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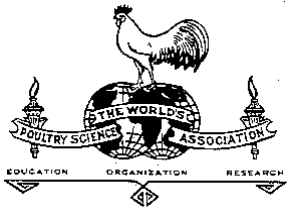

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and

THE WORLD'S POULTRY SCIENCE ASSOCIATION
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WORLDWIDE WATER SCARCITY – WE ARE ALL IN IT TOGETHER

W.G. BOTTJE*, S. DRIDI[1], S. ORLOWSKI[1], M. KIDD[1] and K. LASSITER[1]

Summary

In an increasingly hot, hungry, and crowded world, water scarcity in many parts of the world looms greater each year. Water use efficiency ranging from the broiler to broiler house is a major focus of a comprehensive five year multi-institutional grant†. A large component of the project focused on gut integrity and microbial dysbiosis leading to 'leaky gut'. Studies also investigated using microalgae in broiler diets as an alternative feed protein, and as a platform for engineering algae for production of feed grade enzymes. Educational opportunities included internships, and an international course that had many international guest lectures from leaders in their field and was taken by US, Chinese and Indian students; *The Global Nexus of Food, Energy and Water* (offered by Cornell University). Besides tests and quizzes, the students also participated in debates with teams that incorporated students from each of the home institutions. Two areas that will be highlighted here will be; 1) divergent selection of broilers for water use efficiency, 2) perfecting and managing a fine mist sprinkler system that can reduce water use by 50 to 60% for cooling broiler houses continued with this project. The management of the sprinkler system is key to its success in order to maximize broiler production while reducing water use during periods of hot weather. Although the amount of potential water savings from research presented here may represent only a few drops in the bucket, each drop adds up because we are all in it together.

I. INTRODUCTION

Globally, climate change from green-house gas emissions is producing record breaking years for temperatures, droughts, flooding, fires, and sea level rise. A sign of the times in the US is a program to buyout home owners in South Carolina that have homes in flood prone areas due to sea level rise along the Atlantic coast (Shailer, 2024). Between 2002-2021, it is estimated that droughts and floods resulted in over $1 trillion in economic loss (UNESCO, 2024; NOAA, 2023;WRI, 2020). In the US, housing developments continue to be built in places like Phoenix even though it is becoming increasingly difficult to find water to sustain these developments. On Sept. 3, 2024, Phoenix shattered another heat record by reaching at least 100° F (37.8 C°) for 100 straight days – the previous record was 76 days (Evans, 2024). In the US, 'crazy' ideas resurface every few years. For example, with 20% of the world's freshwater residing in the Great Lakes, why not build a pipeline from Lake Michigan to the US desert southwest as proposed by the chief water scientist at NASA's jet propulsion laboratory (Matheny, 2017)? Or, another stunningly brilliant idea floated by an Arizona State Senator was; Lets pump water from the Mississippi River to the desert southwest (Headley, 2021). Boom: Problem solved! These ideas quite frankly are nuts because of the cost of small physical barriers such as distance (1800 miles) and a little thing called the Continental Divide (i.e. Rocky Mountains). Infrastructure (pipes and pumps) and power needed for the pumps would be enormous. In 2022, the Mississippi River dropped to historic lows (Taylor, 2022), and in 2023, the river dropped so low, that salt water from the Gulf of Mexico threatened the drinking supply in New Orleans; a distance of ~100 miles up-river (Ramirez et al. 2023)

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† USDA NIFA SAS *Empowering the US Broiler Industry for Transformation and Sustainability* (#2019-69012-29905).

In an increasingly hot, hungry, and crowded world, water scarcity looms greater with each passing year. Relationships between food, energy and water are inextricably linked (Figure 1). Factors that alter one of the pillars, will impact the other pillars as well.

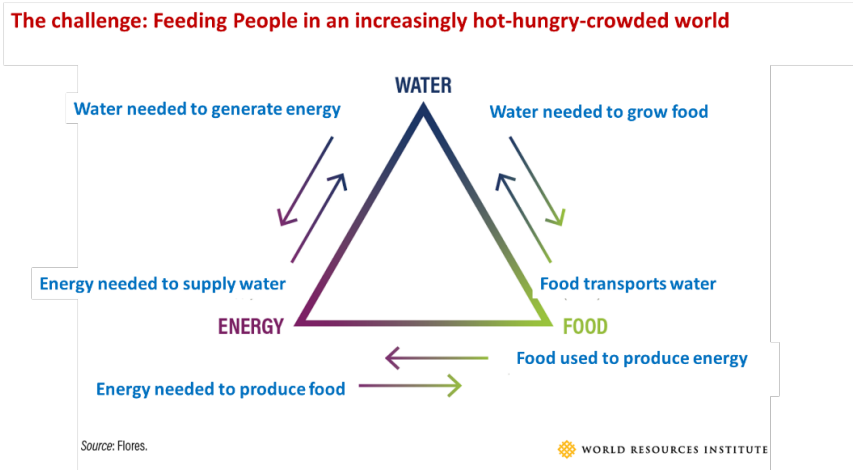

Figure 1 - Three Pillars for Effective Water-Energy-Food Nexus Management | World Resources Institute (wri.org)

Liu et al. (2022) performed a comprehensive meta-data study of the world water needs of 19 of the world's most important grains. They developed a water scarcity index (WSI_{ag}) to assess agricultural water scarcity over a baseline time period (1981-2005) to water needs in the future (2026-2050) under two climate change scenarios; 1) stringent control of greenhouse gas emissions, and 2) a relaxed control of greenhouse gas emissions. WSI_{ag} was determined as follows:

$WSI_{ag} = ET_c/WA$ Total water requirements of 19 crops (ET_c)/ water availability (WA)
$WA = IE \times WA_{blue} + WA_{green}$
ET_c = total water requirements of 19 crops
IE = Irrigation Efficiency
WA_{blue} = ag blue water availability (m^3/month)
WA_{green} = ag green water availability (m^3/month)
Blue Water = irrigation from lakes and streams
Green Water = rainfall and evaporation (the water cycle)

Liu et al. (2022) reported that if stringent green-house gas emission control is enacted, WSI_{ag} % is predicted to increase by 83% between 2026 to 2050. In a relaxed CO_2 emissions scenario, WSI_{ag} % is predicted to increase by 84% during the same time period. The good news: There is very little change in the WSI_{ag}% between the two scenarios. The bad news: There is very little change in the WSI% between the two scenarios.

So, water scarcity is only going to get worse. The majority of ETc reported by Liu et al. (2022) is due to requirements of 6 major crops (wheat, rice, corn, soybean, barley, and cotton). Five of these major crops play a key role in global food security for people and for feeding animals that provide high quality animal protein to feed people. Thus, water scarcity of land areas devoted to these crops will increase. Fallowing this land could reduce water scarcity, but it would exacerbate world hunger and starvation (FAO, 2018).

II. COMPREHENSIVE GRANT IN BROILER SUSTAINABILITY

The USDA NIFA SAS embraced the three 'legs' of the land grant mission of research, teaching, and extension. The project brought together 32 faculty from 8 institutions with an

overarching goal to improve the system wide efficiency of water and nutrient use in broiler production. Objectives were:

1) To enhance water use efficiency in broilers and broiler production systems
2) To improve gut health-microbiome, immunity-disease resistance, and wellbeing of broilers,
3) To develop microalgae as an alternative "greener" feed protein and hydrolytic enzyme supplement,
4) To convert poultry litter and feathers into value-added and environmentally-friendly products,
5) To generate health-promoting chicken for human consumption,
6) To innovate education of the next generation of poultry professionals to lead the industry, and
7) To broadly and rapidly disseminate new technologies to the broiler industry and the general public.

Several of the projects focused on water-related aims are discussed below.

a) Establish a line of water efficient broilers.

The initial step for genetic selection for water efficiency in broilers required development of a water metering device that not only measured accurate water consumption on individual birds (i.e. measure low water flow precisely) but kept the appropriate low water pressure for nipple drinkers. This device was described in Hilz, 2021 and Hilz et al., 2021.
Once the device was validated, divergent selection of broilers for water efficiency was initiated and is now in the 4th generation of selection. Selection was based on water conversion ratio (WCR, weight water consumed/ amount body weight gain, like FCR but wetter). Birds with a low and high WCR phenotypes represent water efficient and inefficient broilers, respectively. A base line of broilers (modern random bred, MRB) has been maintained to compare the effects of selection for LWCR and HWCR to the original broiler population. After 4 generations of divergent selection for WCR, growth performance under thermoneutral and heat stress conditions gene and protein expression analysis of water-homeostasis mechanisms, studies were conducted on HWE, LWE, and MRB broilers in the hypothalamus, kidney, and intestines (upper-lower duodenum) and cecum and assessment of effects of selection on innate immunity and meat quality have conducted (Aloui et al. 2024; Greene et al., 2024; Lassiter et al. 2024a)

b) Microalgae feeding.

Soybean production will need to double by 2050 to meet animal feed production needs in poultry and swine (Ray et al., 2013) a goal that is likely not achievable. Thus, finding ways to replace the protein shortfall will include alternative protein sources or supplementing low crude protein diets with amino acids, is important for maintaining a sustainable poultry industry. One protein source that has been investigated for inclusion in poultry diets is microalgae (*Spirulina platensis*) has been investigated. Among sustainable advantages of microalgae are that; it can be grown in brackish or salt water (not fresh water), water used can be recycled, and it's production does not require fertilizer.

Studies were conducted in male and female broilers that were fed a Control grower diet with 20% crude protein (CP) or diets containing 17% CP formulated with soybean meal alone (LCP) or one in which half of the soybean meal was replaced by *Spirulina platensis* meal (SP-LCP) at 10% of the diet (Mullenix et al. 2021; 2022; Lassiter et al. 2024b). The results indicated that growth performance was reduced in the LCP and SP-LCP groups compared to controls; however, there was no difference between LCP and SP-LCP broilers. The SP-LCP

fed birds had lower bacterial counts in the liver (indicating less bacterial translocation out of the intestines), and lower inflammatory cytokines in the blood. SP-LCP broilers also exhibited enhanced antioxidant levels in muscle, and greater pigmentation of plasma, breast and thigh and skin. Crafton-Wells et al. (2024) reported that there was no difference in growth performance in broilers during the grower period when fed diets containing 0 and 2% algae in combination with 0 and 8% DDGS. They concluded that algae has the potential to be a source of feed protein when fed at 2% or less in the diet. *Spirulina* shows potential for replacing soybean meal but is cost prohibitive in the commercial poultry industry at this time.

c) Reducing water use for cooling broiler houses.

During hot weather, tunnel ventilation and evaporative cooling pads are effective in maintaining thermoneutral temperatures within the house. However, this requires a large amount of water, can raise relative humidity in the house, litter moisture and ammonia in the house (leading to breast and foot blisters). Managing a fine mist sprinkling system combined with allowing house temperatures to increase to 88-90 ºF before fans are turned on can cut water cooling use in half, reduce ammonia, and improve profitability for poultry growers. When used correctly, the fine misting system can reduce body temperature, plasma corticosterone, heat shock protein expression and increase growth performance when compared to a poultry houses using evaporative cooling pads alone. (Liang et al. 2013; 2020; Moon et al., 2022).

d) Education: Global Nexus of Food, Energy and Water:
Engaging students from the US, China, and India.

Students in the Global Nexus Class (offered by Cornell University) US students (mainly from Cornell), two institutions in China and two institutions from India. During the semester, guest lecturers from all over the world would present information in their area of specialty pertinent to food, energy, water, economics, and health. Besides tests, there were also a series of debates on various topics. Debate teams would be made up of students from each of the participating countries/institutions. There would be a con team and a pro team that would debate opposite sides of a given issue. Students on their own time (outside of class time) would have to work together across all the time zone differences to develop their arguments and presentations for the debate. Being able to participate in this course as a guest lecturer with a couple of students from Arkansas has been a very wonderful experience.

e) Potential water savings estimate

My first introduction to the nexus of food, energy and water (Figure 1) came from the Global Nexus class described above. In poultry production, water is needed for efficient growth and water is also needed for evaporative cooling in tunnel ventilated houses in order for poultry to grow efficiently in hot environments. Water is also absolutely essential for growing grain used in poultry feed.

Water use or savings on a large scale (e.g. rainfall) is often presented units of inches (or cm), or acre feet. Large amounts of water may be expressed in millions of gallons or liters or cubic meters, or kilometers – all of which are hard to grasp (in my opinion).

f) Fine mist sprinkling system in broiler houses.

Liang et al. (2013), reported that there was ~20,000 -40,000 gallons of water saved in cooling broilers with fine mist sprinklers compared to conventional evaporative cooling pads*. There are ~25,000 to 29,000 broiler farms in the US.†

Table 1: Units of water‡

1) 1 gallon = 3.754 L water
2) 1 L = 0.2642 gallons
3) 1 m^3 = 1000 L = 266 gallons
4) 1 acre foot = 326,000 gallons = 1.23 ML
5) 1 acre foot = amount of water that covers an acre 1 foot high (12" or 30.5 cm)
6) American football field = ~ 1 acre;
7) Olympic Pool ~ 2 acre ft
   50 x 25 x 2 m = 2500m^3 = 2.5 M L = 660,000 gallons ~ 2 acre feet

Also: 1 hectare = ~ 1 rugby field = 1.4 soccer fields = ? Australian footie fields

Table 2 - Assumptions and Calculations

a) half of these farms are located in SE US = 12,500 farms
b) each farm has 4 and 8 houses per farm = 50,000 to 100,000 houses total
c) two summer-time flocks per house = 100,000 to 200,000 flocks
d) 20,000 gal saved per flock = 2 B to 4 B gal water saved/yr
e) 2B to 4B gal/326,000 per acre ft = 6,135 to 12,270 acre ft of water saved/year.
= football field column of water 1.2-2.3 mile high

Bottom line ($): potential money saved: 2B to 4B gal water x 0.002$/gal[iv] = $4-8M saved/yr

Table 3 - From Aloui et al. 2024. (Table 3, broilers 4-7 wks of age p. 11).

	HWE		HWE		Main Effects		
	TN	HS	TN	HS	HS	Line	Interaction
FCR	1.82±0.02	1.82±0.02	1.91±0.03	1.94±0.05	-	< 0.001	-
WCR	3.14±0.06	4.09±0.07	3.81±0.10	4.89±0.06	< 0.001	< 0.001	-
WI/FI	1.73±0.02	2.26±0.08	1.99±0.03	2.53±0.06	< 0.001	< 0.001	-

g) Divergent selection for water conversion ratio (WCR)

Divergent selection for water conversion ratio (WCR) has been carried out by Orlowski and co-workers for several generations and work has begun to determine the fundamental basis (molecularly- physiologically in these birds (e.g. Aloui et al., 2024; Lassiter et al., 2024). It is generally accepted that water intake is roughly twice as high as food intake. This may vary in response to a number of factors; e.g. heat stress, disease. In Aloui et al. (2024), male broilers from high and low water efficiency (HWE and LWE) lines were placed in environmental chambers and raised under thermoneutral (TN) or cyclic heat stress (HS) from 4-7 wk. FCR, WCR and WI/FI values for this study are shown in Table 1.

The data in Table 2 indicates a large difference (1.50 units) in WCR between the HWE and LWE groups. HWE birds also had significantly greater FCR, indicating that genetic selection for HWE may also have beneficial effects on growth performance.

The difference in water intake (WI) between the HWE and LWE lines was 2.23 kg (L) per bird. If a 10% improvement in WCR was attained by genetic selection in broilers, this

* Studies were done in a 40 x 400 ft broiler house.
† US Poultry Industry Manual - Broilers: scope of the broiler industry | The Poultry Site
‡ For some humor on weights and measures – see Nate Bagartze in Washington's Dream - SNL
iv What is price for tap water in the US - Search

would mean a possible reduction in water intake of 0.223 L (assuming body weight gain did not change). What this might mean regarding water savings is shown in Table 3.

Table 4 - From Aloui et al. 2024. (Table 4, 0-7 wks, p.12 – Main effect of line)

	HWE	LWE	p value
FI (kg/bird)	5.21±0.11	5.58± 0.07	0.0030
WI (kg/bird)	9.94±0.29	12.17± 0.15	< 0.0001
FCR	1.68±0.01	1.76± 0.02	0.0004
WCR	3.23±0.04	4.73± 0.06	< 0.0001
WI:FI	1.92+0.03	2.19+ 0.03	< 0.0001

Table 5 - Potential water savings based on 10% improvement in water conversion ratio.

1 Million bird complex per wk:
- = 0.223 L/bird x 1 M birds = 223,000 L = 59,403 gal saved per wk.
- = 0.223 L/bird x 1 M x 52 weeks = 11.6 ML = 3.1 M gal/yr = 9.5 acre feet/year.
- = $0.002/gal x 3.1 M gal = $6,178.

9 billion broilers per year (US Industry):
- = 0.223L/bird x 9 B birds = 2.01 M L/yr = 5.346 K gal/yr.
- = 5.346 K gal/326,000 gal per acre foot = 1,640 acre ft = football field 1/3 mi high
- = $0.002/gal x 5.346 K gal/year = $1.07 M/yr

h) Water consumed in feed

In the global nexus triangle of food, energy and water (Figure 1), there is a component 'food transports water' in the food-water relationship. It is easy to visualize this by thinking of fruits or vegetables (e.g. watermelon, tomatoes) that contain a lot of water. But there are large ramifications to global movement of water if one considers animal feed. One example of this is a practice in which Saudi Arabia purchased large amounts of acreage in Arizona (US desert Southwest) to grow alfalfa that is shipped to Saudi Arabia to feed their dairy industry[i]. Each month, pelleted alfalfa is loaded onto container ships and sent to Saudi Arabia. Granted – alfalfa pellets would contain far less water than fresh alfalfa, but the amount of water required to grow a ton of alfalfa is approximately 8" of water per acre (2/3 acre foot, 217,442 gallons/acre). So, 100 acres of alfalfa would require 21.7 M gallons of water for a single cutting; 5 cuttings per year would require: 109 M gallons or 334 acre ft per year. (Note: 5 cuttings per year may be an under estimation.[ii]). Fondomonte Farms (which is part Alamari) has purchased 25,000 acres in Arizona and 30,000 acres in Argentina to feed 93,000 dairy cows. It would be physically impossible to ship that amount of water to grow alfalfa in Saudi Arabia. The charge for water rights in Arizona is $75/acre.

So, let's consider the importance of feed conversion ratio (FCR) in growing broilers. FCR continues to improve in the poultry thanks to genetic selection and nutritionists that continue to improve FCR each year. Table 4 presents calculations of additional virtual water required (due to corn and soybeans in the diet) per 0.01 unit of FCR. Assumptions included a 3.48 kg BW, fed a diet containing 60% corn and 30% soybean that require 900 L of water to grow a kg of grain. Using these values, it was calculated that a 0.01 increase in FCR would result in an additional consumption of virtual water of 28 L per bird. This would result in an additional 28 M liters of water consumed in a 1 million bird poultry complex, or 22.88 acre ft over a year, this would amount to 1,190 acre ft per broiler complex.

[i] Amid a water crisis, Arizona is using lots of it to grow alfalfa to export overseas : NPR
[ii] Note: Five cuttings per year may be an underestimation as 9-10 cuttings is possible.
Arizona Agriculture's Amazing Alfalfa!

If this was extrapolated to 9 B broilers processed each year in the US, this would result in an additional consumption of 205,915 acre ft of water per year: or an American football size column of water that is 39 miles high. Conversely, an improvement of 0.01 FCR would represent a savings of 205,915 acre ft per year, representing a fairly significant 'drop in the bucket'.

Table 6 - Determination of additional water consumed per 0.01 increase in FCR.

FCR	-	1.84	1.85	1.86
BW	-	3.48	3.48	3.48
FI	-	6.403	6.438	6.473
kg corn- 60% of diet	-	3.842	3.863	3.883
kg soy - 30% of diet	-	1.921	1.931	1.942
L Water to grow Kg Corn/ bird	900[1]	3458	3477	3495
L Water to grow Kg Soy/bird	900[2]	1729	1738	1748
(L/bird)		5187	5215	5243
L water diff per 0.01 FCR increase per bird			28	28

1 Million Broilers processed per week.	
Additional water consumed (L) per 0.01 FCR	28,000,000
Additional water consumed (gal) per 0.01 FCR	7,458,720
Acre ft/wk per complex	22.88
Acre feet/yr per complex	1,190
Water saved in US (9 B Broilers) in acre ft	205,915
Football field column of water (miles high)	39

[1] Source: How much water is in common foods and products: USGS Water Science School
[2] 5 Most Water Intensive Crops - Claro Energy Private Limited

IV. CONCLUSIONS

The world is being challenged to produce enough food to feed a population of 9 billion people by 2050. High quality animal protein production that is inextricably linked to water (drinking, cooling, and feed) is a vital component of meeting this global need. Here we present a few ideas or *water for thought* on ways to reduce the water footprint and increase water use efficiency in poultry production.

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WATER INTAKE AND ITS RELATIONSHIP WITH FEED EFFICIENCY OF BROILER

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Water is an essential nutrient for poultry and any deviation in water intake indicates issues related to nutrition, health and/or rearing environment. Williams et al. (2013) found that daily water consumption per bird increased over the course of time (1991 – 140.3 mL, 2000-2001 – 160.5 mL and 2010-2011 – 190.5 mL) and water to feed intake ratio (WI:FI) followed a similar pattern (1.90, 1.98 and 2.02, respectively). Thus, fast growing modern genetic strains of birds consume more water. The impact of dietary changes in water intake and WI:FI should be monitored regularly. For this purpose, a real time water measurement system was developed at the University of New England (Sharma et al., 2021). The system consisted of a 1.4-litre reservoir with a microcontroller to monitor water consumption. This paper summarises the results of five experiments to demonstrate the normal water consumption behaviour of broilers and the effects of nutrition on WI:FI and its relationship with feed efficiency.

In Experiment 1, WI:FI was measured in Ross 308 broilers fed an industry standard ration using eight pens and 14 birds/pen. During d 10 to 24, d 24 to 35 and d 10 to 35, the average WI:FI were 2.22, 1.95 and 2.06 respectively.

In Experiment 2, the effect of phytase at four levels (0, 500, 1000 and 1500 FTU/kg) in standard (PC) and down spec (NC with lower Ca (-1.4 g/kg), avP (-1.5 g/kg) and Na (-0.3 g/kg)) diets on WI:FI in broilers was investigated. During d 0 to 24, diet by phytase interaction ($P < 0.01$) was observed in which NC without phytase had a higher WI:FI (2.42) compared to PC without phytase (2.13). The addition of phytase to NC reduced WI:FI of birds by 11.6% to 13.2% (FTU/kg, 0 – 2.42, 500 – 2.14, 1000 – 2.14, 1500 – 2.10) but WI:FI was not affected when phytase was added to PC (FTU/kg, 0 – 2.13, 500 – 2.14, 1000 – 2.14, 1500 – 2.10).

In Experiment 3, the effect of a wheat-soybean meal (SBM) based diet was compared with wheat-SBM-high meat meal (70-110 g/kg), and wheat-SBM-high canola meal (170-190 g/kg) diets on WI:FI in broilers. During d 10 to 32, WI:FI was higher ($P < 0.05$) in high canola meal treatment (2.19) compared to high meat meal (1.87) and SBM (1.99) treatments.

In Experiment 4, the effect of a high sodium (4 g/kg) diet and necrotic enteritis challenge on WI:FI was investigated. There was diet × challenge interaction on WI:FI during d 13 to 20 where the challenge had no effect on WI:FI in the birds fed the normal sodium diet (2.23 vs. 2.25) but increased ($P < 0.05$) WI:FI by 14.1% in the birds fed the high sodium diet (2.62 vs. 2.99).

In Experiment 5, the effect of a reduced protein diet (-20 g/kg crude protein) with the addition of four insoluble fibre sources (sugarcane bagasse at 20 g/kg, lignocellulose at 10 g/kg, oat hulls at 30 g/kg, and soy hulls at 30 g/kg) on WI:FI in broilers was investigated. During d 10 to 35, the reduction in dietary protein decreased ($P < 0.001$) water intake by 11.5 % and WI:FI by 17 points (2.05 vs. 1.88). The inclusion of sugarcane bagasse, oat hulls, soy hulls or lignocellulose at 10-30 g/kg in a reduced protein diet had no effect ($P > 0.05$) on water intake and WI:FI of broilers compared to the reduced protein control diet. Data from this experiment were used to determine a correlation between WI:FI and FCR, and the results showed a linear correlation between the factors ($R^2 = 0.34$, $P < 0.001$). The increase in WI:FI linearly decreased FCR of broilers during 10 to 35 d of age.

Collectively, the results of these experiments demonstrated the effects of nutrition on water intake and WI:FI and the relationship of WI:FI with feed efficiency of broilers. Further work is warranted to investigate the relationship of nutrition, water intake and litter quality.

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EFFECT OF PROCESSING METHODS ON THE QUALITY DETERMINANTS OF CANOLA MEALS

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Summary

Unlike soybean which is mainly processed by direct solvent extraction, canola is normally processed by pre-pressed solvent extraction or hot pressing. Recently, cold pressing has been proposed as a method that can produce canola meals with better nutritional values for non-ruminants. In this study, we looked at the effect of processing methods on the quality determinants of the canola meals. Besides, pre-pressed solvent extraction, cold and hot pressing we also looked at an optimised pressing method whereby the processing temperatures were controlled to below 100ºC to minimise anti-nutritional factors and to maintain high protein solubility. We also investigated a direct solvent extraction method which essentially by-passes the pre-pressing stage where most of the high heat and pressure processing is taking place.

In general, the canola meal produced by cold pressing, optimised pressing and direct solvent extraction gave higher reactive lysine values and had lower furosine content, which would indicate that the amino acid digestibility of these meals could potentially be higher than those produced by hot pressing and pre-pressed solvent extraction. However, the glucosinolate content of cold pressed canola meals is significantly higher at more than double the values of the other canola meals. This may cause problems when canola meal is used at a high inclusion level in non-ruminant feeds.

I. INTRODUCTION

Canola meal, a by-product of canola oil extraction, is an important protein source in livestock feeding. With its high protein content and favorable amino acid profile, it serves as a viable alternative or complement to other protein sources, such as soybean meal and can support high levels of production efficiencies across livestock species (Canola Council of Canada, 2024; Canola Meal Feeding Guide, 2024). However, the quality of canola meal is significantly influenced by the processing methods employed during oil extraction, which can affect its nutritional properties including apparent metabolisable energy (AME), the content of anti-nutritional factors and the availability of essential amino acids.

Traditionally, the principal oil extraction methods for canola are solvent extraction or mechanical pressing. The solvent extraction method uses pre-pressing before solvent extraction. The mechanical pressing method usually involves the use of high temperatures of 110 to 120ºC and high pressure in a screw press or by cold-pressing whereby the canola seed is pressed in a screw press at temperatures below 60ºC. Cold pressing has attracted a lot of attention in recent years as it promises to produce canola meals with a better amino acid digestibility. While each processing method results in different degrees of oil recovery, they also have a big influence on the quality of the meals including protein solubility, the presence of glucosinolate and AME. Therefore, different processing methods will impact the product yields as well as the quality of the oils and meals. The quality of the canola meal is very much the result from the application of the heat, pressure and time during processing, which can influence the formation of Maillard reaction products, such as Amadori compounds. Furosine is one of the amino acids that is produced from Amadori products during the acid hydrolysis step. Thus, furosine can be considered as a marker indicating the extent of heat

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damage to the protein (Pahm et al., 2008; Tekliye et al., 2019). Increasing furosine formation can significantly impact the nutritional quality of canola meal by reducing the reactive lysine content and therefore, its digestibility in animal feeding.

Cold pressing, which does not involve high heat treatment, usually produces canola meals with elevated glucosinolate levels and possibly high myrosinase activity, which can result in the formation of potentially toxic compounds, such as, isothiocyanates, goitrin, nitriles and thiocyanates that interfere with the function of the thyroid gland and can adversely affect growth performance (Khajali and Slominski, 2012; Tripathi and Mishra, 2007). In contrast, pre-pressed solvent extraction will destroy myrosinase activity and reduce glucosinolate levels in canola meal, but due to the use of high pressure, temperature and long processing time, it will also reduce amino acid digestibility and possibly energy content through more extensive Maillard reactions, producing a meal with lower protein solubility. Similarly, hot pressing, which applies even higher pressure and heat, also yields canola meals with reduced protein solubility and lower glucosinolate levels.

Therefore, while cold-pressing produces canola meals with high protein solubility and possibly higher amino acid digestibility and AME, the level of glucosinolate tends to be higher and there is a possibility of myrosinase activity and microbial contamination. Pre-pressed solvent extraction results in lower glucosinolate levels, but the protein solubility of the meal is also lower. In an attempt to improve the nutritional quality of canola meal, two new processing methods have been introduced. One method is an optimised pressing method whereby the temperatures were controlled below 100^{o}C and with lower processing time to maintain high protein solubility/ digestibility while minimising glucosinolate levels. The other method involves the direct solvent extraction of canola seed, which effectively by-passes the pre-pressing stage that accounts for most of the excessive use of heat and pressure. Normally pre-pressed solvent extraction requires canola seed to be heated at 110 to 120^{o}C in a cooker for 30-60 minutes to reduce moisture content down to 4%. Desolventising and toasting processes normally requires 30-45 minutes at temperatures between 65 to 110^{o}C.

Despite the widespread use of canola meal in animal feed, there is limited comparative research on how different processing methods can affect the critical quality determinants of canola meal. This study aims to address this gap by analysing canola meal samples produced using various processing methods. In this study, 25 samples of canola meal, all originating from the same crop year and of Australian origin were collected from five different processing plants using different processing methods and were analysed without further heat treatment. Their analysis included assessments of protein, oil, crude fiber, KOH protein solubility (KOHPS), furosine, reactive lysine, protein as neutral detergent insoluble nitrogen (NDIN) and glucosinolate. These parameters are essential for the understanding of the nutritional quality and value of canola meal in livestock feeding.

II. METHOD

The canola meal samples were analysed for KOHPS as described by Arada and Dale, 1990. Oil content was determined using AOCS Ba 3-38, crude protein using AOAC 992.23 and crude fibre using AOAC 962.09. Glucosinolate levels were measured according to the SN/T 1868-2009 method. Neutral detergent insoluble nitrogen was measured by conducting neutral detergent extraction procedure on the residue (AOAC 2002.04) and subsequently analysing the residue for nitrogen by AOCS Ba 4d-90.

Lysine content of the canola meals was determined by using Waters AccQ•Fluor™ reagent for post-column derivatisation with L-α-Aminobutyric acid as the internal standard. Hydrolysed samples were analysed by high-performance liquid chromatography (HPLC)

equipped with a fluorescence (FLR) detector. Prior to analysis, samples were hydrolysed with 6 *N* HCl for 24 hours at 110°C (AOAC 982.30 E).

For the determination of furosine content (Dong et al., 2019; Pahm et al., 2008), samples were hydrolysed with 10.6 *N* HCl for 24 hours at 110 °C. Following hydrolysis, samples were processed using reversed-phase solid-phase extraction (SPE). A 0.5 mL aliquot of the filtrate was eluted with 3 mL of 3 *N* HCl and evaporated under a nitrogen stream. The dried samples were reconstituted in 3 mL of a mixture of water, acetonitrile, and formic acid and filtered through a 0.45-µm membrane prior to HPLC injection. HPLC analysis was conducted using a mobile phase gradient of 0.1% trifluoroacetic acid in deionised water (mobile phase A) and methanol (mobile phase B). Furosine was detected by HPLC with ultraviolet-visible (UV/VIS) detector.

III. RESULTS AND DISCUSSION

Table 1 - Effects of processing methods of canola meals on quality determinants.

Processing Conditions	Crude Protein (%)	Oil (%)	KOHPS (%)	Glucosinolate µmole/g	Furosine (%)	Reactive Lysine (%)	P as NDIN (%)	Crude Fibre (%)
Cold pressing	31.6^{a}	12.42^{a}	92.1^{a}	17.37^{a}	0.0398^{a}	1.91^{a}	9.44^{a}	11.5^{a}
Pre-pressed solvent extraction	36.8^{b}	2.42^{b}	56.0^{b}	7.45^{b}	0.1500^{b}	1.92^{ab}	12.48^{b}	13.1^{b}
Hot pressing	35.3^{c}	7.05^{c}	68.3^{c}	8.01^{b}	0.1390^{b}	1.79^{c}	13.50^{b}	11.9^{abc}
Optimised pressing	31.4^{a}	12.34^{a}	85.9^{d}	8.32^{b}	0.0426^{a}	2.00^{d}	9.40^{a}	11.8^{acd}
Direct solvent extraction	34.8^{c}	5.24^{d}	59.6^{b}	7.27^{b}	0.0944^{c}	1.99^{bd}	10.96^{ab}	12.6^{bcd}
P-value	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.01

a-d Mean values within a column with different superscripts are significantly different (Tukey test; $p < 0.05$).

As presented in Table 1, pre-pressed solvent extraction, hot-pressing and direct solvent extraction resulted in higher protein content compared to cold pressing and optimised pressing. However, the oil content of the cold pressed and optimised pressed meals were significantly higher. The KOPHS were significantly higher in canola meals processed by cold pressing and optimised pressing compared to the other methods, indicating they have less protein denaturation and heat damage.

Cold-pressed canola meals exhibited significantly higher glucosinolate levels ($p < 0.001$) which were more than two times higher than meals processed by other methods. This is likely due to the absence of heat treatment.

Furosine content, a marker of heat damage of proteins was significantly lower in cold pressed and optimised pressed meals compared with meals processed by pre-pressed solvent extraction and hot pressing ($p < 0.001$). Interestingly, direct solvent extraction also showed significantly lower furosine content ($p < 0.01$) when compared with pre-pressed solvent extraction and hot pressing.

NDIN results for canola meals processed using cold pressing, optimised pressing and direct solvent extraction were not significantly different ($p > 0.05$). However, NDIN values for cold pressed and optimised pressed meals were significantly lower than those from pre-pressed solvent extraction and hot pressing ($p < 0.01$). Cold pressing, hot pressing and optimised pressing also produced meals with a lower fiber content ($p < 0.05$). More important the optimised pressing and direct solvent extraction gave significantly higher reactive lysine content than cold or hot pressing. However, cold pressing and pre-pressed solvent extraction also gave significantly higher reactive lysine content compared with hot pressing. Although

the direct solvent extraction method did not result in significantly higher reactive lysine content compared with pre-pressed solvent extraction, it was numerically higher at 1.99 compared with 1.92 ($p = 0.08$). However, since the furosine content is significantly different between them, we are optimistic that in future, direct extraction will be able to achieve significantly higher reactive lysine levels when compared to pre-pressed solvent extraction.

IV. CONCLUSION

In summary, this study highlights significant differences in canola meal quality produced by different processing methods. The optimised pressing method demonstrated similarities to the cold pressing method in oil extraction efficiency, but it significantly reduces glucosinolate levels while achieving higher reactive lysine contents. Both methods had minimised protein heat damage, as evidence by their lower furosine content and NDIN values. Interestingly, the direct solvent extraction method also gave significantly lower furosine content compared with pre-pressed solvent extraction and hot pressing.

Our study would seem to suggest that there is still a lot of potential to improve on the quality of canola meal through better processing involving the optimum used of heat, pressure and processing time.

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COMBINATION OF CONSENSUS 6-PHYTASE, CARBOHYDRASE ENZYMES AND BACILLUS BASED PROBIOTICS IMPROVES BROILER PERFORMANCE WHEN LOW ENERGY AND AMINO ACID DIETS ARE FED

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Economic concerns continue to force reassessment of the way poultry diets are formulated. Volatility in raw material markets and increasing costs of feed ingredients continue to put pressure on producers. One strategy being employed is to feed lower protein and energy diets to reduce feed costs. The challenge is to be able to do this without compromising bird performance.

The aim of this study was to investigate the effects of feeding a generally low protein and reduced energy wheat-based diets on performance of male broiler chickens and evaluate whether treatment with phytase and enzyme plus probiotic treatments could ameliorate the negative effects of lowering the nutrient density on performance of broiler chickens. A total of 480 male day-old, Ross 308 broiler chicks were allocated to 4 dietary treatments with 10 replicate floor-pens per treatment (12 birds/pen). Diets were wheat/soybean meal-based and fed *ad libitum* as pellet over three phases: starter (1-10 days); grower (11-24 days) and finisher (25-42 days). The positive control (PC) diets were formulated to provide adequate levels of energy and nutrients. The negative control (NC) diets were reduced in ME (150kcal/kg), digestible lysine, methionine, and threonine by 5%, while there was no reduction in Ca and P levels. The NC diets were fed either unsupplemented or supplemented with Consensus 6-phytase at 1000 FTU/kg diet (PHY) or a combination of 1000 FTU/kg consensus 6-phytase, 2000 U xylanase, 200 U amylase and 4000 U protease/kg of diet and a Bacillus probiotic at 300000 cfu/g feed (COMBO). The consensus 6-phytase is derived from a biosynthetic variant of a consensus bacterial phytase gene assembled *via* ancestral reconstruction with sequence bias for the phytase from *Buttiauxella sp*. Bodyweight and feed intake were measured on days 1, 10, 24 and 42 to calculate performance parameters. Data was analysed using ANOVA in JMP, with means separation by Tukeys, with treatment as a fixed effect.

PHY was able to improve final bodyweight by 7.8% ($P<0.05$) versus the NC and restore it to the level of the PC (2627g vs 2437 g and 2549 g respectively). PHY also significantly improved ($P<0.05$) FCR by 9 points (5.4%) versus the NC and restored it to the level of the PC (1.59 vs 1.68 and 1.63 respectively). Supplementation with the COMBO treatment resulted in further significant ($P<0.05$) improvements in final bodyweight versus both the PC and PHY treatment (2728g vs 2549 and 2627 respectively). For FCR, supplementing with the COMBO resulted in 5 points of improvement ($P<0.05$) versus the PHY treatment (1.54 vs 1.59) and 9 points versus the PC (1.54 vs 1.63).

The diets fed in this trial were reduced in energy and amino acids only, no Ca or P downspec was applied. The results demonstrate the ability of PHY to contribute energy and amino acids and restore performance in birds fed low specification diets. Results further demonstrate that despite PHY compensating for the removed nutrients further performance gains can be seen when other additives are used in top of a phytase. The use of enzymes and other additives can facilitate feeding lower specification diets without loss of performance and give clear production gains to producers.

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PROMOTING RESILIENCE: SUSTAINABLE PROTEIN ALTERNATIVES AND LOW-PROTEIN DIETS FOR CHICKEN-MEAT PRODUCTION

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Summary

The use of soybean meal in livestock production, particularly poultry, has sparked global debate due to its links to deforestation and biodiversity. The debate is complicated when balancing sustainability, welfare, economics, and equality. In Australia, chicken meat is the most consumed animal protein and it plays an important role in national food security. Soybean meal is the primary protein source for poultry and efforts to replace imported soybean meal are driven by concerns about supply chain resilience, which was particularly highlighted during the pandemic. Reduced crude protein (CP) diets, where soybean meal is partially replaced by non-bound amino acid (NBAA), have shown potential to reduce nitrogen excretion, ammonia emissions, and improve litter quality. The authors have investigated the use of NBAA in broiler diets, showing that while poultry are less sensitive to digestive dynamics than pigs, there are limits to how much NBAA can replace intact proteins without compromising growth performance. Recent studies comparing soybean meal, whey protein, and NBAA in broiler diets found that an optimal blend of these ingredients led to superior weight gain and feed conversion. The inclusion of locally available protein-rich ingredients, such as canola meal and field peas, can be used as alternatives to soybean meal. Using a combination of NBAA and protein-rich local feed ingredients has shown promising outcomes to completely replace SBM in our recent studies. This approach should be further tested with ingredients beyond canola and field peas.

I. INTRODUCTION

The unfortunate reality is that the Australian chicken-meat industry is hugely reliant on imported feed ingredients and feedstuffs. Many of the feed ingredients including vitamins, methionine, lysine and other feed-grade amino acids, coccidiostats, antibiotic growth promoters, electrolytes and phosphates are totally indispensable. Amongst the feedstuffs, soybean meal is the prime example with importations in the order of 750, 000 tonnes annually. Thus, locally sourced dietary components are essentially confined to wheat, sorghum, canola meal/seed and limestone.

The inclusion of soybean meal in livestock production has been a debatable topic in recent years globally. The key argument focuses on land clearing and deforestation in South America and their impact on biodiversity and climate change. While biodiversity and climate change are both important challenges to address, such debates often overlook: (1) The balance between equality, sustainability, welfare, and economics (Sustainable Development Goals 1, 2, 8, 10, 13, 15); (2) The fact that soybean meal is a by-product of the human food and biodiesel industries; and (3) The impact of urbanisation on farmland and the subsequent effect of farm relocation on land clearing.

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For example, Western Sydney, where the authors are based, lost 9% of its primary production land from 2016 to 2021 and the worst-affected council areas during this period—The Hills Shire, Blacktown, Camden, and Campbelltown—lost 43%, 39%, 26%, and 19%, respectively (Morrison and Piracha, 2022). Localising food production is crucial for food security, as demonstrated during the global pandemic, and for sustainability, as global food miles account for nearly 20% of total food system emissions (Li *et al.*, 2022).

According to Grain Central (2004), Australia harvested a record canola crop of 8.3 million tonnes in 2022-23, but has a canola crush capacity of only 1.2 million tonnes. This indicates that 86% of Australia's crop is exported as whole canola seed, particularly from Western Australia. Therefore, the prospect exists for the Australian chicken-meat industry to utilise far more locally grown canola and other protein-rich feedstuffs as substitutes for soybean meal in broiler diets. Such substitutions, coupled with the development of reduced-crude protein diets, are certainly not without their challenges as will be discussed. Nevertheless, they potentially have the capacity to reduce at least our reliance on expensive, imported soybean meal to marked extents.

The development of reduced crude protein (CP) diets is gaining more and more attention globally where soybean meal is partially replaced by non-bound synthetic or crystalline amino acids (NBAA). Dietary CP reductions were shown to decrease nitrogen (N) excretion and ammonia emissions (Nahm, 2007), enhance litter quality and reduce the incidence of footpad and other lesions (van Harn et al., 2019). Nowadays, supplementation of lysine, methionine and threonine is typical in broiler diets and supplemental amino acids, for instance, reduced the dietary CP content from 356 to 200 g/kg in a typical maize-soybean meal diet (Pesti, 2009). Hence, reduced CP diets is not a new practice, and dietary crude protein can be further reduced when more and more synthetic amino acids are available at affordable prices. With the focus on digestive dynamics, this paper summarises the research outcomes from the Blue Room Team in their efforts to restore growth performance in broiler chickens fed reduced crude protein diets. Additionally, recent efforts to replace imported soybean meal with locally available protein-rich ingredients are also discussed.

II. DIGESTIVE DYNAMICS AND OPTIMAL NBAA INCLUSIONS

Previously, Liu and Selle (2015) concluded that in conventional diets, feed conversion efficiency may be improved by using rapidly digestible protein and slowly digestible starch in broiler diets; more importantly, protein digestion rates have amore pronounced impact on feed conversion efficiency. Non-bound amino acids require no digestion and are immediately available for absorption. Therefore, it is straightforward to treat NBAA as a rapid protein or nitrogen source, and it was hypothesized that increasing NBAA concentrations in diets would enhance growth performance. This hypothesis initially motivated the authors to investigate reduced crude protein diets started with Moss *et al.* (2018) , and the journey has been both fruitful and enjoyable.

The synchrony between protein-bound and non-bound amino acids was a notable research topic in 1970s when synthetic lysine become available. Efforts were put in to investigate whether lysine HCl utilization was compromised by restricted feeding regimes as opposed to *ad libitum* feeding in pigs (Batterham, 1974, Batterham and O'Neill, 1978, Batterham and Murison, 1981), where an interaction between feeding frequency and lysine supplementation for weight gain was reported and higher feeding frequency enhanced utilisation of Lysine HCl. Recently, we investigated the relevance of this consideration in poultry, particularly broiler chickens on *ad libitum* feeding. Yin *et al.* (2019) offered broiler chickens diets with or without 3.5 g/kg lysine HCl, containing either 10.0 or 12.8 g/kg digestible lysine, from 7 to 28 days post-hatch. Meanwhile, birds were given access to diets for

12, 16, or 20 hours per day. Treatment interactions (P > 0.35) between lysine HCl and feed access intervals for growth performance parameters were not observed. The authors concluded that effective lysine HCl utilisation in poultry, irrespective of feeding frequency (unlike in pigs), may stem from anticipatory feeding behaviour, crop and gizzard functionality, shorted retention time and increased episodes of reverse peristalsis.

While it is reassuring that poultry are not as responsive as pigs to changes in digestive dynamics in conventional diets, it remains important to understand whether there is a limit to how much NBAA can be included in broiler diets at higher levels without compromising growth performance. Baker (2009) pointed out that it has been known for well over 20 years that there are limits to how much intact protein can be replaced by free amino acids in terms of achieving maximal weight gain and feed efficiency of broiler chicks.

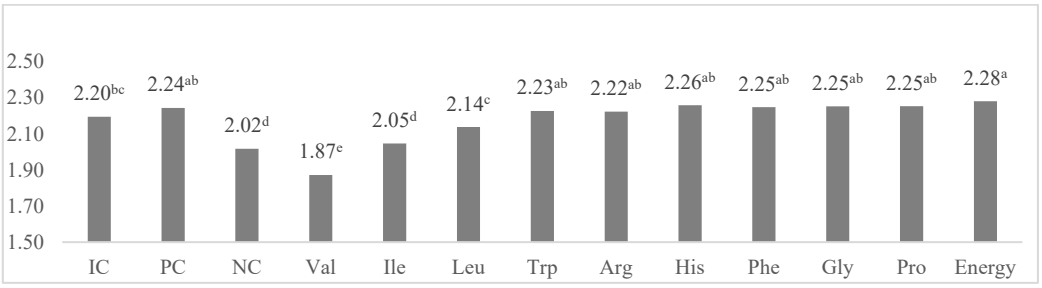
Macelline *et al.* (2022) used a triangular response surface design to compare three diets rich in soybean meal, whey protein concentrates or NBAA to determine the optimal level of NBAA inclusion in wheat-based diets. Superior weight gain and FCR was found in broiler chickens offered an equal blend of soybean meal and whey protein diets. This diet was constituted of 84.3 g/kg whey protein concentrate and 13.4 g/kg NBAA. Quadrative relationships were found between NBAA inclusions and different growth parameters, the mean optimal NBAA inclusion level was 19.23 g/kg across weight gain, feed intake and FCR, above which performance commenced to decline (Chrystal *et al.*, 2021).

Grains with higher inherent protein content may present more challenges when formulating reduced crude protein (CP) diets. This is because, when least-cost formulating iso-energetic and iso-nitrogenous diets, those based on high-protein grains would lead to higher inclusion of cereal grains and NBAA. This was identified as one of the causes of sub-optimal growth performance in wheat-based reduced CP diets compared to maize (Chrystal *et al.*, 2021) and sorghum (Macelline *et al.*, 2023a). A recently completed study by the authors further verified this hypothesis by utilising both high-protein and low-protein wheat grains where reduced CP diets based on high protein wheat depressed growth performance in comparison to the diet based on low protein wheat (unpublished data).

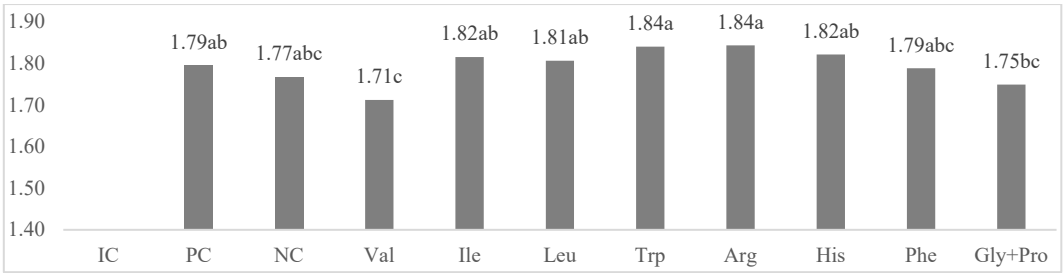
III. AMINO ACID REQUIREMENTS

Two studies were conducted to evaluated the 4th limiting amino acid in reduced crude protein diets based on wheat or maize (Maynard *et al.*, 2020, Maynard *et al.*, 2022). The studies utilised deletion methods, where three control diets were formulated as described in Figure 1, and then each tested amino acid was removed one at a time.

Both studies confirmed the importance of BCAA in reduced CP diets, especially in wheat-based diets where removing Val caused more damage than removing the set of supplemented amino acids. The importance of balanced amino acid profile was further evaluated in Macelline *et al.* (2023c), where different ideal protein ratios may be preferred by conventional and reduced CP diets.



(a)



(b)

Figure 1 - Responses in weight gain when amino acids were deleted one at a time in diets based on wheat (a) and maize (b), IC = Industry Control diet with 210 g/kg CP (Exp 2 did not include IC); PC = Positive Control diet where the CP of this diet was then reduced by 30 g/kg but Lys, TSAA and Thr levels were maintained and NBAA were supplemented to match the amino acid profile of the IC diet; NC = Negative Control where all of the supplemental amino acids, excluding Lys, TSAA and Thr from the PC, were replaced with nonessential N in the form of Glu at the expense of filler to maintain a CP of 186.7 g/kg.

Table 1 - Ideal protein ratios tested.

Amino acid	Ratio A	Ratio B
Lysine	100	100
TSAA	74	75
Threonine	64	66
Tryptophan	16	17
Arginine	104	110
Isoleucine	69	67
Leucine	107	110
Valine	79	77
Histidine	33	35
Phe + Tyr	116	102
Proline	-	140

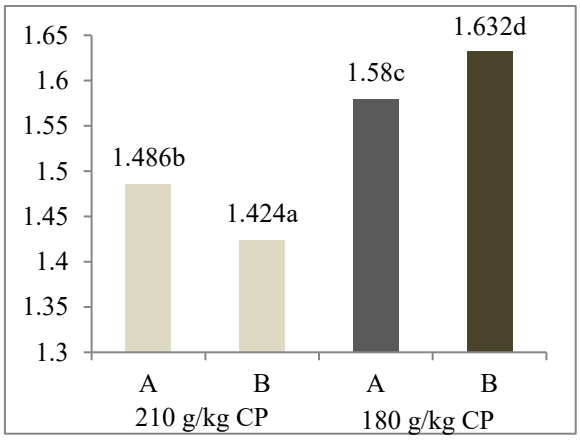


Figure 2 - The impact of ideal protein ratio on FCR in broiler chickens from 14-35 d post-hatch.

IV. PROPOSED SOLUTION

The increased cereal grain and NBAA in reduced CP diets is more likely to lead to imbalance between glucose, protein-bound and non-bound amino acids for absorption and utilisation. This may limit the inclusion of NBAA in the diets; hence, the proposed solution to completely replace SBM is to combine alternative protein sources with a moderate level of inclusion of NBAA. Canola seed and canola meal is produced in large quantities in Australia but their inclusions in broiler diets are capped due to the impact of anti-nutritive factors on growth performance and bird welfare. Our recent study showed that including 5%, 12%, 16%, and 22%

canola products in the starter, grower, finisher, and withdrawal diets, respectively, compromised weight gain, reducing it from 3.73 to 3.60 kg per bird ($P < 0.05$). This reduction was due to decreased feed intake, as no impact on FCR was observed.

Macelline *et al.* (2023b) evaluated the possibility of including 15% canola meal in a reduced CP diets (190 g/kg) to completely remove soybean meal and found that canola meal inclusion did not influence weight gain and FCR ($P > 0.05$) in broiler chickens from 16-35 days post-hatch. Moreover, we tested this approach for the whole production cycle and found that including field peas to diets with moderate CP reduction improved growth performance regardless of the dietary CP levels. These preliminary outcomes are truly encouraging, and the approach should be tested with other available local feed ingredients.

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PRIMING YOUNG BIRDS FOR GUT HEALTH AND RESILIENCE

M.R. BEDFORD[1]

Summary

Minimising enteric disorders and disease is a key goal for any commercial nutritionist, especially in regions of the globe where antibiotic growth promoters are no longer available. Many dietary strategies exist which include optimising nutrient density, digestibility and the use of microbial/immune system modulating additives. Fibre plays a significant role in maintaining the small and in particular the large intestinal microbiota but is mostly overlooked when reviewing intestinal health strategies. This brief review focuses on the types of fibre that the nutritionist should be considering and how a phase feeding strategy should not only take into account the changing needs of the bird as it ages, but also the changing needs of the microbiota if good intestinal health is to be maintained.

I. INTRODUCTION

In a google search the term "Gut health" appeared in 19,400 PDF articles published from 1st Jan 2020 to the 3rd October 2024 and the AI generated summary suggested the main factors influencing gut health included:

1. Diet – Nutrients and additives
2. Water quality
3. Environmental temperature, humidity
4. Management
5. Pathogen exposure
6. Immune system status

Clearly this topic is of great interest and all the factors above are important and need consideration. This is especially the case in regions where antimicrobial growth promoters (AGP's) have either been banned or removed from feeds on a voluntary basis. In such places, the incidence and severity of intestinal disorders and disease has markedly increased, hence the desire to manage gut health has intensified. Animal husbandry (which is involved in factors 2-5 above) plays by far the greater role in determining the likelihood of such disorders, but diet also has an influence. Most dietary interventions intended to manage gut health focus on the avoidance of antinutrients, toxins and excess protein, and the provision of anti-microbial or immune system-stimulatory products. Surprisingly, there is only limited focus on the role that fibre plays in maintaining gut health in broilers (and far more interest in the antinutritive role it can play) and yet it is probably the single most important dietary determinant of intestinal health. One reason for this situation is that the understanding of fibre is poor, made worse by the fact that the routine methods we use for determination of fibre have little value in describing the biological effects it conveys. As such, the fibre content and quality of a diet does not have the same level of scrutiny that other nutrients enjoy, e.g. amino acids. A far better understanding of the dietary content of the different components of fibre and an allocation of biological effect to each is essential if nutritionists are to take full advantage of its value in AGP-free diets. This paper focuses on why fibre is so important, what types of fibre we should consider, and how to ensure that the bird starts well and "learns" how to adapt its microbiota over time and as quickly as possible to utilise fibre as a health promoting substrate.

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II. FIBRE CHEMISTRY

Ideally, the nutritionist should be aware of the fractions of fibre that are rapidly, moderately, and slowly fermented and those fractions which have direct positive (e.g. gizzard development) or negative (e.g. viscosity, accelerating feed transit) effect on the digestive process. Unfortunately, even when the feed industry does consider fibre, it tends to focus on measurements based on methods developed over 150 years ago. This identifies dietary fibre as either crude fibre or nitrogen free extract. Neither of these categories convey any indication of structure or functionality. More sophisticated methods developed in the latter half of last century split fibre components into those fractions remaining after a wash with acid or neutral detergents. This does yield more useful information regarding the quantities of "intransigent" and potentially fermentable fibre components but this information is still quite rudimentary. More recent methods to separate the fibre into:

1. Oligosaccharides
2. Pectins
3. Hemicellulose
4. Cellulose
5. Lignin

This further categorization of fibre is somewhat helpful in identifying whether it might be fermented rapidly, slowly, or not at all, but information is still lacking. Further fundamental characteristics of fibre which influence the effects that it will exert in the intestinal tract of the monogastric are still overlooked. The solubility and size of the fibre along with its complexity of structure have an enormous influence on whether, and if so, where in the intestinal tract, it will be fermented. Each of these characteristics are discussed in more detail below.

III. FIBRE SOLUBILITY AND SIZE

Recent work has demonstrated that only soluble material and extremely small particulates get into the caecum of the chicken (Vanderghinste et al., 2024). The particulates on average are less than 50 microns which limits entry to a very small fraction of the insoluble ileal indigesta. It also suggests that technological processing of the diet likely has little influence on caecal entrance of insoluble fibrous materials. Indeed, to all intents and purposes the caeca should be seen as an organ which mostly processes soluble fibre, thus processes or additives which alter fibre solubility are potential tools for optimising large intestinal health in poultry.

Coupled with the solubility of the fibre is its molecular size. Very small molecules, oligosaccharides, are far more fermentable than their larger counterparts as the latter have to be disassembled before they can be absorbed into the bacterial cell and metabolised (Figure 1.). The larger the molecule, the longer the time needed for disassembly. The initial rate of fermentation is therefore proportionately linked to the size of the molecule but there are two further considerations.

The first is that some oligosaccharides have recently been shown to act as signalling molecules in addition to them being readily fermentable. These "stimbiotics", such as xylo- and arabinoxylo-oligosaccharides (XOS,AXOS) have been shown to dramatically influence the metabolic activity of many significant bacterial species involved in the deconstruction, transport and metabolism of fibre. (Amir et al., 2023). As such they not only increase the capacity of the caeca to degrade and effectively "digest" fibre on behalf of the host, but they also accelerate the development of such a microbiota structure so that the ability to degrade fibre is established earlier in the life of the host. Thus, the evolution of the large intestinal microbiota from a predominantly starch and protein degrading structure to a more stable fibre degrading structure is enhanced and stabilised. This concept is discussed in more detail below.

The second consideration is that very large and soluble polysaccharides, in addition to being sources of more slowly fermented fibre (which is desirable as noted in the next section), they may be detrimental due to their ability (in some cases) to aggregate and form viscous complexes. This can reduce digestibility of all nutrients dramatically and with regards to caecal fermentation and health, the key issue here is whether protein digestion is compromised to the point that the caeca is exposed to excessive soluble protein ingress. Putrefaction of this material can significantly degrade fibre fermentation and result in loss of intestinal integrity and even disease outbreak.

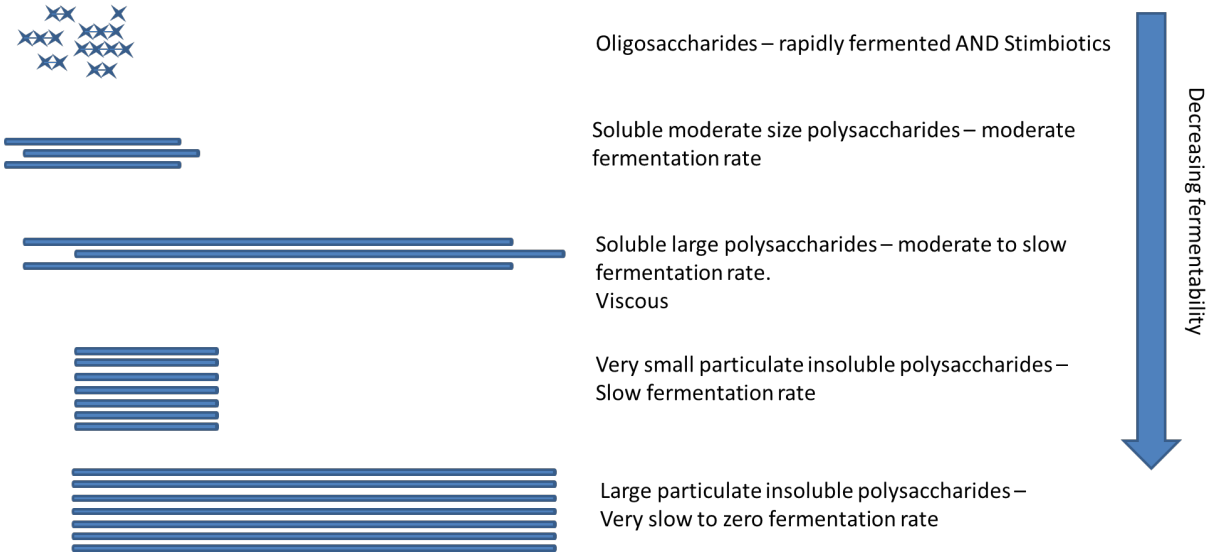

Figure 1 - Schematic of the influence of molecular size and solubility of fibre on its function and fermentability.

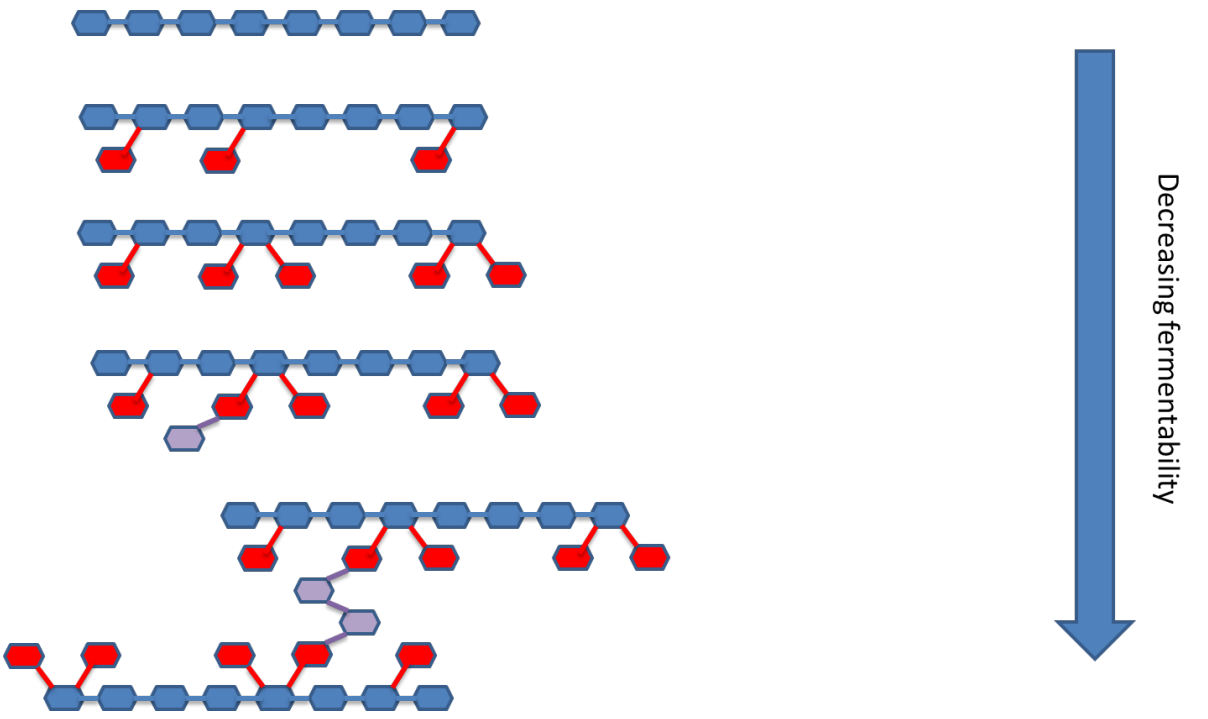

Figure 2 - Schematic of increasingly complex soluble fibre structure starting with an unsubstituted backbone and ending with multiply substituted backbone with phenolic linkages between backbones.

IV. COMPLEXITY OF FIBRE STRUCTURE

The structural complexity of fibre influences the rate at which it can be depolymerised and subsequently metabolised. In general, linear NSP's with very few substitutions can be depolymerised rapidly by just a single, relevant endo-acting enzyme. However, the presence of a few substitutions can limit access of the endo-enzymes to the backbone and thus reduce the rate and extent of depolymerisation. As substitution density and complexity increases, the rate of depolymerisation decreases and the size of the fragments produced increases. Highly substituted fibre demands a significant array of ancillary enzymes that need removal before the backbone can be attacked and the desired oligomers produced. The production of this wide array of ancillary enzymes requires the development of a complex and co-ordinated microbiota which takes time to develop. In general, the most complex fibre structures are fermented only once the "easy to ferment" material has been exhausted. A schematic of fibre complexity is shown in Figure 2.

V. EVOLUTION OF THE HOST MICROBIOTA

At hatch the intestines of the bird are sterile. In modern poultry production the newly hatched chick gets its initial inocula at the hatchery, during transport to the growing sheds and from the sheds themselves including the feeders (and feed) and waterers and numerous other sources. As a result, the structure of the intestinal microbiota is far more variant between individuals and flocks and less suitable for the development of a healthy microbiota compared with nature, where the hens caecal droppings around the nest provide an inocula of relevance to the environment in which the bird has grown. The development of a stable and beneficial microbiota is therefore not assured, and given the absence of prophylactic antibiotic use in many geographies, the opportunities for pathogens to establish are significant. Thus, the strategy for the industry is to ensure a smooth and rapid transition from initial colonizing bacteria to a healthy fibre fermenting community as rapidly as possible.

The evolution of the caecal microbiota is dependent not only upon the initial inocula, but also the substrates to which the bacteria are exposed. The material entering the caeca are the soluble indigesta from the ileum and given the digestive process in the neonate is not fully developed, this means that the caecal residents have a range of substrates to "chose" from, including starch and protein in relative abundance. The availability of such rapidly fermentable substrates favours the development of species which can utilise such material at the expense of those that specialise on more intransigent fibre sources. However, with time the chick significantly increases its ability to digest starch and protein, thus progressively limiting their delivery to the caeca. Coupled with the development of the host digestive capacity, there is also a development of the small intestinal microbiota which effectively remove not only starch and protein but also the more readily fermentable NSP (Davies et al., 2024). At the same time they release soluble NSP from the cell wall structures (Lee et al., 2017) and provide the caeca with more substrate, some of which is highly fermentable (Bai et al., 2024). As the ileal populations mature the supply of soluble fibre to the caeca stabilises with the majority being represented by arabinoxylan, galactan and mannan. Comparison of the sugar composition of ileal soluble fibre with that of the caeca suggests the arabinoxylan is by far the preferred substrate at 42d of age (Lee et al., 2017) and hence the goal should be to develop arabinoxylan fermentation capability as soon as possible. It is most important to recognise that an ideal feed should contain rapidly, moderately and slowly fermented fibre so that all regions of the intestine are presented with substrate. As the bird ages and the caeca mature the availability of greater quantities of slowly fermented fibre may be critical for continued caecal health.

