity, resulting in enhanced nutrient digestibility. They also increase the cell membrane permeability, thus improving the absorption of fats and other nutrients (Jansen *et al*., 2015). Because of these properties, the use of lysolecithins improves growth performance (Wealleans *et al*., 2020; Guo *et al*., 2025) and nutrient digestibility (Jansen *et al*., 2015; Guo *et al*., 2025) of broilers.

However, the studies of lysolecithins in broilers focused mainly on fat sources common globally, including soybean oil and palm oil. There is a lack of research on the effect on CCO, which is commonly used in the Philippines because of its local abundance, cost-effectiveness, and unique nutritional benefits (Tababa, 2023). CCO is rich in medium-chain fatty acids, making it easy for livestock to digest and use as a quick energy source (OilCocos, n.d.). Additionally, the studies on the effect of lysolecithins on reduced dietary energy content are also limited. Thus, the study aimed to determine the effects of lysolecithins supplementation on nutrient digestibility and growth performance of broiler chickens fed diets with CCO.

II. MATERIALS AND METHODS

A total of 400 straight-run day-old Cobb 500 broilers were randomly assigned to five treatments in a randomized complete block design, with pen location as the blocking factor. Each treatment had eight replicate pens, with 10 birds per replicate. The treatments were a standard basal diet (NC), a positive control with 0.2092 MJ less metabolizable energy (PC), PC with emulsifier A

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(EMU), PC with a concentrated lysolecithins-based emulsifier (FRA® LeciMax Dry, Adisseo; LEC) at 0.25 kg/ton (LECld), and PC with LEC at 0.50 kg/ton (LEChd). Diets were given in three phases: booster (0-14 days), starter (15-28 days), and finisher (29-35 days) phases. The NC diets contained 16, 18, and 32 kg/ton CCO and had energy content of 12.27, 12.42, and 12.65 MJ for the booster, starter, and finisher phases, respectively. The PC diets were derived from the NC diets by decreasing the energy level by 0.2092 MJ ME through reduction of CCO. Feed and water were given *ad libitum*.

The nutrient digestibility was conducted using the indicator method, with a 3-day adjustment period and a 3-day collection period. Fecal and feed samples were subjected to chromium analysis, gross energy determination, and crude fat, crude fiber, and ash analyses. For the growth performance, average daily gain (ADG), average daily feed intake (ADFI), and feed conversion ratio (FCR) were measured, and weekly, per-phase, and overall performance were calculated. The data were analyzed using Analysis of Variance (ANOVA), followed by Tukey's HSD Test at $\alpha = 0.05$ for significance and $\alpha = 0.10$ for tendencies.

III. RESULTS AND DISCUSSION

On booster stage, no statistical difference were observed between PC and emulsifiers (EMU, LECld, LEChd) on the energy and nutrient digestibility (Table 1). However, the addition of emulsifiers to finisher diets improved energy digestibility, resulting in higher digestibility coefficients compared to PC ($P < 0.0001$). Moreover, diets supplemented with emulsifiers had metabolizable energy (AME) values comparable to or higher than the NC ($P < 0.05$). These improvements indicate that the reduction of 0.2092 MJ in AME levels in EMU, LECld, and LEChd was offset by enhanced energy digestibility due to emulsifier supplementation. Notably, LECld and LEChd resulted in higher AME levels than NC and PC ($P < 0.0001$).

Similar results were observed by Guo *et al*. (2025), wherein improvement in AME by 1.77 %, AMEn by 1.68 %, crude protein digestibility by 3.97 %, and crude fiber digestibility by 3.30 % were observed in broilers fed diets with LEC. Jansen *et al*. (2015) also reported improvements in the AMEn by 6.30 %, dry matter digestibility by 5.70 %, and nitrogen retention by 15.70 % with lysolecithins supplementation. Lysolecithins improve nutrient digestibility by improving fat emulsification due to increased surface area for lipase activity and by improving nutrient absorption through increased cell membrane permeability (Jansen *et al*., 2015) and increased ion exchanges (Maingret *et al*., 2000).

Table 1 - Energy and nutrient digestibility of chick booster and broiler finisher diets (dry matter basis).

Parameters[1]	Treatments[2]					SEM	P-value
	NC	PC	EMU	LECld	LEChd		
Chick Booster Diets							
COD GE	820.4^{a}	770.2^{ab}	800.1^{ab}	780.9^{ab}	770.7^{b}	1.040	0.0126
AME	14.96^{a}	13.97^{b}	14.72^{ab}	14.44^{ab}	14.14^{b}	0.190	0.0070
COD crude fat	810.5^{a}	720.2^{b}	711.0^{b}	700.2^{b}	680.3^{b}	1.882	0.0005
COD crude fiber	540.7^{a}	410.6^{ab}	410.2^{b}	430.2^{ab}	430.7^{ab}	3.165	0.0323
COD ash	590.2^{a}	480.5^{b}	530.6^{ab}	530.3^{ab}	500.6^{ab}	2.366	0.0433
Broiler Finisher Diets							
COD GE	750.8^{ab}	710.2^{b}	770.9^{a}	800.2^{a}	780.8^{a}	1.124	<0.0001
AME	14.15^{b}	12.77^{c}	14.91^{ab}	15.22^{a}	15.34^{a}	0.213	<0.0001
COD crude fat	731.0	780.0	770.8	760.6	730.4	2.287	0.4853
COD crude fiber	360.9^{a}	160.8^{b}	330.4^{a}	380.9^{a}	340.3^{a}	1.948	<0.0001
COD ash	470.2^{b}	420.2^{b}	450.9^{b}	540.4^{a}	420.7^{b}	1.562	<0.0001

[1]COD (g/kg): Coefficient of digestibility; GE: gross energy; AME (MJ/kg): Apparent metabolizable energy.
[2]NC: standard basal diets; PC: positive control with less 0.2092 MJ metabolizable energy; EMU: PC + Emulsifier A; LECld: PC + 0.25 kg/ton FRA® LeciMax; and LEChd: PC + 0.50 kg/ton FRA® LeciMax.
[a,b,c]Means within the same row with different superscripts are significantly different ($P < 0.05$).

Table 2 presents the growth performance of broiler chickens fed CCO-based diets, with or without emulsifier supplementation. Significant differences were observed during the starter phase (days 11–24). In this period, EMU showed higher ADG compared to LECld (44.60 vs 49.77 g; $P < 0.05$), although LECld remained statistically similar to the other treatments ($P > 0.05$). EMU also exhibited better feed efficiency (lower FCR) than both LECld and LEChd during this phase (1.3770 vs 1.5101 and 1.5145, respectively; $P < 0.05$). In the finisher phase,

Table 2 - Per phase, weekly, and overall growth performance of broiler chickens fed dietary treatments.

Parameters	NC	PC	EMU	LECld	LEChd	SEM	P-value
Per Phase Performance							
Average daily gain, g							
Booster	17.73	17.70	17.84	17.57	17.95	0.54	0.9899
Starter	48.58^{ab}	48.55^{ab}	49.77^{a}	44.60^{b}	47.20^{ab}	1.93	0.0380
Finisher	78.51	79.35	73.32	75.92	79.55	1.52	0.1592
Average daily feed intake, g							
Booster	22.33	21.71	21.91	21.89	21.70	0.45	0.8619
Starter	70.47	70.68	68.08	67.15	71.12	1.44	0.0816
Finisher	128.01	129.36	124.84	122.34	128.90	2.31	0.1254
Feed conversion ratio (feed:gain)							
Booster	1.2623	1.2372	1.2307	1.2474	1.2111	0.02	0.6412
Starter	1.457^{ab}	1.459^{ab}	1.377^{b}	1.510^{a}	1.515^{a}	0.04	0.0122
Finisher	1.632^{ab}	1.631^{ab}	1.7127^{b}	1.6170^{a}	1.623^{ab}	0.03	0.0187
Weekly Performance							
Average daily gain, g							
Week 1	16.42	16.95	16.93	16.35	16.40	0.36	0.5662
Week 2	32.80	30.54	30.79	32.09	32.18	1.27	0.6786
Week 3	47.97^{a}	47.57^{ab}	46.80^{ab}	40.96^{b}	45.76^{ab}	1.99	0.0385
Week 4	72.37	72.37	72.41	67.70	72.25	2.45	0.3098
Week 5	76.32	79.64	73.32	76.51	78.47	2.29	0.3677
Average daily feed intake, g							
Week 1	17.56	16.93	17.17	17.03	16.86	0.46	0.8273
Week 2	52.36	56.05	51.50	49.83	51.77	1.78	0.1796
Week 3	71.90^{a}	70.49^{ab}	68.86^{ab}	65.35^{b}	72.04^{a}	1.62	0.0087
Week 4	83.27	86.01	83.76	78.93	84.05	2.72	0.1959
Week 5	156.61	158.18	153.56	155.18	159.97	2.66	0.4740
Feed conversion ratio (feed:gain)							
Week 1	1.072	0.999	1.013	1.043	1.030	0.02	0.2501
Week 2	1.598	1.870	1.679	1.586	1.614	0.09	0.1652
Week 3	1.505	1.495	1.481	1.623	1.594	0.06	0.3525
Week 4	1.155	1.193	1.157	1.169	1.167	0.03	0.8696
Week 5	2.059	1.996	2.100	2.035	2.049	0.04	0.5981
Overall Performance							
ADG	49.18	49.42	48.05	46.72	49.01	0.99	0.1178
ADFI	75.81^{ab}	77.16^{a}	74.44^{ab}	72.09^{b}	75.80^{ab}	1.19	0.0333
FCR	1.543	1.564	1.550	1.545	1.549	0.02	0.9543

[1]NC: standard basal diets; PC: positive control with less 0.2092 MJ metabolizable energy; EMU: PC + Emulsifier A; LECld: PC + 0.25 kg/ton FRA® LeciMax; and LEChd: PC + 0.50 kg/ton FRA® LeciMax.

[a,b]Means within the same row with different superscripts are significantly different ($P < 0.05$).

LECld showed significantly better FCR than EMU (1.617 vs 1.713; $P = 0.0348$), suggesting that LECld demonstrated improved feed efficiency during the later stage of the production cycle. Similarly, when growth performance was analyzed weekly, significant treatment effects were observed during the mid-trial period.

Overall, LECld had the lowest average daily feed intake over the 35-day period, significantly lower than that of the PC (72.09 vs 77.16 g; $P < 0.05$). Despite the lower intake, the FCR of LECld did not differ from other treatment groups ($P > 0.05$), and LECld achieved the most favorable feed cost efficiency (FCE) value (Table 3).

Table 3 - Feed cost efficiency of broiler chickens fed dietary treatments.

Parameter	Treatments[1]				
	NC	PC	EMU	LECld	LEChd
Feed cost efficiency, PhP	44.71	44.19	44.06	43.91	44.17

[1]NC: standard basal diets; PC: positive control with less 0.2092 MJ metabolizable energy; EMU: PC + Emulsifier A; LECld: PC + 0.25 kg/ton FRA® LeciMax; and LEChd: PC + 0.50 kg/ton FRA® LeciMax

The reduced feed intake with lysolecithins supplementation was also observed in previous studies (Jansen *et al*., 2015; Allahyari-Bake and Jahanian, 2017). This indicates that broiler chickens compensated their feed intake in accordance with the elevated AME value of the diets (Swennen *et al*., 2004). In contrast, Guo *et al*. (2025) found comparable feed intake in broilers fed corn-soybean meal-palm oil-based diets, with 0.3138 to 0.3556 MJ less metabolizable energy and LEC addition, but it resulted in an improvement in the FCR by 3.5 pts. Similar observations on maintained feed intake but better FCR were also observed by Priyatno *et al*. (2025).

The results showed that supplementation of a highly concentrated lysolecithins-based emulsifier, particularly at 0.25 kg/ton, presents a promising nutritional strategy for improving energy utilization and economic efficiency in broiler diets containing CCO. Its efficacy is particularly evident in the finisher phase, suggesting phase-specific benefits aligned with dietary fat content. Further research is recommended to evaluate the effects of lysolecithins on energy digestibility at the starter phase. Additionally, measurement of carcass composition, gut morphology, and nutrient transporter gene expression is recommended to validate the performance.

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EFFECTS OF L-SELENOMETHIONINE ON INCREASING SELENIUM DEPOSITION IN BROILER MEAT

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Selenium (Se) is an essential trace element for both animals and humans, with beneficial effects on antioxidant status, thyroid hormone regulation, anti-cancer properties, improving cardiovascular health and immune function (Bai *et al*., 2025). During the COVID-19 pandemic, research showed that low Se levels at hospital admission was strongly associated with higher mortality risk and more severe disease progression in COVID-19 patients (DuLaing *et al*., 2021). Another paper found that adequate Se status can contribute to supporting immune competence in severely ill COVID-19 associated acute respiratory distress syndrome patients, as survivors showed higher Se levels than non-survivors (Notz *et al*., 2021). This raises the question; can Se help in protecting against high mortality during a future pandemic?
Se levels in plant-based foods depend on selenium in the soil, which shows high variation and can be low in certain countries/regions (e.g. South Australia) (Lyons *et al.,* 2005). The result could be that people in those regions do not consume enough selenium to meet requirements (40-70 µg/day). Therefore, it is important to consider methods to enrich food with Se, for example, *via* animal feed (Combs Jr, J.F. 2001). L-selenomethionine (L-SeMet) is the only form of Se that can be incorporated into animal protein and is expected to increase Se deposition in animal protein when fed to food-producing animals (Combs Jr, J.F. 2001).

A total of 160 male ROSS 308-day-old chicks, from a commercial hatchery, were divided over 40 pens for a 42-day trial period in 2 experimental treatments (20 replicates/treatment).

1. Control diet + 0.2 ppm Se from sodium selenite
2. Control diet + 0.2 ppm Se from L-SeMet (Excential Selenium 4000, Orffa Additives)

On day 42, one bird of average weight was selected from each pen (20 per treatment) for the sampling of 10 g of breast muscle and further analysis of Se deposition via ICP-MS. Data was analyzed with a two-sample t-test.

Broilers supplemented with L-SeMet had a significantly higher deposition of Se (1049.9 µg/kg DM) in the breast muscle than birds supplemented with sodium selenite (418.6 µg/kg DM) ($P < 0.001$). The feed intake for d1-42 (total period) was 115.27 g per day (± 2.41) and 117.12 g (± 2.41) for the sodium selenite and L-SeMet treatment, respectively ($P = 0.59$).

Overall, it can be concluded that the inclusion of L-SeMet in broiler diets can increase Se deposition in meat by 631.3 µg/kg. When consuming 100g of poultry meat, Se-enriched broiler breast meat (1049.9 µg/kg DM, ± 25% DM) could contribute 26.2 µg to the Se intake, around 37-66% of daily requirements. This could provide a solution to overcome Se deficiencies in humans and provide an optimal response against viral infections such as COVID-19.

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EFFECT OF YEAST CELL WALL SUPPLEMENTATION ON THE GROWTH PERFORMANCE OF BROILERS UNDER HIGH STOCKING DENSITY

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High stocking density (HSD) is recognised as one of the most significant stressors in modern broiler production, with the potential to adversely impact bird welfare, feed efficiency, physiological functions, and growth performance (Dong et al., 2025). Among strategies to mitigate the deleterious effects of HSD, nutritional interventions are central. Functional feed additives, including probiotics, prebiotics, and synbiotics, have been investigated for their capacity to attenuate HSD-associated stress and performance losses. However, evidence specifically evaluating yeast cell wall (YCW) products under commercially relevant HSD remains limited. Yeast cell wall products, rich in mannan-oligosaccharides and β-glucans, have shown potential to enhance nutrient utilisation and intestinal health (Teng and Kim, 2018; Lee et al., 2025). Therefore, in the present study, the effect of dietary YCW supplementation on the performance of broiler chickens reared under HSD was evaluated.

A total of 576 one-day-old mixed-sex Ross 308 broilers were randomly allocated to two dietary treatments in a completely randomised design: (1) a basal wheat-soybean meal diet formulated to meet or exceed Ross 308 recommendations (Aviagen, 2019), and (2) the same diet supplemented with 1,000 mg/kg yeast cell wall (YCW; OptiWall®, Lallemand Animal Nutrition, Montreal, QC, Canada). Each treatment comprised 12 replicate floor pens with 24 birds per pen. Each pen contained 24 birds to achieve the maximum stocking density of approximately 28 kg/m² for non-mechanically ventilated sheds, as specified by the Australian Animal Welfare Standards and Guidelines for Poultry and RSPCA recommendations. Birds were floor-reared on clean wood shavings in an environmentally controlled house with *ad libitum* access to feed and water. The trial lasted 28 days. Two feeding phases were used: starter (d 0-10) and grower (d 10-28). Body weight, average daily gain (ADG), average daily feed intake (ADFI), and feed conversion ratio (FCR; mortality-adjusted) were recorded weekly at days 7, 14, 21, and 28. Broiler performance variables (body weight, ADG, ADFI, and FCR) were analysed using one-way ANOVA in R. Statistical significance was set at $P < 0.05$.

The results demonstrated that dietary YCW supplementation improved feed efficiency during the early growth phase. Feed conversion ratio tended to be lower at d 0-7 ($P = 0.0587$) and was significantly lower at d 0-14 ($P < 0.05$). At the same time, ADG at d 0-7 and d 0-14 ($P < 0.05$) and body weight at d 14 were significantly higher in YCW-fed birds ($P < 0.05$). These results indicate that inclusion of 1,000 mg/kg YCW can improve feed efficiency and early growth in broilers reared under high stocking density. Further analyses of metabolic and physiological pathways are ongoing to elucidate the mechanisms supporting these performance responses.

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YEAST CELL WALL POSTBIOTIC AS A FUNCTIONAL FEED ADDITIVE TO SUPPORT BROILER PRODUCTIVITY UNDER BOTH THERMONEUTRAL AND HEAT STRESS CONDITIONS

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Summary

The beneficial effects of dietary supplementation with a yeast cell wall postbiotic were tested in 256 Ross 308 broiler chickens reared under thermoneutral (24 °C) and cyclic heat stress conditions (24 – 36 °C). Chickens were allocated to 32 replicated pens (8 chickens per pen) across two controlled-temperature rooms and fed either a control or a yeast postbiotic-supplemented diet (8 pens per treatment). Growth performance data were collected up to 41 days of age. On day 42, one chicken per pen was randomly selected, slaughtered, eviscerated, and then used to determine carcass and meat quality traits. No significant Diet × Temperature interaction was observed in chicken performances and meat quality traits. Cyclic heat stress significantly impaired the performance of broilers, while supplementing a yeast postbiotic independently improved body weight at both thermoneutral and heat stress conditions. Higher temperatures decreased carcass weight, meat water-holding capacity and shear force, although it did not significantly affect meat quality traits. Despite the lack of interaction between Diet and Temperature, a postbiotic yeast supplementation had the potential to partially alleviate the negative impacts of heat stress in broiler chickens, by restoring some of the suppressed body weight gain during the heat challenge.

I. INTRODUCTION

Heat stress (HS) is an escalating challenge for the livestock industry, with commercial broiler chickens being particularly vulnerable due to their limited ability to cope with high environmental temperatures (Oluwagbenga and Fraley, 2023). Among its detrimental effects, HS significantly affects intestinal physiology and integrity in broilers, compromising overall performance (Rostagno, 2020). As optimal intestinal function is pivotal for high-yielding breeds, there is an increasing interest in cost-effective feed additives that support gut health. Yeast products have gained particular interest, essentially due to their promising effects on birds' intestines and gut microbiota (Roque et al., 2025). However, scientific evidence of their effects on mitigating HS remains limited. Therefore, the present study aimed to investigate the impact of a yeast postbiotic supplementation on growth performance, carcass and meat quality traits of commercial broilers reared under thermoneutral conditions or exposed to cyclic HS.

II. MATERIALS AND METHODS

A total of 256 Ross 308 male broiler chickens were assigned to a 2 × 2 factorial arrangement of treatments, testing two experimental diets (C: Control vs. YEP: yeast postbiotic) under two environmental conditions (TN: Thermoneutral vs. HS: Heat Stress). Chickens were weighed

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on arrival and randomly allocated to 32 pens (8 pens/treatment; 8 birds/pen) and fed either a commercially standard control diet or an experimental diet supplemented with yeast postbiotic following supplier's recommendation for each phase: 250 g/T at starter, 500 g/T at grower, and 250 g/T at finisher phase. Environmental conditions followed Aviagen's guidelines for the starter phase (32 °C during the first week, and a 2 °C temperature drop per week). Heat exposure was implemented in one of the climate-controlled rooms (HS room) starting from the grower phase (day 10). During the HS regimen, the thermoneutral room was maintained at constant temperature (24 °C). In comparison, the HS room followed a cyclic HS program starting from 6:00 with a gradual increase of 2 °C per hour to reach 36 °C at 13:00, then gradually reduced to reach 24 °C at 18:00. Growth performances were assessed on days 10, 28, and 41 of age. All parameters measured were corrected for mortality. At 42 days of age, one bird per pen was euthanised for carcass and meat quality evaluation. After 24 hours of cold storage, the breast muscles were removed and used to determine the ultimate pH, L*a*b* colour values, water-holding capacity (WHC), cooking loss, and toughness (measured by shear force).

III. RESULTS AND DISCUSSION

There was no significant Diet × Temperature interaction for any of the production parameters. Cyclic HS significantly reduced growth performances of broiler chickens ($P < 0.05$; Table 1). At thermoneutral conditions, yeast postbiotic significantly increased the daily weight gain (DWG) and, consequently, the final body weight (FBW), compared to broilers fed with the control diet ($P < 0.05$). However, the yeast postbiotic did not alter the chickens' average daily feed intake (ADFI, $P = 0.862$) or the feed conversion ratio (FCR, $P = 0.308$).

Table 1 - The effect of dietary inclusion of yeast postbiotic on the growth performance of broiler chickens reared under thermoneutral (TN) and heat stress (HS) conditions.

Parameters[1]		FBW g	DWG g	ADFI g	FCR
Diet × Temperature					
Control	TN	3058	75.6	108	1.45
	HS	2799	69.1	105	1.52
YEP [2]	TN	3278	81.1	108	1.40
	HS	2941	72.7	105	1.49
SEM		51.4	1.28	1.45	0.03
P-value		0.449	0.455	0.919	0.806
Main effect of Temperature					
	TN	3168	78.3	108	1.43
	HS	2870	70.9	105	1.51
SEM		35.7	0.89	1.02	0.02
P-value		<0.001	<0.001	0.038	0.001
Main effect of Diet					
	Control	2929	72.3	107	1.49
	YEP[2]	3110	76.9	106	1.44
SEM		35.6	0.88	1.01	0.03
P-value		0.002	0.002	0.862	0.308

[1] FBW: Final body weight; DWG: Daily weight gain; ADFI: Average daily feed intake; FCR: Feed conversion ratio.
[2] YEP: yeast postbiotic postbiotic inclusion was 250 g/T in starter, 500 g/T in grower, and 250 g/T in finisher phase.

Considering the carcass (Table 2), as expected, HS reduced the cold carcass (CC) weight ($P < 0.001$). Among breast meat quality parameters, only WHC and shear force were significantly decreased by HS ($P = 0.001$). The dietary inclusion of the postbiotic yeast had no effect on meat quality traits.

Table 2 - The effect of dietary inclusion of yeast postbiotic on carcass weight (CC) and breast meat physical traits of broiler chickens reared under thermoneutral (TN) and heat stress (HS) conditions.

Parameters[1]		CC g	pH	L*	a*	b*	WHC %	CL %	SF N
Diet × Temperature									
Control	TN	2412	6.09	53.1	2.57	2.39	72.3	21.0	22.0
	HS	2225	6.05	55.2	2.09	2.25	68.0	20.7	16.8
YEP[2]	TN	2521	6.03	53.2	2.29	2.69	72.4	21.1	21.6
	HS	2254	6.01	52.9	2.22	2.39	69.6	22.7	17.2
SEM		38.5	0.08	1.06	0.26	0.24	0.60	1.04	1.35
P-value		0.289	0.851	0.249	0.447	0.731	0.237	0.397	0.762
Main effect of Temperature									
	TN	2467	6.06	53.1	2.43	2.84	72.4	21.1	21.8
	HS	2239	6.03	54.0	2.15	2.32	68.8	21.7	17.0
SEM		26.2	0.05	0.75	0.19	0.16	0.52	0.87	0.93
P-value		<0.001	0.728	0.395	0.306	0.348	<0.001	0.562	0.001
Main effect of Diet									
	Control	2318	6.07	54.1	2.33	2.32	70.2	20.87	19.4
	YEP[2]	2388	6.02	53.0	2.26	2.54	71.0	21.9	19.4
SEM		26.2	0.05	0.75S	0.19	0.16	0.43	0.71	0.93
P-value		0.070	0.553	0.302	0.784	0.334	0.198	0.337	0.994

[1] CC: Cold carcass; WHC: Water-holding capacity; CL: Cooking loss%; SF: Shear force.
[2] YEP: yeast postbiotic postbiotic inclusion was 250 g/T in starter, 500 g/T in grower, and 250 g/T in finisher phase.

IV. CONCLUSION

The results presented hereby showed that the yeast postbiotic effectively improved broiler body weight and growth rate under both thermoneutral and HS conditions, without affecting their average feed intake, feed conversion ratio, or meat quality traits. Despite an insignificant Diet × Temperature interaction, the postbiotic yeast cell wall mitigated some of the growth rate and body weight reduction caused by HS. However, the effect of this postbiotic under HS conditions remains unclear, highlighting the need of further investigations.

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ELEMENTAL SELENIUM LIMITS THE UTILIZATION OF SUPPLEMENTED SELENIUM IN BROILERS

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Selenium (Se) is an essential trace element required for antioxidant defense, controlling inflammation, and growth in poultry. Commercial supplementation practices rely on both inorganic (e.g., sodium selenite) and organic sources (e.g., Se-yeast, selenomethionine). Organic Se is generally considered more bioavailable, but variability in efficacy among sources remains poorly explained (Surai et al. 2018; De Marco et al. 2021). Recent evidence highlights the presence of elemental selenium (Se^0), an insoluble species that may limit Se utilization. This study aimed to evaluate the bioefficacy of diverse Se sources in broilers and to investigate the role of elemental Se in their assimilation.

A total of 120 one-day-old Ross 308 male broilers were randomly assigned to ten treatments (four replicates of three birds). Birds were fed a cereal-soybean basal diet for 14 days supplemented with different selenium sources at 0.3 mg/kg: a negative control (NC, no Se), sodium selenite (SS), three Se-proteinates (Se-Pro1, Se-Pro2, Se-Pro3), two Se-nanoparticles (SeNP1, SeNP2), two Se-yeasts (SY1, SY2), and hydroxy-selenomethionine (OH-SeMet). Feed and water were provided ad libitum. On day 14, breast muscle samples were analyzed for total Se by ICP-AES after acid digestion. Selenium speciation, including selenite, selenate and elemental Se, was determined by HPLC-ICP-MS, while elemental Se was quantified following sulfite transformation into soluble selenosulfate. Statistical analyses were performed by one-way ANOVA ($P<0.05$). Results demonstrated a marked effect of Se source on Se deposition ($P<0.001$). Organic sources, particularly OH-SeMet (1.12 mg/kg), exhibited the highest efficacy, followed by SY2 (0.84) and SY1 (0.57). In contrast, inorganic forms were less effective: SS (0.38), Se-Pro (0.39, 0.32, 0.33) and SeNP (0.29, 0.27). Se-Pro and SeNP were equivalent or inferior to SS ($P<0.001$). A strong negative correlation ($R^2 = 0.74$, $P<0.001$) was found between elemental Se content in inorganic sources and Se deposition in tissues, suggesting that the presence of insoluble Se^0 reduces absorption and subsequent selenoprotein synthesis. Elemental Se has also been detected in Se-yeasts, explaining their lower and variable efficacy compared with chemically defined OH-SeMet.

In conclusion, organic selenium, especially OH-SeMet, is superior for efficient Se tissue deposition. Inorganic forms such as SS, Se-Pro and SeNP are limited by their elemental Se fraction, which negatively impacts bioavailability and utilization.

Table 1 - Selenium deposition in the breast of broilers fed Se deficient diets from 21 -42 days[1].

Treatment	NC	SS	SePro1	SePro2	SePro3	SeNP1	SeNP2	SY1	SY2	OH-SeMet
Se (mg /kg DM)	0.089^{h}	0.379^{d}	0.317^{ef}	0.389^{d}	0.329^{e}	0.291^{fg}	0.268^{g}	0.570^{c}	0.842^{b}	1.123^{a}

[1] Each treatment consisted of four replicates with three birds each (12 birds per treatment). Lowercase letters differ according to the one-way ANOVA ($P<0.05$)

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EFFICACY OF A NEW-GENERATION PHYTASE IN 21 DAYS BROILER CHICKENS COMPARED WITH A REFERENCE PRODUCT ON GROWTH PERFORMANCE, BONE PARAMETERS AND BLOOD BIOCHEMISTRY

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Summary

This broiler study compared a novel phytase with a reference phytase widely used, using growth, bone, and blood parameters as indicators of Ca and P release. The new-generation phytase improved ADG, FCR, and tibia ash values compared with the reference product, achieving maximal bone mineralization from 1000 FTU/kg versus 2000 FTU/kg. Blood haematocrit, haemoglobin, oxygen saturation, total carbon dioxide and bicarbonate confirmed effects of dietary Ca, P, and phytase, with higher doses needed to restore buffering capacity. Overall, the new phytase showed greater efficacy, supporting reduced reliance on inorganic phosphate.

I. INTRODUCTION

The use of exogenous phytase is a common practice in poultry nutrition. However, the application of its nutritional matrix values in feed formulation remains highly variable, despite being critical to addressing environmental and economic challenges faced by the poultry industry. In addition, recent findings have highlighted benefits of phytase beyond mineral release, including a reduction in wooden breast myopathy through improved oxygen transport (Greene et al., 2019). The present study was therefore conducted to evaluate the efficacy of a new generation phytase, compared with a reference product, in terms of phosphorus (P) and calcium (Ca) release, as assessed by growth performance, bone mineralization, and blood biochemistry in broilers from 6 to 21 days of age.

II.MATERIALS AND METHODS

The experimental protocol complied with institutional guidelines and was approved by the Institutional Animal Care and Use Committee (IACUC). A total of 585 male Cobb 500 broilers were randomly distributed to 13 treatments with 9 replicates and 5 birds per replicate Treatments consisted of 5 dietary available P (avP) levels (0.42, 0.35, 0.27, 0.20 and 0.12%) and 4 levels (250, 500, 1000 and 2000 FTU/kg) of 2 different phytases (Quantum Blue -Phytase 1QB and new-generation phytase Quantum Force Phytase 2QF; AB Vista, UK). Two basal diets were formulated and mixed to achieve the expected 5 levels of dietary avP. The 4 levels of dietary phytase were added on top of the 0.12% avP diet. Monocalcium phosphate was used as the source of dietary inorganic P. Dietary Ca was likewise lowered to minimize the risk of mineral imbalance, yielding concentrations of 0.84, 0.76, 0.68, 0.59, and 0.51%, respectively. All birds were raised from day 1 to day 5 on a diet with 0.42% avP and 0.84% Ca prior to being fed the dietary treatments from day six, to avoid excessive mortality and comply with welfare standards. Diets were corn and soybean-meal based and contained rapeseed meal and rice bran to increase dietary phytate-P content to 0.30%, ensuring adequate substrate for the phytase (Table 1). Diets were steam pelleted followed by crumbling and fed *ad libitum* to 21 days of age. 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 euthanised, and then left tibias were excised, and tibia ash percent (TA%) and tibia ash weight

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(TAW,g) measured. A blood analyser (iSTAT, Zoetis) was used to determine Haematocrit (Hct) as an indicator of oxygen transport, haemoglobin (Hb) to assess oxygen transport, oxygen saturation (sO_2) as the fraction of Hb able to bind oxygen and total carbon dioxide (TCO_2) and bicarbonate (HCO_3) as indicators of the buffering capacity. A one-way ANOVA was performed (JMP Pro 16.2) and means separated using Student's T-Test ($P \leq 0.05$). Polynomial orthogonal contrasts (linear, logarithmical, and quadratic) were assessed for P and phytase response.

Table 1 - Basal diet composition.

Ingredient composition, %	High Phosphorus	Low Phosphorus
Maize (corn), %	57.77	60.52
Rice bran, %	2.20	2.20
Rapeseed/Canola, %	2.00	2.00
Soybean meal, %	31.08	30.84
Soya oil, %	2.71	1.79
Limestone, %	1.25	0.98
Monocalcium phosphate, %	1.31	0.00
Others, %	1.68	1.67
Calculated nutrient composition		
Crude Protein, %	20	20
Ash, %	5.38	4.01
Ca (formulated)	0.84	0.51
Total P, %	0.70	0.41
Phytate P, %	0.30	0.30
Available P, %	0.42	0.12
Dig Lys, %	1.12	1.12
Dig M+C, %	0.85	0.85
Dig Thr, %	0.73	0.73
Dig Trp, %	0.21	0.21
Dig Val , %	0.85	0.85
AMEn Adult kcal/kg	3,025	3,025

III. RESULTS AND DISCUSSION

Calcium and total P analysis in all 5 dietary avP treatments agreed with the expected values. The analysed phytase activity for the reference phytase (Quantum Blue) was lower than formulated while higher for the new-generation phytase. Consequently, statistical analyses were performed based on analysed FTU for each phytase to not under or overestimate the effect of the two products. Growth performance (ADG, ADFI) and bone parameters (TAW and TA%) were reduced in a linear, logarithmical and quadratic manner as the dietary Ca and P were decreased ($P < 0.01$). The logarithmical regression provided the best fit (Table 2). Using the comparison between the logarithmical equations for each phytase, we found that Phytase 2QF improved ADFI, ADG and mFCR by 3.1%, 1.3% and 7.4%, respectively, compared to Phytase 1QB. However, TA% exhibited an increase of 3.6%, while TAW demonstrated a more pronounced variation of 23.4%. Tibia ash content (Table 2) ranged from 24.5% to 36.5% across the avP treatments, with no significant difference observed between 0.35% and 0.42% avP ($P > 0.05$), indicating that both levels were sufficient to meet the Ca and avP requirements for bone mineralization. Supplementation with Phytase 1QB yielded the highest tibia ash values at 2000 FTU/kg, whereas Phytase 2QF reached comparable maximal responses from 1000 FTU/kg onwards. Tibia ash weight exhibited a response to dietary Ca and avP levels similar to that of tibia ash percentage. However, supplementation with Phytase 1QB did not achieve the maximal

values observed, whereas inclusion of Phytase 2QF at 1000 FTU/kg resulted in tibia ash weights comparable to those of birds receiving the highest avP level.

Table 2 - Growth performance and bone parameters of broilers fed the different dietary levels of available phosphorus and phytase doses from 6 to 21 days of age.

Parameters	avP,%	FTU/kg	ADG g/d/b	ADFI g/d/b	mFCR g/g	Tibia ash,%	Tibia ash weight, g
avP,%	0.12	0	31.6^{g}	48.5^{g}	1.470^{a}	24.5^{g}	0.508^{h}
	0.20		49.5^{f}	67.6^{f}	1.372^{b}	31.6^{e}	0.795^{g}
	0.27		59.0^{de}	76.7^{de}	1.299^{cd}	33.1^{d}	1.062^{cd}
	0.35		61.7^{c}	80.3^{b}	1.301^{cd}	36.4^{a}	1.117^{bc}
	0.42		62.2^{bc}	78.8^{bcd}	1.267^{ef}	36.5^{a}	1.173^{ab}
Phytase 1, QB, FTU/kg	0.12	250	51.8^{f}	70.0^{f}	1.351^{b}	29.8^{f}	0.830^{g}
		500	56.8^{e}	74.7^{e}	1.316^{c}	31.7^{e}	0.888^{f}
		1000	61.5^{cd}	79.4^{bc}	1.292^{d}	35.2^{b}	0.986^{e}
		2000	62.1^{bc}	79.4^{bc}	1.282^{de}	35.9^{ab}	1.064^{cd}
Phytase 2, QF, FTU/kg		250	58.4^{e}	77.6^{cd}	1.317^{c}	34.1^{c}	1.039^{de}
		500	64.3^{ab}	82.9^{a}	1.292^{d}	35.1^{b}	1.076^{cd}
		1000	66.3^{a}	83.4^{a}	1.260^{f}	36.4^{a}	1.228^{a}
		2000	64.9^{a}	83.1^{a}	1.285^{de}	36.2^{a}	1.146^{b}
ANOVA			< 0.01	< 0.01	< 0.01	< 0.01	< 0.01
avP,%			L,Q,LN	L,Q,LN	L,Q,LN	L,Q,LN	L,Q,LN
Phytase,1QB FTU/kg			L,Q,LN	L,Q,LN	L,Q,LN	L,Q,LN	L,Q,LN
Phytase,2QF FTU/kg			L,Q,LN	L,Q,LN	L,Q,LN	L,Q,LN	L,Q,LN

$^{a\text{-}e}$ Means having different superscripts within the column are significantly different (P < 0.05)
*Linear (L), Logaritmic (LN) and quadratic (Q) represents the significance of the orthogonal contrasts (P < 0.05)

Table 3 - Blood biochemistry markers broilers fed the different dietary levels of available phosphorus and phytase doses from 6 to 21 days of age.

Parameters	avP,%	FTU/kg	Hb, g/dL	Htc, %PCV	sO2, %	TCO2, mEq/L	HCO3, mEq/L
avP,%	0.12	0	8.89^{a}	26.1^{a}	53.3^{d}	29.5^{a}	27.9^{a}
	0.20		7.69^{b}	22.6^{b}	74.4^{abc}	25.9^{bc}	24.8^{bc}
	0.27		7.19^{bc}	21.2^{bc}	72.0^{bc}	28.1^{ab}	26.8^{ab}
	0.35		6.96^{c}	20.5^{c}	73.5^{abc}	23.5^{cde}	22.2^{de}
	0.42		7.25^{bc}	21.3^{bc}	80.7^{a}	21.8^{e}	20.7^{e}
Phytase 1, QB, FTU/kg	0.12	250	7.39^{bc}	21.7^{bc}	76.8^{abc}	25.6^{bcd}	24.4^{bcd}
		500	7.22^{bc}	21.3^{bc}	79.8^{ab}	26.4^{b}	25.0^{bc}
		1000	7.32^{bc}	21.6^{bc}	75.9^{abc}	27.4^{ab}	26.2^{abc}
		2000	7.10^{bc}	20.9^{bc}	77.6^{abc}	23.3^{de}	22.0^{de}
Phytase 2, QF, FTU/kg		250	7.34^{bc}	21.6^{bc}	73.5^{abc}	26.3^{b}	24.9^{bc}
		500	7.24^{bc}	21.3^{bc}	71.9^{c}	27.0^{ab}	25.5^{abc}
		1000	7.09^{bc}	20.9^{bc}	71.9^{c}	25.6^{bcd}	24.2^{cd}
		2000	7.23^{bc}	21.3^{bc}	74.4^{abc}	25.5^{bcd}	24.2^{bcd}
ANOVA			0.005	0.005	< 0.01	< 0.01	< 0.01
avP,%			L,Q,LN	L,Q,LN	L,Q,LN	L,LN	L,LN
Phytase 1 QB FTU/kg			L,Q,LN	L,Q,LN	L,Q,LN	L,LN	L,LN
Phytase 2 QF FTU/kg			L,Q,LN	L,Q,LN	L,Q,LN	L,LN	L,LN

a-e Means having different superscripts within the column are significantly different (P < 0.05)
*Linear (L), Logaritmic (LN) and quadratic (Q) represents the significance of the orthogonal contrasts (P < 0.05)

Blood analytes measured in this study were significantly affected by dietary Ca and P levels ($P < 0.01$) as well as by phytase supplementation ($P < 0.05$; Table 3). The response patterns of the two phytases were similar, with the highest levels of phytase achieving values similar to those observed in birds fed the highest avP diets. While lower phytase doses were sufficient to restore Hb, Hct, and sO_2, higher doses were required to restore TCO_2 and HCO_3. Previous research has demonstrated that phytase, particularly at elevated inclusion levels, increases erythrocyte inositol phosphate concentrations, thereby enhancing systemic oxygen-carrying capacity (Whitfield et al., 2022). This mechanism may also help explain the reduction in the severity of woody breast as reported by Walk et al. (2024).

This study suggests that the P requirements of broilers appear to be lower than current guideline recommendations, which may limit the accurate estimation of phytase avP equivalency, particularly at higher inclusion levels. Nevertheless, the present findings demonstrate greater efficacy of the novel phytase in releasing avP. Moreover, the results highlight the potential to feed broilers without supplemental inorganic phosphate when adequate phytase doses are provided and sufficient phytate-P substrate is available, without compromising growth performance or bone mineralization. This was especially evident with the superior efficacy of the new-generation phytase. In addition, the data support a role of dietary Ca and avP in modulating blood biochemistry and oxygen transport, with phytase supplementation improving oxygenation status in birds subjected to P deficiency especially at higher dose.

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SANGUINARINE-BASED ISOQUINOLINE ALKALOIDS ENHANCE GROWTH PERFORMANCE, GUT HEALTH AND ALTERNATING GUT BACTERIOME AND MYCOBIOME IN BROILERS

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Summary

Sanguinarine-based isoquinoline alkaloid (IQ) has been widely applied to suppress intestinal inflammation, reduce stress, and act as an alternative growth promoter in broiler chickens. This study evaluated the impact of dietary IQ supplementation on alternating bacterime-mycobiome diversity, stress reduction, and gut health improvement associated with growth performance. The results showed that broilers fed the IQ-supplemented diet had significantly higher body weight (BW), body weight gain (BWG), and lower feed conversion ratio (FCR) compared to the control (CON) group ($P < 0.05$). This indicates the positive effect of IQ on enhancing growth performance in broilers. Notably, no significant differences in feed intake were observed between dietary treatments during the grower phase ($P > 0.05$). In addition, broilers in the IQ group had significantly lower serum FITC-d levels on days 35 compared to the CON group ($P < 0.001$). The serotonin-to- corticosterone ratio in broilers fed the IQ diet was significantly higher than in the CON group ($P < 0.001$). Regarding antioxidant and inflammatory responses, broilers fed the IQ diet showed significantly lower expression levels of *IL-4, TNF-α, NF-κB* and *TLR-1* in the ileum at days 14 and 35 compared to the CON group ($P < 0.05$). The relative abundance (%) of cecal bacteriome at the species levels in broilers on days 35 revealed significant differences between birds fed IQ diet and the CON groups. Birds fed the IQ diet exhibited higher abundances of *Bacillus subtilis, Lactobacillus aviaries, Turicibacter sanguinis*, and *Akkermansia muciniphila*, while showing lower abundances of *Methanomassiliicocos intestinalis, Enterococcus cecorum,* and *Escherichia fergusonii*. Additionally, the mycobiome diversity analysis indicated that broilers fed IQ diet had significantly lower abundances of *Aspergillus brunneouniseriatus*, *Aspergillus caesiellus*, *Aspergillus flavus*, *Aspergillus niger,* and *Penicillium dierckxii* compared to the CON group. Overall, dietary supplementation with IQ improved feed efficiency, enhanced growth performance, preserved intestinal integrity, promoted a beneficial bacteriome, reduced stress, and suppressed the presence of mycotoxin-producing fungi in the ileum.

I. INTRODUCTION

The gastrointestinal (GI) tract of broiler chickens hosts a diverse microbial ecosystem, including bacteria (bacteriome)and fungi (mycobiome), which play essential roles in nutrient

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digestion, immune regulation, and intestinal integrity (Apajalahti and Vienola, 2016). The bacterial community is dominated by *Firmicutes*, *Bacteroidetes*, and *Proteobacteria*, where beneficial genera such as *Lactobacillus* and *Bacillus* promote nutrient absorption and pathogen exclusion (Rychlik, 2020). In contrast, the gut mycobiome, composed mainly of *Aspergillus*, *Penicillium*, and *Candida* species, can influence gut health through complex interactions with bacterial populations (Huang et al., 2023). Overgrowth of pathogenic fungi or mycotoxin producers, such as *Aspergillus flavus* and *A. niger*, may compromise intestinal barrier function and immune responses (Maciej et al., 2020). Sanguinarine-based isoquinoline alkaloids (IQ) have emerged as promising natural feed additives that modulate the gut microbiome, reduce inflammation, and improve growth performance in broilers (Khongthong et al., 2024). However, the effects of IQ on fungal–bacterial interactions remain unclear. This study therefore investigates the influence of dietary IQ supplementation on bacteriome–mycobiome diversity, gut integrity, and growth performance in broiler chickens.

II. METHOD

This study evaluated the effects of dietary supplementation with sanguinarine-based isoquinoline alkaloids (IQ) on growth performance, gut health and alternation in bacteriome and mycobiome diversity in broilers. The IQ containing 5.00 mg/g of sanguinarine was used as feed additive. A total of 400 male Ross 308 broiler chickens were randomly assigned to two treatments each with eight replicates and 25 birds per pen: (1) a control group fed a basal diet (CON), and (2) a treatment group fed a basal diet supplemented with IQ at 60 mg/kg of feed (IQ). Pellet-form basal diets were prepared according to the growing stage of the birds: starter (from day 1 to day 21), grower (from day 21 to 35), and finisher (from day 35 to 42). Each nutrient composition was suggested by Ross broiler's guide (Aviagen, 2019). Feed and water were provided *ad libitum* throughout the study. At days 14 and 35, one bird per replicate with total 8 birds per treatment was randomly selected for sample collection. Firstly, fluorescein isothiocyanate-dextran (FITC-d) was delivered by oral administration and after 2.5 h, blood was collected to measure serum FITC-d, and, corticosterol and serotonin levels. Ileum tissue was collected to evaluate the expression levels as fold change (FC) of antioxidative with Toll-like receptor 1 (TLR-1) and inflammatory cytokines, including interleukin-4 (IL-4), tumor necrosis factor-alpha (TNF-α) and nuclear factor κB (NF-κB). Additionally, cecal content samples were also collected for microbiome sequencing of bacteriome (V3-V4 16s RNA) and mycobiome (ITS2). Bacteriome and mycobiome diversity was identified and quantified to determine the abundance with QIIME2 version 2020.11 using DADA2 and Deblur pipeine, respectively. Statistical analysis was performed using the student's *t*-test for growth parameters, two-way ANOVA for gene expression data ($p < 0.05$) and Adonis and DESeq2 methods for microbiome with R. The graph was plotted using GraphPad Prism (version 9.5.0, La Jolla, CA, United States).

III. RESULTS

Broilers fed the IQ diet exhibited significantly higher body weight (BW), body weight gain (BWG), and lower feed conversion ratio (FCR) compared to the CON group ($P < 0.05$), indicating the positive impact of IQ on growth performance in broilers (Table 1). Broilers fed the IQ diet exhibited significantly lower serum FITC-d levels on day 35 compared to the CON group ($P < 0.001$, Figure 1A). This suggests improved gut integrity due to reduced intestinal permeability (leaky gut). Additionally, the serotonin-to-corticosterone ratio in broilers fed the IQ diet was significantly higher than in the CON group ($P < 0.001$, Figure 1B), indicating a reduction in stress levels. The antioxidative stess with *TLR-1* and inflammatory expression of *IL-4, TNF-α, NF-κB* in ileum of broilers fed the IQ diet had significantly lower expression than CON group at days 14 and 35 ($P < 0.05$, Figure 1C-F). These results provided the evidence that IQ suppress the oxdative stress and inflammation in gut. Cecal microbiome analysis found that beta-diversity of IQ diet significantly alternated the bacteriome and mycobiome community

structure when comparing with CON group at days 14 and 35 (*PERMANOVA* $p < 0.001$; Bray–Curtis). It also revealed higher richness (Chao1, Shannon indices) in IQ-fed birds, which exhibited significantly higher relative abundance (%) of species of beneficial bacteria including *Bacillus subtilis*, *Lactobacillus aviaries*, *Turicibacter sanguinis*, and *Akkermansia muciniphila*. Conversely, they had lower abundance of *Methanomassiliicocos intestinalis*, *Enterococcus cecorum*, and *Escherichia fergusonii* compared to the CON group ($P < 0.05$, Figure 2A). Additionally, the mycobiome diversity revealed that broilers fed IQ diet had significantly reduced abundance of *Aspergillus brunneouniseriatus*, *Aspergillus caesiellus*, *Aspergillus flavus*, *Aspergillus niger*, and *Penicillium dierckxii* ($P < 0.05$, Figure 2B) compared to the CON group.

Table 1 - Effects of sanguinarine-based isoquinoline alkaloids (IQ) supplementation on growth performance of broilers for 0-42 days.

Items	Dietary treatment		Pooled SE	*P*-value
	CON	IQ		
Initial BW (day 0) (g)	44.54	43.62	0.121	0.673
BW (day 42) (g)	2,768.47	3,138.01	50.52	<0.001
BWG (g/bird)	2733.65	3100.72	48.12	<0.001
Feed intake (g/bird)	4368.39	4479.94	134.0	0.461
FCR (day 0-42)	1.56	1.44	0.03	0.01
Mortality, (%)	3.64	1.70	0.78	0.047

CON = basal diet; IQ = fed a basal diet supplemented with sanguinarine-based isoquinoline alkaloids at 60 mg/kg feed
FCR = feed conversion ratio; BW = body weight; BWG = body weight gain; SE = standard error.

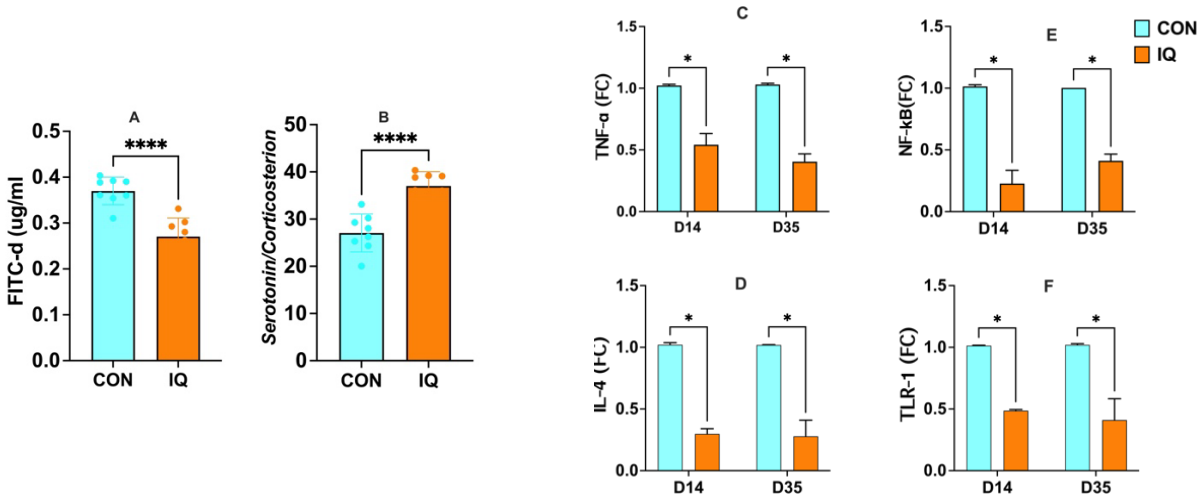

Figure 1 - Serum fluorescein isothiocyanate-dextran (FITC-d)(A), serotonin-to- cortocosterion ratio (B) at 35 days of age, Antioxidative and inflammatory cytokines expression levels as fold change (FC) of tumor necrosis factor-alpha: TNF-α (C) interleukin-4: IL-4 (D), nuclear factor κB: NF-κB (E), and Toll-like receptor 4: TLR-1 (F) in the ileum of broilers at day 14 and 35, Broilers were fed basal diet (CON), or fed a basal diet supplemented with sanguinarine-based isoquinoline alkaloids at 60 mg/kg feed (IQ), different superscripts indicate significant differences between means as determined two-way ANOVA;*($P<0.05$), ****($P<0.0001$).

IV. DISCUSSION

Dietary supplementation with sanguinarine-based isoquinoline alkaloids (IQ) significantly improved growth performance, intestinal integrity, and microbial balance in broiler chickens. The higher body weight gain and lower feed conversion ratio in IQ-fed birds indicate enhanced nutrient utilization and metabolic efficiency, consistent with previous reports that phytogenic alkaloids support intestinal development and digestive function (Khongthong et al., 2023). Reduced serum FITC-dextran levels and serotonin-to-corticosterone ratios further suggest that

IQ alleviated stress and preserved gut barrier integrity, possibly through modulation of the gut–brain axis (Khongthong et al., 2024). The downregulation of *IL-4*, *TNF-α*, *NF-κB*, and *TLR-1* expression indicates that IQ exerts strong anti-inflammatory and antioxidative effects, aligning with findings that sanguinarine inhibits NF-κB activation and cytokine production (Khongthong et al., 2023). IQ supplementation also reshaped the gut bacteriome, enriching beneficial taxa such as *Bacillus subtilis*, *Lactobacillus aviarius*, and *Akkermansia muciniphila*, while suppressing opportunistic bacteria including *Enterococcus cecorum* and *Escherichia fergusonii* (Khongthong et al., 2024). Furthermore, IQ reduced the abundance of mycotoxin-producing fungi such as *Aspergillus* and *Penicillium* spp., indicating possible antifungal or microbiome-stabilizing effects (Apajalahti and Vienola, 2016; Huang et al., 2024). Collectively, these findings suggest that IQ enhances broiler health and productivity by modulating both bacterial and fungal microbiota, highlighting its potential as a natural alternative to antibiotic growth promoters in poultry production.