VI. PRIMING OF THE CAECAL MICROBIOTA

From the points made above, it is clear that the sooner the caecal microbiota are adapted to ferment arabinoxylans, the smoother the transition from the neonatal microbiome to a stable adult microbiome. It has been known for a while that exposure of a broiler to a xylanase from first day of age markedly increases the ability of the caecal microbiome to utilise xylose, XOS, soluble arabinoxylans and even insoluble arabinoxylans (Bedford and Apajalahti, 2018). This clearly suggests that priming of the caecal microbiota is indeed possible, but it is not clear what it is that initiates the change. Prebiotic supply (through degradation and thus dissolution of insoluble NSP coupled with depolymerisation of high molecular weight soluble NSP) has been proposed as one of the 3 key mechanisms by which NSPase's function in addition to viscosity reduction and cell wall degradation. Such supply of additional soluble NSP alone would presumably encourage the growth and activity of NSP degrading bacteria in the caeca, which could explain such effects. Indeed NSPases, particularly xylanases, should be of benefit in diets which are limited in soluble fibre content (eg Maize-soy and in particular sorghum-soy diets). However, whether simply supplying additional substrate is sufficient to drive a smooth caecal population transition before the alternative and rapidly disappearing substrates (protein and starch, e.g.) are exhausted, is not clear. Addition of 0.25% AXOS was shown to enrich Bifidobacteria and improve growth rates of broilers fed wheat-based diet suggesting that quantitatively supplying such a substrate may be all that is needed (Courtin et al., 2008). Indeed, addition of 0.5% AXOS to a wheat-based diet resulted in a significant acceleration of NSP fermentation in broilers compared with the control, suggesting not only was the AXOS fermented but also some of the structural NSP in the diet (Bautil et al., 2020). The AXOS was termed a "kickstarter" as it enabled NSP hydrolysis in the ileum and fermentation in the caecum at far younger ages than could be achieved on the control diet. However, concurrent work had shown such effects were possible with much lower addition rates of XOS – 60g per tonne of feed. This is far too low an inclusion rate to result in a meaningful increment in fermentation activity directly (Ribeiro et al., 2018). Indeed, in this work it was suggested added XOS or AXOS generated by exogenous enzymes may alter the metabolism of the resident microbiota. Recent work has corroborated this hypothesis where it was shown that supply of as little as 50g per tonne of feed of a XOS (DP2-6) markedly increased the presence of SusC and SusE proteins involved in the breakdown of complex polysaccharides, binding of the oligosaccharides produced at the outer membrane and transport across the periplasmic space (Amir et al., 2023). Thus it now appears that some of the products of NSPases may not only be prebiotic substrates, but also signalling molecules or "Stimbiotics" (Gonzalez-Ortiz et al., 2019), which radically alter the ability of the caecal microbiota to ferment fibre when present at very low levels in the diet. Whilst these molecules can be produced by xylanases in wheat based diets, they are not produced in corn based diets (Kouzounis et al., 2021), and hence for the stimbiotic effect in corn based diets, external XOS may have to be added. It is likely that differences in xylan substitutions limit the ability of xylanases to break AX down to the relevant DP2-6 XOS hence stimbiotic oligosaccharides are not produced. Indeed not all wheat samples release XOS when treated with a xylanase (Whiting et al., 2023) and hence XOS should be added as an insurance if the stimbiotic effect is to be guaranteed.

VII. PRIMING – THE CHALLENGE AND PROBLEMS FOR THE INDUSTRY

As the bird and the microbiota mature, the number of different carbohydrate sources entering the caeca become reduced such that at maturity the majority of the fermentable carbohydrate is xylan (Lee et al., 2017). If the caecal resident microbiota fail to adapt in time to use the AX then protein becomes the fermentation substrate of choice (Apajalahti and Vienola, 2016) which results in production of destructive metabolites and increases the chance of disease

outbreak. The challenge is to ensure a rapid and smooth transition towards a microbiota capable of utilising the major fibre substrate in the caeca, namely AX, which can be achieved by educating or kickstarting their metabolism as soon as possible by provision of the necessary signalling molecules, namely the AXOS or XOS with low degrees of polymerisation. Once activated, the challenge for the industry is to maintain an adequate flow of soluble AX into the caeca, since insoluble material will likely not be able to enter due to the significant sieving effects of the caecal villi at the caecal entrance (Vanderghinste et al., 2024). It is proposed that a standard corn or sorghum soy diet may be lacking in this regard due to the paucity of soluble AX in each of these grains and as such the utilisation of a xylanase capable of degrading insoluble AX into soluble AX, particularly in the grower and finisher diets where fermentation capacity is maximised, is likely of great benefit. Thus a combination of a XOS or AXOS with a xylanase likely seems to be the most robust strategy for maintaining caecal health particularly in corn or sorghum based diets and perhaps even in wheat based diets where generation of XOS from a xylanase cannot be guaranteed (Whiting et al., 2023).

VIII. CONCLUSION

Nutritionists need to consider the evolution of the needs of the caecal microbiota in their diet formulations more than ever now that the “cure all”, i.e. prophylactic antibiotics, are no longer available in many parts of the world to deal the upsets that occur when fermentation balance is lost. A long-term approach will likely be most successful whereby the nutritionist not only matches the nutrient contents of the diet to suit the requirements of the bird during each phase, but also considers the quantity and type of fibre that should be present in order to encourage the establishment and development of a beneficial caecal microbiota.

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GOBLET CELL POPULATIONS INCREASE FROM PROXIMAL TO DISTAL SECTIONS OF THE BROILER CHICKEN SMALL INTESTINE

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A key strategy to reduce the reliance on soybean meal in broiler diets lies on improving digestive development, gut integrity and digestive capacity. The epithelial architecture along the small intestine reflects a higher absorptive capacity with longer villi in proximal (i.e., duodenum and proximal jejunum) compared to distal (i.e., ileum) segments (Alshamy *et al*., 2018). However, less is known about the association of intestinal function with epithelial cell profiles. The intestinal epithelium in chickens is a mosaic constructed from at least six cell types differentiating from the precursor stem cells present in the crypts (Shyer *et al*., 2015). Differentiated cells start migrating from the crypt towards the tip of the villi and include goblet cells, Paneth cells, enteroendocrine cells, enterocytes and tuft cells. The relative abundance of each cell type determines the functionality of each segment of the intestine. Thus, identifying the environmental factors that may influence cell differentiation is fundamental to understand and manipulate gut physiology and function. The aim of this study was to profile the cell population in the three main sections of the chicken small intestine, duodenum, jejunum and ileum. It was hypothesized that the relative composition of the cell population in the intestinal epithelia reflects the functional needs associated to its segment.

Six (6), 42-day-old broilers (Ross 308) reared under standard conditions (Aviagen, 2022) were used in this study. Tissue samples from the proximal, medial and distal sections of the duodenum, jejunum and ileum were taken for analysis. Histomorphometry was performed in all sections using H&E staining and OlyVIA image software (Ver.2.9.1), was used to measure villus height (VH), villus width (VW), and crypt depth (CD). In addition, PAS/Alcian Blue special staining was used for localising the goblet cell populations (selected as the first cell type in the characterization). The results showed that the VH was significantly ($P<0.05$) higher in the duodenum (1561.95 ± 218,16 µm) than in the ileum (606.11 ± 63.02 µm), the CD was higher in the jejunum (180.66 ± 16.84 µm) than in the duodenum (83.99 ± 8.54 µm). The villi surface area (VSA) showed a significantly ($P < 0.05$) higher density of villi in the duodenum (662839.86 ± 122947.70 μm^2) compared to the ileum (234808.74 ± 18396.68 μm^2). Goblet cell counts averaged 100.5 ± 10.17 per villus in the proximal duodenum,168.9 ± 46.63 in the medial jejunum and 300.3 ± 21.35 in the distal ileum, with 48.5 ± 10.9 per crypt in the duodenum and 63.4 ± 18.28 per crypt in the ileum, indicating a non-linear increase in the goblet cell density from proximal to distal segments. Relative to the jejunum, the goblet cell counts in the villi were significantly lower in the duodenum ($P = 0.01$) and higher in the ileum ($P < 0.0001$). This variation in goblet cell density suggests regional specialization, with goblet cell numbers increasing from proximal to distal sections of the intestine, reflecting a higher mucus secretion capacity distally where the majority of the microbiota will reside. These differences are compatible with a defence function of mucus production.

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XYLO-OLIGOSACCHARIDES: ARE YOU GETTING WHAT YOU PAID FOR?

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Xylo-oligosaccharides (XOS) are valued for their prebiotic properties. XOS have degrees of polymerisation (DP) ranging from 2 to 15, but it is generally accepted that DP 2-7 (X2-X7) have the highest prebiotic activity. Global demand for XOS products has increased the number of XOS manufacturers and products available. The challenge with measuring the XOS concentrations in these products is that there are also impurities and excipients present that have similar chemical properties to XOS, making purification and quantification difficult. Traditional methods generally overquantify analysed XOS concentration, meaning birds fed these products receive considerably less XOS than anticipated. Unscrupulous manufacturers rely on this, and the fact specific XOS testing is challenging, to supply inferior or fraudulent products. Consequently, the aim of this study was to develop an alternate method, that uses simple techniques and common laboratory equipment, to analyse pure XOS concentration in a range of XOS products. The aspiration is that this alternate method will be readily used by XOS customers to determine XOS concentration of products prior to purchasing.

In this study, the XOS concentration of six products, marketed as XOS supplements for poultry diets, were analysed by both a traditional method and a new proposed alternate method. For the traditional method, acid hydrolysis was used to convert polysaccharides in the sample into constituent monosaccharides, which were then measured by High-Performance Liquid Chromatography (HPLC), and the amount of resulting xylose was used to determine total XOS. This method is unable to measure XOS components only, and impurities in the product can be easily misidentified as XOS. To combat this, an alternative method was developed, in which the sample was subjected to a XOS-specific enzyme (D-Xylosidase), which converts only XOS into xylose. Comparing HPLC results before and after enzyme application allowed the total amount of pure XOS to be determined, by quantifying the xylose concentration. Maltodextrin, a common excipient in XOS products, was also quantified by applying α-amyloglucosidase and measuring glucose concentration. Impurities were unaffected by these enzymes, as confirmed using mass spectroscopy.

Table 1 - Comparison of total xylo-oligosaccharide (XOS) concentration (%) claimed by the manufacturer and measured using the traditional and alternate method, and measured range on degrees of polymerisation (DP) in six different XOS products.

Sample ID	Manufacturer Claim	Traditional Method	Alternate Method
	% Total XOS	% Total XOS	% Total XOS
Sample 1	95	77.6	77.3
Sample 2	35	34.0	28.4
Sample 3	35	46.0	3.0
Sample 4	35	27.8	26.3
Sample 5	70	49.5	52.0
Sample 6	95	65.5	67.8

Table 1 illustrates the results of XOS products tested using traditional acid hydrolysis and the alternative enzyme hydrolysis. Sample 3 showed the largest discrepancy between the two testing methods at 43%. This product contained high DP xylan by-products that were readily hydrolysed with acid to xylose, inflating the total measured XOS concentration. In this same product, added low DP maltodextrin appeared as peaks in the X3-X6 range. The alternate method effectively measured the total pure XOS and can be easily implemented by laboratories with a standard HPLC system.

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REPLACING DIETARY SUPPLEMENTED L-ARGININE WITH GUANIDINOACETIC ACID AND L-CITRULLINE IMPROVES FEED CONVERSION RATIO OF BROILER CHICKENS

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Guanidinoacetic acid (GAA) spares L-Arginine (Arg) when supplemented in the diets of broilers as less Arg would be required to synthesise creatine for which GAA is a precursor. In chickens, L-citrulline (Cit) can also be converted to Arg by the successive actions of two enzymes, argininosuccinate synthetase and argininosuccinate lyase in the kidney and other extrahepatic tissues (Dao et al., 2021). Data are sparse for comparing the effectiveness of GAA and Cit individually or in combination for replacing dietary Arg in broiler chickens. Using a completely randomised design, a 42-day study was conducted with 9 dietary treatments for grower (d 10 to 24) and finisher (d 24 to 42) phases. The first three diets included a control diet (T1) supplemented with 0.24% Arg and two semi-deficient and deficient diets that had 0.12% (T2) and no added (T3) Arg, respectively. Treatments 4, 5 and 6 were constructed by replacing 0.06% of Arg with 0.06% GAA (T4), 0.06% Cit (T5) and a combination (T6) of GAA and Cit, equivalent to 0.12% Arg. Treatments 7, 8 and 9 were made by replacing 0.12% of Arg with 0.12% GAA (T7), 0.12% Cit (T8) and a combination (T9) of GAA and Cit equivalent to 0.24% Arg. Both GAA and Cit replaced Arg at one to one ratio without any uplift designated for their energy contributions in the formulation matrix. Grower and finisher diets contained crude protein levels of 19.6% and 18.2% and digestible Arg of 1.28% and 1.18%, respectively. For the first 10 d of age, all birds received a common starter diet. A total of 864 day-old male off-sex Ross 308 chicks were assigned to 72 pens each accommodating 12 birds. Each diet was replicated 8 times.

Feed intake was not affected by the experimental treatments at any stage of the study. Body weight gain (BWG) remained similar between dietary treatments from d 10 to 24 of age. Birds fed fully deficient diet (T3) had the lowest BWG during the finishing phase and when assessed for the entire study (P<0.001). Birds fed T5 had the highest BWG at the grower phase compared only with the deficient diet (T3) and that of T9. When assessed from d 10 to 42, BWG were similar amongst all the treatments except for the birds fed no added Arg that had the lowest BWG (P<0.001). From d 10 to 24, feeding the Arg deficient diet increased FCR, whereas GAA at both levels (T4 and T8) and a combination of GAA and Arg at 0.12% (T9) decreased the FCR (P<0.0001). From d 10 to 42, the inclusion of GAA or Cit either individually or in combination at both dosages significantly improved FCR compared with the control treatment containing 0.24% Arg (P<0.0001). The performance results indicate that 0.24% supplemental Arg can be replaced by an equal combination of GAA and Cit resulting in approximately 2 points of FCR improvement for the combined grower and finisher phases. Similarly, either GAA or Cit replaced Arg up to 0.12%, leading to a decrease in FCR. Arginine is known to be readily catabolised by the liver, a pathway that can be evaded by Cit. Similarly, GAA can have additional benefits by supplying creatine as a source of energy, particularly in the muscle tissues in addition to sparing Arg. To decipher the mechanisms of action of GAA and Cit, a comprehensive analysis of liver transcriptomics is underway.

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CAECUM BACTERIAL COMMUNITIES RESPOND TO DIETARY GRAIN TYPE AND CRUDE PROTEIN LEVELS AND CORRELATE WITH BROILERS' GROWTH

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The symbiotic interactions between host and gut microbes are crucial for poultry production. The caecum, a pair of blind-ended pouches open into the junction of the small and large intestines, hosts a diverse bacterial community with versatile metabolic capabilities (Shang et al., 2018). Our previous study showed that in maize-based diets replacing soybean meal-based crude protein (M-RCP) with synthetic free amino acids (FAA) did not compromise growth and production compared to diets with a standard CP level (RCP: 165 vs. SCP: 222 g/kg). In contrast, replacing CP with FAA in wheat-based diets (W-RCP) led to significantly lower weight gain and an increased feed conversion ratio (FCR) compared to its standard CP counterpart (W-SCP) (Chrystal et al., 2021). The objective of this preliminary study was to understand the impact of grain type and CP level on caecal microbiomes and their relationship to growth performance.

16S rRNA sequencing-based analysis was performed on caecal content from male, off-sex (parent line) Ross 308 chicks fed four experimental diets (reduced/standard CP, maize/wheat-based) from 7 to 35 days post-hatch as stated in Chrystal et al., (2021). The four diet treatment groups had significantly different caecal microbiota at study completion. M-RCP resulted in the highest bacterial diversity, richness, and evenness compared to all other treatments. Both cereal types and CP levels significantly impacted the community structure.

The bacterial classes with the highest relative read abundance across all dietary groups were Clostridia (> 50%), Gammaproteobacteria (> 20%), and Bacilli (> 10%). Using a multivariate generalised linear model, we identified numerous amplicon sequencing variants (ASVs) enriched in maize-based (M) and wheat-based diets (W), as well as in RCP or SCP diets. Notably, the mean relative abundance of M- and SCP-enriched ASVs positively correlated with weight gain (Spearman's $rs = 0.431$, $p < 0.001$; $rs = 0.553$, $p < 0.001$; respectively) and reduced feed conversion ratio (FCR) ($rs = -0.316$, $p = 0.004$; $rs = -0.521$, $p < 0.001$; respectively). Conversely, the mean relative abundance of W- and RCP-enriched ASVs showed negative correlations with weight gain ($rs = -0.285$, $p = 0.01$; $rs = -0.188$, $p = 0.091$, respectively) and was associated with increased FCR ($rs = 0.248$, $p = 0.025$; $rs = 0.319$, $p = 0.004$, respectively). Among the potential growth-promoting ASVs (M- and SCP-enriched), four ASVs were classified as *Limosilactobacillus reuteri*, known as a probiotic (Jiang et al., 2023). In contrast, the W- and RCP-enriched ASVs including potentially saccharolytic *Clostridium saccharolyticum* (synonym: *Fusicatenibacter sp900543115*, 5 ASVs) (Murray et al., 1982) and bacteriocin-producing *Ligilactobacillus agilis* (3 ASVs) (Yoo et al., 2023).

These findings suggest that specific gut bacterial species respond to dietary composition and are associated with poultry growth performance, offering potential for microbiota-targeted interventions to improve digestive health and production in poultry.

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USING PROTEOGENIC AMINO ACIDS TO MODULATE PATHOGENIC BACTERIAL GROWTH IN THE INTESTINAL MICROBIOME OF BROILER CHICKENS

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The gut health of chickens is supported by a balanced microbiota and is damaged by the overgrowth of pathogenic bacteria (Chalvon-Demersay et al., 2021). The concept of "aminobiotics" emerged from the observation that commensal and pathogenic bacteria utilise and sense amino acids in distinct ways (Beaumont et al., 2022). This suggests the potential to use amino acids to selectively promote beneficial bacteria while suppressing the growth of pathogenic bacteria. Our objective is to evaluate the effects of additive amino acids on the growth of intestinal bacteria. The hypothesis is that supplementing amino acids can stimulate or inhibit bacterial growth.

In total, eight avian pathogenic *Escherichia coli* (APEC) isolates from clinical cases of colibacillosis in broiler chickens were tested. Bacterial growth in broth medium with or without the addition of 1 g/L of each amino acid was measured in absorbance (OD600). Each isolate was tested in triplicates. Subsequently, four growth parameters (maximum density, maximum growth rate, lag time and initial growth time) were calculated in R using the package gcplyr (Blazanin 2024). Student's T-test or Mann-Whitney test was used to compare the mean value of each trait for normal distributed and non-normal distributed data, respectively. Cysteine completely inhibited the growth of APEC (p <0.05). Histidine and phenylalanine significantly ($p < 0.05$) delayed the initial growth time by 2.6 and 1.5 hours. In contrast, asparagine, aspartic acid, glutamic acid, glutamine, isoleucine, proline and tryptophan significantly ($p < 0.05$) shortened the lag time and initial growth time by 1 to 2 hours. Lysine significantly ($p < 0.05$) shortened the lag time and increased the growth rate. of the APEC

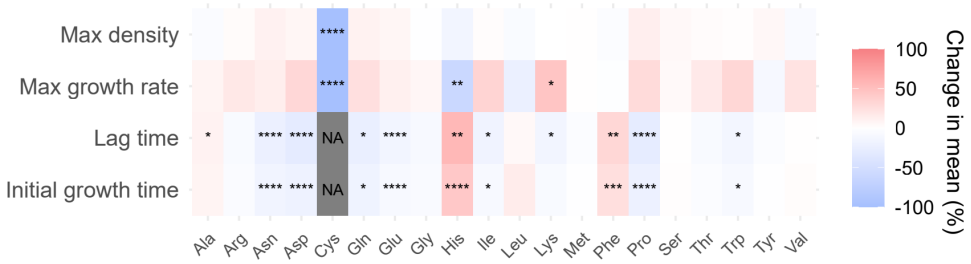

Figure 1 - Changes in the mean values in each growth trait of APEC in the presence of 20 amino acids. *$p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ and **** $p < 0.0001$.

In summary, cysteine, histidine and phenylalanine exhibited inhibitory effects on APEC while asparagine, aspartic acid, glutamate, isoleucine, lysine and proline demonstrated promoting effects on the growth of APEC.

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INCORPORATING AN ESSENTIAL OIL AND SAPONIN BLEND AS PART OF AN EFFECTIVE COCCIDIOSIS MANAGEMENT PROGRAM

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Summary

Coccidiosis continues to be a top challenge in the poultry industry and growing evidence of reduced sensitivity to traditional anticoccidials like ionophores and chemicals drive the need for solutions. Here we evaluate different programs for managing coccidiosis utilizing traditional anticoccidials and an essential oil and saponin blend under different challenge conditions in broilers.

I. INTRODUCTION

The estimated cost associated with coccidiosis is between 10 and 16 billion USD globally (Blake et al., 2020). The economic cost associated with coccidiosis is not only derived from the cost of therapeutics and prophylactics such as ionophore and chemical coccidiostats, but also the associated and performance loss. *Eimeria* spp., can also be predisposing factors to secondary bacterial challenges that could also increase the economic impact. For several decades, chemicals, ionophores and chemical/ionophore blends have been available and used to control coccidiosis. Given the lack of new coccidiostat development and the increase in anticoccidial resistance observed in *Eimeria,* producers have been seeking alternative solutions to managing coccidiosis. *Eimeria* spp. directly damage intestinal integrity which is associated with poorer nutrient digestion and absorption and inflammation (Souza et al., 2024). Therefore, managing coccidiosis can consider both the direct and in-direct challenges of *Eimeria* spp. infections.

Both saponins (Alghirani et al., 2022) and essential oils derived from oregano (Guar et al., 2018) have anti-parasitic properties whereas citrus oil has been demonstrated to be anti-inflammatory (Yang et al., 2023). Specific essential oils and saponins were selected and formulated into an essential oil + saponin blend (EOS) based on their functional properties that support birds through a coccidiosis challenge. This EOS blend was evaluated under various *Eimeria* or *Eimeria* + *Clostridium perfringens* challenge models and compared to traditional coccidiosis management strategies.

II. METHOD

Two studies were conducted to evaluate a commercially available product (AccuGut^{TM} C.1, dsm-firmenich, Animal Nutrition and Health, Switzerland) compared with traditional coccidiosis management programs. This commercially available product contains a proprietary blend of phytogenic compounds from oregano, citrus, and *Yucca schidigera*. Study 1 utilized Cobb 500 males in a 42-day trial with 4 treatments and 7 pens per treatment containing 25 birds per pen raised on used litter. The dietary treatment groups are outlined in Table 1 and dietary treatments were supplemented on top of a corn-soybean meal based basal diet. All challenged groups were given 5,000 oocysts *Eimeria maxima* orally per bird on day 14 and 1.0×10^8 CFU of *Clostridium perfringens* per bird applied in the bottom of tube feeders on day 19, 20 and 21.

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Study 2 utilized Ross 308 males in a 35-day trial with 6 treatments and 10 pens per treatment containing 25 birds per pen raised on fresh litter. The dietary treatment groups are outlined in Table 2. All challenged groups were given 21,800 oocysts *Eimeria acervulina,* 13,300 oocysts *Eimeria maxima,* 5,200 oocysts *Eimeria tenella* orally per bird on day 14. For both studies, performance metrics, mortality, and lesion scores on day 21 were recorded. Data were analyzed utilizing the Glimmix procedure of SAS (Study 1) and JMP (Study 2) with significance reported for $P < 0.05$.

Table 1 - Study 1 treatment groups by feeding phase.

Treatment	Day 0 – 21	Day 21 – 35	Day 35 – 42
Challenged control (CC)	Basal diet	Basal diet	Basal diet
CC + Nicarbazin to Narasin (N-N)	Nicarbazin	Narasin	Narasin
CC + Nicarbazin to EOS (N-EOS)	Nicarbazin	EOS	EOS
CC + EOS (EOS)	EOS	EOS	EOS

Nicarbazin (125 ppm); Narasin (60 ppm); EOS = essential oil + saponin (125 ppm).

Table 2 - Study 2 treatment groups by feeding phase.

Treatment	Day 0 – 21	Day 21 – 35
Non-challenged control (NCC)	Basal diet	Basal diet
Challenged Control (CC)	Basal diet	Basal diet
CC + Salinomycin (S)	Salinomycin	Salinomycin
CC + Nicarbazin/Narasin to Salinomycin (NN-S)	Nicarbazin/Narasin	Salinomycin
CC + Nicarbazin/Narasin to EOS (NN-EOS)	Nicarbazin/Narasin	EOS
CC + EOS (EOS)	EOS	EOS

Salinomycin (60 ppm); Nicarbazin/Narasin (40 ppm Narasin + 40 ppm Nicarbazin); EOS = essential oil + saponin (125 ppm)

III. RESULTS

In Study 1 for the 42-day period, N-N, N-EOS, and EOS reduced ($P < 0.05$) the 42-day FCR compared to the CC whereas body weight gain was increased ($P < 0.05$) for N-N and N-EOS compared to CC and EOS. Lesions scores were reduced ($P < 0.05$) in birds fed N-N (0.14), N-EOS (0.29), and EOS (0.43) compared to the CC (0.90) and there was no statistical difference in mortality although numerically less in N-N, N-EOS, and EOS treatments.

In Study 2 for the 35-day period, the CC had numerically increased mortality (4.09%) compared to the NCC (1.36%) whereas each of the other treatments were intermediate and not statistically different from each other: S (2.27%), NN-S (0.45%), NN-EOS (0.45%) and EOS (0.91%). Body weight gain was reduced ($P < 0.05$) by 23 g and FCR was increased ($P < 0.05$) by 13 points for the CC compared to the NCC. Birds that received a dietary treatment (S, NN-S, NN-EOS, or EOS) were numerically intermediate the NCC and CC and were not statistically different from each other.

IV. DISCUSSION

The challenge model in Study 1 was effective in producing lesions associated with necrotic enteritis which is one potential secondary challenge associated with coccidiosis. The lesion scores observed in the CC were relatively mild as was the mortality that were confirmed with lesions (2.85% for the CC). These would indicate the model used in this study was relatively mild; however, the performance impact was greater than anticipated. Based on the Cobb 500 guidelines, males should have a body weight gain of 3.5 kg and cumulative feed conversion of 1.53 by 42-days. In Study 1, CC birds body weight gain was 2.2 kg with a cumulative feed conversion of 1.94 which is a 37% and 27% reduction, respectively, compared with the breed

standard. Although we cannot deduce if the performance reduction was solely based on challenge, it is a relatively large deviation from the performance guideline. Incorporating Nicarbazin in the starter feed with either EOS or S inclusion in the grower and finisher phases was effective in increasing body weight gain and reducing feed efficiency for the 42-day period indicating that both programs could be effective solutions. Nicarbazin has continued to be an effective solution against coccidiosis; however, the mode of action is poorly understood (Noack et al., 2019). There is also some evidence to suggest that there may be development of reduced sensitivity to nicarbazin in some regions (Kraieski et al., 2021).

In Study 2, two commonly used programs (Salinomycin in the starter and grower) and a Nicarbazin/Narasin blend in the starter and shuttled to Salinomycin in the grower) were evaluated and compared with a Nicarbazin/Narasin blend in the starter shuttle to EOS in the grower and EOS in the starter and grower. The challenge model used in this study was slightly more severe compared with Study 1 in terms of mortality, but less in terms of performance with only a 12% reduction in body weight gain and 7% reduction in FCR. Given that all of the mitigation strategies had a response that was intermediate in value compared to the NCC and CC, we can conclude that these programs were effective in increasing performance in this study.

Kraieski et al. (2021) also concluded that FCR appears to be a good indicator for sensitivity for anticoccidials. This is in agreement with Souza et al. (2024) that demonstrated the relationship between *Eimeria* challenge on intestinal integrity and the associated impact on nutrient digestion and absorption, inflammation, and susceptibility to other diseases that would ultimately reduce the efficiency of which nutrients and energy are utilized for growth.

V. CONCLUSION

By incorporating the essential oil and saponin blend in a shuttle or standalone program, birds showed improved body weight gain and feed efficiency compared to the challenged control groups. The increased prevalence of anticoccidial resistance in *Eimeria* further drives the need to incorporate rotation (from one production cycle to the next) and shuttle (within one production cycle) programs and other solutions like essential oil and saponin blends that can be considered as part of effective coccidiosis management programs.

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IMPACT OF PAST, PRESENT, AND FUTURE FEED ADDITIVES ON THE CHICKEN GUT ECOSYSTEM: THEIR ROLE IN PRODUCTIVITY AND SUSTAINABILTY

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Summary

Optimal gut health is of vital importance to the performance of poultry to be able to perform to their genetic potential. Understanding a 'healthy gut' requires knowledge of the functional interactions of all components of the enteric ecosystem: the host, the microbiota, and the environment. The connections between these diverse physiological features of the enteric ecosystem underscore the extent of areas encompassed by the gut and the difficulty in correlating specific components of gut health with the ability to regulate poultry performance. Antibiotics used as growth promoters (AGP) have successfully controlled dysbiosis and enteropathogens for the past 70 years. However, the recent increase in worldwide non-AGP poultry production is challenging the industry in management, health, and animal welfare. Therefore, there has been a major focus on the development of alternatives to antibiotics that have concentrated on those directed towards the microbiota especially including pre-, pro-, and postbiotics. However since, the host is under selective pressure to ensure that the microbiota becomes and remains beneficial, there is now an increased focus on how the host can exert control over their microbiota and the development of novel antibiotic alternatives that can be directed towards modulating immunity, barrier function, the gut-brain axis, and intestinal epithelial functional metabolism.

I. INTRODUCTION: INTESTINAL ECOSYSTEM

A functionally reliable and healthy gut is of vital importance to the modern broiler to be able to perform to their utmost genetic potential and maintain sustainable production (Kogut et al., 2022b, 2022c). A healthy gut requires functional interactions between the components of the intestinal ecosystem: the host, the gut microbiota, and the environment, especially diet, but including mycotoxins, infections, and temperature (Barron and Young., 2021). The connections between these diverse physiological and ecological features of the gut emphasize the difficulty in correlating specific components of gut health with the ability to regulate poultry performance (Stanley et al., 2013). As an ecosystem, the interactions between the microbiota and the avian immune system regulate multiple pathways in enteric homeostasis and disease (Broom and Kogut, 2018; Zenner et al., 2021). The microbiota composition and biogeography of microbial communities in the gut are shaped by the constitutive functions of the host components of ecosystem (immune response and epithelial cells), while, in return, the microbiota influences the induction, development and training of the host's immune system (Belkaid and Hand, 2014; Ducatelle et al., 2023). This intimate interaction induces the protective responses to pathogens, prevent commensals from over exploiting host resources, and maintain the regulatory pathways that mediate the sustained tolerance to the commensal microbes.

Commercial poultry are unique in the establishment of the microbiota and gut development. In current commercial production of chickens, chick[1]s are hatched without the presence of adult hens in a clean, sanitary hatchery environment (Zenner et al., 2021; Ducatelle et al., 2023) although it is now known that some transfer of maternal microbiota into the embryo does occur (Ding et al.,2017; Lee et al.,2019). Consequently, the commercially hatched chicks are more reliant on environmental sources of microbes to colonize the intestine which soundly affects the diversity of the gut microbiota (Mancabelli et al., 2016). Moreover, the development

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of the gut in neonatal chicks is hindered by the delay of access to feed for 1-3 days post-hatch (Kpodo and Proszkowiec-Weglarz, 2023). Lastly, neonatal poultry exhibit a transient susceptibility to infectious diseases during the first week of life. This susceptibility is largely due to a qualitative impairment of the avian innate and acquired host defenses characterized by 1) the general failure of T cells to proliferate and secrete cytokines; 2) a reduced ability to produce immunoglobulin; and 3) a functional inefficiency of heterophils and macrophages for the first 7-21 days of life for chickens and turkeys (reviewed in Kogut, 2022a).

In modern broiler production, the first week post-hatch represents about 25% of the post-hatch production period, so managing gut health during this period is central to overall broiler production. Therefore, the establishment of a healthy and functionally beneficial neonatal intestinal microbiota in poultry is unlikely and linked to exposure to environmental microbes in the hatchery, transport trucks, and on the farm. Consequently, the normal development, maturation, and programming of the immune system and other physiological functions involved in early life intestinal homeostasis is arguably affected (Kers et al., 2018). It is generally agreed that it takes about 3 weeks for the microbiota to reach a stable, mature state (Carrasco et al., 2019) although this can vary greatly depending on farm management and bird genetics (Zhao et al., 2013; Oakley et al., 2014). The slow development of functional efficiency of the chicken innate system mention above, is undoubtedly related to the 3-week development of a mature gut microbiota (Kogut, 2019; Kogut, 2021b, 2022).

II. ANTIBIOTIC GROWTH PROMOTERS AND THE GUT ECOSYSTEM

For over 70 years subtherapeutic concentrations of antibiotics as feed additives were used for improving growth promotion in food-producing animals including poultry (Castanon,2007). However, increased antibiotic resistance in food animals has led to the complete ban on antibiotic growth promoters (AGPs) in animal feeds by the European Union in 2006 (E.U. regulation 1831/2003/EC; European Commission, 2003), China in 2020 and subsequent FDA request for the voluntary removal of medically important AGPs from animal feed in the United States (Thanner et al., 2016). The US federal government has begun enforcing the Food and Drug Administration Veterinary Feed Directive in 2017 that significantly reduced the availability and usage of antibiotics as growth promoters in farm animals (FDA 2017).

Notably, no consensus, based on scientific evidence, has ever described as the mechanism(s)of action of AGPs. Remarkably different antibiotics with diverse mechanisms and targets of action, induced growth promotion and increased feed efficiency in poultry. Evidence thus far has separated the effects of AGPs into two basic gut ecological targets, the microbiota and the host.

Several hypotheses have evolved to explain how subtherapeutic levels of AGPs affect the microbiota: (1) limiting opportunistic pathogens and subclinical infections (Brussow, 2015); (2) reduced nutrient competition by microbiota (Kohl et al., 2018); (3) alteration or reduction of microbiota metabolites that regulate signaling pathways (Foley et al., 2019; Plata et al, 2022) and (4) enrichment of the cecal microbiota with butyrate and lactic acid-producing bacteria (Kairmi et al., 2024).

Alternatively, AGPs may function by directly reducing the energy cost of constant low grade inflammatory response in the gut resulting from persistent interactions with infectious and non-infectious environmental stressors (nutrient, physical, chemical, biological stimuli) (Kogut et al., 2018; Dal Pont et al., 2021, 2022c). Thus, AGPs promote an immunometabolic balanced environment in the intestine that promotes the growth of beneficial commensals and increases the production of beneficial metabolome including small chain fatty acids, indole, and bile salts (Fouard et al., 2021; Bansai et al., 2020) which consequently increases

intestinal enzymatic activity, metabolism, and energy harvesting which results in weight gain increased production.

III. MICROBIOTA-CENTERED ALTERNATIVES TO ANTIBIOICS

The intestinal microbiota and microbiome embody a biologically active organ within the gut that provides functional benefits to the host. Manipulating the intestinal microbiota has served as the most promising therapeutic paradigm; albeit not a new concept for the poultry industry as evidenced by competitive exclusion where newly hatched chickens could be protected against colonization by *Salmonella enteritidis* by dosing a suspension of gut contents derived from healthy adult chickens (Rantala and Nurmi, 1973). This concept of adding beneficial bacteria to the intestine has led to the development of probiotics and prebiotics. Unlike the host genome, which is rarely manipulated by xenobiotic intervention, the microbiome is readily changeable by diet, ingestion of antibiotics, infection by pathogens and other host- and environmental-dependent events. The plasticity of the microbiome has been implicated in numerous disease conditions, and an unfavorable alteration of the commensal structure of gut microbiota is referred to as dysbiosis; this includes a reduction in the number of tolerogenic bacteria and an over-growth of potentially pathogenic bacteria (pathobionts) that can penetrate the intestinal epithelium and induce diseases in certain genetic or environmental context.

Feed additives that affect the microbiota taxonomic composition have received a massive amount of attention in research over the last two decades with many commercial products approved and used in the field (Carrasco et al., 2019; Yadav and Jha, 2019; Ayalew et al., 2022). Most commercially available microbiota-centric feed additives are used to prevent enteric bacterial infections, growth promoters, or stabilizers of the microbiota during dysbiotic conditions (Carrasco et al., 2019; Yadav and Jha, 2019; Ayalew et al., 2022; Zhou et al., 2023). However, an ongoing issue is that many of the studies on these products in poultry have failed to establish a cause-effect relationship between the physiology, growth promotion, and reduction in enteric infections to changes in the taxonomy in the gut microbiota (Kogut, 2019, 2022a). Thus, the open question left is whether changes in the gut microbiota are a consequence or a cause of the growth promotion and/or the reduction of enteric infections.

Alternatives to antibiotics can be divided into biological and chemical additives that have varied impacts on the chicken. Biological additives include the ‘biotcs’ (pre- pro-, post-, syn-) and exogenous enzymes have been directed to modulation of the microbiota composition, growth promotion, and pathogen prevention (Yadav and Jha, 2019; Ayalew et al., 2022). Chemical additives include organic acids essential oils, and phytochemicals (Kikusato, 2021; Urban et al., 2024; Yadav et al., 2024). The list of the predominant microbiota-centric alternatives to antibiotics and their mechanisms of action are summarized in Table 1.

IV. HOST-DIRECTED ALTERNATIVES TO ANTIBIOTICS

As discussed above, the paradigm is that the microbiota is the primary effector of the host gut physiology by providing nutrients, protecting the host from infection, and promoting immune development and maturation that is critical for a healthy gut (Kogut, 2019; Carrasco et al., 2019; Kogut, 2022). However, ecologically speaking, the host is under selective pressure to ensure that the microbiota becomes and remains beneficial. This pressure is mediated by several host controlled mechanisms that can affect the microbiome biology. In a new review, Wilde and colleagues described several host-derived mechanisms focused on the mammalian microbiome that can be the basis to develop novel alternative strategies to manipulate the microbiota of poultry (Wilde et al., 2024).

Table 1 - Microbiota-centric alternatives to antibiotics.

Approach	Rational	Mechanism(s) of action
Prebiotics	Supply complex food product not digestible by host to stimulate specific members of microbiota	Provide nutrients, prevent pathogen adhesion, stimulate host immunity, improve gut barrier function
Probiotics	Replace a presumed "missing" organism(s)	Competitive exclusion, release antimicrobial factors, stimulate host immunity, improve gut barrier function
Synbiotics	Supply a complex of a microbe or microbes with a prebiotic meant to be used by these organisms to replace a missing function in a microbiome	The prebiotic substance used in the synbiotic selectively favors the growth and metabolite production of probiotics.
Postbiotics	Any substance released by or produced through the metabolic activity of the microorganism, which exerts a beneficial effect on the host, directly or indirectly	Improve potency of active microbes**:** immunomodulatory, anti-inflammatory, antioxidant
Exogenous feed enzymes	Diets that contain different anti-nutritive factors that impede normal digestion and nutrient absorption	Digest different anti-nutritive factors (non-starch polysaccharides) that impede normal digestion & nutrient absorption; supports growth of beneficial bacteria
Phytobioitcs	the plant-derived products: (1) herbs (flowering, nonwoody, and nonpersistent plants), (2) botanicals (entire or processed parts of a plant such as roots, leaves, and bark), (3) EOs (hydrodistilled extracts of plant volatile compounds), and (4) oleoresins (extracts based on nonaqueous solvents)	Growth promotion, gut morphology reinforcement, nutrient digestion/absorption, biofilm control, immunomodulation; anti-oxidant activity
Organic acids	Those acids built on a carbon skeleton, known as carboxylic acids, which can alter the physiology of bacteria, causing metabolic disorders that prevent proliferation and cause death.	The supplementation of organic acids at the right high doses in animal feed can increase the bodyweight, improves feed conversion ratio and reduces colonization of pathogens in the intestine.

The four main host-directed mechanisms include the immune system, the intestinal barrier function (physical and chemical), the gut-brain axis (neuroimmune control of gut transit and peristalsis), and the intestinal epithelial cell physiology (Wilde et al., 2024) (Table 2). Coincidentally, the understanding the mode of action and modulation of these host control mechanisms were a primary focus of the European Union's Horizon research and innovation program in the 2022 STAR-IDAZ International Research Consortium on Animal Health report on the Research Roadmap Development for Alternatives to Antibiotics (Star-Idaz, 2022)).

Table 2 - Host-Directed Mechanisms to Control Microbiota.

Approach	Rational	Mechanism of Action	Modulation
Immune response	Innate: use PRRs[1] to bind MAMPs[2] Acquired: identify & change chemical ligands by Ag specific receptors	Innate: production of AMPs[3], cytokines; Trained immunity: epigenetic & metabolic reprogramming of innate immune cells Acquired: sIgA, cytokines	• Segmented filamentous bacteria • PRR agonists and antagonists (β-glucan, oxidized low-density lipoprotein • Cytokines, • Dectin-1, mannans, yeast fraction • Nanobodies • Extracellular vesicles
Barrier function	Physical barrier: Limit microbial contact with host cell; Allow metabolites to pass both directions Chemical barrier	Tight junction (TJ) proteins seal paracellular space Mucus, AMPs, IgA	• Dietary (tryptophan, fiber, glutamine, Vitamin D, SCFA, polysaccharides) • Extracellular vesicles
Neuroimmune interactions: Gut-brain axis	Bidirectional biochemical communication between neurons and immune cells regulate homeostasis and disease.	Bidirectional signaling cytokine receptors on neurons; Neuro-transmitter receptors on immune cells. Neurotransmitter production by microbiota	• Neurotransmitters, • MAMP agonists, • DREADDs[4],
Epithelial cell function/metabolism	Sense/respond to microbial and immune cell signals (mitochondrial signaling platform) Impose ecological control mechanisms: shape spatial and nutritional niches throughout gut Specialized functional cells	Paneth cells: production of AMPs Goblet cells: mucin secretion Enteroendocrine cells: secrete hormones regulating gut physiology Enterocytes: absorptive (nutrient/H_2O) and metabolic functions (mitochondria)	• Microbial metabolites (Butyrate, 2^o bile acids) • Growth factors (EGF, KGF) • Pharmaceuticals that target epithelial cell metabolism: modify mitochondrial function/dysfunction • Nanobodies • PRR agonists and antagonists • Cytokines

[1]PRRs = pattern recognition receptor
[2]MAMPs = microbial associated molecular patterns
[3]AMPs = antimicrobial peptides
[4]DREDDS = designer receptors exclusively activated by designer drugs

a) Immune response

An effective host immune response to pathogens in the earliest stages of infection is a critical determinant of disease resistance and susceptibility. One promising approach involves host-

directed immunomodulatory therapies, whereby natural mechanisms in the host are exploited to enhance therapeutic benefit. The objective is to initiate or enhance protective antimicrobial immunity while limiting inflammation-induced tissue injury. The advantages of modulating the innate response are threefold: (1) induction is rapid, (2) non-specificity of the response allows for cross-protection against unrelated pathogens, and (3) different levels of therapeutic potential, i.e., prophylactic affects, adjuvant effects, systemic and local protection, and multiple immune cellular targets (reviewed in Kogut, 2022a).

The concept of 'trained immunity' or innate immune memory has emerged and challenged conventional paradigms of T and B cell-mediated adaptive memory. Essentially, trained immunity is induced after either a primary infection or innate modulation and confers protection independently of T or B cells, is mediated by innate immune cells, and increases resistance to infection by the same and other pathogens (Verwoolde et al., 2020; Subhinnasinghe et al., 2024; Yoshimura et al., 2024). Trained immunity differs from immune priming since after recovery from infection, ate immune responses do not return to the steady-state level due to the epigenetic and metabolic reprogramming of innate immune cells rather than the short-lived change of state seen in immune priming. Stimulation of the innate immune response with prominent microbial components that activate pattern recognition receptors (PRRs) offer therapeutic and adjuvant potential in poultry since they not only directly stimulate arrange of cell types but can give rise to robust adaptive responses through driving maturation of antigen presenting cells.

b) Intestinal barrier

The intestinal epithelium is a critical component of a communications network that is essential for transmitting signals generated in response to infection with microbial pathogens to cells of the innate and acquired immune systems in the underlying intestinal mucosa (Winkler et al, 2007; Artis, 2008). Intestinal epithelial cells are in a continuous state of response to the normal microbial ecology and through their products, regulate the composition of this community. Because of these functions, the epithelium is considered as a "microbial sensor" (Artis, 2008). Like professional immune cells, recognition of structural components of microbes by epithelial cells is a primary influence on the development of immune responses. Specifically, toll-like receptors (TLRs) and the NOD-like receptors (NLRs) are required for microbial recognition, gut homeostasis, and induction and regulation of the innate and adaptive immune responses (Kim et al., 2004; Rakoff-Nahoun et al., 2004). Recognizing components of microbes' triggers both innate and adaptive immune responses that eliminate pathogens to shape the intestinal microbiota, including the synthesis of antimicrobial peptides, pro-inflammatory cytokines and chemokines (Trinchieri and Sher, 2007), as well as the secondary anti-inflammatory responses required for the resolution of inflammation (He et al., 2007). Thus, modulating the intestinal barrier in poultry would have valuable consequences on the gut development, health and performance of the birds.

c) Neuroimmune interactions: gut brain axis

The gut-brain axis is a bidirectional communication system connecting both gut and brain across the intestinal epithelial barrier (IEB) and blood-brain barrier by neural, neuroendocrine, immune, and metabolic systems (Cao et al., 2021; Jadhav et al., 2022; Beldowska et al., 2023). The intricate interplay between the nervous and immune systems is vital for maintaining tissue balance and combating diseases. Signaling molecules and pathways, including cytokines, inflammatory mediators, neuropeptides, neurotransmitters, chemoreceptors, and neural pathways, facilitate this complex communication. They establish feedback loops among diverse immune cell populations and the central, peripheral, sympathetic, parasympathetic, and

enteric nervous systems within the intestine. Further, the neuroimmune barrier is also very involved in the transit of material through the intestine. Smooth muscle regulates the contractions of peristalsis and is mediated by neurons of the enteric nervous system.

d) Epithelial cell functional metabolism

The intestinal epithelial cells (IELs) are vital facilitators of intestinal homeostasis by controlling microbiome physiology by functionally supporting the establishment of an immunometabolic environment imposing ecological control mechanisms' (Byndloss et al., 2018). Besides the physical barrier described above, the intestinal epithelial barrier contains several specialized cell types to influence the composition of the microbiota population such as Paneth cells which produce antimicrobial peptides in the small intestine, and goblet cells which secrete mucin. The gut also contains a large number of enterochromaffin cells (endocrine cells that produce serotonin) are dispersed among the intestinal epithelial cells of the chicken (Hiramatsu, 2020) The gut endocrine cells secrete peptides signaling substances into the lamina propria of the gut lining, where they have regulatory activity on the enteric nervous system (ENS) and the afferent and efferent nerve fibers of the central nervous system (CNS), in particular the autonomic nervous system (reviewed by Hiramatsu, 2020). These cells regulate several functions of the gastrointestinal tract, including sensation, motility, secretion, absorption, local immune defense, and even food intake (by affecting the appetite). In the large intestine, the epithelial cells use β-oxidation of butyrate to consume local oxygen to induce epithelia cell surface hypoxia and sustain luminal anaerobiosis required by the beneficial anaerobes that ferment fiber (Byndloss et al., 2018; Wilde et al., 2024). Besides their barrier functions, the IELs function by responding to cytokines and chemokines produced by the immune cells which regulate barrier function, but also influence microbiota composition. Further, the IELs sense and respond to microbial signals by secreting various immunological mediators to modulate the host immune response (Peterson and Artis, 2014).

V. CONCLUSION

As poultry meat and products are expected to increase to meet the protein demands of an expanding world population, so to must the commercial poultry industry meet the challenges of physiological and environmental sustainability. Genetic selection for production traits are likely to be limited for further increases. Therefore, modulating and/or improving the health of the intestine is of utmost importance in meeting these challenges. A shift in gut health initiatives from a microbiota-centric regulation of the host physiology to a more host-directed approach of modulating multiple gut-microbiota axes (gut-brain, gut-lung, gut-muscle) seems to be a more practical and measurable means of making progress to meet the challenges of the future.

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BENEFICIAL IMPACT OF A COMMERCIAL TRIPLE-STRAIN *BACILLUS*-BASED PROBIOTIC ON CECAL COLONIZATION OF *SALMONELLA ENTERITIDIS* IN CHALLENGED LAYER PULLETS

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Summary

Salmonella Enteritidis (SE) in layers represents a significant challenge in the poultry industry, directly impacting food safety for consumers. As a primary source of Salmonellosis outbreaks from egg consumption, effective control of SE is critical. A commercial triple-strain *Bacillus*-based probiotic was tested to determine its effect on *Salmonella* colonization in the ceca of commercial layer pullets. Potential for reduction in *Salmonella* colonization was assessed by prevalence and enumeration following oral inoculation of a selected nalidixic acid resistant SE strain. Two treatments were tested, each containing 128 day-of-hatch LSL layer pullets. On top of a standard diet, the treatments were: 1) No supplement (Control, CON), and 2) Probiotic (PRO, 1.6×10^6 CFU/g of finished feed). Environmental swabs were collected from each experimental group and tested to ensure freedom from SE prior to challenge. At 21 days of age, the SE challenge strain was inoculated orally at a dose of 3.3×10^8 CFU/bird. Pullets from each experimental group (n=32) were euthanized at 6-, 10-, 14-, and 18-days post infection (dpi). Contents from the ceca were aseptically collected for prevalence and enumeration of SE. Data were analyzed using GraphPad Prism 10.0.2 (GraphPad Software LLC, San Diego, CA). Significant differences ($P<0.05$) were identified by ANOVA of log transformed SE counts. No differences in prevalence of SE positive ceca following oral inoculation were observed between treatment groups at 6-, 10-, 14-, and 18-dpi ($P>0.05$). Cecal SE counts in the PRO group were not significantly different from CON at 6- or 10-dpi. However, significantly lower SE counts in the ceca of the PRO group were observed at 14-dpi ($P=0.038$) and 18-dpi ($P=0.019$) compared to CON. SE counts were 1.24 and 1.34 logs lower than CON at 14- and 18-dpi, respectively. In conclusion, supplementation of the triple-strain *Bacillus*-based probiotic resulted in lower cecal counts of SE compared to those birds not on an effective probiotic. SE counts were reduced to 5.6% and 4.7% of the control group at 14- and 18-days post inoculation, respectively.

I. INTRODUCTION

Salmonella continues to be a predominant cause of foodborne illnesses globally. Salmonella is a Gram-negative facultatively anaerobic, rod-shaped bacterium that exhibits flagellation and belongs to the Enterobacteriaceae family (Brenner et al., 2000). Salmonellosis is an infection caused by bacteria from the genus Salmonella. Exposure occurs through the consumption of contaminated food or water, or through contact with infected animals or their environment. Common sources may include undercooked meat, eggs, and unpasteurized dairy products. Several strategies are employed to control Salmonella in poultry production. Salmonella Enteritidis has been widely acknowledged as a significant causative agent of foodborne illness, predominantly associated with the consumption of table eggs. While these interventions have proven effective to a certain extent, the egg industry continues to seek additional pre-harvest food safety measures and technologies that can be implemented at the farm. Consequently, pre-harvest food safety emerges as a critical aspect that plays a pivotal role in the overall quality

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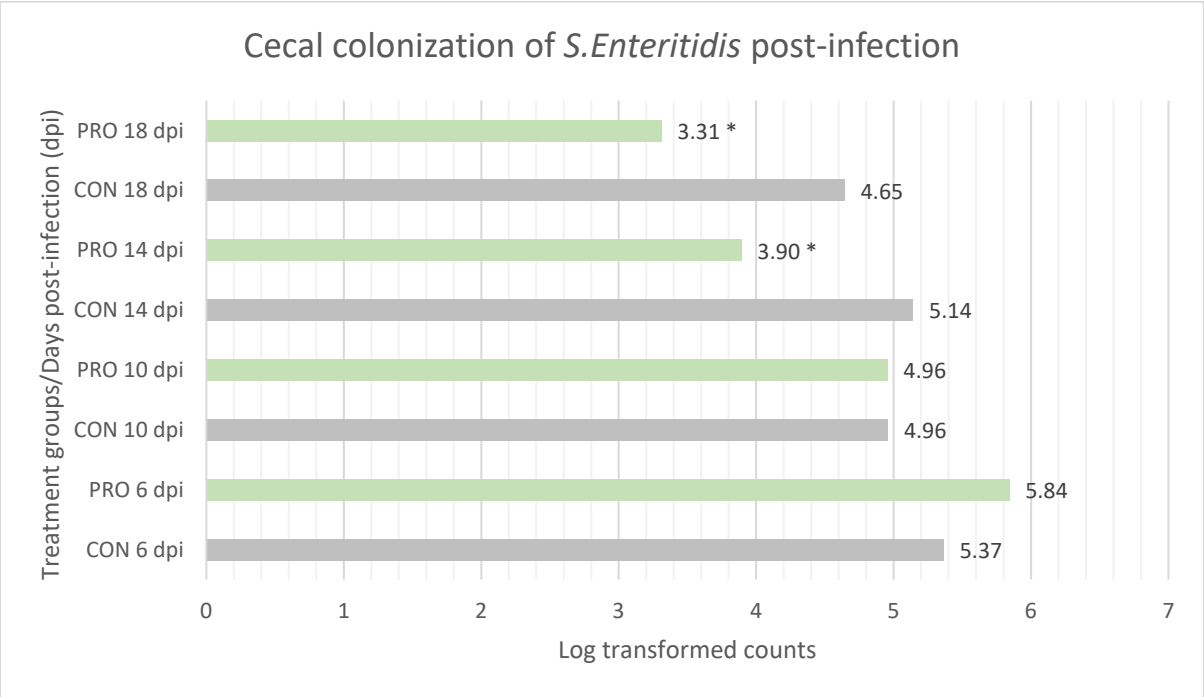
and safety of poultry products (Van Immerseel et al., 2002). It encompasses a range of strategies and practices aimed at minimizing the presence of pathogens and contaminants in poultry flocks prior to processing. These measures include appropriate feed and water hygiene, pest management, biosecurity protocols, vaccines, and the use of veterinary drugs and feed additives, all of which contribute significantly to reducing the risk of foodborne illness. (Trampel et al., 2014). Among various feed additives, probiotics offer a promising option for producers, presenting an effective strategy to mitigate Salmonella and improve food safety (Van Immerseel et al., 2002). These beneficial bacteria are widely employed in poultry production to support bird health, optimal performance, and overall well-being. Spore-forming bacteria such as Bacillus spp. have gained attention because of their thermotolerance, environmental stability, and adaptability to harsh conditions. Bacillus spp. can exert their beneficial effects on the host via mechanisms such as competitive exclusion, improved intestinal barrier function, immune modulation, and digestion and nutrient absorption (Abd El-Hack et al., 2020). Furthermore, considering concerns regarding overuse of antibiotics and the subsequent risk of pathogenic antibiotic resistance, probiotics have garnered attention in animal agriculture. They are increasingly employed as alternatives to antibiotics, aiming to support normal gut functions and inhibit potentially harmful bacteria commonly implicated in foodborne illness (Van Immerseel et al., 2002). Given that consumption of eggs is a primary source of Salmonella outbreaks, effective control of SE in layer chickens is crucial. Thus, a study was conducted to evaluate the effects of a commercial triple-strain Bacillus-based probiotic on cecal colonization with SE in commercial layer pullets.

II. METHOD

Day-old chicks were placed in cage units with wire floor raised on stainless steel decks. Each experimental group was assigned to a single cage unit. Pullets were randomly assigned to 2 groups, each containing 128 day-of-hatch LSL layer pullets. A commercial probiotic product (GALLIPRO® FIT, Novonesis) consisting of two different strains of Bacillus subtilis (*B.subtilis* 597 and 600) and one strain of *B. amyloliquefaciens* (*B. amyloliquefaciens* 516) was used in the trial. The lyophilized probiotic was mixed in the feed for a final concentration of 1.6×10^6 CFU/g feed. On top of a standard diet, the treatments were: 1) No supplement (Control, CON), and 2) Probiotic (PRO, 1.6×10^6 CFU/g of finished feed). Screening for SE negative status was conducted by collection and testing of environmental swabs 7 days prior to challenge. Each experimental group was fed a single batch of assigned diet throughout the entire duration of the study. Pullets received Salmonella challenge 21 days after placement in experimental cage units, during which experimental groups were fed appropriate diets with or without the test item. Pullets were provided with fresh potable water *ad libitum*. Each pullet received 0.5 ml of nalidixic acid resistant strain of SE inoculum containing 3.3×10^8 CFU. Challenge doses were administered orally once to pullets at the age of 21 days using a dosing syringe and gavage tube. Environmental swabs were collected from each experimental group and tested to ensure freedom from SE prior to challenge. Each swab was placed in sterile sampling bag and gloves were changed between samples. Swabs were also collected 7 dpi to verify SE shedding associated with the experimental infection. At 6, 10, 14 and 18 days post-infection, 32 pullets from each experimental group were euthanized by cervical dislocation. Cecal pouches were aseptically collected from individual birds and transported to the laboratory on ice packs for immediate analysis. Data were statistically analyzed using GraphPad Prism software version 10.0.2 (GraphPad Software LLC, San Diego, CA). Significant differences ($P \leq 0.05$) among means of experimental groups were identified by analysis of variance of log transformed SE counts.

III. RESULTS AND CONCLUSIONS

Environmental swabs collected from experimental groups prior to challenge tested negative for *Salmonella*. Environmental swabs collected at 7 dpi were all positive for SE confirming shedding associated with the experimental challenge. Cecal SE counts in CON group did not decline throughout the duration of experimental infection. Cecal SE counts in the PRO group were not significantly different from CON at 6 or 10 dpi. However, significantly lower SE counts in the ceca of PRO supplemented group were observed at 14 dpi ($P = 0.038$;) and 18 dpi ($P = 0.019$; Figure 1) compared to controls. SE counts were 1.24 and 1.34 logs lower than controls at 14 and 18 dpi, respectively.



* P < 0,05

Figure 1 - Mean counts (log10 CFU/g) of SE in cecal contents of pullets challenged at 21 days of age as influenced by dietary treatments at 6, 10, 14, 18 dpi.

The number of SE positive ceca was numerically lower in PRO-fed birds at 14 and 18 dpi compared to CON (Table 1). However, the prevalence of positive birds between experimental groups was non-significant at all sampling points.

Table 1 - Prevalence of SE positive ceca following oral gavage of 3.3×10^8 CFU to 21 days old pullets.

Days Post-Challenge	Tissue	CON	PRO
6-dpi	Ceca	32/32 (100%)	32/32 (100%)
10-dpi	Ceca	31/32 (97%)	30/32 (94%)
14-dpi	Ceca	32/32 (100%)	28/32 (88%)
18-dpi	Ceca	30/32 (94%)	26/32 (81%)

Values represent the number of SE positive birds/total number of birds tested.

In conclusion, feed supplementation with the triple-strain *Bacillus*-based probiotic resulted in an accelerated clearanceof SE compared to control birds. SE counts were reduced to 5.6% (1.24 logs lower) and 4.7% (1.34 logs lower) of the Control group at 14- and 18 days post-infection, respectively. This study outcome reinforces the findings of prior *in-vitro* and

in-vivo research, affirming the probiotic's significant contribution to improving food safety in poultry production.

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A DOSE-RESPONSE STUDY WITH ARGININE IN OVO FEEDING SHOWS SIGNS OF TOXICITY BEYOND 60 MG/EGG IN BROILER CHICKENS

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The susceptibility of newly hatched chicks to diseases is partially associated with immature digestive and immune system. The in ovo feeding (IOF) principle was developed in part to improve embryonic development (Uni and Ferket 2004). Feeding 6 mg/egg arginine (Arg) in ovo improved growth and immune development of 21-day old broilers, through Arg-NO signalling pathway (Gao et al., 2018). The aim of this study was to find the optimum IOF arginine dose to enhance broiler embryonic development supporting their long-term growth, health and wellbeing. It was hypothesized that embryonic development could be evaluated in relation to hatchability and day-old chick quality, without negative effects from Arg IOF.

Fertile Ross 308 eggs (n = 300) were distributed among six Arg doses considering Arg solubility range in saline (n = 50), T1: control (0.9% saline without arginine), T2: 20 mg/egg, T3: 40 mg/egg, T4: 60 mg/egg, T5: 80 mg/egg and T6: 100 mg/egg. Following the standard IOF procedure (Uni and Ferket 2004), amniotic fluid of day 17.5 embryo was supplemented with 1 mL of treatment. Hatchability, chick quality (navel score, deformity, bodyweight; body weight (BW) and length), and organ weights (n=10; residual yolk, heart, liver, intestine, gizzard and proventriculus) were recorded at hatch. Data were analysed using PROC GLM in SAS 9.4 with Turkey's honest significant difference test for multiple comparison.

Hatchability decreased ($P < 0.001$) in the group that received the 100 mg/egg Arg dose while chicks in the groups beyond 60 mg/egg Arg IOF were poorer in quality ($P \leq 0.05$) in terms of deformity and navel score compared to the control group. Body or organ weights were not affected ($P > 0.05$) by the treatments. Body length at hatch was reduced ($P \leq 0.05$) in 80 and 100 mg/egg Arg IOF group compared to control group. Skeletal muscles of the deformed chicks fed Arg at 60, 80 and 100 had a blackish-green coloration, indicating ammonia toxicity. In conclusion, IOF of Arg at doses ≥ 60 mg/egg showed negative impacts on embryonic development. Investigation on the metabolic basis for such negative impact is underway.

Table 1 - Hatchability and day-old chick quality of broilers after a dose-response study of in ovo feeding of arginine at day 17.5 of the incubation period.

Arg dose (mg/egg)	Hatchability (%)	Deformed birds (%)	Average navel score	BW(g) SEM=0.45	Body length (cm) SEM=0.10
00	94^{a}	2^{c}	1.26^{c}	47.35	18.14^{a}
20	90^{ab}	9^{c}	1.53^{ab}	47.46	17.90^{ab}
40	88^{ab}	9^{bc}	1.40^{bc}	47.24	17.95^{ab}
60	88^{ab}	24^{ab}	1.76^{a}	46.67	17.73^{ab}
80	92^{ab}	41^{a}	1.72^{ab}	45.93	17.48^{b}
100	78^{b}	36^{a}	1.64^{ab}	45.93	17.46^{b}
P value	<0.001	<0.001	0.008	0.993	<0.001

Navel score: 1=completely closed, 2= not completely closed, 3= not completely closed and black button appearance. Within a column, values followed by different lower-case superscripts are significantly different ($P < 0.05$).

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EFFECTS OF DIETARY SUPPLEMENTATION OF LAURIC AND BUTYRIC ACID GLYCERIDES ON THE PERFORMANCE AND INTESTINAL HEALTH OF BROILERS IN A SUBCLINICAL NECROTIC ENTERITIS CHALLENGE MODEL

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Summary

Short- and medium-chain fatty acid-based products are often used as gut health supporting feed additives in broiler production. While several of these products have individually been reported to improve intestinal health and performance parameters in enterically challenged broilers, not much information is available on their combined use and on the optimal dosage and timing of dietary inclusion. We therefore set up an experiment where broilers were raised in a subclinical necrotic enteritis model and we supplemented their feed with different levels of glycerides of butyric and lauric acid in starter and grower diets, to evaluate their effect on performance and health. We found that supplementation of butyric glycerides in the starter and grower period, combined with lauric glyceride supplementation in the grower period, reduced signs of dysbacteriosis. This effect was not enhanced if lauric glycerides were additionally added to the starter diet.

I. INTRODUCTION

Necrotic enteritis (NE) is a significant health challenge in poultry, causing substantial economic losses globally. Subclinical forms of NE are particularly problematic, as they often go unnoticed while negatively impacting bird performance and intestinal health. The use of dietary supplements, such as short- and medium-chain fatty acids, has been explored as a potential intervention to mitigate these effects. For example, butyric acid (C4, as it contains four carbon atoms) is recognized for its effects on gut development and wound healing, while lauric acid (C12) is known for its antimicrobial properties *in vitro*, especially against Gram-positive bacteria such as *C. perfringens*. (Sikandar et al., 2017; Lieberman et al., 2006; Skrivanova et al., 2006).

As there is little information on the optimal combination of butyric- and lauric-based additives in enterically challenged broilers, the aim of this study was to investigate the effect of glycerides of C12 (FRA® C12, Adisseo) and glycerides of C4 (FRA® Butyrin Hybrid, Adisseo) on the performance, intestinal gross lesions and cecal microbiota in broilers subjected to a subclinical NE challenge model (Tsiouris *et al*., 2015).

II. MATERIALS AND METHODS

In brief, birds were raised on a wheat/rye diet, with fishmeal replacing soybean meal as the main protein source during the grower period. A live Gumboro vaccine was administered at day 16 and a tenfold dose of attenuated live coccidiosis vaccine on day 19. For four consecutive days (days 20-24), three times a day, $4x10^8$ CFU of a $netB^+$ *C. perfringens* strain (#56) was given to the birds via oral gavage.

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A total of 256 one-day-old broiler chicks were randomly allocated to four treatment groups, with four replicates per group (n = 16), as outlined in Table 1.

The birds' performance was assessed by measuring body weight and feed intake and calculating feed conversion ratio (FCR) at days one, 19, 27 and 35. At days 24 and 35, three birds per replicate group were euthanized and their intestines were scored for dysbacteriosis (on a 0-10 scale, as described in Teirlynck *et al*., 2011) and NE gross lesions (on a 0-4 scale, as described in Truscott and Sheikhly, 1977). Additionally, ceca were aseptically removed and analyzed for *C. perfringens*, *E. coli*, *Lactobacillus* spp., and *Bifidobacterium* spp. levels by culturing and enumerating 10-fold dilutions on selective media.

The effect of dietary treatments on performance parameters and log-transformed bacterial counts was analyzed via ANOVA and a *post-hoc* Tukey's test. Statistical analysis of the gross lesion scores was performed by Chi-squared tests applied with SPSS as well as with the use of GraphPad Prism (version 9.1.2 for Windows®, GraphPad Software, San Diego, CA, USA). The level of significance was set at $p \leq 0.05$.

Table 1 - Overview of the different treatment groups and additive inclusions.

Group	Challenge	Additive: Type	Additive: Inclusion level (g/kg)		
			Starter day 1 - 17	Grower day 17 - 23	Finisher day 23 - 35
A	No	-	-	-	-
B	Yes	-	-	-	-
C	Yes	Glycerides of C4	1	1	-
		Glycerides of C12	-	2	-
D	Yes	Glycerides of C4	1	1	-
		Glycerides of C12	2	2	-

III. RESULTS AND DISCUSSION

Average daily feed was significantly lower in Group B and C as compared to group A during the period from day 19 to day 27 (Table 2). Birds in group D exhibited a numeric increase in weight gain compared to the other experimental groups during the period from day 19 to day 27, as well as over the entire trial period. No statistically significant differences were observed for the other performance parameters. On day 24, the average NE gross lesion scores and cecal *C. perfringens* counts were significantly higher in the challenged groups compared to the negative control group (Table 3). No significant differences were found for other evaluated bacteria across the groups. By the end of the trial (day 35), group B had a higher dysbacteriosis score than group A (Table 3). However, the glyceride-treated groups (C and D) showed reduced dysbacteriosis scores, comparable to those observed in group A (Table 3).

To summarize, the addition of butyric glycerides to the starter and grower diet, complemented with lauric glyceride supplementation in the grower period, lowers the challenged-induced dysbacteriosis score. However, this reduction was not correlated with an improvement of necrotic lesion scores or a reduction of post-challenge caecal *C. perfringens* counts. The exact mechanisms underlying the improvement of dysbacteriosis scores therefore remain to be elucidated.

Table 2 - Performance data of the experimental groups.

	Group A	Group B	Group C	Group D	P
ADWG, day 1-19, g/day	25	27	26	27	0.240
ADWG, day 19-27, g/day	87	81	84	94	0.123
ADWG, day 27-35, g/day	102	97	105	104	0.459
ADWG, day 1-35, g/day	58	56	59	61	0.292
ADFI, day 1-19, g/day	35	37	36	38	0.342
ADFI, day 19-27, g/day	90 b	84 a	82 a	85 ab	**0.024**
ADFI, day 27-35, g/day	128	129	130	135	0.749
ADFI, day 1-35, g/day	57	58	56	59	0.497
FCR, day 1-19	1.52	1.52	1.60	1.53	0.484
FCR, day 19-27	1.32	1.32	1.29	1.28	0.750
FCR, day 27-35	1.42	1.49	1.38	1.47	0.446
FCR, day 1-35	1.42	1.45	1.42	1.43	0.630

ADWG = average daily weight gain; ADFI = Average Daily Feed Intake, FCR = Feed Conversion Rate. Statistical differences are indicated with different letters in the same row.

Table 3 - NE gross lesion and dysbacteriosis scores of the experimental groups.

	Group				P
	A	B	C	D	
Avg. caecal *C. perfringens*, day 16, log 10	2.52	2.66	2.51	2.51	0.980
Avg. caecal *C. perfringens*, day 24, log 10	2.32^{b}	5.21^{a}	5.47^{a}	5.87^{a}	< 0.001
Average necrotic lesion score, day 24	0.75^{b}	2.00^{a}	1.60^{a}	1.85^{a}	< 0.001
Average necrotic lesion score, day 35	0.60	0.80	0.75	0.84	0.332
Average dysbacteriosis score, day 24	2.85	2.80	2.65	2.90	0.892
Average dysbacteriosis score, day 35	3.85^{b}	5.26^{a}	3.80^{b}	3.94^{b}	0.001

Statistical differences are indicated with different letters in the same row.

In conclusion, in the applied model, butyric and lauric glycerides reduced feed intake in the day 19-27 period, without a statistically significant effect on weight gain and FCR. In addition, the dietary combined inclusion of these products increased resilience against signs of dysbacteriosis, when administered before and during the challenge. Further research is needed to uncover the underlying mechanisms.

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BUTYRIC AND VALERIC GLYCERIDES BLEND PREVENTS ADVERSE IMPACTS OF EIMERIA CHALLENGE ON PERFORMANCE AND REDUCES OOCYST LOADS IN BROILER CHICKENS

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Summary

Whilst organic acids are well known for their antibacterial and antifungal effects, but their potential effectiveness against coccidian parasites has not been extensively explored. The aim of the study was to evaluate the impacts of a butyric and valeric glyceride blend product (BVg) in mitigating the negative impacts of coccidiosis in broiler chickens. A total of 960 mixed-sex d-old Cobb 500 chicks were randomly allocated to 2×2 +1 design. Five treatments groups included: 1) unchallenged control (UC)- birds not challenged and fed with a standard diet; 2) challenged control (CC) – birds challenged with *Eimeria* and fed a standard diet; 3) BV- birds challenged with *Eimeria* and fed a standard diet supplemented with a blend of butyric and valeric glycerides; 4) AntS- birds challenged with *Eimeria* and fed a standard diet supplemented with salinomycin; and 5) ABV- birds challenged with *Eimeria* and fed a standard diet supplemented with both salinomycin and BVg. Coccidiosis was induced on d 9 via oral gavage with *Eimeria* spp. Performance parameters were determined on d 8, d 19, d 28 and d 35. Four birds/pen were euthanized on d 16 for ileal digesta collection, and excreta samples were collected on day 20 for oocyst enumeration. Results showed reduced WG and increased FCR in CC group compared to the UC group during challenge period (d8-19) indicating a successful induction of challenge. An interaction between BVg and salinomycin was observed for FCR in the challenge and entire experiment period, where BVg reduced FCR, only when administered alone (without antibiotic) and its effect was not evident when combined with antibiotics. Furthermore, BVg reduced oocyst counts in challenged birds compared to the CC group, demonstrating anticoccidial effects similar to those in the AntS and ABV groups. These findings suggest that BVg may be a promising nutritional strategy for mitigating the adverse effects of coccidiosis, with potential to support gut health and enhance production efficiency in broilers.

I. INTRODUCTION

Coccidiosis, caused by protozoan parasites of the genus *Eimeria*, is a globally significant economic disease, particularly in poultry, where it incurs substantial costs due to production losses and treatment expenses. As of 2016, the global annual cost of coccidiosis in poultry production was estimated at US $ 11.53 billion (Blake et al., 2020). Anticoccidials and vaccines which have been traditionally used as effective strategies to control coccidiosis for extended periods of time, are now facing challenges such as the emergence of drug-resistant strains and regulatory bans on certain coccidiostats and ionophores. These limitations have prompted a growing demand for safer, nutritional-based alternatives to manage coccidiosis. Among the potential alternatives, organic acids (OAs) have gained attention for their proven benefits on gut health and performance. While OAs are well established as antibacterial and antifungal agents, their effectiveness against coccidian parasites remains underexplored. Previous research suggests that OAs may reduce gut pH and creates an unfavorable environment for

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oocyst development (Aristimunha et al., 2016). Specifically, butyric acid has been shown to inhibit sporulation and damage oocysts in a dose-dependent manner in vitro (Zurisha et al., 2021). However, valeric acid is a relatively new organic acid, has been studies far less and its effects on gut health and performance remain largely unknown. Considering these facts, this study aimed to evaluate the potential effects of butyric and valeric acids glycerides blend (BVg) on growth performance and oocyst counts of broiler chickens under mild coccidiosis challenged.

II. METHOD

A total of 960 d-old Cobb 500 chicks were allocated into 60 floor pens (16 birds/pen) birds. The experiment used a randomized design with five treatments, each replicated 12 times. The five treatments were: UC (unchallenged control), CC (*Eimeria*-challenged control having basic diet); BV (CC + blend of butyric and valeric glycerides (Gastrivix™ Avi, Perstorp Waspik BV, Netherlands) at 500, 500, and 250 g/ton in the starter, grower and finisher phases, respectively); AntS (CC + Salinomycin at 60 g/ton active compound in all phases); and ABV (CC + Salinomycin + BVg (similar dose as BV). Wheat, soybean meal and sorghum-based diets were formulated to meet the nutritional requirements of Cobb 500 birds. The diets were provided in three phases: starter (d 0-8), grower (d 8-19), and finisher (d 19-35). Challenged birds were gavaged with 1 mL/bird *per os Eimeria* spp. vaccine containing *E. acervulina* 5,000, *E. maxima* 5,000 and *E. brunetti* 2,500 oocysts on d 9, while UC group received PBS at 1 mL/bird as a sham treatment. Pen weight and feed intake were measured on arrival (d 0) and on d 8, 19, 28 and 35, and the data was used to calculate the weight gain (WG) and feed conversion ratio (FCR).

On day 16, four birds/pen were randomly selected, electrically stunned and euthanized by cervical dislocation, followed by dissection. Pooled ileal digesta samples from two birds were collected for oocyst count. On day 20, pooled freshly voided excreta droppings were collected for oocyst enumeration. A two-way ANOVA was used to analyze the main and interaction effects of BVg and salinomycin in challenged groups (CC, BV, AntS, ABV). A one-way ANOVA with Tukey's HSD compared all groups, including the unchallenged control (UC). The *Eimeria* oocyst counts data were analyzed using the non-parametric Kruskal-Wallis test as the data were not normally distributed. Significance was set at $P < 0.05$.

III. RESULTS

The effects of different treatments on broiler growth performance under coccidiosis challenge are presented in Table 1. During the challenge phase (d 8-19), and the entire experimental period (d 0-35), the *Eimeria* challenge significantly reduced WG, while increasing FCR ($P < 0.01$) in birds in the CC group compared to the UC group. An interaction was observed during both the challenge phase (d 8-19) and overall experimental period (d 0-35), where BVg supplementation improved FCR ($P = 0.019$ and $P = 0.017$, respectively), only when administered alone (without antibiotics), and did not show its effect when combined with salinomycin.

Table 2 outlines the impact of experimental treatments on the *Eimeria* oocyst counts in ileal digesta (d 16) and in excreta (d 20). Birds in the CC group had significantly higher oocyst in both the ileal digesta and excreta compared to the UC group ($P < 0.001$). Salinomycin supplementation effectively reduced oocyst counts relative to the CC group ($P < 0.001$), but the reduction was not significantly different from the UC, BV and ABV groups. Supplementation with BVg significantly decreased oocyst counts in ileal digesta compared to the CC group, demonstrating anticoccidial effects similar to those observed in the AntS and

ABV groups. However, the combination of BVg and anticoccidial (ABV) did not provide any additional benefit beyond what was observed with BVg or anticoccidial alone (P > 0.05).

Table 1 - Effect of BVg on growth performance of broilers under coccidiosis challenge

One-way ANOVA	BVg[3]	Salinomycin[4]	D (8-19)		D (19-35)		D (0-35)	
			WG (g)	FCR[2]	WG (g)	FCR	WG (g)	FCR
CC	No	No	462[b]	1.539[a]	1646[c]	1.573[a]	2264[b]	1.519[a]
BV	Yes	No	476[b]	1.467[b]	1654[bc]	1.555[ab]	2289[b]	1.485[b]
AntS	No	Yes	632[a]	1.327[c]	1747[a]	1.518[b]	2532[a]	1.424[c]
ABV	Yes	Yes	624[a]	1.350[c]	1736[ab]	1.518[b]	2513[a]	1.431[c]
UC			649[a]	1.294[c]	1735[abc]	1.543[ab]	2542[a]	1.428[c]
SEM[1]			13	0.016	22	0.010	29	0.007
P-value			<0.001	<0.001	0.002	0.006	<0.001	<0.001
Main effect (2×2)								
BVg								
-			548	1.431	1701	1.544	2403	1.469
+			550	1.407	1699	1.534	2406	1.457
SEM			10	0.012	15	0.008	20	0.005
Salinomycin								
-			469[b]	1.500[a]	1655[b]	1.561[a]	2283[b]	1.500[a]
+			628[a]	1.338[b]	1745[a]	1.517[b]	2526[a]	1.426[b]
SEM			10	0.012	15	0.008	20	0.005
P-value								
BVg			0.891	0.225	0.945	0.440	0.934	0.109
Salinomycin			<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
BVg × Salinomycin			0.076	0.019	0.628	0.460	0.406	0.017

[a-c]Values within a column with different letters differ significantly ($P < 0.05$).[1]SEM: standard error of means. [2] FCR were based on standardized DM of 88%.[3]BVg: butyric and valeric glycerides at 500, 500, and 250 g/ton in the starter, grower and finisher phases, respectively. [4]Salinomycin at 60 g/ton active compound in all phases.

Table 2 - Effect of BVg on oocyst counts of broilers under *Eimeria* challenge.

Treatment	Oocyst/g	
	Ileal digesta (d 16)	Excreta (d 20)
UC	00[c]	00[b]
CC	19890[a]	26171[a]
BV	11136[b]	11335[ab]
AntS	6946[bc]	154[b]
ABV	4810[bc]	63[b]
SEM[1]	1593	3151
P-value	<0.001	<0.001

[a-c]values within a column with different letters differ significantly ($P < 0.05$).
[1]SEM: standard error of means.

IV. DISCUSSION

The findings showed that the BVg supplementation improved FCR in the challenged birds, however the positive effect of BVg was only visible when used alone in the diet. Additionally, BVg effectively reduced oocyst counts, demonstrating anticoccidial effects similar to the AntS and ABV groups. The induced coccidiosis in the CC group resulted in lower WG and increased FCR compared to the UC group, confirming a successful induction of coccidiosis. It is well-documented that both clinical and subclinical coccidiosis cause severe damage to intestinal epithelium, leading to impaired nutrient absorption and reduced growth performance in broiler

chickens (Chapman, 2014). Salinomycin supplementation improved both performance and oocyst load compared to the CC group to the levels similar to the UC group. Salinomycin, an ionophore and potent coccidiostat, exerts its effect on coccidian parasites by disrupting ion gradients across the cell membrane of the parasites (Antoszczak et al., 2019), thereby preventing intestinal damage, reducing oocyst shedding and improving overall performance. The growth performance improvements observed with BVg may be attributed to the enhanced gut development, improved digestibility, and modulation efficiency of gut microflora, inflammation and immunity by organic acids as reported earlier (Khan et al., 2022). Furthermore, butyrate, a component of BVg, acts as an energy source for enterocytes, supports gut mucosal health, and promotes epithelial proliferation and differentiation contributing to better nutrient absorption (Canani et al., 2011). Although BVg did not significantly affect WG or FCR during the finisher phase (d 19-35), the observed shift in FCR from CC group towards levels seen in both antibiotic supplemented groups may suggest a potential for improving feed efficiency under certain conditions. The reduction in oocyst counts in the BVg group may be attributed to its ability in lowering gut pH, creating an unfavourable environment for oocyst survival. Additionally, previous studies have shown that butyric acid damages the *Eimeria* oocysts *in vitro*, and reduces lesion scores and oocyst counts, leading to improved broiler performance (Zurisha et al., 2021). In conclusion, the reduction in oocyst numbers in the BVg-treated group likely led to less severe intestinal damage from parasitic invasion into enterocytes, ultimately enhancing overall performance. The lack of synergy between salinomycin and BVg suggests that their combined effects may not be additive under the conditions tested. Further research should be focused on optimizing dosages of Bvg and also examining the molecular mechanisms underlying its observed benefits to further clarify its role in mitigating coccidiosis and enhancing broiler productivity.

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INFLUENCE OF *BACILLUS SUBTILIS* PROBIOTIC ON GUT INTEGRITY AND GENE EXPRESSION IN BROILERS

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Summary

Antimicrobial resistance (AMR) poses a global threat to both human and animal health, highlighting the urgent need for safe alternatives to in-feed antibiotic growth promoters (AGPs). Probiotics from the genus *Bacillus* have shown promise as potential substitutes. This study investigates the effects of a *B. subtilis* probiotic (BP-13) on intestinal epithelium integrity, growth performance, and ileum gene expression in broilers under a leaky gut challenge model. BP-13 significantly improved epithelial integrity, as measured by the transepithelial electrical resistance (TEER) assay compared to the control group ($P < 0.05$). No significant differences in growth performance were observed between birds fed the control diet and those supplemented with BP-13. However, after 42 days of continuous supplementation, transcriptomic analysis revealed that BP-13 supplementation led to significant alterations in host pathways, including the downregulation of pathways related to cholesterol metabolism, inflammation, gastroenteritis, and hypertension, and the upregulation of pathways associated with memory, angiogenesis, and lipid concentration.

I. INTRODUCTION

The use of AGPs in commercial poultry production has been gradually restricted and banned in some countries due to growing concerns over AMR and the potential for cross-contamination between poultry and humans, which can lead to complicated infections (Manyi-Loh et al., 2018). As AGP use declines, research has surged into alternatives, such as improved biosecurity measures and production practices (Yu et al., 2022). However, the removal of AGPs has been linked to gut dysbiosis and microbial diseases in poultry, underscoring the need to support host-microbiota interactions to maintain gut health, immunity, and overall productivity (Rinninella et al., 2019). Among alternative products, *Bacillus* spp. probiotics have shown notable benefits, including preventing gastrointestinal tract disorders, improving gut integrity, reducing ammonia excretion, and promoting beneficial microbiota (Li et al., 2023), making them a promising substitute for AGPs.

This study investigates the effects of a *Bacillus*-based probiotic on broilers challenged with dexamethasone (DEX)-induced leaky gut, a model for intestinal barrier dysfunction linked to inflammation and chronic diseases (Le et al., 2023). Previous similar study suggests that *Bacillus* probiotics improve performance, gut morphology, and microbiota in non-challenged broilers (Bromfield et al., 2024; Horyanto et al., 2024). By using RNA sequencing (RNA-seq) to analyse transcriptome profiles, this study aims to identify key genes and pathways contributing to enhanced health, microbial balance, and gut integrity in DEX-challenged broilers.

II. METHOD

a) TEER Assay

The TEER of intestinal epithelial barrier integrity was measured using cellZscope® (nanoAnalytics GmbH, Germany), simulating in vivo conditions. This technique assesses TEER in monolayers of various cell types cultured on semi-permeable membranes. The supplemented cell culture medium

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included DMEM, 1% L-Glutamine, Streptopenicillin, and 10% FBS. HT29 cells (human colon adenocarcinoma) were cultured on TC inserts (Sarstedt, Germany) in 24-well plates for three weeks, incubated at 37.5°C and 5% CO_2 with medium changes every alternate day.

For *B. subtilis* BP-13 (Bioproton Pty Ltd., Australia) inocula preparation, 300 µL of overnight cultures were diluted in DMEM to an OD of 0.5 using a Nanodrop™ One (ThermoFisher Scientific). TEER was measured every 1.5 hours for 24 hours across six replicates per group. The electrodes were sterilised and balanced as per the cellZscope® manual. A blank control was concurrently measured under similar conditions.

b) Animal Trial and Management

The study was approved by Central Queensland University's Animal Ethics Committee (approval 0000023123). ROSS 308 broiler chicks were divided into two groups: a control fed a commercial basal diet (CTR), and a group supplemented with *B. subtilis* (BP-13) (3×10^8 CFU/kg of complete feed). BP-13 was incorporated into the same commercial basal diet at 500 g/t and thoroughly mixed using a commercial-grade mixer to ensure homogeneity and uniform distribution throughout the feed. From days 28-35, DEX was supplemented at 0.6 ppm, following Horyanto et al. (2024) and Vicuña et al. (2015). Broiler performance parameters, such as body weight (BW), average daily gain (ADG), average daily feed intake (ADFI), and feed conversion ratio (FCR) were measured weekly. Data were analysed using GraphPad Prism.

c) RNA Sequencing and Analysis

Approximately 100 mg of ileum tissue was homogenised in TRIsure (Bioline, UK), and RNA was extracted using the Isolate II RNA Mini Kit (Bioline). Sequencing libraries were prepared with TruSeq RNA Library Prep Kit v2 (Illumina) and sequenced on Illumina NovaSeq 6000. RNA-seq data were processed using the nf-core RNAseq pipeline (Ewels et al., 2020), with QC conducted via FastQC, Trim Galore, and other tools. Genome alignment was performed with STAR, and differential expression analysis used DESeq2, with pathway analysis through IPA (QIAGEN).

III. RESULTS

a) TEER Assay

The TEER assay was used to select BP-13 as a candidate probiotic for the subsequent broiler trial. Figure 1 shows the TEER values recorded over 24 hours for both the CTR and BP-13 groups. A statistically significant difference ($P < 0.0001$) was observed in the mean TEER values between CTR and BP-13, with BP-13 showing higher values (25.02 vs. 30.87 Ω.cm²).

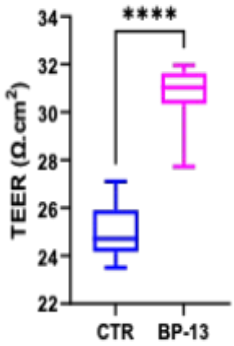

Figure 1 - The average TEER values of CTR and BP-13 groups measured in $\Omega.cm^2$; **** = $P < 0.0001$. The values represent the mean of six replicates per treatment group.

b) Animal Performance

There were no significant differences in production performance parameters between the CTR and BP-13 groups, including BW, ADG, ADFI, and FCR. Mortality rates in both groups were similar and remained within the expected range of less than 5%. Additionally, both groups performed above the standards outlined in the ROSS 308 Broiler: Performance Objectives.

c) RNA-seq

i) Pathway Analysis

The datasets obtained from Ingenuity Pathway Analysis (IPA) were cleaned, and pathways meeting the criteria of $P < 0.05$ and an absolute z-score > 1.2 are illustrated in Table 1. Pathways are color-coded for clarity: orange indicates activation, and blue indicates inhibition. Among the most activated pathways were sperm motility, eNOS signaling, endocannabinoid neuronal synapse pathway, VEGF family ligand-receptor interactions, and 3-phosphoinositide biosynthesis. Conversely, superpathway of cholesterol biosynthesis, cholesterol biosynthesis pathways (I, II, and III) and the antioxidant action of vitamin C were among the most inhibited.

Table 1 - The ingenuity canonical pathways were identified by applying -log (P < 0.05) and absolute z-score > 1.2. Pathways are indicated by colour: orange for activation and blue for inhibition. The horizontal orange line indicates the threshold (P < 0.05, absolute z-score > 1.2).

Ingenuity Canonical Pathways	-log(p-value)	z-score
Superpathway of Cholesterol Biosynthesis	5.59	-2.646
Antioxidant Action of Vitamin C	2.52	-2.236
Cholesterol Biosynthesis I	3.84	-2
Cholesterol Biosynthesis II (via 24,25-dihydrolanosterol)	3.84	-2
Cholesterol Biosynthesis III (via Desmosterol)	3.84	-2
Sperm Motility	1.56	1.633
eNOS Signaling	1.59	1.633
Endocannabinoid Neuronal Synapse Pathway	1.62	1.633
VEGF Family Ligand-Receptor Interactions	1.96	1.633
3-phosphoinositide Biosynthesis	1.94	1.667
Netrin Signaling	1.25	2
Superpathway of Inositol Phosphate Compounds	2.31	2.111
Phospholipases	2.57	2.236
Endothelin-1 Signaling	1.23	2.646

ii) Regulatory Networks

The regulatory networks and transcriptomic effects of BP-13 probiotic supplementation on genes, diseases, and functions were analysed. The predicted master regulators for upregulated genes are AIRE and SSTR2, which activate genes such as AKR1B10, HBEGF, and TLR2. These changes contribute to reduced inflammation in the gastrointestinal tract, lower risk of gastroenteritis and chronic inflammatory disorders, and improvements in memory, angiogenesis, lipid concentration, organism survival, and molecular secretion.

IV. DISCUSSION

TEER is a validated assay for assessing gut barrier integrity and permeability in vitro. Under stress, increased gut permeability disrupts epithelial integrity, allowing the passage of macromolecules, endotoxins, and pathogens (Kim et al., 2023). Figure 1 shows significantly higher TEER values in *B. subtilis* (BP-13), consistent with findings by Gu et al. (2014), who reported *B. subtilis* enhancing TJ integrity, increasing ZO-1 expression, and protecting against deoxynivalenol (DON)-induced

barrier dysfunction. Prior to DEX treatment, growth performance, including FCR, did not show significant differences, likely because the broilers exceeded standard growth benchmarks, making further improvements difficult to observe. However, broiler performance was significantly impaired during DEX treatment.

Transcriptomic analysis revealed downregulation of cholesterol biosynthesis pathways and upregulation of phospholipases (Table 1). Phospholipases are important for lipid metabolism, oxidative stress management, and bacterial membrane disruption (Giresha et al., 2022). Efficient lipid metabolism is crucial for energy balance, particularly in laying hens, as it influences ovulation, reproduction, and inflammation (Ceciliani et al., 2018). Poor lipid metabolism can lead to fatty liver syndrome, negatively impacting liver function and health. BP-13 also activated pathways associated with inositol phosphate degradation, which improves nutrient absorption and reduces phosphorus excretion, vital for sustainable poultry production (Selle et al., 2023).

BP-13 activated the 3-phosphoinositide biosynthesis pathway, involved in growth factor signaling, cell proliferation, and glucose uptake (Alessi & Downes, 1998). Memory and cognition-related pathways, such as endocannabinoid signaling, and cardiovascular health pathways, including endothelin-1 and eNOS signaling, were also upregulated, which regulate vasoconstriction and vasodilation (Yang et al., 2010).

The IPA bioinformatics software summarised the regulatory networks influenced by BP-13, showing significant inhibition of inflammation and gastrointestinal disorders. Environmental stressors like heat or excessive dietary fiber/protein can trigger inflammation, leading to dysbiosis and gut leakage (Hrncir, 2022). This study demonstrated BP-13's capacity to reduce inflammation and enhance gut integrity.

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EVALUATNG DIFFERENT APPLICATION STRATEGIES OF A SAPONIN-ALUMINOSILICATE BLEND IN BROILERS CHALLENGED WITH *EIMERIA*

M. BRINK[1], M. SINCLAIR[1] and S. SRINONGKOTE[2]

Coccidiosis is one of the most frequently occurring disease challenges affecting intensive poultry production and has a significant economic impact on the poultry industry with costs estimated at USD 0.20 per bird (Blake et al., 2020). Globally, the use of natural feed additives has gained much attention as an alternative to replace anticoccidial drugs in broilers. The aim of this study was to evaluate the potential to reduce the use of salinomycin by evaluating the impact of different application strategies of a natural blend consisting of *Quillaja saponaria* extract (rich in saponins) and an aluminosilicate on performance, intestinal lesions and morphology, and oocyst excretion in broilers challenged with *Eimeria* spp.

A total of 600 one-day-old Ross 308 male broilers were divided over six treatments, each with 10 replicate floor pens (using rice hulls as bedding material), and reared for 42 days: positive control with no *Eimeria* challenge and no anticoccidials (PC); negative control challenged with *Eimeria* with no anticoccidials (NC); NC with 60 mg/kg of salinomycin from 0-35d (Sal 35d) and no anticoccidial from 35-42d; NC with 200 mg/kg of a saponin-aluminosilicate blend (Excential Sapphire Q, Orffa Additives B.V.) from 0-42d (SQ 42d); NC with 60 mg/kg of salinomycin from 0-28d and with 200 mg/kg of the saponin-aluminosilicate blend from 0-42d (Sal 28d + SQ 42d); NC with 60 mg/kg of salinomycin from 0-28d and with 200 mg/kg of the saponin-aluminosilicate blend from 28-42d (Sal 28d + SQ 28-42d). All treatments, except the PC, were orally challenged with mixed *Eimeria* spp. on day 14 (1 mL per bird containing a total of 15000 sporulated oocysts of *E. tenella*, *E. maxima*, and *E. acervulina*). Body weight (BW), body weight gain (BWG), feed intake (FI) and mortality corrected feed conversion ratio (FCRm) were measured from 0-10d, 10-28d, and 0-42d. At 21, 35, and 42 days of age, one bird per pen were euthanised for intestinal scoring of coccidiosis and necrotic enteritis lesions. From the same birds, jejunal samples were collected to evaluate villus height (VH), crypt depth (CD), and their ratio (V/C) at 21 and 35 days of age. On days 21, 35, and 42, fresh excreta samples were collected and pooled from each pen to determine the number of oocysts shed per gram of excreta (OPG). Results were analyzed using ANOVA with significance set at 0.05. In case of significant effects, Duncan's multiple range test was used to determine differences among treatment means.

For the overall period from 0 to 42 days, broilers receiving only salinomycin for 35 days (Sal 35d) had a higher BW and BWG ($P < 0.001$) than the PC, NC, and SQ 42d groups, but a similar BW and BWG to both the Sal + SQ groups. The NC and SQ 42d groups had the lowest BW and BWG, with PC being intermediate. The FI was highest for the Sal 35d treatment, but similar to the Sal 28d + SQ 42d group ($P = 0.024$). The FCRm was best for the PC, Sal 35d, and Sal + SQ groups (non-significant for the four groups), and the worst for the NC, with the SQ 42d treatment being intermediate ($P < 0.001$). No differences in coccidiosis and necrotic enteritis lesion scores were noted among treatments ($P > 0.05$ for all). For OPG, significant differences were observed only at 21 days of age. The lowest oocyst shedding was observed for PC, Sal 35d, and the Sal + SQ groups and the highest shedding for NC treatment, with SQ 42d being intermediate ($P < 0.001$). At 21 days, V/C was highest for PC and lowest for NC and SQ 42d, with the Sal 35d and Sal + SQ groups being intermediate ($P < 0.001$). At 35 days, these differences disappeared ($P = 0.07$) and V/C tended to be higher for PC compared to the other groups.

The application strategy of a saponin-aluminosilicate blend affects broiler performance, intestinal health, and *Eimeria* pressure. In this study, treatment with salinomycin significantly improved performance and intestinal health of broilers challenged with *Eimeria*, however, when the saponin-aluminosilicate blend was fed in combination with salinomycin for the full rearing period, or fed only during the finisher period, the inclusion of salinomycin could be reduced from 35 to 28 days.

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EFFECTS OF QUILLAJA AND YUCCA COMBINATION PRODUCT AT DIFFERENT INCLUSION LEVELS AND DURATION OF USE IN BROILERS SUBJECTED TO INTESTINAL CHALLENGE MODEL

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Summary

Several scientific publications and commercial field experience indicate positive effects of *Quillaja saponaria* and *Yucca schidigera* combination product (Magni-Phi®) on broiler performance when exposed to enteritis challenge from specific pathogens or stress factors such as feed deprivation. The current study aims to investigate its effects at different inclusion rates and phase of feeding on intestinal health and performance in a floor pen trial model, mimicking field non-specific enteritis infection model, compared to an infected untreated control (IUC). When fed to broilers subjected to intestinal challenge the product reduced mortality, intestinal inflammation, *C. perfringens* and *E. coli* intestinal counts, *Eimeria* oocyst excretion (oocyst per gram – OPG) and *Salmonella* spp. incidence; and improved significantly the zootechnical performance (body weight and body weight gain, FCR and EPEF) and processing characteristics (improved carcass and breast yield and reduced abdominal fat) in a dose dependent manner with larger improvements achieved at higher supplementation level. In both supplementation levels, better results have been observed when product was provided for 0-42 days or 0-28 days of age, while when product was supplemented only for 29-42 days of age period, improvements were marginal or none. Thus, a conclusion can be made that the optimum effect of the product can be achieved when supplemented during the whole grow-out period or at least in the first four weeks of life. This is in line with the previously proposed mode of action through improved immunity development, reduced inflammatory tissue damage, and faster recovery, rather than direct effect on pathogens.

I. INTRODUCTION

Previous research indicates positive effects of *Quillaja saponaria* and *Yucca schidigera* combination product (QYP), containing a minimum of 3.5% triterpenoid (Quillaja) saponins and typically 0.8-1.0% of total polyphenols expressed as gallic acid equivalent (Magni-Phi®) on broilers' performance. The above effect is witnessed either when birds are exposed to enteritis challenge from certain pathogens like Eimeria and Clostridia (Bafundo et al., 2020, Bafundo et al., 2022) or stress factors such as feed deprivation (Osho et al., 2022). The current study aims to investigate the effects of QYP at different inclusion rates and phase of use on intestinal health and performance in a floor pen trial model, mimicking field enteritis infection, compared to an infected untreated control (IUC).

II. METHODS

A total of 5616 as hatched day-old Ross 708 broilers were allocated to 9 treatments as per Table 1 with 12 replicates each and 52 birds per pen. In order to mimic a natural field enteritis infection, birds

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were placed on used litter containing coccidia, *C. perfringens, E. coli*, *Salmonella* spp., and other pathogens. Additionally, before birds' placement, litter was supplemented with 100 000 oocysts/bird, primarily *E. acervulina* and *E. maxima*. Mortality, body weight (BW), body weight gain (BWG), feed conversion ratio (FCR), European Poultry Efficiency Factor (EPEF), and BW coefficient of variation (CV) were measured at 42 days. At 28 days BW was measured as well. At 21 days and 42 days, 4 birds per pen were humanely sacrificed and scored for intestinal inflammation as follows: 0 – no lesions found; 1 – mild hyperemia, but no cell sloughing or mucous; 2- moderate hyperemia and /or mild cell sloughing; 3 – severe hyperemia and/or severe cell sloughing and 4 – actual necrosis or bleeding observed. The same birds were also sampled for cecal *C. perfringens* and *E. coli* count and *Salmonella spp*. incidence.
The study was conducted according to Good Clinical Practice and approved by the AHPharma Animal Ethical Committee.

III. RESULTS

An overview of intestinal health parameters is provided in Table 1. At day 21 intestinal inflammation scores, *E. coli* counts, *Salmonella* spp. incidence, and oocyst per gram (OPG), were significantly reduced in the QYP supplemented birds correlating to the inclusion rate with significant differences for the different inclusions. *C. perfringens* counts were reduced only when QYP was provided at 500 g/t. The same dose dependent effect was observed at day 42 of age. QYP demonstrated residual effect for intestinal inflammation, *C. perfringens* counts, *Salmonella* incidence and OPG: groups that were supplemented during days 0-29 only, showed significant improvement compared to IUC at day 42.

Table 1 - Overview of intestinal health parameters: intestinal inflammation lesion scores and pathogen loads.

Treatment	Intestinal Inflammation		*C. perfringens* (CFU/g $\log_{10}$)		*E. coli* (CFU/g $\log_{10}$)		*Salmonella* Incidence (%)		*Eimeria spp.* OPG ($\log_{10}$)	
	d 21	d 42	d 21	d 42	d 21	d 42	d 21	d 42	d 21	d 42
IUC	1.79^{a}	1.80^{a}	4.42^{a}	4.82^{a}	6.73^{a}	6.69^{a}	83.33^{a}	84.17^{a}	6.60^{a}	7.06^{a}
QYP 250 g/t 0-28d	0.79^{b}	0.40^{b}	4.15^{a}	3.54^{bc}	6.13^{b}	6.65^{a}	56.25^{b}	49.17^{bc}	5.95^{b}	5.99^{b}
QYP 250 g/t 29-42d	1.75^{a}	0.44^{b}	4.49^{a}	3.68^{b}	6.70^{a}	6.04^{b}	79.16^{a}	47.50^{bc}	6.76^{a}	6.12^{b}
QYP 250 g/t 0-42d	0.77^{b}	0.48^{b}	4.11^{ab}	3.59^{bc}	5.89^{b}	6.10^{b}	52.08^{b}	43.33^{c}	6.21^{b}	5.96^{b}
QYP 500 g/t 0-28d	0.13^{c}	0.11^{c}	3.56^{c}	3.42^{c}	5.09^{c}	6.20^{b}	16.67^{c}	22.50^{d}	5.44^{c}	6.07^{b}
QYP 500 g/t 29-42d	1.83^{a}	0.06^{c}	4.46^{a}	2.85^{d}	6.73^{a}	5.35^{c}	83.33^{a}	55.83^{b}	6.74^{a}	5.53^{c}
QYP 500 g/t 0-42d	0.13^{c}	0.06^{c}	3.55^{c}	2.87^{d}	5.05^{c}	5.37^{c}	27.08^{c}	26.67^{d}	5.49^{c}	5.39^{c}
QYP 250 g/t 0-28d 500 g/t 29-42d	0.77^{b}	0.07^{c}	4.29^{a}	2.85^{a}	5.96^{b}	5.39^{c}	45.83^{b}	55.83^{c}	6.16^{b}	5.45^{c}
QYP 500 g/t 0-28d 250 g/t 29-42d	0.06^{c}	0.13^{c}	3.73^{bc}	3.75^{b}	4.90^{c}	6.05^{b}	16.67^{c}	44.17^{bc}	5.27^{c}	6.12^{b}

Means with different letters in the superscripts are significantly different, $P \leq 0.05$. Statistical analysis deploying ANOVA and Fisher's LSD test to separate treatments in different statistical groups was applied

An overview of zootechnical performance and processing characteristics is provided in Table 2. Mortality was significantly reduced in all treatment groups with a dose dependent effect (Fig. 1). The effect on mortality was not different for 0-28 and 0-42 days supplementation and significantly better compared to 29-42 days supplementation for both inclusion rates. Body weight at day 28 was significantly improved proportionally to the inclusion rate in line with the intestinal health parameters. The same inclusion rate correlation persisted at day 42. The best effect for both inclusion rates 250 and 500 g/t respectively, was achieved in groups supplemented during the whole grow-out period 0-42 days (Fig. 2). Birds supplemented for the 0-28 days period provided residual significant improvement at day 42, while groups

supplemented for 29-42 days period only, did not provide significant improvement of FCR (Fig. 3).

Table 2 - Overview of zootechnical results and processing characteristics.

Treatment	Mortality (%)	BW (g)	BW (g)	BW CV	BWG (g)	FCR	EPEF	Carcass yield %	Breast yield %	Adom. fat %
	d 0-42	d 28	d 42	d 42	d 0-42	d 0-42	d 0-42	d 42	d 42	d 42
IUC	7.99^{a}	1293^{c}	2490^{g}	12.81^{a}	2453^{g}	1.890^{a}	278^{e}	70.94^{d}	25.57^{de}	1.49^{ab}
QYP 250 g/t 0-28d	3.47^{c}	1371^{b}	2580^{ef}	11.07^{bc}	2543^{ef}	1.851^{abcd}	312^{c}	70.88^{d}	25.51^{e}	1.46^{abc}
QYP 250 g/t 29-42d	5.56^{b}	1293^{c}	2547^{f}	12.83^{a}	2510^{f}	1.877^{ab}	297^{d}	73.18^{bc}	26.08^{c}	1.44^{bc}
QYP 250 g/t 0-42d	2.78^{cd}	1371^{b}	2621^{d}	10.52^{bcd}	2584^{d}	1.838^{bcde}	323^{b}	72.86^{c}	26.03^{cd}	1.49^{ab}
QYP 500 g/t 0-28d	1.74^{de}	1410^{a}	2691^{ab}	10.03^{d}	2653^{ab}	1.798^{ef}	342^{a}	74.75^{a}	26.76^{ab}	1.46^{abc}
QYP 500 g/t 29-42d	6.42^{b}	1297^{c}	2581^{e}	12.92^{a}	2543^{e}	1.861^{abc}	301^{d}	75.53^{a}	26.37^{bc}	1.50^{a}
QYP 500 g/t 0-42d	0.69^{e}	1371^{b}	2700^{a}	9.77^{d}	2663^{a}	1.794^{f}	351^{a}	74.36^{ab}	27.19^{a}	1.44^{bc}
QYP 250 g/t 0-28d 500 g/t 29-42d	2.78^{cd}	1371^{b}	2655^{c}	11.33^{b}	2618^{c}	1.821^{cdef}	331^{b}	74.74^{a}	26.76^{ab}	1.42^{c}
QYP 500 g/t 0-28d 250 g/t 29-42d	0.69^{e}	1406^{a}	2660^{bc}	10.32^{cd}	2623^{bc}	1.814^{def}	342^{a}	74.34^{ab}	26.86^{a}	1.45^{abc}

Means with different letters in the superscripts are significantly different, P ≤ 0.05. Statistical analysis deploying ANOVA and Fisher's LSD test to separate treatments in different statistical groups was applied

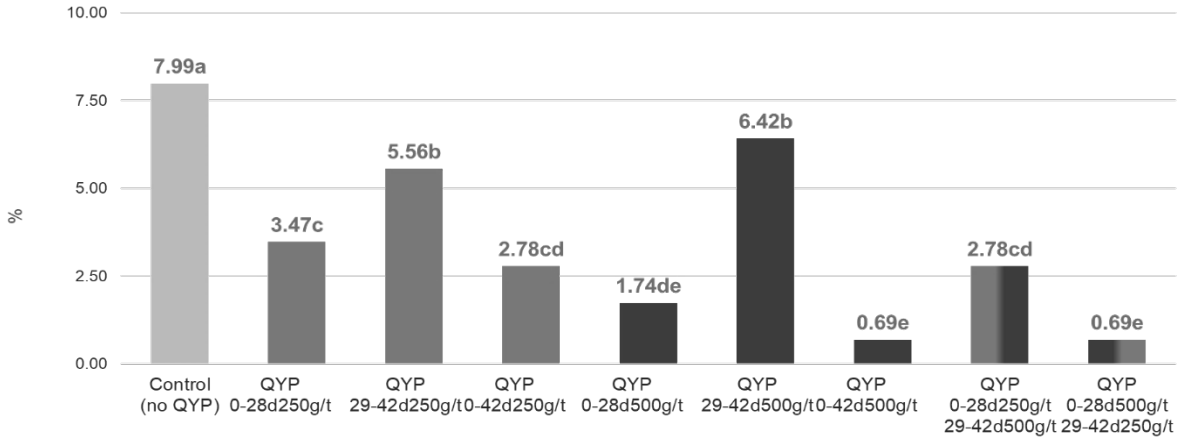

Figure 1 – Mortality (%) 0-42 days. Means with different letters in the superscripts are significantly different, P ≤ 0.05. Statistical analysis deploying ANOVA and Fisher's LSD test to separate treatments in different statistical groups was applied.

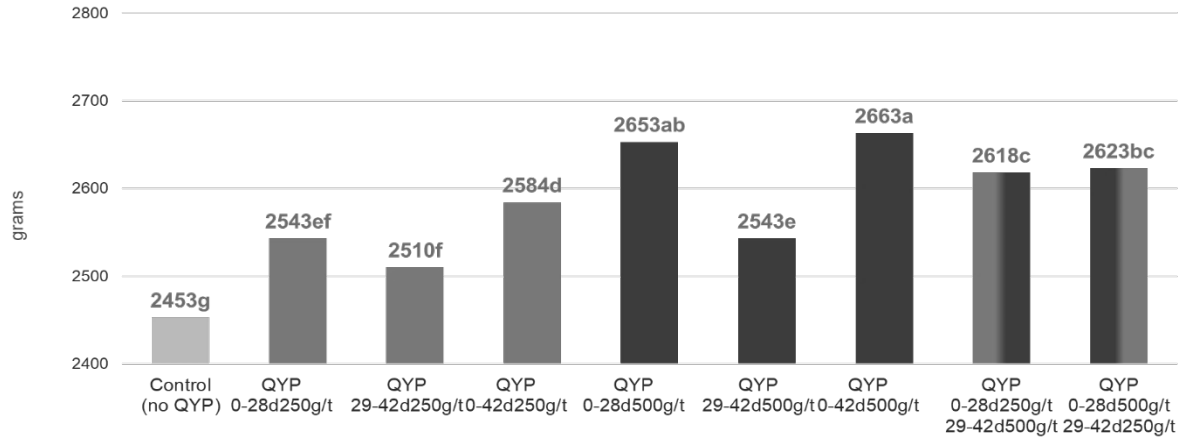

Figure 2 – Body weight (g) gain 0-42 days. Means with different letters in the superscripts are significantly different, P ≤ 0.05. Statistical analysis deploying ANOVA and Fisher's LSD test to separate treatments in different statistical groups was applied.

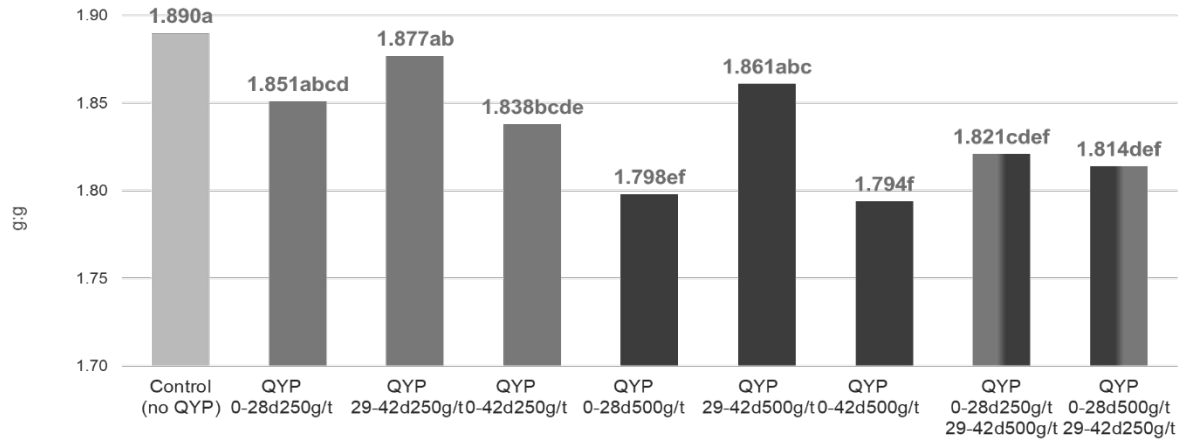

Figure 3 – Feed conversion ratio 0-42 days. Means with different letters in the superscripts are significantly different, P ≤ 0.05. Statistical analysis deploying ANOVA and Fisher's LSD test to separate treatments in different statistical groups was applied.

IV. DISCUSSION

The results of the current trial are in line with previous reports from Bafundo et al. (2021) and Bafundo et al. (2022) that QYP provides a dose dependent improvement of performance, inflammation, or pathogen related parameters in broilers in both pathogen challenge and non-challenge models. In the current study QYP significantly reduced mortality, intestinal inflammation, *C. perfringens* and *E. coli* intestinal counts, *Eimeria* oocyst excretion and *Salmonella* spp. incidence. It also significantly improved the zootechnical performance (BW, BWG, FCR and EPEF), and processing characteristics. These effects were dose dependent. Larger improvements were achieved at the higher inclusion rate, with 500g/t providing better response compared to 250g/t. A positive effect was demonstrated when QYP was supplemented 0-42 days or at least 0-28 days of age. When product was provided only for the 29-42 days of age period, results were not significant for most of the parameters measured. Thus, a conclusion can be made that the optimum effect of the product can be achieved when supplemented during the whole grow-out period or at least in the first four weeks of life. The prolonged period of supplementation required for maximizing the effect of QYP could be explained with the previously proposed mode of action by Stanev et al., (2024) through improved immunity development, reduced inflammatory tissue damage, and faster recovery, rather than direct effect on pathogens.

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RECENT ADVANCES IN BROILER BREEDER NUTRITION

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Summary

Broiler breeder nutrition has received little focus over the past few decades compared to meat and egg-type poultry. However, significant genetic progress over the same period, coupled with welfare challenges associated with ever-increasing feed restrictions during rearing and production, have emphasised the need for focused broiler breeder research now and into the future. Recent development of precision feeding equipment for broiler breeder research, and primary breeder company assistance in this research, is in the process of investigating differences in rearing growth profiles, liveweight at light stimulation and throughout the production cycle. The effect of manipulation of rearing and laying liveweight profiles on lifetime breeder and progeny performance are receiving attention. Additionally, welfare concerns have re-ignited efforts to investigate substantial dilution of breeder diets during rearing. Instructively, diet dilution illustrates that a range of different diets can be successfully offered to broiler breeders since they are reared to a target growth profile, simply requiring the amounts of feed offered to be adjusted, so the targets are achieved. However, energy supply and amino acid balance remain a critical aspect of broiler breeder nutrition and utilisation of an ever-increasing amount of non-protein-bound amino acids in breeder diets requires further elucidation. The notionally "essential" and "non-essential" amino acids need to be considered in balanced protein research for both rearing and production. Strategies to increase early egg size and maintain eggshell quality in lay continue to receive attention. The role of fatty acids in yolk deposition, particularly linoleic acid, together with vitamin and trace mineral status of the egg and progeny are important. Other feed additives, such as exogenous enzymes, guanidinoacetic acid, organic acids, phytogenic compounds, prebiotics, probiotics, postbiotics, antioxidants etcetera, have not been well researched in broiler breeders but are receiving attention, particularly within internal primary breeder organisations. There is a dearth of broiler breeder research in Australia despite a growing poultry industry, that exports both grandparent and parent stock in substantial volumes into the Asia-Pacific region. Future regional research is essential to support future sustainability of poultry production and growth in the region.

I. INTRODUCTION

Continued genetic progress at the broiler chicken level has led to increasing challenges at the broiler breeder level. Primary breeder companies strive to gain continued genetic advancement at both progeny and breeder level. For example, the genetic progress over the next four years (Aviagen, 2024) can be accurately quantified, since this is the approximate timeline from pedigree production through to broilers placed in the field (Table 1). Over the past four decades, targeted selection of broiler breeders has been accompanied by a decline in body lipid in the progeny since feed conversion ratio (FCR) improvement favours lean gain over lipid gain. Selection for increased feed intake has led to leaner broilers, due firstly to the differences in water content of body lipid (200 g/kg) and that of protein (800 g/kg) (Leenstra and Cahaner, 1991) and, secondly, the energy cost of lipid deposition (10.9 kcal/g) compared with lean tissue deposition (8.63 kcal/g) (Lopez et al., 2007). Whilst data on relative fat pad measurements in broiler breeders is dependent on the age of the bird, and the dietary energy content of the feed offered, the decline in fat pad weights over the past 50 years has been substantial (Figure 1).

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Table 1 - Anticipated annual genetic gain in the performance of broiler chickens and broiler breeders from 2024 to 2028 (Aviagen, 2024a).

Trait	Range of annual change
Broiler	
Growth (g)	40 to 45
FCR (g feed/g liveweight)	-0.020 to 0.025
Eviscerated yield (g/kg)	1.0 to 1.5
Breast yield (g/kg)	1.0 to 1.5
Leg defects (%)	-0.15 to -0.20
Mortality (%)	-0.10 to 0.20
Breeder	
Hatching eggs per hen housed	0.15 to 0.20
Hatchability (%)	0.20 to 0.25

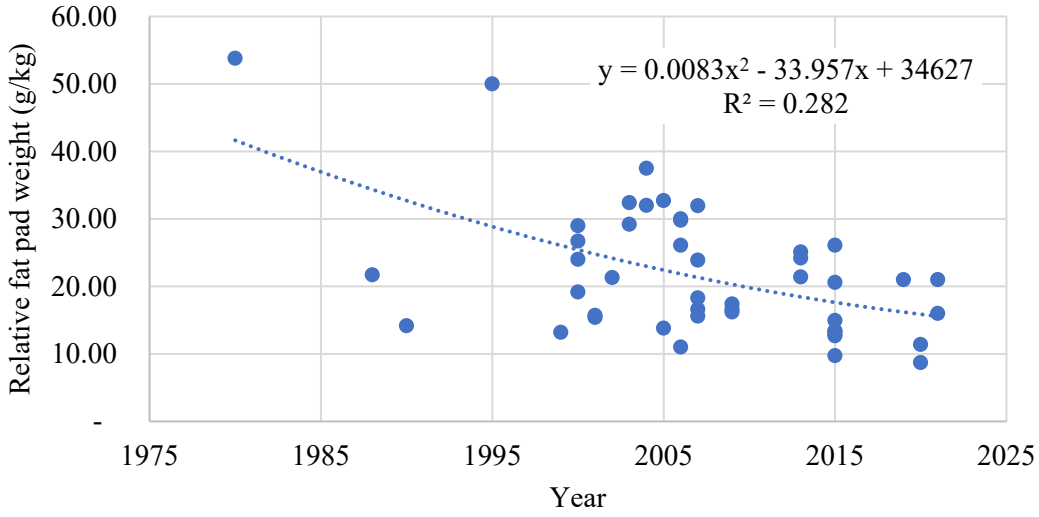

Figure 1 - Change in relative fat pad weights in broiler breeders over the past 50 years in g/kg of bodyweight.

The intensity of feed restriction has increased in broiler breeders over the past decades raising welfare concerns (Afrouziyeh et al., 2023; Carney et al., 2022). A comprehensive study at the University of Alberta (Carney et al., 2022) indicated that the feed required during rearing of broiler breeders was 99.4% of ad-libitum in 1957, 57.0% in 1978, 29.5% in 1995 and 24.9% in 2015. For the past five decades, feed restriction has been necessary during rearing and laying periods to reduce obesity and allow for normal reproductive performance in broiler breeders (Richards et al., 2010). More recently, Afrouziyeh et al., (2023) investigated growth profiles in broiler breeder hens relaxed to between 5 and 20% above the Ross 708, Aviagen published target. Compared with Primary Breeder standard growth, heavier hens produced progeny with improved FCR and increased gastro-intestinal health. Additionally, reducing the severity of growth restriction during prepubertal and early pubertal growth improved male progeny growth performance.

II. RELAXING BROILER BREEDER GROWTH RESTRICTION

The development of a precision feeding system (Zuidhof, 2018) has facilitated research on varying growth profiles for broiler breeders. There are at least 3 biologically relevant growth phases in broiler breeder rearing including prepubertal, pubertal and post-pubertal, defined by different Gompertz functions (Zuidhof, 2020). Broiler breeder hen body composition changes

during rearing and there is a fat-free tissue threshold that must be reached prior to pubertal development (Zuidhof, 2020; Kwakkel et al., 1995). Unrestricted laying hens have a total bodyweight gain mainly in the prepubertal phase of between 80.2 and 83.5% of mature bodyweight, whilst feed-restricted broiler breeders are only 49.4% for hens and 40.1% for roosters (Grossman and Koops, 1988). In Zuidhof (2020), the triphasic model was superior to both the mono- and diphasic models with all parameters highly significant ($P < 0.001$, $R^2 = 99.96$) accompanied by a low root mean square error. The concept of prepubertal lean growth followed by pubertal growth spurt of reproductive organs and, finally long-term tissue accretion has been recognised in humans and animals (Zuidhof, 2020). The difference between commercial layers (for table eggs) and broiler breeders in prepubertal growth suggests that the natural growth pattern of the latter has been altered and commercial implications of different growth trajectories can only be speculated about since there is a dearth of data available. Broiler breeder research suggests that feed restriction to reach current target weights in Ross 308 parent stock may be reaching a limit whereby some pullets have insufficient body lipid to undergo sexual development (van der Klein et al., 2018a,b; Zuidhof, 2018, 2020). Therefore, new targets for rearing are required and the triphasic model is suitable as a tool to aid in this pursuit. Afrouziyeh et al. (2021) observed similar bodyweights from zero to six weeks post-hatch and, whilst age at first egg was similar for standard versus 20% increase in prepubertal weight gain (175.7 and 175.6 days respectively), the heavier reared birds produced 110 compared with 105 eggs to 42 weeks post-hatch. Increasing weight gain during the prepubertal and early pubertal phases reduces hunger during rearing and laying. Additionally, relaxed feed restriction improved egg weights and mass and, this has implications for greater utilisation of eggs early in lay.

III. DIET DILUTION

Dilution of breeder diets is not a new concept and, just under 20 years ago, Sandilands et al. (2005) used oat hulls as a diluent in breeder diets to alleviate chronic hunger and associated welfare concerns. Oat hulls were included at either 300 or 400 g/kg from two to 20 weeks post-hatch and offered *ad-libitum* while the positive control was quantitatively restricted. Noticeably, the quantitatively restricted birds exhibited object pecking and excessive water intake that was absent in the *ad-libitum* fed birds. Birds reared on diets diluted with 400 g/kg oat hulls reached target weights close to the standard and spent 30 to 38% of their time feeding whilst those that were restricted consumed their feed within 2 to 4 hours. Welfare concerns in western Europe have reignited research on dilution of breeder rearing diets including straw by up to 500 g/kg of the diet (Aviagen, 2024b). Diets were diluted for protein and energy only and, vitamins and minerals were maintained at normal diet levels. Broiler breeders offered diluted diets were much calmer, doubled feed clean-up time and had improved feather cover and flock uniformity. However, water intake, litter volume and mortality increased, due to constipation. Interestingly, in Sandilands et al., (2005) diet dilution also caused an increase in mortality. Reducing dilution to 100 g/kg resulted in reduced mortality. Unfortunately, the cost of qualitative restriction is far higher than conventional, quantitative restriction, accompanied by associated reductions in feedmill throughput due to pelleting diluted diets (Table 2).

Qualitative feed restriction during rearing and modest dilution (50 g/kg) during lay appears to benefit broiler breeders in terms of egg production and, ultimately number of chicks per hen housed. However, more research is warranted on the effects of feed dilution on the progeny of the broiler breeders offered these diets.

Table 2 - Advantages and disadvantages of a 180 g/kg diet dilution with wheat straw during broiler breeder rearing.

Rearing period	Advantageous	Neutral	Disadvantageous	Comments
Uniformity	✓	✓		Delayed lighting from 20 to 21 weeks
Body weight profile		✓		Slower into production
Calmer birds	✓			Less aggression during rearing
Abnormal behaviour	✓			Reduced object pecking
Feathering	✓			Noticeably better
Mortality			✓	Improved in subsequent trials
Feed clean-up time	✓			Doubled over normal
Manure volume			✓	Increased FOM[1]
Constipation			✓	Start dilution early
Feed allocation above dilution			✓	Energy cost of FOM?
Feedmill tonnes per hour			✓	High fibre diets slow throughput
Fine particles			✓	Poorer pellet quality
Cost of feed			✓	Total cost above standard feeding
Farmer acceptance	✓			Good welfare outcomes
Logistics			✓	Additional feed volumes
Water consumption	✓		✓	Increased but less aggressive
Litter quality		✓		Similar to standard feeding

[1]Faecal organic matter

IV. UTILISATION OF ENERGY AND PROTEIN

Broiler breeders are normally reared to a growth profile set by primary breeders, allowing a wide range of diets, differing in nutrient density, to be offered. However, energy intake remains a key parameter in published breeder performance objectives. In a critical review of the energy content of broiler diets and feed ingredients, Mateos et al., (2019) concluded that none of the methodologies utilising values from tables, or equations based on nutrient analyses, would provide accurate estimates under practical situations suggesting that nutritionists should use their own experience to estimate the energy of feed and feed ingredients. Subsequently, it has been noted that there was lack of clarity about the origin of the metabolizable energy (ME) values in most databases due to issues relating to ME values generated across various laboratories, using different ME systems, bioassays, and techniques (Wu et al., 2020). The complexity of determining accurate ME values for feed and feed ingredients is due to dietary energy resulting from the oxidation of carbohydrates, protein and lipid, with a possibly erroneous assumption that energy is linearly additive in complete poultry diets (Kleyn and Chrystal, 2020). ME is usually measured in broiler chickens or in caecectomised roosters and the data extrapolated to broiler breeders. However, the metabolisability of energy (ME to gross energy ratio) differs owing to production stages, ages and species in poultry (Noblet et al., 2022) suggesting ME is largely a function of the animal rather than the feed it consumes. Instructively, Cozannet et al., (2010) observed that the ratio of gross energy (GE) to apparent ME, adjusted for nitrogen retention (AMEn) differed between roosters (65), broilers (77) and laying hens (87). Also, the ME:GE ratio has not been well established for restrict fed broiler breeders with the European Table of Energy values for Feedstuffs (1989) based largely on data from adult roosters, being primarily adopted by leading enterprises for AMEn recommendations. A model developed in Hubbard Hi-Yield hens and Peterson males for ME utilisation at various temperatures (Rabello et al., 2006) estimated ME requirement (kJ/bird/day) to be as follows:

$$ME = kgW^{0.75}(806.53 - 26.45T + 0.50T^2) + 31.90G + 10.04EM$$

Where $kgW^{0.75}$ is body weight (kg) raised to the power 0.75, *T* is temperature (°C), *G* is weight gain (g) and *EM* is egg mass (g). These authors estimated that the efficiency of energy utilisation for egg mass and weight gain was 60% and, the basal ME values used for feed were from the Brazilian Tables for Poultry and Swine (Rostagno et al., 2000). The Brazilian tables are based largely on the total excreta collection method (Hill and Anderson, 1958) determined in broilers of different ages. Using calorimetry systems, Caldas et al., (2018) observed that 79% of the energy intake at peak egg production was used for maintenance. These authors also noted that heat production was a significant portion of maintenance energy and quantified this at 100 kcal/$kgW^{0.75}$ at the onset of lay increasing to 106 kcal/$kgW^{0.75}$ by week 59 at termination of the trial.

In work done nearly two decades ago, Nonis and Gous (2006) examined the response of broiler breeders to either protein-bound amino acids or supplementation with L-lysine HCL and DL-methionine. Replacing dietary protein with increasing amounts of free lysine and methionine, up to 2.3 g/kg feed, had no effect on feed intake, bodyweight gain, egg weight or efficiency of lysine utilisation, but reduced the crude protein content in the diet up to 3.3 percentage units and improved the efficiency of protein utilisation by 22.3%. However, for each g increase of non-bound amino acid content/kg above this level, the rate of lay and egg output decreased by 3.0% and 2.5 g per day, respectively, and the efficiency of methionine utilisation declined by 4.3%. Current wheat- soyabean meal-based parent stock peak lay diets in Australia typically contain up to 4.72 g/kg of non-bound amino acids and a concomitant decline in protein-bound amino acids. More recently, De Paula Dorigam et al., (2016a) used summit dilution diets to model the lysine requirement of broiler breeder hens based on daily nitrogen retention and efficiency of dietary lysine utilisation. The range of analysed dietary lysine was 2.30 to 12.18 g/kg in this study. These authors modelled retention of nitrogen for maintenance (NMR) and maximum theoretical nitrogen retention (NRmax) in broiler breeder hens. NMR values were 239.1 (31–35weeks post-hatch) and 271.7 mg/B*W*$kg^{0.67}$ per day (46–50weeks post-hatch) and the authors hypothesized that endogenous nitrogen loss was negatively correlated with egg production. The threshold value of NRmax was 1684 mg/B*W*$kg^{0.67}$ at peak production (31–35weeks) and decreased to 1484 mg/BW$kg^{0.67}$ from 46 to 50 weeks of age. The derived optimum lysine intake was 915 mg/day for broiler breeder hens from 31 to 35 weeks, with a nitrogen retention of 1371 mg/B*W*$kg^{0.67}$ per day that corresponds to 0.81 times the NRmax value. In close agreement with Rostagno et al., (2011), the optimal dietary lysine of an 11.70 MJ/kg AMEn diet was 5.41 g/kg at 169 g/d feed intake and 6.78 g/kg at 135 g/d feed intake (31 to 35 weeks of age) and decreased to 5.37 g/kg (163 g/d feed intake) or 6.74 g/kg (130 g/d feed intake) in broiler breeder hens from 46 to 50 weeks of age. The Aviagen (2021) Parent Stock Nutrient Specifications are in reasonable agreement with these values at dietary digestible lysine values of 6.2 g/kg for breeder 1 to 32 weeks, 5.6 g/kg from 33 to 50 weeks and 5.2 g/kg post 50 weeks of age. In a follow-up study, De Paula Dorigam et al., (2016a) investigated the ideal amino acid ratios, by deletion method, for broiler breeder hens (with lysine as the reference amino acid) and concluded that these were 86% for methionine + cysteine, 23% for tryptophan, 80% for threonine, 113% for arginine, 90% for valine, 91% for isoleucine, 133% for leucine, 108% for phenylalanine + tyrosine, 94% for glycine + serine and 35% for histidine.

V. LINOLEIC ACID

Avian species are unable to synthesize linoleic acid which is why it is considered an essential fatty acid (Kleyn and Chrystal, 2020). However, minimum required levels in broiler breeder diets have not been well documented in the literature. The most recently published Nutrient

Requirements of Poultry (NRC, 1994) noted that deficiencies were seldom encountered but, in male broiler breeders, a shortage may have a negative impact on spermatogenesis and adversely affect fertility. Also, 10 g/kg for adult birds is sufficient but levels above this may be required for maintaining satisfactory egg weights. Current commercial recommendations have a minimum linoleic acid level of 12.5 g/kg in rearing and male feed and, 20.0 g/kg for laying breeder hens. The effect of diet linoleic acid level on egg weight, egg composition, hatchability, chick weight, yolk sac percentage relative to chick weight and yolk fatty acid composition of broiler breeders in Ross 305, from 27 to 40 weeks of age, were evaluated using two diets with two levels of linoleic acid. Egg weight and linoleic acid yolk composition were higher for diet with 19.3 g/kg of linoleic acid than for diet with 14.8 g/kg (59.5 g vs 59.0 g and 195 vs 155 g/kg, respectively). There were no linoleic acid effects on yolk, albumen, eggshell, yolk sac percentage, hatchability or chick weight (Ribeiro et al., 2007). Variations in the fatty acid content of the yolk could contribute to variation in hatchability and growth of broiler breeder progeny (Yilmaz-Dikmen and Sahan, 2009). These authors observed a decline in egg yolk deposition of linoleic acid from 21.6 g/kg at 28 weeks of age to 13.87 by 65 weeks of age ($P < 0.01$). In hens over 50 weeks post-hatch, decreases in myristic and linoleic fatty acids were negatively correlated with late embryonic mortality, and hatchability ($P < 0.05$) suggesting that dietary linoleic acid may need to be increased as broiler breeders age.

VI. EXOGENOUS ENZYMES

A plethora of feed additives are available on the market, including exogenous feed enzymes, although these have not been well researched in broiler breeders. The inclusion of xylanase in wheat-based diets and β-glucanase in barley-based diets is common across all types of poultry including broiler breeders. However, the use of phytase in broiler breeder diets is not universally accepted. Decreasing non-phytate phosphorus (NPP) by 1.0 g/kg and adding 500 FTU of phytase in 6-week old broiler breeder pullets reared in battery cages decreased faecal P by 17% during a 48-h collection study (Lilburn et al., 2004). In another study on Ross 308 broiler breeders from 22 to 64 weeks post-hatch, diets were offered containing 3.7 g/kg NPP or reduced to 2.7 g/kg NPP with the addition of 500 FTU of phytase or a further reduction to 1.9 g/kg NPP and 0.9 g/kg NPP plus 500 FTU of phytase (Plumstead et al., 2007). The P content of litter showed no effect when NPP was reduced by 1.0 g/kg and phytase was included. Decreasing NPP without adding phytase reduced litter P by 18%. During the laying period, a reduction of NPP from 3.7 to 0.9 g/kg with added phytase, the water-soluble P in litter reduced by 42%. Hen day egg production was highest on the lowest NPP diet with phytase, but fertility decreased when the dietary NPP was reduced below 3.7 g/kg. More recently, data on broiler breeders with 500 FTU phytase added during rearing or laying or throughout the growth and production phases resulted in an increase ($P < 0.05$) in rearing mortality (Aviagen, 2024b). The layer phase phytase treatment had a trend ($P = 0.08$) toward higher mortality whilst phytase offered over the lifetime of the birds was also higher ($P < 0.05$). The highest causes of hen mortality were cannibalism and congested lungs followed by peritonitis and, synovitis and tenosynovitis in roosters. Egg production was lower in broiler breeders offered phytase throughout their growth and laying phase. These data would suggest that the matrix values used for the phytase were too high (1.80 g/kg Ca and 1.73 g/kg NPP, respectively). The control diets had an estimated phytate phosphorus of 3.4 g/kg.