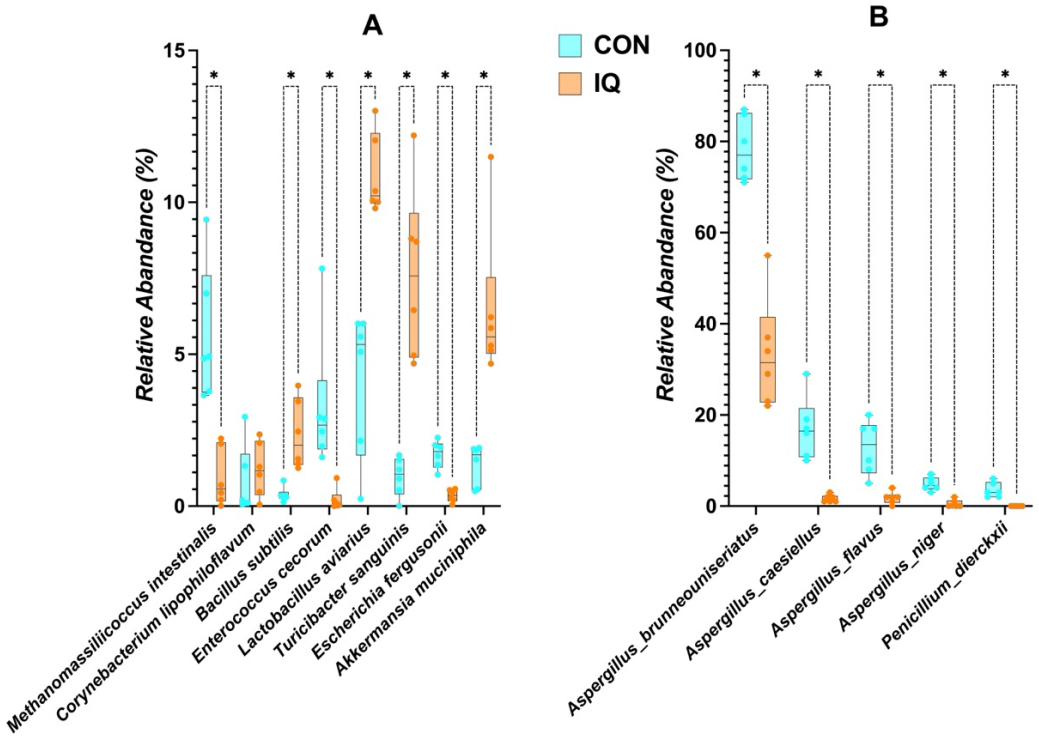

Figure 2 – Significant species abundance *(%)*of ileum bacteriome (A) and mycobiome species (B) in broilers at day 35, broilers were fed basal diet *(*CON*)*, or fed a basal diet supplemented with sanguinarine-based isoquinoline alkaloids at 60 mg/kg feed (IQ), different superscripts indicate significant differences between means as determined by Mutiple t-test; * (P<0.05).

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POST-PEAK PRODUCTION BENEFITS OF A FEED SUPPLEMENTATION WITH A TRIPLE-STRAIN *BACILLUS*-BASED PROBIOTIC IN DYSBIOSIS-CHALLENGED LAYERS

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Summary

Layer producers have been navigating through a range of challenges stemming directly from the genetic progress with hens now laying for longer periods and being more feed efficient. However, all those genetic improvements do come with a few trade-offs such as a reduced persistency in laying cycles overtime, an impaired eggshell quality and a reduced physiological resilience. The latter makes the hens more sensitive to pathogenic infections and can then lead to dysbacteriosis problems that will negatively affect gut integrity, digestion and therefore productivity and egg quality. The aim of the study was to assess the effect of a feed supplementation with a commercial triple-strain Bacillus-based probiotic on productivity and egg quality of dysbiosis-challenged hens throughout a period of 30 days (i.e. D0-D30). Two hundred and eighty eight Lohman Brown hens of 40 weeks of age were randomly allocated to 6 different pens of 48 layers (one pen per treatment group). The challenge consisted in inducing dysbiosis in the gut of layers by applying a 10 days antibiotic treatment (based on Enrofloxacin at 10mg/kg feed and Amoxicillin at 20mg/kg feed) through the diet as from D10 to D20. The birds were all on the same mid-lay diet and allocated to 6 treatment groups: a negative control group (NC) not challenged, a positive control challenged, a probiotic group (PRO30) challenged and continuously supplemented with a commercial triple strain Bacillus-based probiotic (1.6×10^6 CFU/g finished feed) from D0 to D30, a second probiotic group (PRO10) challenged but supplemented with the same probiotic from D20 to D30 only, a phytogenic group (EO30) challenged and fed with a combination of essential oils and organic acids (D0-D30) and a second phytogenics group (EO10) challenged but fed with the same combination as EO30 but only during the last 10 days of the experiment (D20-D30). The antibiotic administration affected intestinal morphology but did not affect intestinal permeability nor the counts of enterobacteria. PRO supplementation to laying hens led to significantly ($p<0.05$) higher intestinal villus length-to-crypt depth ratio compared to negative and positive controls: at D30 of the experiment in the duodenum, at D10 , D20 and D30 in the jejunum and at D20 and D30 in the ileum. The proportion of dirty eggs, being one of dysbiosis markers in layers, was relatively high in the study but was significantly lower in PRO30 birds compared to all the other treatment groups ($p<0.05$) except NC. The laying rate was also improved in PRO30 ($p<0.05$) compared to EO and PRO10. Egg weight was not significantly different among treatment groups. A significant reduction on average daily feed intake was found in hens in PRO and EO compared to hens in NC, PC and EO10 groups ($P = 0.01$). In conclusion, continuously feeding layers with the triple strain Bacillus-based probiotic during one month and 10 days before a dysbiosis challenge did enhance post-peak egg production and feed efficiency while improving the cleanliness of eggs through improved gut morphology and greater surface area for nutrient absorption.

I. INTRODUCTION

After peak production, laying hens are increasingly exposed to pathogenic and management-related challenges that can disrupt intestinal health. The high metabolic demand and continuous

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egg output reduce physiological resilience, making birds more susceptible to opportunistic pathogens such as Clostridium perfringens, E. coli, and Salmonella. These imbalances often trigger dysbacteriosis and/or an alteration of gastrointestinal functionality which can compromise gut integrity, reflected in shortened villi, deeper crypts, and reduced absorptive capacity. The consequences extend beyond the intestine: impaired nutrient utilization and inflammation lead to dirty eggs due to increased intestinal leakage, a decline in laying rate, reduced feed efficiency, and, in severe cases, elevated mortality. Today's highly productive layers are more susceptible to compromised egg cleanliness which is a significant concern for producers because they affect both food safety perception and egg marketability. Post-peak of production, layers face increased sensitivity to dysbacteriosis, a condition impairing gut integrity and nutrient absorption with negative implications on bird productivity and egg quality. Therefore, a robust and diverse intestinal microbiome is crucial for boosting the resilience of hens during the production cycle. Among feed additives, Bacillus-based probiotics can be particularly effective to impact microbiome robustness due to the ability of specific strains to enhance enzymatic digestion (amylases, proteases, lipases), stabilize fermentation, and produce antimicrobial metabolites controlling opportunistic bacteria such as *Clostridium perfringens* and *E. coli*. These effects preserve intestinal morphology, increase villus length-to-crypt depth ratio, and improve barrier function. The main objective of the study was to investigate the potential benefits of supplementing feed with a commercial triple-Strain Bacillus based probiotic on gut health, productivity and egg safety in dysbiosis-challenged layers.

II. METHOD

In this challenge study, Two hundred and eighty eight Lohman Brown laying hens of 40 weeks of age were randomly distributed to 6 pens. Each pen represented a treatment group and was composed of 48 layers. The aim of the challenge was to induce dysbacteriosis conditions within the bird's digestive tract by treating the hens with antibiotics during 10 days as from D10 of the experiment i.e. D10-20 . The treatment consisted of supplementing the diet with Enrofloxacin at 10mg/kg feed and Amoxicillin at 20mg/kg feed and was aimed at impairing the balance and diversity of the gut microbiota. The birds were all on the same diet and allocated to 6 treatment groups: a negative control group (NC) not challenged, a positive control challenged, a probiotic group (PRO30) challenged and continuously supplemented with a triple strain Bacillus-based probiotic (GalliPro® Fit, Novonesis; dose:1.6×10^6 CFU/g finished feed) from D0 to D30, a second probiotic group (PRO10) challenged but supplemented with the same probiotic from D20 to D30 only, a phytogenic group (EO30) challenged and fed with a combination of essential oil compounds (eugenol, thymol and piperine) and organic acids (benzoic acid)at the dose of 300 g/t feed and another phytogenics group (EO10) challenged but fed with the same combination as EO30 but only during the last 10 days of the experiment (D20-D30). The effect of the probiotic on gut health of layers was assessed by measuring gut morphology in three different compartments (duodenum, jejunum, ileum) as well as intestinal permeability and microbial abundance of enterobacteria and Bacilli in caeca. Regarding histopathology, 5 birds per group were randomly selected and euthanized by cervical dislocation for necropsy and collection of duodenum, jejunum and ileum samples at three different timepoints (D10, D20 and D30 of experiment). For the histological evaluation, the samples were collected and immediately fixed in formaldehyde 10% for at least 24 h. These samples were placed in a circulator and treated with different concentrations of alcohol and toluene. They were then embedded in paraffin and cut in 3 µm slices which were stained with hematoxylin and eosin. All the slides were scanned (Aperio AT2 from Leica Biosystems) and digitally analyzed (Aperio ImageScope v12.4.0.5043 from Leica Biosystems, São Paulo, SP, Brazil). The villus height (from the tip of the villi to the villus crypt junction), villus width (from one side to other in the midpoint of the villus), crypt depth (defined as the depth of the invagination between adjacent villi), and crypt

width (from one side to other of the crypt) were measured. Also, VH:CD ratio and absorptive surface were calculated Membrane permeability was measured by using the FITC-labeled dextran method on 5 birds per treatment groups at D1, D10, D20 and D22 of the experiment. Finally, 5 birds per pen were sacrificed at D10, D20, D25 and D30 for measuring the cecal counts of enterobacteria. The proportion of dirty eggs (DE), average daily feed intake (ADFI), egg production (EP) and egg weight (EW) were also measured per treatment group (pen) during the whole study period of 30 days. Regarding the statistical analysis for multiple comparison, grouping Information using the multiple Fisher LSD Method and 95% confidence was performed using the statistical program SAS 9.2. ($p<0.05$).

III. RESULTS & DISCUSSION

No significant differences in intestinal permeability was observed before start (D1), after 10 days of supplementation of additives (D10), after 10 days of antibiotic administration (D20) and at 2 days post antibiotic administration (D22). No significant differences in intestinal counts of Enterobacteria across treatment groups were observed at any time during the study. Except for the duodenal and ileal tissue samples at D10, the villi length: crypt depth ratio differed significantly between treatment groups (Table 1). PC had significantly smaller villi : crypt ratio than NC except in jejunum at D10. Postponing the supplementation of additives (PRO10, EO10) after antibiotic administration challenge i.e. D20-D30? led to significantly smaller villi : crypt ratios compared to the additives' supplementation during the entire study (PRO30, EO 30 i.e. D0-D30), except for EO30 in ileum at D30. The probiotic supplementation during the entire study (PRO30) led to a significantly larger villi : crypt ratio compared to NC.

Table 1 - Gut Morphology - villi height : crypt depth ratio in duodenum, jejunum and ileum at D10, D20 and D30.

	Treatments	Duodenum			Jejunum			Ileum		
		D10	D20	D30	D10	D20	D30	D10	D20	D30
	n/group (N total)	5(30)	5(30)	5(30)	5(30)	5(30)	5(30)	5(30)	5(30)	5(30)
T1	NC	7.3	7.2	7.8^{b}	9.4^{b}	9.3^{c}	10.0^{b}	7.0	6.9^{b}	7.0^{b}
T2	PC	7.3	6.2	6.7^{c}	9.6^{b}	7.3^{d}	7.7^{c}	7.2	3.9^{c}	4.1^{c}
T3	PRO 30	9.7	8.8	8.3^{a}	14.8^{a}	12.7^{a}	13.3^{a}	8.6	8.1^{a}	8.2^{a}
T4	PRO 10	7.2	6.2	7.3^{b}	9.5^{b}	7.3^{d}	4.0^{d}	7.2	3.9^{c}	4.3^{c}
T5	EO 30	9.3	8.5	7.9^{b}	13.5^{a}	11.4^{b}	12.5^{a}	8.0	7.5^{ab}	7.3^{b}
T6	EO 10	7.3	6.4	6.6^{c}	9.3^{b}	7.3^{d}	7.5^{c}	7.1	4.2^{c}	7.3^{b}
	P-Value	0.32	0.06	0.01	0.03	0.02	0.01	0.72	0.01	0.01

Except for the ileal tissues at D10, the villi width differed significantly between treatment groups. However, while PC and NC did not differ significantly in villi width, postponing the supplementation of additives (PRO10 and EO10) after the challenge led to significantly lower villi width compared to the additives' supplementation during the entire study (PRO30 and EO30), except for EO30 in ileum at D30. Finally, additives' supplementation during the entire study (PRO30 and EO30) led to significantly wider villi than NC, except in ileum tissue samples at D30 where only PRO30 led to significant difference versus the negative control. Based on the significant difference in dimensions of the intestinal microvilli (length and width), the birds continuously fed with the probiotic (PRO30) had greater surface area for absorption of nutrients compared to those challenged birds that were fed with any feed additive (PC). For example, the surface area of duodenal villi was 43% greater in PRO30 compared to PC. A significant reduction of dirty eggs percentage (DE) was found in hens that received probiotic throughout the whole trial period (PRO30) compared to the positive control group (PC) and to hens supplemented with phytogenics (EO30 and EO10; $P = 0.01$). EO supplementation following antibiotics administration (EO10) did significantly increase the

percentage of DE compared to NC and PC (EO10 *vs*. NC and PC, $P = 0.01$). Besides the negative impact on gut morphology, the high proportion of DE during this one-month trial period also indicates that dysbiosis may have successfully been induced by the 10-day antibiotic administration (Table 2).

Table 2 - Proportion of dirty eggs after 30 days (100 x [Number of dirty eggs in a study period]/[number of eggs in the study period]); a, b, c = $P < 0.05$.

Treatment groups	NC	PC	PRO 30	PRO 10	EO 30	EO 10
Dirty eggs, %	8.69^{bc}	11.58^{b}	8.18^{c}	9.71^{ab}	10.74^{b}	16.01^{a}

Regarding zootechnical endpoints, a significant reduction of the average daily feed intake (ADFI) was found in hens in PRO30, PRO10 and EO30 compared to NC and PC (P = 0.01) while a significant drop of the laying rate was observed in PRO10 and EO30, compared to PRO30, NC and PC (P = 0.01). Finally, Egg weight was not significantly different among groups (P = 0.27).

Table 3 - Eggs production after 30 days (100 x [Number of eggs in a study period]/[number of layer-days in the study period]).

Treatment groups	NC	PC	PRO 30	PRO 10	EO 30	EO 10
Eggs production, %	88.81^{a}	85.95^{a}	87.86^{a}	83.33^{b}	78.54^{b}	85.95^{a}

IV. CONCLUSION

The in-feed antibiotic challenge did affect the intestinal morphology of hens but did not impact gut permeability nor cecal counts of enterobacteria. The overall percentage of dirty eggs was relatively high in the study which is also a marker for dysbiosis in commercial layers. Thus, the microbiota disturbance that the antibiotic treatment may have initiated seemed to have impaired gut health in challenged birds. The continuous feed supplementation with the triple-strain Bacillus-based probiotic from D1 did sustainably favor egg cleanliness and maintain standard productivity level by potentially helping the hen to cope with dysbacteriosis conditions through greater intestinal villi and related increased nutrient digestibility. In conclusion, applying the probiotic as preventive sustainable solution in a challenged environment showed potential to optimize post-peak productivity of commercial layers and improve egg safety and marketability.

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SUPPLEMENTING A HIGH-DOSE OF PROTEASE IN THE STARTER DIET OF BROILER CHICKENS CHALLENGED WITH ESCHERICHIA COLI IMPROVED GROWTH PERFORMANCE AND INTESTINAL MORPHOMETRICS

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Summary

Protease enzymes are traditionally used at standard doses in poultry diets to improve protein utilization, but at elevated inclusion levels they may also exert benefits unrelated to protein digestion, particularly on gut structure and resilience during enteric challenges (Wang et al., 2008; Barbosa et al., 2022). Administering a high dose of protease early in life is important, as this phase is characterized by low endogenous enzyme secretions, high dietary protein supply, and increased susceptibility to intestinal challenges (Barbosa et al., 2022). This study evaluated whether feeding broilers a higher dose of protease during the starter phase, followed by conventional doses in later phases, could improve performance and intestinal morphology under an *Escherichia coli* (*E. coli*) challenge. A total of 480 one-day-old Cobb 500 chicks were allocated to two dietary treatments over a 42-day period. Birds received either a standard corn–soybean meal diet (CON) or the same diet supplemented with protease (CON + P) at 400 g/t in the starter phase and 125 g/t in the grower and finisher phases. Each treatment was fed to 12 replicate pens of 20 birds. All birds were exposed to an enteric challenge by placing chicks in pens containing litter sprayed with 100 mL of *E. coli* at placement. Growth performance was monitored through body weight (BW), BW gain (BWG), and feed conversion ratio (FCR). On day 14, samples of duodenum, jejunum, and ileum (one bird/pen) were collected to measure villus height (VH), crypt depth (CD), and VH:CD ratio. Data were analyzed by T-test with significance declared at $P < 0.05$ and approaching significance at $P < 0.10$. High-dose protease supplementation resulted in greater final BW at day 42 ($P < 0.05$), with improvements ($P < 0.05$) in BWG and FCR compared with the CON group. Birds receiving the CON + P also tended to show increased duodenal VH ($P < 0.1$). Overall, these findings indicate that protease supplementation, particularly at higher doses in early life, can offer benefits beyond nutrient release. By supporting intestinal development and improving nutrient utilization, protease helps broilers maintain growth and efficiency when exposed to factors that challenge gut health.

I. INTRODUCTION

Protease enzymes are increasingly incorporated into commercial broiler diets due to their ability to hydrolyze proteins and long-chain peptides, releasing shorter peptides and amino acids that can be readily utilized by the bird (Chowdhury et al., 2021). Beyond improving nutrient digestibility, protease supplementation can indirectly support intestinal health by reducing the flow of undigested protein to the hindgut. This limits the substrates available for pathogenic bacteria to ferment, thereby lowering their potential to proliferate and negatively affect bird performance (Windey at al., 2012). Recent studies further suggest that protease, when included at higher doses, may exert direct effects on gut health and resilience. These benefits include improvements in intestinal morphology, modulation of pathogenic populations, and enhanced tolerance to enteric stressors, likely through multiple interacting

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mechanisms (Ghazi et al., 2002; Windey et al., 2012; Chowdhury et al., 2021). The starter phase in broiler chickens is characterized by high dietary protein levels and immature digestive and immune functions, which may result in inefficient protein utilization and increased susceptibility to enteric challenges. During this period, birds are also frequently exposed to pathogenic bacteria such as *Escherichia coli* (*E. coli*) from the hatchery and brooding environment, potentially leading to impaired performance and early mortality (Joseph et al., 2023). Supplementation with a higher dose of protease during this critical phase may be particularly advantageous for improving nutrient utilization and supporting intestinal health (Windey et al., 2012). However, limited information is available regarding the effects of high protease supplementation in the starter phase on growth performance and intestinal development in broilers under enteric pathogen challenge. The present study was therefore designed to evaluate the effects of feeding broilers a high dose of protease during the starter phase, followed by regular doses in the grower and finisher phases, under *E. coli* challenge conditions. Outcomes were assessed in terms of growth performance and intestinal morphology.

II. METHOD

An experiment was conducted to evaluate the effects of feeding broilers a high dose of protease during the starter phase, followed by regular doses in the grower and finisher phases, on growth performance and intestinal morphology under *E. coli* challenge.

Table 1 - Ingredient composition of control (CON) diets[1].

Composition (%)	Starter (d 0-14)	Grower (d 15-28)	Finisher (d 29-42)
Corn	63.0	67.6	72.4
Soybean meal (46% CP)	30.3	26.1	21.8
Meat and bone meal	3.86	3.12	2.61
Soybean oil	0.61	1.06	1.17
Limestone	0.85	0.85	0.82
Salt	0.30	0.30	0.30
Broiler Premix[2]	0.20	0.20	0.20
DL-Methionine	0.40	0.34	0.30
L-Lysine	0.24	0.22	0.20
L-Threonine	0.18	0.15	0.13
Choline chloride (60%)	0.04	0.04	0.04
Phytase[3]	0.01	0.01	0.01
Calculated nutrient composition, %			
Metabolizable energy (kcal/kg)	3,040	3,112	3,166
Crude Protein	22.0	19.9	17.9
Crude Fat	3.37	3.78	3.88
Crude Fiber	2.23	2.17	2.11
Digestible Lysine	1.22	1.08	0.95
Digestible Methionine	0.68	0.60	0.53
Digestible Cysteine	0.25	0.24	0.22
Digestible Threonine	0.82	0.73	0.65
Digestible Tryptophan	0.21	0.19	0.17
Digestible Isoleucine	0.80	0.72	0.64

[1]Protease was included 'on top' of control diet at 400 g/t in the starter diet, and 125 g/t in the grower and finisher diets.
[2]Broiler premix was included to supply the following per kg diet: Vit. A, 5484 IU; Vit. D3, 2643 ICU; Vit E,11 IU; Menadione sodium bisulfite,4.38 mg; Riboflavin, 5.49 mg; D-pantothenic acid, 11 mg; Niacin, 44.1 mg; Choline chloride, 771 mg; Vit B12, 13.2 ug; Biotin, 55.2 ug; Thiamine mononitrate, 2.2 mg; Folic acid, 990 ug; Pyridoxine hydrochloride, 3.3 mg; I, 1.11 mg; Mn, 66.06 mg; Cu, 4.44 mg; Fe, 44.1 mg; Zn, 44.1 mg; Se, 300 ug.
[3]Quantum Blue, AB Vista, UK

A total of 480 one-day-old Cobb 500 broiler chicks were randomly allocated to two dietary treatments: (1) a corn–soybean meal-based control diet (CON), or (2) the control diet supplemented with protease (CON + P). Protease was included at 400 g/t in the starter phase (days 1–14) and 125 g/t in the grower (days 15–28) and finisher (days 29–42) phases (Table 1). Each treatment consisted of 12 replicate floor pens with 20 birds per pen, and birds were reared until 42 days of age. Feed and water were provided *ad libitum* throughout the trial. Litter in each pen was sprayed with 100 mL of *E. coli* suspension (3×10^8 CFU) prior to chick placement to induce an enteric challenge. Growth performance was assessed at the end of the 42-day trial, including body weight (BW), body weight gain (BWG), average daily feed intake (ADFI), and feed conversion ratio (FCR). On day 14, one bird per pen was sampled (total 12 birds/treatment), and tissue sections from the duodenum, jejunum, and ileum were collected to determine villus height (VH), crypt depth (CD), and the villus height-to-crypt depth ratio (VH:CD). Data were analyzed using independent T-tests, with statistical significance declared at $P < 0.05$ and trends considered at $0.05 \leq P < 0.10$.

III. RESULTS

Birds receiving protease at a high dose in the starter phase, followed by regular doses in the grower and finisher phases showed greater ($P < 0.05$) final BW, BWG, and improved ($P < 0.05$) FCR compared to birds fed the CON diet (Table 2). There was also a tendency ($P < 0.10$) for protease supplementation to improve the duodenal VH in birds at day 14 relative to the CON group (Table 2).

Table 2 - Growth performance and intestinal morphometrics of birds fed experimental diets.

		CON	CON + P	SEM	*P*-value
Growth Performance	Initial BW, g	42	42	0.15	0.98
	Final BW, g	3,058[b]	3,176[a]	34.58	0.03
	D 0 - 42 BWG, g	3,017[b]	3,135[a]	34.57	0.03
	D 0 - 42 FCR	1.52[b]	1.50[a]	0.004	0.03
Duodenum (D14)	VH, µm	1,479[y]	1,576[x]	39.32	0.09
	CD, µm	238	227	9.04	0.42
	VH:CD	6.58	7.20	0.27	0.13
Jejunum (D14)	VH, µm	520	540	24.61	0.57
	CD, µm	163	154	7.70	0.38
	VH:CD	3.34	3.57	0.15	0.29
Ileum (D14)	VH, µm	285	346	26.19	0.12
	CD, µm	124	109	8.23	0.21
	VH:CD	2.52	2.99	0.21	0.14

[a,b] Means within the same row with different superscripts differ significantly ($P < 0.05$).
[x,y] Means within the same row with different superscripts differ significantly ($P < 0.10$).
CON = control diet; CON + P = control diet supplemented with protease at a high dose in the starter phase followed by regular doses in the grower and finisher phases.
BW = body weight; BWG = body weight gain; FCR = feed conversion ratio; VH = villus height; CD = crypt depth; VH:CD = villus height to crypt depth ratio; SEM = standard error of mean.

IV. DISCUSSION

Protease is recognized for its ability to enhance protein and amino acid digestibility in poultry diets (Lu et al., 2020), thereby supporting muscle deposition and overall growth. Beyond nutrient utilization, protease supplementation may also contribute indirectly to gut health. By reducing the amount of undigested protein reaching the hindgut, protease limits the substrates available for pathogenic bacteria such as *E. coli* (Chowdhury et al., 2021). This reduction in fermentable protein likely explains the mitigated effects of the enteric challenge observed in

the CON + P group, as indicated by their growth performance. Consistent with this, Chowdhury et al. (2021) reported lower *E. coli* counts in the ileum of broilers when higher dietary protease levels were provided. Protease has also been associated with extra-proteinaceous effects, including enhanced resilience to enteric stress and improved intestinal health (Barbosa et al., 2022). These benefits may stem from increased amino acid availability for mucin synthesis, degradation of antigenic proteins, and reduction of protein-based anti-nutritional factors that negatively influence gut integrity (Barbosa et al., 2022). Administering a high dose of protease early in life is particularly relevant, as this phase is characterized by low endogenous enzyme secretion, high dietary protein supply, and heightened susceptibility to intestinal disturbances (Chowdhury et al., 2021). In line with previous findings, there was a tendency for protease supplementation to improve intestinal morphology in broiler chickens (Lu et al., 2020). Although these effects were not statistically significant in the present trial, the modest improvements observed suggest that protease may aid recovery in birds exposed to pathogenic stressors. In summary, providing a high protease dose during the starter phase, followed by regular inclusion in later phases, appears to help broilers better withstand enteric challenges. This feeding strategy not only enhances growth performance but also supports intestinal structure and resilience, enabling birds to recover more effectively under disease pressure compared with challenged controls.

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ENERGY DEPENDENT REGULATION OF PERFORMANCE, INTESTINAL MORPHOLOGY AND GENE EXPRESSION IN BROILERS SUPPLEMENTED WITH GLUCOSE OXIDASE

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Summary

Dietary energy, is a key driver of poultry performance, regulating immune function, thermoregulation, and maintenance. Glucose oxidase (GOD) is an aerobic dehydrogenase enzyme, that catalyses conversion of β-D-glucose to gluconic acid, producing hydrogen peroxide. Supplementation of GOD in animal feed has shown to improve performance, intestinal barrier function and nutrition absorption capacity. This study investigated the interactive effects of dietary energy level and GOD supplementation on performance, intestinal morphology and gene expression in broilers. A total of 384-day-old mixed sex Cobb-500 chicks were allocated to a 2 × 2 factorial design, with two energy levels (Standard: based on Cobb-500 broiler specifications and low: 80 kcal below Standard diet), with or without GOD supplementation (200g/T), each with eight replicates. Performance parameters were measured for the overall trial period (0-35). On day 35, jejunum samples were collected for histomorphology and mRNA expression of nutrient transporters (*ASCT1, GLUT2* and *LAT1*), enzyme production (*APN*) and tight junction proteins (*JAM2*, *CLDN5* and *OCLN).* Data were analysed using two-way ANOVA with Tukey's test for paired comparison. Results showed that, although no significant interaction was observed between energy level and GOD dose for BWG and jejunal VH:CD ratio, GOD significantly improved BWG in overall experimental period and showed tendency for improving jejunal VH:CD ratio regardless of energy level. These results aligned with jejunal gene expression results, where low energy with GOD supplementation upregulated the expression of *GLUT2*- supports nutrient transport and *CLDN5*- indicates barrier integrity. GOD also upregulated the expression of *JAM2* irrespective of energy level, which also regulates integrity. However, GOD downregulated the expression of *OCLN*, *APN* and *LAT1* only in the birds fed Standard energy diets. These findings suggest that GOD may be a promising feed additive with potential to improve nutrient absorption and barrier function and enhance performance when dietary energy is insufficient.