VII. CONCLUSIONS

Broiler breeder research is uncommon in Australia although, there is an attempt to revisit this in both Queensland and New South Wales. Most of the current research in broiler breeders is carried out by the primary breeder companies themselves and is seldom published in peer-

reviewed Journals. Extrapolating data from full fed commercial layers to restrict-fed broiler breeders is fraught with problems since the broiler breeder has a bodyweight almost double that of commercial layers and a lower egg output. Unlike broiler chickens, breeder research is complex, costly and time-consuming. Research needs to be targeted at both hens and roosters and, the multi-faceted outcomes need to include welfare parameters (including feather cover), egg production, shell quality, fertility, hatchability and performance of the progeny. Whilst primary breeder companies will continue to do internal research to aid in updating management and nutrition guides, there is a need for independent research in the Oceania geographical region that withstands peer-review scrutiny and is ultimately published in reputable Journals.

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REDUCING DIETARY PROTEIN FROM 16 TO 12% DOES NOT HAVE A NEGATIVE IMPACT ON BROILER BREEDER PERFORMANCE

B.C. RAY[1], A. KUMAR[1], S. NIKNAFS[1] and E. ROURA[1]

The poultry industry's dependency on soybean meal as a primary source of protein in practical feed formulations poses a challenge to sustainability in Australia. Implementing a low crude protein (CP) / low soybean meal diet with amino acid balance can potentially diminish this dependence (Chrystal et al., 2021). However, significant protein reductions can compromise bird performance and negatively affect chicken meat production. This study aimed to assess how varying dietary CP levels in broiler breeders affect egg-laying performance. We hypothesized that by reducing dietary CP levels down to 12% and supplementing crystalline /synthetic amino acids to meet the broiler breeder's requirement, it is possible to maintain optimal egg production performance.

A total of 103 (91 hens and 12 roosters), 36-week-old Ross 308 broiler breeders were randomly allocated to twelve pens with seven or eight hens and one rooster per pen. The birds were fed a standard diet for one week before feeding the experimental diets. Three iso-energetic experimental mash diets were fed for three weeks (36-38 weeks of age) consisting of feeds formulated to contain 16, 14, and 12% CP. All the diets were formulated to meet or exceed the essential nutrient requirements including amino acids. The experimental diets were feed-restricted for both male and female birds according to the age of the breeders as per Ross 308 breeder guidelines (Aviagen, 2021). Egg production and egg weight were recorded daily, and the hen-day egg production, egg mass, and feed conversion ratio were calculated. Birds were weighed at the beginning and end of the experiment, and feed and water consumption were recorded. At the end of the feeding trial, blood samples were collected from four hens of each replication pen for plasma amino acid analysis. Data were analysed using PROC GLM (SAS 9.4).

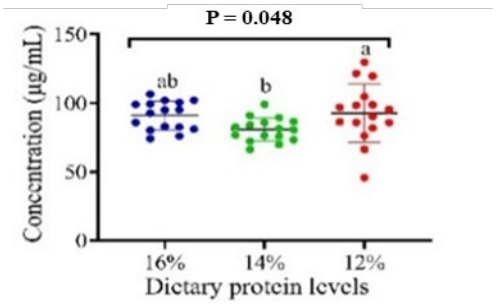

Figure 1 - Plasma concentration of glutamic acid from broiler breeders fed three dietary CP levels (n=16 per treatment). Each dot in the scatter plot indicates the values of each observation. Error bar indicates mean with standard deviation. [ab] Different letters indicate significant differences among treatments at the stated probability level.

Results showed that dietary CP levels did not significantly affect (P = 0.845) hen-day egg production, egg weight, egg mass output, feed conversion ratio, body weight (or weight gain), or water consumption. Similarly, no significant differences between groups were observed in abnormal eggs (double yolk, pinholes, blood spot, and elongated). Plasma amino acid profile showed a significant difference (P = 0.048) in glutamic acid which was significantly higher in the 12% CP-fed group compared to 16% or 14% groups (*Figure 1*).

In conclusion, dietary CP levels did not affect the egg production performance and body weight of broiler breeders. However, a low CP diet significantly increased blood level of glutamic acid. The latter may reflect a deficiency in non-essential amino acids worth further investigation.

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TOWARDS AN *IN VITRO* DIGESTION MODEL BASED ON *EX VIVO* ANALYSIS OF CHICKEN DIGESTA

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Optimizing feed digestibility in chickens is key to improving feeding efficiency, metabolism, immunity and overall bird health (Borda-Molina et al., 2018). Feed digestibility is routinely assessed using in vivo trials that require specialized facilities and many birds which is expensive and time-consuming. A standardized in vitro method would reduce the need for these costly trials. Although a wide range of research has been conducted in this area (Sharma et al., 2022), most studies are based on the human gastrointestinal conditions without considering the importance of particle size and dry matter.

This study aims to develop a model that can predict digestion results. It is hypothesized that by analyzing the dry matter and particle size distribution from different digestive sections of chicken in vivo digesta, an in vitro digestion model can be developed. It will also test whether it is necessary to develop different models for different bird ages. Using such a model, changes in feed composition, particle size or the addition of exogenous enzymes could be evaluated quickly and accurately, prior to confirmatory animal trials on feeds selected by in vitro screening.

Digesta was collected from the gizzard, duodenum, jejunum, ileal, large intestine and caeca of chicken at different ages (7 days old, 28 days old and 42 days old) on different diets. The dry matter of the digesta and feeds were determined by freeze drying. To assess the particle size distribution at each site, the dried digesta were successively sieved through 1.18mm, 600µm, 425µm, 212µm, 106µm and 53µm sieves. The sizes 425µm and below were observed using polarized light microscopy to identify residual feed structures in the digesta of each site. The total starch and protein content of digesta samples of gizzard, jejunum and ileal at different ages were measured and compared with feed to define the changes along the digestive tracts.

Analysis of chicken digesta across different ages and different diets reveals the following: 1) Dry matter remains consistent in the same digestive tract section across all three ages; 2) Particle size distribution in the same section is similar regardless of age or diet; 3) Feed particle size decreases significantly after passing through the gizzard, with a marked increase in smaller particles. The jejunum and ileum contain significantly more small particles than the gizzard, though no significant difference in particle size distribution is observed between the jejunum and ileum. Based on the dry matter and particle size distribution in the gizzard and jejunum, it will be possible to develop a single in vitro digestion method to accurately predict in vivo digestion outcomes across different growth stages of chickens.

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FEEDING METHIONINE HYDROXYL ANALOGUE CHELATED ZINC, COPPER AND MANGANESE TO BROILER CHICKENS REDUCES MINERAL EXCRETION

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Zinc (Zn), copper (Cu), and manganese (Mn) are important trace minerals for metabolism, growth, and the general health condition of broiler chickens (Bao et al., 2007). Inorganic trace minerals including Zn, Cu and Mn are typically used at high levels in diets to compensate for their low bioavailability (Bao et al., 2010; Zhao et al., 2010), but they are usually contaminated with fluorine and cadmium (Bhagwat et al., 2021). Additionally, the metabolism and absorption of inorganic trace minerals may be reduced by antagonisms among inorganic mineral sources (Lopes et al., 2017; Bhagwat et al., 2021) and excess inorganic trace minerals in excreta is potentially harmful to the bird and environment (Zhao et al., 2010). Organic trace minerals are acknowledged to be a more readily available source of trace elements compared to conventional inorganic alternatives (Byrne and Murphy, 2022). However, reports comparing the effects of Cu as a growth promoter using inorganic Cu salts versus reduced rate of organic Cu sources in the literature are scarce. Given the increasing Cu price in the market and possible negative effects of high dietary Cu supplementation, it is necessary to reassess optimal Cu level and source in meat-chicken diets. Therefore, this study investigated the effects of dietary supplementation of mineral methionine hydroxyl analogue chelates (MMHAC MINTREX®, Novus International, Inc.) with zinc (Zn), copper (Cu) and manganese (Mn) bound by methionine hydroxyl analogues in a 2:1 chelated molecule. This study reports the effects of treatments on the excreta mineral and nitrogen content of broilers and further explores environmental impacts (emissions (CO2; CH4; N2O), the total t CO2e/farm, and the total t CO2e/farm per kilo live weight gain) via a life cycle assessment (LCA). Approval for the study was granted by the UNE Animal Ethics Committee under approval number ARA23-004. A total of 384 day-old Ross 308 male chicks were randomly distributed to 4 dietary treatments, with 8 replicate pens of 12 birds per pen. The treatments were (1) Inorganic trace mineral control diet containing ZnSO4 110 ppm, $CuSO_4$ 16 ppm, MnO 120 ppm (ITM), (2) MMHAC Zn 40 ppm, Cu 10 ppm, Mn 40 ppm (MMHAC10), (3) Inorganic trace mineral $ZnSO_4$ 110 ppm, tribasic copper chloride 125 ppm, MnO 120 ppm (TBCC125), and (4) MMHAC Zn 40 ppm, Cu 30 ppm, Mn 40 ppm (MMHAC30) over starter (d0-10), grower (d10-21), and finisher (d21-42) phases. The Poultry Greenhouse Accounting Framework V1.45 from the Primary Industries Climate Change Centre (PICCC, 2023) was utilised in the LCA to determine the emissions (CO_2; CH_4; N_2O), the total t CO_2e/farm, and the total t CO_2e/farm per kilo live weight gain in a simulation of a total of 320,000 broiler chickens across 3 grow-out periods of 42 d each. Birds offered MMHAC at both levels had significantly lower Zn and Mn levels in the excreta compared to those fed the other diets on d10, d16, d21, d28 and d 42 ($P < 0.001$). Excreta nitrogen was similar between the dietary treatments. Due to the improved live gain of the TBCC125 and MMHAC30 treatments, the lowest total t CO_2e/farm per kg live gain was achieved by the MMHAC30 treatment followed by the TBCC125 treatment. The improvement in environmental sustainability (total t CO_2e/farm per kilo live weight gain) for the MMHAC30 treatment was in the order of 5.54%. Therefore, supplementation of MMHAC as a replacement for inorganic minerals to broiler diets may reduce the burden of chicken-meat production on the environment.

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COMPARING TRADITIONAL FORMS OF SUPPLEMENTAL COPPER TO A MONOVALENT COPPER ON BACTERIA CONTROL

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Summary

Enterococcus cecorum is an opportunistic pathogen found in the gastrointestinal tract of chickens, being a growing concern in the broiler industry, as it causes locomotion disorders and septicemia. The objective of this study was to compare different copper sources on *E. cecorum* growth. The sources of Cu compared in this trial were copper sulfate (CuS), Cu amino acid (AA) chelated (CuAA), tribasic Cu chloride (TBCC), and monovalent Cu (CR). The *in vitro* study used the strain DSM 20682 of *Enterococcus cecorum*. BHI medium was used for preparatory and growth media during the test. The medium was saturated (10g Cu/L) with the Cu products by mixing. After centrifugation, supernatants were autoclaved and used as growth media. Microtiter plates were sealed with gas-tight plastic film (PCR-grade) and incubated anaerobically at 37 °C in triplicate. Growth was monitored by turbidity measurements every 5 min. Minimal inhibitory concentrations (MIC) were determined after 24h incubation. Resulting growth data were examined for start of growth according to moving average determination. A one way-factorial ANOVA with Bonferroni post-hoc test was used to determine significant differences between products. The results of MIC showed that 64 µg of Cu from CR per mL are enough to stop *E. cecorum* growth, while 128 µg of CuAA and more than 256 µg of TBCC or CuS are necessary. The growth kinetics was measured below lethal concentration (32 µg/mL) and was observed that *E. cecorum* starts to grow after 6h in the media with divalent sources, while it starts to growth after 11h in the media with monovalent Cu source. In conclusion, monovalent copper showed stronger ability to inhibit the growth of *Enterococcus cecorum* compared to all other Cu sources, being a tool to reduce the risk of Enterococci related disorders in broilers.

I. INTRODUCTION

Enterococcus cecorum is an opportunistic pathogen found in the gastrointestinal tract of chickens, being a growing concern in the broiler industry, as it causes locomotion disorders and septicemia (Schreier et al., 2022). This gram-positive coccus is found in the gastrointestinal tract, where it first colonizes the intestine within the first week of life; then translocate to the bloodstream affecting several extraintestinal organs; reaching then skeletal sites via the blood; finally causing lameness and paresis (Souillard et al., 2022).

Maintaining optimum gut integrity can help to avoid *E. cecorum* leakage, especially during the first week of the broiler's life. In this context, the antimicrobial effect of copper (Cu) is well recognized and its supplementation can control the growth of bacteria (Dollwet, 1985). It can be sourced from divalent forms (sulfates, chlorides, carbonates, …) or from a monovalent source. Studies have shown that monovalent Cu presents stronger antibacterial effect than the divalent Cu on *Streptococcus mutans* (Dunning et al., 1998), on *E. coli* and *Staphylococcus aureus* (Saphier et al., 2018), among other pathogens. The objective of this

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study was to compare the antibacterial properties of different copper sources on *E. cecorum* growth.

II. METHOD

This trial used four Cu sources, three of them divalent Cu sources and one monovalent source. The divalent sources were Cu sulfate (CuS; 25% Cu; Manica Cobre S.L., Spain), Cu amino acid chelated (CuAA; 10% Cu; AvailaCu®, Zinpro, USA), and tribasic Cu chloride (TBCC; 54% Cu; IntelliBondC®, Selco, USA). The monovalent Cu tested was CoRouge® (CR; 75% Cu; Animine, France).

The in vitro study used the strain DSM 20682 of *Enterococcus cecorum*. BHI medium was used for preparatory and growth media during the test. The medium was saturated (10g Cu/L – corrected according to the Cu concentration in each product) with the Cu products by mixing. After centrifugation, supernatants were autoclaved and used as growth media. Total Cu was determined using atomic absorption spectrometry and Cu concentrations ranged from 0 – 256 µg/mL total Cu.

Microtiter plates were sealed with gas-tight plastic film (PCR-grade) and incubated anaerobically at 37 °C in triplicate. Growth was monitored by turbidity measurements every 5 min. Minimal inhibitory concentrations (MIC) were determined after 24h incubation. Resulting growth data were examined for start of growth according to moving average determination. A one-factorial ANOVA with Bonferroni post-hoc test was used to determine significant differences between products.

III. RESULTS

Table 1 shows the observed minimal inhibitory concentrations (MIC) of *E. cecorum* for copper sources. The results of MIC showed that 64 µg of Cu from CR per mL are enough to stop *E. cecorum* growth, while 128 µg of Cu from CuAA and more than 256 µg of Cu from TBCC or CuS are needed ($P<0.05$).

Table 1 - Minimum Inhibitory Concentration (MIC) of copper products on *E. cecorum.* Different letters in the same column indicate a statistically significant difference ($P<0.05$).

Product	MIC, µg/mL	Amount of product to achieve MIC, µg*
CR	64[c]	85
CuAA	128[b]	1,280
TBCC	>256[a]	>474
CuS	>256[a]	>1,024

*Amount of product to achieve the MIC value to to stop the growth of *E. cecorum*, according to the Cu concentration in each product (CR, monovalent copper; TBCC, tri-basic copper chloride; CuAA, Cu amino acid chelated; CuS, copper sulfate).

The growth kinetics was measured below lethal concentration (32 µg/mL; half of CR MIC) and was observed that *E. cecorum* starts to grow after 6h in the media with divalent sources, while it starts to grow after 11h in the media with monovalent Cu source. In summary, at this sublethal concentration, the growth of *E. cecorum* starts first for TBCC source, followed by CuAA and CuS sources, and latest by CR source ($P<0.05$).

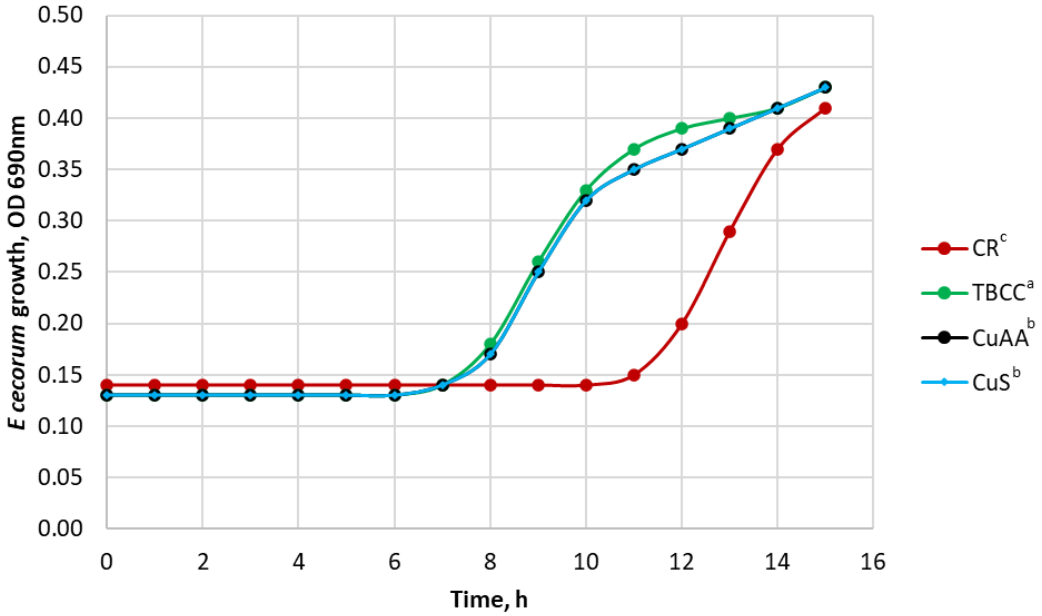

Figure 1 - Growth kinetics of *E. cecorum* at sublethal concentration (32 µg/mL) of Cu products (CR, monovalent copper; TBCC, tri-basic copper chloride; CuAA, Cu amino acid chelated; CuS, copper sulfate). Different letters indicate a statistically significant difference in the time it takes for the bacteria to start growing (P<0.05).

IV. DISCUSSION

The minimum inhibitory concentration is the lowest concentration of an antibacterial agent (expressed in µg/mL) which completely prevents visible growth of the test strain of an organism. The highest MIC found in this study can be related to the ionic form of Cu in CR. It has been known that the antibacterial effects of Cu vary depending on its redox state, with the monovalent form (Cu^{+}) demonstrating stronger efficacy in anaerobic conditions compared to the divalent form ($Cu2^{+}$). Saphier et al. (2018) found that monovalent Cu was more effective than divalent Cu at acidic pH in inhibiting both gram-positive and gram-negative bacteria, with the minimum inhibitory concentration (MIC) for *E. coli* and *Staphylococcus aureus* being half that of divalent Cu.

This also explains why, when following the kinetics of growth, bacteria take longer to start to grow in a media with monovalent Cu in comparison to divalent Cu. Dunning et al. (1998) found that monovalent Cu showed higher antibacterial activity on *Streptococcus mutans*, compared to divalent Cu, which was ineffective. His study showed that after 25 minutes *S. mutans* decreased by half when monovalent Cu was present, but no changes were observed up to 50 minutes in the media with divalent Cu. With this background, the supplementation of monovalent Cu could help to control *E. cecorum* growth, reducing the leakage of *E. cecorum* from gut to extraintestinal organs.

The range of Cu values tested in this study (between 32 µg of Cu/mL for growth kinetics to 64 µg of Cu/mL in the MIC evaluation) are in line with the range of values found in the gastrointestinal tract of broilers. Hamdi et al. (2018) observed that the concentrations of soluble Cu in the ileum are between 8-10 µg of Cu/mL when Cu is supplemented at 15 ppm, and between 67-74 µg of Cu/mL when Cu is supplemented at 150 ppm. We can assume that the values tested in our study correspond to a dietary Cu supplementation between 15 and 150 ppm.

The higher *in vitro* antibacterial effect of monovalent Cu found in our study was also observed *in vivo*. A study testing 150 ppm of monovalent (CR) and divalent (CuS) Cu in broiler

diets, from 1 to 42d of age, observed that the supplementation of monovalent Cu established changes in the gut microbiota by regulating the bacterial population in the ileum (Forouzandeh et al., 2021). This modulation of microbiota was raised as the hypothesis to explain the positive impact on broilers' growth performance when birds received monovalent Cu (2.59kg versus 2.47kg of final BW).

In conclusion, monovalent copper showed stronger ability to inhibit the growth of *Enterococcus cecorum* compared to all other Cu sources, being a tool to reduce the risk of Enterococci related disorders in broilers.

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PROTEIN TURNOVER IN BROILER CHICKENS

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Summary

Protein turnover is the dynamic balance between deposition and degradation of whole-body proteins. A positive balance, or protein accretion, is a fundamental driver of weight gain and growth in broiler chickens. This succinct review considers the impacts reducing dietary crude protein concentrations might have on protein turnover, particularly protein degradation.

I. INTRODUCTION

Protein turnover is the continuous flux between deposition and degradation of whole-body proteins, including skeletal musculature. A positive balance in protein turnover equates to net protein synthesis, resulting in protein accretion and growth. The express rates of gain presently exhibited by broiler chickens is largely driven by protein accretion of skeletal muscle, which represents nearly 70% of the carcass weight of birds (Weimar et al., 2022). Protein accretion is straightforward, but superficially protein degradation seems counter-productive, which led Reeds (1989) to question the rationale for the evolution of such a process. However, Glickman and Ciechanover (2002) aptly described protein degradation as "destruction for the sake of construction". The principal route via which proteins are degraded is the ubiquitin–proteasome pathway. This pathway degrades intracellular proteins to destroy misfolded or damaged polypeptides to prevent the accumulation of non-functional, potentially toxic proteins (Goldberg, 2003). Somewhat surprisingly, there does not appear to be any suggestions that protein in skeletal muscle is degraded to meet amino acid deficiencies. The protein turnover rate in broiler chickens of 30.2 g/kg body weight$^{0.75}$ per day is nearly double the average rate of 16.8 g/kg body weight$^{0.75}$ per day in mouse, rat and man (Muramatsu. 1990). However, relatively little research into protein turnover in broiler chickens has been reported. This is unfortunate because protein turnover well could be the fundamental determinant of growth performance and, interestingly, it appears that protein degradation is the more important element in the dynamic balance (Sunde et al., 1984). The lack of research probably reflects the fact that quantifying protein turnover is not straightforward. The justified quest to develop reduced-crude protein (CP) diets places a focus on protein turnover as, almost by definition, a reduced-CP diet challenges net protein accretion.

II. NET PROTEIN SYNTHESIS

Few studies have investigated protein turnover in broiler chickens, but Klasing et al. (1987) compared protein synthesis and protein degradation in muscles from fast and slow-growing birds. Fractional degradation rates were 12% to 19% slower in broiler chicks than in slow-growing Single Comb White Leghorn birds. Protein turnover in the *Pectoralis major* muscle in chickens selected for divergent growth rates was investigated by Tesseraud et al. (2000). Net protein synthesis was greater in fast versus slow-growing broilers at both 2.5 (108 versus 47 mg/day) and 4 (161 versus 67 mg/day) weeks of age by a 2.4-fold factor. These researchers concluded that heavier *Pectoralis major* weights in rapidly growing birds were essentially a consequence of decreased protein degradation. Protein turnover in skeletal muscles was determined in broiler chickens that were offered 250 or 200 g/kg CP
maize, wheat and soybean meal-based diets from 35 to 42 days post-hatch by Temim et al. (2000). In this study, dietary CP reduction retarded absolute protein synthesis rates by 17.6% (1689 versus 2049 mg/day) and absolute protein breakdown rates by 18.6% (902 versus 1108 mg/day) in the

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Pectoralis major in birds, indicating a 50 g/kg dietary CP reduction declines net protein synthesis rate by 16.4% (787 versus 941 mg/day). Thus, the Temim et al. (2000) study supports the contention that dietary CP reductions have a deleterious impact on protein turnover.

III. FEATHERING AND INSULIN

Serine, glutamic acid, proline, leucine and cysteine comprise nearly 50% of the 957 g/kg protein in feathers (Stilborn et al., 1997). However, mature feathers do not have a blood supply and their shedding constitutes a loss of constituent amino acids as they cannot be degraded and returned to the systemic pool. Broiler chickens are usually considered to be hyperglycaemic and resistant to insulin. However, broilers are sensitive to insulin *in ovo* and for the first three to four weeks of age (Franssens et al., 2014). Thus, young broilers may retain their insulin sensitivity and respond to the anabolic effects of insulin supressing protein degradation (Tessari, 2024).

IV. METABOLIC ACIDOSIS

Metabolic acidosis has been shown to accelerate protein degradation rates by stimulating the ubiquitin-proteasome pathway in rats (Mitch et al., 1994). However, the catabolism of amino acids in birds offered reduced-CP diets, particularly methionine, cysteine and cationic amino acids, has an acidifying impact because they generate endogenous acid (H^+) production (Poupin et al., 2012). For example, a dietary excess of 30 g/kg cysteine was shown to trigger metabolic acidosis resulting in mortalities in broiler chickens (Dilger and Baker, 2008). Also, one gram of dietary lysine·HCl increases dietary acid levels by 7 mEq/kg DM of acid (Patience, 1990), which is of importance given that increased inclusions of lysine HCl are typical in reduced-CP diets. Metabolic acidosis, induced by ammonium chloride, was shown to increase amino acid oxidation and protein breakdown in humans (Reaich et al., 1994). Acid-base homeostasis and amino acid metabolism are closely intertwined in animal nutrition (Patience, 1990). Interestingly, the Ibrahim et al. (2024) study, found that glutamine and asparagine inclusions in broiler diets permitted higher dietary non-bound amino acid levels in reduced-CP diets before growth performance or nitrogen accretion was compromised. This was attributed to glutamine and asparagine attenuating challenges to acid–base homeostasis via a compensation of metabolic acidosis.

V. 3-METHYLHSTIDINE

Quantifications of protein turnover in broiler chickens are not straightforward, particularly in a practical feeding study. However, determinations of 3-methylhstidine concentrations in excreta are considered indicative of protein degradation rates in skeletal muscle (Munro and Young, 1978), although reservations about the accuracy of this approach have been expressed (Rennie and Millward, 1983). However, plasma 3-methylhstidine concentrations may be a more reliable indicator (Shiraishi et al., 2023). It is relevant that Hocking and Saunderson (1992) reported that reducing CP from 150 to 100 g/kg in poultry diets increased 3-methylhistidine excretion by 15.7% (54.4 versus 47.0 µmol/day), which indicates that dietary CP reductions trigger increases in protein degradation rates. Therefore, an evaluation of L-carnitine inclusions in reduced-CP diets in which plasma 3-methylhstidine concentrations are determined should prove instructive.

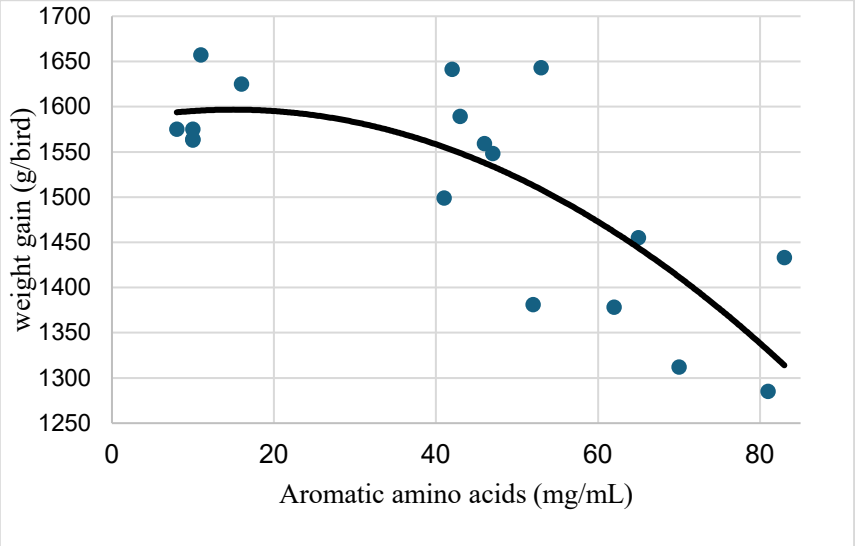

Figure 1 - Quadratic relationship (r = 0.778; P = 0.00095) between plasma concentrations of free aromatic amino acids (phenylalanine plus tyrosine) and weight gains in birds offered 160 g/kg CP diets with 0, 75 and 150 g/kg L-carnitine inclusions from 7 to 33 days post-hatch.

VI. L-CARNITINE

Dietary inclusions of L-carnitine (0, 75, 150 mg/kg) in in diets based on a sorghum-wheat blend with CP contents of 220, 190 and 160 g/kg from 7 to 33 days post-hatch were evaluated by Greenhalgh et al. (2022). L-carnitine inclusions in 220 and 190 g/kg CP diets did not have marked impacts on growth performance. In contrast, 150 mg/kg L-carnitine in 160 g/kg CP diets significantly improved weight gains by 15.9% (1593 versus 1374 g/bird) and FCR by 3.16% (1.564 versus 1.615). The aromatic amino acids, phenylalanine and tyrosine, have been extensively used to monitor rates of protein turnover in skeletal muscle because their metabolism is restricted to their roles as substrates for protein synthesis and products of proteolysis (Williams et al., 1981). Remarkably, in the Greenhalgh et al. (2022) study, 150 g/kg L-carnitine reduced free systemic plasma concentrations of phenylalanine by 69.8% (6.5 versus 21.5 mg/mL) and tyrosine by 84.3% (10.8 versus 68.8 mg/mL). From retrospective analyses, increased plasma concentrations of phenylalanine (r = 0.736; P = 0.003) and tyrosine (r = 0.780; P < 0.001) were associated with quadratically depressed weight gains. Collectively, the two aromatic amino acids were quadratically related to declining weight gains (r = 0.778; P < 0.001) as shown in Figure 1. Pivotally, there are indications that L-carnitine suppresses protein degradation. In Sprague-Dawley rats, L-carnitine noticeably depressed concentrations of ubiquitinated proteins in skeletal muscle indicating a diminished degradation of myofibrillar proteins by the ubiquitin-proteasome pathway in Keller et al. (2013). More recently, L-carnitine was shown to significantly retard fractional protein breakdown rates in Labrador Retrievers (Varney et al., 2020). The likelihood is that dietary L-carnitine concentrations declined with the reductions in dietary CP to the point they were limiting and responses to dietary L-carnitine were declared. While not conclusive, it is quite possible that retarded rates of protein degradation, as reflected in reduced aromatic amino acid plasma levels, were responsible for the positive growth performance responses. L-carnitine (β-hydroxy-γ-trimethyl-aminobutyrate) is a quaternary amino compound that plays an obligatory role in fatty acid metabolism and has muti-functional purposes in poultry (Adabi et al., 2011). The biosynthesis of L-carnitine is from lysine and methionine and L-carnitine transports long-chain fatty acids from the cytosol into the mitochondria where ß-oxidation of fatty acids takes place (Buyse et al., 2001). L-carnitine, has been shown to suppress fat deposition in broiler chickens (Xu et al., 2003) and it may counteract the effects of NH_3 intoxication (Kloiber et al., 1988). For these reasons, coupled with possible impacts on protein turnover, further evaluations of L-carnitine in reduced-CP broiler diets do appear justified.

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OPTIMIZING NUTRITION AND MANAGEMENT TO ENHANCE PRODUCTIVITY IN MODERN LAYING HENS: FROM REARING TO PEAK PRODUCTION

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The genetic advancement of modern laying hens has greatly improved their production efficiency, characterized by enhanced feed conversion, higher egg yields, and prolonged laying persistence. These developments are accompanied by a trend toward reduced body weight, requiring precise management to avoid excess weight gain, particularly during key developmental phases. Establishing optimal body weight profiles, especially by week 5, is critical for unlocking the flock's productive potential. Research indicates that early weight gains correlate with higher production persistence, reduced mortality, and increased egg numbers later in life.

The rearing phase, spanning from 0 to 18 weeks, plays a foundational role in determining long-term productivity. Nutrition during this period must support growth and uniformity, with crumble starter diets recommended for the first 5 weeks to promote consistent development. A gradual transition to mash diets over two weeks helps optimize feed consumption behavior. Between 12 and 18 weeks, monitoring weight gain becomes paramount, as excessive gains can result in delayed production onset, compromised shell quality, and increased mortality. This period demands careful regulation of feed intake to maintain body weight within optimal ranges, avoiding metabolic disorders that could jeopardize future performance.

As hens enter the pre-lay and early production phases, calcium management becomes a focal point. Diets incorporating coarse limestone support shell quality by building reserves necessary for sustained production. A strategic increase in feed intake is required to achieve peak production while preserving body reserves. Controlling egg weight also necessitates preventive measures, with adjustments in amino acid and energy levels implemented before reaching the desired target egg weight. This proactive approach minimizes the risk of oversized eggs and potential production losses.

Cage-free production systems introduce additional complexities, including heightened energy requirements due to increased activity and environmental variability. To address these demands, dietary energy is typically increased by 5–10%, and higher levels of methionine and lysine are provided to support production and maintain feather quality. Insoluble fiber is particularly beneficial in these systems, promoting gastrointestinal health, enhancing satiety, and controlling body weight while reducing aggressive behaviors. Behavioral and environmental challenges, such as feather pecking and higher exposure to parasites, necessitate targeted interventions, including nutritional adjustments and regular health monitoring.

Shell quality remains a cornerstone of sustainable egg production. Achieving an optimal balance of shell thickness and flexibility is supported by efficient calcium metabolism and the inclusion of essential minerals such as zinc, copper, and manganese. Nutritional refinements, including the use of insoluble fiber and precise amino acid profiles, further enhance production performance. Managing nutrient excesses, particularly in reactive hens, is critical to prevent adverse effects such as oversized eggs and metabolic stress.

Across all systems, the meticulous management of nutrition and body weight profiles from day one to peak production is essential for achieving the full productive potential of modern laying hens. This comprehensive approach not only ensures optimal performance but also supports sustainability and profitability in diverse production environments. By addressing the unique needs of each production system, producers can maximize the productivity and welfare of their flocks while meeting the demands of modern egg production.

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A COMPARISON OF HEN PERFORMANCE DURING AN EXTENDED LAYING CYCLE BASED ON HEN BODY WEIGHT

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Summary

Commercial egg-laying hens that have incremental increases in body weight (BW) throughout the laying phase are prone to irregular persistency of lay and the production of large eggs with poor shell quality. They continue to accumulate abdominal fat through the laying cycle which typically ceases when they are 72-78 weeks of age (WOA). The table egg industry is moving towards extending the laying cycle until 100 WOA. In this study, the performance of Hy-line Brown hens, grouped based on BW, was assessed through to 100 WOA. The hens were housed in individual cages in a high-rise layer shed under 16 h light, with *ad libitum* access to feed and water from 17.5-100 WOA. They were retrospectively allocated to four BW quartiles at 62 WOA, i.e. quartile 1 (Q1), the lightest hens, BW 1.99 kg to quartile 4 (Q4), the heaviest hens, BW 2.53 kg. Hen BW and egg weight (EW) had been measured at 72 and 100 WOA and cumulative feed intake (FI), cumulative egg production (EP), cumulative egg mass (EM) and cumulative feed conversion ratio (FCR) were calculated for each BW Quartile from 17.5 WOA until 61, 72 and 100 WOA. Egg characteristics of Haugh unit (HU), relative dry shell weight, shell thickness and shell breaking strength were measured at 76-80 and 96-100 WOA. At 62, 72 and 100 WOA the BW of hens in each quartile were different with Q1 BW matching the breed standard BW for age. At 100 WOA average BW was 2.04, 2.24, 2.40 and 2.64 kg for Q1, Q2, Q3 and Q4 respectively ($P < 0.05$). Concurrently EW was 63.5, 63.9, 63.9 and 66.8 g; cumulative FI was 64.1, 65.1, 67.8 and 70.2 kg; cumulative EP was 509, 513, 518 and 482 eggs, cumulative EM was 28.9, 29.4, 30.1 and 28.3 kg, and cumulative FCR was 2.23, 2.23, 2.27 and 2.70 kg/kg respectively. At both 76-80 and 96-100 WOA eggs from Q4 birds were the heaviest but with the lowest HU. At 96-100 WOA the shell of eggs from Q4 hens was numerically ($P = 0.067$) thinner than the eggshell of Q1-Q3 hens. There were no differences in relative dry shell weight nor eggshell breaking strength. These outcomes support management of egg-laying hens to maintain their BW at breed standard recommendation for age. i.e. Quartile 1. Compared to heavier hens, Q1 BW will achieve the most efficient production of good quality eggs.

I. INTRODUCTION

Hen body weight (BW) impacts feed intake (FI), feed efficiency and egg characteristics (Harms et al., 1981). Egg weight and egg production (EP) are central to commercial egg-laying hen performance and FI is pivotal to these outputs. However, higher FI is not necessarily associated with higher EW (Anene et al., 2021) or egg production (Cerolini et al., 1994; Lacin et al., 2008). However, higher hen BW is generally associated with higher FI, poorer feed efficiency (Lacin et al., 2008; Akter et al., 2018) and an increase in the number of abnormal eggs (Anene et al., 2021).

Studies of individually housed hens held under similar management and dietary conditions have shown considerable variation in their BW, FI, and feed efficiency (Akter et al., 2018;

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Muir, 2023). These variations can be evident early in the laying period, persisting through to mid-lay i.e. 40 WOA (Anene et al., 2021) and late lay i.e. 69 WOA (Muir et al., 2022). Given the interest of the egg industry in extending egg production cycles to when hens are 100 WOA (Bain et al., 2016) and the inclination for heavier hens in the Australian industry (Muir et al., 2023a), evaluation of BW effect on FI, EP, FCR and egg quality through an extended egg production period is required.

Data on hen performance and egg quality through an extended laying cycle of hens became available from a study that involved several lighting and feeding treatments during rearing (Muir et al., 2023b; Muir et al., 2024). Analysis of BW data when the hens were 62 WOA identified hens of a range of BW with each rearing treatment group. This presented an opportunity to explore the impact of differences in BW on performance and egg quality to the end of an extended laying cycle. The main study followed the original experimental design until hens were 100 WOA (Muir et al., 2024). Then, irrespective of their rearing treatments, the flock was retrospectively divided into four BW groups or quartiles (Q), based on BW at 62 WOA. The data were then analysed to assess the effect of hen BW in late lay on cumulative FI, EP, egg mass (EM), FCR and egg characteristics throughout an extended laying cycle.

II. MATERIALS AND METHODS

Hy-line Brown hens were housed in individual cages (25x50x50 cm) in a high-rise layer shed under 16 h light/24h. From 17.5 until 100 WOA they had *ad libitum* access to layer diets appropriate for their stage of EP. Each hen's individual EP, FI and the weight of each egg was measured. Subsequently EM and FCR were calculated. At 62 WOA, all 426 hens had been weighed. Body weight was used to retrospectively allocate hens to four, BW quartiles (Q). The BW range, average BW and number of hens in each quartile are presented in Table 1. At 62, 72 and 100 WOA hen BW and EW were determined and cumulative FI, EM and FCR from 17.5 to either 61, 72 or 100 WOA were determined for each BW quartile (Table 2). At 76-80 and 96-100 WOA 12 eggs from each of the BW quartiles were assessed for Haugh unit (HU), shell thickness, relative dry shell weight and shell breaking strength, as described by Muir et al. (2023a). All data were analyzed using a One-way ANOVA on generalised linear model procedure of STATISTICA Version 6 (Statsoft Inc. 2003). Means were separated by Tukey's honestly significant difference test. Statistical significance was set at $P < 0.05$.

Table 1 - Hen allocation to body weight quartiles at 62 weeks of age.

Quartile	Average body weight (kg)	Body weight range (kg)	Number of hens
1	1.99	1.70 ≤ 2.12	107
2	2.17	> 2.12 ≤ 2.24	106
3	2.36	> 2.24 ≤ 2.40	107
4	2.53	> 2.40 ≤ 2.9	106

III. RESULTS AND DISCUSSION

The allocation of hens to BW quartiles based on their BW when 62 WOA generated four distinctive groups, ranging from the lightest Q1, average BW 1.99 kg to Q4, average BW 2.53 kg (Table 1). Each quartile contained a similar number of hens. The BW of the quartiles were also different from each other at 72 and 100 WOA ($P < 0.001$; Table 2). The BW of Q1 hens corresponded with the Hy-line Brown recommended weight for age i.e. 2.01 kg at 72 WOA compared to recommended 1.91-2.03 kg and, 2.04 kg at 100 WOA compared to recommended 1.92-2.04 kg (Hy-line Brown Management Guide, 2018). At both ages the BW for Q2, Q3 and Q4 continued to be above breed standard weight for age.

Table 2 – Hen body weight, feed intake, egg production and feed conversion for BW quartiles at 62 WOA.

Observation	Age (weeks)	Body weight quartile (lightest to heaviest)				SEM	*P*-value
		Quartile 1	Quartile 2	Quartile 3	Quartile 4		
Body weight (kg)	62*	1.99^{d}	2.17^{c}	2.32^{b}	2.53^{a}	0.007	<0.001
	72	2.01^{d}	2.19^{c}	2.33^{b}	2.58^{a}	0.008	<0.001
	100	2.04^{d}	2.24^{c}	2.40^{b}	2.64^{a}	0.094	<0.001
Cumulative feed intake (kg) (from 17.5 weeks of age)	61	33.6^{d}	35.1^{c}	35.8^{b}	37.9^{a}	0.160	<0.001
	72	42.0^{d}	43.7^{c}	44.8^{b}	47.4^{a}	0.234	<0.001
	100	64.1^{c}	65.1^{c}	67.8^{b}	70.2^{a}	0.508	<0.001
Cumulative eggs produced (from 17.5 weeks of age)	61	279	281	281	279	0.908	0.445
	72	347	352	350	345	2.16	0.083
	100	509^{a}	513^{a}	518^{a}	482^{b}	5.84	<0.001
Cumulative egg mass (kg) (from 17.5 weeks of age)	61	16.2^{c}	16.5^{ab}	16.6^{abc}	16.9^{a}	0.011	<0.001
	72	20.1	20.6	20.7	20.7	0.201	0.070
	100	28.9^{ab}	29.4^{ab}	30.1^{a}	28.2^{b}	0.235	0.050
Cumulative FCR (from 17.5 weeks of age)	61	2.07^{b}	2.13^{b}	2.16^{b}	2.25^{a}	0.012	<0.001
	72	2.14^{b}	2.13^{b}	2.17^{b}	2.33^{a}	0.033	<0.001
	100	2.23^{b}	2.23^{b}	2.27^{b}	2.70^{a}	0.059	<0.001
Egg weight (g/d)	61	60.2^{c}	60.9^{bc}	62.2^{ab}	63.4^{a}	0.410	<0.001
	72	60.2^{c}	60.8^{bc}	62.1^{b}	64.3^{a}	0.439	<0.001
	100	63.5^{b}	63.9^{b}	63.9^{b}	66.8^{a}	0.669	0.003
Haugh unit	76-80	99.0^{a}	97.1^{a}	94.1^{ab}	89.9^{b}	1.82	0.004
	96-100	95.6^{a}	88.9^{ab}	86.0^{b}	85.1^{b}	2.37	0.014
Dry shell weight (%)	76-80	9.73	9.94	9.75	9.73	0.142	0.658
	96-100	9.15	9.26	9.29	9.00	0.190	0.713
Shell thickness (mm)	76-80	0.368	0.364	0.382	0.364	0.006	0.116
	96-100	0.359	0.352	0.367	0.345	0.006	0.067
Eggshell breaking strength (g)	76-80	3.85	3.72	4.07	3.78	0.105	0.105
	96-100	3.60	3.59	3.79	3.48	0.103	0.225

*BW measured at 62 WOA but production data were taken from 61 WOA.
[a,b,c,d] Rows without a common superscript are significantly different at $P < 0.05$.

During each period, i.e. from 17.5 to either 61, 72 or 100 WOA, Quartile 1 hens were the lightest BW group and consumed less feed in total ($P < 0.001$) compared to Q3 and Q4. The higher FI of heavier hens has also been reported by others including Lacin et al. (2008) and Muir et al. (2023a). Further, the correlation between cumulative FI and BW when hens were 100 WOA was significant ($r=0.52$; $P < 0.005$). Egg production through to 100 WOA was similar for Q1, Q2 and Q3 hens but Q4 hens produced fewer eggs than the former quartiles ($P < 0.001$). Feed not used for hen maintenance and egg production is stored as abdominal fat, leading to ongoing weight gain, whereby the larger hens become obese, with more irregular egg quality and compromised hen health (Anene et al., 2021).

Cumulative EM was highest in Q4 hens at 61 WOA, and in Q3 hens at 100 WOA, with no differences when hens were 72 WOA. At 100 WOA the higher cumulative EM of Q3 hens was different to Q4 cumulative EM only. Cumulative EM produced by Q1 and Q2 hens to 100 WOA was not different to Q3 or Q4 hens, nor to each other. From 17.5 – 100 WOA the cumulative FCR of the Q1, Q2 and Q3 hens were similar, all being lower than the cumulative FCR of Q4 hens ($P < 0.001$). This corresponds with other recent studies where lighter ISA Brown hens had lower FCR than their heavier counterparts at 55 WOA (Akter et al., 2018) and 69 WOA (Muir et al., 2022).

The heavier hens (Q4) produced the heaviest eggs, reflecting the observations of Muir et al. (2022) but contrary to Akter et al. (2018). Further, the heavier eggs of Q4 hens had the lowest HU (P = 0.014; Table 2). Aligning with Muir et al. (2022), eggshell breaking strength and relative shell weight did not differ due to hen BW. However, at 96-100 WOA, the eggshells of the heavier Q4 hens were thinner (approaching significance, P = 0.067) compared to the eggshells of the hens in Q1, Q2, and Q3.

IV. CONCLUSIONS

Overall, compared to lighter hens, heavier hens consume more feed but without a corresponding increase in egg production. While their eggs are heavier, their FCR is notably poorer. Hence it is recommended that hens are maintained close to the breed standard weight for age throughout an extended laying cycle. This will optimise FI and FCR while maintaining good egg size and eggshell quality without compromising egg production.

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PERFORMANCE, EGGSHELL QUALITY AND BONE DENSITOMETRY OF WHITE LAYING HENS SUPPLEMEMENTED WITH AMINO ACID-COMPLEXED MINERALS FROM 78 TO 98 WEEKS OF AGE

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Summary

This study evaluated the effects of supplementing microminerals zinc (Zn), manganese (Mn), copper (Cu), iron (Fe), and selenium (Se), while replacing their inorganic mineral (IM) source in the diet, on the performance, eggshell quality and bone densitometry of laying hens. A total of 400 Lohmann White laying hens from 78 to 98 weeks of age were housed in cages and assigned to 4 treatments in a completely randomized design with 10 replicates and 10 birds per experimental unit. Treatments consisted of 4 diets in which the IM source was fully replaced by a source of amino acid-complexed microminerals (AACM), with supplementation levels reduced to 70%, 50%, and 40% of the IM supplementation, respectively, as follows: T1 - IM - Control diet, only IM sources at the following levels: 60 ppm Zn, 60 ppm Mn, 7 ppm Cu, 40 ppm Fe, 0.2 ppm Se, and 2.0 ppm Iodine (I); T2 - AACM70% with 42 ppm Zn, 42 ppm Mn, 4.9 ppm Cu, 28 ppm Fe, 0.14 ppm Se, and 1.4 ppm I; T3 - AACM50% with 30 ppm Zn, 30 ppm Mn, 3.5 ppm Cu, 20 ppm Fe, 0.10 ppm Se, and 1.4 ppm I; and T4 - AACM40% with 24 ppm Zn, 24 ppm Mn, 2.8 ppm Cu, 16 ppm Fe, 0.08 ppm Se, and 1.4 ppm I. Orthogonal polynomial contrasts (linear and quadratic effects) of AACM levels were used to determine their impact ($P < 0.05$), and Dunnett's test was performed between IM and AACM groups ($P < 0.05$). Results showed that reducing AACM supplementation linearly improved egg production, egg weight, egg mass, and feed conversion ratio. The AACM40% diet increased egg production by 8% and egg weight by 1.7% compared to the control. Hens receiving AACM40% produced the thickest eggshells ($P < 0.01$), 4.2% thicker than those receiving IM. AACM treatments improved tibia density with AACM40% treatment increasing medial density by 21% compared to IM. In conclusion, supplementation with AACM for layers from 78 to 98 weeks of age improves their performance and egg and bone quality traits.

I. INTRODUCTION

Trace minerals are essential in poultry diets as they participate in bone metabolism, enzymatic reactions, hormone, immune system function, tissue synthesis, and other physiological functions (Santos et al., 2024). To prevent deficiencies, these nutrients should be supplemented in diets at optimal levels that meet mineral requirements (NRC, 1994). However, factors such as bioavailability, mineral interactions, and the presence of dietary antagonists should be considered when determining inclusion rates. In addition, excessive mineral supplementation can also be detrimental, leading to adverse effects, and environmental pollution. These compounds can interact with molecules such as phytate, folic acid, and tannins in the gastrointestinal tract, dissociating into active cations and forming insoluble complexes, thereby becoming unavailable to the animal (Goff et al., 2017). This results in varying mineral's stabilities, ratios, sizes, and stereochemistry, all of which directly impact their solubility, reactivity, and absorption (Byrne and Murph, 2022; Gao et al., 2014). Unlike IM, amino acid-complexed microminerals (AACM) do not interact with other compounds because of their

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stability coefficients, as the 1:1 chelation process protected them from dietary antagonists (Ashmead, 1993). The AACM uses active transport mechanisms facilitated by AA ligands for absorption (Gao et al., 2014). Furthermore, these characteristics indicate that AACM supplementation levels can be lower than IM sources, and the inclusion rates required for AACM may help mitigate toxicity risks associated with high IM levels. When high volumes are ingested, saturation of cellular metal binding transporter proteins can occur, increasing free ionized metal concentrations, which can cause tissue damage. The toxic effects vary depending on the specific trace element in question, the total amount of that element in the diet, the age and condition of the animal and dietary conditions (Thompson et al., 1991). The toxic effect of a trace element can also be the cause of a secondary deficiency of another trace element The purpose of this study was to investigate the effects of the total replacement of IM sources by Zn, Mn, Cu, Fe, and Se AACM in in laying hen diets on performance, eggshell quality and bone densitometry.

II. MATERIALS AND METHODS

A total of 400 Lohmann White laying hens from 78 to 98 weeks of age were housed in cages and assigned to 4 treatments in a completely randomized design with 10 replicates and 10 birds per experimental unit. Treatments consisted of 4 diets in which the inorganic mineral (IM) source was fully replaced by a source of amino acid-complexed minerals (AACM), with supplementation levels reduced to 70%, 50%, and 40% of the IM supplementation, respectively, as follows: T1. IM - Control diet, only IM sources at the following levels: 60 ppm Zn, 60 ppm Mn, 7 ppm Cu, 40 ppm Fe, 0.2 ppm Se, and 2.0 ppm I; T2 - AACM70% with 42 ppm Zn, 42 ppm Mn, 4.9 ppm Cu, 28 ppm Fe, 0.14 ppm Se, and 1.4 ppm I; T3 - AACM50% with 30 ppm Zn, 30 ppm Mn, 3.5 ppm Cu, 20 ppm Fe, 0.10 ppm Se, and 1.4 ppm I; and T4 - AACM40% with 24 ppm Zn, 24 ppm Mn, 2.8 ppm Cu, 16 ppm Fe, 0.08 ppm Se, and 1.4 ppm I. Calcium iodate was used as I source for all groups. Phytase enzyme was added at 600 FTU/kg of feed in all diets. Feed and water were available *ad libitum* throughout the experimental period. The environmental conditions inside the facility were monitored daily, with an average temperature of 27ºC and relative humidity of 81%.

a) Laying performance, Eggshell thickness and Tibia densitometry

To evaluate laying performance, eggs were collected daily, and all produced eggs were counted and weighed, and feed leftovers were weighed weekly. From the collected data, the laying rate, average daily egg mass, average daily feed intake, and feed conversion ratio to egg mass and to dozen eggs were then calculated. For eggshell thickness, each 28-day period, 30 eggs were collected over 3 days (3 eggs per experimental unit) for the evaluation. The eggshells were air-dried for 48 h before weighing and their thickness measurements were performed using a digital caliper. For tibia densitometry, the right tibia of 5 birds per treatment were collected at the end of the trial. Bone densitometry was performed using Hi Speed FXI CT scanner equipment. Cross-sectional images were acquired using Dicom® software to estimate individual bone radiodensity values at the 3 diaphysis cut levels (proximal, medial, and distal).

b) Statistical Analysis

Normality and homoscedasticity assumptions were tested for analysis of variance. Orthogonal polynomial contrasts (linear and quadratic effects) of AACM levels were used to determine their impact ($P < 0.05$), and Dunnett's test was performed between IM and AACM groups ($P < 0.05$). Version 9.4 of SAS software was utilized (SAS, 2009).

III. RESULTS

The results are summarized in Table 1 and Figure 1.

Table 1 - Performance of laying hens fed different sources and levels of Zn, Mn, Cu, Fe and Se from 78 to 98 weeks of age.

Treatment	ADFI, g	EO, %	EW, g	EM, g	FCR-EM, g/g	FCR-DZ, g/dz	ST, mm
IM	105	73.6[b]	67.9[b]	50.0[b]	2.075[a]	1.671[a]	407[b]
AACM70%	105	76.7[a]	67.9[b]	52.0[b]	2.012[a]	1.645[a]	410[b]
AACM50%	104	78.6[a]	68.1[b]	53.1[a]	1.967[a]	1.607[a]	419[b]
AACM40%	103	80.8[a]	69.1[a]	55.3[a]	1.868[b]	1.555[b]	424[a]
MEAN	104	77.1	68.3	52.6	1.960	1.610	415
SEM	0.368	0.780	0.169	0.566	0.016	0.012	2.000
P values							
Dunnet	0.154	0.022	0.026	0.004	0.001	0.023	0.005
Linear[1]	0.054	0.044	0.011	0.014	<0.001	<0.001	0.003
Quadratic[1]	0.674	0.801	0.275	0.605	0.403	0.722	0.623

ADFI, average daily feed intake; EO, egg output; EW, egg weight; EM, egg mass; FCR-EM, feed conversion ratio for egg mass; FCR-DZ, feed conversion for dozen eggs; ST, shell thickness; SEM = standard error of the mean.
[1]Orthogonal polynomial contrast; * within columns differ from Dunnett's test ($P < 0.05$) .

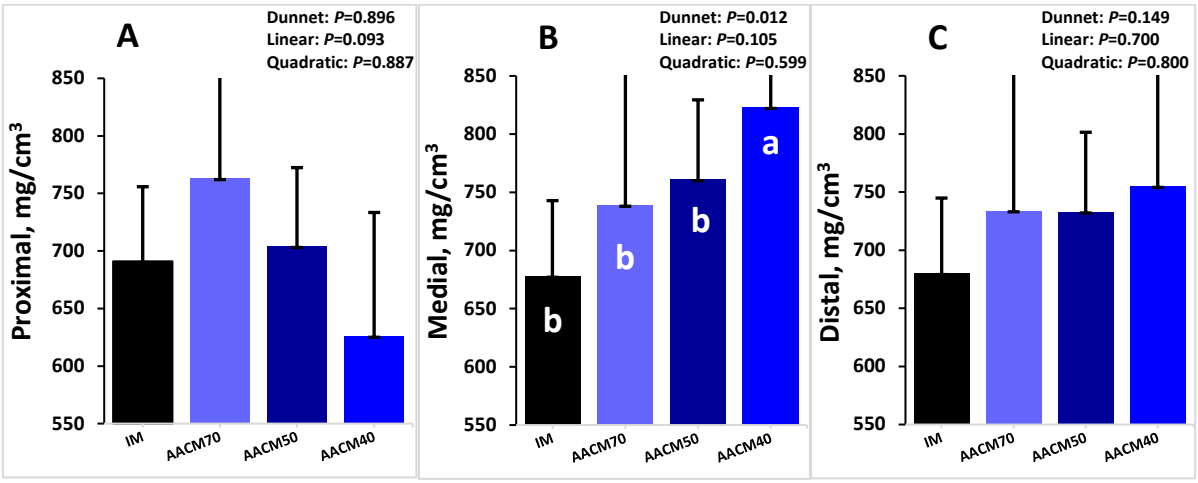

Figure 1 - Proximal (A), medial (B), and distal (C) tibial densitometry from laying hens fed different sources and levels of Zn, Mn, Cu, Fe and Se from 78 to 98 weeks of age. [ab]Differ by Dunnett's test ($P < 0.05$).

IV. DISCUSSION

This research supports evidence that supplementation of lower levels of AACM into laying hen diets leads to improved zootechnical performance, shell quality and bone characteristics when compared to the use of IM, with the optimal inclusion of AACM was 40% in relation to IM levels.

Complexed trace minerals are known to exhibit enhanced absorption and retention in the body, allowing animals to better utilize the minerals to support vital biological processes (Gao et al., 2014; Pereira et al., 2020). In the case of egg-laying hens, boosted bioavailability of minerals such as Zn, Mn, Cu, Se, and Fe from AACM supplementation improve performance efficiency (Pereira et al., 2020). These minerals serve as essential cofactors for enzymes and hormones that regulate oocyte development and ovarian follicle growth. With more robust mineral nutrition, hens can sustain optimal reproductive performance to meet the heavy demands of daily egg production (Medeiros-Ventura et al. 2023; Santos et al., 2024).

Nonetheless, the treatment with 40% trace mineral supplementation resulted in a thicker eggshell, indicating that an excess of these nutrients may adversely affect the structural formation of the eggshell, with laying hens potentially unable to maintain trace element homeostasis at high dietary levels. Similarly, IM treatment, which is characterized by the low bioavailability of Mn and Zn, was also harmful to eggshell formation. The homeostatic balance observed in the AACM40% treatment indicates a more efficient utilization of Mn and Zn for eggshell synthesis.

Moreover, our study highlights the role of Zn, Cu, Mn, and Fe in bone quality. The AACM40% treatment resulted in improved medial density, emphasizing the dependence of bone quality on the ideal and bioavailable amounts of these minerals. Xiao et al. (2014) and Zhang et al. (2017) demonstrated the involvement of Mn- and Cu-dependent enzymes, such as glucuronyltransferases and lysyl-oxidases, in the formation of collagenous bone matrix. Furthermore, Florencio-Silva et al. (2015) noted that Zn-activated enzymes such as phosphatases and carbonic anhydrase play a crucial role in regulating bone deposition and reabsorption. Balogh et al. (2018) highlighted the importance of Fe in maintaining the balance between bone formation and deformation. Overloading and a lack of Fe can cause osteoclast activity and bone losses, implying that the optimal level of Fe is crucial for bone homeostasis. Higher Fe concentrations in IM sources may also result in poorer bone quality. This occurs because excess Fe can limit Ca sequestration to mitochondria in the liver and disrupt bone homeostasis. This change is characterized by greater reabsorption induced by osteoclasts and reduced bone production caused by osteoblasts (Balogh et al., 2018).

In conclusion, this study demonstrated that replacing IM with lower AACM inclusion levels in laying hen diets improves productive performance, feed efficiency, eggshell quality, and tibial bone density. The optimal supplementation levels are: 24, 24, 2.8, 16, 0.08, and 0.8 ppm of Zn, Mn, Cu, Fe, Se, and I, respectively, corresponding to 40% of IM supplementation.

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NUTRITION AND FEEDING STRATEGIES IN EXTENDED EGG PRODUCTION IN DIFFERENT PRODUCTION SYSTEMS

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Summary

To successfully reach a stable and prolonged egg laying period of ~100 weeks, laying hens need optimal management and well-balanced nutrition. Proper feeding management and lighting regimes for the pullets are essential for achieving the expected growth curve and target body weight before the onset of laying. In conventional egg production, synthetic amino acids, enzymes and other additives are used to optimize feed formulation, allowing for precise nutrient and energy provision tailored to the hen's genotype and productive stages.
However, further research during an extended production period of up to 90-100 weeks is necessary in order to obtain a more basic understanding of especially amino acids requirement of layers in the different stages during their production period.

Conventional layer diets typically include cereals, various protein crops, oils, minerals and vitamins. In organic egg production, the use of synthetic amino acids and certain protein sources like soybean meal is prohibited, challenging the formulation of well-balanced diets. Research into new protein sources, such as mussel meal, grass protein, microalgae, and insects, seems promising for improving nutrient balance. Protein is a vital nutrient in layer diets, providing essential amino acids like methionine and cystine. The daily amino acid requirements of hens are influenced by various factors, including genotype, age, and diet composition. Laying hens also require energy for growth, maintenance, and daily egg production. Hens with outdoor access have higher energy needs due to increased physical activity and temperature variations. Furthermore, calcium and phosphorus are key minerals in layer diets, essential for bone development and eggshell formation, and even more important at extended egg laying, where eggshell quality limits how long the laying hens can be kept in production. A Danish study demonstrated that it is possible to reduce the phosphorus content considerably in organic poultry feed without impairing production results by using strategic calcium allocation. This approach allows hens to regulate their own calcium intake, maintaining good bone strength and welfare. The study highlights the potential for optimizing feeding strategies to enhance both productivity and animal welfare in egg production. Furthermore, reduced dietary phosphorous will have a positive effect on the environment due to lower phosphorous excretion, supporting a more sustainable egg production.

I. INTRODUCTION

Poultry is considered a very important animal-based food, both economically and as a source of protein, vitamins, and minerals from eggs, egg products, and meat. From an environmental and climatic point of view, poultry production is considered to be very competitive with other animal-based food productions, both in the conventional and organic production (Istiak and Khaliduzzaman, 2022).

The poultry breeds for egg production today have been selected over many generations for a high production rate and high egg quality, which has resulted in egg laying breeds with efficient feed utilization and a low body weight (Bain et al., 2016; Pottgüter, 2016). Based on

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this development, there has been an increasing interest in recent years to extend the production period up to 90-100 weeks to obtain a more sustainable egg production. To ensure a high level of hen welfare during a prolonged egg production period, factors such as managing feed intake, pullet growth, and the energy-protein ratio to ensure high productivity and good health are important (Nys 2015). Egg production in conventional housing systems, free-range, or organic systems is very different in aspects of both housing, nutrition and welfare (Bryden et al. 2021). More open housing systems in free-range and organic production with access to outdoor areas influence the behavior of the hen, with the hen being more physically active, which increases the energy required for maintenance and influences amino acid requirements and has an impact on their bone health (Tauson, 2005; Van Krimpen et al., 2015; Regmi et al., 2016). Protein, amino acids and calcium and phosphorus are the most important nutrients in balanced diets for laying hens to ensure optimal egg production and egg quality during a long production cycle (Korver, 2023; Sinclair-Black et al., 2023). By understanding these differences, it should be possible to tailor the management practices to optimize the welfare, health, productivity, and egg quality of laying hens in both conventional and organic systems for extended laying periods of up to 100 weeks.

II. REARING PULLETS

The focus of this paper will be on the laying hen. However, to ensure successful egg production for a prolonged period, optimal conditions for the rearing of the pullets are very important and a prerequisite. The diet must include the proper combination of protein, amino acid composition, energy, and minerals, which changes during the rearing period in order to sufficiently cover the nutrient requirements for the pullet during growth. For the transition to laying facilities, the pullet should achieve as a minimum the standard target weight recommended by the breeding companies as this can reduce the risk of a low feed intake at the beginning of the laying period, which is important for egg production efficiency, egg weight, and FCR (Bain et al., 2016; Pottgüter, 2016).

III. LAYING HENS

a) Feeding and available ingredients

Feed is the most significant cost in egg production, hence, layer diets are designed to meet nutrient requirements at the lowest possible cost, with synthetic amino acids playing a crucial role in balancing diets. The inclusion levels of dietary ingredients depend on their availability and quality, with conventional layer diets typically comprising cereals, various protein crops (e.g., soy products/beans, other legumes, sunflower cake), oils, minerals, and vitamins. In conventional egg production, the use of synthetic amino acids and exogenous enzymes such as phytase and cell wall-degrading enzymes optimizes feed formulation (Blair et al. 1999; Keshavarz and Austic, 2004; Sureshkumar et al., 2023). This allows for precise nutrient and energy provision tailored to the laying hen’s genotypes and productive stages. In organic production, the use of exogenous enzymes, synthetic amino acids, and certain protein sources like soybean meal is prohibited, complicating the formulation of well-balanced diets. Diets for layers in organic production utilize many different protein sources like peas, faba beans, lupins (Blair, 2018), albeit in smaller amounts due to their lower methionine content compared to soybeans, sunflower and rapeseed cake.

Introducing new protein sources with more optimal amino acid profiles for both conventional and organic layers could enhance diet balance and reduce protein surplus, particularly in organic diets. Research in new protein sources for poultry includes mussel and starfish meal (Van der Heide et al., 2021), microalgae (Thapa 2020; Olsen et al., 2021), grass

protein (Hammershoj and Johansen, 2016; Santamaria-Fernadez and Lübeck, 2020), and insects (Józefiak et al., 2016; Dörper et al. 2024).

b) Protein, amino acids, and energy

Protein is essential in layer diets with the amino acid profile, especially methionine and cystine, being crucial. A well-balanced amino acid profile meets the hen's requirement for maintenance and egg production, while avoiding protein oversupply. Daily amino acid requirements depend on feed intake, which is influenced by genotype, age, diet composition, and housing system. Breeding companies provide specific recommendations for protein, amino acids, minerals, and energy between different production systems and genotypes, and consider higher energy needs for hens in alternative housing systems like free-range and organic (e.g. Hendrix, Dekalb).