I. INTRODUCTION

Achieving maximum growth performance while maintaining intestinal health is becoming more challenging due to increased feed costs and a growing emphasis on limiting the use of antibiotics in animal feed (Durunna et al., 2005). Since energy is one of the primary components in most animal feeds and accounts for 70% of expenditures, it should be used as efficiently as possible to achieve the desired economic results in the manufacture and formulation of chicken feed (Noblet and Van Milgen, 2004). Reducing dietary energy is a widespread strategy to lower feeding cost (Donohue and Cunningham, 2009), although such reductions often have a negative impact on growth performance and feed efficiency. Nutritionists and producers are constantly searching for feed additives to enhance the overall health and digestive efficiency of the birds. There has been interest in the potential of enzymes such as glucose oxidase (GOD) to influence the intestinal environment and increase feed intake (Wu et al., 2019). GOD is an aerobic dehydrogenase enzyme, mainly produced by *Aspergillus*

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niger and *Penicillium glaucum* (Müller, 1928). It oxidizes β-D-glucose to gluconic acid while producing hydrogen peroxide as a byproduct. Gluconic acid act as an acidifier in the intestinal tract while hydrogen peroxide has bactericidal effect. Previous studies have shown that GOD can improve intestinal barrier function and ability to absorb nutrients (Liu et al., 2020). Given these potential benefits, the present study aims to examine if glucose oxidase improves performance and gut health in broilers, particularly when dietary energy is insufficient.

II. METHOD

A total of 384-day-old mixed-sex Cobb-500 broiler chicks were randomly allocated to 32-floor pens in a 2 × 2 factorial design with 8 replicates per treatment (12 birds / pen). The factors included: energy level (Standard: based on Cobb-500 Standard and low: 80 kcal below Standard diet), and glucose oxidase supplementation (0, 200g/T). All diets were wheat-soybean meal based and were provided in the 3 phases: starter (d0- 8), grower (d8-19) and finisher (d19-35). Body weight gain (BWG) was calculated for overall period (d0-35). On d35, jejunal tissues were collected and stored in 10% buffered formalin for histological assessments. The collected tissues were sectioned, processed and villus height (VH) and crypt depth (CD) were measured with a minimum of 10 villi and associated crypts randomly chosen for measurements according to protocol published by Kumar et al. (2021). For gene expression, about 2 cm of the proximal jejunum tissue was excised and collected in 2 mL Eppendorf tubes containing RNA later. The tissue sections were kept at 4°C for 4 h and then stored at -20°C until the extraction of RNA. Total RNA was extracted from each jejunal sample, quantified, assessed for integrity and cDNA synthesis was done using SensiFast cDNA synthesis kit (Meridian Bioscience, Sydney, NSW, Australia). The qPCR analysis was performed with cDNA template in duplicates using the SensiFAST SYBR No-ROX kit (Meridian Bioscience, Sydney, NSW, Australia) with a real-time PCR machine (Rotor-Gene Q, QIAGEN GmbH, Hilden, Germany). Primers targeting specific genes of interest related to nutrient transporters (*ASCT1*= Alanine, serine, cysteine, and threonine transporter; *GLUT2*= Glucose transporter-2; *LAT1*= Large neutral amino acid transporter-1), enzyme production (*APN*=Aminopeptidase N) and gut integrity (*JAM2*=Junctional adhesion molecule-2; *CLDN5*= Claudin- 5; *OCLN*= Occludin) were used from previously published papers (Gharib-naseri et al., 2021). Data were analysed by two-way ANOVA with Tukey's test for paired comparison. Significance was set at $P < 0.05$ and declared a tendency to be different with $0.05 < P < 0.10$.

III. RESULTS

The overall BWG of broilers from day 0 to 35 and jejunal VH: CD ratio at d35 in response to dietary energy level and GOD supplementation are shown in Table 1. No significant interaction was found between energy level and GOD dose (P>0.05). However, supplementation with GOD led to significantly higher BWG (2615 vs. 2543; P<0.05) and tended to improve jejunal morphology (VH:CD) (P = 0.063), irrespective of dietary energy level.

Effects of energy level and GOD dose on jejunal expression of nutrient transporters, tight junction proteins and enzyme production are shown in Table 2. Significant interactions between dietary energy and GOD supplementation were observed for *GLUT2*, *APN*, *LAT1*, *OCLN*, *CLDN5* (P < 0.001) and *ASCT1* (P < 0.05). For *GLUT2,* GOD supplementation effected the Standard and low energy diets in opposite directions, causing a downregulation in birds fed with Standard energy diets, but an upregulation in the birds fed with low energy diets. On the other hand, *ASCT1* was significantly downregulated only in birds fed with un-supplemented low energy diets. In contrast, GOD supplementation downregulated the expression of *OCLN*, *APN*, and *LAT1* only in birds fed with Standard energy diets. However, GOD significantly increased *CLDN5* expression only in birds fed low energy diets. Furthermore, *JAM2* was upregulated in Standard energy diets regardless of GOD supplementation (P < 0.01) and GOD supplementation also upregulated *JAM2* expression irrespective of energy level (P < 0.01).

Table 1- Performance (BWG) and jejunal histomorphology in broilers in response to energy and glucose oxidase dose level.

Energy (kcal/kg)	GOD dose (g/T)	[1]BWG (d0-35) (g)	Histomorphology (d35) [2]VH:CD
Standard	0	2585	10.1
	200	2630	11.5
Low	0	2500	8.86
	200	2601	11.2
Main effects			
Energy Level	Standard energy	2608	10.8
	Low energy	2550	10.0
[3]GOD dose	0	2543[b]	9.48
	200	2615[a]	11.3
P-value	Energy	0.107	0.443
	Dose	0.044	0.063
	Energy x Dose	0.421	0.599

[1]BWG: body weight gain; [2]VH:CD- Villus height and crypt depth ratio [3]GOD dose- glucose oxidase @ 0, 200g/T respectively in each treatment; [a,b] values within a row with different letters differ significantly ($P < 0.05$).

Table 2- Effects of energy level and GOD dose on jejunal gene expression on d 35.

		Nutrient transporters			Enzyme production	Tight junction proteins		
Energy (kcal/kg)	GOD dose (g/T)	*ASCT1*	*GLUT2*	*LAT1*	*APN*	*JAM2*	*CLDN5*	*OCLN*
Standard	0	1.509[a]	2.014[a]	2.129[a]	1.791[a]	1.318	1.535[a]	1.749[a]
	200	1.100[ab]	0.798[bc]	0.777[b]	0.882[b]	1.817	1.243[ab]	0.818[b]
Low	0	0.729[b]	0.594[c]	0.774[b]	0.724[b]	0.467	0.565[c]	0.745[b]
	200	1.076[ab]	1.514[ab]	1.039[b]	1.147[b]	1.067	1.025[b]	1.153[b]
Main effects								
Energy	Standard energy	1.305	1.406	1.453	1.336	1.568[a]	1.389	1.284
	Low energy	0.903	1.054	0.907	0.935	0.767[b]	0.795	0.949
[1]GOD dose	0	1.119	1.304	1.452	1.257	0.892[b]	1.050	1.247
	200	1.088	1.156	0.908	1.014	1.442[a]	1.134	0.986
P-value	Energy	0.018	0.094	0.001	0.012	0.000	0.000	0.007
	Dose	0.847	0.472	0.001	0.112	0.001	0.378	0.032
	Energy x Dose	0.025	0.000	0.000	0.000	0.723	0.000	0.000

[1]GOD dose- glucose oxidase @ 0, 200g/T respectively in each treatment; [a,b,c] values within a row with different letters differ significantly ($P < 0.05$).

IV. DISCUSSION

The findings showed that GOD supplementation significantly improved BWG during the overall period of the experiment, regardless of energy level. Previous work has shown that gluconic acid, a key metabolite produced by GOD, can improve growth performance in poultry (Liu et al., 2020) and other studies have indicated the addition of GOD in feed can improve weight gain in male broilers aged 22 to 42 days (Meng et al., 2021). Also the observed tendency of higher ratio of villus height to crypt depth, could reflect a partial improvement in intestinal absorption function that reveals the morphological structure and functional state of the gut (Jiang et al., 2024; Meng et al., 2021). In this study, GOD supplementation increased the jejunal expression of *CLDN5* in birds fed low energy diets, which is supported by previous findings. Tight junction proteins, such as *CLDN5*, control the functioning of the intestinal barrier. It has been shown that dietary GOD supplementation may enhance the expression of tight junction genes, indicating improved gut integrity (Liu et al., 2020; Zhao et al., 2022). Additionally, in

this study, GOD supplementation significantly upregulated the expression of *JAM2* irrespective of energy level that can indicate an improved barrier function. *JAM2* tight junction proteins are essential for preserving cell adhesion and controlling tight junction permeability in a variety of tissues, especially in endothelial cells that make up the blood-brain barrier and other vascular structures (Schottlaender et al., 2020). Furthermore, in this study, low energy diets supplemented with GOD increased *GLUT2* gene expression, which is aligned with a previous study with supplementation of exogenous multienzymes, where higher *GLUT2* expression of supplemented group was shown in broiler chickens fed a low-energy diet (Saleh et al., 2018). *GLUT2*, plays an important role in transporting monosaccharides, including glucose, fructose, galactose, and mannose, across the intestinal basement membrane into the bloodstream. These monosaccharides are then transported to the liver, where they undergo metabolism and contribute to glucose production, which is then released into the bloodstream and used throughout the body (Mueckler and Thorens, 2013). Overall, GOD appears to improve BWG and have partial positive effects on jejunal histomorphology (VH:CD), possibly through the regulation of genes, particularly when dietary energy is limited. This could suggest a compensatory mechanism that promotes barrier function and nutrient absorption under energy-restricted feeding conditions.

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TARGETED EARLY GUT COLONISATION IN BROILERS USING PROBIOTIC *PEDIOCOCCUS SP.*

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Summary

Industrial chicken production disrupts natural gut colonisation, as hatchery practices limit microbial transfer from the hen to the chick and increase the likelihood of colonisation with harmful microbes. Early gut colonisation with beneficial microbes is essential for immune system development, pathogen resistance, and growth. This study evaluated the effects of supplementing the first feed with a *Pediococcus sp*. isolate in Ross 308 broilers versus normal feed and later challenge with Dexamethasone. Chicks were assigned to three groups, challenged control (unsupplemented basal diet with dexamethasone at week 4), unchallenged control, (unsupplemented basal diet), and *Pediococcus*-treated (PS) (supplemented basal diet with Dexamethasone challenge at week 4) groups, receiving probiotic-fermented feed only for the first 48 hours. At week 4, challenged groups were exposed to dexamethasone to induce leaky gut, and gut microbiota were analysed.

While bird growth, feed efficiency, and microbial diversity were unaffected, PS birds showed enrichment of potentially beneficial taxa (*Faecalibacterium*, *Candidatus arthromitus*, and *Brachybacterium*). In contrast, controls harboured potential pathogens (*Escherichia-Shigella*), as well as potentially beneficial genera like *Lactobacillus*, *Terrisporobacter*, *Romboutsia*, *HT002*, and *Corynebacterium*. Early supplementation with *Pediococcus sp.* influenced the long-term gut microbiota, suggesting that probiotics administered at hatch can enhance broiler gut health.

I. INTRODUCTION

Industrialisation of chicken production increases efficiency and output, but introduces welfare challenges such as overcrowding, stress, poor air quality, and limited displays of natural behaviour (Fu et al., 2022, Ivulic et al., 2022, Moekti, 2020). In hatcheries, eggs are separated from hens and incubated under controlled conditions, which limits microbial exposure from the hen and disrupts natural colonisation of the chick's gut. Eggshell microbes, derived from the hen and its environment, partially influence colonisation of the gastrointestinal tract (Ding et al., 2017). However, hatcheries' strict biosecurity measures and the use of chemical disinfectants reduce pathogens but also eliminate beneficial microbes, exposing chicks to environmental bacteria after hatching (Stanley et al., 2013). Early gut colonisation with beneficial microbiota is essential for supporting immunity, preventing pathogen establishment, and enhancing growth and welfare (Rubio, 2019, Song et al.,2022). Probiotics are a promising strategy for controlling microbial colonisation of the gut, improving feed efficiency, and enhancing overall health in broilers (Baldwin et al., 2018, Cox et al., 2015).

This study isolated and characterised several putative probiotic strains from healthy chickens. After a range of culturing and *in vivo* experiments, we selected an isolate of *Pediococcus sp*. as a candidate probiotic for early gut colonisation. As a lactic acid genus, *Pediococcus* species have been widely utilised in food fermentation and have been demonstrated to enhance feed conversion in chickens (Harun et al.,2024, Noohi et al.,2016). In addition to lactic acid, *Pediococcus* species produce antimicrobial peptides that inhibit pathogens and regulate immune responses through toll-like receptor pathways (Lan et

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al.,2020). Here, we present a study evaluating the effects of supplementing the first feed with a *Pediococcus* sp. isolate in broilers.

II. METHOD

To investigate the ability of a *Pediococcus* isolate to alter the course of gut colonisation, 264 Ross-308 chicks were hatched on the premises and randomly assigned into three treatments: control (Unchal_Ctr), control challenged (Chal_Ctr), and challenged *Pediococcus* (PS), with 11 birds per pen and eight pens per treatment.

Basal diet was sterilised, 3% of sterile water was thoroughly mixed using a food mixer, and 40 ml of 24-hour grown *Pediococcus* culture was mixed into the feed and placed in the incubator at 37 degrees and 40% humidity for 24 hrs. After 24 hours, the feed was taken out of incubator and dried at room temperature for 3 days. Colony count after drying the feed was 4.7 x 10^9 viable CFU per gram of feed

Pediococcus-fermented feed was provided only in the first feed for 48 hours, while control groups received basal diet. After 48 hours, the feed was replaced with a basal diet. All pens received a basal diet until week 3. During week 4, all groups except the unchallenged control were treated with dexamethasone to induce leaky gut (Vicuña et al., 2015). Performance data were collected weekly. At day 28, the birds were euthanised, and samples of cecum content, ileum content, ileum mucosa, and jejunum content were collected for microbiota analysis. The V3-V4 regions of the 16S rRNA gene were sequenced using the Illumina MiSeq platform and analysed with QIIME2, followed by Phyloseq and Microeco R packages.

III. RESULTS

There were no statistically significant differences in body weight gain and feed efficiency, cecal short-chain fatty acids (SCFAs) production, or alpha and beta diversity between the Chal_Ctr and PS.

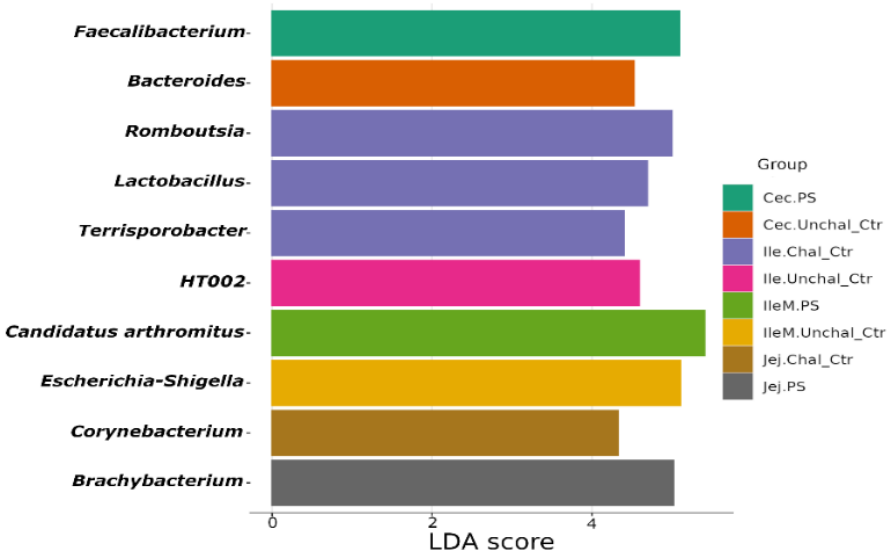

Figure 1 - LEfSe analysis identified a reduction of *Echerichia* in the ileum mucosa and an increase of *Faecalibacterium* in the cecum of *Pediococcus* colonised birds.

Linear discriminant analysis Effect Size (LEfSe) identified distinct biomarker genera associated with each group across different intestinal regions (Figure 1). In the cecum, the

genus *Faecalibacterium* was associated with PS treatment, whereas *Bacteroides* served as a biomarker for Unchal_Ctr. Ileum Chal_Ctr was characterised by the genus *Romboutsia*, *Lactobacillus* and *Terrisporobacter*, while UnChal_Ctr's distinctive genus was *HT002*. The genus associated with the Ileum mucosa for PS was *Candidatus arthromitus*, while the UnChal_Ctr genus was *Escherichia-Shigella*. In the jejunum, the markers for PS were *Brachybacterium* and for Chal_Ctr were *Corynebacterium*.

IV. DISCUSSION

LEfSe identified distinct biomarker genera associated with each treatment across different intestinal regions. In the cecum, *Faecalibacterium*, a known butyrate producer (Sakamoto et al., 2022) was particularly related to the PS treatment. In contrast, *Bacteroides*, linked to increased weight gain and improved feed conversion (Farkas et al., 2022, Zhang et al., 2025), was representative of the Unchal_Ctr microbiota. In the ileum, *Romboutsia*, *Lactobacillus*, and *Terrisporobacter*, all associated with host-beneficial effects such as competitive pathogen exclusion, influencing metabolic environment in the gut, and protecting against oxidative stress (Chen et al., 2020, Zhang et al., 2024), were characteristic of Chal_Ctr, while the Unchal_Ctr had *HT002* (family *Lactobacillaceae*), a beneficial commensal and possible probiotic in chicken (Xu et al.,2024). The ileal microbiota of challenged and unchallenged birds showed distinct group-specific signatures, indicating that the challenge had a stronger effect on community composition than *Pediococcus* supplementation at the ileum lumen. Although LEfSe analysis did not identify dominant indicator genera for the *Pediococcus* group in ileal digesta, the ileal mucosa was characterised by *Candidatus Arthromitus*, a segmented filamentous bacterium associated with enhanced mucosal immunity and epithelial maturation (Hedblom et al., 2018, Liu et al., 2023). Although mechanistic data in chickens are still emerging, recent evidence suggests that this bacterium attaches itself to the intestinal epithelium without provoking inflammation and triggers an immune response that can block pathogens from colonising the epithelial receptors in the ileum (Choi et al., 2025). In contrast, *Escherichia-Shigella,* a genus-level taxonomic group that includes pathogens like avian pathogenic *Escherichia coli*, characterised the ileum mucosa of the Unchal_Ctr. In the jejunum, *Brachybacterium*, a non-harmful genus linked to litter phosphorus content, also described as a phosphate-accumulating probiotic (Muyyarikkandy et al., 2023), served as the biomarker for PS treatment, while Chal_Ctr was characterised by *Corynebacterium*, a commensal genus associated with carbohydrate metabolism and feed utilisation (Wen et al., 2021).

Colonisation of the gut begins at hatch when there's an empty ecological niche; the microbes that first colonise establish themselves, modifying the environment to favour the same type of bacteria by producing secondary metabolites that may impede the growth of different species. After the first two days, the microbial community evolves rapidly, achieving stability within the first three weeks (Franco et al., 2023). By providing *Pediococcus sp*. at hatch, we can still observe the effect four weeks after, especially in the ileum mucosa and cecum, where the characteristic beneficial bacterium *Candidatus arthromitus, and Faecalibacterium* are established. In contrast, control birds are colonised by beneficial bacteria, such as *Bacteroides*, in the cecum, as well as by a known conditional pathogen, *Escherichia-Shigella* taxonomic group, in the ileum mucosa. Although this conditional pathogenic group was present, the birds were healthy with no signs of infection.

In conclusion, the addition of *Pediococcus* sp. to the broiler feed during the first 48 hours resulted in long-term effects and altered the gut microbiota community dynamics. Providing probiotics in hatchery could be a promising strategy to improve gut health by promoting early gut colonisation with beneficial species capable of outcompeting pathogens during the colonisation process.

ACKNOWLEDGEMENTS: The study was part of the AgriFutures Chicken Meat Consortium – Nutrition, Gut Health and Environment project (PRO-016111), which is funded and supported by the AgriFutures Chicken Meat Program with its associated levy payers, five research providers, seven industry partners and nine international universities.

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DIET- AND STRAIN-DEPENDENT SPORE OUTGROWTH KINETICS OF BACILLUS-BASED PROBIOTICS IN BROILER FEEDS

L. BAUER[1], S. MOLCK[2], B. JAYARAMAN[3] and A. HUESER[2]

Summary

This study investigates how diet composition influences the efficacy of spore-forming *Bacillus*-based probiotics in poultry, with a focus on the transition from spore to metabolically active vegetative cells. Previous research identified the duodenum as the critical site for spore germination and outgrowth. Therefore, the study introduces a standardized spore outgrowth assay using in-vitro digesta from the duodenum compartment (dFeed) of the DAISy model from starter and finisher broiler diets representative for eight regions globally to test ten commercially available *Bacillus*-based probiotics. Results show that spores outgrew faster in starter than in finisher feeds. Additionally, feed formulations affected the outgrowth speed of spore-forming probiotics. *Bacillus* CECT 5940 spores coming from an improved fermentation process consistently achieved the fastest and most reliable outgrowth across all tested diets, while other probiotic products displayed greater variability and, in some cases, insufficient outgrowth speed relative to feed retention time. The findings highlight feed composition as a key but previously underappreciated factor affecting probiotic performance.

The authors propose that rapid outgrowth should be a primary selection criterion for spore-forming probiotics in poultry, as it maximizes the number of active cells during the limited retention time in the gut, supporting improved animal health and productivity under diverse dietary conditions.

I. INTRODUCTION

Bacillus-based probiotics are increasingly used in poultry for gut health, immunity, and performance. Unique is their ability to exist as spores or vegetative cells: *Bacillus* species benefit from spore stability during feed processing and shelf life, while vegetative cells provide probiotic benefits after activation in the gastrointestinal tract (GIT). Consistent, rapid transition from spore to active cell (further described as outgrowth speed) is essential for product efficacy but may vary widely due to differences in production processes and probiotic strains. This study examines how different diet compositions could affect the speed and consistency of outgrowth testing ten different *Bacillus*-based probiotics. Additionally, a high-throughput spore outgrowth assay was introduced to assess outgrowth rates across products and feeds.

II. MATERIALS AND METHODS

For this study representative starter and finisher broiler feed formulations were collected from eight regions (Europe, South Africa, Egypt, Brazil, China, North America, India, and the Philippines) globally, resulting in sixteen different diets to measure the consistency and time of outgrowth of spore-forming probiotics products under various dietary conditions. The feeds showed differences in raw materials used (Europe – corn, soybean meal (SBM), wheat, rapeseed meal, soy oil; South Africa – corn, SBM, corn gluten meal (CGM); Egypt – corn, SBM, soy full fat, wheat middlings, CGM; Brazil – corn, SBM, meat and bone meal (MBM); China – SBM, rice, corn, wheat, CGM, peanut meal, cottonseed meal, poultry fat; North America – corn, SBM, CGM, poultry fat; India – corn, SBM, rice polish, deoiled rice bran, soy oil and the Philippines

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– corn, SBM, wheat, palm oil, MBM, rice bran) and in the respective regional raw material quality and nutrient composition. All sixteen feeds were pre-digested up to the duodenum (dFeed) by using a Dynamic Avian Intestine in-vitro System (DAISy). The experimental setup of the DAISy model includes compartments representing the crop, proventriculus, gizzard, duodenum/jejunum, ileum, and caecum to simulate the gastrointestinal tract (GIT) of poultry (Hüser and Nerenz, 2024). At duodenum conditions the digestion process in the DAISy model can be stopped and the entire in-vitro digesta can be lyophilized and stored upon usage as a medium for feed-specific spore outgrowth testing. This approach allows for an evaluation of spore outgrowth in duodenum like conditions and providing a controlled medium to assess the impact of different feed formulations. Spore outgrowth assays need to be conducted with duodenum like digesta medium as reported in earlier research from our group (Bauer et al., 2026). Within the GIT, the duodenum is the only segment where spore germination and outgrowth can take place (Bauer et al., 2026; Koopmann et al., 2022).

In total, ten different commercially available *Bacillus*-based spore-forming probiotics were collected and used in the study. The shelf life for all products was confirmed and valid at the time of testing. Those *Bacillus* spore probiotics were standardized to 1E+6 CFU/g and incubated in 384-well plates containing the respective dFeed and the metabolic indicator reagent resazurin (PrestoBlue™, Thermo Fisher, Waltham, USA). Upon irreversible reduction, resazurin converts from a non-fluorescent oxidized form to a reduced fluorescent form, with the degree of reduction correlating to the metabolic activity of vegetative cells. Fluorescence was measured every 10 minutes with a TECAN Spark multimode microtiter plate reader (Tecan Group Ltd., Männedorf, Switzerland) to monitor metabolically active vegetative cells. The time between assay initiation and reaching a predefined fluorescence threshold—set at a level corresponding to two times the mean blank signal measured at the respective kinetic time point (Armbruster et al., 2008)—was recorded as the relative outgrowth speed for each probiotic.

III. RESULTS

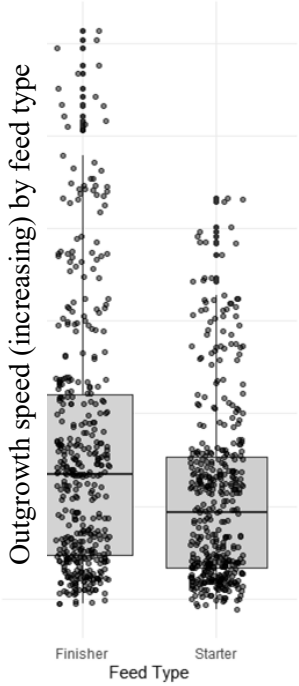

Figure 1 - The average outgrowth speed of nine different Bacillus-based probiotics in starter vs. finisher feeds (average of starter and finisher feeds, respectively) representative for the regions Europe, South Africa, Egypt, Brazil, China, North America, India, and the Philippines.

The outgrowth speed of probiotics was compared between pooled starter and finisher feeds from all products and regions. Probiotics grew significantly faster in starter feeds than in finisher feeds (Wilcoxon rank sum test, W = 111311, P < 0.001) (Figure 1).