In a comprehensive review by Macelline et al. (2021), different aspects and challenges related to identifying the optimal amino acid requirements for laying hens were discussed. With the possibility to include synthetic amino acids in layer diets, the feed can be formulated with a lower content of crude protein level, which is beneficial for the environment as a reduced nitrogen excretion in manure is expected. Different results have been reported in literature about the effect of lower protein diets on e.g. performance and egg weight, where some studies found an inferior performance and other studies did not. It was also concluded in the review (Macelline et al., 2021) that increased use of different synthetic amino acids in low protein diets may affect protein digestion and alter amino acids requirements. Egg production changes during the production period, and in extended laying, the amino acid requirement can be expected to be higher for older hens. Reported studies in the literature are often carried out over a short production period and are therefore not representative, necessitating further research during an extended production period of up to 80-100 weeks.

Laying hens need energy for growth, maintenance, and egg production. Hens with outdoor access are more active and face varying temperatures, leading to higher energy needs for optimal egg production (Al-Saffar and Rose, 2002; Elwinger et al., 2008; Van Krimpen et al., 2015). Increased feed intake due to higher maintenance needs requires adjustments in other dietary nutrients, including amino acids. Van Krimpen et al. (2015) found that methionine intake is influenced by the diet's energy content and the ambient temperature, with different recommendations for winter and summer periods to achieve optimal laying performance.

c) Calcium and phosphorous

Calcium (Ca) and phosphorus (P) are key nutrients in layer diets, as these minerals have many essential roles, especially in bone development and eggshell formation (Etches 1987; Bar 2009). This is due to the large demand for Ca during egg production, where an average of about 2.2 g of Ca is deposited in the shell of each egg (Kebreab et al., 2009). It is, however, not possible to merely feed the hen with large amounts of Ca as it is dependent on the presence of P in its available form (AP) (Etches, 1996). These two minerals are intertwined in their deposition in the bones where they are stored as calcium phosphate in a Ca:AP ratio of 2.2:1 (Scott et al., 1982, Kebreab et al., 2009). During egg formation, Ca and P are released from the medullary bone, and P is excreted to a high extent into the environment, as only a small amount of calcium phosphates are included in the eggshell. In contrast, Ca is reabsorbed and used for eggshell formation. This means that the dietary need for P is much lower than that of Ca (Etches, 1996).

The excretion of P is increased further by the lack of availability of P from cereals (Poulsen et al., 2019). Cereal grains are the major ingredients in layer diets, but they contain phytate, which binds the P and makes it almost indigestible for monogastric animals (Maenz and Classen, 1998; Sommerfeld et al., 2020). In conventional production systems, exogenous

phytase is added to the diet, which cleaves phytate, thereby making the P bound in it readily digestible for layers (Singh, 2008). This is unfortunately not possible in organic egg production system as phytase derives from an industrial production process based on genetically modified microorganisms (GMO), which are prohibited.

d) Egg production and quality in extended laying

Nutrition is, together with hen health, genotype, sanitary conditions, and age, an important factor that determines egg production and egg quality (Roberts 2004). Egg quality describes a range of internal and external characteristics, of which the focus here will be on the egg size and shell parameters.

Egg weight is positively correlated with body weight during the onset of lay (Bouvarel, 2011) and nutrition can influence egg weight during the egg production period. It is well known that in modern laying breeds the egg weight increases rapidly during the first period of lay, often with a target to reach 55-60 g (Pottgüter, 2016), hereafter reaching a steady state, but still with a slightly positive rate of increasing in egg weight, which is aimed to remain below 63 g (Pottgüter, 2016). Eggs produced by hens beyond 80 weeks will be larger in size, and as the shell weight increases at a lower rate than the rest of the egg components; albumen and yolk, the resulting shell-percentage, shell thickness and consequently the shell strength are negatively impacted by the longer egg laying period.

There are few studies on how to counteract or prevent a decline in shell quality in extended egg laying. However, as it will be the shell quality that limits the length of egg laying, it is key to manage towards high shell quality parameters, otherwise a too high number of cracked eggs and thereby food waste will result in an unsustainable production, both economically and environmentally. The protein and especially the methionine and sulphur containing amino acids content of the diet has significant influence on egg production (Harms, 2001; Hammershoj and Steenfeldt, 2005; Steenfeldt and Hammershøj, 2015; Van Krimpen et al., 2015). The balanced amino acid composition of the diet is typically the first parameter to control the egg size.

For high eggshell quality, the strength of the eggshell is key, and the mineral supply of Ca and P via the feed is essential. There has been research supporting the link between production system, bone mineralization, and eggshell quality, as production systems where hens are less physically active have both poorer bone strength and eggshell strength (Ferrante et al., 2009). More recent studies have looked into the relationship between housing systems and egg quality and find that several factors influence egg quality in addition to housing systems, such as genetics, age, season, nutrition, health conditions, and management (Pires et al., 2021; Roderiguez-Mengod et al. 2024). Eggs from hens at the age of 47-50 weeks on diets with similar nutritional composition showed the eggs from organic production to have significantly higher shell thickness and shell percentage compared to conventional production, however, with interactions to hen breed (Roderiguez-Mengod et al. 2024).

However, one of few studies on extended laying of up to 105 weeks, where bone mineralization and shell quality are in focus (Alfonso-Carrillo et al., 2021), does conclude that hens high in egg production and in eggshell quality parameters have poorer bone quality. Furthermore, there appears to be no clear dependency in correlation between bone and egg/eggshell parameters, which is confirmed by Dunn et al. (2021). Hence, bone and egg production/eggshell quality are independent and should be aimed to be improved separately in extended laying with nutrition and genetics as primary management tools.

e) Future perspectives on using strategic calcium feeding and reduced dietary phosphorus in extended laying

Ca and P are key minerals to support optimal egg production, egg quality, and bone strength; however, current feeding practice and P level recommendations for laying hens may lead to an oversupply of P and thereby an increased P-excretion, which has a negative effect on the environment. Furthermore, Ca given during the day, where there is no eggshell formation, can increase the P requirement (Etches, 1996). In a review by Li et al. (2017), it was discussed if current layer diets were formulated in excess of Ca and P, and it was concluded that the concentration of both Ca and P could be decreased in layer diets without negative effect on egg production, egg quality or welfare. Normal practice is to increase dietary Ca with increasing hen age to maintain a proper eggshell quality, but in the mentioned review it was stated that keeping the same content of Ca and P in layer diets from start of lay up to 80 weeks did not affect egg production or quality. The review was based on different studies from conventional production systems, and the use of phytase was considered an important dietary tool to ensure productivity with reduced P in the diets.

A recent study in Denmark with organic laying hens (manuscript under preparation) aimed to investigate the effects of lowered Ca level during the day but with access to a coarse Ca source from the afternoon until morning (i.e. strategic Ca allocation) combined with reduced dietary P in organic laying hens from 30 weeks-of-age (WOA) to 80 WOA. 1200 Dekalb White hens were fed one of four levels of P (0.20-0.35% AP) and one of two Ca feeding strategies (100% of the Ca included in compound feed or low dietary Ca combined with strategic Ca allocation at 4:30 PM to 7:30 AM) (Table 1). Production data were collected continuously throughout the experimental period. Hen weight and plumage quality were obtained seven times during the study. Measures on bones' breaking strength were carried out at 28, 42 and 80 weeks.

Table 1 - Distribution of Ca in the 8 experimental diets.

	Ca exclusively from compound feed	20% Ca from compound feed + strategic Ca allocation
0.35% AP	A	E
0.30% AP	B	F
0.25% AP	C	G
0.20% AP	D	H

AP: available phosphorus

The hens fed the four diets with separate allocations of Ca had a high level of egg production throughout the study of up to 80 weeks (average (av.) > 90%), independent of the P level (Figure 1). However, hens fed the lowest level of P (0.20% AP) with Ca exclusively in the compound feed had decreased egg production at the end of the study (av. 85% from 70-80 weeks), the feed conversion ratio increased (70-80 weeks; 2.45 kg feed/kg egg vs. 2.25-2.30), which resulted in weight loss. The highest levels of P (0.30 and 0.35% AP) with Ca exclusively in the compound feed resulted in higher egg production (av. 91% from 70-80 weeks) compared to the diet with 0.20% AP.

Over the course of the study, strategic Ca allocation resulted in a higher egg weight compared to providing all Ca in the compound feed (63.1 vs. 62.5g), especially for the lowest P level from 70-80 weeks (63.2 vs. 61.6g). There was no effect of treatment on eggshell strength, neither positive nor negative. For all treatments, even the lowest P treatments, the hens had high plumage quality, and the mortality was very low (av. 2.5% for the study period).

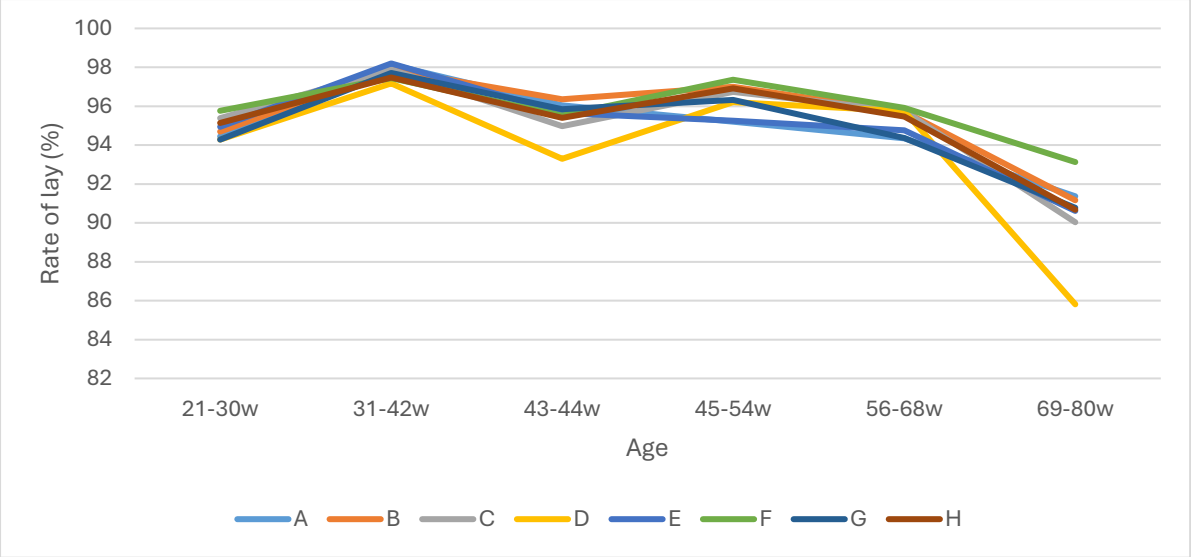

Figure 1 - Egg production up to 80 weeks for 8 treatments A-H (details shown in table 1). Age:21-30 weeks (starter period) – in-door; 31-42 weeks (in-door); 43-44 weeks (moving out-door); 45-80 weeks outdoor (69-80 weeks: winter feeding).

The study proved that it is possible to reduce P-content in the feed considerably without impairing production results if coarse Ca is allocated as a separate source, which indicates that hens regulate their own Ca intake with this feeding strategy. The results on measurement of the bone's breaking strength showed that there was no difference between treatments at the different ages, and it was concluded that it is possible to reduce P in the feed to a very low level and at the same time maintain good bone strength, which is positive for welfare. Phytase was not added to the diets in the Danish study, as it is not permitted in organic poultry production. However, it seems that by introducing the strategic Ca allocation, there was no need for phytase to obtain optimal productivity of up to 80 weeks.

In a recent paper by Ruhnke et al (2021) Ca was offered to Isa Browns hens in a combination of dietary Ca and by giving access to a limestone grit as a separate Ca source. The dietary Ca was included at 3 levels; 40, 30, and 20 g/kg, and all groups had free access to a limestone supplement. The experiment lasted from 19-24 weeks of age, so with young hens. The hens were individually caged, and the results showed overall that egg production was not affected, but eggshell quality was negatively influenced by the decreased content of Ca in the diets. Interestingly the study showed that the voluntarily intake of the limestone supplement differed to a high degree among the hens, indicating that not all hens were able to compensate for a lowered dietary Ca content by increasing the limestone intake, and it indicates that some hens are maybe not able to adapt to a separate Ca allocation strategy. However, the study by Ruhnke et al (2021) was carried out in individual cages, and it is most likely that hens in groups can stimulate each other to eat more of the freely available Ca source. More research in this area is needed and important as it has perspectives for a more sustainable production by the possibility to decrease the dietary P content.

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ALTERNATIVE INGREDIENTS TO REPLACE SOYBEAN MEAL IN COMMERCIAL BROILER CHICKEN PRODUCTION

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Summary

A 42-d trial was conducted to investigate alternative ingredients including canola meal, sunflower meal, wheat gluten and meat and bone meal to replace soybean meal (SBM), fully or partially, in broiler chicken feed. Experimental diets were formulated to gradually replace SBM with the alternative ingredients (supplemented with crystalline amino acids) in both standard crude protein (STD-CP) and reduced crude protein (RCP) diets, and their effects on growth performance, meat quality, immune response, and gut morphology were studied. The results indicate that alternative protein sources exhibit no significant differences in growth performance and feed efficiency compared to soybean meal when chickens fed with RCP. However, replacing SBM fully or partially when chickens fed STD-CP diet, showed negative impact on growth performance and feed utilisation. The findings suggest that reducing CP and inclusion of crystalline amino acids, canola meal, sunflower meal together with meat and bone meal or wheat gluten meal can totally replace SBM without compromising growth performance. The potential reason for the reduced growth performance when canola meal, and sunflower meal replace SBM in STD-CP may be the inaccurate data on amino acids digestibility coefficients which requires fresh research and updated values, as no negative impact of replacing SBM was observed with the inclusion of crystalline amino acids with high digestibility in RCP diets.

I. INTRODUCTION

The Australian feed industry heavily relies on imported soybean meal (SBM) as a protein source for animal feed, particularly for the poultry industry, making it vulnerable to fluctuations in global SBM prices, international commodity trades, supply chain disruptions, and other external factors. To address these challenges, there is growing interest in developing alternative protein sources that can replace or reduce the reliance on imported SBM. Using some local alternative ingredients to replace SBM in the feed, such as canola meal (rapeseed meal) with 35–42% CP, the Australian feed industry can improve the resilience and sustainability of poultry production, reduce import activities and the carbon footprint associated with imported SBM, and support the domestic agricultural sectors. The aim of this project was to evaluate the efficacy of alternative ingredients including canola meal, and sunflower meal together with some other protein-rich ingredients as substitutes for SBM in standard (STD-CP) and reduced crude protein (RCP) diets for commercial broiler chicken production.

II. METHOD

A 42-day trials was conducted to test the potential of different feed formulation where SBM was gradually replaced with alternative ingredients in STD-CP and RCP diets. Five hundred- and twelve Day-old male broiler chickens (ROSS 308), purchased from Aviagen and transferred to the QASP Facility, at Gatton Campus, University of Queensland. All birds were weighed at arrival and randomly allocated to floor pens. Birds were provided with *ad libitum* feed and water throughout

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the entire trial and feed was formulated for three phases (starter 1 – 10 d; 11 – 28 d; 29 – 42 d). Chickens were fed with a standard starter feed for the first 10 d and experimental diets were started from 11 d. A SBM-corn-wheat basal diet was formulated at 2 different CP levels: STD-CP; (based on recommendations) and RCP (– 1.5 % less than STD-CP + crystalline amino acids). Trial was a 2 × 4 factorial arrangement with 2 CP levels and 4 dietary formulations, 8 replicate pens per experimental diet and 8 birds in each (n=64). The four experimental diets, repeated for both STD-CP and RCP levels (generating 8 experimental diets), included: Control Diet: Corn-wheat-soybean meal; Canola meal replacing 25% SBM; Canola meal + Sunflower meal replacing 50% of SBM; Canola meal + Sunflower meal replacing 100% SBM. Wheat gluten meal, meat and bone meal, and crystalline amino acids were used to satisfy amino acid requirements, when soybean meal was replaced at 50 or 100%. All the experimental diets were isocaloric and isonitrogenous (iso-aminoacidic) as presented in Table 1. Feed intake, body weight gain, and feed conversion rate (FCR) were recorded for each phase and total period (1-42 d). On day 42, after recording all the performance data, five bird per pen were euthanized and various samples were collected for further analyses.

Table 1. Calculated nutrient composition and digestible amino acid content of experimental grower and finisher diets.

Diet Composition	Grower Experimental Diets[1]							
	Standard Crude Protein				Reduced Crude Protein			
	D1	D2	D3	D4	D1	D2	D3	D4
Calculated Nutrients Composition								
Crude Protein (%)	20.00	20.00	20.00	20.00	18.50	18.50	18.50	18.50
Calcium (%)	0.78	0.78	0.78	0.99	0.78	1.05	0.78	0.96
Available Phosphorous (%)	0.40	0.40	0.40	0.44	0.40	0.40	0.40	0.45
AMEn broiler (kCal / kg)	2950	2950	2950	2950	2950	2950	2950	2950
Total Lys.	1.18	1.21	1.21	1.22	1.17	1.18	1.20	1.21
Dig. Lys	1.08	1.08	0.83	0.83	1.08	1.08	0.85	0.85
Dig. Met	0.51	0.48	1.08	1.08	0.53	0.54	1.08	1.08
Dig Met+ Cys	0.81	0.81	0.81	0.81	0.81	0.81	0.81	0.81
Dig Thr	0.73	0.73	0.73	0.73	0.76	0.76	0.76	0.76
Dig Trp	0.21	0.20	0.18	0.18	0.18	0.18	0.18	0.18
Dig Val	0.86	0.86	0.86	0.86	0.86	0.86	0.86	0.86
Dig. Ile	0.78	0.75	0.72	0.72	0.72	0.72	0.72	0.72
Dig. Leu	1.20	1.15	1.13	1.13	1.13	1.13	1.13	1.13
Diet Composition	Finisher Experimental Diets							
	Standard Crude Protein				Reduced Crude Protein			
	D1	D2	D3	D4	D1	D2	D3	D4
Calculated Nutrients Composition								
Crude Protein (%)	18.50	18.50	18.50	18.50	17.00	17.00	17.00	17.00
Calcium (%)	0.78	0.78	0.78	0.96	0.78	0.78	0.78	0.78
Available Phosphorous (%)	0.40	0.40	0.40	0.45	0.40	0.40	0.40	0.40
AMEn broiler (kCal / kg)	3100	3100	3100	3100	3100	3100	3100	3100
Total Lys.	1.09	1.11	1.12	1.13	1.08	1.10	1.11	1.10
Dig. LYS	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
Dig. Met	0.46	0.45	0.45	0.48	0.48	0.47	0.47	0.49
Dig Met+ Cys	0.75	0.75	0.75	0.75	0.75	0.75	0.75	0.75
Dig Thr	0.68	0.68	0.68	0.68	0.68	0.68	0.68	0.68
Dig Trp	0.19	0.18	0.17	0.17	0.18	0.18	0.18	0.18
Dig Val	0.80	0.80	0.80	0.80	0.80	0.80	0.80	0.80
Dig. Ile	0.72	0.68	0.67	0.67	0.67	0.67	0.67	0.67
Dig Leu	1.09	1.05	1.05	1.05	1.05	1.05	1.05	1.05

[1] D1-D4: experimental diet D1: Corn-wheat-soybean meal; D2: Canola meal replacing 25% SBM; D3: Canola meal + Sunflower meal replacing 50% of SBM; D4: Canola meal + Sunflower meal replacing 100% SBM.

III. RESULTS

Table 2 presents the overall growth performance of the chickens on trial 1. As reflected in the diet × CP level interactions, replacing SBM fully or partially with canola meal and sunflower meal had no negative effects on final body weight (FBW), average daily gain (ADG), average daily feed intake (ADFI), and feed conversion ratio (FCR) in RCP diets, however, it did reduce FBW and ADG when chickens fed STD-CP (P<0.05).

Table 2 - The effect of canola meal and sunflower meal gradually substituting soybean meal on production performance in commercial broiler chicken.

Factor	FBW (g)	ADG (g)	ADFI (g)	FCR
	d42	Total	Total	Total
CP level[1]				
STD	3070	72	107[b]	1.49
RCP	3106	73	109[a]	1.50
SEM	15.69	0.37	0.61	0.01
Diet[2]				
D1	3160[a]	74[a]	110	1.47
D2	3077[b]	72[b]	108	1.50
D3	3065[b]	72[b]	108	1.50
D4	3051[b]	72[b]	108	1.50
SEM	22.19	0.53	0.86	0.01
CP level × Diet				
STD×D1	3197[a]	75[a]	110	1.46
STD×D2	3037[b]	71[b]	107	1.50
STD×D3	3050[b]	72[b]	108	1.51
STD×D4	2997[b]	70[b]	106	1.50
RCP×D1	3124[ab]	73[ab]	110	1.49
RCP×D2	3116[ab]	73[ab]	110	1.50
RCP×D3	3080[ab]	72[ab]	108	1.49
RCP×D4	3105[ab]	73[ab]	110	1.51
SEM	31.39	0.75	1.21	0.01
P Value				
CP	0.11	0.11	0.05	0.52
Diet	<0.01	>0.01	0.48	0.08
Diet × CP	0.03	0.03	0.22	0.14

[1] STD-CP: Following crude protein / essential amino acids recommendations; RCP: STD-CP – 1.5%.
[2] D1-D4: experimental diet D1: Corn-wheat-soybean meal; D2: Canola meal replacing 25% SBM; D3: Canola meal + Sunflower meal replacing 50% of SBM; D4: Canola meal + Sunflower meal replacing 100% SBM.
a-b: values with different superscripts are significantly different (P<0.05).

IV. DISCUSSION

The results of the trial indicated that canola meal with sunflower meal, together with wheat gluten meal, meat and bone meal, and crystalline amino acids can fully replace SMB when the dietary CP level is slightly reduced (1.5% less than recommendations). Although, replacing SBM with the alternative ingredients when chickens fed STD-CP diets impaired growth performance, indicating that probably the amino acid digestibility coefficients in alternative ingredient may be inaccurate for the current breed of broiler chickens and require revisiting. The findings of the current study align with those of Naseem et al. (2006) and Min et al. (2011), indicating that canola meal can be incorporated at levels up to 25% without compromising the growth performance of broilers. Furthermore, our results are also in line with the observations made by Nascimento et al. (1998), reporting a slight reduction in feed consumption as the inclusion of canola meal increased during the finisher phase. Similar to canola meal, the use of sunflower meal in large quantities are limited in chicken feed due to the lower metabolisable

energy content and higher fiber content compared to SBM and substantial quantities may negatively impact growth performance and feed utilization as reported in previous studies (Bell, 1993; Chibowska et al., 2000; Ologhobo, et al., 1991). However, other studies, such as Abid et al. (1990), have successfully incorporated up to 20% sunflower meal in broiler chicken diets, with no adverse impact on growth rates. In conclusion, up to 15 – 20% canola meal, 5 – 7% sunflower meal can be used in the feed formulation, when supplementing with meat and bone meal and crystalline amino acids, exhibiting a great potential to replace SBM fully in slightly reduced CP diets, contributing to enhanced sustainability, reduced carbon footprint, and support domestic agriculture sector, however, further research is required to update the amino acid digestibility coefficients of alternative ingredients including canola, and sunflower meal for current broiler chicken breeds. In addition, potential processing techniques could be studied to improve the digestibility of the alternative ingredients to fully replace SBM in commercial broiler chicken diets.

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NUTRITIONAL VALUE OF GRAIN LEGUMES COMPARED TO SOYBEAN MEAL: STANDARDISED ILEAL DIGESTIBILITY

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Digestible nutrient values are widely used in diet formulation for better estimation of nutrient bioavailability (Osho et al., 2019). However, published data on standardised ileal digestible (SID) values of grain legumes, potential alternative to soybean meal (SBM), is limited. The present study determined SID of faba bean, lupin, lentil, chickpea, field pea and SBM for growing broilers using the direct method.

Five grain legumes and SBM (solvent extracted) were used to develop test diets. Australian-grown, grain legumes (with hulls), and SBM were sourced from commercial suppliers. A portion of each of the 5 grain legumes, but not SMB, was steam conditioned twice at 80°C for 15 seconds, then air dried. Twelve dextrose-based test diets (nitrogen-free; SBM; and each raw and heat-treated grain legume) were formulated to contain 15% crude protein and fed in mash form to 384, 21-d old chicks randomly allocated to 4 replicate cages (8 birds per cage). Basal endogenous amino acid losses were measured using a N-free diet for calculation of SID of amino acids (AA). All diets contained titanium dioxide at 5 g/kg as an indigestible marker. On day 24, all birds were euthanised by intravenous injection of sodium pentobarbitone and ileal digesta was collected from the lower half of the ileum. Proximate analysis and antinutritional factors were determined using standard procedures. Analysis of variance was conducted on SID data and orthogonal contrasts were used to make comparisons of interest, e.g., raw against heat-treated for each grain legume.

Trypsin inhibitor activity in SBM was greater than in raw and heat-treated lentil, faba bean and lupin, but lower than that in field pea and chickpea. Phytic acid content in grain legumes was lower than in SBM. Concentration of crude protein and AA in grain legumes were about half that in SBM.

Digestibility of all AA and nitrogen in SBM meal was greater than in field pea ($P < 0.05$), but not in lentil and lupin. No effect ($P > 0.05$) was observed for heat treatment on SID of AA for lentil, lupin and field pea, but inconsistent effects were observed for chickpea and faba bean. Among raw grain legumes, standardised digestible protein content was highest in lupin and lowest in field pea (262.5 and 156.2 g/kg dry matter, respectively). Digestible AA concentrations were lowest for methionine and cysteine in raw and heat-treated legumes. Grain legumes could replace some SBM in broiler diets, but an additional protein source will be needed to offset the high protein content in SBM.

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USE OF FIELD PEAS AND NON-BOUND AMINO ACIDS IN BROILER DIETS REPLACING SOYBEAN MEAL

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The Australian chicken meat industry relies on imported soybean meal (SBM) as a primary protein source in broiler diets. This raises concerns about the economic viability and long-term sustainability of its inclusion. Therefore, the industry intends to reduce the dependency on SBM by utilizing locally available feed ingredients. For instance, canola meal is routinely included in Australian broiler diets to partially replace SBM. However, its inclusion is often capped in broiler diets due to its antinutritive factors including glucosinolates, sinapine, phytate and fiber or non-starch polysaccharides. Legumes such as field peas have been utilized as a rotation crop and are available to be included in poultry diet. Non-bound amino acids (NBAA) are routinely included in poultry diets to meet amino acid requirements for broiler chickens without introducing antinutritive factors that exist in plant-based ingredients. Thus, this study aimed to evaluate the feasibility of replacing SBM with a combination of field peas and NBAA in wheat-canola-based broiler diets.

A total of 300 Ross 308 mixed sex broiler chickens were used in a 3 × 2 factorial design experiment, with three levels of SBM inclusions (conventional, medium, and low/nil) to have conventional dietary crude protein (CP) and 1% and 2% reduction of CP from conventional CP diets with or without field peas. Field pea inclusions were set at 50, 80,100,120 g/kg in starter, grower, finisher and withdrawal phases, respectively. Each dietary treatment was offered to 10 replicates of 5 birds per bioassay cage from 0 to 42 days post-hatch.

As a main effect, field peas inclusions increased body weight by 2.83% (3459 versus 3557 g/bird, $P < 0.05$), body weight gain (BWG) by 2.87% (3421 versus 3519 g/bird, $P < 0.05$), and improved FCR by 3.1 points (1.518 versus 1.487, $P < 0.05$) from 0 to 42 days post-hatch. Importantly, SBM reductions in diets did not influence the growth performance. There were no interaction or main effects observed for carcass yields and relative fat pad weights.

These results may be associated with the wheat inclusions in diets where weighted wheat inclusions to diets linearly correlated to BWG ($r = -0.883$; $P = 0.020$) as expressed in the following equation: y (BWG)= 4180 - 1.01* weighted wheat inclusion. Therefore, improved performance associated with pea included diets could stem from low wheat content. In those diets comparing with non-pea diets wheat inclusion reduced by 8.33% in starter, 7.10% in grower, 8.79% in finisher and 13.11% in withdrawal phases. Moreover, solvent canola meal (SCM) inclusion in all phases was also quadratically correlated to FCR ($r = 0.926$; $P = 0.020$) as expressed in the following equation: $y(FCR) = 1.551 - 0.004 \times$ weighted SCM inclusion + 4.959×10^{-5} weighted SCM^2 inclusion. Likewise, diets formulated with pea accommodates low SCM inclusions to diets compared to non-pea diets which also could be a possible reason for improved performance in broilers offered pea included diets than non-pea diets. Overall, this study demonstrates that replacing SBM with field peas and NBAA in wheat-canola-based broiler diets may be feasible without compromising growth performance.

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HIGH INCLUSION OF WHEAT DISTILLER'S DRIED GRAINS WITH SOLUBLES IN BROILER CHICKEN DIETS

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Wheat distiller's dried grains with solubles (wDDGS) are a valuable alternative ingredient for poultry rations. However, the inclusion rate is often limited to 12 % in broiler feed due to their high non-starch polysaccharides (NSP) contents. In the present study, high wDDGS-containing diets were offered to broiler chickens with or without carbohydrases. One-day-old Cobb 500 mixed-sex broiler chickens were assigned to five dietary treatments fed as three growth phases (starter: d 0 – 10, grower: d 10 – 21 and finisher: d 21 – 35), with eight replicates of 16 birds per treatment (n = 640). The dietary treatments were (1) PC: corn-soybean meal-based diet, (2) NC: a high-wDDGS diet, (3) XG: NC + xylanase and β-glucanase (Natugrain® TS; xylanase 560 TXU/kg and β-glucanase 250 TGU/kg), (4) M: NC + mannanase (Natupulse® TS; 800 TMU/kg) and (5) XG+M, NC supplemented with all three enzymes. The inclusion rates of wDDGS were 11, 19 and 22% of wDDGS in the starter, grower and finisher diets, respectively. Male bird % of each pen was used as a covariate during statistical analysis. Overall (d 0 – 35), birds fed the NC diet presented 11% lower weight gain and worse FCR by 0.24 points ($P < 0.001$) than those fed the PC diet, whereas M or XG+M improved these measurements relative to the NC diet (Table 1). At d 35, enzyme supplementation improved ileal digestibility of both soluble ($P < 0.001$) and insoluble ($P = 0.018$) NSP compared to non-supplemented birds, with XG+M leading to more pronounced improvements. Caecal butyrate ($P = 0.003$) and total short-chain fatty acids ($P = 0.026$) concentrations measured at d 35 were increased upon enzyme supplementation than the PC diet, with XG+M leading to a significant increase. The PC diet resulted in a higher caecal isobutyrate level ($P < 0.001$) compared to the other treatments.

Our results show that supplemental carbohydrase successfully countered wDDGS's anti-nutritive effects on growth performance, with supplementation of M or XG+M producing greater improvements in growth performance. It is noted that wDDGS inclusion likely stimulated fibre fermentation, numerically increasing butyrate levels, and suppressed protein fermentation, lowering branched fatty acid level in the hindgut, even in the absence of supplemental enzymes. The GIT adaptation to fibre could be further enhanced with the aid of carbohydrases as shown in our study, in which XG, M or XG+M improved ileal NSP utilisation, resulting in either numerically or statistically better weight gain compared to non-supplemented birds. Collectively, wDDGS can replace conventional feedstuff up to 11 – 22 % in broiler diets without compromising growth performance when supplemented with carbohydrases.

Table 1 - Effects of dietary treatments on growth performance, ileal non-starch polysaccharide (NSP) digestibility and caecal short-chain fatty acid (SCFA) production.

	Growth performance (d 0 – 35)			Ileal digestibility (%) at d 35		Caecal SCFA level (µmol/g) at d 35		
Item	Weight gain (g)	Feed intake (g)	FCR (g/g)	Soluble NSP	Insoluble NSP	Butyrate	Isobutyrate	Total
PC	2,491^{a}	3,758	1.51^{b}	-83.9^{c}	-6.0^{b}	14.6^{b}	0.98^{a}	95.3^{b}
NC	2,218^{b}	3,888	1.75^{a}	-10.2^{b}	11.1^{ab}	18.3^{ab}	0.72^{b}	104.7^{ab}
XG	2,350^{ab}	4,030	1.72^{a}	-2.5^{ab}	19.9^{a}	20.3^{ab}	0.64^{b}	112.3^{ab}
M	2,481^{a}	3,916	1.58^{b}	-0.5^{ab}	7.6^{ab}	21.6^{ab}	0.60^{b}	111.8^{ab}
XG+M	2,446^{a}	3,915	1.60^{b}	16.5^{a}	18.8^{a}	22.7^{a}	0.58^{b}	115.5^{a}
SEM	28.6	42.5	0.019	6.36	2.82	0.76	0.03	2.25
P-Value	0.006	0.394	<0.001	<0.001	0.018	0.003	<0.001	0.026

^{ac}Means (n = 8) in a row followed by a different superscript are significantly different ($P < 0.05$).

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EVALUATION OF APPARENT ILEAL AMINO ACID DIGESTIBILITY OF GUAR KORMA MEAL IN MALE AND FEMALE BROILERS

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Summary

By product of guar gum industry are gaining popularity as a poultry feedstuff. This experiment evaluated the apparent ileal amino acid digestibility of guar korma meal in male and female broilers. A total of 240 male and female broiler day old chicks were provided guar korma based diet with 6 replicates each having 20 birds. Assay diet which contained 18% crude protein as the only source of nitrogen from test ingredient were provided ad-libitum from day 22 to 29. The statistical method used was a one-way ANOVA. Prominent amino acids observed in guar korma were arginine and isoleucine. Except for arginine, the AIDC of all determined amino acids was higher ($P<0.05$) in male than female. Overall, the AIDC of amino acids were low compared with soybean meal.

I. INTRODUCTION

Conventionally used ingredients are the main source of protein in poultry diets. However, shortage and escalating cost make poultry farming uneconomical in developing countries. Continuous efforts are therefore prompted in search of viable alternate protein rich ingredients. The potential economical alternative protein sources include rape seed meal (RSM), sunflower meal (SFM) and guar meal (GM) (Ullah, Ahmed, Nisa, & Sarwar, 2016). Guar korma meal is a byproduct of the guar gum industry. It is considered a relatively cheap high-protein meal source that, depending upon the type of fraction, contain 33 to 50 % CP (Saeed et al., 2017).Despite being a rich source of protein its use as a protein supplement has been limited in poultry due to the presence of deleterious factors like gum (galactomannans), anti-trypsin factor, and anti-vitamin E factor (Dinani, Tyagi, Wani, Ongmoo, & Sharma, 2020).

Guar Seed consists of three major components; These include seed coat (15%) . he endosperm component (36 to 40%), and the last and major component of germ (44 to 48%) (Hussain, Rehman, & Khalid, 2019). The germ is the major part which has most of the protein while the endosperm contains the galactomannan gum (which is mostly used in the industrial application). It is high in essential amino acids such as arginine and isoleucine. However, the information about the digestibility of amino acids in guar meal is scarce (Hussain et al., 2019). The addition of guar meal as a partial replacement for soybean meal in poultry diets may be a useful economic strategy for decreasing feed costs. Furthermore, it is rich in arginine but a deficit in lysine, methionine, threonine, isoleucine, and leucine (Rama Rao, Prakash, Raju, Panda, & Murthy, 2014) compared to SBM.

II. MATERIALS & METHODS

A total of 240-day-old broiler male and female chicks were divided into 2 groups of male and female chicks. Each group had 6 replicates with 20 birds per replicate. All chicks were kept and reared under controlled environmental conditions. Both groups were offered a commercial starter diet containing 23% crude protein (Aviagen 2019) from day 1 to 12 and grower diet containing 21.5% crude protein,

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from day 13 to 21 days. On day 22, test diets were offered ad-libitum. The variation in mean body weight among replicates was ±10 g.

Test diet was formulated representing guar korma meal as the only source of nitrogen making the net crude protein level in final feed up to 18%. The inclusion level of GKM in diet was adjusted on crude protein (CP) basis so that dietary crude protein represents breed standard of Aviagen recommendation (Ravindran et al., 2005). Corn starch and dextrose were also used as energy source in these diets. Titanium O2, an inert marker, was added at 0.3% to each diet (Nalle & Ravindran, 2021) from day 21 to 28 to determine the AID.

Table 1 - Experimental Diet for Apparent Ileal Amino Acid Digestibility of Guar Korma Meal.

Ingredient g/kg	Diet
Guar Korma Meal	380.0
Dextrose	260.0
Corn Starch	272.0
Soya Oil	40.0
DCP 18%	19.0
Limestone	13.0
Sodium Bicarbonate	3.0
Sodium Chloride	3.0
Choline Chloride	3.0
Vitamin Premix	2.0
Mineral Premix	2.0
Titanium Oxide	3.0
Total	1000.00

On day 29, 10 male & 10 female birds from each replicate were euthanized by intravenous injection, Ketamax (Ketamine hydrochloride). Digesta was collected from terminal ilium which is a portion of small intestine start from Vitelline diverticulum to 40 mm proximal to ileo-cecal junction (Bandegan et al., 2009) by gently flushed with distilled water. Digesta was immediately stored at -20°C followed by freeze drying (Kong and Adeola, 2014). Dried samples were then ground to pass through 0.5 mm sieve and stored in plastic bags at -4°C for further analyses (Bandegan et al., 2009). Test ingredient, assay diet and dried ileal digesta samples for each replicate were analyzed for total amino acid contents at Masters Lab, Netherlands, (accredited by RvA Dutch Accreditation Council (L172)), with HPLC. Same Samples were analyzed for Dry Matter, Ether Extract, CF, Crude Protein, and ash contents (AOAC, 2006 method). Titanium dioxide (digestibility marker) in all test diets and digesta samples were analyzed with UV spectrophotometer, a method prescribed by (Morgan, Scholey, & Burton, 2014). One-way ANOVA was used to analyze the data. Differences were considered significant at ($P < 0.05$).

The apparent ileal digestibility coefficient (AIDC) of amino acids was calculated as shown below:

$$AIDC = (AA/TiO_2)_{diet} - (AA/TiO_2)_{ileal} /(AA/TiO_2)_{diet}$$

III. RESULTS

The proximate composition and total amino acid contents in guar korma meal are summarized in Table 2. Most prominent amino acids observed on guar korma meal are arginine, leucine, and isoleucine, respectively (Table 3). The most limiting amino acids observed in guar korma meal is cysteine, followed by methionine. Proximate and amino acid concentration in reference material was in line with the published data (Ullah et al., 2016).

Results of apparent ileal digestibility (AID) of crude protein and determined amino acids are presented in Table 4. The AIDC of Lys, Met, Cys, Thr, Iso, Leu, Gly, Val and His was high (P<0.05) in male compared with the female counter part. Among the determined AA, arginine was the one which did not exhibit significant (P > 0.05) differences between sexes. The highest average apparent amino acid digestibility was in Arg (71.36) in males followed by His (60.7). The lowest digestible value was observed for Cys (16.02) in female broilers. Crude protein digestibility also revealed significant difference (P < 0.05) between male vs. female (50.1 vs. 40.4).

Table 2 - Analyzed Total AA and Nutrient Composition (g/kg) for Test Ingredient and Assay Diet.

Nutrient	Guar Korma Meal	Diet 1
Moisture	59.0	111.0
Dry Matter	941.0	889.0
Crude Protein	481.5	211.0
Crude Fat	30.0	75.0
Ash	45.0	50.0
Crude Fiber	35.0	15.0
Lysine	18.8	6.8
Methionine	8.9	3.2
Cysteine	7.6	2.7
Threonine	17.4	6.0
Arginine	61.4	6.3
Isoleucine	18.9	22.2
Leucine	35.2	6.8
Glycine	26.8	12.8
Valine	22.9	9.7
Histidine	13.0	8.3

Table 3 - TAA Composition in Crude Protein % (As is Basis).

	LYS	MET	CYS	THR	ARG	ILE	LEU	GLY	VAL	HIS
TAA	1.88	0.89	0.76	1.74	6.14	1.89	3.52	2.68	2.29	1.30
% to CP	3.914	1.854	1.570	3.612	12.743	3.925	7.317	5.561	4.763	2.706

CP = Crude protein, Lys = lysine, Met = methionine. Cys = cysteine, Thr = threonine, Arg = arginine, Ile = isoleucine, leu = leucine, Gly = glycine, Valine = valine, His = histidine.

Table 4 - Apparent ileal digestibility Coefficients of guar meal.

Sex	CP	Lys	Met	Cys	Thr	Arg	Ile	Leu	Gly	Val	His
M	50.1[a]	39.9[a]	47.9[a]	25.8[a]	34.9[a]	71.4[a]	51.7[a]	51.3[a]	45.6[a]	48.5[a]	60.7[a]
F	40.4[b]	29.4[b]	37.4[b]	16.0[b]	22.9[b]	70.6[b]	41.6[b]	38.8[b]	35.6[b]	38.0[b]	54.4[b]
SEM	2.20	2.40	2.45	2.30	2.73	0.26	2.30	2.80	2.25	2.38	1.60
P Value	<0.001	<0.001	0.002	0.004	0.001	0.126	<0.001	<0.001	<0.001	<0.001	0.019

Values in the same row with different superscripts are significantly different (P<0.05).
Where, M = Male, FM = female, CP = Crude protein, Lys = lysine, Met = methionine. Cyc = cysteine, Thr = threonine, Arg = arginine, Ile = isoleucine, leu = leucine, Gly = glycine, Valine = valine, His = histidine,
SEM = standard error mean, SID = standard ileal digestibility

IV. DISCUSSION

The outcome of this research can be helpful in formulating feed using guar korma with known digestible values. Our results confirmed that it is very rich in some essential amino acids especially Arg and isoleucine while moderate amounts of the other essential amino acids which help the

nutritionist to formulate least cost balance diets keeping the ideal amino acids ratio as described by (Dari, Penz, Kessler, & Jost, 2005). It was hypothesized that there is a difference in nutrient utilization of male and female broilers. Our results reveled that male broiler exhibited higher AID of amino acids compared with the female counterpart. A relatively higher basal metabolic rate (BMR) of male broiler may explain higher nutrient requirements and nutrient digestibility (MISKI & QUAZI, 1981). The AID of essential amino acids in guar korma was low compared with the other major protein sources like soybean, canola, and sunflower-meals.

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THOUGHTS ON DIET ENERGY- DEFINITIONS, REQUIREMENTS AND ALTERNATIVES

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Summary

Energy is by far our most expensive nutrient yet surprisingly we never receive guarantees when we trade ingredients or manufacture diets. Energy level dictates feed intake, and so knowledge of diet energy levels is a prerequisite to setting specifications of most other nutrients. We have gravitated to almost universal use of AMEn in describing requirements and for diet formulation, and so unfortunately have glossed over the potential of using DE for establishing the relative diet value of ingredients. The validity of using AMEn vs AME is discussed relative to current needs of poultry nutritionists. To-date we have not established real-time NE values for ingredients, rather trending to predictive assessments based on various estimates of energy yielding components and generalized estimates of digestibility or metabolizability. Almost 50% of diet energy is used for maintenance, so minimizing this component is an obvious direction to improving efficiency.

I. INTRODUCTION

Globally we spend more than $100 billion on the energy provided by our poultry diets. Unlike the situation with most other nutrients, we have little assurance of the energy within purchased ingredients and merely an estimate of the energy within formulated diets that are delivered to the farm. Energy is obviously a complex entity, provided by all organic components of a diet. Most of the energy comes from carbohydrates and proteins with minor variably contributions from fat. Depending on the energy evaluation system used, then energy values of ingredients and diets can be influenced by bird status. Currently, the only accurate and precise way to estimate energy is via some type of bioassay involving live birds, which is both expensive and time consuming. To-date we have had limited success in estimating energy from prediction equations, *in vitro* assays or from rapid physical methods of analysis.

Apart from being expensive, the energy component of the diet is significant since it is the main driver of feed intake. Like most animals, birds eat to their energy requirements and so setting diet energy levels during formulation has a profound effect on establishing the proportional levels of other nutrients such as amino acids. White-egg layers are perhaps the most accurate in their adjustment to feed intake based on diet energy concentration. Changing diet energy level, by design or by fault, causes an almost immediate change in feed intake so as to normalize energy intake. The same situation applies to broilers if they are truly under ad-lib feeding conditions, which is a rare occurrence considering commercial stocking density, environmental conditions and pellet quality. Knowledge of diet energy level is therefore critical in establishing all other nutrient levels considered in formulation. Following are discussions on various systems of energy evaluation, a brief overview of energy requirements and finally alternative options for energy needs by the bird.

II. ENERGY EVALUATION

For the last 50 years or so we have used the schematic of energy definition to describe possible options for evaluation (Fig.1)

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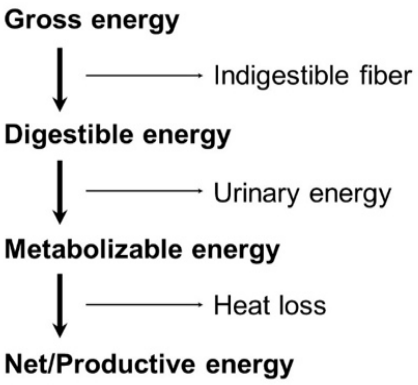

Figure 1 - Schematic of energy definitions.

We invariably dismiss GE since it represents total heat of combustion. However, for QC programs we need to identify "outliers" and so GE can give us a rapid indication that perhaps an ingredient is different to the norm, and so warrants further investigation. DE is likewise dismissed as being impractical to measure since the bird excretes urine and faeces combined, and so measurement needs either surgical modification of the bird or sampling from the distal intestine. On the other hand, we have embraced DAAs as a standard in describing ingredients and diets that are now routinely used in formulation. It is somewhat surprising that we did not pursue DE in the same manner. DE has the major advantage over ME and NE of being totally unencumbered by bird status and general feeding status. DE is arguably the most appropriate system of evaluating and ranking individual ingredients.

Metabolizable energy has become the system of choice in evaluating ingredients and diets and virtually all recommendations from commercial breeding companies are as ME. Although we talk about ME in fact we are usually referring to AMEn. The 'A" prefix refers to the "Apparent" status of these values, since there are always endogenous energy losses from a bird regardless of feed intake. The industry briefly looked at TMEn that accounts for endogenous losses. Such losses are quite minor, unless the bird is given very limited quantities of feed. With ad-lib intake, endogenous losses are in the order of 2-3%, which has a minor impact in the calculation of retained energy. Such endogenous losses are inevitable and must be "paid-for" at some stage in the calculation of energy retention, a situation that occurs with AMEn. To some extent endogenous losses will vary with ingredient and is usually higher for ingredients with higher levels of fibre (so contributing to their lower energy capture).

The suffix "n" in AMEn refers to nitrogen retention status and is perhaps of more significance. During a bioassay, birds will retain nitrogen to varying degrees and so this impacts urinary energy output. The greater the nitrogen retention, the higher the AME value. To reduce such variance, the common practice is to correct all assay calculations based on the assumption that no nitrogen is retained i.e. zero nitrogen retention. Each gram of N retained, if it appeared in the urine would add 34.4 kJ to the excreta energy. This rather drastic measure has some rational basis in that all N/protein is eventually used as an energy source during protein turnover. Labelled N fed to the broiler at 7d will not be found in the body at 42d for example. However, during a 4-5d bioassay, obviously all N consumed will not be excreted in a healthy bird. The zero-retention basis also had a legitimate basis when introduced in the 1960s since nutritionists were formulating diets for multiple farm species that had inherently different rates of N retention. However, it seems somewhat illogical to derive energy values accepting the situation that the bird is N-free and has not grown, or that the laying hen has not produced eggs. One advantage of using adult roosters in such ME bioassays is that they are usually close to zero-N balance and so the correction is a physiological norm. However, we know that age of bird impacts AME values (Lopez and Leeson,2007). The n-correction will obviously penalise

protein-rich ingredients more so than cereals. Since broiler nutritionists rarely need to formulate for multi species that perhaps have variable N-retention, then perhaps the use of uncorrected values (AME) is more appropriate for today's more focused poultry nutrition. Lopez and Leeson (2008) estimated both AME and AMEn for maize and soybean meal with different ages of broiler (Table 1)

Table 1 - AME and AMEn of maize and SBM (Lopez and Leeson, 2008).

	Bird age	AME MJ/kg	AMEn MJ/kg	Δ
Maize	9-12d	14.4	13.7	-4.8%
	30-33d	14.0	13.7	-2.1%
SBM	9-12d	10.0	9.3	-6.8%
	30-33d	10.7	9.2	-14.1%

As suspected, the correction for N retention penalises SBM by 7-14% compared to just 2-5% with maize. Abdollahi et al (2021) showed comparable differences for SBM. In taking this concept of AME for broilers one step further, Lopez and Leeson (2008) showed that broilers grown on diets formulated to AME, rather than conventional AMEn, had slightly inferior growth rate (-20g) and inferior feed efficiency (+0.02) although the cost per kg of body weight gain was reduced by 5%.

Formulating to AME does however pose some practical issues. Because N retention varies with age, then age-related AME values are likely needed to fully capitalize on this system. The other major obstacle currently, is that all requirement values, whether commercial or independent, are based on AMEn. In essence AME is a step closer to NE, which is touted as being the ultimate system of energy evaluation. Measuring AME or AMEn necessarily involves a live bird bioassay during which the GE of feed eaten, and resultant excreta are measured. This bioassay is straightforward in assessing diets but becomes much more complicated in assessing individual ingredients. Ingredients are substituted for basal diets or for glucose and then the ingredient value calculated from regression analysis to 100% inclusion of the test ingredient. The greater the distance from actual inclusion (e.g. 20%) level to 100% the greater the standard error of prediction. High test inclusion levels are challenging with high-fibre or high-CP ingredients and especially so for fat where maximum inclusion rarely gets above 15% of any test diet. For ingredients used at exceptionally low inclusion levels, such as amino acids, a bioassay becomes virtually impossible and so we rely on predictive estimates (van Milgen *et al*, 2020). Since energy comes from organic components there is potential for estimation via NIRA. Valdez and Leeson (1992a,b,c) clearly showed that NIRA has potential to predict AMEn of both diets and ingredients and was especially useful for assessing fats and oils. However, as in any NIRA measurement, prediction is reliant on robust calibration that includes calibration samples covering the entire range of anticipated AMEn.

The final potential evaluation system to consider is Net Energy. When digested energy is utilized there will be varying degrees of inefficiency associated with "rearrangement" of components necessary for ultimate deposition as fat or protein in eggs or the body of meat birds. There will also be inefficiency from a production viewpoint in that energy will be used for non-productive purposes, generally termed maintenance- with adult broiler breeders for example the overwhelming use of energy is for maintenance. Of most economic significance is the proportion of energy deposited as protein (Nep) or fat (Nef). It is exceptionally difficult to measure NE and to date there is virtually no published information on the direct assessment of NE of ingredients for poultry. A bioassay involves first measuring AMEn and subsequently either energy deposition as fat and protein measured by measuring egg and/or carcass (body) fat and protein. Alternatively, one can estimate maintenance energy from measurement of RQ

in a respiration chamber to yield NE (but not Nep and Nef). Measuring NE of diets is complicated and measuring NE of individual ingredients is a very daunting challenge. Consequently, most research on NE involves using various regression or prediction equations based on "digestible" nutrient content etc., a concept that we know does not work very accurately for AMEn. A further thought is that the NE of an ingredient or diet is as much influenced by the skill of the nutritionist during formulation, as it is the bird's use of diet energy.

III. ENERGY REQUIREMENTS

Regardless of how energy requirements are calculated, the nutritionist must set specific energy levels during formulation. This situation is somewhat confounded with the bird's ability to alter feed intake to regulate energy intake. Table 2 shows the ability of both layers and broilers to adjust feed intake in response to diet energy concentration.

Table 2 - Energy and feed intake of broilers (from Gopinger *et al* 2017) and layers (from Ribeiro *et al* 2014) when fed diets varying in AMEn.

Broiler 22-42d	Feed intake (kg)	Energy intake (MJ)
11.92 MJ/kg	3.91	46.9
12.34 MJ/kg	3.75	46.4
12.76 MJ/kg	3.65	46.4
13.18 MJ/kg	3.57	46.9
13.60 MJ/kg	3.50	47.7
Layers	Feed intake (g/d)	Energy intake (MJ/d)
11.30 MJ/kg	97.6	1.10
11.60 MJ/kg	95.2	1.10
11.90 MJ/kg	92.5	1.10
12.20 MJ/kg	91.4	1.12
12.60 MJ/kg	86.9	1.09

Although broilers have voracious appetites, they are still able to alter feed intake in response to a vast range of diet energy (assuming they can eat *ad libitum*). The almost perfect adjustment of feed intake in caged white-egg layers can be used to advantage in monitoring diet energy concentration, assuming there is accurate measurement of daily feed intake. Energy concentration is therefore more of an economic decision rather than an attempt to meet specific goals of performance. Because energy concentration impacts feed intake, then this value dictates intake of all other nutrients. The concept of maintaining an energy: protein ratio still applies to modern birds, albeit more likely replaced by consideration of energy: dig lysine. For broilers, increasing energy concentration in diets adequate in AAs invariably results in improved feed efficiency. However, adding energy to broiler diets deficient in protein/AA (even if balanced) usually has the opposite effect (Aftab,2019).

Although there are various equations used to predict energy requirements, they invariably fail due to adequately estimate underlying feed intake. In broilers, feed intake is affected by the complex interaction involving stocking density, environmental temperature, pellet quality/size and lighting program. The situation is perhaps less complicated for layers and especially broiler breeders (Teofilo *et al*, 2023) where we control feed intake. Table 3 shows expected energy intake of broilers, layers and breeders.

Table 3 - Expectations of on-farm energy intake.

Broiler	Energy intake (MJ)
0-7d	2.05
8-14d	4.60
15-21d	7.95
22-28d	11.51
29-35d	15.06
36-42d	18.00
0-42d	59.17
White Egg Layer	Daily energy intake (MJ)
24 weeks	1.17
44 weeks	1.21
100 weeks	1.26
Broiler breeder	
25 weeks	1.76
35 weeks	1.86
65 weeks	1.76

The broiler is now slightly more efficient than is the laying hen in terms of energy input per unit of "commercial" gain- for a 3 kg broiler at 42d, efficiency is around 18.8 kJ AMEn/g bodyweight gain, while for layers at peak egg mass around 44 weeks of age, a 59.5 g daily egg mass converts to around 20.5 kJ AMEn/g egg. This change has undoubtedly been caused by the propensity of modern broilers to deposit more muscle (at just over 4 MJ/kg) and less fat (at around 37 MJ/kg).

Energy requirements will obviously be impacted by environmental temperature. At temperatures much below 26ºC the bird will eat more feed to maintain body temperature. Conversely, they will eat less at higher temperatures, assuming that they are not panting which is a very energy demanding process. Measuring house temperature at first glance seems to be a straightforward task. Thermometers or temperature probes can be positioned at bird height and records collected daily. However, there is usually considerable fluctuation in temperature throughout the day. Without exact control on house temperature, how do we reconcile this temperature fluctuation in trying to calculate changes in maintenance energy and feed needs. A good example occurs with broiler breeders, since we control feed intake and so we can accommodate fluctuating environmental conditions as they occur. The traditional approach has been to simply take an average of all readings or the average of the high and low daily temperatures e.g. (26º+14º)/2 = 20°C. However, breeders do not behave in a similar manner during the day compared with nighttime darkness. During the day most breeders are rarely in contact with other birds and so the air around them is at a temperature very similar to that recorded on the thermometer. When lights are switched off however, birds invariably sit down and are usually huddled close to their flock-mates. Sitting, rather than standing, will reduce heat loss of the bird, while huddling as a group has a great insulating effect. This behavioural change in the bird has the effect of lessening the impact of the cooler night temperatures. Simply averaging high and low temperatures, to calculate feed need, may therefore be inaccurate. Leeson and Summers (2005) proposed calculation of Effective Temperature for use in calculating energy requirements.

$$\textit{Effective temperature} = \frac{\textit{(daytime high temperature x 2) + (nighttime low temperature)}}{3}$$

For the example detailed above, Effective Temperature becomes 24ºC rather than 20ºC. Table 4 uses this concept and describes increases in daily energy allowance needed for adult

breeders maintained at various temperatures. This same concept can be used to balance energy requirements for any age or class of bird.

Table 4 - Increase in energy allowance of adult breeders to accommodate lower environmental temperature (kJ AMEn/d)

Daytime	Nighttime Temp. (°C)							
Temp. (°C)	26	24	22	20	18	16	14	12
26	0	12	21	38	46	58	71	83
24			46	59	71	83	96	104
22				84	96	104	117	130
20					117	129	142	154
18						154	167	180
16							192	200
14								225

IV. ENERGY ALTERNATIVES

There are obviously no alternatives to provision of diet AMEn from the vast array of ingredients now available to the industry. The use of exogenous enzymes helps to improve DE, and formulation is designed to optimize nitrogen utilization. We can however consider various options for improving energy utilization by reducing waste in terms of Heat Increment and/or overall maintenance energy use. Figure 2 is a schematic of energy use by layers and broilers with emphasis on FMR maintenance needs.

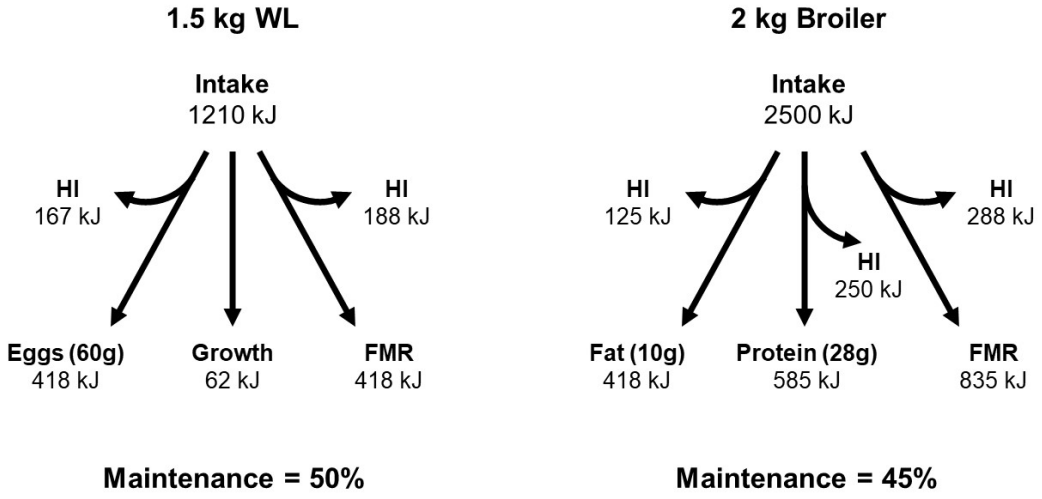

Figure 2 - Energy partitioning of Layers and Broilers.

Regardless of high sustained output of our commercial strains today, we still have at least 45-50% of AMEn intake being used for "non-productive" purposes in terms of maintenance energy needs. Maintenance is comprised of the essential Fasting Metabolic Rate plus activity, plus inefficiency associated with nutrient utilization. Table 4 previously described the energetic inefficiency related to raising birds outside the thermal neutral zone, and long-term energy use will decline as globally most countries embrace controlled environment housing systems. Non-cage systems for layers (and now broilers?) obviously impose an inefficiency in energy utilization associated with variable loss in production and greater maintenance need for activity. Hendrix (2022) propose an increase in energy requirement of at least 10% for free-range vs caged layers. For the Australian layer industry, which embraces

free-range production of layers, this represents an additional $17.5mAUD of feed energy, which seems incongruous with current interest and quantification re sustainability.

So called Heat Increment relates to the inefficiency associated with "re-arrangement" of nutrients by the liver in converting from a profile provided by the feed to that required for production of muscle, adipose tissue and eggs. Re-arrangement of proteins are most costly in energy need, with fats being the most efficiently utilized. In this regard, reducing crude protein, while sustaining DAA levels will always increase NE capture from the diet. However, this approach is not always economical as well as metabolically inefficient, often due to failure to balance DEB and the fact that large quantities of free synthetic amino acids may not be used with 100% efficiency, likely associated with quantity vs time of "imbalanced" supply arriving at the liver.

For broilers the reason for using pelleted diets is to reduce maintenance energy cost associated with feeding activity. The classic work of Leo Jensen at the University of Georgia showed that improvement in feed efficiency resulting from pelleting feed was not due to "improved" digestibility of heated starch, but rather an 18% reduction in the time spent feeding. Each 1% improvement in pellet quality (% of intact pellets in the feed) is equivalent to +13 kJ/kg of AMEn. Today, pelleted diets are most often used for broilers, although we often underestimate the ability of birds to consume large pellets at younger ages. Obviously the larger the pellet, the less time spent feeding. Table 5 shows the relative number of pellets consumed by broilers as pellet diameter and length are increased.

Table 5 - Number of pellets consumed to provide comparable daily feed intake for 30d broilers.

	Pellet Length	
Pellet Diameter	4 mm	6 mm
3mm	580	390
4mm	330	220
5mm	210	140

The same concept applies to layers and can be important where feed intake is compromised due to heat distress. In general, we need to limit the impact of maintenance to direct diet energy to productive purposes.

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COMPARATIVE EFFECTS OF LIQUID AND DRY LYSOLECITHIN MIXTURES ON GROWTH PERFORMANCE, PROFITABILITY AND ENVIRONMENTAL IMPACT IN BROILERS

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Summary

Supplementation of a combination of lysolecithin, a synthetic emulsifier, and monoglycerides (LEX) in liquid (LEL) and dry (LED) form to broiler diets with different energy levels was investigated to determine their effect on performance and environmental impact of poultry production. 1248 day-old Ross 308 broilers were assigned to one of six treatments in a 2x3 arrangement (with normal and low energy content diets with no LEX, LED or LEL) for a 42-day study. Each treatment consisted of 13 pens of 16 birds each. Results showed a higher ($p<0.05$) cumulative bodyweight gain with LEX supplementation (NE CON=2718 g, NE+LED=2829, NE+LEL=2895, LE CON=2722, LE+LED=2787, LE+LEL=2893; P=0.0331). An increased feed intake was observed for the LE diets, however cumulative FCR of LE+LED and LE+LEL were similar to the NE control (1.649 NE CON, 1.664 LE+LED, 1.656 LE+LEL; $p>0.05$), suggesting LEX enabled the birds to compensate for the energy gap.

I. INTRODUCTION

Previous studies have shown that nutrient absorption enhancers based on lysolecithin are effective tools to improve dietary energy and protein availability (Boontiam et al., 2019) – especially when supplemented in combination with other potentiating compounds such as synthetic surfactants and monoglycerides (LEX, Wealleans, 2020). LEX is also able to contribute directly to the emulsion and hydrolysis of dietary fats (Michels, 2023), thereby releasing amino acids, carbohydrates and other nutrients from the fat matrix.

LEX supplementation results in improved villus height (Gazahlah et al., 2021; Brautigan et al., 2016) and increased absorptive capacity, driving improvements in nutrient digestibility beyond lipid absorption (Wealleans et al., 2020). These non-lipid related effects may explain recent work by Ghazalah et al. (2021), which showed that the ability of LEX to improve feed efficiency and growth, both in 'on top' and reformulated diets, is not only linked to the utilization of added fat in high-density diets, but can also improve the performance and profitability of birds fed low-energy diets containing no added fat.

To date, no studies have investigated the impact of reformulating broiler diets with LEX on the carbon footprint and other environmental impacts of broiler production. Therefore, the present study aimed to compare LEX supplementation as either a dry or liquid formulation, on the growth performance and carbon footprint of broilers fed either normal or low energy diets.

II. METHOD

The accommodation and care of animals used in this study was in accordance with Directive 2010/63/EC (EC, 2010) and European Commission Recommendation 2007/526/EC. The protocol and all procedures used in this experiment were approved by the Kemin Industries review process (KAE-21-46). The trial was conducted at the facilities of Roslin Nutrition, UK. 1248 day-old male Ross 308 broilers were procured from P.D Hook Hatchery, Cote, Oxfordshire. All birds were individually weighed on arrival, then randomly assigned to six dietary treatments with 13 pen replicates of 16 birds per treatment.

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Two basal control diets were formulated: a normal energy (NE) control, formulated to meet nutrient requirements (Aviagen, 2019), and a low energy (LE) control with a 90-100 kcal reduction in energy, dependent on phase, compared to the NE. NE and LE diets were isonitrogenous and provided balanced mineral and AA profiles. Energy was diluted by the addition of rapeseed meal in LE diets (3.50-4.33%) and a reduction in soya oil (-0.60-0.94%) and full fat soybeans (-2.40-1.50%). A further four experimental diets were created by the addition of a combination of lysolecithin, synthetic emulsifier and monoglycerides (Lysoforte® EXTEND, Kemin Europa N.V., Herentals, Belgium; LEX) in either dry or liquid form to both the NE and LE controls. The dry formulation (LED) contained the active ingredients on a limestone carrier and was added to the feed at 500 g/t, the standard commercial rate of supplementation. The liquid formulation (LEL) contained the active ingredients in a rapeseed oil carrier and was added to the feed at 300 g/t. Feed intake (FI) and bird weight (pen basis) were measured on a weekly basis. For statistical analysis, the pen/replicate was considered the experimental unit. No outlier data were identified or excluded from the dataset. Data were analysed using the Fit Model capability of JMP 16 (SAS Institute, Cary, NC), as a 2x3 factorial with dietary energy level (NE, LE) and lysolecithin supplementation (none, LED, LEL) as the main factors.

To compare the economic impact of the dry and liquid applications of the combination of lysolecithin, synthetic emulsifier and monoglycerides to normal or low energy diets, a cost-benefit calculation was made based on real performance data, the weighted average of feed cost based on real feed intake, as per Godov et al. (2024). The same model was used to assess the effect of treatment on environmental impact, using calculated values for carbon footprint, water use and Land Use Change. Values for feed carbon footprint (in kg CO_2 eq/t), water use (in m^2 deprivation/t) and Land Use Change (in Pt/t) were calculated using the GFLI 2.0 database (ReCiPe method, economic allocation). These values were calculated for all feed phases and for all treatments.

Profitability and environmental impact numbers were calculated using treatment performance averages, and therefore were not subject to statistical analysis.