The outgrowth speed of different Bacillus-based probiotics differed between the different regional representative feed formulations. Among eight different diets, spores of *Bacillus* CECT 5940 showed the fastest outgrowth, which was used as the reference point (set to zero) for comparisons in the benchmark test.

For the analysis, all samples from starter and finisher feeds of all *Bacillus*-based probiotics were pooled. The regional representative feed formulations affected the outgrowth speed of probiotics (Kruskal-Wallis test, chi-squared = 101.06, df = 7, P-value < 0.001). Overall, fastest spore outgrowth was found in feeds from Brazil, South Africa and Philippines. Spore outgrowth was slower in feeds from India, Egypt, North America, China, and Europe (Figure 2).

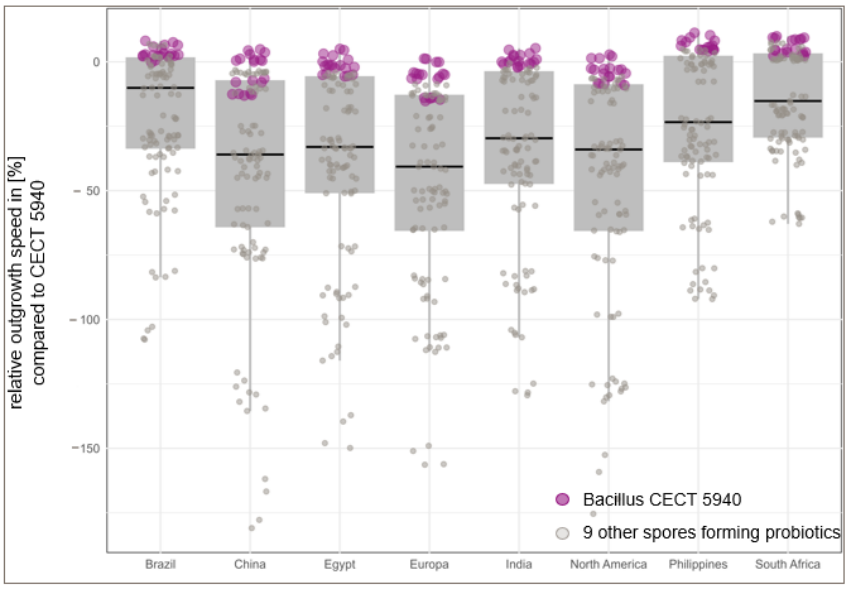

Figure 2 - Outgrowth speed of ten different Bacillus-based probiotics in eight different feed compositions (average of starter and finisher feeds, respectively) representative for the regions Europe, South Africa, Egypt, Brazil, China, North America, India, and the Philippines.

Bacillus CECT 5940 spores consistently exhibited the most rapid outgrowth in both starter and finisher feeds from all eight regions. In contrast, other spore-forming probiotics products displayed greater variability and slower outgrowth speed, with some strains requiring more time for outgrowth than the typical feed retention time in the GIT of broilers.

IV. DISCUSSION

Bacillus-based spore-forming probiotics are widely used in poultry for their stable spores, which convert to active cells in the gut and produce metabolites (Bernadeau et al., 2017). Product effectiveness depends on factors like *Bacillus* strain and spore stability in feed and storage. This research also highlights spore outgrowth time as a crucial factor.

This study found that feed composition plays a key role in the shift from spore to active cell for spore-forming probiotics. Starter feeds generally had higher amounts of crude protein and added amino acids, resulting in greater amino acid density than finisher feeds. On the other hand, finisher feeds contained more carbohydrates and fat. These findings suggest that the availability of amino acids may help trigger germination and growth, which is supported by other researchers (Artzi et al., 2021).

The DAISy system and dFeed medium were utilized as platforms to assess probiotic outgrowth under simulated gut conditions. *Bacillus* CECT 5940 spores were observed to exhibit rapid and consistent outgrowth across various regional representative diet formulations, whereas outgrowth speed of other *Bacillus*-based products was slower, inconsistent, and dependent on diet composition.

Outgrowth speed is suggested as a key criterion for selecting probiotics in poultry production, since rapid activation increases the number of active cells present during the short feed retention period in the chicken gut. This can influence colonization and pathogen inhibition, as only metabolically active cells are capable of reproduction and exerting probiotic effects.

V. CONCLUSION

This study demonstrates that diet composition significantly influences the outgrowth kinetics of Bacillus-based spore-forming probiotics in poultry. Starter feeds, characterized by higher amino acid density, consistently promoted faster spore activation compared to finisher feeds. Among the tested strains, Bacillus CECT 5940 exhibited the most rapid and reliable outgrowth across all diets, highlighting strain-specific differences in performance. These findings underscore the importance of considering both feed formulation and outgrowth speed as critical criteria for probiotic selection to maximize efficacy within the limited gastrointestinal retention time in broilers.

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NUTRIENT TRANSPORT AND BARRIER PROPERTIES CHANGE WITH CELL-LINEAGE COMPOSITION IN THE SMALL INTESTINE OF BROILER CHICKENS

F. DÍAZ-AVILÉS[1], M. NAVARRO[1], P. BOGERE[1], M. NASEEM[2], C. PALMIERI[2] and E. ROURA[1]

The intestinal epithelium combines the nutrient absorption and barrier defence functions with a highly dynamic renewal and differentiation of cell lineages. The epithelial architecture and cell-lineage composition varies along the small intestine, reflecting segment-specific functions: proximal regions (duodenum/jejunum) with long villi and cell profiles including a higher proportion of enterocytes generally support greater absorptive capacity than the distal ileum (Diaz-Aviles et al., 2025), . Early nutritional programming through in-ovo feeding practices or early post-hatch feeding, have been shown to enhance digestive tract development and improve gut function (Jha et al., 2019). However, the mechanistic links between epithelial structure, cell composition, and gut function outcomes such as absorptive capacity remain poorly understood. We hypothesised that enterocyte-rich epithelium (i.e., duodenum) preferentially supports amino acid uptake compared to goblet cell-rich distal segments (i.e., ileum) which prioritize barrier and mucosal defence.

Duodenum, proximal jejunum, distal jejunum and distal ileum from eight 35-day-old Ross 308 broilers fed a standard grower diet were collected without the serosa layer, and placed in an Ussing chamber, the mucosal and serosal faces were separated as two independent hemi-chambers perfused with Krebs solution continuously bubbled with carbogen, held at physiological temperature. Electrodes connected via agar–KCl salt bridges were used to measure transepithelial potential (Vt) and passed current to clamp Vt at 0 miliVolts, enabling continuous short-circuit current (Isc) recording. Transepithelial resistance (Rt) was obtained from brief voltage pulses and expressed as Transepithelial Electrical Resistance (TEER) = Rt × tissue area. After stabilization, L-Lysine, L-Glutamate, Lys-Lys, Lys-Lys-Lys, and glucose (positive control) were applied to the mucosa. Responses were recorded as time courses (baseline to ~20 min post-addition) and summarized as ΔIsc and ΔTEER relative to pre-addition baselines. Treatments were randomized across chambers and birds. The results showed regional variations in the intestinal basal Isc. The Isc was significantly different in the proximal jejunum when compared to the duodenum, distal jejunum, and distal ileum ($P < 0.0001$). The TEER resulted in a higher Vt in duodenum than proximal jejunum, distal jejunum, or distal ileum ($P < 0.0001$). Distal ileum exceeded proximal jejunum ($p=0.022$), while distal jejunum did not differ from proximal jejunum or distal ileum. These results indicate that the epithelial functionality changes by region. An elevated Isc in proximal jejunum indicates a high electrophysiological activity compatible with higher nutrient absorption activity, whereas higher TEER in duodenum —and relatively elevated TEER in distal ileum— signals tighter epithelial sealing. Thus, within the chicken small intestine we observed the highest nutrient absorption capability in the medial jejunum while tightened barrier function proximally and distally (i.e., duodenum and ileum, respectively). Overall, these results support the hypothesis that influencing cell differentiation in the intestinal mucosa has the potential to improve the efficiency of feed/nutrient digestibility and absorption.

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ADAPTIVE METABOLIC RESPONSES TO A LOW CRUDE PROTEIN DIET IN BROILER CHICKENS

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One of the priorities in improving the sustainability of broiler production in Australia is to decrease the dependence on imported soybean meal (SBM). Lowering dietary crude protein and supplementing with synthetic amino acids is one of the studied strategies. However, a decrease of 25% or more crude protein, cannot be completely compensated by supplementing adequate essential amino acid (EAA) levels, which inevitably leads to reduced growth rates. The objective of this experiment was to determine the adaptive metabolic responses underpinning decreased growth rates in broilers fed a low CP (LCP) diet compared to a standard CP (SCP) diet. We hypothesized that an LCP diet would impair growth performance of birds due to unmet non-essential amino acid (NEAA). A total of 96 Ross-308 male broiler chickens were fed a commercially standard starter (0–9 days) and grower (10–28 days) diets. On day 10, broilers were split into two groups of 48 chickens and were randomly assigned an SBM-free LCP (15.5%) or an SBM-based SCP (21.5%) diets. Both grower diets were iso-energetic (ME 3050 kcal/kg, dig. Lys 1.18%) and iso-amino acidic in EAA. Alanine (a NEAA) was used to maintain the diets iso-nitrogenous. Body weight (BW) was recorded weekly. On day 28, six birds per diet were euthanized and blood collected for metabolomics. Growth data were analyzed in SAS 9.4. We applied PLS-DA (MetaboAnalyst.ca) and integrated enrichment/pathway analysis to identify differential metabolites. The LCP group showed a lower ADG than the SCP group. Birds fed the LCP diet gained 60.7 g/day versus 76.6 g/day for the SCP group, with average daily feed intake of 103.5 g and 106.5 g, respectively. FCRs were 1.70 and 1.39 for LCP and SCP, respectively. The PLS-DA showed a clear separation between the LCP and SCP. The top 15 differential metabolites and the corresponding enriched pathways in the LCP diet are shown in Figure 1. The LCP diet group showed downregulation in riboflavin, purine, and amino acid metabolic pathways, including lower levels of riboflavin, xanthylic acid, aspartic acid, proline, and valine, and upregulation of indolelactic acid, 4-chloro-3,5-dinitrobenzotrifluoride, and selenocystathionine. In conclusion, the SCP diet supported superior broiler growth than the LCP diet showing a disruption in plasma metabolic profiles associated mainly with NEAA (aspartic acid, proline and cysteine) and valine metabolism. The lower levels of aspartic acid and proline may, in turn, affect arginine availability in the LCP diet which confirms prior findings published from our group (Khatun et al, 2025).

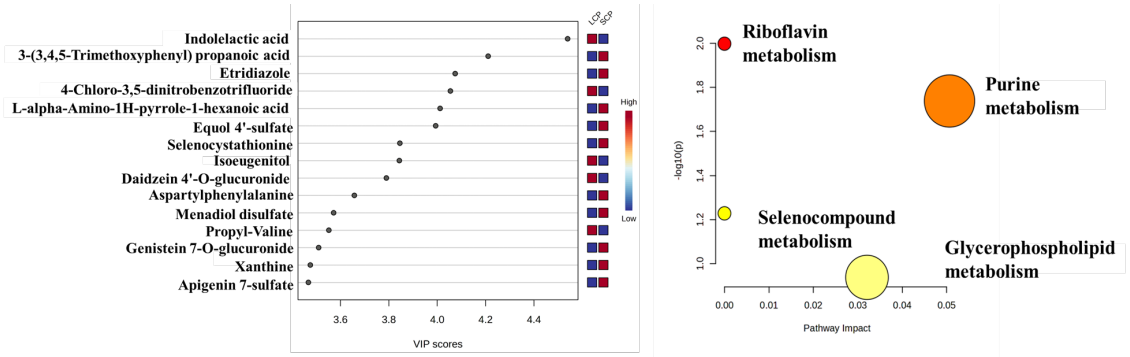

Figure 1 - Top 15 metabolites ranked by VIP scores differentiating between LCP and SCP groups (left). A bubble plot of integrated enrichment and pathway analysis (right).

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SEQUENTIAL PROFILING OF DIETARY CARBOHYDRATES

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Carbohydrates are the primary component of poultry diets, serving as the main energy source and playing a key role in gut health and microbial composition. The structural complexity of carbohydrates presents an ongoing challenge for comprehensive analysis, particularly when evaluating fibre interventions and feed enzyme strategies. Current assays used to measure carbohydrates include gravimetric assays to determine bulk fibre values, enzymatic–colourimetric assays to measure starch and β-glucans, and liquid chromatography-mass spectrometry assays to quantify xylo-oligosaccharides (Morgan et al., 2019). The method developed by Englyst and Cummings (1984) remains the most widely used technique for evaluating non-starch polysaccharides. In this assay, starch is removed, and then the fibre is portioned into soluble and insoluble fractions, followed by acid hydrolysis, allowing for analysis of the constituent monosaccharides. This analysis provides comprehensive detail on the components of fibre but overlooks critical structural features that influence physiological outcomes. To obtain a comprehensive analysis of carbohydrates, each of these assays detailed above must be conducted independently, which is both time-consuming, requires large amounts of sample and increases the potential for errors. Consequently, the aim was to develop a technique that integrates these assays into a single, stepwise procedure.

We have developed a sequential profiling workflow that differentiates enzyme-susceptible from resistant fractions, quantifies soluble and insoluble pools, and identifies intermediate oligosaccharides with prebiotic relevance. From a single sample, carbohydrates are progressively released using targeted enzymes and mild acid hydrolysis, with hydrolysates characterised at each stage, and gravimetric monitoring ensuring complete material recovery. Importantly, the workflow is modular, enabling the inclusion of additional selective steps to interrogate specific structural features in greater detail. Figure 1 presents a workflow of this method.

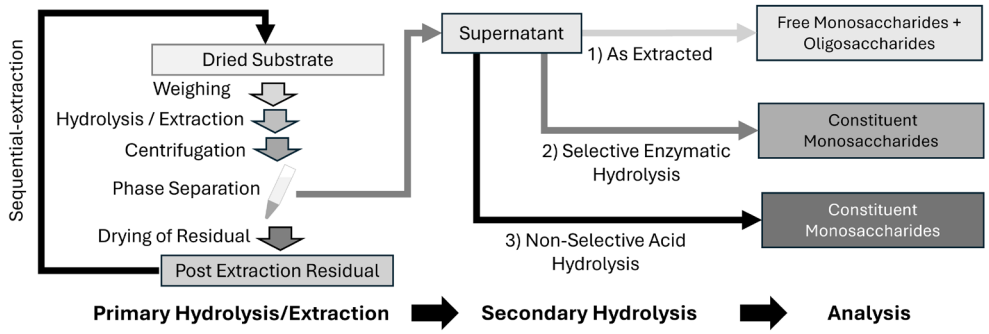

Figure 1 - Flowchart of carbohydrate sequential profiling workflow.

The proposed method has been applied to diet and ileal samples and could also be used on raw materials before formulation. It shows that tailored enzymatic strategies yield more detailed profiles than existing techniques, giving poultry nutritionists clearer insights into feed enzyme activity and the degree of dietary fibre utilisation.

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REDUCING DIETARY NET ENERGY IN BROILERS UNDER THERMONEUTRAL AND HEAT STRESS CONDITIONS

C. XIE [1], E. KIM[1], S. MACELLINE[1], J. LI[1], M. TOGHYANI[1], R. BAREKATAIN[1] and S.Y. LIU[1]

Lowering dietary energy can reduce metabolic heat production, potentially improving broiler performance under heat stress. As the net energy (NE) system accounts for heat loss during metabolism, it offers a more accurate measure of usable energy. This trial evaluated whether low-NE diets enable broilers to sustain growth when subjected to heat stress.

This study design followed a 2 × 2 factorial arrangement with 2 levels of NE (Control vs. low) and exposure to heat stress (no or yes). Off-sex male Ross 308 birds were randomly allocated to one of four treatments in a floor pen facility (10 pens/treatment, 7 birds/pen). A common starter feed was fed from 0 to 13 days post hatch. The experimental basal diets were formulated based on wheat and soybean meal to meet Ross 308 nutrient specifications from 14 to 35 days post hatch. The 0.42 MJ/kg reduction in NE was achieved by diluting the diets through the inclusion of wheat bran and lowering crude protein by 10 g/kg. All diets were formulated to maintain a similar balance of digestible essential amino acids and minerals. From day 25 to 35, half of the birds were subjected to cyclic heat stress (33±1°C for 8 h from 0900 to 1700), while the non-heat stressed controls were maintained at 23°C. Humidity averaged 54% RH under heat stress and 81% in thermoneutral conditions.

There was no interactive effect of NE level and heat stress on broiler growth performance nor carcass measurements (Table 1).

Table 1 - Growth performance over the experimental period (14 to 35 days post-hatch).

Main effects	BW g/b		FI	FCR	BWc FCR	Carcass g/kg BW	
	D14	D35	g/b	g/g	g/g	*P. Major*	Fat pad
NE							
Control	501.6	2515	2629	1.307[a]	1.323[a]	151[b]	9.96
Low	502.3	2549	2619	1.280[b]	1.289[b]	168[a]	9.54
Heat Stress (HS)							
No	501.7	2604[a]	2690[a]	1.280[b]	1.278[b]	162	9.92
Yes	502.3	2460[b]	2557[b]	1.307[a]	1.334[a]	157	9.58
Source of variation *P value*							
NE	0.457	0.102	0.641	0.018	0.019	0.003	0.246
HS	0.514	<0.001	<0.001	0.017	<0.001	0.260	0.340
NE × HS	0.201	0.525	0.543	0.168	0.203	0.641	0.168

Means for main effects are based on pooled replications across the treatments.
[a-b] Means within a column not sharing a superscript differ significantly at the *P* level shown for the main effects.

Heat stress reduced feed intake ($P < 0.01$) and body weight (BW; $P < 0.01$), leading to a higher feed conversion ratio (FCR; P = 0.017) by day 35. When corrected to body weight, this difference in BWc FCR was amplified ($P < 0.01$). Heat stress did not affect any of the measured carcass yields ($P > 0.05$). Birds offered low NE diets presented lower FCR (P = 0.018) and higher breast muscle yield (P = 0.003). In summary, these results indicate that while heat stress compromised growth performance even with reduced NE diets. Dietary NE reduction alone did not compromise production and is a feasible way to reduce feed costs. Additionally, reduced NE diets enhanced muscle yields without increasing fat deposition, indicating potential economic benefits.

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USING DAILY DATA TO DETERMINE FACTORS INFLUENCING MARKET BODY WEIGHT

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Efficient production of poultry is a challenging task, subject to the influence of multiple factors. Amongst these factors is the control of temperature. In summer, temperatures can increase in the house causing heat stress. Inadequate temperature regulation during these challenging periods can cause severe losses (Lara and Rostagno, 2013; Wasti et al., 2020). Although this is a widely known phenomenon, estimates of the loss associated with uncontrolled temperatures in field conditions are scant. Hence, a retrospective analysis was conducted using daily production data from a European poultry company to identify the influential factors and specifically the impact of heat control associated with suboptimal end weights in broiler chickens.

Data was collected between 2018 and 2021 using *Aviapp*®, a software platform developed by Huvepharma®. In total results of 240 flocks, reared on 3 different farms (A: 97, B: 71, C: 72) and 13 different houses were available for the analysis. Information on water intake (ml/bird), daily temperatures (max, min), breed (3 types, mixed, A, B), hatchery, flock density, and feed mill were available. Numerical variables were averaged by week. Several derived variables were included as well, like the average deviation from the reference water intake, the weekly change in water intake, and the difference between minimum and maximum temperatures. A lasso regression was first applied to determine the most influential factors, followed by a multiple linear regression using the selected variables. All analysis were performed in R version 4.3.3.

Significant associations were found between the parent age, water intake in the 2nd week, the breed, stocking density, and maximum temperatures in the 5th week. The total variability explained by the final model (R^2) was equal to 0.60.

When the age of the parents increases with 1 week (parent age ranged from 24 weeks to 99 weeks), the end weight on average is 2.4 g/bird better (95% CI [0.65, 4.21]. In addition, a 1 mL/bird increase of water intake in week 2 increase the end weight by an average of 6.8 g (95% CI [4.70, 8.53]. No such correlations were observed for water intake during week 4. Increasing stocking density (range 16.5 – 22.2 birds/m²) was associated with a reduction in bodyweight of 44.3 g, 95% CI [24.3, 62.2]). The average maximum in-house temperature during week 5 was negatively correlated with the end weight (temperature range 23 - 34 °C). Each 1°C increase was associated with 16.6 g (95% CI [8.98, 24.3]) reduction in body weight. Installation of a cooling system in 2019/2020 led to a lower increase of temperatures in the house in the spring of 2020, resulting in a reduced difference in performance between seasons. In 2019 the average bodyweight in spring was significantly lower (142 g, 95% CI [18.1, 266.5] compared to the summer, whereas in 2020 the average difference was 51 g higher (95% CI [-86.0, 188.07].

This study highlights the multifactorial nature of broiler performance and provides quantitative insights to support data-driven optimisation of production practices.

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EFFECT OF AGE AND SEX ON INDIVIDUAL GROWTH PERFORMANCE IN FARM-RAISED COMMON PHEASANTS (PHIASIANUS COLCHICUS)

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Common pheasants (*Phasianus colchicus*) live in the wild and they are also raised on farms for their meat (Daszkiewicz and Janiszewski, 2020). A significant problem is the survival rate of pheasants bred on farms and then released into the wild. In natural conditions, pheasants only take flight when stressed or due to other natural causes, mainly moving around on foot. The mass of muscles involved in pheasant locomotion may be important for their survival after release. Age has a significant effect on carcass tissue composition in birds and knowledge in this area can be helpful in optimising the age of birds intended for restocking or the age of slaughter in the case of carcass production for consumption (Murawska, 2016).The aim of the study was to determine changes in the growth of tissue components and the distribution of muscle tissue in in common pheasants raised on a farm in an extensive system.

The experiment was performed on male and female pheasants kept on a farm in Lesy a rybniky mesta Ceske Budejovice s.r.o., Czech Republic. Between days 10 and 150 of age, at 10-day intervals, 16 birds (sex ratio 1:1, a total of 240 birds) were selected randomly from the flock and slaughtered, and their carcasses were dissected. A statistical analysis involved the determination of arithmetic means ($\overline{x}$) and standard deviations (±SD) for the traits analyzed in the study, and the significance of differences in mean values between the sexes and age groups. Data were subjected to crossed two-way analysis of variance. Significant differences were determined by Duncan's multiple range test at a significance level of $P < 0.05$.

Between 10 and 150 days of age, the body weight of males and females increased 20-fold and 19-fold, respectively ($P<0.01$). From 40 days of age until the end of rearing, males had a higher body weight than females ($P <0.01$). Muscle tissue growth was confirmed in ♂ up to 130 days of age and in ♀ up to 140 days of age ($P<0.01$). Leg muscle mass increased up to day 110 in birds of both sexes ($P< 0.01$), while breast muscle mass increased up to day 130 in males and day 120 in females ($P <0.01$). Analysis of the percentage of meat from individual carcass parts in the body weight of pheasants revealed numerous age-sex interactions. Between the 10th and 150th day of the birds' life, proportion of meat from pectoral and dorsal muscles in the total weight of this tissue component increased ($P <0.01$).The proportion of breast meat increased by 3.1 % in ♂ and by 8.6 % in ♀, and the proportion of meat from the dorsal region increased by 4.2 % in ♂ and by 1.6 % in ♀. The proportion of meat from the legs, wings, and neck decreased in both males and females ($P <0.01$), as follows: legs – by 5.1 % and 5.6%, respectively; wings - by 1.4 % and 2.1 %, respectively; neck - by 1.0 % and 1.6 %, respectively.

In conclusion, the leg muscles, as the main locomotive muscles in pheasants, finish growing faster than the breast muscles, which are more desirable in terms of the consumption of carcasses.

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25-HYDROXY VITAMIN D_3 FROM FERMENTATIVE ORIGIN IMPROVES BONE TRAITS IN LATE-STAGE LAYING HENS

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Summary

Laying hens of 61 weeks of age were supplemented with 25-hydroxy vitamin D_3 of fermentative origin to assess the impact on different bone traits. The supplementation was formulated into the diet by exchanging 50% of the original International Units (IU), coming from vitamin D_3, with the corresponding level of 25-hydroxy vitamin D_3. The reformulation was compared to standard basal diets without a 25-hydroxyvitamin D_3 source, but with the same total IU of vitamin D (3000 IU/kg feed). After 15 weeks of supplementation, bone parameters including bone volume, surface and density were significantly improved in the treatment group ($P < 0.05$).

I. INTRODUCTION

Vitamin D is well-known for its involvement in calcium metabolism and bone strength (Khan *et al*., 2021), but other bone parameters are often neglected in animal trials. At the same time, adapting the evaluated parameter to the specific animal species could help to better interpret the impact of a certain management intervention. The trial at hand is an example thereof: classic parameters like bone strength are important for late-stage hens, but other bone parameters such as volume and density might be more important in regards to bones as a prime calcium source for egg development - even though these other parameters are not directly linked to bone strength. Much research has focused on which vitamin D metabolite is preferred in poultry nutrition. Unfortunately, research comparing supplementation with a vitamin D_3 control at the same level of IU is limited, as the treatment is often added on-top instead. Thus, an experiment was conducted with the level of total IU per kg of final feed kept the same in both the control and the treatment group to better investigate the preferred vitamin D metabolite.

II. METHOD

On a commercial farm, a total of 24,862 Hy-Line Brownmax hens were included in the study and divided into two groups: a control, receiving a basal standard diet with a total vitamin D level of 3000 IU per kg of feed, and a treatment group, receiving the same basal standard diet with the same level of vitamin D IU but with 50% coming from 25-hydroxy vitamin D_3. A 25-hydroxy vitamin D_3 of fermentative origin was used (Bio D®, Huvepharma®). The final supplemented IU was calculated by converting every 1 µg of 25-hydroxy vitamin D_3 into 80 IU. Animals were housed in groups of eight in wire cages (90 cm x 46 cm x 38 cm) and supplemented with the respective diets for 15 weeks, starting when the animals were 61 weeks of age. Water and feed were provided *ad libitum* throughout the study. Three hens per cage were randomly sacrificed at 72 weeks of age, after which both femoral bones per animal were harvested and analysed using micro-CT scanning. Using SAS, data was statistically analysed using a generalized linear model.

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III. RESULTS

The bone volume, surface, surface density and connectivity density were significantly higher in the treatment group than in the control group (Table 1; $P < 0.05$). At the same time, total porosity decreased in the treatment group.

Table 1 - Effect of 25-hydroxyvitamin D_3 supplementation on bone micro-CT parameters of late-stage laying hens.