III. RESULTS

Across the whole study, application of both LED and LEL resulted in significantly increased body weight gain (Table 1). There was no significant effect of energy level (P=0.8021) or interaction between energy level and LEX supplementation (P=0.9242). FCR results revealed a significant effect between treatments for the energy level of the diets, as shown in Table 2, with the LE treatments having significantly increased feed conversion ratios. Likewise, there was a statistical trend toward the LEX treatments having improved FCRs compared to the untreated controls (P=0.0747).

Table 1 - Effect of supplementing dry LEX (LED) or liquid LEX (LEL) to normal and low energy diets on body weight (BW) and body weight gain (BWG) in broilers.

	Normal Energy			Low Energy			SEM	P-value		
	CON	LED	LEL	CON	LED	LEL		Energy	LEX	En*LEX
BW day 0	43.44	43.64	43.88	43.49	44.15	43.01	0.4061	0.7759	0.4970	0.2730
BW day 10	261	268	273	264	271	276	3.2545	0.2842	0.0034	0.9941
BW day 21	908	915	941	910	912	908	7.9126	0.0960	0.1671	0.0934
BW day 42	2766	2873	2938	2761	2831	2936	61.028	0.8007	0.0332	0.9269
BWG* day 0-10	217	225	233	221	227	230	3.1668	0.2559	0.0026	0.9877
BWG day 10-21	647	647	668	646	640	632	7.8407	0.0352	0.7417	0.0977
BWG day 21-42	1853	1958	1997	1855	1920	2027	58.075	0.9714	0.0435	0.8559
BWG day 0-42	2718	2829	2895	2722	2787	2893	60.994	0.8021	0.0331	0.9242

Table 2 - Effect of supplementing dry LEX (LED) or liquid LEX (LEL) to normal and low energy diets on feed intake (FI) and feed conversion ratio (FCR) in broilers.

	Normal Energy			Low Energy			SEM	P-value		
	CON	LED	LEL	CON	LED	LEL		Energy	LEX	En*LEX
FI day 0-10	278	270	285	266	278	283	5.4071	0.6302	0.0863	0.2257
FI day 10-21	902	900	916	908	934	937	9.8896	0.0217	0.1220	0.3790
FI day 21-42	3298	3375	3410	3411	3424	3563	61.568	0.0529	0.1319	0.7230
FI day 0-42	4479	4545	4611	4584	4636	4783	67.771	0.0400	0.0737	0.8376
FCR day 0-10	1.279	1.203	1.245	1.203	1.223	1.215	0.0201	0.1062	0.4147	0.0851
FCR day 10-21	1.394	1.392	1.373	1.405	1.460	1.485	0.0174	<.0001	0.2256	0.0287
FCR day 21-42	1.784	1.726	1.710	1.874	1.786	1.765	0.0343	0.0237	0.0356	0.8719
FCR day 0-42	1.649	1.607	1.595	1.698	1.664	1.656	0.0208	0.0027	0.0747	0.9560

Improved FCR is a major driver of increased profitability in broiler production. In the present study, the income over feed cost (IOFC) of the normal energy control treatment was considered as the reference point for comparison. IOFC (AUD/1000 birds) was affected by treatment (Table 3). The best profitability was seen for LEL supplementation in an LE diet, which earned $283.18 and $190.61 (AUD/1000 birds) more than NE and LE controls, respectively.

For environmental impacts, NE diets resulted in higher kg CO_2 and Land Use Change per bird than LE diets, as shown in Table 3, with diets reformulated for lower nutrient density presenting lower environmental impacts on a per tonne basis. As LED and LEL supplementation reduced FCR, environmental impact was also reduced on a per bird basis: compared to the NE control, LED and LEL in LE diets reduced CO_2 output by 0.29 and 0.31 kg per bird, respectively. Water use was not substantially changed by diet or LEX supplementation.

Table 3 - Effect of supplementing dry LEX (LED) or liquid LEX (LEL) to normal and low energy diets on feed costs, profitability and the carbon footprint of production.

	Normal Energy			Low Energy		
	CON	LED	LEL	CON	LED	LEL
Feed costs, $/t feed	333.62	335.46	335.34	319.23	321.16	320.97
Income Over Feed Costs, $/t feed	2084.99	2247.19	2333.63	2177.56	2257.88	2368.17
Kg CO_2 equivalent/t feed	2650.15	2651.17	2651.44	2449.55	2451.14	2451.00
Land Use Change, Pt/t feed	320244	320278	320259	307162	307144	307167
Water use, m^3 deprivation/t feed	58.25	59.47	59.80	56.80	58.01	58.35
kg CO_2 eq/bird	11.87	12.05	12.23	11.23	11.36	11.72
kg CO_2 eq/kg LW	4.37	4.26	4.22	4.13	4.08	4.05
Net difference in kg CO2/kg LW		-0.11	-0.14	-0.24	-0.29	-0.31
Land use (Pt / bird)	1434.1	1455.7	1476.7	1408.3	1423.9	1469.2
Land use (Pt / kg LW)	527.6	514.6	510.1	517.4	510.9	507.8
Net difference per in Land use in pt/kg LW		-13.1	-17.5	-10.2	-16.7	-19.8
Water use (m^3 deprivation/ ton product)	0.261	0.270	0.276	0.260	0.269	0.279
Water use (m^3 deprivation / kg LW)	0.096	0.096	0.095	0.096	0.096	0.096
Net difference. m^3/kg LW		-0.0004	-0.001	-0.0003	0.0005	0.0005

IV. DISCUSSION

In conclusion, the present study investigated the impact of lysolecithin-based nutrient absorption enhancers, assessing both dry and liquid formulations, on broiler performance, and profitability.

The research confirmed the efficacy of lysolecithin in enhancing nutrient digestibility, resulting in improved body weight gain and feed efficiency, especially notable in the liquid formulation.

Economic analysis revealed that both dry and liquid formulations led to increased profitability compared to non-supplemented diets, with the liquid formulation showcasing slightly enhanced financial benefits. Sustainability assessment showed improved measurements of environmental impact, due to changes in the feed formulation during reformulation to lower energy and performance improvements in the bird. While LE diets were less environmentally costly than higher density diets, the effect of LEX supplementation on the environmental footprint per bird was more pronounced in NE diets. While this study showed numerical advantages for the liquid formulation, it established that supplementing broiler diets with LEX, regardless of formulation, significantly improves profitability and the present, limited, environmental impact of production.

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EFFECTS OF GRAIN AND FAT SOURCES ON MEAT CHICKEN GROWTH PERFORMANCE OFFERED DIETS WITH REDUCED METABOLIZABLE ENERGY IN STARTER AND GROWER

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It has been confirmed that reducing dietary metabolizable energy (ME) by 0.42 and 0.31 MJ/kg in starter and grower phases of meat chickens does not compromise body weight gain (BWG), growth rate or feed efficiency at 42 days of age (Toghyani et al., 2024). However, in those studies all the diets were wheat-based with canola oil. The current study investigates if grain or supplemental fat sources affect or interact for growth parameters when same level of ME reduction is applied to starter and grower diets. The experimental design comprised a 3×3 factorial array of treatments with three types of grain (wheat, sorghum and barley) and three supplemental fat sources (canola oil, poultry and beef tallow). Each of the 9 dietary treatments was replicated 8 times in floor pens with 25 birds per replicate. The diets were fed for starter (0-10 days) grower (10 to 22 days), finisher (22 to 35 days) and withdrawal (35 to 42 days) phases. The ME in starter and grower diets were formulated to 12.03 and 12.45 MJ/kg, which were 0.42 and 0.31 MJ/kg lower than Ross 308 recommendations, respectively. In finisher and withdrawal, ME was 12.97 and 13.18 MJ/kg, respectively. Growth performance results (0-42 days) grouped by grain type are presented in Table 1.

Table 1 - The effect of grain source on broilers growth performance (0-42 d).

Treatments	BWG g/b	FI g/b	FCR g/g	Age to 2.5 kg
	0-42 D	0-42 D	0-42 D	Day
Wheat	3463	5381	1.554[a]	30.3
Sorghum	3410	5388	1.581[b]	30.8
Barley	3404	5314	1.562[ab]	30.9
SEM	21.1	29.0	0.006	0.19
P -value	0.099	0.148	0.025	0.093

There were no significant effects of grain or supplemental fat sources on BWG or FCR during the starter and grower phases. There were no interactions of grain and supplemental fat sources on overall growth performance and age to reach 2.5 kg live weight. Fat sources did not impact growth performance ($P > 0.05$). Grain source affected the overall FCR (0-42 d), as birds offered wheat-based diets exhibited significantly more efficient FCR compared to birds fed sorghum-based diets ($P < 0.05$). In commercial meat chicken diets, sorghum has traditionally been considered inferior to wheat due to the presence of several antinutritional factors. Key among these are kafirin, a prolamin protein, and phenolic compounds, which together hinder the efficient digestion and utilization of sorghum starch (Selle et al., 2021). In conclusion, these findings confirm that reducing dietary ME by 0.42 MJ/kg in the starter and 0.31 MJ/kg in the grower phases does not compromise growth performance, even when diets are formulated with grain and supplemental fat sources other than wheat and canola oil, respectively.

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INTERACTIVE EFFECTS OF DIETARY NUTRIENT DENSITY AND BREED ON GROWTH PERFORMANCE OF STRAIGHT-RUN BROILERS

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The current trial is the last in a series of four feeding studies. The first three studies confirmed the viability of decreasing metabolizable energy (ME) by 0.42 and 0.31 MJ/kg in the starter and grower diets without compromising growth performance of Ross 308 broilers (Toghyani et al., 2024). However, while Ross 308 is a prominent breed in broiler production, Cobb 500 is also widely used in Australia, with distinct nutrient requirements. This study was conducted to assess whether Cobb 500 broilers would exhibit a similar response to lower ME diets as Ross 308 birds. The study design followed a 3 × 2 factorial arrangement with 3 nutrient densities and 2 breeds. The diets were formulated to either Ross 308 (Aviagen 2022), Cobb 500 (Cobb Vantress 2022) or Ross 308 with reduced ME by 0.42 and 0.31 MJ/kg in starter and grower diets, respectively, and increased ME by 0.11 MJ/kg in withdrawal diet named as AgriFutures specs (AGF) fed to either Ross 308 or Cobb 500 birds.

On placement, Cobb 500 birds were heavier than Ross 308 by 1g/b ($P < 0.01$) and continued to have higher BW compared to Ross 308 throughout the experimental period. There was no interactive effect of breed and dietary nutrient density on BWG, FI, FCR or age to 2.5 kg of BW. Table 1 summarises the impact of the main effects on overall growth performance.

Table 1 - Growth performance over the entire feeding trial.

Main Effects	BWG g/b	FI g/b	FCR g/g	BWc FCR g/g	Age to 2.5 kg, d
Breed					
Cobb	3484[a]	5310[a]	1.524[a]	1.327[a]	30.1[a]
Ross	3302[b]	4834[b]	1.464[b]	1.304[b]	31.8[b]
Nutrient Density (ND)					
AGF	3398	5048[b]	1.485[b]	1.305[b]	30.9
Cobb	3395	5169[a]	1.522[a]	1.343[a]	31.0
Ross	3386	4999[c]	1.475[b]	1.298[b]	31.0
Source of variation *P value*					
Breed	<0.001	<0.001	<0.001	0.001	<0.001
ND	0.772	<0.001	<0.001	<0.001	0.760
Breed × ND	0.302	0.090	0.540	0.666	0.287

Each value for each treatment represents the mean of 8 replicates of 45 birds each.
[a-c] Means within a column not sharing a superscript differ significantly at the $P < 0.05$ level for the treatment effects and at the P level shown for the main effects.

An interaction between breed and diet specs resulted in higher feed cost $/kg BW in Cobb 500 birds fed Ross 308 specs compared to Ross 308 birds fed Ross 308 specs, but with Cobb 500 specs Ross 308 birds recorded the lowest feed cost $/kg BW and feed cost $/bird ($P < 0.01$).
In summary, these results indicate that nutrient density of the diets did not affect BWG and growth rate. However, similar FCRs were obtained with AGF and Ross 308 specifications but Cobb 500 specifications resulted in an FCR higher by 5 points than both Ross and AGF. Although there wasn't an interactive effect of breed and diet specs on BWG, Cobb birds gained an average of 182 g/b more BW than Ross 308 birds ($P < 0.01$).

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EFFECT OF GLUCOSE OXIDASE ON PERFORMANCE OF BROILERS FEEDING NORMAL AND LOW ENERGY DIETS

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Energy is required for homeostasis, growth and locomotion in poultry. It is a major cost of broiler feed and the cost of energy yielding ingredients has recently increased because of different reasons. Glucose oxidase (GOD) is an aerobic dehydrogenase which is primarily produced by fungi and insects, oxidizes β-D-glucose into gluconic acid and produces hydrogen peroxide. By decreasing intestinal pH and improving activity of digestive enzymes, gluconic acid acts as an acidifier in the intestinal tract (Wu et al., 2019). It is hypothesized that this enzyme may increase the availability of nutrients and improve performance of birds. Therefore, a 2 × 4 factorial arrangement of treatments was employed to examine the effects of glucose oxidase in different doses on the performance of broilers fed normal and low energy diets. A total of 806-day-old Cobb-500 chicks were assigned to 8 treatments with 8 replicates each. Treatments involved administering GOD at 0, 200, 400, and 600 g/T across diets of normal and low energy levels. Birds and feed were weighed on d 0, 8, 19, 28 and 35 and average body weight gain (WG), feed intake (FI), and feed conversion ratio (FCR) were calculated. Data were analysed by two-way ANOVA with Tukey's test.

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

Performance		Day 0-8		Day 8-19		Day 0-35		Fat pad	Litter
Energy	God Dose	[1]FCR	[2]FI (g)	[1]FCR	[2]FI	[1]FCR	[2]FI	%	moisture %
Normal	0	1.062^{a}	203	1.270	1001	1.458	3793^{ab}	0.89	31
	200	1.017^{c}	201	1.291	1028	1.450	3836^{ab}	0.97	30
	400	1.013^{c}	199	1.289	1008	1.448	3751^{ab}	0.93	27
	600	1.036^{abc}	203	1.295	1013	1.462	3799^{ab}	0.86	29
Low	0	1.038^{abc}	204	1.292	1026	1.464	3678^{b}	0.82	32
	200	1.033^{bc}	203	1.301	1033	1.483	3878^{ab}	0.85	34
	400	1.036^{abc}	209	1.294	1049	1.493	3925^{a}	0.82	33
	600	1.054^{ab}	209	1.320	1028	1.479	3911^{a}	0.88	30
Main effects	Normal energy	1.032	202^{b}	1.286^{b}	1013^{b}	1.454^{b}	3795	0.91	29^{b}
	Low energy	1.040	206^{a}	1.302^{a}	1034^{a}	1.479^{a}	3848	0.84	32^{a}
GOD dose	0	1.050^{a}	204	1.281^{b}	1013	1.461	3736	0.86	32
	200	1.025^{b}	202	1.296^{ab}	1031	1.466	3857	0.91	32
	400	1.025^{b}	204	1.291^{ab}	1028	1.470	3838	0.88	30
	600	1.045^{a}	206	1.308^{a}	1021	1.470	3855	0.87	30
P-value	Energy	0.066	0.043	0.011	0.018	0.000	0.151	0.053	0.008
	Dose	0.000	0.713	0.021	0.489	0.703	0.073	0.733	0.382
	Energy x Dose	0.002	0.506	0.576	0.546	0.179	0.045	0.516	0.144

[1,2] FCR: feed conversion ratio; FI: feed intake. [a-c] values within a column with different letters differ significantly ($P < 0.05$).

Results indicated a significant interaction in FCR during the 0-8 period, where GOD dosages of 200 and 400g/T significantly improved FCR in birds fed with normal energy diets. However, during the 8-19 period, birds fed GOD had higher FCR, particularly when supplemented at higher dose, suggesting that the enzyme maybe more effective in younger birds when administered at low to medium doses. During the d0–35 and d8-19 periods, birds on normal energy diets consistently demonstrated lower FCR ($P < 0.05$) compared to those on low energy diets. Additionally, a significant interaction between energy level and GOD dosage was observed on d0-35 on FI ($P < 0.05$) with increase noted at 400 and 600 g/T, only in birds fed with low energy diets. There was a tendency ($P= 0.053$) for increased fad pad percentage and a significantly lower litter moisture ($P<0.01$) in birds fed with normal energy diets. No significant changes of WG were observed during the whole period of experiment ($P > 0.05$, data not shown). This study suggests the potential of GOD supplementation at 200 and 400 g/T to enhance feed efficiency during the starter phase for birds fed normal energy diets. However, further research is required to investigate the mechanisms underlying these observations, including effects on intestinal morphology and gut health, to better understand its impacts over the entire production cycle.

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ENHANCING PRODUCTIVITY THROUGH NET ENERGY APPLICATION IN BROILERS

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Summary

Net energy (NE) is accepted as a more precise energy system in farm animals and yet it is not widely practiced in the broiler industry mainly due to the sophisticated nature of running the trials and validating NE values. Therefore, a huge effort is needed to provide a reliable NE prediction system to enhance widespread implementation of NE in poultry. A trial with 23 complete diets was conducted to determine the broiler's NE requirements, NE values of complete diets, and NE of 13 raw ingredients used in those diets. An equation that predicts NE from apparent metabolizable energy (AME), crude protein (CP), ether extract (EE), and neutral detergent fiber (NDF) was proposed. A series of large-scale trials that mimic field conditions were conducted to validate the optimum levels of NE and relative ratio to digestible lysine (dig. Lys). Compared to the AME system, NE favors the use of alternative raw ingredients that create opportunities for feed cost savings and enzyme application without compromising the growth performance of the birds. Crude protein requirements in NE diets were lower than those of the ME diets. Consequently, a reduction in excreta nitrogen (N) load, improved N retention and reduction in incidence of footpad dermatitis (FPD) were observed. Changes in carcass composition are yet to be seen. Field application of NE further proved to achieve lower feed costs and increased use of alternative raw ingredients. In contrast to the ME system, with the aid of assessment platforms for sustainability and carbon footprint, the NE system is found to be more sustainable under different situations, including the increased use of local/ regional alternatives ingredients and lower deposition of nutrients including N to the environment. Evidence of alteration in gut microbiota in response to nutrient compositions and/or ingredients was observed and a couple of bacterial biomarkers were identified. Besides, blood biomarkers related to energy metabolism are also in the process of being validated mainly for field applications. Improvement in flock uniformity and reduction in FPD incidence could be seen as a positive impact on flock health in general. By providing a more precise energy system, NE diets also leave less resources for pathogenic bacteria in the hind gut. Assessment of pathogen load related to the NE system is still in process. In conclusion, the net energy system in broilers creates a huge opportunity to save feed production cost and efficient use of alternative raw ingredients without adverse impact on growth performance. It is now in the stage of large-scale field application in broilers.

INTRODUCTION

Global food security and sustainability are shaping the industries to utilize the resources efficiently while animal feeds alone account for 38 % of total cereal used (FAO, 2024). In broiler farming, energy is the major cost of production and hence optimizing the energy efficiency of broilers is of great importance. In healthy chickens, beyond the default apparent metabolizable energy (ME), around 20 – 25 % of AME is lost as the heat increment (HI) resulting from the thermic effect of nutrient metabolism and physical activities (Carré et al., 2014; Noblet et al., 2023; Van der Klis and Kwakernaak, 2008). It is therefore the net energy (NE) system, that includes HI in calculation, is regarded as the more precise energy system. Studies on NE in broilers has been dated as early as 1970s (De Groote, 1974) and there are difference approaches upon estimating the more precise energy system for poultry.

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Nevertheless, industrial application of NE in broilers are in the early stage compared to swine industry, mainly due to the facts that assessment of NE values are complicated and laborious in nature, literature reports are less available compared to ME values, and there is less room for NE to take advantage when the diets rely heavily on traditional ingredients such as corn-soy based diets (Noblet et al., 2023). A number of NE studies on broilers have been published and there are promising reports of NE over ME as an energy system for broilers (Carré et al., 2013; Cerrate et al., 2019; Wu et al., 2019). The present work tries to highlight the effort to implement the NE system in broilers starting with the trial to generate NE prediction equations, followed by semi-commercial scale validation trials and field application in the industry.

NET ENERGY PREDICTION FOR BROILERS

In order to determine the birds' NE requirement, NE values of feeds and feed ingredients, a study was conducted in broiler grower chickens from 18 – 23 d of age using 23 diets. Open circuit indirect calorimetry method was used to determine the total heat production (HP) and heat increment (HI) of the birds housed inside 12 respiratory chambers. The 23 diets generated a wide range of chemical compositions (33.6% to 55.3% for starch; 20.8% to 28.4% for CP, 2.7% to 10.6% for ether extract (EE) and 7.0% to 17.2% for NDF, all on DM basis) with low correlation between the major chemical composition as well as between the levels of raw ingredients included. Thirteen raw ingredients, namely, corn, cassava pellet, broken rice, paddy rice, wheat, animal protein, full fat soybean, soybean meal, canola meal, rice bran, palm kernel meal and palm kernel oil, were chosen as the representative ingredients both regionally and globally. Each of the 23 diets were tested with a minimum of 8 replicates and a total of 235 data were analyzed by multiple linear regression models. The NE requirement for broilers at growing phase was 1.29 MJ/kg metabolic body weight/day with the average efficiency of GE for AME at 79.4% and AME for NE at 76.6%. Net energy value for the feed was 11.71 MJ/kg of feed where NE was positively influenced by ether extract (EE). On the other hand, crude protein (CP) and neutral detergent fiber (NDF) both negatively impact on NE values of the diets. Among a set of NE prediction equations generated, the following equation was proposed to use:

$$NE\ (MJ/kg.\ DM\ basis) = 0.815*AME - 0.026*CP + 0.020*EE - 0.024*NDF$$

It was found that NE values measured in the present study were in good agreement with the prediction values proposed by Wu et al. (2019) while the measured values in the current study were around 2% higher than those predicted by Wu (Figure 1).

Among the 13 ingredients tested, NE value was highest in palm kernel oil, as expected. Interestingly, paddy and broken rice were found to have higher NE/AME than corn, indicating potential alternatives to replace corn. Canola meal exhibited lowest in NE/AME among 13 ingredients. Table 1 summarizes the composition of the 23 diets and NE values from the study. Details of the finding were previously reported (Tay-Zar et al., 2024).

VALIDATION OF NET ENERGY APPLICATION IN BROILERS

Subsequent trials were conducted to validate the NE values in the semi-commercial setup, mainly adjusting the optimum NE, CP and digestible lysine (dig. Lys) levels/ ratios. Diets composed of different levels of NE, CP and dig. Lys as well as different ratios of CP to NE and NE to dig. Lys were prepared in five trials. Each of the five trials used over 2000 birds in floor pens that resembled the field conditions. A standard ME diet was included in each trial as a reference. To formulate the NE diet, in the very first step, a standard ME diet was created, and NE value of that diet was used as the standard NE value. Likewise, CP to NE ratio and NE to dig. Lys ratio of standard ME diet was used in standard NE diet. Table 2 shows the comparison

Table 1 - Summary of dietary nutrient compositions and NE values in broilers.

Item	Mean	Min.	Max.	P-value (diet)
Nutrient composition, % DM basis				
CP	24.2	20.8	28.4	-
EE	6.5	2.7	10.6	-
Starch	43.1	33.6	55.3	-
CF	3.9	2.2	6.6	-
ADF	5.7	3.3	8.8	-
NDF	10.7	7	17.2	-
Energy balance, kJ/kg $BW^{0.70}$ per day				
AME intake	1685	1509	1861	<0.001
HP	842	759	939	0.252
HI	394	311	492	0.252
NE intake	1291	1129	1494	<0.001
Energy values, MJ/kg DM				
AME	15.28	13.74	17.06	<0.001
AMEn	14.31	12.76	15.95	<0.001
AMEs	15.11	13.67	16.84	<0.001
NE	11.71	9.99	13.69	<0.001
Energy utilization, %				
AME/GE	79.4	73.4	86.7	<0.001
AMEn/GE	74.4	68.0	81.4	<0.001
AMEs/GE	78.5	72.7	85.3	<0.001
NE/AME	76.6	70.6	81.9	<0.001
NE/AMEn	81.8	75.2	86.9	0.022
NE/AMEs	77.4	71.1	82.8	<0.001

AME = apparent metabolizable energy; AMEn = AME corrected for zero nitrogen retention; AMEs = AME standardized (corrected with N retained in the body equal to 60% of N intake); HP = heat production; HI = heat increment; NE = net energy; $BW^{0.70}$ = metabolic BW (kg) as a means of daily metabolic BW values over 5 d.

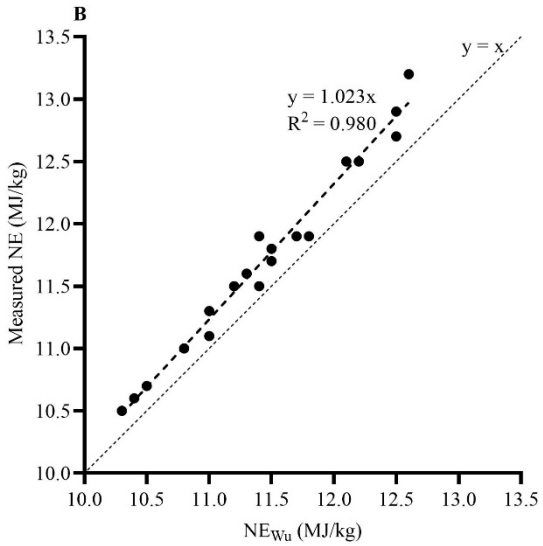

Figure 1 - The relationship between measured NE values of 23 diets from the present study and NE values predicted according to Wu et al. (2019).

of standard ME diet and standard NE diet for broiler growers. In contrast to ME diets, the resulting NE diets were around 25 – 80 kcal lower in ME per kg of feed. Moreover, CP was around 0.5% lower in NE diets. Growth performance, incidence of footpad dermatitis (FPD), flock uniformity and N retention were measured. The requirement of CP in NE diets was lower than those in ME diets. Those trials consistently indicated the advantages of NE over AME system when traditional corn-soy diets were replaced with alternatives raw ingredients. In particular, NE diets favored the use of paddy and co-products, a major cereal grown in South and East Asia regions. Without compromising the growth performance, the NE system reduced feed production cost as well as lowering CP requirements in the diets (Table 3). In contrast to ME diets, improved N efficiency was observed in birds given NE diets which might be due to the lower CP levels and increase use of AAs. Flock uniformity and FPD scores were also improved in birds NE diets.

Table 2 - A comparison of standard ME diet and standard NE diet.

Item	Standard ME diet	Standard NE diet
Ingredients, %		
Corn	50.8	10.5
Soybean meal	27.5	26.2
Paddy	-	15.0
Broken rice	6.1	32.5
Full fat soybean	5.0	5.0
Cassava pellet	5.0	5.0
Palm oil	1.9	1.7
Others[1]	3.7	4.1
Nutrient composition (as is)		
AME, MJ	12.89	12.54
NE, MJ	10.25	10.25
CP, %	20.0	19.0
dig. Lys, %	1.2	1.2
EE, %	5.3	4.0
NDF, %	7.0	3.8
dig. Lys/ME, (g/MJ)	0.93	0.96
dig. Lys/NE, (g/MJ)	1.17	1.17
Feed cost, %[2]	100.0	98.1

[1]Minerals, salt, amino acids, phytase, NSPase and vitamins.
[2]Feed production cost relative to standard ME diet (100%).

NET ENERGY APPLICATION IN THE FIELD

Following the validation trials, NE was introduced in the field, starting from the farms under closed monitoring. The first round of field applications included 16 houses with a total of 320,600 broilers fed with either NE diets or ME diets. Growth performance was observed at the end. While there was no difference in terms of growth performance between the two groups, feed production cost was lower (1%) for NE diets. Overall carcass quality, assessed at the meat processing plant, was higher in birds fed with NE diets than those fed with ME diets. This is the promising step for the field application and more data will be available as more commercial farms will be included in the NE assessment.

Table 3 - Comparison of growth performance between standard ME diets and standard NE diets in broilers.

Items	Standard ME diet	Standard NE diet	SD	SEM	P-value
0 -14d					
FI, g/b	487	488	7	3	0.82
ADG, g/b	31.9	32.1	0.3	0.1	0.50
FCR	1.091	1.088	0.012	0.005	0.84
FCG, %	100.0	99.6	-	-	-
15 - 28d					
FI, g/b	1706	1756	35	14	0.06
ADG, g/b	95.3	97.6	1.9	0.8	0.13
FCR	1.279	1.285	0.011	0.004	0.56
FCG, %	100.0	99.2	-	-	-
29 - 35d					
FI, g/b	1275	1281	11	5	0.54
ADG, g/b	121.4	124.2	4.9	2.0	0.55
FCR	1.503	1.476	0.050	0.020	0.56
FCG, %	100.0	97.3	-	-	-
0 - 35d					
FI, g/b	3472	3533	36	15	0.01
ADG, g/b	75.3	76.9	1.0	0.4	0.02
FCR	1.317	1.313	0.012	0.005	0.71
FCG[1], %	100.0	98.7	-	-	-

[1]Feed cost per BW gain relative to standard ME diet (100%). FI = feed intake, ADG = average daily gain, FCR = feed cost per gain corrected to mortality.

FEED ENZYMES AND OTHER ADDITIVES

Application of NE system in broilers attracts use of alternative raw ingredients which are economical and often less competitive for industry use and human food. In many cases, alternative raw ingredients like industry by-products are high in less nutritious compounds such as fibers which monogastric animals struggle to utilize efficiently. In such conditions, use of enzymes like single or multi NSP enzymes improve the overall nutrient efficiency of the diet. In contrast to ME system, NSP enzymes prove to have higher potential in NE system. From a series of NSP enzyme trials in broilers, it could be concluded that efficiency of AME for NE is more obvious than the efficiency of GE for AME (Figure 2) which is more reflective to the growth performance of the birds.

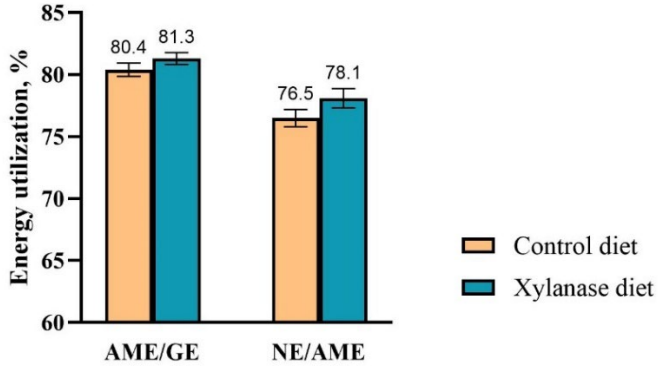

Figure 2 - Effect of xylanase on energy utilization of broilers age between 18 – 23d (n = 18 per treatment).

From the literature, application of multi carbohydrase claimed to benefit both in AME and NE systems (Morgan et al., 2019; Musigwa et al., 2021). Furthermore, the NE system was found to be responsive to probiotics as well where improved immune status in healthier flocks might play a role in reduction of heat energy loss related to immune response. It has been proved that *Bacillus subtilis* has the ability to improve the energy efficiency in broilers (Goodarzi Boroojeni et al., 2018; Upadhaya et al., 2019). More work is needed to validate the use of other major enzymes like phytase, proteases as well as other feed additives under NE system.

NET ZERO AND SUSTAINABILITY GOALS

Current efforts of NE application in industry mainly focus on feed cost saving and economic gain. Globally, many regions are heavily relying on imported raw materials like corn and soybean. Such practice not only adds to the logistic costs, but also raises the concerns for sustainability issues. By providing more flexibility in raw ingredient selection, NE encourages increased use of local and less competitive kinds of raw ingredients. In the above trials, paddy and co-products were heavily used when there was a global price hike of corn. Even in the situation like no alternative raw ingredients are available, NE system still proves to be as efficient as AME system.

ONE HEALTH APPROACH

Data collected during the semi-commercial scale validation trials and commercial applications reveal that flock uniformity was improved when fed with NE diets compared to ME diets. On the other hand, the incidence of FPD and fecal N load were reduced, which are indicators of overall improvement in health and welfare of animals. As a consequence, improved immune response and less medical intervention were observed. Use of blood biomarkers further strengthens the evidence that the NE system contributes significantly to the One Health approach. As a more precise energy system, NE diets have been found to reduce the bacteria that interfere with healthy gut functions. A large data set of gut microbiota collected during the NE prediction trial reveals the possibility of dietary intervention to modulate gut health and reduce pathogen load.

CONCLUSION

A series of validation trials and ongoing field application prove the effectiveness of the NE prediction equations for the industry. Key contributions from NE system are reduction in feed production cost, increased use of alternative raw ingredients, potential of low CP diets and increase use of AAs and enhanced application of feed enzymes such as NSP enzymes. Recently, a set of NE prediction equations for both complete diets and ingredients were proposed by Noblet et al. (2024) by compiling 50 diets from the three NE studies with similar methodology. Collectively, a more sustainable way of farming and healthier flocks are the ultimate achievements to be implemented. More efforts to bring the NE database for raw ingredients and fine tuning of digestible values of nutrients would further bring the NE system to be more reliable and sustainable.

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PREVALENCE AND PREVENTION OF ENTEROCOCCUS AROUND THE WORLD

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Summary

Emerging pathogenic *Enterococcus cecorum* and *Enterococcus faecalis* are causing important losses to the broiler industry worldwide because of the presentation of systemic disease, high mortality, and increased morbidity mainly related to leg problems leading to bad productive performance. Also, two additional concerns have been reported: leg problems frequently associated with broilers' welfare and reports of EC/EF exhibiting high resistance to antibiotics. Pathogenic EC has been isolated mainly from broiler chickens older than 2 weeks, while EF causing mortality has been isolated from embryos and early mortality (1-5 days old broiler chickens).

I. INTRODUCTION

Bacteria of the genus *Enterococcus* are negatively impacting poultry production worldwide. Although this genus has more than 60 species, emerging pathogenic *Enterococcus cecorum* (EC) and *Enterococcus faecalis* (EF) are the two species causing significant economic losses in the poultry industry worldwide, especially in the broiler sector. Reports of disease caused by EC/EF come from Australia, Belgium, Bulgaria, Canada, the Czech republic, Denmark, France, Germany, Hungary, Iran, Malaysia, the Netherlands, Norway, Poland, Scottland, South Africa, and the United States of America (USA) (Aarestrup et al, 2000, Braga et al., 2016, Borst et al., 2016; Dolka et al., 2017, Tremblay et al., 2017; Jung et at., 2018, Dunnam et al., 2023; Karunarathna et al., 2022; Pulido Landinez et al, 2023; Souillard et al., 2022; Arango et al., 2024). Additionally, the Poultry Research and Diagnostic Center (PRDL) of the College of Veterinary Medicine of Mississippi State University in the USA has diagnosed this bacterium in samples from Colombia, Costa Rica, and Guatemala throughout the PRDL diagnostic service.

Regarding public health, bacteria from the *Enterococcus* genus could be considered a potential foodborne agent and or a zoonotic agent. Human infections with EC are rare, but EF infections are considered nosocomial. Resistance to antibiotics has increased the alert level because a multidrug resistance character of enterococci, especially some EF strains isolated from humans, has shown resistance to vancomycin. Poultry products have not been linked yet to human infections. Interestingly, a case of EC and *Gallibacterium anatis* in an immunocompromised person was reported in Japan. The origin of this infection with EC was not related to chicken meat or any other poultry product (Kodana et al., 2025).

II. THE AGENT

The current *Enterococcus* genus includes the enterococcal members previously classified with the group D streptococci. Based on molecular analysis and genetic techniques, this new genus was named *Enterococcus* to draw attention to its intestinal origin. Today, 61 species are recognized. These bacteria are gram-positive cocci, facultatively anaerobic. The species frequently isolated from poultry include E*. faecalis, E. cecorum, E. faecium, E. durans, E hirae, E. gallinarum, and E. casseliflavus*. It was first described as a normal microbiota in chickens *(Gallus gallus domesticus*). However, over the past two decades, pathogenic EC and EF strains

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have emerged within the commercial poultry industry (Jung et al., 2018; Procop et al., 2020; Dunnam et al., 2023).

III. THE DISEASE IN CHICKENS

The reports of disease caused by EC and EF have increased exponentially over the last four years. Most of the time, clinical signs are not observed in acute disease. However, in some cases, the clinical presentation could be related to the site of infection, including lameness and depression. There are no specific reports of morbidity and mortality available. However, some authors report that EC infections can cause morbidity as high as 35 % and mortality of 15%. In general, the performance is affected in chickens infected with pathogenic EC, and there are reports of increased embryo mortality and low hatchability related to EF. Figure 1 shows the behavior of these bacteria in necropsy cases and samples received at the PRDL (Figure 1).

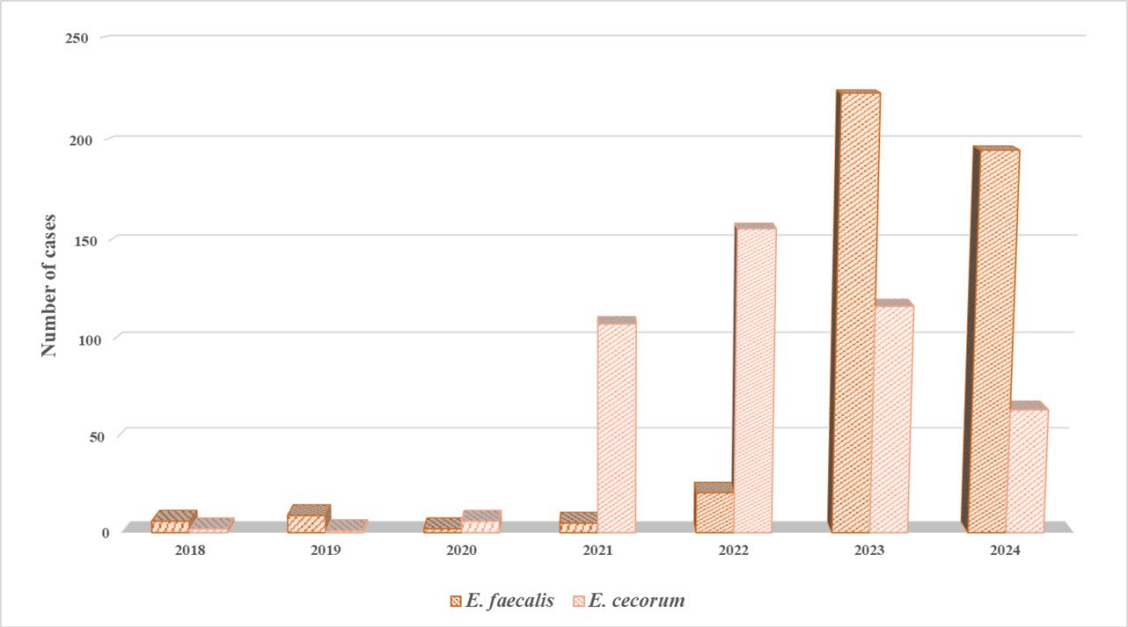

Figure 1 - *Enterococcus cecorum* and *Enterococcus faecalis* affecting chickens: PRDL cases 2018 to November 2024.

According to Dunnam et al. (2023), the main gross lesions caused by pathogenic EC include pericarditis and perihepatitis with whiteish exudates, multifocal hepatitis, femoral head necrosis (FHN), and vertebral osteoarthritis (VOA)/osteomyelitis (Figure 2). For cases where EF is involved, omphalitis, pericarditis, perihepatitis, and multifocal hepatitis have been identified as the main lesions during necropsy of embryos 19 to 21 days and chickens younger than 7 days (Figure 3) (Pulido et al., 2023).

These bacteria have implications for the birds' health and their productive performance. It is also important regarding animal welfare, especially due to the lameness problems observed in chickens affected by arthritis, femoral head necrosis, and leg muscle damage. A marked increase in mortality and condemnations in broiler plants has been reported worldwide in recent years (Jung et al., 2018; Ramirez et al., 2023). Additionally, this bacterium shows high antibiotic resistance, which has generated alarm regarding public health due to the potential presence of resistant *Enterococcus*, especially in chicken meat (Dunnam et al., 2023).

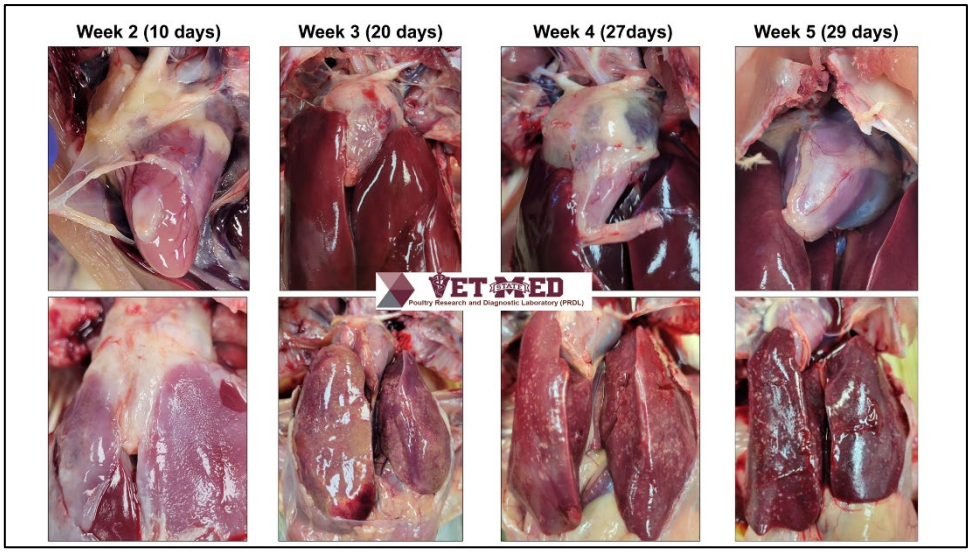

Figure 2 - Macroscopic lesions observed in PRDL necropsy cases of chickens positive for *Enterococcus cecorum*. Mild to severe pericarditis with white exudates, perihepatitis with white exudates, and multifocal hepatitis. PRDL cases from 2018 to November 2024. Pictures courtesy of Dr. Martha Pulido-Landinez.

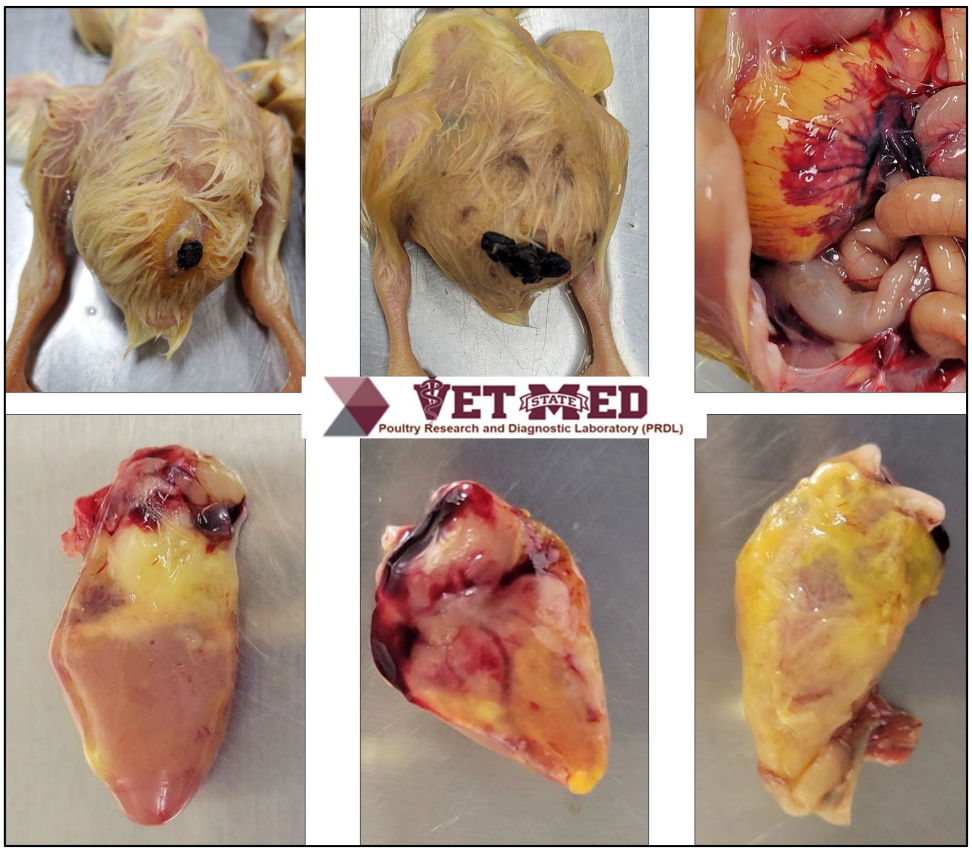

Figure 3 - Macroscopic lesions observed in PRDL necropsy cases of chickens positive to *Enterococcus faecalis.* Navel pathology, omphalitis, and severe pericarditis in PIPs and chickens from 0 to 5 days. PRDL cases 2018 to November 2024. Pictures courtesy of Dr. Martha Pulido-Landinez.

IV. FOUR LEARNED LESSONS THROUGHOUT FOUR YEARS DEALING WITH THE ENTEROCOCCUS EMERGING TREND

1. EC/EF causing current problems: an increase in case numbers was observed after the pandemic. What happened during the pandemic that may have changed EC/EF behavior? Situations like staff reduction, increased incubation of floor/dirty eggs, less cleaning frequency at the hatchery and/or farm, and biofilm growth (among others) could cause better conditions for EC/EF adaptation to poultry environments and chickens. Breaking Enterococcus adaptation up is needed. Better cleaning and sanitation programs at the hatchery and chicken farms are required.

2. EF is mainly found in eggs and embryos. This bacterium is usually used as a fecal contamination indicator. Is egg fecal contamination increasing? Has there been an increase in the number of floor eggs sent to the hatchery? The type of incubator could be important? multistage incubation with less frequent cleaning and disinfection could be involved. Are hatcheries getting old, causing difficulties in cleaning them?

3. EF/EC presentation is age-dependent. Is it important for Enterococcus control? Yes! EF in eggs and/or baby chickens means the genus *Enterococcus* is present in them. So, EC may cause problems later.

4. The number of EC and EF cases at the PRDL has shown a seasonal pattern. Is EC presentation temperature-dependent? Apparently, this is the case. EC/EF presentation increases during warm months. Supporting this idea, some unpublished studies by the University of Arkansas show that the bacteria from the genus Enterococcus increase in the gut when the chickens are housed at high temperatures (Uribe et al. 2024; not published data).

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COMMENSALLY DELIVERED RECOMBINANT IMMUNITY AGAINST ZOONOTIC BACTERIA IN CHICKENS

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Among the various zoonotic microorganisms, *Campylobacter jejuni* is one widely studied bacterial species, which is associated with human gastroenteritis. Infections caused by contaminated undercooked poultry meat represents about 50–70% of the global *Campylobacter* infections cases when compared to other types of meat (Igwaran and Okoh, 2019). The intestinal tract of chickens has favourable growth conditions for commensal bacteria *C. jejuni*. Infection caused by *C. jejuni* can lead to gastroenteritis in humans, which causes abdominal cramps, fever, and diarrhea (Igwaran and Okoh, 2019). Despite decades of research, traditional vaccine approaches against *C. jejuni* in chickens have not yielded the desired results of reduced colonisation as *C. jejuni* are of commensal nature and do not illicit a host immune response required to clear the bacteria (Pumtang-on et al., 2021). Evidence has shown a reduction of *C. jejuni* load by 2 log CFU in chickens can lead to reduction of zoonotic transmission in humans by 30 times (Lin, 2009).

Nanobodies are the antigen-binding domains of heavy chain antibodies found in *Camelid* species and have various advantageous features such as their small size, stability, improved tissue penetration and can be used as potential tools to control *C. jejuni* colonisation (Vanmarsenille et al., 2017). Anti-*Campylobacter* nanobodies do not require conformational processing such as post-translational modifications and are suited for expression in live bacterial vector candidates against *C. jejuni* by targeting key antigens in commensal bacteria which normally inhabit chickens.

Fifteen recombinant anti-*Campylobacter* nanobodies targeting flagella (Fla), outer membrane protein (OMP) and ATP synthase (ATP)-binding cassette transporter protein (pglK) in *C. jejuni* have been designed. Immunoglobulin A hinges have been *in silico* modelled with AlphaFold2 based on *Gallus gallus* sequence and fused to anti-*Campylobacter* nanobodies to form dimeric protein.

Of the fifteen designed recombinant anti-*Campylobacter* nanobodies, four candidates (OMPNb84, OMPNb24, FlaNb67, pglKNb87) showed significant binding to *C. jejuni* total protein extract when compared to controls in ELISA and western blots. OMPNb84, OMPNb24, FlaNb67 have shown significant differences in mean fluorescent intensity on binding to *C. jejuni* in Flow cytometry ($p < 0.05$) experiments compared to controls. Of the four lead candidates, immunofluorescence microscopy confirmed the interaction of OMPNb84 with the bacterial cell surface of *C. jejuni* cells and was visualised using Incucyte™ S3 imaging system (Sartorius). Further to binding experiments, nanobody functionality was demonstrated using motility assays and the four lead candidates demonstrated significant inhibition ($p < 0.05$) in *C. jejuni* motility halos when compared to controls. Primary cell culture of duodenal epithelial cells from E19 chicken embryos to develop an air-liquid interface (ALI) model is currently underway to further characterise selected nanobodies and their ability to inhibit colonisation of *C. jejuni* in vitro. Lead candidates will be transferred to bacterial expression plasmids for expression in live bacterial vectors to determine their ability to affect *C. jejuni* colonisation in chicken gastrointestinal environment both in vivo and ex vivo.

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EVALUATION OF INFLUENZA A VIRUS RESTRICTION BY DUCK TRIM PROTEINS

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Ducks are an important ecological reservoir and resistant host of influenza A virus (IAV) (Webster et al., 1992), making them a valuable model for studying IAV resistance and tolerance. In contrast, chickens are highly susceptible to IAV and often experience more severe outcomes (Alexander et al., 1986). Tripartite motif (TRIM) proteins, a diverse family with antiviral functions, are known for species-specific antiviral roles (Stremlau et al., 2004). We characterized the TRIM gene repertoire in the domestic mallard duck (*Anas platyrhynchos*) and compared it to that of chickens. Ducks possess 57 TRIM proteins, compared to 52 in chickens, divided into 12 subfamilies based on their C-terminal domains (Campbell et al., 2023). Among these, C-IV TRIM proteins are often associated with immune responses. We identified clusters of C-IV TRIM genes that varied between species in the avian major histocompatibility complex (MHC) region (Campbell et al., 2023). It remains unclear how the duck TRIM gene repertoire is regulated in response to highly pathogenic (HPAI) and low-pathogenic (LPAI) avian influenza viruses.

To determine which duck TRIM genes are upregulated in response to IAV, we analyzed previous RNA-sequencing data from ducks infected with HPAI and LPAI (Campbell et al., 2021). Due to level 2 biosecurity limitations, follow-up studies utilized LPAI, while prior HPAI studies were conducted at St. Jude's Children's Hospital. We selected several TRIM genes for functional testing in primary duck embryonic fibroblasts (DEF) and the immortalized chicken fibroblast cell line DF-1. TRIM genes were overexpressed in both DEF and DF-1 cells, followed by LPAI infection. To assess viral replication, cells were stained with anti-nucleoprotein (NP) conjugated to green fluorescent protein (GFP) and analyzed using flow cytometry 24 hours post-infection. In a second experiment, TRIM genes were overexpressed in DEF cells and infected with LPAI, then fixed before staining for the viral proteins non-structural protein 1 (NS1), NP, polymerase basic protein 1 (PB1), and polymerase basic protein 2 (PB2). Confocal microscopy visualized the interaction and colocalization of TRIM proteins with these viral proteins.

Many MHC-linked TRIM genes are upregulated in response to HPAI. Notably, TRIM206, a TRIM gene found in ducks but absent in galliform birds, was upregulated by both HPAI and LPAI. Overexpression of TRIM206 restricted IAV replication in DEF but not DF-1 cells. Analysis of microscopy results found TRIM206 colocalized with IAV NP, not PB1, PB2, or NS1.

These findings highlight the evolutionary divergence of TRIM gene-mediated antiviral defenses between ducks and chickens, emphasizing the role of TRIM proteins in species-specific IAV infection outcomes. Our results demonstrate the importance of TRIM proteins, particularly TRIM206, in shaping immune responses to IAV infections. Understanding how TRIM206 modulates viral replication in ducks enhances our knowledge of natural resistance mechanisms and identifies key genes that contribute to ducks' resilience, which are absent in more susceptible species like chickens.

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DUST CHALLENGE MODEL TO TEST THE SUCCESS OF LIVE *SALMONELLA* VACCINE

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Summary

Layer chickens were vaccinated with live attenuated *Salmonella* Typhimurium vaccine using an industry standard protocol supplemented with an additional vaccine dose at week 15. Chickens were challenged with 10^6 or 10^8 CFU of wild-type *Salmonella* Typhimurium (ST) using dust at week 17 post-hatch. Vaccination at week 12 via intramuscular route resulted in a significant increase of antibody response. In the vaccinated chickens, very low levels of vaccine were detected prior to wild-type ST infection. The shedding profile of wild-type ST showed detection in all unvaccinated challenged chickens, while loads were significantly ($P < 0.05$) lower in vaccinated chickens. The load of wild-type ST in organs at weeks 1 and 2 post-challenge showed that vaccination provided better protection to chickens challenged with 10^6 CFU wild type ST. Overall, the data showed that vaccination with live attenuated ST vaccine ST can reduce shedding of wild-type ST introduced via dust.

I. INTRODUCTION

Consumption of contaminated eggs and egg products leads to human salmonellosis (McLure et al., 2022). *Salmonella* can be isolated from layer farm environments at different stages of production both in free range and cage systems (McWhorter & Chousalkar, 2019, 2020). *Salmonella* in dust on egg farms is an important source of infection (McWhorter & Chousalkar, 2019, 2020). Multiple strategies are used to mitigate *Salmonella* on egg farms including biosecurity, clean feed, egg washing and vaccination. In Australia, *Salmonella* vaccination is now widely practiced in the egg industry. The commercially available vaccine is recommended to be administered at day 1 via coarse spray, then in drinking water at week 4 and via intramuscular injection at week 12 of flock age. Previous studies have shown that the intramuscular injection at week 12 results in a significant increase of serum antibody level (McWhorter & Chousalkar, 2018; Sharma et al., 2018). The current study investigated the role of *Salmonella* vaccination in response to two different doses of wild-type *Salmonella* challenge in layer chickens receiving standard vaccine doses and an additional dose at week 15 of age.

II. MATERIALS AND METHODS

a) Rearing of layer chicks, vaccination and wild-type *Salmonella* challenge.

This experiment was approved by the Animal Ethics Committee at the University of Adelaide (approval number S-2020-076). Hy-Line brown layer chicks were hatched, and female chickens ($n = 95$) were reared as per Hy-Line Management Guide. The details of the treatment groups are presented in Table 1. The chicks were vaccinated at day 1 and week 4 via drinking water and at week 12 post-hatch via intramuscular injection as per manufacturer's protocol of Vaxsafe ST. At week 15 post-hatch, selected treatment groups received an additional dose of vaccine via drinking water (Table 1). Cloacal swabs were collected at fortnightly intervals from week 1-14 and then weekly until week 19 of flock age for testing vaccine shedding using

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culture method (McWhorter & Chousalkar, 2018). Wild-type *Salmonella* Typhimurium PT9 doses were prepared at 10^6 and 10^8 colony forming units (CFUs) per g of poultry-farm dust (Khan et al., 2024) and sprinkled in each pen containing 5 or 6 chickens at week 17 post-hatch (Table 1). Control groups received 1 g of sterile dust.

Table 1 - Treatment group details of layer chickens.

Treatment	Vaccination status	Wild-type *Salmonella* challenge dose
Unvaccinated and unchallenged control	Nil	Nil
Unvaccinated challenged control 10^6 ST	Nil	10^6 CFU/pen
Unvaccinated challenged control 10^8 ST	Nil	10^8 CFU/pen
Vaccinated 3 doses control	Yes, standard protocol	Nil
Vaccinated 4 doses control	Yes, additional dose at 15 weeks of age	Nil
Vaccinated 3 doses and challenged 10^6 ST	Yes, standard protocol	10^6 CFU/pen
Vaccinated 3 doses and challenged 10^8 ST	Yes, standard protocol	10^8 CFU/pen
Vaccinated 4 doses and challenged 10^6 ST	Yes, additional dose at 15 weeks of age	10^6 CFU/pen
Vaccinated 4 doses and challenged 10^8 ST	Yes, additional dose at 15 weeks of age	10^8 CFU/pen

b) Vaccine and wild-type *Salmonella* shedding profile of chickens.

Vaccine shedding was monitored by testing cloacal swabs at every 2 weeks from weeks 2-14 and then weekly from week 16 – 19 post-hatch using culture method. At the same timepoints, blood was also collected for *Salmonella* Group B ELISA (McWhorter & Chousalkar, 2018). Wild-type ST was isolated by culture method (McWhorter & Chousalkar, 2018). To quantify both vaccine and wild-type ST from chickens, caecum, spleen. lungs and liver were collected at weeks 18 and 19 post-hatch and processed for bacteriology (McWhorter & Chousalkar, 2018). ST load per gram of tissue (Log_{10} CFU) data were analysed in GraphPad Prism using non-parametric analysis. Level of significance was determined by Fishers Least Significant Difference at $P < 0.05$.

III. RESULTS

a) Vaccine detection through cloacal swabs and chicken antibody response.

The vaccine shedding data showed that higher shedding level (61%) was detected at week 2 post-hatch. From week 4 until week 14, the shedding level remained < 20% and at week 14, vaccine was detected only in 14% of the vaccinated chickens. At week 16, the vaccine shedding level in the treatment group that received an additional dose of vaccine was 80%. Post-challenge, the vaccine shedding was mainly recorded in the vaccine control groups that received an additional dose of vaccine. Antibody response was significantly higher after intramuscular injection of vaccine and an additional dose of vaccination resulted in higher antibody titre at week 19 post-hatch.

b) Wild-type *Salmonella* shedding level in faeces and quantification from organs.

Irrespective of the inoculum dose (10^6 vs 10^8 CFU), ST was detected in the cloacal swabs of all the positive control chickens at 24 hr post-challenge. Within the vaccinated and ST challenged chickens, ST detection was significantly ($P < 0.05$) lower in the vaccinated chickens that received an additional dose of vaccine and were challenged with 10^6 CFU. At weeks 1 and

2 post-challenge, consistently lower levels of ST were detected in the vaccinated groups. Load of wild-type ST was quantified from organs at weeks 1 and 2 post-challenge (corresponding to weeks 18 and 19 post-hatch). Overall, the load was significantly lower in the vaccinated challenged groups compared to the unvaccinated positive control treatment groups (Figure 1 a – d). For liver, the load was significantly lower in the vaccinated and challenged with 10^6 and 10^8 CFU compared with the positive control treatment groups (10^6 and 10^8 CFU) both in weeks 18 and 19 post-hatch (Figure 1a). For spleen, among the vaccinated and challenged groups, *Salmonella* load was numerically lower in the treatment groups that received 10^6 CFU compared with 10^8 CFU challenge dose (Figure 1b). At week 18 post-hatch, low load of *Salmonella* (< 1 log) was detected in the lungs of both vaccinated and challenged groups (Figure 1c).

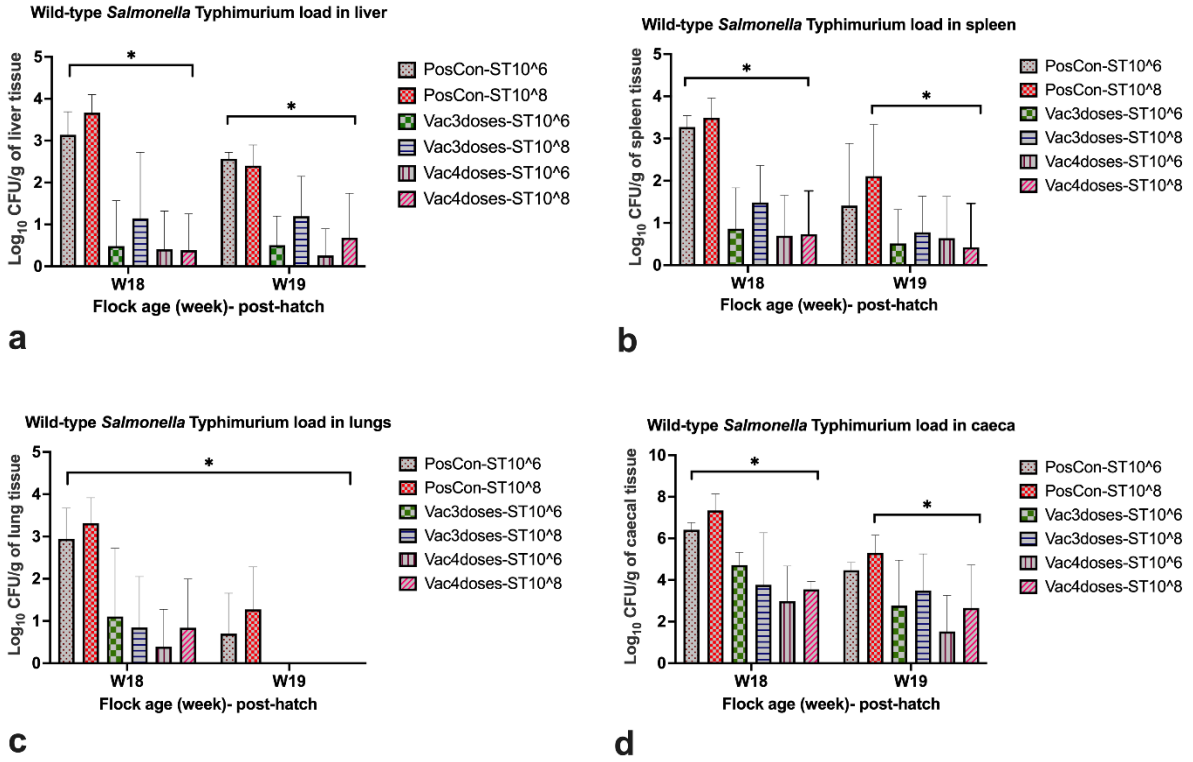

Figure 1 - Load of wild-type *Salmonella* Typhimurium in organs of layers at weeks 18 – 19 post-hatch. *Salmonella* load in (**a**) liver; (**b**) spleen; (**c**) lungs and (**d**) caeca. Level of significance at P value < 0.05 between the positive controls (10^6 or 10^8 CFU) and the remaining treatment groups has been depicted by the significance bar with asterisk.

However, at week 2 post-challenge, no *Salmonella* was quantified from any of the vaccinated and challenged groups (Figure 1c). In caecal tissue, at week 18 post-hatch, the mean load in the vaccinated and challenged treatment groups was approximately 2 log lower than the positive control groups (Figure 1d). At week 19 post-hatch, the load of *Salmonella* was significantly different mainly between the positive control 10^8 CFU and the vaccinated and challenged treatment groups (Figure 2d). Overall, chickens received 10^6 CFU showed numerically lower load compared with chickens that received 10^8 CFU.

IV. DISCUSSION

In this study, a dust infection model was developed for challenge with wild type ST. Significantly lower count of wild-type ST in the vaccinated groups compared with the positive controls demonstrated that vaccination reduced the bacterial load of wild-type ST in chickens.

Previous studies have shown that dust in poultry sheds contains a significant load of *Salmonella* (McWhorter & Chousalkar, 2020) indicating that pullets can be infected with dust containing viable *Salmonella* after introduction to the production farm. Under experimental conditions, it has also been shown that dust carrying 10^3 CFU of wild-type ST is sufficient to infect layer chickens (Khan et al., 2024). Collectively, the dust model data in this and previous studies highlights the role of residual dust in infecting birds with *Salmonella*. Therefore, it is important to regularly remove the dust from poultry sheds.

Previous studies used ST challenge doses > 10^8 CFU/bird (Groves et al., 2016; McWhorter & Chousalkar, 2018). In the current study, challange doses were 10^6 or 10^8 CFU/g of dust per pen to simulate field conditions. One-week post-challenge, 40% of chickens that received an additional dose of vaccine at week 15 were ST positive, compared with chickens that received 3 doses of vaccine (60%) and unvaccinated controls (100%). At week 2 post-challenge, lower detection of wild type ST in cloacal swabs was observed in birds that received 3 and 4 doses vaccine while the positive control groups (both 10^6 and 10^8 CFU) remained consistently 100% positive. A significantly higher antibody response was observed after intramuscular injection showed the importance of week 12 vaccination. The additional dose of vaccination resulted in an elevated antibody titre observed at week 19 post-hatch. This suggests that an additional dose of vaccine at week 15 could reduce ST shedding in birds, during the transition from rearing to potentially infected production sheds. Further examination of the duration of immunity following a 4^{th} vaccination should provide important information for the health of flocks during lay.

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VARIANT STRAINS OF IBV ARE STILL EMERGING IN VACCINATED LAYER FLOCKS

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Summary

Infectious bronchitis virus (IBV) is an ongoing concern for the layer industry because of its impact on egg production, despite the availability of vaccines. The present study received tissue samples from a free-range laying farm at the Infectious Disease Laboratory at the Sydney School of Veterinary Science. Total RNA was extracted from each sample, and the N-3'UTR region of the virus was targeted with a reverse transcription (RT) polymerase chain reaction(PCR). The amplicons were sent for sequencing to confirm the nature of these strains. Four of the six positive samples rendered good-quality sequences, which had moderate similarity to A3, VIC S, I and S vaccines. The analysis also revealed closer proximity to GV strains than GI-5, GI-6, GIII-1, GIII-2 and GIII-3 genotypes, although phylogenetic analysis showed that they formed a separate cluster from published GV strains.

This study confirmed that variants of IBV are still emerging within Australian layer farms. It is recommended that a national IBV surveillance program be established to characterise the strains circulating the country and identify emerging strains. IBV surveillance can then support the development of new IBV vaccines, which will be more effective against emerging variants than the existing vaccines developed several decades ago.