Parameters	Control	Treatment	SEM	*P* value
Bone volume, mm^3	264.60	332.90	18.96	0.044
Bone surface, mm^2	1371.85	1756.45	73.76	0.010
Bone surface density, 1/mm	1.70	2.22	0.08	0.005
Total porosity, %	67.06	57.63	3.11	0.076
Connectivity density, $1/mm^3$	0.54	1.87	0.31	0.025

IV. DISCUSSION & CONCLUSION

The results demonstrate the added value of supplementing late-stage laying hens with 25-hydroxy vitamin D_3, with a significant improvement in bone parameters. This is in line with previous work elaborating on the use of 25-hydroxy vitamin D_3 to increase bone volume and surface area (Chen *et al.,* 2020; White *et al.*, 2023). These parameters fit in with the impact of vitamin D on effective bone development and mineralization. Additionally, the decrease in total porosity and increase in connectivity density suggest increased mineral accumulation, indicating that the bones of hens receiving 25-hydroxy vitamin D_3 were denser and thus contained more minerals than those of the control group. As both bone strength and eggshell formation are linked to these parameters, these results underline the commercial importance of using the right vitamin D metabolite to fulfil animals' vitamin needs. Notably, all of the above was achieved without increasing the total level of supplemented IU. In other words: 25-hydroxy vitamin D_3 was more effective compared to vitamin D_3 to improve these parameters.

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NUTRIENT CONTENT OF RE-USED LITTER FROM A FREE-RANGE BROILER SYSTEM

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It is well established that broiler chickens consume litter (Malone et al., 1983; Eser et al., 2022). In Australia, using new bedding for every growth cycle in the meat chicken industry is common practice. However, some Australian broiler farms reuse litter, a practice that is common in many countries, including the USA, as it reduces the need for new bedding materials. The used litter is heaped into piles or windrows, where it generates heat via microbial activity, which kills pathogens and viruses (Abougabal, 2019). Accordingly, under suitable management practices, litter re-use presents a promising strategy for reducing the economic and environmental burdens associated with litter management in Australia. The litter retains nutrients excreted by the previous flock, which may be supplying additional nutrients to the subsequent flock housed on the re-used litter. However, the extent to which the composting process alters litter nutrient content remains unclear. Consequently, the aim of this experiment was to determine the nutrient content of re-used litter and compare it to new litter and litter at the end of a growth cycle (day 42), to evaluate the potential nutritional content of re-used litter and the impact of composting on its nutrient composition. Litter samples were collected from a commercial free-range broiler shed in Western Australia. Samples were collected from 10 locations in the shed, including near feeders and pop-holes, on the day of bird arrival (day 0) and at day 42. On day 42, the litter was removed, composted for 5 days, and sampled to represent 're-used' litter. Dry matter and pH were analysed in the day 0, day 42 and re-used samples immediately post-collection. Protein, energy, fibre, ash and mineral concentrations were analysed in triplicate in the samples. Univariate analysis, using IBM SPSS Statistics 30, was used to compare the nutrient content across the three samples, with differences considered significant at $P < 0.05$

Table 1 - Analysed nutrient content of litter samples collected from a commercial free range broiler farm on arrival (day 0) and at bird age day 42, and reused (composted) litter.

	Day 0	Day 42	Re-used	*P*-value
Dry Matter	88.44^{a}	66.97^{b}	64.18^{c}	<0.001
pH	5.87^{c}	8.23^{a}	7.38^{b}	<0.001
Energy (MJ/kg)	19.25^{a}	17.03^{b}	16.53^{c}	<0.001
Protein (%)	3.33^{c}	19.70^{b}	21.28^{a}	<0.001
Fibre	79.54^{a}	21.50^{b}	15.29^{b}	<0.001
Ash (%)	1.30^{b}	9.80^{a}	9.78^{a}	<0.001
Cu (ppm)	50.61^{c}	102.61^{b}	137.56^{a}	<0.001
Fe (ppm)	150.10^{b}	216.22^{b}	1004.24^{a}	<0.001
P (ppm)	12.42^{c}	434.87^{b}	1757.29^{a}	<0.001
Zn (ppm)	0.00^{c}	3091.81^{b}	34773.44^{a}	<0.001
Ca (ppm)	759.71^{c}	2534.36^{b}	5646.23^{a}	<0.001
K (ppm)	772.39^{c}	5047.31^{b}	8663.74^{a}	<0.001
Na (ppm)	507.69^{c}	1892.27^{b}	3929.23^{a}	<0.001

Table 1 illustrates that re-used litter exhibited higher protein and mineral content, but lower energy and fibre concentration, compared to new litter. Composting increased the protein and individual mineral content and decreased the pH, energy and dry matter concentration of the litter, but had no impact on fibre or total ash content. This suggests that housing new birds on re-used litter may provide some nutritional benefits. The broader potential effects of re-used litter on gastrointestinal health and microbiota require additional research.

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NUTRIENT CONTENT OF EGG-RELATED WASTE FROM THE BROILER INDUSTRY

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The broiler industry generates substantial quantities of egg-related waste. This waste is commonly discarded, posing environmental and biosecurity challenges. However, egg-related waste is rich in nutrients and bioactive compounds, presenting opportunities for recycling into value-added products. Despite this potential, there has been limited research in characterising the nutrient composition of egg-related waste from the broiler industry, hindering its utilisation. Consequently, in this study the nutrient composition of post-hatch eggshells, unhatched eggs (embryonic mortality prior to hatch) and unfertilised eggs (no viable embryo) from the broiler industry was analysed and compared, to provide foundational knowledge for potential strategies to valorise this waste. Three samples of each of the three different streams of waste were collected from a commercial hatchery in Western Australia. The dry matter, energy, protein, fat and ash content of the samples was analysed in triplicate per sample. Univariate analysis, using IBM SPSS Statistics 30, was used to compare the nutrient content across the three waste-streams, with differences considered significant at $P < 0.05$.

Table 1 - Analysed nutrient content of post-hatch eggshells, unhatched eggs and unfertilised eggs obtained from a commercial broiler hatchery.

	Post-Hatch Eggshells	Unhatched Eggs	Unfertilised Eggs	*P*-value
Dry Matter (%)	97.86 ± 0.08^{a}	34.94 ± 0.17^{b}	33.37 ± 2.07^{b}	<0.001
Energy (kcal/100g)	36.53 ± 2.62^{b}	389.54 ± 2.17^{a}	378.96 ± 8.34^{a}	<0.001
Protein (%)	8.86 ± 0.61^{c}	39.43 ± 0.61^{a}	31.13 ± 2.16^{b}	<0.001
Fat (%)	0.12 ± 0.03^{c}	25.76 ± 0.25^{b}	28.27 ± 0.96^{a}	<0.001
Ash (%)	87.69 ± 1.81^{a}	10.73 ± 0.40^{c}	16.91 ± 0.63^{b}	<0.001

Table 1 illustrates that unhatched and unfertilised eggs are rich sources of energy, protein and fat. Unhatched eggs had higher protein and lower fat and ash content, but similar energy and dry matter content, compared to unfertilised eggs. Post-hatch eggshells had a higher ash and dry matter content, and lower energy, protein and fat content, compared to the other products. Several pathways exist for the utilisation of this egg-related waste. The high mineral and protein content suggests potential as fertilisers or as a mineral supplement or source of high-quality protein and amino acids for animal and human feed, including poultry, at a time when alternative ingredients are attracting significant interest. However, further research is required to evaluate processing methods and economic feasibility, with particular attention to mitigating biosecurity risks from microbial contamination and disease transmission (i.e. *Salmonella*), while maintaining nutrient integrity. Other applications include using eggshell powder in bioplastics or as fillers in biodegradable composites (Gacem *et al*., 2025), as a low-cost adsorbent for heavy metals or catalyst in chemical processes, and as an additive in ceramics and cement to enhance strength (Iftikhar *et al*., 2024). Moreover, eggshell membrane has potential applications in the pharmaceutical and nutraceutical industries, because of its high protein content, including collagen, glycosaminoglycans and bioactive peptides (Kulshreshtha *et al*., 2022). Further research is needed to develop practical methods for removing membranes from eggshells. In conclusion, this preliminary analysis highlights the potential to convert a by-product from the broiler industry into a valuable resource.

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BLACK CUMIN SEED MEAL TO PARTIALLY REPLACE SOYBEAN MEAL AND ENHANCE SUSTAINABILITY OF BROILER PRODUCTION

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Summary

A 42-day feeding trial was conducted to evaluate the potential of black cumin seed (*Nigella sativa*) meal (BCSM), a by-product of black cumin seed oil extraction, as a partial replacement for soybean meal (SBM) in broiler diets. A total of 256 Ross 308 male chicks were randomly assigned to four dietary treatments: control, control + 2% black cumin seed (BCS), control + 2% BCSM, and control + 4% BCSM. These inclusion levels (2% and 4% of the total diet) equate to approximately a 10-20% replacement of SBM. Growth performance, feed efficiency, and meat quality were assessed. Inclusion of 2% or 4% BCSM maintained average daily gain, feed conversion ratio, and final body weight, with no adverse effects on meat quality traits, including colour (L*, a*, b*), cooking loss, drip loss, or shear force. However, breast muscle pH was modestly higher in the 2% BCS group, although the biological relevance appears to be limited, as other quality parameters remained unaffected. Improvements in average daily gain during the grower phase suggest more efficient nutrient utilisation at moderate BCSM inclusion levels. Birds fed 2% BCS also showed improved feed efficiency, consistent with the functional properties of seed bioactive compounds. Overall, the results indicate that BCSM can be safely included at up to 4% of the diet without compromising broiler performance or product quality. Its utilisation offers a sustainable strategy to reduce reliance on imported SBM while adding value to a locally available by-product that would otherwise be wasted in landfill.

I. INTRODUCTION

The Australian poultry industry is highly dependent on soybean meal (SBM) as the principal protein source in broiler diets. However, domestic soybean production is constrained by climatic and agronomic factors, necessitating the importation of more than one million tonnes annually, primarily from South America (Kandel et al., 2025). This reliance exposes producers to fluctuations in global supply and market prices, while the environmental footprint of soybean cultivation, including deforestation, land-use change, and greenhouse gas emissions, further challenges the sustainability of SBM as the primary protein source for livestock (Greenhalgh et al., 2020). To secure a more resilient and sustainable protein supply chain, interest has shifted toward evaluating novel, locally available protein sources and agricultural byproducts. Oilseed meals are particularly promising, as they are produced during oil extraction and often discarded. Black cumin seed (*Nigella sativa)* meal (BCSM), a by-product of oil production, contains residual protein, fibre, and bioactive compounds, making it a potential replacement for SBM (Seidavi et al., 2020). In Australia, BCSM is locally produced but remains largely underutilised, and its evaluation is therefore aligned with sustainability goals aimed at reducing waste and broadening protein sources in poultry nutrition. Its utilisation could reduce feed costs and divert waste streams into productive use. In addition, black cumin seed itself contains thymoquinone and other bioactive compounds with antimicrobial, antioxidant, and immunomodulatory properties (Navidshad et al., 2025). Previous studies have reported beneficial

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effects of dietary BCSM inclusion on broiler performance traits, along with enhanced antioxidant status, immune response, and meat quality, as well as reduced pathogenic bacterial populations (Fathi et al., 2023). While the focus of the present study was on BCSM as a sustainable protein ingredient, whole seed was also included at 2% to assess potential functional benefits. This trial evaluated the effects of partially replacing SBM with BCSM at 2% and 4%, alongside 2% whole seed, during the grower-finisher phase in Ross 308 broilers. It was hypothesized that inclusion of up to 4% BCSM would replace SBM without compromising growth, feed efficiency, and meat quality, supporting a more resilient and sustainable protein supply for Australian poultry.

II. METHOD

The experimental procedures were approved by the Animal Ethics Committee of The University of Queensland (Approval No: 2023/AE000309) and were conducted in accordance with the Australian Code for the Care and Use of Animals for Scientific Purposes. A 42-day feeding trial was conducted at the Queensland Animal Science Precinct (QASP), Gatton Campus, The University of Queensland. A total of 256 one-day-old male Ross 308 broiler chicks (Aviagen) were individually weighed and randomly allocated to 32 floor pens with eight birds per pen. Birds were reared under controlled environmental conditions and provided *ad libitum* access to feed and water throughout the experiment. Black cumin seed and meal were supplied by Hab Shifa (VIC 3803, Australia). The trial comprised four dietary treatments in a completely randomized design: (1) control diet (corn-wheat-SBM basal diet), (2) control + 2% black cumin seed (BCS), (3) control + 2% BCSM, and (4) control + 4% BCSM. These inclusion levels (2% and 4% of the total diet) equate to approximately a 10-20% replacement of dietary SBM. Each treatment was replicated eight times. Diets were formulated to be isocaloric and isonitrogenous, meeting or exceeding Ross 308 nutrient recommendations. A commercially formulated starter diet was fed from day 1 to 14, with experimental diets introduced from day 14 to 42. Growth performance was assessed per pen at the end of each phase (starter, grower, finisher) and cumulatively. Variables measured included body weight, average daily gain (ADG), average daily feed intake (ADFI), and feed conversion ratio (FCR). On day 42, one bird per pen was euthanised to collect carcass and breast muscle samples. Meat quality parameters (pH, colour, cooking loss, drip loss, shear force) were determined using standard analytical procedures (Bromfield et al., 2021). Data were analysed using the General Linear Model (GLM) procedure in SAS (SAS Institute Inc., Cary, NC). The pen was considered the experimental unit for performance, and individual birds were considered for meat quality.

III. RESULTS

The effects of dietary treatments on growth performance and feed efficiency are summarized in Table 1. From day 1 to 14 (pre-treatment period), no significant differences were observed among groups. During the grower phase (d 14-28), birds fed 2% or 4% BCSM exhibited higher ADG (83.4 and 82.4 g/d, respectively) compared with the control (79.5 g; $P = 0.04$). FCR also differed ($P = 0.03$), with the 2% black cumin seed (BCS) group showing numerically improved FCR (1.39) compared with the control (1.41). From day 28 to 42 and over the entire 42-day period, ADG, ADFI, and FCR were not significantly affected by treatments. The average final body weight at 42 d ranged from 2754 g (control) to 2819 g (4% BCSM), with no significant differences among groups.

Table 1 - Effects of dietary black cumin seed meal (BCSM) and black cumin seed (BCS) on the performance of broiler chickens during the different growth phases[1].

Items[2]	Treatments[3]				SEM[4]	*P*-value
	Control	2% BCSM	4% BCSM	2% BCS		
Average final body weight (g)	2754.56	2805.66	2818.57	2766.72	32.17	0.45
Starter (1-14 d)						
ADG, g/d	30.28	29.47	28.83	30.19	0.91	0.65
ADFI, g/d	37.55	37.79	36.94	37.65	0.67	0.81
FCR	1.25	1.28	1.28	1.25	0.03	0.69
Grower (14-28 d)						
ADG, g/d	79.46^{ab}	83.37^{a}	82.44^{ab}	79.05^{b}	1.25	0.04
ADFI, g/d	112.34	115.5	115.84	115.42	1.64	0.41
FCR	1.41^{ab}	1.39^{a}	1.41^{ab}	1.46^{b}	0.02	0.03
Finisher (28-42 d)						
ADG, g/d	99.57	100.35	102.96	101	1.28	0.30
ADFI, g/d	162.43	166.25	166.96	163.12	1.43	0.08
FCR	1.63	1.66	1.62	1.62	0.02	0.54
Overall (1-42 d)						
ADG, g/d	66.37	67.6	67.93	66.66	0.78	0.45
ADFI, g/d	104.11	106.51	105.78	105.4	1.00	0.4
FCR	1.57	1.58	1.56	1.58	0.01	0.57

[1]Values are means of eight replicates per treatment (8 birds per pen).
[2]ADG = average daily gain; ADFI = average daily feed intake; FCR = feed conversion ratio.
[3]BCSM = black cumin seed meal; BCS = black cumin seed.
[4]SEM = standard error of the mean.
Means within a row with different superscripts differ (P ≤ 0.05).

Carcass characteristics, including live weight, warm carcass weight, cold carcass weight, and breast muscle weight, were not significantly affected by dietary treatments (Table 2). Likewise, breast meat quality indices, including colour values (L*, a*, b*), cooking loss, drip loss, and shear force, showed no treatment-related differences. However, breast pH was influenced by diet (P = 0.04), with birds fed 2% BCS exhibiting a higher pH (5.71) compared to the control (5.48). The values for 2% and 4% BCSM (5.60 and 5.50, respectively) were intermediate.

Table 2 - Effects of dietary black cumin seed meal (BCSM) and black cumin seed (BCS) on carcass and breast meat quality of broilers at 42 d.

Items	Treatments[1]				SEM[2]	*P*-value
	Control	2% BCSM	4% BCSM	2% BCS		
Live weight, g	2818.00	2866.75	2880.75	2918.13	78.70	0.84
Warm carcass weight, g	2572.75	2618.88	2634.75	2650.63	74.54	0.89
Cold carcass weight, g	2198.25	2249.50	2272.00	2291.25	67.20	0.78
Breast weight, g	531.00	526.00	554.50	549.25	26.26	0.84
Breast pH	5.47^{b}	5.60^{ab}	5.50^{b}	5.71^{a}	0.06	0.04
Breast colour L*	47.22	45.96	46.64	46.24	0.77	0.68
Breast colour a*	1.50	1.91	1.82	1.65	0.17	0.39
Breast colour b*	3.54	3.35	3.57	3.18	0.18	0.40
Cooking loss, %	0.25	0.20	0.23	0.24	0.01	0.12
Shear force, N	22.86	20.89	20.49	23.57	1.61	0.48
Drip loss, %	10.89	8.88	19.43	10.47	1.01	0.53

[1]BCSM = black cumin seed meal; BCS = black cumin seed.
[2]SEM = standard error of the mean.
Means within a row with different superscripts differ significantly (P ≤ 0.05).

IV. DISCUSSION

The present study evaluated the potential of black cumin seed meal (BCSM), a by-product of oil extraction, as a partial replacement for soybean meal (SBM) in broiler diets. Results showed that inclusion of 2% or 4% BCSM maintained growth performance, feed efficiency, carcass yield, and meat quality, supporting its suitability as a sustainable protein ingredient. Our findings are consistent with previous reports, which show that moderate levels of BCSM do not impair growth performance or feed efficiency. Fathi et al. (2023) observed improvements in body weight gain and FCR at 40–60 g/kg BCSM, while Jahan et al. (2015) and El-Kashef (2020) also reported no adverse effects on performance when SBM was partially replaced by BCSM. Similarly, Zaazaa et al. (2023) demonstrated that BCSM enhanced antioxidant status and immune responses without compromising productivity. In our study, numeric improvements in ADG during the grower phase indicate that BCSM can support efficient nutrient utilisation when included up to 4%, confirming its potential as a practical SBM replacer in Australian conditions.

Carcass traits were unaffected by dietary treatments, aligning with earlier findings (Fathi et al., 2023) that BCSM inclusion does not alter dressing percentage or breast muscle yield. Most meat quality parameters, including colour values (L*, a*, b*), drip loss, cooking loss, and shear force, were also unaffected by BCSM or BCS inclusion. However, breast muscle pH showed a modest dietary effect, with slightly higher values in birds fed 2% BCS compared with controls. This result suggests a potential influence of seed-derived bioactive compounds on post-mortem muscle metabolism; however, the biological relevance appears limited, given that other quality traits remained unchanged. Overall, the data indicate that residual bioactive compounds in BCSM, while beneficial for antioxidant capacity (Kumar et al., 2017), do not adversely affect muscle deposition or meat quality at the inclusion levels tested.

In addition, the comparison with whole black cumin seeds highlighted their functional properties, notably improved feed efficiency at a 2% inclusion rate, consistent with reports that seed phytochemicals enhance gut health and immune function (Khan et al., 2012; Seidavi et al., 2020). Nonetheless, from a sustainability perspective, the meal represents greater practical value as it is a locally available by-product currently underutilised.

Overall, this study demonstrates that BCSM can replace up to 4% of SBM in broiler diets without impairing performance or meat quality. Its utilisation supports circular economy principles, reduces dependence on imported SBM, and provides a pathway toward more sustainable poultry production in Australia.

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BROILER PERFORMANCE ON LUPIN AND FABA BEAN-BASED STARTER DIETS

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To reduce dependence on soybean meal in broiler diets, alternative ingredients for broiler production need to be investigated. The present study investigated effect of lupin and faba bean inclusion in starter diets on broiler growth, nutrient utilisation and digestive tract development.

Five wheat-based diets using soybean meal (control diet) and lupin or faba bean at 100 or 200 g/kg inclusion were formulated based on standardised ileal digestible amino acid values to meet Aviagen recommendations (Aviagen, 2022). Lupin and faba bean were sourced from a commercial supplier in Australia. Diets were steam pelleted at 75°C. A total of 320, one-day-old male broilers (Ross 308), obtained from a commercial hatchery, were individually weighed and randomly allocated to 40 pens. Each pen had eight birds, and eight replicated pens per treatment. Birds were raised in floor pens until d-14 when they were moved to enriched cages to facilitate excreta collection. Fresh and clean water was readily available, and feed was provided *ad libitum* throughout the experiment period (1- 21 d).

Feed intake (FI) and body weight of birds were recorded on pen basis at weekly intervals. Feed conversion ratio (FCR) was corrected for mortality. On d 21, all birds were euthanised by intravenous injection of sodium pentobarbitone and ileal digesta was collected from the lower half of the ileum. Two birds from each cage, euthanised for ileal collection, were selected, weighed and used for measurements of digestive tract sections.

At 100 or 200 g/kg grain legume inclusion, weight gain of birds fed the soybean meal-based diet was not different ($P > 0.05$) to those fed faba bean, but was lower ($P < 0.05$) than birds fed lupin. Birds fed 100 or 200 g/kg faba bean had the lowest ($P < 0.05$) FI, while the soybean meal fed birds had the highest ($P < 0.05$) FCR.

Coefficient of apparent ileal digestibility (CAID) of nitrogen (N) for birds fed soybean meal was lower ($P < 0.05$) than those fed grain legumes, apart from the 100 g/kg faba bean fed birds that had CAID of N not different to birds fed soybean meal. The CAID of starch in soybean meal fed birds was not different to those fed faba bean, but lower ($P < 0.05$) than lupin fed birds. Grain legume fed birds had CAID of fat not different to soybean meal fed, except for birds fed 200 g/kg faba bean that had greater fat digestibility. Except for birds fed a diet containing 100 g/kg faba bean, grain legume fed birds had greater ($P < 0.05$) relative empty weight of gizzard and proventriculus.

The inclusion of grain legumes enhanced nutrient utilization which could be partly due to improved gizzard and proventriculus development. In general, the study showed that inclusion of lupin or faba bean up to 200 g/kg in broiler diets supports nutrient utilisation and good growth performance

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IMPACT OF GRADED LEVELS OF PALM KERNEL MEAL ON GROWTH PERFORMANCE AND NUTRIENT DIGESTIBILITY IN ROSS 308 BROILERS

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The rising volatility in the prices of maize and soybean meal, the primary energy and protein sources in poultry diets have intensified the search for cost-effective, locally available feed alternatives. Among such alternatives, palm kernel meal (PKM), a by-product of the palm oil industry, has gained attention due to its wide availability and moderate crude protein content (Azizi et al., 2021) yet its use in broiler diets remains limited. Thus, this study aims to evaluate the effects of graded levels of PKM on growth performance, nutrient digestibility, and organ weight in broilers.

A total of 810 a day-old Ross 308 chicks (BW: 44.59 ± 0.42 g) were randomly assigned to 3 dietary treatments: 0%, 4%, or 8% PKM, with 15 replicates of 18 birds/pen. Diets were formulated to meet NRC (1994) requirements and offered in three phases: Growth performance [body weight gain (BWG), average daily feed intake (ADFI), and feed conversion ratio (FCR)] nutrient digestibility, and visceral organ weights were measured at the end of day 35. Data were analyzed using the GLM procedure in SAS and orthogonal polynomial contrasts were tested. $P < 0.05$ was set as significant and $0.05 < P < 0.10$ was set as tendency

Table 1 - Effect of PKM on growth performance and nutrient digestibility in broilers on day 35.

Items	PKM			SEM[1]	P -Value[2]	
	0 %	4 %	8 %		Linear	Quadratic
Growth performance						
BW, g	2006	1978	1934	29	0.064	0.805
ADG, g	56.0	55.2	53.9	0.8	0.068	0.755
BWG, g	1959	1930	1886	29	0.065	0.801
FI, g	2835	2817	2783	38	0.327	0.865
FCR	1.450	1.458	1.479	0.014	0.189	0.723
Nutrient digestibility (%),						
Dry matter	71.44	70.71	69.75	1.45	0.340	0.937
Nitrogen	69.41	68.87	67.59	1.16	0.219	0.764
Energy	70.28	69.50	68.53	1.29	0.313	0.945

[1] Standard error of means; [2] $P < 0.10$ was considered as tendency.

Increasing inclusion levels of PKM in broiler diets showed a tendency ($P < 0.10$) to reduce BW, ADG, and BWG on day 35. However, FI and FCR were not affected, suggesting that feed efficiency remained stable even with PKM inclusion. The slight reduction in growth performance may be attributed to the higher fiber content and lower amino acid digestibility of PKM compared to conventional protein sources. Nonetheless, nutrient digestibility and organ weights were unaffected, indicating that PKM inclusion did not compromise nutrient absorption or physiological development. Our finding supports the use of PKM as sustainable alternative protein source, particularly in regions where SBM are expensive or limited.

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DIGESTIBILITY STUDY OF BLACK SOLDIER FLY LARVAE RELATIVE TO SOYBEAN MEAL SHOWED POSITIVE RESULTS IN PULLETS

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Soybean meal is a widely used source of protein owing to its balanced essential amino acid profile (Banaszkiewicz, 2011). However, due to the semi-arid climate of Australia, soybean cultivation in the country remains minimal, and the demand for soybean is fulfilled via imports from Argentina. However, this also gives rise to further environmental implications, including transport-related carbon emissions and the impacts of land-use change for soybean cultivation (Copley et al., 2023). This study explores the use of feed protein sources using soybean meal in pullets reared for egg production. The focus was in commonly cultivated Australian crops such as canola and lupins, which possess protein levels comparable to soybeans, as well as black soldier fly larvae (BSFL), an ingredient that can be sustainably produced through the recycling of food waste. It was hypothesized that sustainable protein sources such as BSFL could exhibit similar nutrient utilization efficiency in pullets compared to conventional soybean feeds.

A digestibility trial involving 96 Hy-line brown pullets (15 weeks) was conducted. The birds were allocated into four independent dietary treatment groups, differing in the main protein sources: soybean meal (control), canola meal, lupin meal or black soldier fly larvae meal. The trial was conducted in 2 phases; the first phase was the substitution phase (conducted for 1 week), with a focus on apparent metabolizable energy, where the pullets were fed with a formulation consisting of 70% basal feed formulation with a substitution of 30% of the various protein ingredients. The second phase was the experimental diet phase (conducted for 3 days) where the birds were fed semi-purified diets containing each separate alternative protein ingredient with the inclusion of celite as an indigestibility marker, required to accurately estimate the apparent ileal nutrient digestibility. The, total tract apparent digestibility, proximate amino acid and proximate crude protein levels of the pullets were also planned to be analyzed.

Table 1: Dry matter digestibility and performance parameters.

Parameter	Dietary Treatment				SEM	P-value
	SBM	CM	LM	BSFL		
ADFI (g/d)	75.17^{b}	56.50^{c}	98.64^{a}	65.42^{bc}	3.45	0.0001
ADG (g/d)	12.22^{b}	-8.61^{c}	21.39^{a}	7.78^{b}	2.56	0.0001
FCR	6.15^{ab}	-6.57^{c}	4.61^{b}	8.41^{a}	1.70	0.0001
DMD1 (%)	72.46^{a}	64.73^{b}	63.29^{b}	71.04^{a}	0.80	<0.001
DMD2 (%)	74.93^{a}	60.82^{b}	66.60^{ab}	76.85^{a}	4.48	0.008

This could indicate that BSFL could be a potential alternative protein source to replace soybean meal due to the similar level of dry matter digestibility. However, further analysis should be conducted to elucidate the specific level digestibility of crude protein and amino acid between each of the protein sources.