I. INTRODUCTION

Infectious bronchitis (IB), produced by the IB virus (IBV), an avian coronavirus, is known to produce drops in egg production and quality (oviduct tropism) and directly (urinary tract tropism) or indirectly (respiratory tract tropism) increase mortality in both broilers and layers. Furthermore, despite the low mortality due to their non-nephropathogenic forms, they can indirectly produce a high impact on the farm's profitability. A synergistic effect between IBV and other pathogens, like mycoplasmas (Bwala *et al.*, 2018; Landman and Feberwee, 2004; Nunoya *et al.*, 1995) and avian pathogenic *E. coli* (APEC) has been demonstrated due to the alteration of the immune response induced by IBV (Matthijs *et al.*, 2009). Also, chickens previously or simultaneously exposed to IBV exhibit a higher tracheal replication rate of *Mycoplasma gallisepticum* (Chu and Uppal, 1975). IBV can produce subtle but considerable losses for the egg industry. The respiratory symptoms might go unnoticed if the infection occurs during rearing. However, if the strains have tropism for the reproductive tract, the damage induced by IBV is permanent, and that flock might never reach their maximum genetic potential (Chousalkar *et al.*, 2009; Chousalkar and Roberts, 2007). At that stage, it would be hard to correlate that poor performance with the IBV infection. In other cases, IBV infection during production can reduce egg production or quality. Although poultry companies rely on vaccines to prevent its occurrence, IBV represents a constant problem for the poultry industry due to the emergence of variants not covered by current vaccines. This study aims to identify, isolate, and characterise IBV isolates in Australia to test the protection conferred by the current IBV vaccines against the strains circulating in the country.

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II. METHOD

Origin of the samples: The samples were collected at a free-range layer farm in February 2024. Six hens (1 to 6) were sampled post-mortem; the samples taken were tracheal and cloacal dry swabs, a piece of magnum and both caecal tonsils. Hens 7 to 8 were sampled alive, taking exclusively tracheal and cloacal dry swabs. All samples were packed in a temperature-sealed container with cool packs and sent to the Infectious Disease Laboratory in Camden Campus, The University of Sydney. Immediately after arrival, the samples were stored at −80 °C until further processing.

Sample processing and RNA extraction: The cotton tip of each dry swab was cut into a 1.5 ml tube containing 350 µL of lysis buffer supplemented with 1% β-mercaptoethanol (β-ME) and vortexed for 30 seconds. For the tissue samples (magnum and caecal tonsils), approximately 30 mg of each tissue was transferred to 1.5 mL tubes containing 600 µL of RLT lysis buffer supplemented with 1% β-ME and incubated for at least 4 hours. Then, the total RNA was extracted from all samples using the RNeasy Plus kit (QIAGEN, Australia) using the manufacturer's instructions for tissue samples with slight modifications, including an increased time of spinning, extra incubation times during the elution and passing the final elution flow-through twice.

RT/PCR-qPCR: All samples were subject to reverse transcription using Superscript IV reverse transcriptase following the manufacturer's instructions with slight modifications and using Oligo$(dT)_{18}$ as a primer (Thermo Fisher Scientific, Australia). Then, a polymerase chain reaction (PCR) was performed using Taq-polymerase following the manufacturer's instructions, and All-F and Del1-R primers (Quinteros *et al.*, 2024). This PCR amplifies a region of the IBV genome between the 3' end of the nucleocapsid gene and the untranslated region located at the 3' end of the genome (N-3'UTR). The PCR solutions of all samples were then run in a 2% agarose gel, and all those who exhibited a band of the estimated size were deemed positive for IBV.

Sequencing and sequence analysis: The PCR products were sent to the Australian Genome Research Facility (AGRF, Sydney, Australia) and sequenced using the Sanger technology with termination dye, according to AGRF's protocols. The sequences (multiple alignments and phylogenetic trees) were analysed using the Geneious Prime software package, version 2024.0.7. All IBV nucleotide sequences used in this study are available in the GenBank database (accession numbers are available upon request to the corresponding author).

III. RESULTS

RT-PCR: According to the PCR results, six samples (three from caecal tonsils, two from cloacal swabs and one from a tracheal swab) produced an amplicon of the expected size (approximately 400 nt in length). The samples were named sequentially according to the nomenclature proposed by Jackwood (2012).

Sequence analysis: Four PCR amplicons sent for sequencing produced a good-quality sequence using both forward and reverse primers. Each amplicon's pair of sequences (F and R) were identical, except for 4 ambiguities left as such in the consensus sequences. Each of those amplicon's consensus sequences (Q1/24, Q3/24, Q4/24 and Q6/24) were aligned with those of each IBV vaccine available in Australia (VIC S, I, S and A3) and an outlier (V18/91). A heatmap depicting the nucleotide identities can be found in Figure 1. The lowest level of similarity was with V18/91, but none of the vaccines exhibited a high level of similarity with any of the isolates. Only Q6/24 showed a moderate to high similarity with the A3 vaccine.

When compared with the reference strain for each Australian genotype (according to Quinteros et al. (2021)), all variants' highest identity was achieved with the GV reference strain N1/03 (Figure 2). These sequences were then aligned and compared with the GV strains.

According to the distance heatmap, the new variants' highest nucleotide identities were strains 18, N1/03, and V1/07 (Figure 3).

	V18/91	VIC S vaccine	I vaccine	S vaccine	A3 vaccine	Q1/24	Q3/24	Q4/24	Q6/24
V18/91									
VIC S vaccine	36								
I vaccine	26.5	100							
S vaccine	26.7	99.7	99.7						
A3 vaccine	36.6	84	78.8	79					
Q1/24	**28.8**	**67.5**	**77.6**	**77.8**	**82.3**				
Q3/24	**27.8**	**69.3**	**76.2**	**76.4**	**86**	**94.5**			
Q4/24	**29.4**	**68.7**	**79**	**79.2**	**83.3**	**97.3**	**94.9**		
Q6/24	**26.2**	**76.8**	**78**	**78**	**93.6**	**93.1**	**92.6**	**94.1**	

Figure 1 - Percentage of nucleotide identity between a 579 nt segment of the N-3'UTR of the Australian IBV vaccines and the isolates obtained in the present study (bold font).

	N1/62 (GI-5 ref)	VIC S (GI-6 ref)	N1/88 (GIII-1 ref)	Q3/33 (GIII-2 ref)	V18/91 (GIII-3 ref)	N1/03 (GV ref)	Q1/24	Q3/24	Q4/24	Q6/24
N1/62 (GI-5 ref)										
VIC S (GI-6 ref)	81.8									
N1/88 (GIII-1 ref)	36.3	40.1								
Q3/33 (GIII-2 ref)	37.9	41.3	86							
V18/91 (GIII-3 ref)	36.8	40.5	71.3	74.7						
N1/03 (GV ref)	94.8	80	28.9	30	28.2					
Q1/24	**78.9**	**66.8**	**29.9**	**30.5**	**28.9**	**96.9**				
Q3/24	**82.4**	**69**	**29**	**30.2**	**28.8**	**95.5**	**91.8**			
Q4/24	**80.3**	**68**	**29.7**	**30.8**	**29.5**	**98.6**	**97.3**	**92.2**		
Q6/24	**90.5**	**76.1**	**27.4**	**28.8**	**26.9**	**95.1**	**93.1**	**93.6**	**94.1**	

Figure 2 - Percentage of nucleotide similarity between a 573 nt segment of the N-3'UTR of reference strains from each genotype infectious bronchitis virus and the isolates obtained in the present study (bold font).

Finally, phylogenetic trees were constructed using the previous alignments (Figure 4). It shows that all sequences identified in the current study are located far from the vaccine strains (Figure 4A), closely related to GV strains (Figure 4B) but forming a separate clade (Figure 4C).

	Q1/13	N4/03	N4/02	18	N1/03 (GV ref)	V1/07	N5/03	Q1/24	Q3/24	Q4/24	Q6/24
Q1/13											
N4/03	95.2										
N4/02	95.2	99.3									
18	94.1	99.1	99.1								
N1/03 (GV ref)	94.6	99.5	99.5	99.5							
V1/07	94.6	99.5	99.5	99.5	100						
N5/03	95.4	99.5	99.5	99.3	99.8	99.8					
Q1/24	**79.6**	**81.9**	**81.6**	**96.5**	**96.9**	**96.9**	**81.9**				
Q3/24	**83.2**	**85.4**	**85.2**	**95**	**95.5**	**95.5**	**85.6**	**89.6**			
Q4/24	**81**	**83.3**	**82.9**	**98.1**	**98.6**	**98.6**	**83.3**	**96.4**	**90.4**		
Q6/24	**91.5**	**93.1**	**92.9**	**94.6**	**95.1**	**95.1**	**93.4**	**93.1**	**93.6**	**94.1**	

Figure 3 - Percentage of nucleotide similarity between a 561 nt segment of the N-3'UTR of GV genotype infectious bronchitis virus isolates and the isolates obtained in the present study (bold font).

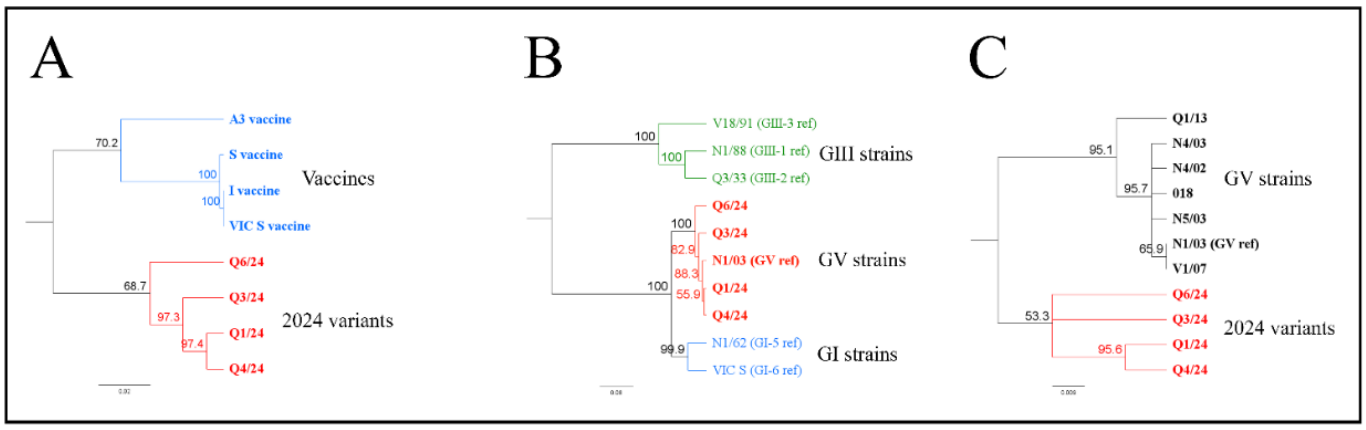

Figure 4 - Phylogenetic trees comparing a 400-nt segment of the N-3'UTR of infectious bronchitis virus of poultry as amplified from samples collected in 2024 with A, the vaccine strains available in Australia; B, the reference strains of each Australian genotype, according to Valastro *et al.* (2016); C, the GV group strains as in Valastro *et al.* (2016) and Quinteros *et al.* (2021).

IV. DISCUSSION

The results demonstrate that the genotype GV, which emerged in the early 2000s, might still be prevalent. Moreover, this genotype continues to evolve, so new IBV variants emerge in Australia that differ from the current vaccine strains. To further confirm the genotype of these variants, analysis of the rest of the genome seems highly important, especially the complete sequence of the S1 glycoprotein, which is the one inducing protective antibodies in the host and a genotype determinant. It is also evident that a constant IBV surveillance program, such as the one conducted in the USA (Jackwood and Jordan, 2021), is highly important to detect early the emergence of variants not protected by the vaccines and reduce the incidence and negative effects of IBV in poultry production in Australia. Such a study will determine the characteristics of the new isolates and the level of protection conferred by the current vaccines against the most recent isolates to evaluate the need to produce a new vaccine for the country.

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MICROBIOME-HOST INTERACTION IN AVIAN PASTEURELLOSIS

Y.S. BAJAGAI[1], F. PETRANYI[1] and D. STANLEY[1]

Summary

This study investigates the impact of avian pasteurellosis, caused by *Pasteurella multocida*, on gut microbiota and host gene expression in chickens. Diseased and healthy birds from a free-range layer farm were sampled, and digesta from the jejunum, ileum, and cecum were collected for microbiota analysis and liver tissue for transcriptomic analysis. The study found significant alterations in the gut microbiota of infected birds, including an increased abundance of *Escherichia-Shigella,* raising the possibility of co-infection with pathogenic *E. coli,* and decreased levels of beneficial genera, such as *Bifidobacterium* and *Lactobacillus*. Transcriptomic analysis revealed activation of pathways related to cell proliferation, differentiation, and immune responses, notably involving the cytokine TGFB1.

I. INTRODUCTION

Fowl cholera, or avian pasteurellosis, is a highly contagious bacterial disease caused by the gram-negative pathogen *Pasteurella multocida*, affecting various avian species, including chickens, turkeys, and ducks. It poses significant economic challenges to the global poultry industry due to high mortality rates, reduced egg and meat production, and increased management and veterinary costs. The disease can impact both broiler and layer chickens, especially in free-range production systems (Zhang et al., 2004, Singh et al., 2014).

Clinically, avian pasteurellosis manifests either as an acute form with septicemia and high mortality or as a chronic form with localised symptoms such as respiratory distress, anorexia, mucoid discharge from the mouth, and swollen wattles and footpads. Despite extensive research on its diagnosis and pathogenesis, understanding host-pathogen interactions, particularly the impact on host microbiota and metabolic pathways, remains incomplete.

This study aims to develop a comprehensive understanding of the disease's impact by integrating data on the effect of the pathogen on intestinal microbiota and host gene expression andmetabolic pathway disruptions by transcriptomic study of liver tissue.

II. METHOD

This study was approved by the Central Queensland University Animal Ethics Committee (Approval No. 0000022879). A suspected outbreak of avian pasteurellosis was identified on a free-range commercial layer farm in Queensland, Australia. Before initiating any treatment, ten diseased and ten healthy birds were euthanised, and digesta samples from the jejunum, ileum, and cecum were collected for microbiota analysis, along with liver tissue for host gene expression analysis. Laboratory testing performed in a commercial diagnostic laboratory confirmed *Pasteurella* infection in the diseased birds, while control birds tested negative. DNA and RNA were extracted from digesta and liver tissues using established protocols (Yu and Morrison, 2004). The V3–V4 region of the 16S rRNA gene was amplified using specific primers and sequenced on the Illumina MiSeq platform. RNA samples were sequenced on the Illumina NovaSeq 6000 platform.

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The 16S rRNA gene sequencing data were processed with QIIME2 using the DADA2 (Callahan et al., 2016) for quality filtering, denoising, and chimera removal. Taxonomic classification was performed using the SILVA v138.1 database. Statistical analyses and visualisation were conducted in R using Phyloseq, Vegan, and Microeco packages. RNA-seq data were processed with the nf-core/rnaseq pipeline (Ewels et al., 2020), including quality control, alignment to the chicken genome (bGalGal1) using Spliced Transcripts Alignment to a Reference (STAR), and quantification of transcript and gene expression with Salmon. Differentially expressed genes were identified using DESeq2 and analysed with Qiagen Ingenuity Pathway Analysis (IPA) to elucidate biological functions and pathways.

III. RESULTS

a) Effect of avian pasteurellosis on gut microbiota.

Avian pasteurellosis significantly altered the gut microbiota composition in the jejunum, ileum, and cecum of infected birds compared to healthy controls, with the most pronounced changes observed in the cecum. PERMANOVA of weighted unifrac distances confirmed significant differences between infected and healthy birds in all gut sections (jejunum, P = 0.02; ileum, P = 0.02; cecum, P = 0.001).

In the cecum, the genus *Rikenellaceae RC9 gut group* showed a strong positive correlation with disease status (r = 0.87, P < 0.001), while *Bifidobacterium* (r = –0.62, P = 0.003) and *Enorma* (r = –0.59, P = 0.006) were negatively correlated. At the phylum level, Bacteroidota (r = 0.68, P = 0.001) and Proteobacteria (r = 0.73, P < 0.001) were positively associated with infection, whereas Actinobacteriota was negatively correlated (r = –0.54, P = 0.01).

Notable microbial shifts included increased *Campylobacter* in the jejunum and *Fusobacterium* in the ileum of diseased birds, along with a decrease in *Rikenellaceae RC9 gut group* in the cecum. *Escherichia-Shigella* significantly increased across all gut sections in infected birds, while *Lactobacillus* and *Bifidobacterium* decreased. LEfSe analysis identified genera characterising infected birds (e.g., *Rikenellaceae RC9 gut group, Escherichia-Shigella*) and healthy birds (e.g., *Bifidobacterium*, *Lactobacillus*).

Microbiota richness, measured by the Chao1 index, was significantly reduced in the cecum of diseased birds (P = 0.03), though no significant differences were found in other gut sections.

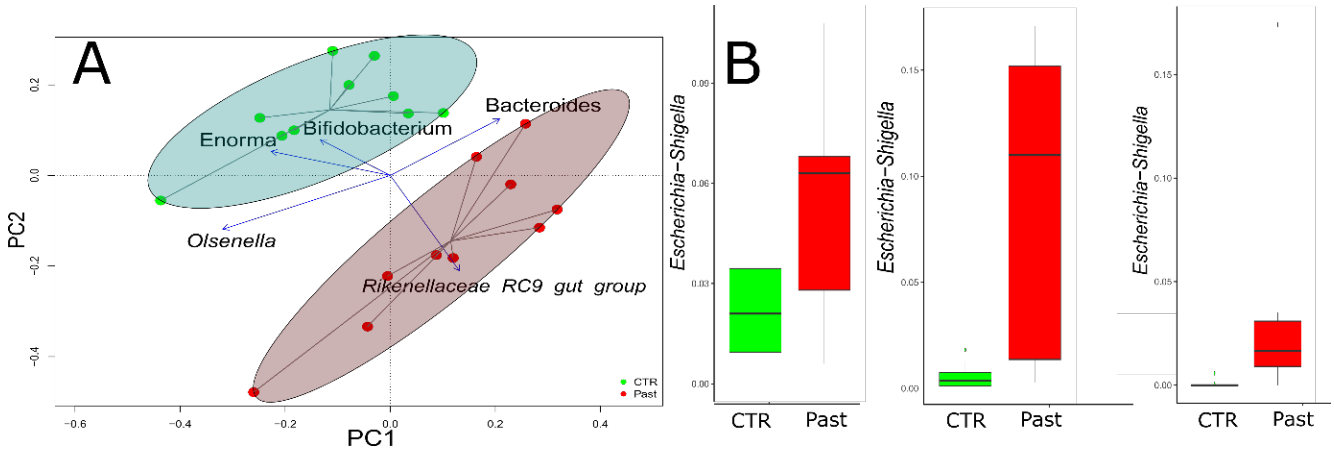

Figure 1 - Impact of avian pasteurellosis on intestinal microbiota A) Ordination of samples with principal component analysis B) Relative abundance of *Escherichia-Shigella* in jejunum, ileum and cecum.

b) Host gene expression and pathway analysis.

Pasteurella infection significantly impacted host gene expression in liver tissue. Pathway analysis revealed activation of several canonical pathways, notably Molecular Mechanisms of Cancer (activation z-score = 6.09, P = 0.001), RHO GTPase Cycle (z = 5.91, P = 0.000001), and S100 Family Signaling Pathway (z = 5.66, P = 0.003).

Disease and biofunction analyses highlighted associations with the pathways that are also involved in cancer, organismal injury, endocrine disorders, gastrointestinal disease, and neurological disease, indicating systemic effects of the infection. Top toxicity functions affected included increased levels of lactate dehydrogenase and albumin, cardiac hypertrophy, liver steatosis, hepatic fibrosis, and renal injury, highlighting the widespread physiological impact of *Pasteurella* infection.

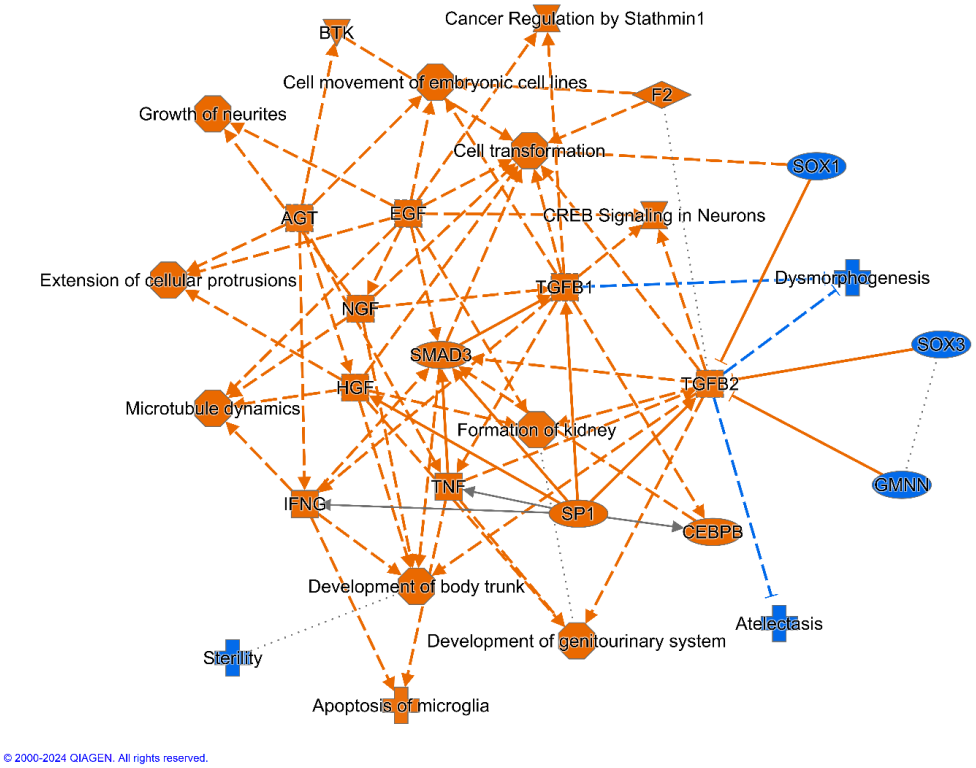

Figure 2 - Graphical summary of differential transcriptomic responses in liver tissue between *P. multocida* infected and control birds, highlighting key pathways and molecules identified by Ingenuity Pathway Analysis (IPA). The orange colour indicates activated, and the blue colour indicates inhibited molecules and functions.

IV. DISCUSSION

The significant alterations observed in microbial communities and host metabolic pathways highlight the systemic nature of *Pasteurella multocida* infection and its extensive effects on poultry health.

Our findings revealed a marked shift in the gut microbiota of infected birds, characterised by an increase in potentially pathogenic bacteria and a decrease in beneficial microbes. At the phylum level, there was a significant increase in Proteobacteria and Bacteroidota and a reduction in Actinobacteriota in diseased birds. The increase in Proteobacteria is particularly noteworthy, as this phylum includes many opportunistic pathogens and is often associated with poor intestinal health and inflammation (Shin et al., 2015). Similarly, the rise in Bacteroidota, specifically the genus *Bacteroides*, suggests a shift

in the microbial balance that may impact nutrient absorption and immune modulation. Conversely, the reduction of Actinobacteriota, especially *Bifidobacterium*, indicates a loss of beneficial microbes that play crucial roles in immune regulation and maintenance of gut barrier function (O'Callaghan and Van Sinderen, 2016).

The significant increase in the genus *Escherichia-Shigella* across all gut sections of diseased birds indicates gut dysbiosis and raises the possibility of co-infection with pathogenic *Escherichia* coli. Since *Escherichia-Shigella* includes various strains of *E. coli*, some of which are pathogenic to poultry and some are zoonotic, the elevated abundance of this genus suggests that *E. coli* may play a role in the observed disease pathology alongside *Pasteurella multocida*. Further studies are required to identify the specific species and strains involved. Nevertheless, it may be prudent to consider the possibility of co-infection with other pathogens, such as *E. coli*, when diagnosing and treating *Pasteurella multocida* infections in poultry.

The transcriptomic analysis revealed significant changes in host gene expression in the liver of infected birds. Notably, pathways related to cell proliferation, differentiation, and apoptosis were highly activated, including the Molecular Mechanisms of Cancer and RHO GTPase Cycle pathways. The activation of these pathways suggests that *P. multocida* infection may induce cellular stress responses and alter normal cellular functions. The upstream regulator analysis identified TGFB1 as the most significantly activated regulator. TGFB1 is a multifunctional cytokine involved in regulating immune responses and maintaining tissue homeostasis (Li et al., 2006). Its activation implies a role in modulating inflammation and immune responses during infection. The influence of TGFB1 on target molecules such as ACVR1, CCL20, and RUNX2 highlights its involvement in cellular proliferation and immune cell recruitment. The inhibition of specific microRNAs (e.g., miR-291a-3p and mir-8) suggests a complex regulatory network affected by infection. These microRNAs typically regulate gene expression post-transcriptionally, and their inhibition could lead to the overexpression of target genes involved in the immune response and cellular regulation (O'Connell et al., 2010).

The identified toxicity functions, such as increased levels of lactate dehydrogenase and liver steatosis, indicate potential organ-specific toxicities resulting from the infection. These systemic effects emphasise the severity of avian pasteurellosis and its possible long-term consequences on poultry health.

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PATH MODELS FOR SPOTTY LIVER DISEASE

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Summary

The epidemiology of Spotty Liver Disease (SLD) has begun to be explored in Australia. It is known that the causative organisms (*Campylobacter hepaticus* or *C. bilis*) may be present in birds and their environment in cage-free facilities without producing clinical outbreaks of SLD. Hence SLD is considered to be a multifactorial condition, requiring the interaction of several factors with the host and the causative organism for disease to be expressed. Epidemiological surveys conducted over 2019-2022 have identified several key determinants that contribute to outbreaks of SLD. These include the presence of a scratch area, the use of natural ventilation systems and a higher nest density. Findings from three epidemiological surveys have been combined to produce causal models describing the coincidental presence of several identified key factors that illustrate strong evidence and rationale for the clinical expression of SLD.

I. INTRODUCTION

Spotty Liver Disease (SLD) causes mortality and egg production drops in free-range layer farming (Courtice et al., 2018). The causative agent of SLD has been identified as *Campylobacter hepaticus* (Van et al., 2017). Three analytical epidemiological surveys have been conducted across cage-free layer operations in Australia to improve the understanding of the epidemiology of SLD in cage-free layers (Gao et al., 2023a, 2023b; Groves et al., 2024a). The studies searched for statistical associations between facility and management practices with the occurrence of SLD. Factors included house design and furniture, ventilation systems, floor space, feeder and drinker space and types, nest type and space allowance, slat design, range size, and many bird performance parameters. C. *hepaticus* is a necessary cause of SLD (the causative organism must be present for SLD to occur) but with a multifactorial disease the presence of other factors is required for the disease to be clinically expressed.

The aim of this study was to combine results from all three epidemiological studies to produce path models that can lead to clinical outbreaks of SLD under Australian conditions and thus to facilitate further experimental work on this disease. The hypothesis was that SLD expression is modified by management and environmental factors and that, by removing or modifying one factor within a path model, the expression of SLD could be avoided or minimized.

II. METHOD

Three formal epidemiological surveys were conducted in Australia over 2019 to 2022 from flocks up to 40 weeks of age. These involved extensive housing and management questionnaires and collection of cloacal swabs, faeces and/or dust for detection of *C. hepaticus* by PCR from each flock. Recorded findings were cross tabulated against occurrence of SLD. Initial comparisons used Pearson's Chi-square analysis for categorical factors and Student's t-tests for continuous data. Multiple logistic regression was used to define statistically important variables and to account for confounding and interaction. The individual results of these

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surveys have been previously published (Gao et al., 2023a, 2023b, Groves et al., 2024b). Key determinants from each survey were combined into theoretical models which constitute different sufficient causes. JMP® 18.0.0 was used for logistic regression analyses in each survey. Models were constructed following Martin et al. (1987) and Thrusfield (2003) by incorporating key contributory factors for each path to the disease. Calculation of the contribution to proportion of disease outcomes were calculated from each survey outcome.

III. RESULTS AND DISCUSSION

The initial survey identified the presence of a scratch area as a major risk factor for SLD, with every such house being a clinical SLD case (Gao et al., 2023a). Transmission route for *C. hepaticus* is regarded as fecal-oral (Phung et al., 2022) and hence the greater exposure to faeces afforded by a scratch area explains this finding, while full slat coverage of the floor provides separation from much fecal material inside the house and thus can be somewhat protective against SLD. Of the fully slatted houses, 45% had clinical SLD, but the sample size remaining was only 11 flocks which restricted further findings. Hence surveys 2 and 3 (Gao et al., 2023b; Groves, 2024) were conducted to evaluate other factors in either fully slatted houses or houses with a scratch area. These latter surveys identified the use of natural ventilation, and the density of birds related to nest space (birds/ m^2 nest space) as further key determinants in both fully slatted and partially slatted houses. Tunnel ventilation appeared as a protective factor in both systems. Cage-free farms can be of several types: conventional "flat deck" design, where a central nest box system is flanked by slats which may either reach the shed walls (fully slatted) or terminate before the wall and allow a scratch area on the floor; or, an "aviary" style house capable of holding more birds due to utilization of vertical space, including a central "system" of tiers catering for feeding, nesting and sleeping sections. Aviaries invariably allow birds to contact the floor which is essentially a scratch area. Flat deck or aviary styles can be run as barns, with birds confined to the shed, or as free-range where birds may leave the house through pop holes.

The consistent findings from the surveys allowed three theoretical path models to be generated.

- Model #1 – a cage-free house with a scratch area, using natural ventilation, where C. *hepaticus* is present.
- Model #2 – a cage-free house with a scratch area, using tunnel ventilation but where nest density exceeds 102 birds/ m^2 and *C. hepaticus* is present.
- Model #3 – a cage-free house which is fully slatted, using natural ventilation where nest density exceeds 112 birds/ m^2 and *C. hepaticus* is present.

Data from the surveys indicated that model #1 was responsible for 44% of SLD cases, model #2 resulting in 20% of cases and model #3 was involved in 36% of SLD cases in the field.

The importance of the ventilation system is consistent with expression of the disease in warmer weather and with the observation that severity of the disease can be markedly reduced if shed temperature is able to be lowered at the onset of symptoms (Garcia and Courtice, 2024).

SLD is predisposed by a cage-free housing system. The expression of the disease is enabled by the presence of a scratch area and by sub-optimal temperature control under natural ventilation conditions. Higher nest density is a reinforcing factor for the expression of the disease. Under exposure to the specified combination of these factors, SLD is precipitated by the presence of the necessary cause (*C. hepaticus*).

The findings presented here allow better understanding of the reasons for the variations in occurrences of SLD and provide advice to producers in techniques to control the disease.

Modification of houses to decrease SLD is understandably difficult and expensive but guidance is provided in some measures to reduce the problem and towards the design of layer housing going forward. These causal models represent the epidemiological understanding of SLD under Australian conditions to the extent of current knowledge. It is hoped that they form a basis for the addition of further information as it becomes available.

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REINFECTION OF CHICKENS WITH *CAMPYLOBACTER HEPATICUS*, THE CAUSE OF SPOTTY LIVER DISEASE

S. EASTWOOD[1], T.B. WILSON[2], P.C. SCOTT[2], R.J. MOORE[1] and T.T.H. VAN[1]

Summary

This study investigated the ability of chickens to resist and recover from reinfections of *Campylobacter hepaticus*, the causative agent of Spotty Liver Disease (SLD). One hundred and twenty layer chickens were allocated to 10 groups and exposed to varying challenges of *C. hepaticus*. Positive control groups developed typical liver lesions, while negative control groups showed no signs of infection. Interestingly, birds that were reinfected multiple times showed reduced liver lesions, and some groups did not develop lesions despite the presence of *C. hepaticus*, suggesting that repeated exposure enhanced resistance. The study also found a significant increase in spleen-to-body weight ratios following the first infection, indicating a strong immune response. However, the ratios decreased significantly after repeated exposures. The findings suggest promising opportunities for developing vaccines or implementing management strategies to mitigate SLD in poultry.

I. INTRODUCTION

Spotty Liver Disease (SLD) is a significant bacterial disease in poultry. It is characterised by liver damage including multi-focal, 1-2 mm grey-white lesions, reductions in egg production of between 10 and 35% and mortalities of up to 15% in affected flocks (Courtice et al. 2018; Peckham 1958). The move from cages to free range and barn systems has coincided with increasing incidences of the disease, presumed to be at least partly due to the faecal-oral route of infection. SLD is a global concern, and it has been reported in Australia, Costa Rica, Germany, Jordan, New Zealand, UK, and the US in recent years with anecdotal reports from many other countries (Crawshaw et al 2015; Crawshaw et al., 2021; Gregory et al., 2018; Günther et al., 2023; Hananeh et al., 2021; Quesada-Vásquez et al., 2023; Van et al., 2016). In 2016, *C. hepaticus* was confirmed as the cause of the disease and in 2022, *C. bilis*, was also confirmed to cause the disease, although it was less frequently reported (Phung et al., 2022).

Flocks sometimes experience multiple outbreaks of SLD. It is not clear whether the same birds are reinfected or if different birds within the flock become infected. This study aims to determine if infection offers any protection against subsequent infection—specifically, whether only naive birds are susceptible, or if no significant protection is provided by previous exposure, leaving all birds susceptible. The information obtained will provide critical insights for developing effective management strategies, including vaccination protocols and biosecurity measures, to minimize the risk of reinfection in commercial poultry flocks.

II. METHODS

An animal trial was carried out at the Scolexia Animal Research Facility (SCARF, Keilor East). The animal experimentation was approved by the Wildlife and Small Institutions Animal Ethics Committee of the Victorian Department of Economic Development, Jobs, Transport and Resources (approval no. 14.16). A total of 120 commercially cage-reared, 19-week-old Hyline Brown birds were allocated into 10 groups (12 birds per group) for the trial. All birds were confirmed to be free of *C. hepaticus* and *C. bilis* through PCR testing on their cloacal swabs,

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following the method described by Van et al., 2017. Cultures of *C. hepaticus* HV10^T were grown as described in Phung et al. (2021) and used to challenge the birds at various timepoints. Birds were dosed orally 1, 2, or 3 times (with 6 weeks between each challenge) with 1 mL of Brucella broth containing approximately 1 × 10^9 CFU/mL of *C. hepaticus*. Birds were sacrificed six days after infection unless otherwise stated. Group 3 was challenged twice; Group 5 was challenged once and necropsied 7 weeks after the challenge. Group 7 was challenged three times, and Group 9 was challenged twice, with the challenges 12 weeks apart. Groups 2, 4, and 8 served as the negative control groups, while groups 1, 6, and 10 were the positive control groups for the first, second, and third challenges, respectively, with each group being challenged only once. At necropsy, the spleen and body of each bird were weighed, and SLD lesions on the liver were recorded. Bile samples were also collected to assess the recovery of *C. hepaticus* after each exposure timepoint using the method described by Van et al. (2017).

III. RESULTS AND DISCUSSIONS

As expected, 10-12 birds in the positive control groups (Group 1, 6 and 10) presented typical liver lesions, ranging in number from 5 to more than 1000 lesions (Table 1). No liver lesions were observed in the negative control groups (Group 2, 4 and 8). After two reinfections six weeks apart, three birds from Group 3 presented with a few (one to five) liver lesions and nine birds had no liver lesions. Birds challenged three times (Group 7) did not have any liver lesions at necropsy. Similarly, the Group 9 birds, that received two challenge 12 weeks apart, did not have any liver lesions at necropsy. The results imply that the birds developed some level of immunity that prevented the manifestation of liver lesions, following repeated exposure to the pathogen.

C. hepaticus was isolated from all the challenged groups, except for Group 1, where isolation was not attempted. Interestingly, *C. hepaticus* was isolated from bile samples in 10 out of 12 birds 7 weeks after a single exposure (Group 5), although none of the birds displayed liver lesions, presumably having healed. This demonstrates that the birds can recover from infection, probably by an immune response that protects the liver, but obviously does not clear the bacteria from the gall bladder.

Table 1 - Liver lesions observed and the presence of *C. hepaticus* in bile samples.

Group*	G1	G3	G5	G6	G7	G9	G10
Liver spots/number of birds infected	10-500/12	1-5/3	0	5->1000/10	0	0	50->1000/10
C. hepaticus isolated from bile/12 birds per group	Not done	10/12	11/12	8/12	11/12	12/12	11/12

* Groups 2, 4, and 8 were negative control groups—no liver lesions were observed, and *C. hepaticus* was absent in all birds; therefore, they are excluded from the table. Groups 1, 6 and 10 were positive control groups; Group 3 was challenged twice; Group 5 was challenged once, with necropsy conducted 7 weeks post-challenge; Group 7 was challenged three times; and Group 9 was challenged twice, with a 12-week interval between challenges.

The body weight and spleen weight of each bird were measured, and the spleen-to-body weight ratio was calculated to evaluate whether there was an association between total body weight and spleen weight during the reinfection stages. Six days after the first infection, the spleen-to-body weight ratio (P=0.0102) significantly increased compared to the untreated groups (Group 10 vs. control group) (Figure 1). Two birds from Group 10 and three birds from Group 6, the positive control groups had the highest spleen weights and also exhibited the most

liver lesions. A significantly increased spleen-to-body weight ratio was also observed following *Salmonella* infection (Huang et al., 2022). Second and third infections had reduced spleen/body weight ratios.

Twelve weeks between reinfection timepoints (Group 9) resulted in a mean average drop of 0.4% compared to untreated birds (P=0.0003) (Figure 1). After three consecutive reinfections, the mean ratio for Group 7 decreased by 0.3% (P=0.0183). The reduction of the reduced spleen/body weight ratios in the second and third infection group reflects the adaptive immune response and the physiological changes that occur during and after *C. hepaticus* infections.

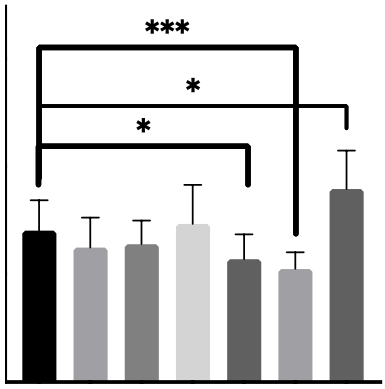

Figure 1 - Spleen to body weight ratios presented as a percentage. Groups are as follows: untreated control group, secondary reinfections (Group 3), a 'long term' single infection (Group 5), two positive controls (Group 6 &10), third reinfections (Group 7) and staggered secondary reinfections (Group 9). Percentages are represented as mean ± SD, n=12 birds per group. The birds were similar in age. Unpaired t-test using Welch's correction was used when P=0.05. Correlation was determined using Pearson with a 95% confidence level.

In conclusion, this study demonstrates the ability of chickens to resist and recover from reinfections of *C. hepaticus*. The results suggest that the recurrence of SLD in flocks may occur because not all birds become infected during the first exposure and therefore remain susceptible to subsequent infections. The findings are important for developing effective management strategies in poultry farming, as they highlight the potential for enhanced disease resilience in flocks. Additionally, these results show promise for the development of a possible vaccine for SLD. Although no immunological analysis is presented here, it is hypothesised that the ability of birds to resist liver lesion formation upon reinfection is driven by immunological mechanisms.

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EFFECT OF YEAST CELL WALL SUPPLEMENT ON THE PERFORMANCE OF BROILER CHICKENS UNDER CHRONIC HEAT STRESS

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Heat stress and Avian pathogenic *Escherichia coli* (APEC) pose significant challenges to the global poultry industry. Heat stress adversely impacts the welfare, productivity, and immune response of broilers and laying hens (Lara and Rostagno, 2013). Additionally, stress is a key predisposing factor for APEC infections in broilers (van Limbergen et al., 2020). Mannan-oligosaccharides (MOS), prebiotics derived from the yeast cell wall of *S. cerevisiae*, enhance the immune response of broilers and reduce pathological lesions caused by *E. coli* infection (Fadl et al., 2020).

The aim of this study was to examine the impact of dietary supplementation with a commercial yeast cell wall product (YCW) on the performance of broilers subjected to chronic heat stress (constant temperature of +32°C) and an APEC challenge. A total of 440 day-old, male Cobb broiler chickens were allotted to four treatments, each treatment group contains 10 replicates with 11 birds per replicate. The birds were fed either control diets (Negative Control (NC) and Challenged Control (CC)) or diets supplemented with YCW at 1000 mg/kg for the starter phase and 500 mg/kg for the grower and finisher phases (Negative YCW (NYCW) and Challenged YCW (CYCW)). On day 14, two groups (CC and CYCW) were exposed to an intestinal APEC challenge. Feed (a three-phase pelleted diet) and drinking water were provided *ad libitum*. Body weight (BW) was recorded weekly for each pen. Cumulative feed intake (FI) was measured for each feeding phase. Average daily gain (ADG) and average daily feed intake (ADFI) were calculated. Data were analysed using a General Linear Model with two fixed factors (diet and challenge) and their interaction (SPSS v24.0).

The APEC challenge significantly reduced growth performance, with BW dropping from 2679 g in the non-challenged group to 2565 g in the challenged group. Additionally, ADFI decreased by 2.7%, leading to a notable increase in the feed conversion ratio by 0.03 units for the challenged birds. Mortality rates were 1.8 times higher in the APEC-challenged birds. The European Efficiency Factor (EEF) was significantly lower in the challenged birds (367.4) compared to the non-challenged ones (401.7). There was a tendency for a significant interaction between diet and challenge for ADFI, with the CC group having a lower ADFI than the other three groups (NC: 95.4 g/day; CC: 90.7 g/day; NYCW: 97.4 g/day; CYCW: 97.0 g/day). The groups receiving YCW had the lower mortality rate than the untreated groups (1.82% combined groups receiving YCW vs. 7.27% for the untreated group). The EEF was significantly improved by 27 units for the YCW birds.

The findings from this study carried out under chronic heat stress conditions indicate that supplementing broiler diets with YCW enhances their performance and survival rates. Additionally, it helps mitigate the adverse effects of APEC challenges on average daily feed intake (ADFI).

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COMPARISON OF LITTER, SUBJECTIVE MOISTURE AND FRIABILITY SCORES TO MEASURED LITTER MOISTURE CONTENT

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Summary

Describing and scoring litter moisture and friability can be achieved using consistent terminology as defined in the *Litter Guide*, which was developed for the Australian chicken meat industry. In this study, we compared the *Litter Guide's* litter scores to litter moisture content (%, determined with a standardised oven-drying method). Demonstrating the relationships between litter scores and moisture content (%) may reduce the need to oven-dry litter samples and enable more regular assessment of litter quality, with the added benefit of assessing litter friability.

I. INTRODUCTION

Moisture content and friability are important properties that are commonly used to describe 'litter conditions' and 'litter quality'. They influence how chickens interact with the litter and affect litter thermal properties; microbiome; odour and ammonia production; the weight and volume of spent litter; in-shed relative humidity, ventilation and heating requirements; chicken comfort, and risks associated with pathogens, contact dermatitis, foot and joint health.

Researchers measure litter moisture content by drying the litter in an oven for about 24 hours until it is completely dry. They then calculate the relative weight of the water compared to the total weight of the litter, reporting this as the percentage moisture content. Poultry growers, on the other hand, do not collect samples of litter from their sheds and determine the oven-dried moisture content. Instead, they assess and describe the litter condition qualitatively based on its appearance, feel, smell and friability.

Meaningful assessment of litter moisture is challenging because conditions vary throughout the shed, at various depths (from the litter surface to the earth/concrete floor) and over time (within each day and over the course of the grow-out). But what measure of litter moisture relates to the risks associated with wet litter? Should the focus be on specific places in the shed, times of the day/batch, how much of the floor is affected and should there be any different approach to assessing friable or caked areas? Is the moisture of small areas of the wettest litter the most important, or is the minimum moisture content of the driest areas? Research papers investigating litter conditions often report the shed-average litter moisture content, for example, 25%, but in reality it would likely have ranged from 15% to 45% at any point in time, and almost certainly changed hour-to-hour and day-to-day. If 25% is regarded as the threshold for wet litter (Collett, 2012), how should growers assess litter moisture and then respond with management actions to minimize potential risks?

Litter friability is another property that is commonly associated with litter quality. It is important because it affects how easily the chickens can 'work' the litter (Lister, 2009). Keeping litter 'working' is important for breaking up and diluting fresh excreta as well as accelerating litter drying by bringing moist litter to the surface where the water is removed by ventilation. Friability tends to be measured qualitatively (using descriptors) on a spectrum from completely friable to completely caked/capped/crusted. Trying to quantify litter friability (with an absolute measurement) is difficult because it would require quantifying a combination of parameters inducing particle size, aggregation, compaction and cohesiveness.

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To provide easy-to-use and consistent descriptors of litter moisture and friability, a litter assessment method was developed by a committee of Australian poultry industry representatives and researchers (AgriFutures Australia and DAF Qld, 2020) and is called the *Litter Guide*. It uses a matrix table (Figure 1) that enables litter to be assessed in terms of moisture (using the descriptive terms ‘dry’, ‘moist’, and ‘wet’) and friability (using the descriptive terms 'friable’, ‘clumping’, and ‘caked’). The unique combination of each moisture and friability description relates to an ‘overall litter score’ from 1 to 5 (Figure 2) that in a general sense relates to: (a) potential risks associated with the litter condition for the chickens; (b) the urgency required for corrective actions; and (c) the likelihood for the litter condition to deteriorate and require corrective action. The overall litter condition score should not be applied directly as an absolute assessment of risk or trigger for action because the quantity and duration of litter being described by the litter score needs to be factored in, as does any potential risk associated with undertaking corrective actions.

The objective of a recent research study (Dunlop and Pepper, 2023) was to determine relationships between litter condition scores (as described in the *Litter Guide*) and gravimetric wet-basis moisture content (%, determined by the oven drying method).

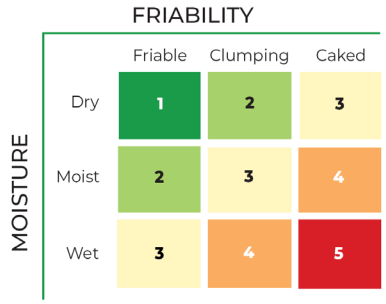

Figure 1 - Litter assessment scoring matrix (AgriFutures Australia and DAF Qld, 2020).

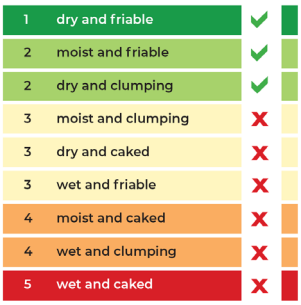

Figure 2 - Litter scores (1–5) and their descriptors. The ✓ or X indicates if corrective action is required.

II. METHOD

Litter samples were assessed on selected occasions at 22 meat chicken farms with chicken age ranging from 14 d to 51 d. No formal experimental design was used, rather, this was an observational study aimed at assessing the range of litter conditions found in commercial meat chicken farms. More specifically, the focus of this activity was to compare the relationships between quantitative, laboratory-based determination of litter moisture content (%) and qualitative, rapid assessments using moisture and friability scores. Potential variability between the assessment methods because of different farms, in-shed sampling location, bedding material types, chicken age, litter reuse or individual assessor’s scoring were not investigated because commercial application of litter scoring would not be able to adjust based on these and many other affecting factors.

Litter was based on pine or hardwood bedding materials and some had been reused for multiple grow-out cycles. On each occasion, litter samples were collected for moisture content analysis, with litter conditions scored in terms of moisture and friability using the *Litter Guide* definitions. Litter was assessed before any kind of mechanical disturbance (e.g. litter tilling or catch-out events). Litter was collected from discrete locations or from four sampling transects (two in the front half of the shed and two in the rear). At the transects, multiple small samples were collected from the surface 1–2 cm of the litter along each transect. The surface litter was collected because chickens directly interact with the surface. These samples were then combined to create a representative sample of the respective area within the shed. For each

transect, additional samples were collected of visibly dry, and visibly damp litter (or from under the drinker lines if there was no visibly damp litter). For each discrete or transect litter sample, moisture content (%) was determined by oven drying the samples to constant weight at 105 °C.

The litter assessment process was performed by two people, with a single score being agreed by consensus for each sampling location. Litter moisture, friability and overall score were compared to the measured moisture content. Data were analysed using Genstat (2022). General linear models were used to assess the differences and relationships between the measured variables, and whether any factors had additional influences. In all models, the different sheds, batch-ages and locations were pooled into the random error term, as there was no interest in compartmentalising these effects.

III. RESULTS

There were highly significant, positively correlated relationships between each of the litter scores and litter moisture content (%) ($P < 0.001$). Litter moisture scores tended to increase with moisture content (%). Transitions from *dry* to *moist* scores occurred at 26–30% moisture content and *moist* to *wet* occurred at 38–40% moisture content (referring to the 25th to 75th percentile range displayed by the boxes in Figure 3). Similar differences occurred for the friability scores, with transitions from *friable* to *clumping* occurring at 27-28% moisture content and *clumping* to *caked* occurring at 38–39% moisture content (Figure 3).

Litter scores tended to increase with moisture content as they transitioned from *dry and friable* (score 1) to *wet and caked* (score 5) (Figure 4). Most litter in this study had a score of 1 or 2, and there were only limited occurrences of scores 3, 4 or 5.

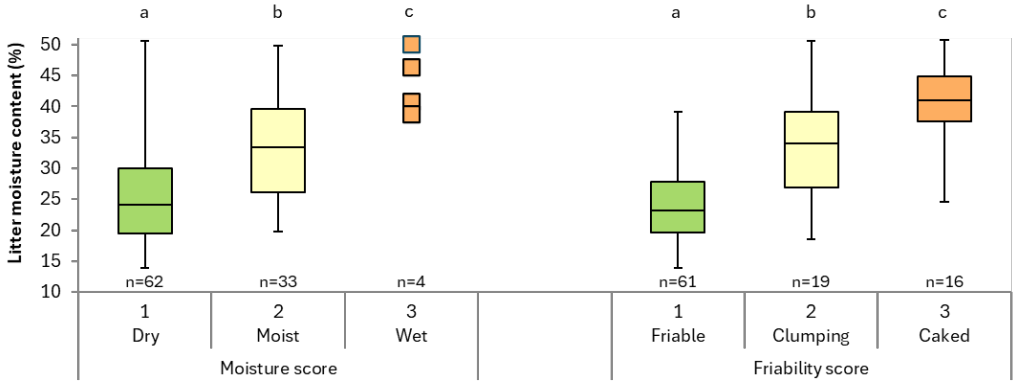

Figure 3 - Boxplot showing moisture content (%) for litter moisture and friability scores. Moisture scores and friability scores with a common letter had means that were not significantly different ($P<0.05$) (Boxes represents the 25th to 75th percentile; the line in the middle of the box is the median value and the whiskers represent the maximum and minimum values. Individual data points were shown if there was insufficient data to produce a box with whiskers).

IV. DISCUSSION

The significant, positively correlated relationships between moisture content (%) and the litter scores described in the *Litter Guide* support the scores to be used to assess litter conditions on commercial farms or research situations. The litter described as '*dry and friable*' (score 1), with some occurrences of '*dry and caked*' or '*moist and friable*' (score 2), is very likely to have moisture content less than 25–35% and therefore considered to be 'good litter' (Lister, 2009) and not require urgent corrective actions.

Based on our observations, the low occurrence of litter with scores 3, 4 or 5 was due to growers actively managing litter to keep it dry and friable. Scores 2, 3 and 4 showed wide ranges of moisture content from approximately 18% to 50%, which was not surprising given

that they included multiple litter combinations from '*dry*' through to '*wet*' and each of the friability scores. For describing the general risks, management requirements and likely persistence of litter conditions, we suggest that the 1 to 5 score is probably sufficient; however, in research settings, there would be benefits to recording the individual moisture and friability scores, or assigning a unique score for each litter combination in the *Litter Guide* matrix (Figure 1), for example, assigning score 1 to '*dry and friable*' up to score 9 for '*wet and caked*'. We suggest that in research trials, moisture content (%) should still be the primary measure of litter moisture, but moisture and friability scoring should be used to record additional information about temporal, daily or spatial variability of litter conditions that may be influential in health, welfare, performance or microbial outcomes being measured.

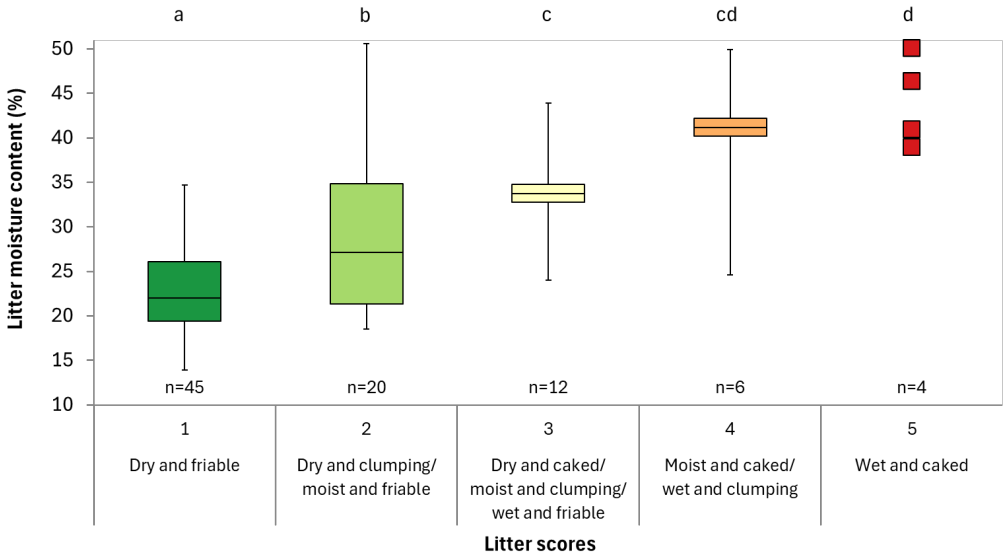

Figure 4 - Boxplot showing moisture content (%) for litter score. Litter scores with a common letter had means that were not significantly different (P<0.05) (see note in Figure 3 for interpreting boxplots).

While this study focused on comparing the relationship between litter moisture content (%) and scores for litter moisture, friability and overall condition, future studies should focus on relating litter scores to measurable health, welfare and production outcomes.

ACKNOWLEDGEMENTS: We thank the AgriFutures Australia Chicken Meat Program and the Department of Agriculture and Fisheries, Queensland Government for funding this research (PRJ-011502) and supporting the production of the *Litter Guide.* We also thank the people who contributed to developing the litter guide and the growers and integrators who contributed to this research.

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MECHANICAL HARVESTING OF BROILERS DOES NOT INCREASE PRE-SLAUGHTER MORTALITY OF MEAT CHICKENS COMPARED TO MANUAL CATCHING

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Catching and transporting meat chickens must done correctly to ensure the catching conditions are optimised to reduce stress on the birds and people involved. Mechanical harvesters have the potential to improve work conditions for catchers and reduce the costs of catching (Monch et al., 2020). However, there are concerns that mechanical catching may increase stress levels in meat chickens, culminating in an increase in pre-slaughter mortality (Jacobs et al., 2017; Mönch et al., 2020). Therefore, this experiment aimed to compare pre-slaughter mortality, expressed as "dead on arrival" (DOA) after transport to the processing house between conventional manual catching and machine harvesting under Australian commercial conditions. This project did not require animal ethics approval as the data was collected as part of normal commercial practices and analysed retrospectively. Commercial flocks (free-range n = 732; conventional n = 13,933) of Ross308 meat chickens (n = 68,539,177) were transported from commercial broiler farms (n = 69) to one of two processing plants in Victoria over 15 months (January 2023 – March 2024). Data were recorded by industry personnel for each flock that was transported including the catching method (manual n = 7070; mechanical = 6863), duration of catching, loading and transportation, environmental conditions, age of the birds, average body weight, time off feed, and the number of DOA. Climatic conditions at the farm and lairage, were sourced from the Bureau of Meteorology including average daily temperature, rainfall and relative humidity. Transport temperatures ranged between -1.7 °C to 41.1 °C. Data were analysed using RStudio. Risk factors (n = 28) were included in a random forest analysis with 500 trees to determine variables that likely impact DOA. At each split, five variables were tested from a pool of randomly selected candidates, with an mtry value of 6 (i.e., six variables were randomly selected as candidates at each split). The random forest model explained 41% of the variance in DOA. A forward feature selection indicated that 11 variables contributed to the model prediction: average body weight, duration on truck, lairage duration, duration catching to kill, duration feed withdrawn, average daily rain, average daily temperature (maximum and minimum), average daily relative humidity (maximum and minimum), as well as the cumulative number of pickups per crew, cumulative number of birds caught per catching crew and the cumulative average weight of birds caught per crew. A subsequent General Linear Model, on cube-transformed DOA was performed. DOA was not related to the catching method ($P > 0.05$). The factors that were found to contribute to DOA were increased average body weight, duration on truck, duration from catching to kill, lairage duration and minimum average daily temperature ($P < 0.05$). While significant effects were identified as risk factors for DOA, average DOA was low (0.32% ± SEM) and below company QA welfare thresholds. While the low rate of DOA reflects effective practices, we identified variables for further investigation for continual improvements to chicken welfare. These findings suggest that efforts should focus on identifying areas where changes to current practices could have the greatest impact to further reduce mortality and enhance welfare and economic outcomes.

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FEATHER-DIRECTED BEHAVIOURS ARE CONTEXT-SPECIFIC AND LIKELY HAVE VARYING IMPACTS ON MEAT CHICKEN BREEDER WELFARE

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Feather licking (FL) has been reported in meat chicken breeder flocks and has been proposed to be a redirected foraging behaviour that may lead to feather pecking (FP). However, FL is underreported in the parent stock literature and a thorough description and aetiology is lacking. A useful first step in understanding the implications of feather-directed behaviours (FDB) for bird welfare and the breeder industry is to develop a working definition. We aimed to develop context-specific definitions of FL from behavioural observations of meat chicken breeders in commercial conditions. Specifically, we aimed to develop a discrete description of FL that differed from preening, feather pecking, feather pulling and feather eating. This project was approved by the University of Melbourne Animal Ethics Committee (#27336-41404-2). Bird behaviour was recorded in eight commercial sheds: four production (6,000 – 10,000 birds/shed) and four rearing (2 cockerel and 2 pullet (6,000 – 10,000 birds/shed) sheds across two states (Victoria and South Australia). Stocking density was maintained below 29.5 kg/m² under Australian animal welfare legislative requirements. Two video cameras were mounted on each side of each shed. Weekly scan samples were collected in both rearing (4 – 18 weeks of age) and production (20 – 40 weeks of age) at four time points; 15 minutes before feeding, 1 hour after feeding, 5 hours after feeding and 15 minutes before lights were turned off. Focal sampling was conducted at time points when FL was previously observed during scan sampling (n = 17 time points; 4 focal birds per time point; 5.6 hours of observations) in production sheds and rearing (cockerels only). Focal sampling monitored the behaviour of the first five birds to be observed feather licking or feather pecking for 5 continuous minutes.

In rearing birds, FL was a rapid, repetitive pecking-type behaviour, and nearly half of the behaviours preceding FL in rearing were active (locomotion = 42.9%). In production birds, FL was gentle with short infrequent bouts (9.9 - 11.5 s) and most (57.7%) of the behaviours that preceded FL were inactive (sitting = 34.6%; standing = 23.1%), 15.4% were preceded by preening. Over 95% of initiators and recipients were female, which may reflect a sampling bias with a high female-to-male ratio (10:1) in the production flock. FL was rarely observed in the cockerel-rearing flock on Farm 1, but FP was seen every week (n = 88 occurrences overall) with most focal birds (80 - 100% birds) displaying the behaviour at least once. FL was seen frequently during cockerel rearing on Farm 2, with bout lengths increasing from an average of 2.4 s in week one to 45.1 ± 17.5 s in week 15. FL bouts ranged between 0 - 11 times per five-minute observation period, and each lasted an average of 11.5 ± 1.8 s. FL predominately targeted the tip of the tail (53.8 – 91.4%). Conversely, feather pecking (FP) typically targeted the back (7.1 - 34.5 %) and wings (6.7 – 34.5%). Recipients did not move away in response to FL, but approximately 20% of the time, the recipient stopped their behaviour after being licked. We provide two working definitions for FL: gentle FL in production and repetitive gentle FL in rearing. Both behaviours differed from FP.

We developed these definitions based on context and the repetitive nature of the behaviours. Providing clarity around these FDBs will provide better insight into the causation, implications and potential prevention of such behaviours in the future. Further research is required to understand the welfare implications of FL for both initiators and recipients.

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AVIAN INFLUENZA IN AUSTRALIA – PAST, PRESENT AND FUTURE

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Summary

Australia experienced its largest recorded high pathogenicity avian influenza (HPAI) outbreak due to H7N3 virus in May-June 2024. The outbreak affected free-ranged and housed egg production across multiple premises of a large commercial chicken layer farm located in Meredith, Victoria. During the outbreak investigations, HPAI due to a different virus of H7N9 subtype was also detected in a separate but commercially linked premise in Terang, Victoria. Around the same time in June 2024, H7N8 HPAI was detected in a mixed free-ranged and housed chicken layer farm in the Hawkesbury region in New South Wales (NSW). This H7N8 virus strain also affected an epidemiologically linked free-ranged meat chicken farm in NSW as well as a barn production layer farm in the Australian Capital Territory (ACT). Several non-commercial premises in NSW and one in the ACT located within the control areas were also impacted. These HPAI outbreaks led to the destruction of approximately two million birds. Epidemiological and virus phylogenetic investigations determined that three HPAI virus strains, A(H7N3) and A(H7N9) in Victoria and A(H7N8) in NSW/ACT, were each closely but independently related to Australian lineage H7 low pathogenicity avian influenza (LPAI) viruses previously detected wild bird samples in Australia. The apparent occurrence of independent spillovers of H7 LPAI viruses from wild birds into commercial poultry leading to the concurrent emergence of three different H7 HPAI variants is unprecedented in Australia. This paper describes the relationship between H7 LPAI and HPAI viruses in wild birds and Australian poultry. The epidemiological picture of H5 LPAI viruses is also presented to highlight the different AIV dynamics observed at the wild bird and poultry interface in Australia. Australia and Oceania remain the only continental region that has not been impacted by the ongoing Eurasian-lineage H5N1 HPAI panzootic, with concerns of potentially devastating impacts to both locally farmed poultry and unique wildlife populations if introduced. The key factors leading to a perceived elevated risk for the potential introduction of an exotic H5 HPAI virus lineage into Australia is discussed.

I. INTRODUCTION

From 1976 to 2020, eight HPAI outbreaks in commercial chicken flocks have been reported in Australia, all due to H7 subtype viruses (Table 1). In 2024, three further H7 HPAI outbreaks have occurred, two events in Victoria and one in New South Wales/Australian Capital Territory. In contrast, subtype H5 HPAI has never been reported in Australia, although multiple low pathogenicity AIV detections of various subtypes have been recorded in different states through opportunistic or passive surveillance (Scott et al., 2020).

Wild water birds, in particular waterfowl species, are the natural reservoir and transmission hosts of LPAI viruses. The birds most associated with the distribution of AIV belong to the orders Anseriformes that includes the waterfowl, and Charadriiformes that includes waders and gulls. Australia and the broader Oceania region serve as a sink for AIV genetic diversity (Wille et al., 2022). In Australia, AIV across 14 detected haemagglutinin (HA) and nine neuraminidase subtypes originate from incursions that occur infrequently from the greater Eurasian and North American virus gene pools. The ecological dynamics of AIV in Australia are strongly influenced by its endemic regional waterfowl species. Australia lies outside the typical flyways of long-distance migratory waterfowl from the northern

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hemisphere. Instead, waterfowl species in Australia are nomadic with a range distribution limited to the Australo-Papuan region and with non-structured movements aligned with water availability within this range (McCallum et al., 2008). Although waterfowl plays an important role in sustaining the AIV reservoir within Australia especially for certain subtypes, the long-distance migratory shorebirds and waders of the order Charadriiformes are likely the main source of introduction of new AIV diversity via the East Asian-Australasian flyway (Wille et al., 2022). Once in Australia, AIVs circulate in wild birds as isolated Australian lineages that can persist for many decades, or they are replaced by new introductions.

Table 1 – High pathogenicity avian influenza outbreaks in Australian poultry from 1976 to 2024 associated with subtype H7 viruses[a].

Date (mm/yyyy)	Virus subtype	Location[b]	Number affected farms	Production type
01/1976	H7N7	Keysborough, Victoria	2	Mixed meat and caged layer chicken farms.
05/1985	H7N7	Bendigo, Victoria	1	Mixed layer, meat and breeder chicken farm.
07/1992	H7N3	Bendigo, Victoria	2	Meat chicken farm and sero-positive duck farm.
12/1994	H7N3	Lowood, Queensland	1	Layer chicken farm.
11/1997	H7N4	Tamworth, NSW	3	Meat chicken farms and emu farm.
11/2012	H7N7	Maitland, NSW	1	Semi-free range layer chicken farm.
10/2013	H7N2	Young, NSW	2	Mixed free range and caged layer chicken farms.
07/2020	H7N7	Lethbridge, Victoria	3	Free range layer chicken farms, and mixed free range, housed and caged layer chicken farm.
05/2024	H7N3	Meredith, Victoria	7	Mix of free range, caged and housed layer chicken farms, and duck farm.
05/2024	H7N9	Terang, Victoria	1	Mixed free range and caged layer chicken farm.
06/2024	H7N8	Western Sydney, NSW and ACT	3	Mixed free range and housed layer chicken farm, free range meat chicken farm (NSW) and housed layer chicken farm (ACT).

[a]Modified from (Scott et al., 2020).
[b]NSW, New South Wales; ACT, Australian Capital Territory.