In conclusion, BSFL can partially replace soybean meal without impairing pullet performance, as BSFL was able to maintain dry matter digestibility in pullets at a comparable level to soybean meal without compromising the health of the pullets. However, the BSFL availability of supply and cost warrant further investigation.

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YEAST-DERIVED COMPONENTS REDUCE SALMONELLA IN VACCINATED AND UNVACCINATED LAYING HENS

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To reduce the potential of foodborne illness occurrence, feed additives, in egg layer diets, that can reduce the prevalence and load of *Salmonella* have become widely utilized in *Salmonella* control programs. Hydrolyzed yeast and yeast culture, derived from *Saccharomyces cerevisiae,* contain mannan oligosaccharides that bind to pathogens, such as *Salmonella* Enteritidis, and can be an effective feed additive in a *Salmonella* control program, along with biosecurity, sanitation, and vaccination.

These research trials were conducted to evaluate the effect of dietary hydrolyzed yeast + yeast culture from *Saccharomyces cerevisiae* (HY/YC; Cascade supplied by Natural Biologics, Inc., Newfield, New York) on *Salmonella* prevalence and load in vaccinated (Trial 1) and unvaccinated (Trial 2) egg layers. Forty-eight, 25 weeks-of-age laying hens were allotted to two treatments with 12 replicate cages per treatment and two hens per cage in Trial 1. All hens were orally vaccinated with a live, attenuated *Salmonella* vaccine (*Salmonella* Typhimurium, *Salmonella* Enteritidis, *Salmonella* Heidelberg) at day of hatch and eight weeks of age. The treatments were: Challenged control and Challenged HY/YC (100 g/ton). Hens were fed a corn-soybean meal mash diet formulated to meet their nutrient requirements. Following a four-week adaptation period, layers were orally challenged with 1 mL of inoculum at 10^9 CFU/mL of nalidixic acid resistant *Salmonella* Enteritidis. At seven days post-challenge (30 weeks-of-age), all layers were sacrificed, and ceca were collected for determination of *Salmonella* Enteritidis prevalence and load (via BAX® System SalQuant® Real-Time PCR Assay. In Trial 2, 54 50-weeks-of-age laying hens were allotted to three treatments with nine replicate cages per treatment and two hens per cage. The treatments were: Non-challenged control, Challenged control, and Challenged HY/YC (100 g/ton). The methods were the same as in Trial 1 except the challenge dose was $2x10^9$ CFU/mL of nalidixic acid resistant *Salmonella* Enteritidis, the layers were not vaccinated for *Salmonella,* and samples were plated on XLT4 agar to determine *Salmonella* load .

In Trial 1 (vaccinated layers), seven days post infection, *Salmonella* prevalence was 95.8% for the Challenged control layers and 91.7% for the Challenged HY/YC layers. Cecal *Salmonella* load was numerically lower ($P > 0.05$) for Challenged HY/YC layers (10.5 CFU/g and 0.170 log CFU/g) than for Challenged control layers (94.8 CFU/g and 0.441 log CFU/g; Table 1). In Trial 2 (unvaccinated layers), *Salmonella* prevalence was 0, 55.56, and 33.33% for Non-challenged control, Challenged control, and Challenged HY/YC layers, respectively. *Salmonella* load was reduced by 1 log when layers were fed HY/YC (2.615 log CFU/g HY/YC vs. 1.594 log CFU/g Challenged control; $P = 0.122$; Table 1).

Both *Salmonella* vaccinated and unvaccinated laying hens had numerically lower *Salmonella* prevalence and load when they were fed HY/YC compared to Control fed layers. Thus, including a feed additive to reduce *Salmonella* in layers can further reduce *Salmonella* prevalence and load, than a vaccine alone. The HY/YC can be utilized as part of a *Salmonella* prevention program to reduce the incidence of *Salmonella* contamination and foodborne illness.

Table 1 - The effect of yeast-derived components on *Salmonella* load of layers under a *Salmonella* Enteritidis challenge.

	Trial 1, log CFU/g	Trial 2, log CFU/g
Challenged control	0.441	2.615
Challenged HY/YC	0.170	1.594
P-value	0.667	0.122

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ENHANCED CONTROL OF *SALMONELLA* AND *ESCHERICHIA COLI* BY PARAFORMIC ACID COMPARED TO FORMIC ACID AND FORMALDEHYDE: IN VITRO AND FEED CONTAMINATION STUDIES

M. LEJA[1] and Y.F. ZHAI[2]

Summary

This study evaluated the antimicrobial potential of paraformic acid compared to formic acid and formaldehyde, through *in-vitro* and feed contamination trials. Study 1(*in-vitro*: Minimum Inhibitory Concentration (MIC)) compared paraformic acid liquid (70%), formic acid (94%) and formaldehyde (40%) in the control of poultry-derived strains of *E. coli* and *Salmonella*. Formaldehyde treatment showed the lowest MIC across all strains while paraformic acid showed lower MIC compared to formic acid for three *Salmonella* serovars (Newport, Enteritidis, Infantis: MIC 0.2% vs 0.8%), paraformic acid achieved the same MIC level (0.2%) as formic acid for the remaining *E. coli* and *Salmonella* serovars. Study 2 (in-feed contamination model) evaluated two types of paraformic acid at different rates of inclusion (paraformic acid liquid at 0.1% and 0.2%; paraformic acid powder at 0.15% and 0.3%). 0.2% paraformic acid liquid consistently achieved the highest microbial reduction, lowering the *E. coli* counts from 4.3 x10^3 CFU/g to 9.5 x10^2 CFU/g at 6 h and 4.5 x 10^2 CFU/g at 12 h ($p < 0.05$ and *Salmonella* counts from 1.32 x 10^5 CFU/g to 4.22 x 10^4 CFU/g at 6 h, and 1.59 x 10^4 CFU/g at 12 h ($p < 0.05$). Paraformic acid powder (0.15%, 0.3%) showed inconsistent or minimal net reduction compared to control at 12 h, indicating lower efficacy than the liquid form.

I. INTRODUCTION

Ensuring the microbiological safety of animal feed is critical not only for livestock health but also for the safety of meat, milk, and eggs entering the human food chain (Davis et al., 2003). Feed ingredients may harbor chemical contaminants for example mycotoxins as well as bacterial pathogens capable of colonizing food-producing animals such as cattle, poultry, swine, and turkeys, which serve as reservoirs for pathogenic organisms including *Campylobacter spp*., *non-Typhi Salmonella enterica*, *Shiga toxin–producing E. coli*, and *Listeria monocytogenes* (Wang et al., 2023).

Among these, *Salmonella* remains a leading cause of foodborne disease worldwide, responsible each year for an estimated 150 million illnesses and 60000 deaths (Jung et al., 2022). In chickens and other avian species, both horizontal and vertical transmission can occur without overt clinical signs, increasing the risk of contaminated meat and eggs entering the human food chain (Antunes et al., 2016).

Diarrhea caused by *E. coli* poses a significant challenge in animal production, especially among newborn animals. Infections in the digestive tract can lead to stunted growth, higher mortality rates, decreased milk production, and weakened immune responses in both livestock and poultry (Barros et al., 2023; Kim et al., 2022; Wang et al., 2024).

Both formic acid and formaldehyde have been used in animal feed to prevent *Salmonella* spp. and *Escherichia coli* contamination. However, formaldehyde's irritancy and associated human-health hazards led to regulatory bans in several regions, notably in the European Union since August 2018. While formic acid confers both acidification and antimicrobial benefits, its inherent corrosivity and pungency create occupational safety and equipment-integrity concerns in feed mills. Paraformic acid contributes similar acidifying and antimicrobial properties with

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substantially reduced corrosivity and irritancy, making it more amenable to routine, large-scale application in feed mills (Zhong et al., 2023; Zhou et al., 2025). This study evaluates the ability of paraformic acid, formic acid and formaldehyde to reduce *E. coli* and *Salmonella* strains both *in vitro* and in an in-feed contamination trial.

II. METHODS

Study 1(*in-vitro*: minimum inhibitory concentration (MIC)), the (MIC assay was performed following the method ISO 20776-1:2019 via broth microdilution in Mueller-Hinton II at 37 ℃ with no visible growth at 24 h as the endpoint. Three antimicrobial agents were evaluated: 1) paraformic acid liquid 70% (trade name: Megacid-FL, Provided by Numega Nutrition Pte. Ltd, Singapore), 2) formic acid (94 %) and 3) formaldehyde (40 %). Study 2 (in feed contamination model) Mashed poultry feed was contaminated with 10^4 CFU/g *E. coli* and 10^5 CFU/g *Salmonella*) as control (CON). 0.1% and 0.2% paraformic acid liquid, and 0.15% and 0.3% paraformic acid powder were added to CON to form four additional treatments. Feed samples from each treatment were used to perform the *E. coli* and *Salmonella* count via the horizontal method for the enumeration of microorganisms (ISO 4833-1:2013) at 6 or 12 h post-treatment under 25℃ and 65 % RH in a PN-EN ISO/IEC 17025:2018 accredited facility.

Data were analyzed by one-way ANOVA (SPSS 25.0; $p < 0.05$). Significant differences are indicated by letters (a, b, c); same letters denote no significant difference.

III. RESULTS

Paraformic acid liquid exhibited stable antimicrobial efficacy comparable to formic acids against various strains of *E. coli* and *Salmonella* isolated from commercial poultry, with formaldehyde producing the lowest MIC among treatments (Table 1). Interestingly, paraformic acid liquid had higher antibacterial effects compared to formic acid against *Salmonella* Newport, *Salmonella* Enteritidis, and *Salmonella* Infantis, with distinctly lower MIC values (0.2 % vs. 0.8 % respectively).

Table 1 - MIC of paraformic acid versus formic acid and formaldehyde *in-vitro* (Study 1).

Strain	Evaluated sources		
	Formic acid	Formaldehyde	Paraformic acid (liquid)
E. coli 1578 (Turkey)	0.2 %	<0.1 %	0.2 %
E. coli 1546 (Broiler)	0.2 %	<0.1 %	0.2 %
E. coli 1559 (Broiler breeder)	0.2 %	<0.1 %	<0.1 %
E. coli 1562 (Commercial layer)	0.2 %	<0.1 %	0.2 %
E. coli 1542 (Broiler breeder)	0.4 %	<0.1 %	0.2 %
Salmonella Newport	0.8 %	<0.1 %	0.2 %
Salmonella Enteritidis	0.8 %	<0.1 %	0.2 %
Salmonella Infantis	0.8 %	<0.1 %	0.2 %
Salmonella Senftenberg	0.2 %	<0.1 %	0.2 %
Salmonella Coeln	0.2 %	<0.1 %	0.2 %

In the in-feed contamination study, 0.2% paraformic acid liquid demonstrated a significant antimicrobial effect. *E. coli* counts decreased from initial 4.3 x 10^3 CFU/g to 9.5 x10^2 CFU/g at 6 h, and to 4.5 x 10^2 CFU/g at 12 h ($p < 0.05$). *Salmonella* counts were reduced from initial 1.32 x 10^5 CFU/g to 4.22 x 10^4 CFU/g at 6 h, and to 1.59 x 10^4 CFU/g at 12 h ($p < 0.05$). In contrast, 0.1% paraformic acid liquid and paraformic acid powder (0.15% and 0.3%)

exhibited only inconsistent or marginal net reductions versus controls at 12 h (Figure 1) for both *E. coli* and *Salmonella*.

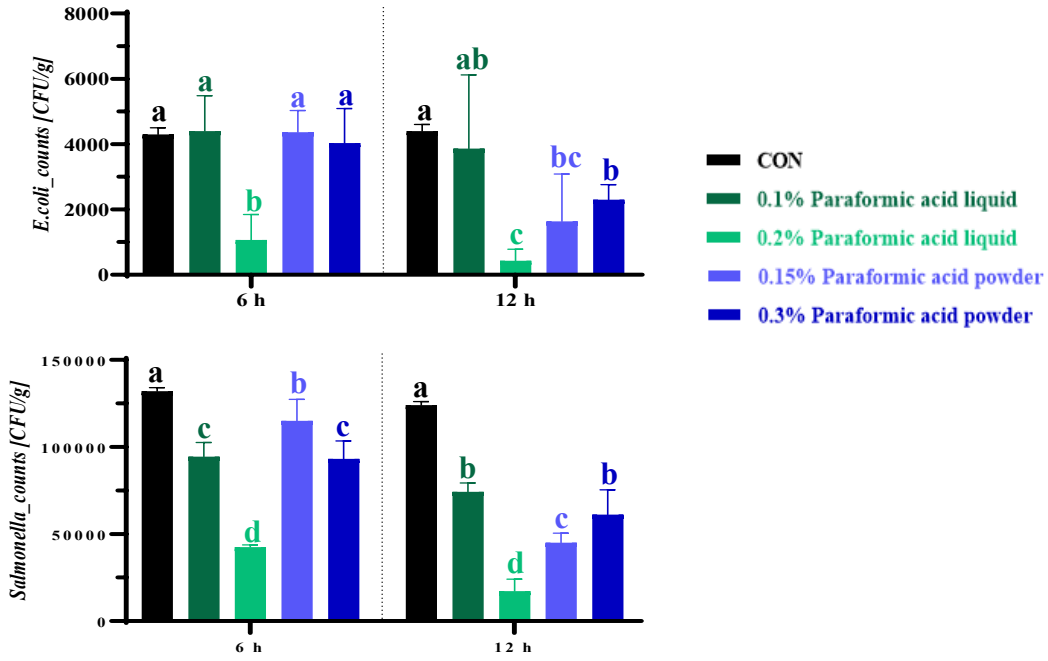

Figure 1 - Suppression efficacy of liquid and powder paraformic acid on *E. coli* and *Salmonella* in contaminated feed at 6 and 12 h post treatment (Study 2).

IV. DISCUSSION

Organic acids have been proposed as one of the potential alternatives to replace antibiotics as growth promoter (Dittoe et al., 2018; Huyghebaert et al., 2010). Formic acid was a popular organic acid due to its strong antimicrobial properties. Gómez et al. (2019) evaluated the antimicrobial activity of six different organic acids (formic acid, propionic acid, sodium butyrate, sodium heptanoate, sodium salt of coconut fatty acid distillates), six essential oils (cinnamaldehyde, thymol, carvacrol, clove essential oil (EO), oregano EO and black pepper EO) and formaldehyde against *E. coli*, *C. perfringens* and *Salmonella spp.* in swine feed, finding that formic acid exerts the strongest MIC against *E. coli* and *Salmonella*, while sodium salt of coconut fatty acid was most effective against *C. perfringens*. The outer membrane of Gram-negative bacteria contains protein channels within its lipid bilayer that permit only low-molecular-weight compounds to diffuse into the cytoplasm (Galdiero et al., 2012). Interestingly, formic acid, chemically known for being the simplest carboxylic acid with low molecular weight (Ricke et al., 2020) has the capacity to easily diffuse through the outer membrane and disrupt the growth and survivance of gram-negative bacteria such as *E. coli* and *Salmonella.*

Although the formaldehyde treatment was more effective than formic acid and paraformic acid in controlling the *E. coli* and *Salmonella* in the MIC study, formaldehyde exhibits significant toxic effects, including reproductive failure and carcinogenicity effects. Farm workers who handle feed treated with formaldehyde as a preservative, may be exposed to it primarily through inhalation, and potentially through contact with their skin and eyes (Mantovani et al., 2022).

Paraformic acid, a new formic acid derivative, is a dimer formed from two formic acid molecules and obtained through a polymerization process (Li et al., 2022). Based on the results from study 1, 0.2% paraformic acid liquid shows lower MIC in specific *Salmonella* serovars

compared to formic acid. Furthermore, 0.2% paraformic acid achieved statistically significant reductions in both E. *coli* and *Salmonella* within 12 h in contamination feed.

Direct comparisons between study 1 and study 2 were not made as their designs and agents differ, but study 1 generated dose-ranging data that informed dose selection for the paraformic acid in study 2. The findings highlight paraformic acid, as a promising feed hygiene solution with potential applications in reducing microbial contamination and improving food safety in poultry production systems.

V. CONCLUSION

In study 1 (*in vitro*), 0.2% paraformic acid liquid exhibited lower MIC than formic acid for specific *Salmonella* serovars, while formaldehyde showed lowest MIC overall. In study 2 (in feed contamination model), 0.2% paraformic acid liquid produced significant reduction in both poultry-derived strains of *E. coli* and *Salmonella* at 12 h after treatment.

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DO WE KNOW THE TRUE GROWTH POTENTIAL OF MODERN BROILERS?

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The growth potential of broiler chicken has increased tremendously over the past few decades and continues to do so today. Studies have shown that genetic selection contributes around 85-90% of the improvement in growth performance, while nutritional changes make up the remaining (Havenstein et al., 2003). This genetic progress is illustrated by the fact that a strain of chicken unselected since 1957 reaches a body weight (BW) of 905 g by day 56, while today's broilers typically attain a BW of 4714 g at the same age, an increase of 420% (Aviagen, 2022).

Renema et al. (2007) described the improvement in growth potential of male broilers (Hubbard) from 1972 to 2005 using the equation $y = 0.0372x + 1.3637$ (R^2=0.968), where x denotes the number of years since 1972, and y the day 42 BW. Extrapolating to the year 2025, the model projects a day 42 BW of 3335 g. Broilers currently attain a slaughter BW (2300 g) by day 33-35, however, at the current rate of genetic improvement, by the year 2034, broilers are predicted to reach a slaughter BW before 29 days of age (Tavárez & Solis de los Santos, 2016). On the other hand, Tallentire et al. (2018) stated that genetic improvement may be reaching its limit, and increasing growth rates to their apparent biological limit will reduce the time taken for birds to reach their slaughter weight by only 1.2 days from the current 34-35 days.

We have recently completed a trial as part of a master's thesis project at Massey University, New Zealand, where the growth rates establish a new benchmark for the growth potential of modern broilers, with performance far surpassing current records. Broilers (Ross 308) in our study achieved a final (day 35) BW of 3302 g, with an FCR of only 1.226. This translates to a 35% increase in BW and a 20% reduction in BW-corrected FCR compared to Aviagen's (2022) performance objectives. The birds reached their final body weight more than 7 days ahead of the breeder target. To our knowledge, it is for the first time that performance that exceeds the breed standard by this magnitude has been reported, raising questions about the true growth potential of modern broilers.

It is certain that progress made through selection will eventually slow down, and growth performance will reach its biological limit. As an example, if genetic selection were to continue improving growth at the rate previously observed, by the year 2078, birds will reach their slaughter BW on the day of hatch (Korver, 2023). Although our results show that genetic improvement is progressing at a faster rate than expected, they also indicate that at such a rapid rate of genetic progress, the biological limit of birds may be reached sooner than anticipated. This raises an important question: where will the broiler industry turn once the primary driver of its progress reaches its limit? Ultimately, once genetic improvement for growth reaches its biological limit, the broiler industry will no longer continue to progress at the remarkable rate observed over the past decades.

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PREDICTING BROILER GROWTH PERFORMANCE THROUGH MACHINE LEARNING ANALYSIS OF BLOOD BIOMARKERS RELATED TO HEAT STRESS AND METABOLIC IMBALANCE

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Summary

A machine learning–based growth prediction model integrating blood biomarker data was used to evaluate broiler performance and support precision management strategies. In this study, blood biomarker analysis conducted in June 2024 revealed low bicarbonate (HCO_3^-, 17–20 mmol/L) and total carbon dioxide (TCO_2, 15–20 mmol/L) levels, indicating heat stress and associated acid–base imbalance. Concurrently, elevated chloride (110–115 mmol/L) and anion gap (iGap, 18–20 mmol/L) values suggested metabolic disturbances that may impair nutrient utilization and growth. Growth predictions during this period were reduced, consistent with the observed physiological imbalance. Following recommendations generated by the machine learning system, flock management and nutrition were adjusted by reducing stocking density, increasing ventilation and limiting chloride in drinking water. A second sampling in October 2024 showed improved biomarker profiles and higher predicted growth performance, which were consistent with the actual growth results. These findings demonstrate that machine learning–driven decision tools enable timely interventions that optimize broiler health, growth, and overall productivity.

I. INTRODUCTION

Broiler production in tropical regions faces unique challenges due to the constant exposure of birds to high ambient temperatures and humidity. Climate stress, particularly heat stress, is widely recognized as one of the most significant environmental factors limiting growth performance and welfare in broilers (Soleimani et al., 2011). Under such conditions, the physiological ability of chickens to maintain thermal and metabolic homeostasis is compromised, leading to impaired feed intake, reduced nutrient utilization, and increased susceptibility to disease (Quinteiro-Filho et al., 2010). Blood biomarker analysis has emerged as a valuable diagnostic tool for assessing the physiological consequences of heat stress. Alterations in bicarbonate (HCO_3^-) and carbon dioxide (CO_2) levels provide evidence of acid–base imbalance, which is a hallmark of respiratory alkalosis in heat-stressed broilers. These changes are often accompanied by disruptions in electrolyte balance, including elevated chloride concentrations and altered anion gap (iGap) values, further exacerbating metabolic instability and reducing growth efficiency (Borges et al., 2014). In the present study, blood biomarker profiling was combined with a machine learning–based growth prediction model to evaluate the impact of environmental stressors on broiler performance. This study was conducted and presented as an initial case assessment rather than a replicated, controlled experiment.

II. METHOD

Commercial broiler (Ross 308) flocks (n = 50) from Suphanburi province, Thailand were monitored under standard production conditions, with blood samples collected at 2, 6, 9, 16, 20, 28, and 37 days of age during two samplings on 20th June (flock was raised during 17th May – 20th June) and 4th October (flock was raised 31st September – 4th October)2024. Suphanburi situated in central Thailand, experiences a hot and humid rainy season from May to October, with constantly temperatures ranging from 32 to 35 °C and relative humidity of 75–85 %. On-site blood analysis of 21 biomarkers including glucose, sodium (Na^+), chloride (Cl^-), potassium (K^+), pH, TCO_2, HCO_3^-, ionized calcium, total calcium (Ca), hematocrit, hemoglobin, total protein, uric acid, aspartate

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aminotransferase (AST), creatine kinase (CK), albumin, globulin, iGap and total carotene, were performed using VetScan VS2 (Avian/Reptilian profile rotor), i-Stat Alinity (Chem8+ profile cartridge), and i-Check carotenoid (whole blood carotenoid buffer) devices. Results were uploaded to the Verax™ cloud-based digital analysis platform for automated benchmarking, scoring, and growth prediction modeling. The growth prediction model, built from the Verax™ Commercial Broiler Database (reference range, DB) (≈10,000 farms worldwide), uses supervised machine learning to create a regressor model that associates body weight, gender, and genetics with blood biomarker profiles. The biomarker reference ranges, or DB were developed using only healthy birds and as such can be used to detect derangements associated with sub-optimal nutrition, health or environmental management. The shaded area of the reference range represents the normal variability observed in healthy birds, typically defined as the standard deviation (SD) around the mean biomarker value. Birds with biomarker levels within this range are considered to have normal physiological status, while values outside the shaded area indicate potential deviations related to stress, health, or management factors. Deviations from optimal biomarker reference ranges result in lower predicted growth scores, which provided color-coded risk scores and management recommendations. Following machine learning recommendations, flock management adjustments (reduced stocking density, improved ventilation and water chloride limitation) were applied in the subsequent flock cycle, and biomarkers were reassessed in October. Additionally, 41 necropsy evaluations covering skeletal, hepatic, renal, respiratory, and gastrointestinal systems were conducted. Correlations between biomarkers and pathology were assessed via the digital analysis platform.

III. RESULTS

Blood biomarker data and production records were analyzed using an digital analysis platform, which applies machine learning algorithms to benchmark biomarker trends and generate predictive growth scores. Key deviations were observed in June 2024, with TCO_2 and HCO_3^- below reference ranges,DB (17–20 mmol/L and 15–20 mmol/L in respectively), indicating putative hyperventilation and associated acid–base imbalance. Further, chloride and iGap were elevated (110–115 and 18–20 mmol/L in respectively) relative to the benchmarking database, suggesting metabolic derangement. By October 2024, all four biomarkers had closer result to database (TCO_2 24–25 mmol/L, HCO_3^- 22–25 mmol/L, chloride 110–112 mmol/L and iGap 17–19 mmol/L), reflecting improved physiological stability (Figures 1–4). These changes were consistent with enhanced flock health and supported by the machine learning–predicted growth score, which improved from low (red/yellow) in June to high (green) in October (Figure 5). The predicted growth score aligned with actual performance trends, showing that in June the flock grew below the ROSS 308 standard (Aviagen, 2022), whereas by October, actual performance slightly surpassed the breed benchmark (Figure 6).

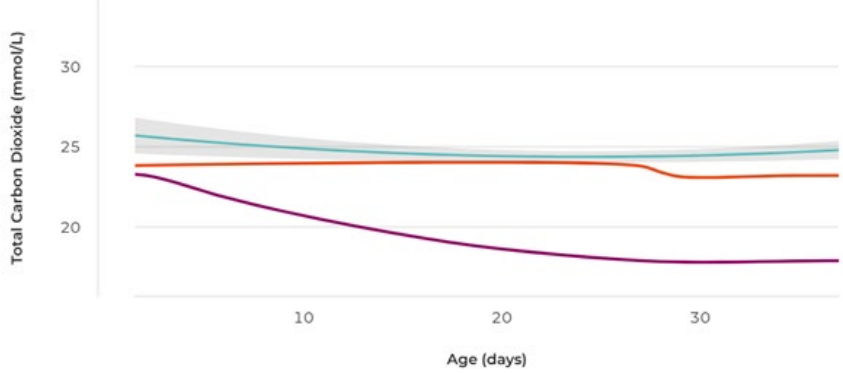

Figure 1 - Blood TCO_2 level in bird age 2, 6, 9, 16, 20, 28 and 37 days compared with database (DB). DB, June result and October result.

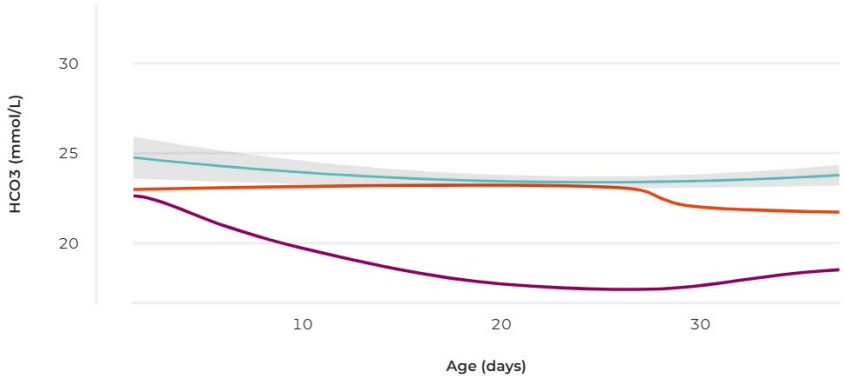

Figure 2 - Blood HCO_3^- level in bird age 2, 6, 9, 16, 20, 28 and 37 days compared with database (DB). DB, June result and October result.

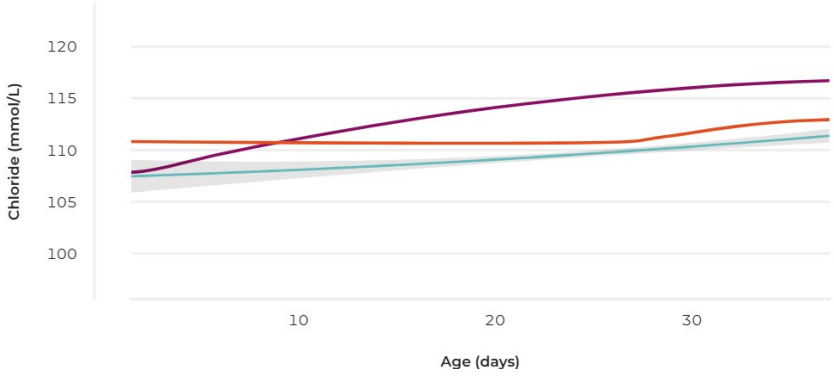

Figure 3 - Blood chloride level in bird age 2, 6, 9, 16, 20, 28 and 37 days compared with database (DB). DB, June result and October result.

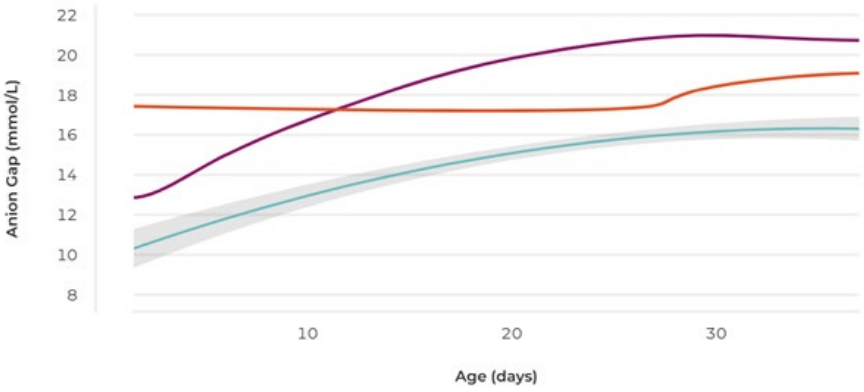

Figure 4 - Blood iGap level in bird age 2, 6, 9, 16, 20, 28 and 37 days compared with database (DB). DB, June result and October result.

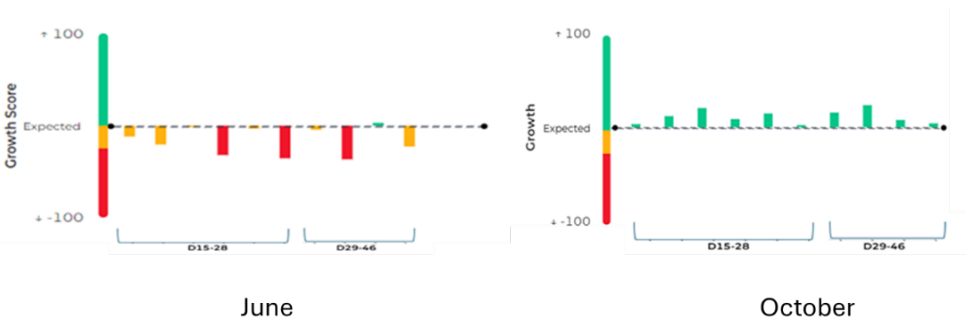

Figure 5 - The growth prediction model of bird at 2, 6, 9, 16, 20, 28and 37 days old in October 2024 and June 2024. Green color is expected growth score, yellow color is moderate growth score, and red color is lower expected growth score.

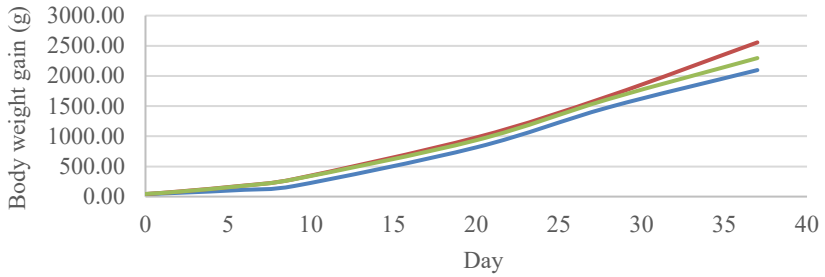

Figure 6 - The body weight gain (g) of bird at 2,6, 9, 16, 20, 28 and 35 days old in —October 2024 and —June 2024 compared with —Ross 308 standard growth.

IV. DISCUSSION

The machine learning–based growth score integrated 21 blood biomarkers together with breed, weight, sex, and age to assess flock performance. In June 2024, key biomarkers; Cl^-, TCO_2, HCO_3^-, and iGap deviated from reference ranges, indicating heat stress–related respiratory alkalosis with metabolic compensation. These findings align with previous studies showing that acid–base disturbances under thermal stress (32–38 °C) can impair nutrient utilization and growth performance (Soleimani et al., 2011; Borges et al., 2004; Quinteiro-Filho et al., 2010). Corrective measures, including reducing stocking density, enhancing ventilation, and limiting chloride levels in drinking water, were implemented, leading to improved physiological stability and flock performance. By October 2024, biomarker levels had normalized and corresponded with higher predicted growth scores. Although elevated chloride and iGap values were observed in 8–12-day-old chicks, these were likely due to sampling stress and did not affect growth, as early performance (0–14 days) was primarily influenced by nutrient density, amino acid balance, and genetics rather than minor electrolyte variations (Moran, 2001; Attia et al., 2018). Additionally, the predicted growth score showed a strong association with actual body weight gain, confirming the potential of this analytical approach to accurately identify growth performance. This study applied all key recommendations simultaneously to evaluate improvements in prediction accuracy and biomarker responses. However, future research should test each intervention independently to determine its specific effectiveness in alleviating heat stress and metabolic imbalance. Nonetheless, as an unchanged control flock was not assessed alongside the modified flock in October 2024, further studies are required to validate these findings under comparable farm conditions. Overall, integrating blood biomarker monitoring with machine learning provides a practical decision-support tool for detecting heat stress and guiding management interventions to sustain flock health and productivity (Akbarian et al., 2016; Livingston et al., 2020).

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DEVELOPMENT OF NOVEL COMPOUNDS FOR TREATMENT AND PREVENTION OF COCCIDIOSIS IN CHICKENS USING A *TOXOPLASMA GONDII IN VITRO* MODEL

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Summary

Coccidiosis (caused by *Eimeria* spp) is an important disease in poultry systems causing significant financial impact. We have established a chicken coccidiosis (*Eimeria*) model to test promising novel compounds identified through our drug screening pipeline, which uses a related parasite, *Toxoplasma gondii*, as a model organism for *in vitro* testing, circumventing the inability to complete the *Eimeria* lifecycle *in vitro*. Two lead compounds from our *T. gondii in vitro* screening have shown potentially promising biological activity in a pilot study run using our chicken coccidiosis (*Eimeria*) model.

I. INTRODUCTION

Eimeria species, the aetiological agents causing coccidiosis in poultry, are responsible for substantial costs in poultry systems, estimated at ~£10.4 billion world-wide in 2016 (Blake et al., 2020). Anticoccidial chemoprophylactic agents are consistently in use, particularly in intensive poultry systems, leading to the detection of multiple resistant strains of *Eimeria* (Abbas et al., 2011). With the availability of effective chemoprophylactic agents remaining critical for the ongoing management of this important disease, it is important that new safe and cost-effective drugs are developed that can replace existing treatments where resistance is prevalent and production losses are observed.

Given the ongoing inability to complete the lifecycle of *Eimeria* species *in vitro*, and the close genetic relatedness of *Toxoplasma gondii* and *Eimeria* spp, *T. gondii* has been used as a model organism for *in vitro* screening of drug efficacy against *Eimeria* (summarised by Kim and Weiss, 2004). To this end, we screened a library of analogues of the anti-coccidial drug robenidine against *T. gondii.* We have now established a chicken coccidiosis model to test the *in vivo* efficacy of our most potent lead compounds against *Eimeria* species.

II. METHOD

a) Drug screening using *T. gondii* model

Our *T. gondii in vitro* model uses a recombinant RH strain that expresses yellow fluorescent protein (Tg RH-Tub-eYFP)(Gubbels et al., 2003), grown in Human Foreskin Fibroblasts

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(HFF's). Robenidine analogues were dissolved in DMSO (10 mM stock solution) Drug screens were undertaken according to established protocols (Gancheva et al., 2025) in 96 well plates, at 37°C, with fluorescence of all wells being measured using a PHERAstar® FSX HTS Microplate Reader (BMG LABTECH) at 72 hours post infection. Fluorescence measurements were corrected for background (uninfected control) and then growth was expressed as a percentage of the positive control. Compounds that inhibited growth by >80% with initial screens at a concentration of 1µM were selected for determining the half-maximal inhibitory concentration (IC_{50}) using a serial dilution (1:2) of drug concentrations. Assay plates were measured for fluorescence at 72 hours with non-linear regression used to generate curves and IC_{50} values (GraphPad Prism 9, GraphPad Software LLC). Robenidine analogues with IC_{50}s against *T. gondii* <500 nM were selected for assessment of host cell toxicity against HFF cells (Gancheva et al., 2025). HFF cytotoxicity IC_{50} values were determined using a serial dilution (1:2) of drug concentrations. The compound Selectivity Index (SI) was calculated by dividing the Host cell (HFFs) cytotoxicity IC_{50} by parasite (*T. gondii*) IC_{50}.

b) Coccidiosis drug trial

Field isolates of *Eimeria* spp were obtained from local producers and were submitted for speciation PCR (Birling Laboratory, Bringelly NSW) and sporulated prior to the trial, resulting in a mixed population of *E. acervulina, E. tenella* and *E. maxima.* Day old broiler chicks (Cobb) were obtained from a commercial hatchery for use in the steps below (ethics approved by University of Adelaide Animal Ethics Committee, approval number S-2024-073, approved on 06/09/2024). Oocysts were first propagated; day old chicks housed in cages in the poultry facility until seven days old when they were inoculated with up to 10,000 total oocysts (proportion in original sample was *acervulina* > *mitis* > *tenella* > *maxima*). At 14 days of age, the birds were humanely euthanised via cervical dislocation, with oocysts harvested via gastro-intestinal tract mucosal scrapings, from duodenum to colon.

c) Pathogenicity trial

After propagation, the pathogenicity of the included *Eimeria* species was established, using three groups of four chicks inoculated with 1 * 10^5, 5 * 10^5 and 1 * 10^6 total oocysts per bird in each group, at seven days of age as per propagation above. At 14 days old, birds were humanely euthanised and necropsy performed to assess intestinal coccidiosis lesions, which were scored on a four-point scale (Johnson and Reid, 1970). The inoculum for the main trial was then designed to produce maximum average lesion scores of three for *E. acervulina*, two for *E. maxima* and three for *E. tenella*.

d) Drug efficacy and safety pilot study

The pilot study was conducted using our two lead Robenidine analogues. Starting with day old chicks as above, chicks were randomly allocated into five groups (three chicks per group).
At six days old, chicks began receiving drug treatment by oral gavage, three times per day for the remaining duration of the study. Given the commercially applicable dose would be approximately 50 mg/kg of feed, the daily dose rate was calculated using average feed consumption for average bodyweight per group. At seven days old, chicks were given the inoculum as described above and at 14 days old, chicks were humanely euthanised via cervical dislocation, and necropsy performed as per pathogenicity step above. Fresh faeces were collected from each group daily and oocyst counts performed.

III. RESULTS

a) Drug screening using *T. gondii* model

A total of 338 novel compounds generated from robenidine were screened, of which 15 inhibited in vitro growth by more than 80%. Of these, nine compounds had IC_{50} values below 320 nM and were subjected to host cell cytotoxicity testing. Robenidine 6 was found to be the most potent and Robenidine 7 had the best combination of potency and limited host-cell toxicity (Table 1).

Table 1 - Results for top 2 robenidine analogues (RA) from drug screening.

Compound	*T.gondii* IC_{50} (nM)	Selectivity Index (HFF IC_{50}/Tg IC_{50}
RA6	< 100	25
RA7	< 150	998

b) Coccidiosis drug trial

Table 2 - Results of Robenidine compound efficacy trial.[a, b, c]

Treatment Group	Bird #	Lesion Score (gut location)				Eimeria species lesions			Avg. total weight gain (at day 12)	Pooled oocyst count (at day 12)
		Ant.	Mid.	Post.	Caec.	Acer.	Ten.	Max.		
Positive Control (Robenidine)	1	4	2	0	0	+	-	-	215g	462,273
	2	3	2	0	0	+	-	-		
	3	2	1	0	0	+	-	+		
Unchallenged Control	4	0	0	0	0	-	-	-	251g	113
	5	0	0	0	0	-	-	-		
	6	0	0	0	0	-	-	-		
Negative Control (challenged untreated)	7	2	2	0	2	+	+	-	147g	507,429
	8	3	2	0	1	+	+	-		
	9	1	1	0	3	+	+	-		
RA6 treated	13	3	3	0	3	+	+	-	145g	14,471
	14	2	2	0	3	+	+	-		
	15	3	3	0	3	+	+	-		
RA7 treated	10	3	3	0	3	+	+	-	142g	56,328
	11	3	3	0	2	+	+	-		
	12	2	2	0	2	+	+	-		

[a] Lesion scores are described by severity per region of the gut (0 – 4) and by presence of characteristic lesions by *Eimeria* species (+ or -)
[b] Due to the pathogenicity of our *Eimeria* species isolates, humane euthanasia was performed at 12 days old, due to welfare criteria being met ahead of the planned 14-day conclusion.
[c] The small number of oocysts in the unchallenged control is consistent with residual contamination between counts rather than infection.

Table 2 summarises the results of our pilot *in vivo Eimeria* treatment trial using our two lead Robenidine analogues (RA6 and RA7) compared to the parent compound Robenidine. Some variation in lesion scores is expected due to the small number of birds in each group leading to individual variations having an outsized impact (including more oocysts shed in positive control group). *Eimeria maxima* lesions were minimal as this species is known to be overgrown by other species that share gastro-intestinal tract predilection sites in mixed inoculums (Jenkins et al., 2008), *E. acervulina* in our case. The most notable results were that robenidine (positive control) promoted weight gain and eliminated *E. tenella* lesions but had no effect on other gastro-intestinal tract lesions or oocyst shedding, whereas our novel compounds caused a 9- and 35-fold reduction in oocyst shedding respectively, without any improvement in gastro-intestinal tract lesions scores or improvement in weight gain under the conditions tested.

IV. DISCUSSION

This pilot study has shown promising results for the utility of our *T. gondii in vitro* model as a first step in identifying anticoccidial compounds for use in chickens. The two lead robenidine analogues identified from the *in vitro T. gondii* screens showed biological activity against *Eimeria* in chickens, creating a substantial decrease in oocyst shedding, which alone has the potential to impact infectious spread in commercial broiler systems. However, it is important to note two limitations of coccidia challenge via gavage model we used in this preliminary trial; (1) infection by gavage has the potential to overwhelm a drug's capacity to control symptoms/lesions and (2) treatment by gavage also effects how *Eimeria* developing in the gut is exposed to the drug. Moving forward, we plan to expand our model to include floor pen trials and will have promising compounds added to feed to assess the effectiveness of drugs in a commercially viable delivery system. Despite this simplified study design, we have successfully demonstrated the biological activity of our two lead robenidine analogues in our chicken coccidiosis model.

We have established a pipeline to screen and prioritise anticoccidials for testing against the economically important *Eimeria* spp. parasites and have additional leads outside the two highlighted here prioritised to test for efficacy against *Eimeria in vivo*. Our Eimeria *in vivo* model is established for efficacy testing of in-house leads, and is also available for efficacy testing of drug, antibody, vaccine and other *Eimeria* control measures of research and industry partners. Our combined models show promise for future identification of novel anticoccidials for use in broiler systems and demonstrate potentially useful efficacy of two lead drugs in preliminary experiments.

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THE EFFECT OF INTELLIGENT RED LIGHT-EMITTING DIODE LIGHTING SYSTEM ON LAYING HEN PERFORMANCE

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Summary

The study aimed to investigate the effect of installing an intelligent red light-emitting diode lighting system on egg production in farm scale. The experiment was conducted on White Leghorn hens aged from 48 to 74 weeks preceded by a three-week adaptation period (wk 45-47) . The results obtained in the aviary experimental hen house (E) were compared with retrospective results in the control house (C) with incandescent and white lighting. The average body weight of the laying hens (E: 1802 g ±6.2; C: 1798 g ±16.6; p = 0.893) and the daily feed consumption per 1 hen (E: 122 g ±5.3; C: 118 g ±3.8; p = 0.769) in E and C house did not differ statistically. The layer from hen house E laid approximately 1 egg more per week (6.04 ±0.23) than the layer in hen house C (5.05 ±0.25) and the difference was statistically significant (p = 0.001). The average share of large eggs (L) in hen house E (39.02 ±2.45 %) was significantly higher (p = 0.001) compared to hen house C (35.29 ±5,52 %), especially in the last weeks of the analyzed period. The tested lighting system may prove to be a convenient and effective tool in precise poultry production.

I. INTRODUCTION

According to available studies, the use of red light may improve egg production because the longer wavelengths in red light can penetrate the skull and stimulate hypothalamic photoreceptors and reproduction in birds (Poudel et al. 2022, Lewis and Morris, 2000). Additionally, the use of Light-Emitting Diodes (LEDs) is recommended as they are economically viable, sustainable, and have a longer useful life (Moreira et al. 2025, Tez & Akşit 2024). Considering the above arguments, it was decided to design the intelligent red light-emitting diode lighting system and investigate its effect on farm scale, in comparison with the traditional incandescent white lighting system used in the hen house.

II. METHOD

The red light-emitting diode (RLED) intelligent lighting system (ILS) for laying hens was designed by our research team. In this project, we used the following parameters: red light spectrum 635 nm; red and white light ratio 100 : 50 ; temperature of colour = 1278 Kelvin; colour rendering index = 69.8; light intensity 15 lux; 14 hours of light; remotely control and monitoring – IPOX NMVS-2.0 software. The RLED+ILS system was installed in the hen house with an area of 802 sq m. It was commercial aviary henhouse, with no natural light, and an automatically controlled mechanical ventilation system to ensure standard temperature and humidity conditions for laying hens. White Leghorn hens' weekly performance results (hen weight, feed intake, egg production, egg weight) were monitored in the period from 48 to 74 weeks of age preceded by a three-week adaptation period (45-47 wk). The following egg weight

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categories were used: L (large: 63-73 g), M (medium: 53-63 g) and S (small: < 53 g). The results obtained in one experimental hen house (E: 7,000 laying hens) were compared with identical retrospective results collected in the same one control house (C: 7,000 laying hens) with incandescent and white lighting (13 lux, 60 W, 2 rows × 17 bulbs). The control (C) and experimental (E) data were collected under the same conditions (line and origin of hens, age of hens, season – from May to November, management practices and nutrition, indoor environmental conditions, stocking density 8,7 hen/m^2) by the same operator, using the same forms and techniques. The production results of the laying flocks were analyzed statistically using STATISTICA ver. 13.3 software (TIBCO Software Inc., Tulsa, OK, USA, 2017). The normality/non-normality of distribution was determined by the Shapiro–Wilk test. The collected data were processed by Student's t-test. The results were presented as means and the standard deviation (±SD). The statistical significance was estimated at $p \leq 0.05$.

III. RESULTS

In the analysed period (48-74 weeks of age), the average body weight of the laying hens and the daily feed consumption in the E and C hen house and did not differ statistically (Table 1). The laying hens from hen house E laid approximately 1 egg more per week ($p = 0.001$) than the layers in hen house C (Table 1). The average egg weight per layer from hen house E was 1.67 g lower ($p = 0.003$) in comparison with hen house C (Table 1). In the analysed period the part of S eggs in hen house E was not present, unlike in hen house C ($p = 0.001$), while in hen house C they amounted to about 1.9% (Table 1). In the period of weeks 48-52, the share of M eggs in hen house E was lower compared to hen house C, and from the 53 week it raised. From week 64, a decreasing trend in the share of M eggs was noticed in the hen house E. The share of M eggs for the entire analysed period was lower ($p = 0{,}001$) in henhouse E (60.71 ±6.62) compared to henhouse C (63.08±2.30). The average share of L eggs in hen house E (39.29 ±2.5) was significantly higher ($p = 0{,}001$) compared to hen house C (35.02 ±3.5), especially in the last weeks of the analyzed period (Figures 1-2). The improvement in egg production is likely to be reflective of the lighting treatment, however more replication is required to confirm this observation.

IV. DISCUSSION

In the analysed period, the average body weight of the laying hens and the daily feed consumption in the experimental (E) hen house with the RLED+ILS lighting program and in the control (C) house were comparable. Similarly, Tez and Akşit (2024) study on the Lohmann brown strain, there was no significant difference in feed consumption during red LED light use. The layer from hen house with the RLED+ILS lighting program laid approximately 1 egg more per week (Table 1). Moreira et al. (2025), using red LED lighting in a laboratory scale (Hy-Liner® strain), also found that laying hens under red LED lamps showed a trend of greater egg production and average egg weight. During the period under discussion, the share of L eggs in the total number of eggs showed an upward trend. The average share of large eggs (L) in hen house E was significantly higher compared to hen house C, especially in the last analyzed weeks. Gongruttananun & Pannapat (2012) noticed that photostimulation by red light resulted in an acceleration of sexual development in Thai native hens compared to hens exposed to full-spectrum lighting; however, live performance and egg production, were not affected whatever the light treatment.

Table 1 - Selected production parameters in the control (C) and experimental (E) poultry houses in the period from 48 to 74 weeks of age of the laying hens (mean±SD).

Specification	Statistical measures	Group		p-*value*
		C	E	
Body weight (g)	$\bar{x}$	1802.0	1798.0	0.893
	SD	6.20	14.60	
Daily feed consumption (g/hen)	$\bar{x}$	122.0	118.0	0.769
	SD	5.26	3.80	
Number of eggs per week from the laying hen (number/1 hen)	$\bar{x}$	5.05	6.04	0.001
	SD	0.25	0.23	
Egg weight (g/egg)	$\bar{x}$	68.66	66.99	0.003
	SD	2.05	1.88	
Share of S eggs in the total number of eggs laid (%)	$\bar{x}$	1.90	0.00	0.001
	SD	0.161	0.00	
Share of M eggs in the total number of eggs laid (%)	$\bar{x}$	63.08	60.71	0.001
	SD	2.30	3.62	
Share of L eggs in the total number of eggs laid (%)	$\bar{x}$	35.02	39.29	0.001
	SD	5.52	2.45	

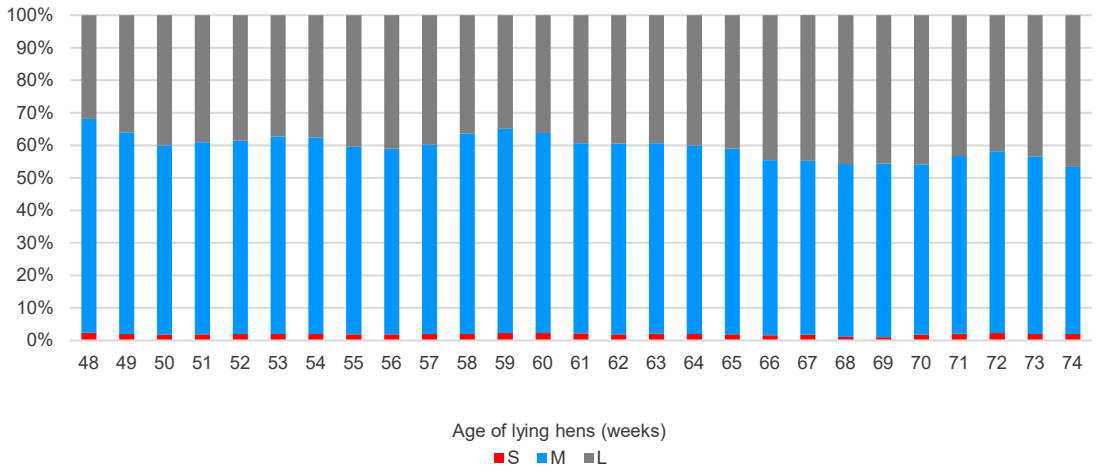

Figure 1 - Share of eggs in individual weight classes (S – small, M – medium, L- large) in the total number of eggs in control (C) henhouse in individual weeks (%, wk 48-74).

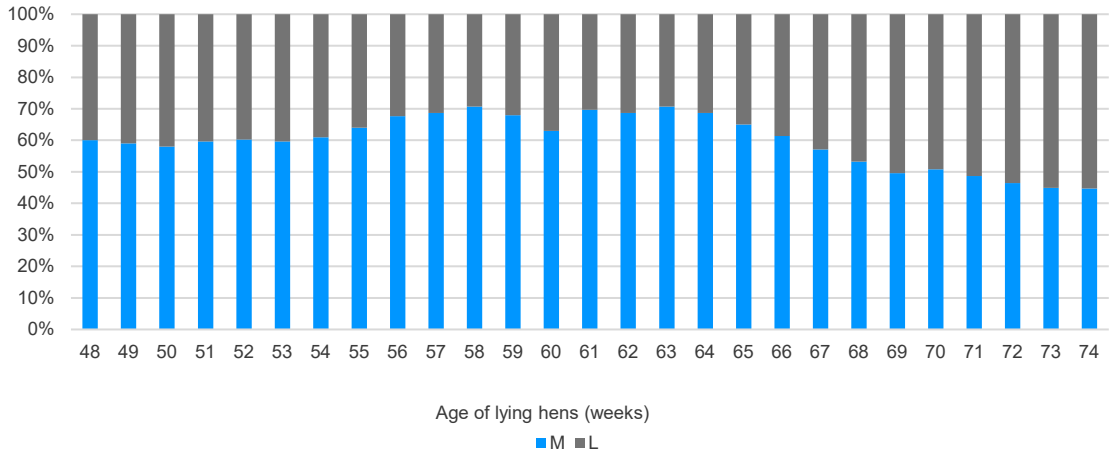

Figure 2 - Share of eggs in individual weight classes (S – small, M – medium, L- large) in the total number of eggs from one hen in experimental (E) henhouse in individual weeks (%, wk 48-74).

In conclusion, the used RLED+ILS lighting system seemed to be more beneficial in terms of production efficiency (average production, as well as the share of individual classes in the total number of eggs laid) than the traditional system with incandescent and white lighting, however more replication is required to confirm this results . It should be emphasized that this is a pilot study on a farm scale, and despite the efforts mentioned above to collect data under the same conditions, this study has limitations that should be considered when drawing conclusions, as two flocks may differ in various ways that were not assessed.

In our opinion, the light colour must be correlated with its intensity and colour temperature. Thanks to its wide range of lighting parameters and the ability to quickly and remotely adjust them (without the need to enter the flock or from a distance, using remote computer control system), the tested lighting system may prove to be a convenient and effective tool in precision poultry production. For this reason, we plan to continue this research on a broader scale.

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