II. AVIAN INFLUENZA A(H7) IN AUSTRALIA

All 11 H7 HPAI outbreaks reported in Australian poultry to date are associated with a single H7 virus lineage that has been present in Australia since the mid-1970's (Australian Government Department of Agriculture, Fisheries and Forestry, 2024; Wille et al., 2022). H7 LPAI virus detections from wild bird samples from 2007 to the present indicate that the same H7-HA lineage has had a continuous circulation, along with linear genetic evolution, in Australia for at least 50 years. This has been accompanied by sporadic virus spillovers to commercial poultry and emergence of HPAI variants that have caused independent HPAI outbreaks in poultry farms in at least three Australian states, including three separate outbreaks from May-June 2024 (Table 1). In each reported outbreak, the HPAI virus was contained and limited to the affected poultry flocks, with no further detection following effective response and resolution of the outbreaks. Highly pathogenic H7 virus has not been detected in wild birds in Australia, with the exception of an isolated positive common starling (*Sterna vulgaris*)

passively sampled within an HPAI infected poultry shed during the 1985 H7N7 outbreak in Victoria (Nestorowicz et al., 1987), believed to be contaminated from infected poultry or the outbreak environment rather than the source of infection (Scott et al., 2020). However, LPAI H7 viruses continue to be detected in Australian wild waterfowl and wild bird environmental samples through the national wild bird AIV surveillance (Grillo et al., 2015; Wille et al., 2022).

III. AVIAN INFLUENZA A(H5) IN AUSTRALIA

High pathogenicity avian influenza associated with H5 subtype AIV has never been reported in Australia. Like H7 LPAI viruses, H5 LPAI viruses are sustained in wild water birds in Australia as a distinct localised lineage, detected since 2007 in the case of H5 (Hansbro et al., 2010; Wille et al., 2022). In this time, there has been occasional evidence of H5 LPAI virus in Australian poultry including A(H5N3) detected in free-range farmed ducks in Victoria in 2012 and in backyard ducks and chickens in Western Australia in 2013 (Scott et al., 2020). More recently in 2020, LPAI A(H5N2) was also detected in farmed turkeys in Victoria (Agriculture Victoria, 2024). Analysis of available virus sequences shows that these are related to the same Australian H5 lineage. Due to indistinct clinical expression or co-morbidities, it is unclear whether these H5 LPAI detections were directly associated with disease. However, HPAI virus has never emerged from this lineage.

Interestingly from April 2023, LPAI H5 detections in wild bird samples from different Australian state and territory jurisdictions have been characterised as belonging to a novel incursion of Eurasian lineage H5 into the continent (Wille et al., 2024). This recently introduced H5 lineage has reassorted with other local AIV gene segments and appears to have competitively excluded the previous Australian H5 lineage, which has not since been further detected. There is concern that this currently circulating H5 LPAI virus lineage may have greater potential for mutation into high pathogenicity following transmission in poultry, although it has not been detected in local domestic birds to date.

IV. CONCLUSIONS

At the time of writing Australia and Oceania remain the only continental region where the panzootic Eurasian clade 2.3.4.4b H5N1 HPAI has not occurred (Food and Agriculture Organization of the United Nations, 2024). The change in epidemiology and ecology of this HPAI H5 virus lineage to a sustained reservoir with rapid widespread dissemination in wild birds, along with the greater potential impacts to local wild and domestic bird populations if introduced, have led to an elevation of risk for introduction to Australia (Wildlife Health Australia, 2023). Although Australia has remained resilient to HPAI virus introduction from the northern hemisphere via migratory waterfowl, regional nomadic bridging species and seabirds could present alternative fronts of incursion of clade 2.3.4.4b H5N1 viruses. The presence of this HPAI H5N1 lineage in neighbouring Southeast Asian countries (Wibawa et al., 2024), and its detection in wild birds and marine mammals in Antarctica and its territories increase the potential risks to Australia (Scientific Committee on Antarctic Research, 2024). In recognition of these risks, the Australian Government has recently announced the investment of approximately $100 million into enhancing national preparedness and response capability (Australian Government Department of Agriculture, Fisheries and Forestry, 2024). In addition to the threat of exotic HPAI, H7 AIV with potential to become HPAI in poultry continues to circulate endemically in local waterfowl populations. The introduction and subsequent widespread distribution of a novel Eurasian lineage LPAI H5 also pose an unknown but potential threat to Australian poultry, possessing a low pathogenicity HA cleavage site motif recognised as a possible precursor to H5 HPAI viruses (Wille, Grillo et al., 2024). This LPAI H5 and the recent detection of a novel North American lineage H10 subtype AIV into Australia

(Wille, Broz et al., 2024), together with the heightened risks posed by panzootic H5N1 HPAI, highlight the importance of an ongoing and strengthened national AIV wild bird surveillance program in contributing to avian influenza vigilance and preparedness.

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IDENTIFICATION OF MEAT QUALITY AND FLAVOUR SUBSTANCES IN KOREAN WOORIMATDAG NO. 2 CHICKEN BREAST MEAT DURING COLD STORAGE

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As global demand for high-quality chicken meat increases, consumers are becoming more discerning in their choice of chicken. In Korea, Korean Native Chicken (KNC) is valued for its superior taste and texture and is traditionally consumed on special occasions. To date, the KNC meat market has been led by several selectively bred lines, such as the Hanhyup and Woorimatdag breeds. These are the result of decades of targeted breeding efforts by the Korean government and companies, focusing on commercial traits such as growth efficiency and sensory quality. In 2012, the Korean government initiated the "Golden Seed Project" to identify genetic resources and promote sustainable KNC production while preserving genetic diversity (Jin et al., 2017). Accordingly, the National Institute of Animal Science in Korea developed the Woorimatdag No. 1 breed, a product of three-way cross breeds between native and adapted KNCs, with the aim of improving taste quality and growth performance (Kim et al., 2012). Although this breed is rich in flavor and bioactive compounds, it is still less marketable due to low growth performance. To overcome this, Woorimatdag No. 2 (WRMD2) was developed by crossbreeding Brown KNC/Rhode Island Red females with Black Cornish/Brown Cornish males. This breeding strategy reduced the rearing time while maintaining taste and nutritional value of KNCs (Kim et al., 2012). However, an in-depth study of meat quality and flavor characteristics of WRMD2 has not been sufficiently conducted.

Therefore, the aim of this study was to identify the quality and flavor compounds of breast meat from market-sized WRMD2 (n=72, 12 weeks old) and commercial broiler (CB) (n=72, 6 weeks old) and their relationship during up to 7 days of aerobic cold storage. After slaughter, breast meat was separated, then directly packaged using polystyrene trays and low-density polyethylene film and stored at 4℃ for 7 days. For each storage day (day 1, 3, 5, and 7), 18 samples were randomly collected and subjected to further analysis. The pH and drip loss increased during storage, and WRMD2 had a significantly lower pH and higher drip loss than CB. Creatine, anserine, and carnosine levels gradually decreased during storage. WRMD2 showed a significantly higher anserine content and a lower carnosine-to-anserine ratio than CB. Taste related compounds, inosine monophosphate (IMP) and guanosine monophosphate (GMP), levels decreased during storage. The IMP and GMP content were significantly higher in CB than in WRMD2 on day 1 and days 1-3, respectively. WRMD2 had significantly higher levels of polyunsaturated fatty acids, especially C20:4n6 and C22:6n3, than that of CB. Multivariate analysis identified several volatile compounds, including methyl salicylate on day 1, dodecanal on day 3, naphthalene on day 5, and 2,4-decadienal on day 7, as potential indicators to discriminate between WRMD2 and CB on each day of storage. Correlation analysis revealed five key meat quality traits, including drip loss, aerobic plate count, and anserine, which are strongly related to taste and aroma compounds such as IMP, GMP, 1-octen-3-ol, and hexanal.

Our findings contribute to a comprehensive understanding of the distinction of meat quality and flavor compounds between WRMD2 and CB, which may help improve the meat quality and commercial scope of native chicken.

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EFFECTS OF A LYSOLECITHIN PRODUCT ON THE PEFORMANCE, EGG PRODUCTION RATE AND EGG QUALITY OF END-OF-LAY HENS UNDER HEAT STRESS

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Heat stress (HS) has been a major challenge for poultry production especially in laying hens aged over 48 weeks. Exposure to elevated temperatures (>28 ℃) and humidity (>80%) can significantly increase layers' panting times and disrupt their metabolic and hormonal processes (Teyssier et al., 2022). In the meantime, feed digestion, nutrient absorption and deposition are also negatively affected. For instance, secretions of endogenous enzymes, such as lipases and proteases, and bio-emulsifier as bile salt are greatly reduced under HS conditions (Kim et al., 2024). The expression level of calcium transporter protein (calbindin) in both the small intestine and oviduct is significantly declined in layers under HS (Kim et al., 2024). The physiological challenges imposed by heat stress will negatively affect feed conversion ratio (FCR) and reduce body weight. At last, HS also disrupts nutrient deposition in eggs and egg formation processes, including the deposition of minerals, amino acids and lipids, etc. Together with the impaired overall health conditions under HS, egg production rate decreases and mortality rate increases, leading to an increased incidence of egg quality issues, such as dirty, cracked or broken eggs (Teyssier et al., 2022 and Kim et al., 2024). Improving nutrient digestion, especially fat utilisation in layers may offset many negative impacts in old layers under HS, because it could help increase available energy and nutrients for egg production, and gut health by reducing fermentation of undigested nutrients in the hindgut. Therefore, the application of bio-emulsifiers is getting popular and has proven to be effective in layer production (He et al., 2023). In the current study, the effect of a lysolecithin product with a high lysophospholipids (LPL) content (FRA® LeciMax Dry, Adisseo NL B.V., Netherlands) on laying hen (Lohmann Brown) performance, egg production rate, egg quality and mortality under heat stress conditions was conducted on a commercial farm in Chengdu, China. The trial was carried out in summertime with the average temperature ranging from 28 to 36 ℃ and average humidity higher than 82%. A total of 57,774 laying hens (59-66 weeks) were allocated to 2 treatments including a control group that received a commercial layer diet with 6% crude fat, and a treatment group that received the same commercial diet with 500ppm LPL added over the top. During 7-wk trial period, feed and water were provided *ad libitum*. Feed intake, FCR, egg production rate, number of broken eggs, egg weight and mortality were determined per day. At 66 weeks of age, the treatment group showed a lower feed intake (-2.0%, $P<0.05$) and FCR (-12.0 points, $P<0.05$), and a significantly lower number of broken eggs (-19.7%, $P<0.05$). Numerical improvements in egg weight and laying rate were observed as well. But there was no difference in mortality rates between the two groups. In conclusion, using an emulsifier based on lysolecithin can improve laying hen performance, egg production rate and egg quality under HS conditions.

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FLOOR EGGS IN AUSTRALIAN FLOCKS OF CAGE-FREE BROWN EGG-LAYING HENS

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Summary

During recent years cage-free egg production systems have increased in numbers throughout Australia, and currently dominate Australian egg sales. However, with increasing consumer demand for protein, cage-free egg farming faces the challenge of meeting the increasing demand for food. Mislaid or floor eggs (FE), which are laid outside of the designated nest boxes, may limit the potential to increase productivity and are a challenge for cage-free egg farmers. This scoping survey study, which included 39 flocks, was designed to explore factors that influence FE prevalence in cage-free egg systems within Australia. The percentage of FE ranged from 0.01% to 17%. There was a notable increase in labour costs for flocks with higher levels of FE ($p = 0.04$). Additionally, flocks in sheds which utilised tunnel ventilation had significantly lower FE prevalence compared to sheds that used other forms of ventilation ($p = 0.0127$). There was a negative correlation between flock size and number of FE and, the farmer's acceptable level of FE ($r = -0.4993$, $p = 0.001$; $r = -0.4870$, $p = 0.001$ respectively). This suggests that flock size plays an influential role in FE prevalence. Additionally, flocks experiencing higher FE values can expect it will affect labour related costs. This study emphasizes the variability of FE laying, which is affected by various factors related to the design and management of cage-free systems.

I.INTRODUCTION

The production of fresh table eggs plays a crucial role in meeting the global demand for food. The Australian egg industry is shifting towards cage-free systems, including free range and barn laid systems, which accounted for 71.7% of egg sale volume in 2023 (Australian Eggs, 2023). Traditionally, caged systems can achieve a more efficient use of resources per unit of production (Summer, 2011). Therefore, egg production in cage-free systems raises challenges for productivity and food safety compared to traditional caged systems (Summer, 2011). Floor eggs are also a major challenge for cage-free systems. They can represent a significant loss of up to 10% of total daily egg production. They also require intensive labour for staff to encourage the movement of hens towards the nesting boxes as well as any floor egg collection (Bist et al., 2023; Brannan & Anderson, 2021; Vroegindewwij et al., 2018).

Environmental factors within sheds, such as ventilation and temperature control, can influence laying behavior and egg production. Under stressful environmental conditions (for example hot or poorly ventilated sheds), hens avoided upper levels of the shed; concurrently with a higher incidence of eggs laid on the floor areas (Biswal et al., 2022). Furthermore, small egg producers face financial constraints that limit their ability to invest in advanced monitoring and management practices, potentially exacerbating FE issues when compared to larger operations (Dhillon & Moncur, 2023; Rada & Fuglie, 2019).

Recent findings on FE in Australian flocks (Ciarelli et al., 2024) were opportunistic evaluations and not drawn from studies specifically designed to evaluate FE. Therefore, purpose designed studies to explore possible relationships between FE and features of the cage-

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free systems, including breed-specific behaviours, environmental stressors, and management practices are required. By improving our understanding of factors that contribute to the incidence of FE targeted solutions for the minimization of FE can be implemented to optimise egg production efficiency while meeting evolving consumer and regulatory expectations. Hence a survey was designed to capture a snapshot of the current demographics of cage-free egg production in Australia. The incidence of FE together with flock size, housing system, ventilation system and the impact of FE on on-farm labour costs was ascertained.

II. METHOD

Initially mediated through Australian Eggs, a not-for-profit company providing marketing and research & development (R&D) services for Australian egg farmers each participant received an information statement about the study, an outline of the survey questions and a consent form. Once consent was received the farmer was contacted and completed a short 16 question phone-based survey that established features of the farm system and shed design, flock demographics (i.e. breed, age, size), floor egg prevalence at peak lay and flock health status.

Survey responses were entered into REDCap, a secure web application for building and managing online surveys and databases. Each farm and flock had a unique identifier. Data were separated by flock, i.e. where a farm had multiple flocks, a separate survey was completed for each flock. Farms were not identifiable in the output and the original data is encrypted and stored securely in REDCap. The survey responses were tabulated automatically using REDCap 'Data Export' function. T-tests, correlation and regression equations were generated using SPSS. The data are presented as mean values ± standard error of means. Statistical significance is set at $p < 0.05$.

II.RESULTS

This study encapsulated data from 39 flocks within Australia. Their locations included New South Wales (n=29), Queensland (n=5), Tasmania (n=2) and Western Australia (n=3). Among these 39 flocks the majority identified as a free-range system (n=31) followed by cage-free (n=2) and pasture (n=2). The production system was not identified for 4 flocks. There were 3 hen breeds being Hy-Line Brown (n= 15), Lohmann Brown (n=5) and ISA Brown (n=19). There was no significant difference between FE prevalence (%) for the three breeds (p= 0.49) (Table 1). Flock size varied, ranging from 200 to 33300 hens.

The percentage of floor eggs at peak lay ranged between 0.01-17%, with a mean of 3.53% and median 2.49%. The level of floor eggs at peak lay that the farmer identified as being acceptable ranged from 0.20-10%, with a mean of 4.48% and median 2.00%.

Across the 39 flocks, 9 (23%) experienced an increase in labour costs due to the level of floor eggs, with no effect on labour costs in the remaining 30 flocks ($p = 0.04$). The average incidence of FE in the former was 5.95%, and 2.81% in the latter.

When flock size was broken into quartiles (Q) from smallest to largest, the occurrence of FE at peak lay was; Q1= 7.20%, Q2=3.77, Q3=1.70 and Q4=1.26%, illustrating a negative correlation of FE with flock size ($y = 6.1268-0.0002*x$; 0.95 confidence interval, $r = -.50$, $p = 0.001$) (Table 1). That is, as FE at peak lay increased, flock size decreased. Similarly, the level of FE at peak lay considered to be acceptable by the farmer had a negative correlation with flock size ($y = 18850.8718-2261.5721*x$; 0.95 confidence interval, $r = -0.49$, $p = 0.001$). That is, the acceptable level of FE at peak lay increased as flock size decreased.

The type of shed ventilation impacted the level of FE. Specifically, flocks in sheds which were ventilated tunnel (mechanical) had significantly lower FE prevalence compared to sheds that were ventilated by other mechanisms ($p = 0.0127$) (Table 1).

Table 1 - Floor egg prevalence in flocks housed in sheds with or without tunnel ventilation, flock size between quartiles and hen breeds.

Factor	Mean FE	Standard deviation	N	P-value
Tunnel Ventilation (no)	4.86[a]	4.72	23	
Tunnel Ventilation (yes)	1.64[b]	1.55	16	0.013
Flock size (Q1, 200-3000)	7.20[A]	6.06	10	
Flock size (Q2, 5000-10000)	3.77[AB]	2.19	10	
Flock size (Q3, 10000-20000)	1.70[B]	1.61	10	
Flock size (Q4, 24100-33300)	1.26[B]	1.24	9	0.0018
Hy-Line Brown	4.37	4.98	15	
Lohmann Brown	4.09	7.28	5	
ISA Brown	2.73	1.52	19	0.490

[ab and AB] rows with different superscripts are different at $p<0.05$. *N* = number of flocks.

IV. DISCUSSION

Consistent with other research this study found the proportion of FE from cage-free egg-production systems to vary significantly between 0.01-17%. Earlier scientific evidence from Dorminey et al. (1970) reported large variation in FE of the same flock, ranging from 3.5 up to 22.9%. Hence, to maintain consistency between flocks the level of FE at peak lay was used in this survey. The variability in the levels of FE is likely due to multifaceted factors including the design and management of a cage-free system.

As flock size increased, FE prevalence and the level of FE that was acceptable to the farmer also decreased. For the flocks involved in this survey, the larger flocks had lower incidence of FE ($p=0.005$). Smaller enterprises, in contrast, may face challenges in managing FE due to more limited finances for investment in data collection, technology and research (Oliveria et al., 2022). This can also result in less stringent monitoring and fewer interventions for the minimization of FE (Blasch et al., 2022; Mizik, 2022). Overall, adaptability, research, and technology play crucial roles in egg production efficiency, with larger farms benefiting from better resources and more rigorous data collection practices.

It is not surprising that the farming operations that reported an increase in labour costs to address FE also reported higher levels of FE than those that did not experience an increase in costs due to FE. Other research supports this notion as FE must to be collected manually, which is labour intensive and time consuming, creating a financial burden for the business (Chai et al., 2023). Additionally, collecting eggs can account for up to 37% of the work of a farm hand (Matthews & Sumner, 2015). Oliveira et al. (2019) indicated that 5% FE is not uncommon in a cage-free system, while others report 10% (Chai et al., 2023), or as high as 28% (Ciarelli et al., 2024). Therefore, FE are a cost to the farming operation, in both direct costs and lost product.

Flocks housed in sheds with mechanical tunnel ventilation produced less FE. Tunnel ventilation maintains a lower temperature during hotter ambient climates compared to naturally ventilated sheds (Silva et al., 2013), and the airflow facilitates convective heat loss from the surface of the bird's body (Tong et al., 2019). Without appropriate ventilation, the presence of heat stress has detrimental consequences on a bird's productive efficiency, health and welfare (Biswal et al., 2022). Under conditions of heat stress birds will prioritise biological functioning, and thermoregulation to reduce their core body temperature (Farag & Alagawany, 2018), spending less time walking and using enrichments (i.e. perches and ramps) and more time drinking and resting (Biswal et al., 2022). This can increase the likelihood of FE as birds utilise the floor areas and avoid more elevated areas including the nesting boxes.

This survey is the first phase of a larger study designed to identify solutions for the mitigation of FE in cage-free egg production systems. A subsequent, more in-depth survey of these flocks is currently being undertaken, with results to follow.

ACKNOWLEDGEMENT: We thank Australian Eggs for funding this project and the egg farmers that participated in the survey.

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EFFECT OF DIETARY ELECTROLYTE BALANCES ON PERFORMANCE AND EGG QUALITY IN LAYING HENS

K.J. LEE[1,2], T.X. LI[1,2] and I.H. KIM[1,2]

In birds, acid–base balance is affected by some factors, such as environmental condition, diet and metabolism, which alter the regulation of pH in the blood and tissues (Adekunmisi and Robbins, 2009). The monovalent ions Na+, K+ and Cl− play a crucial role in maintaining chicken's acid–base homeostasis. Previously, Gezen et al. (2005) reported that excessive dietary Cl^- (by NH4Cl supplementation) reduces blood pH and $HCO3^-$ concentrations and cause decreased of eggshell quality. DEB value ranging from 220 to 260 mEq/kg (Koreleski et al. 2011) has been shown to influence the productive performance in poultry. In contrast, Senkoylu et al. (2005) reported that increasing DEB from 176 to 242 mEq/kg did not improve the egg laying performance of laying hens from 22 to 32 weeks of age. Based on the above literatures, we hypothesized that 180 to 220 mEq/kg DEB ranges might be benefit for enhancing laying hen's performance.

A total of 360 Hy-Line Brown laying hens, aged 28 weeks were used to examine the effect of dietary electrolyte balances on performance and egg quality in laying hens. The hens were randomly assigned to three dietary treatment groups, with 10 replicates per group and 12 birds per replicate. During the 12-week trial, the DEB in the control (CON) group was maintained at 180 mEq/kg, while the experimental groups were adjusted to 200 mEq/kg (Group 1) and 220 mEq/kg (Group 2) using sodium bicarbonate. Birds had *ad libitum* access to water and diet. Hen day egg production, shell quality (thickness, strength, cracked egg ratio), and egg weight were determined. Data were analyzed using the general linear model in SAS and orthogonal polynomial contrasts were tested and the $P<0.05$ was set as statistically significance.

Table 1 - Effect of DEB on laying hens egg quality.

Items	CON	Group1	Group 2	SEM	P-value	
					Linear	Quadratic
Week 4						
Egg weight, g	62.7	63.3	64.0	1.0	0.037	0.125
Eggshell Strength, kg/cm^2	4.17	4.21	4.42	0.06	0.003	0.236
Eggshell Thickness, mm^{-2}	37.5	38.9	40.0	0.9	0.044	0.924
Week 8						
Eggshell Strength, kg/cm^2	4.12	4.23	4.40	0.9	0.005	0.747
Eggshell Thickness, mm^{-2}	37.4	38.7	40.0	0.9	0.034	0.885

Compared to CON and group 1, group 2 birds showed linearly increased ($P < 0.05$) egg weight eggshell strength, and eggshell thickness at the end of week 4 and 8. However, there was no difference found on egg production and eggshell quality throughout the trial. Based on the result, we infer 220 mEq/kg DEB would be more suitable for improving egg weight and the production performance of laying hens.

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COMPATIBILITY OF IN OVO APPLICATIONS OF ESSENTIAL OILS AND MAREK'S DISEASE VACCINE: A CHICKEN EMBRYO FIBROBLAST MODEL

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One of the main constraints in broiler chicken production is the vulnerability of chicks immediately after hatch. Essential oil (EO) supplements have been widely studied during the post-hatching period, showing positive effects on gut health, among other modes of action in broilers. However, post-hatching applications come too late for a high percentage of chicks experiencing problems at hatch. In ovo interventions have been studied to improve embryonic development. Recently, in ovo applications of EO have gained momentum showing positive impact on gut development but also potential toxicities in broiler chickens (Khashkheli et al., 2024, Niknafs et al., 2024). Marek's disease vaccine is widely administered in the chicken industry when embryos are 17 or 18 days old. This study aims to investigate the feasibility of adding EO as a supplement to the commercial Marek's disease vaccine. The main objective of this study is to identify the optimal type and dose of individual EOs to be administered in combination with the Marek's disease vaccine. It was hypothesized that EOs of interest will be compatible with the Marek's disease vaccine at the standard dose of application in ovo of 50 ppm.

Cell-based screening assays were employed as the most effective method prior to in vivo testing (Yang, Zhang et al. 2024). In this study, chicken embryo fibroblasts (CEF) were used to represent the chicken embryo, while herpesvirus of turkeys (HVT) was used as a proxy of the Marek's disease vaccine, with the goal of investigating the feasibility of adding EO as a supplement to the cell-associated HVT with minimal side effects on the HVT infection.
Multiple doses (0-6400 PPM) of 8 EOs were firstly tested individually for their toxicity in CEF alone using the MTT assay. Three EOs (black pepper, ylang-ylang, and clary sage) that showed a repeatable breakpoint ($P < 0.05$) with acceptable toxicity ($P > 0.05$) over the first five days of the experimental period were selected for further experiments combining HVT-infected CEF (0.01 MOI). Based on breakpoints observed in previous trials, further 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay using more segmented EO doses (0-400 PPM) were conducted to confirm the metabolism of HVT-infected CEF. Meanwhile, the HVT infection rate was determined by measuring the green fluorescent protein (GFP) signal from GFP-labeled HVT.

The results showed that the acceptable toxic doses for CEF alone were 200 ppm, 100 ppm, and 50 ppm for black pepper, ylang-ylang, and clary sage, respectively. As expected, tolerance levels decreased when HVT was introduced, reducing the doses to 100 ppm, 25 ppm, and 12.5 ppm, respectively. The combined results from the fluorescent reader and MTT assays in HVT-infected CEF showed that black pepper EO had the least effect on the HVT infection rate, supporting the feasibility of adding EO as a supplement to the commercial Marek's disease vaccine. These results have implications for potential commercial adoptions since it shows that some EOs can be safely supplied in ovo together with the Marek's disease vaccine.

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LITTER TILLING CONTRIBUTES TO SHORT-TERM INCREASES OF IN-SHED AMMONIA CONCENTRATION

M. DUNLOP[1] and C.-M. PEPPER[1]

Summary

The majority of Australian meat chicken growers regularly till their litter to improve its friability and keep it 'working'. Tilling releases a surge of water and ammonia for several hours afterwards. In this study, we quantified the surge of ammonia released after tilling and found that the surge was greater and more persistent later in the grow-out, and when litter had higher moisture content. Growers can manage the in-shed ammonia concentration by increasing ventilation, especially in the hours after tilling, and may need to maintain it at a slightly higher rate for 24 hours after tilling to keep ammonia concentration below 15 ppm.

I. INTRODUCTION

Litter tilling (also known as litter conditioning, stirring, turning, flipping, harrowing or rotary hoeing) uses machinery to improve litter friability by reducing clumps and caking with a cutting or pulverizing action. A survey of Australian meat chicken growers found that nearly 90% of growers were regularly tilling their litter (Pepper and Dunlop, 2022). When litter is friable, the chickens can more easily 'work' the litter (Lister, 2009), which is the process that incorporates fresh excreta into the litter by chicken activity. Litter tilling helps to keep litter working and simultaneously helps to reduce the occurrence of both excessively wet or caked litter and excessively dry or dusty litter. This of course depends on the proportions of wet and dry litter that are present, how well the litter is mixed and redistributed, and how effective subsequent ventilation is to evaporate water from the litter.

The mechanical action of breaking up and stirring caked and clumpy litter has the effect of reducing compaction, increasing litter porosity and surface area. Each of these actions increase the release of water vapour and gases from the litter. Ammonia (NH_3) is one gas of particular interest due to the potentially harmful effects that it can have at high concentrations. Tilling or disturbing the litter has also been reported to cause a 'spike' in NH_3 due to the increase in litter porosity and surface area (Malone and Marsh Johnson, 2017). By July 2025, poultry growers will need to ensure that NH_3 concentration is kept below 15 parts per million (ppm) or take immediate action to reduce it (DAFF, 2022). NH_3 concentration can be reduced by management practices that either reduce how much NH_3 is released from the litter or by increasing ventilation to dilute it. Release of NH_3 from the litter is reduced when litter is acidic ($pH < 7.0$), when the litter is dry and litter temperature is reduced (Elliott and Collins, 1982; Mou *et al.*, *in-press* 2024). Changing litter pH requires the addition of acidifying chemicals that aren't routinely used in Australia, and cooling litter isn't feasible. The only control that growers have on NH_3 production is to maintain dry litter condition.

In this study (Dunlop and Pepper, 2023), we investigated how tilling affected NH_3 emissions from the litter and contributed to the in-shed NH_3 concentration at chicken level.

II. METHOD

NH_3 measurements were conducted at four commercial meat chicken farms, two of which used new bedding materials (pine shavings) throughout the shed and two that used new bedding in

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the brooding area of the shed and reused litter in the non-brooding area. Litter tilling was performed at each shed by the farm staff on their routine tilling days and using their normal equipment, which included tractor-powered tilling implements (purpose built for tilling), rotary hoes or power harrows. Tilling was conducted approximately weekly at 12-14 d, 19-21 d and 26-28 d. At one farm, tilling was also performed immediately after a thinning-out operation on day 36, in the empty section of the shed where the catching crew had been working.

In-shed NH_3 concentrations were measured with an NH_3 sensor (Figure 1; 0–100 ppm NH_3 range, DOL 53, dol-sensors A/S, Denmark) and were recorded with a data logger (HOBO UX120-006M; Onset Computer Corporation, Bourne, MA, USA). The sensor was mounted on a tripod with a height-adjustable arm that enabled the sensor to be positioned close to chicken head height (but slightly above the chickens to avoid damage to the sensor).

The rate of NH_3 released from the litter (NH_3 flux) was measured using an isolation chamber (Figure 2). Measurements were performed at two dry locations and two wet locations per shed for each sampling event. The chamber was designed and operated according to the requirements of AS/NZS 4323.4:2009, with instrument-grade compressed air used to sweep the chamber at 5.0 L/min. The method for using the chamber was modified by installing an NH_3 sensor (0-100 ppm, DOL 53) in the chamber to directly measure the NH_3 concentration. The chamber remained in place on the litter surface until the concentration reached steady state. Occasionally, the concentration in the chamber exceeded the maximum range of the NH_3 sensor. When this happened, colorimetric NH_3 detection tubes and a sampling pump (3La tube (2.5–220 ppm NH3) and 3M tube (2–1000 ppm NH3) with GV-100 sampling pump; Gastec Corporation, Japan, https://www.gastec.co.jp/en/) were used after a 24 min equilibrium period to measure the NH_3 concentration inside the chamber. NH_3 measurements were conducted before tilling, immediately after tilling, 1.5-3.0 h after tilling and 24 h after tilling.

Figure 1 - In-shed sensor stand with data logger and sensors to measure ammonia (NH_3), temperature and relative humidity.

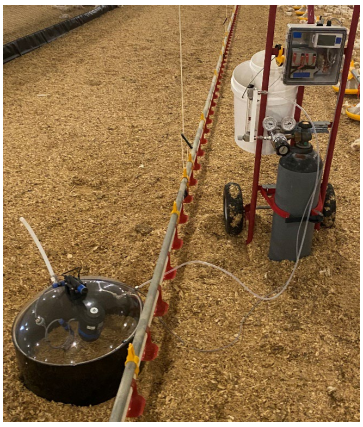

Figure 2 - Isolation chamber with ammonia (NH_3) sensor to measure emissions from the litter.

Data were analysed using general linear models with fixed effects of chicken age, litter location (wet or dry), bedding material (pine, hardwood or reused) and sample timing (before, immediately after tilling, hours after tilling).

III. RESULTS

In shed NH_3 concentrations increased during and after tilling ($P = 0.05$) but started to reduce shortly after tilling and in most cases were less than 15 ppm after one hour (Figure 3). NH_3 concentration continued to decrease and by approximately 3 hours after tilling, the

concentration stabilised at a value that was slightly higher than the pre-tilling concentration. At some farms, a surge in NH_3 concentration was observed approximately 12 hours after tilling, at approximately 9–11 pm. At farms where ventilation activity was recorded by the ventilation controller, the increase in NH_3 concentration coincided with a reduction in night-time ventilation rate. NH_3 concentration was higher in sheds with re-used litter ($P < 0.001$) and increased with chicken age ($P < 0.001$). When chickens were 12-14 d old, the in-shed NH_3 concentration never exceeded 15 ppm, not even during tilling. After 19 d of the grow-out, NH_3 concentration increased above 15 ppm during and after tilling, with the concentration tending to be higher and persist for longer after tilling as the grow-out progressed.

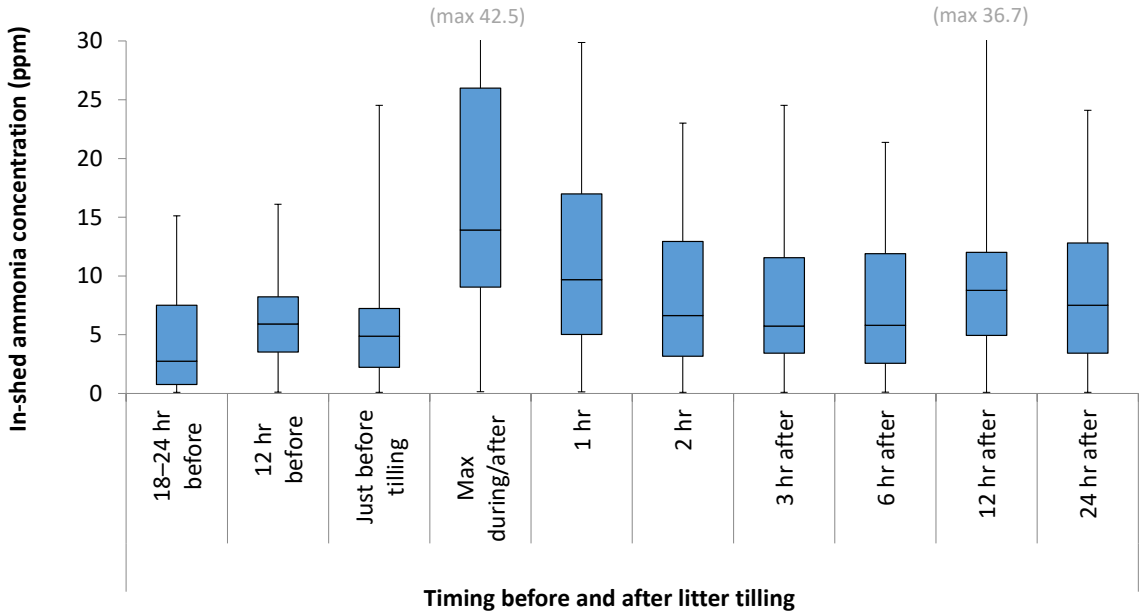

Figure 3 - In-shed ammonia (NH_3) concentration before and after tilling.

Each of the growers increased ventilation at the commencement of tilling but varied in how much they increased it and for how long. Some increased it by about 20% while others put the shed into full tunnel ventilation. Some growers reverted to normal ventilation soon after finishing tilling while others maintained higher levels of ventilation for hours afterwards. Decisions made by growers about increasing ventilation depended on chicken age, stocking density, weather conditions and their previous experiences. We observed that NH_3 concentration generally tended to stay lower when the grower greatly increased ventilation and maintained it for longer after tilling. The effects of litter conditions on in-shed NH_3 were more difficult to determine because each shed contained a range of litter conditions in terms of moisture and friability, and higher ventilation rates masked the effects of litter conditions. The isolation chamber was therefore used to investigate the relationship between litter conditions, especially moisture content, and NH_3 production.

NH_3 emissions from the litter were significantly affected by litter moisture content, day of the grow-out and by tilling ($P < 0.001$ for each of these main effects). NH_3 emissions increased during the grow-out (Figure 4) and were frequently greater from damp litter locations (e.g. under drinkers) where moisture content was greater than 25% (Figure 5). NH_3 emissions from the litter increased during tilling and for a short duration afterwards. The temporal pattern was similar to in-shed NH_3 concentrations (Figure 3) and peak emissions were 0.8–6.8 times greater than pre-tilling emissions from dry litter and 0.6–8.1 times greater from damp litter (5^{th} to 95^{th} percentile values). This related to a two-way interaction between day of the grow-out and tilling ($P = 0.01$), with small increases in NH_3 concentration occurring in early stages of

the grow-out and greater increases occurring later.

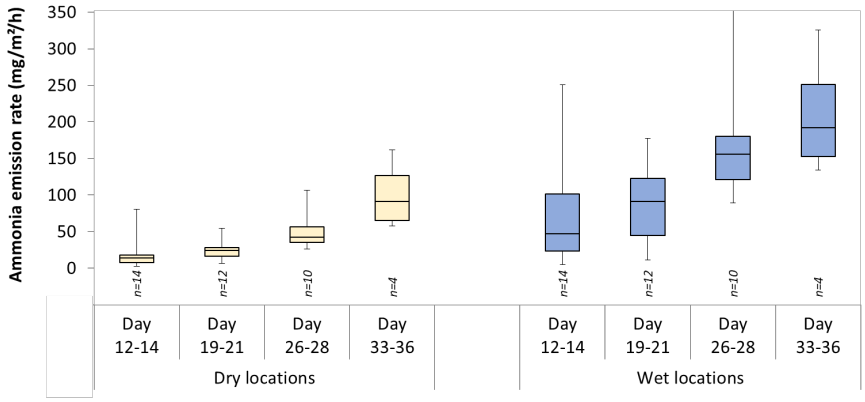

Figure 4 - Emission rates from ammonia (NH_3) from undisturbed pre-tilled litter, and the day after tilling for wet and dry litter locations.

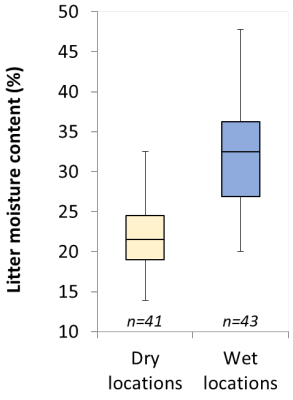

Figure 5 - Moisture content of dry and wet litter.

IV. DISCUSSION

Litter tilling is an effective management practice for improving litter friability and reducing litter moisture content, but it will create a surge in the amount of ammonia (NH_3) released from the litter. The surge generally subsides after 2–3 hours, but NH_3 concentration is likely to remain slightly elevated for 24 hours after tilling. The amount that it increases and how long it persists depends on many factors. NH_3 surges will be greater and longer lasting from the third week of the grow-out and when litter has higher moisture content. Growers can effectively manage the in-shed NH_3 concentration during and after tilling by keeping litter as dry as possible and by increasing ventilation rate. They should also be aware of the need to monitor conditions and further increase ventilation if needed, especially during the first night after tilling. Where possible, growers should perform litter tilling in the mid-morning and ventilate as much as possible for the rest of the day to maximise the release of moisture and NH_3 from the litter.

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TRANSGENERATIONAL INFLUENCE OF VARIED DIETARY PROTEIN ON BROILER EMBRYONIC ADAPTATION

K.W. LAI[1], X. TAN[1], B.C. RAY[1] and E. ROURA[1]

The first week of life for a broiler chick is a critical phase of life. The transition during hatching from embryo to chick necessitates a huge amount of adaptation time due to a shift in nutrition metabolism (Yerpes et al., 2020). Maternal feeding practices and dietary patterns correlated directly with progeny eating habits are observed in broiler breeder production practices, parental feeding choices and availability or scarcity of nutrients acted as an environmental influence on the innate preferences and feeding habits of the resultant progeny (Jha et al, 2019). This transgenerational effect was prevalent in various studies in chickens fed with varied selenium diets. In this study we aim to explore the embryonic adaptation of chicks in response to varied crude protein to energy ratio in the maternal diet. This is based on the hypothesis that day old chicks from low versus high crude protein maternal groups show protein-sparing adaptive metabolic changes, and decreased organ weights at hatch.

In order to interrogate the role of transgenerational mechanisms in nutrient metabolism, two groups of Ross 308 broiler breeders (maternal) were fed diets consisting of high (16%), or low (12%) protein content. An organ weight assessment was then conducted on the resultant progeny, comparing the mass differences between various organ types of both groups. A significant mass increase was observed in the whole intestine weight in the high protein group compared to the low protein group. After which, the proteomic profile present in the jejunum of the progeny at hatch were purified and analysed using whole tissue protein extraction and ZenoTOF mass spectrometry, to determine levels of differentially associated proteins associated with nutrient metabolism.

A significantly increased whole intestine weight ($P = 0.0234$) was observed and a significant upregulation of 677 differentially abundant proteins were quantified (adj. $P < 0.00001$) in the high protein group. In addition, the enrichment analysis showed that most upregulated abundant proteins present in the progeny fed with a high protein diet (16%) were significantly associated with metabolic pathways determining carbohydrate, amino acid and fatty acid metabolism denoted in the KEGG pathway database as; “Glycolysis/Gluconeogenesis”, “Glutathione metabolism”, “Pyruvate metabolism”, “Citrate cycle (TCA cycle)”, “Tryptophan metabolism” and “Fatty acid degradation” (adj. $P < 0.05$). Additional analyses also revealed metabolic pathways involving xenobiotic metabolism (through cytochrome P450). These may indicate that excessive amino acids provide unnecessary N that needs to be catabolized. In addition, the C chain of the excess amino acid might be metabolized for energy use.

In conclusion, the resultant progeny of broilers fed with a high crude protein diet (16%) is able to facilitate adaptive metabolic pathways, thereby leading to possible improvements in newly-hatched chick resilience against the metabolic toll during the transition from yolk to solid feed.

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LOWERING THE CRUDE PROTEIN IN MATERNAL DIETS DECREASED INTESTINAL WEIGHT AND INCREASED DEAD-IN-SHELL OF THE DAY-OLD BROILER CHICK PROGENY

B.C. RAY[1], A. KUMAR[1], S. NIKNAFS[1] and E. ROURA[1]

Lowering dietary crude protein (CP) content in broiler chickens is a desirable strategy to decrease dependence on imported soybean meal and decrease nitrogen excretion to the environment. However, low CP may lead to compromised performance, highlighting the need to improve dietary protein utilization efficiency. Maternal nutrition directly influences the early-stage development of chicks through embryonic programming (Santana et al., 2023). Thus, this study aimed to explore the effects of maternal programming on embryonic development in fertile eggs and its potential to enhance protein utilisation in broiler progeny. We hypothesized that feeding broiler breeders a low-protein diet (e.g., 12%) would not adversely affect fertility, hatchability, and embryonic development of eggs and condition the progeny to better adapt to low CP and low-soybean meal diets, leading to improved performance under such feeding regimes.

A total of 587 Ross 308 broiler breeder eggs from 16%, 14%, and 12% dietary CP-fed groups (207, 192, and 188 eggs, respectively) were collected and incubated. At hatching, all the chicks were weighed, and 12 one-day-old chicks from each dietary treatment group were euthanised. Their proventriculus, gizzard, heart, liver, whole intestine, and residual yolk were collected, weighed, and expressed as percentages relative to the body weight of day-old chicks. From the remaining chicks, 270 good-quality day-old chicks free from any abnormalities (n=90 per treatment) were selected, and chicks from each dietary treatment of broiler breeders received either standard (23% CP), medium (20.5% CP), or low (18.0% CP) dietary protein levels for seven days. Standard diets were formulated according to breeders' recommendations (Aviagen, 2022) to meet all the nutrient requirements. Medium and low CP diets were supplemented with synthetic amino acids to meet the birds' amino acid requirements. There were nine progeny treatments with six replications per treatment, with five chicks per pen in a 3×3 factorial design (3 maternal CP levels × 3 progeny CP levels). Data were analysed using PROC GLM (SAS 9.4).

The results showed that fertility and hatchability of eggs from breeders fed either 16%, 14%, or 12% CP levels were not significantly ($P > 0.05$) affected. However, higher dead-in-shell was found in eggs from breeders fed 12% CP compared to the 14% or 16% CP-fed groups ($P = 0.04$). Day-old chicks from breeders fed a 12% CP diet had lighter-weight intestines compared to those chicks from breeders fed diets containing 14% or 16% CP ($P = 0.0025$). No significant differences ($P > 0.05$) were observed for body weight or other organ weights at hatch. A significant correlation was found between different organs on the first day of post-hatch. The post-hatch feeding trial demonstrated that the main effect, "maternal CP levels", had no significant effects on the growth performance of progeny. In contrast, the main effect, "progeny CP levels", had a significant effect ($P < 0.05$) where 23% and 20.5% protein levels improved final body weight, weight gain, and FCR than the 18% protein-fed group. The interaction between maternal and progeny CP levels had no statistical significance for the measured parameters at seven days of age.

In conclusion, the hypothesis was accepted for fertility and hatchability but rejected for embryonic development and progeny adaptation. Low CP maternal diets resulted in a higher incidence of dead-in-shell embryos and lighter-weight gastrointestinal tracts in day-old chicks. The maternal programming based on dietary CP levels did not affect early post-hatch growth, but low CP in progeny diets resulted in poor performance.

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DIETARY ARGININE REQUIREMENT IN LOW CP DIETS MAYBE HIGHER THAN PREVIOUSLY ASSESSED IN STANDARD CP CONTENTS IN CHICKENS

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The improvement in the sustainability of broiler production is a goal that requires a multifactorial and incremental approach. The reduction of dietary crude protein (CP) and the decrease in the reliance on (imported) soybean meal have been identified as key components to achieving this goal. Reduction in dietary CP is a target that can only be achieved with a precise understanding of the requirement of all the twenty proteinogenic amino acids. Essential amino acids (EAA) must be provided through the diet at a level that has been thoroughly studied usually expressed as the ratio to digestible Lys (Aviagen, 2022). However, potential variations in these optimal Lys ratios have not been properly studied under low CP diets. The objective of this experiment was to test dietary requirements for nine EAAs in chickens fed a low CP diet using a double-choice (DC) model. It was hypothesised that chickens have the capability to select the proportion of two diets (one deficient and one excess) to perfectly balance the dietary requirement.

Forty-eight male Ross-308 broiler chicks were fed a common starter diet during the adaptation period (0–7 days). On day 7, chicks were individually caged with ad libitum access to two feeders and water. A DC training occurred from days 7 to 9. The DC tests started at day 10 and finished at day 30 (testing 20 days). Each chicken was offered two low CP diets (15.5%) based on a basal soy-free formulation containing wheat and canola as main ingredients (AMEn 3050, Lys 1.18%). The DC model consisted of one of the diets having 20% excess and the other 20% deficiency of one of the 9 EAA Arg, His, Ile, Leu, Lys, Met, Thr, Trp, and Val tested. Alanine was used to maintain the diets iso-nitrogenous. There was a total of 10 test sessions, of 2 days (48 hours). Each session had 4 or 5 replicates of each treatment distributed following a randomized Latin-square design. At the end of the trial, each amino acid and control groups had been tested by all the chickens once (this is 48 replicates). Feed preference and amino acid appetite were analysed using R software, with paired comparisons to the control made using the Tukey's test. Figure 1 shows the two-days average preference results for nine EAA and the control group relative to the 50% preference neutral value. The results showed that eight of the EAAs tested and the control group did not show any significant preference. The Arg supplemented group showed a significant preference ($P<0.05$) for the 20% excess group on both days indicating that Arg requirement in low CP diets may have been underestimated in the literature (based on standard CP contents).

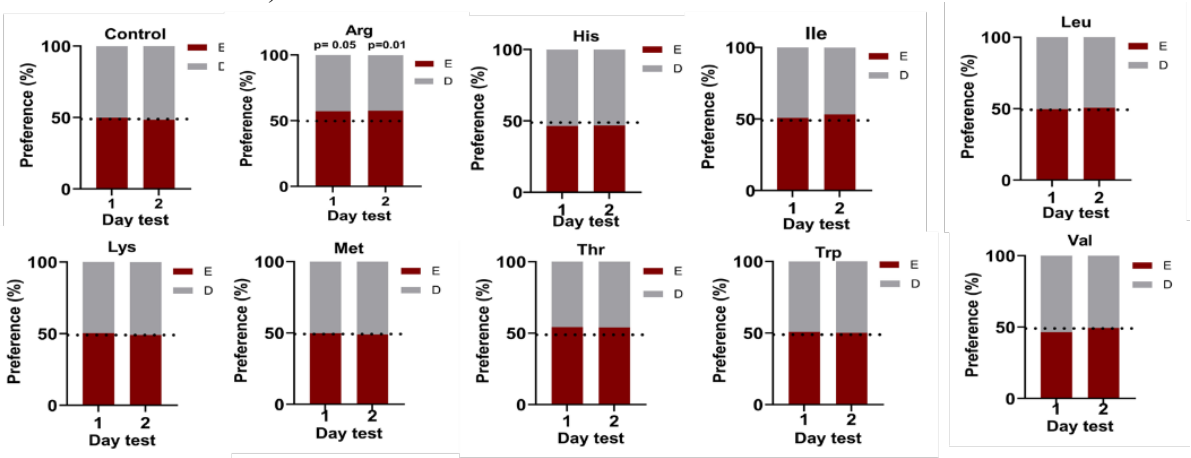

Figure 1 - Two-day average preference tests of EAA offered in 20% excess (red) or 20% deficient (grey) in a low CP (15.5 %) diet. The p value indicates statistical significance relative to the 50% preference neutral value.

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SUSTAINABLE POULTRY PRODUCTION: THE REFERENCE POSTBIOTIC FOR REDUCING THE CLIMATE CHANGE EFFECTS OF POULTRY PRODUCTION

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Summary

Life Cycle Assessments (LCAs) are widely accepted as comprehensive and accurate in evaluating the environmental impact of a commercial product. This analysis takes into account the production both of the product under evaluation as well as the production systems it is used in. A recent study was conducted to evaluate a yeast postbiotic which has been shown to have positive effects on efficiency, immunity, pathogen load and overall production. The results of this assessment showed that the postbiotic reduced the overall carbon footprint of broiler production systems by 8.4% versus 2 challenged groups.

I. INTRODUCTION

Poultry meat production is intrinsic in providing affordable and accessible protein sources to a growing global population. All forms of agriculture are being pressured to disclose and reduce their effects on the environment and natural resource use.

Life Cycle Assessments (LCAs) are widely accepted as comprehensive and accurate in evaluating the environmental impact of a commercial product. They take into account the production of both the product under evaluation as well as the production systems it is used in. With LCA, climate change effect of poultry meat production systems has been shown to be substantially lower than red meat counterparts, but since broiler farming is a volume driven business, the industry as a whole contributes substantially to climate change. This impact will be compounded by the current growth rate of the industry which is predicted to be around 1.4% between 2020 and 2030 versus only the 0.3% cumulative average growth rate experienced between 2010 and 2020.

In this study, we estimated that in modern production systems, the poultry meat industry contributes 4kg CO2 equivalents per kg meat. By evaluating the climate change contributions of each part of the overall production cycle, the potential sustainability benefits of improving nutrition and health could be evaluated.

In this study, we evaluated the potential climate change benefits of using a postbiotic that was designed to enhance feed efficiency and reducing pathogen loads. Postbiotics represent preparations of inanimate microorganisms and/or their components that can confer health benefits to the host body such as through improved gut heath and by binding certain types of bacteria such as Clostridium & Salmonella species. The tested product was a yeast cell wall product that contained consistent levels of mannans and β-glucans.

II. METHOD

In order to evaluate and reduce carbon equivalent emissions, the entire life cycle of broiler birds needed to be investigated from the starting point of crop cultivation, through the feed manufacturing process and ending with the production of 1kg live weight of a broiler bird. LCA was used to assess environmental impacts of particulate matter formation; disease incidence; acidification of water; water use; measurable direct carbon footprint which includes fossil fuel use, biogenic fuel use and land use change; land allocation; eutrophication of water

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sources -marine, terrestrial and freshwater bodies and finally depletion of abiotic resources and fossil fuels.

This study comprised 3 biotic (*Clostridium perfringens*) and 3 abiotic (heat stress) trials, which were combined to evaluate the effects of these challenges as well as mitigation ability of a yeast postbiotic (Safmannan®, supplied by Phileo by Lesaffre, France) on climate change. The method of comparative analysis used was the EF3.1 adapted method which is underwritten by the European Platform on Life Cycle Assessment.

The positive control comprised a group of birds in each biotic or abiotic stress group which was exposed to the stressor but did not receive nutritional supplementation. The test groups were supplemented with the postbiotic under the exact same conditions as the positive control group in each case. Results were compiled from all 6 studies and compared.

Table 1 - Test versus Control Groups.

Negative Control: No Supplementation; No Challenge	**Positive Control 1:** Clostridium Challenge	**Positive Control 2:** Heat Stress Challenge	**Test Group 1:** Supplementation; No Challenge	**Test Group 2:** Clostridium Challenge with Yeast Postbiotic Supplementation	**Test Group 3:** Heat Stress Challenge with Yeast Postbiotic Supplementation

Results were obtained from 6 different trials conducted previously by Phileo by Lesaffre and supplied to BLONK Consultants for further analysis.

III. RESULTS

According to the National Chicken Council, USA, feed production equates to approximately 74% of total carbon emissions per lifecycle of broiler birds (Fig 1). Heating and power consumption add a further 14.7%; transport of birds from the hatchery to the farm and again from the farm to the slaughterhouse contributes another 1.7%; 1.3% is allocated to waste management and the remainder of the total carbon output lies with housing emissions and other various outputs.

The results from the comparative study showed that on average, the positive control group had an 8.4% higher climate change effect than the group of birds tested using the yeast postbiotic. This equated to 0.19 kg CO2 equivalents per kg of live weight.

This 8.4% improvement was attributed to reduction in FCR as well as land use associated carbon outputs. These were seen to be 0.1 kg CO2 equivalents per kg of live weight for each of these measurements. The treatment contributed to a 7.2% improvement in FCR versus the positive control group for *Clostridium perfringens* infection and a 6.6% improvement when compared to the heat stress control group. In the absence of challenge, the treatment reduced the FCR by 6.9% when compared to negative controls.

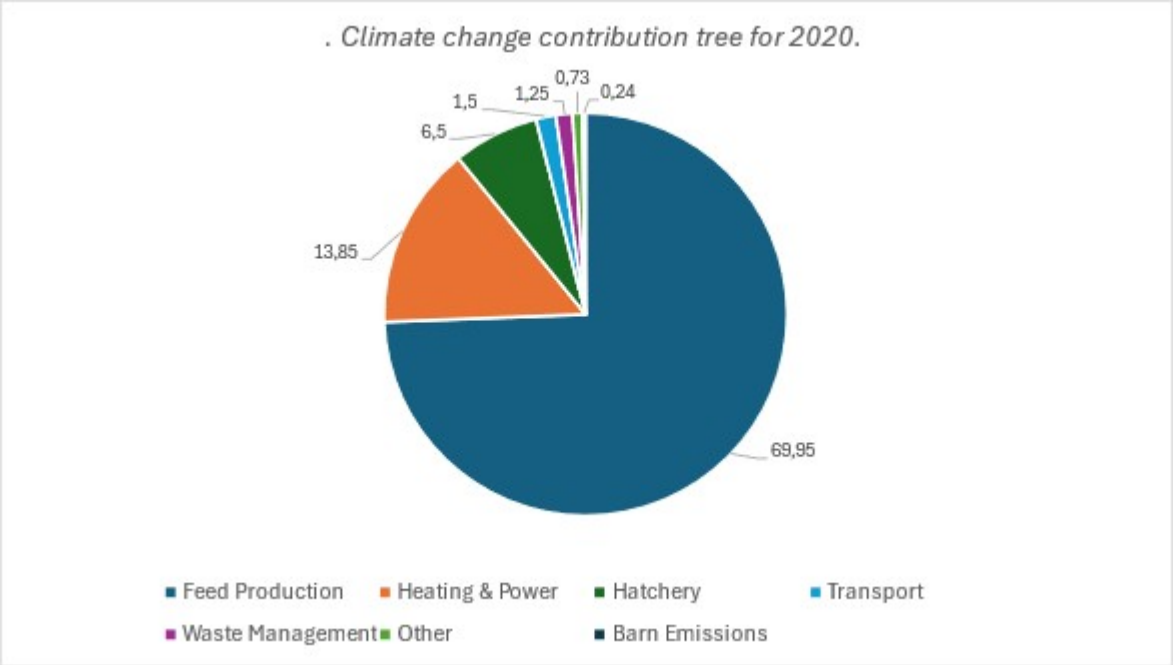

Figure 1 - Climate change contribution tree for 2020. Values reported as percentage. National Chicken Council USA (2020).

When evaluating the impact categories of the yeast postbiotic versus positive control groups, the following results were obtained:

Table 2 - Categorized Improvements with Yeast Postbiotic Supplementation.

CO2 Emission/Climate Change	8.4%
Acidification	12.5%
Particulate Matter	11.4%
Marine Eutrophication	9.5%
Freshwater Eutrophication	8.6%
Terrestrial Eutrophication	12.6%
Land Use	8.7%
Fossil Fuel Resource Use	7.9%
Water Use	7.7%

IV. DISCUSSION

Many factors contribute to the overall climate change contribution of a broiler production system. Feed consumption is at the forefront of these factors, contributing to almost three quarters of the total carbon output of a broiler operation.

It is known that certain pathogenic challenges such as infection by *Clostridium perfringens* and metabolic challenges such as heat stress decrease the efficiency of birds by causing dysbiosis, damaging gut integrity, altering bird feeding behavior and causing nutrient reallocation within the body. This reduction of feed efficiency causes the carbon contribution of affected production systems to increase, mainly due to increased relative feed contribution.

The effects of these challenges in driving up FCR were demonstrated as 2 positive control groups in this study. By improving the feed efficiency and associated water consumption of challenged broilers, the probiotic allowed them to cope better with temperature challenges and pathogens and the overall carbon equivalent output per kg live weight was reduced.

By supporting the intrinsic mechanisms of the broiler body to cope with stress and produce optimally, as well as protecting it from pathogens, three focus pillars, people, planet and profit are met. People—both producers and consumers—benefit from higher production efficiency; broiler enterprises can achieve higher levels of profit due to enhanced efficiency and carbon emissions from such enterprises are lower, contributing to responsible custodianship of the planet.

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TRIBUTYRIN RELEASE KINETICS AND ITS EFFECTS ON GUT HEALTH AND PERFORMANCE OF COMMERCIAL BROILERS

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Summary

Two studies were conducted to evaluate the release kinetics of tributyrin (TB) and the effects of dietary supplementation of the TB on gut health and growth performance of broilers raised up to 42 days. In Exp 1, it was hypothesized that release of TB would not occur in the first compartment of gut model and hence, it was expected that butyric acid from TB was released as soon as pancreatic lipase introduced in the second compartment of gut model [Dynamic Avian Intestine *in vitro* System (DAISy)]. In Exp 2, it was hypothesized that TB supplemented birds would perform better than negative control birds. In Exp 2, a total of 4,400 one-day old Vencobb 430 broiler chicks were randomly distributed to five dietary treatments with 8 replicate pens/treatment containing 110 chicks each. Experimental diets were as follows; (i) Negative control (NC) – corn-soybean meal-based diet; (ii) Positive control (PC) – [NC + chlortetracycline 15%]; (iii) NC + TB [500 g/MT in starter; 250 g/MT in grower & finisher] (TB1); (iv) NC + TB [500 g/MT in starter and grower; 250 g/MT in finisher] (TB2); (v) NC + TB [1000 g/MT in starter; 500 g/MT in grower; 250 g/MT in finisher] (TB3). Results from the *in vitro* study demonstrated that TB was not cleaved in the first compartment of DAISy, whereas, in duodenum and jejunum, TB was gradually cleaved into dibutyrin, monobutyrin, butyric acid and glycerol. Feeding study results indicated birds fed TB1, TB2 and TB3 groups had improved ($P < 0.05$) final body weight compared to NC (2.30 kg, 2.32 kg and 2.32 kg versus 2.19 kg). The FCR of TB2 was significantly improved ($P < 0.05$) compared to NC (1.67 versus 1.72). Broiler production index in TB1, TB2 & TB3 was better ($P < 0.05$) than NC (294, 302 & 293 versus 263). Carcass yield and breast meat yield, respectively in TB groups (TB1, TB2 & TB3) were 10% and 16% higher ($P < 0.05$) than in NC. Dietary TB3 supplementation had improved ($P < 0.05$) gut morphology (villus height & villus height to crypt depth ratio) compared to other dietary treatments. In conclusion, dietary TB2 supplementation (500 g/MT in starter and grower; 250 g/MT in finisher) could support improved gut integrity, and also improved overall performance in broilers.

I. INTRODUCTION

Gut health is considered as an ultimate criterion for the overall health and welfare in broilers, which directly impacts performance and productivity (Ducatelle et al., 2023). For the past decade, nutritionists have been exploring different approaches to maintain gut integrity and modulation of gut function in broilers (Zhu et al., 2021). In this regard, butyric acid has been demonstrated to play a potential role in modulation of gut microbiota and integrity in broilers (Gong et al., 2021; Hu et al., 2021). However, due to butyric acid's unpleasant smell and rapid absorption in the upper gastrointestinal tract, its application in poultry diets is limited (Gong et al., 2021). To overcome these drawbacks, tributyrin (TB) was developed, which is a triglyceride containing three molecules of butyric acid (Gong et al., 2021; Hu et al., 2021). The first study hypothesized that TB would not release in the first gut compartment but would release butyric acid upon introducing pancreatic lipase in the second compartment. The second study hypothesized that dietary TB supplementation would perform better than control groups. The first study assessed dry TB's

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release properties, while the second examined TB supplementation's impact on gut health and performance in broilers.

II. MATERIALS AND METHODS

a) Experiment 1

The experimental setup of the first study involved the use of a Dynamic Avian Intestine in vitro System (DAISy) (Locher, 2021) to simulate the gastrointestinal tract (GIT) of poultry. The DAISy model included compartments representing the crop, proventriculus, gizzard, duodenum/jejunum, ileum, and caecum. A standard grower feed was introduced without TB supplementation (Control) and with 18 mmol TB (SpeoCare^{TM} T60, Evonik Operations GmbH, Germany) supplementation (TB) into the DAISy model. Pancreatic juice enzymes were added to the duodenum/jejunum compartment to simulate digestion. Samples were collected from various compartments at different time intervals to measure the concentration of butyric acid, glycerol, mono-, di-, and tributyrin by Liquid Chromatography Mass Spectrometry Time of Flight (LC-MS TOF). From those measurements release properties of the dry TB could be calculated.

b) Experiment 2

A total of 4,400 one-day-old Vencobb 430 broiler mixed-sex chicks were randomly distributed to 5 dietary treatments with 8 replicates containing 110 chicks each. Birds in each treatment were housed in a floor pen containing paddy husk litter. Basal diets (starter, grower and finisher) were based on corn and soybean meal. Experimental diets were as follows: (i) Negative control (NC) – Corn-soybean meal-based diet ; (ii) Positive control (PC) – (NC + Chlortetracycline 15%); (iii) NC + TB (500 g/MT in starter; 250 g/MT in grower & finisher) (TB1); (iv) NC + TB (500 g/MT in starter & grower; 250 g/MT in finisher) (TB2); (v) NC + TB (1000 g/MT in starter, 500 g/MT in grower; 250 g/MT in finisher) (TB3). Three phase feeding programs were used, starter (d 0 to 14); grower (d 15 to 28) and finisher (d 29 to 42). Feed (mash form) and water were provided *ad libitum*. Feed intake and body weight gain (BWG) were measured. Broiler production index (BPI) was calculated as follows: ((survivability x average daily gain)/feed conversion ratio)) x 10. On d 42, 40 birds (8 birds per treatment; 1 bird per replicate pen) were euthanized by cervical dislocation for determination of gut morphology (villous height (VH), crypt depth (CD) and VH:CD). Carcass yield and breast meat yield (BMY) of the birds (1 bird per pen) was measured on day 42. BMY% was calculated as follows: (Breast meat weight /final BW) x 100. The response criteria measured were BWG, FCR, BMY and BPI. Data were analysed as one-way ANOVA using SAS 9.4. Mean values were separated by Tukey's test and statistical significance was declared by $P < 0.05$.

III. RESULTS

a) Experiment 1

TB was not cleaved in the first compartment of the DAISy model simulating the upper GIT including crop, proventriculus and gizzard (HCl + Pepsin addition). In the duodenum/jejunum compartment pancreatic juice (pancreatin + carbonate + bile salts) was added and TB was gradually cleaved into dibutyrin, monobutyrin, butyric acid and glycerol. After pancreatin addition tributyrin disappeared immediately, whereas significant amounts of diester were measurable only a few minutes after pancreatin was added. After 45 minutes, which corresponds to chicken gut segment jejunum, beginning of the ileum, a stable level of metabolite concentrations was reached, which indicated that the lipase cleavage was completed. Butyric acid concentration at this point was in the range of 20 mM. In the DAISy model, ileum microbiota did

not metabolize the released butyric acid, as shown by stable butyric acid levels. There is no nutrient absorption simulation in the in-vitro model. This indicates that the butyric acid released in the chicken intestine can be completely absorbed by the enterocytes. There is no competition with the commensal bacteria.

b) Experiment 2

Data on growth performance and carcass quality are presented in Table 1. The results indicated that tributyrin at different levels (TB2 & TB3) of supplementation significantly improved ($P < 0.01$) BWG compared to the NC and PC. The FI of TB3 was higher ($P < 0.01$) than NC and PC groups. The FCR of TB2 was significantly improved ($P < 0.01$) compared to NC and FCR was numerically lower with TB supplementation than PC. Birds fed TB2 supplementation had higher CY (12%) and BMY (17%) compared to NC. TB2 had better BPI compared to NC (302 versus 263). Data on gut morphology are presented in Table 2. Dietary tributyrin supplementation increased gut morphology (villous height & villous height to crypt depth ratio, VHCD) compared to NC.

Table 1 - Effects of dietary tributyrin supplementation on growth performance and carcass quality in broilers (42 d).

Diet	FBW, kg	FI, g	FCR	CY, g	BMY, g	Mortality, %	BPI
NC	2.19^{c}	3.76^{b}	1.719^{a}	1562^{b}	503^{b}	11.82	263^{b}
PC	2.23^{bc}	3.76^{b}	1.684^{ab}	1642^{a}	525^{b}	8.44	280^{ab}
TB1	2.25^{ab}	3.84^{ab}	1.672^{ab}	1712^{a}	594^{a}	8.57	294^{a}
TB2	2.28^{a}	3.87^{ab}	1.666^{b}	1777^{a}	606^{a}	7.53	302^{a}
TB3	2.28^{a}	3.88^{a}	1.673^{ab}	1742^{a}	607^{a}	9.61	293^{a}
P-value	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05	> 0.05	< 0.05

FBW: Final body weight; FI: feed intake; FCR: feed conversion ratio; CY: carcass yield; BMY: breast meat yield; BPI: broiler production index. ^{ab} Means within column not sharing a common suffix are significantly different at $P < 0.05$.

Table 2 - Effects of dietary tributyrin supplementation on gut morphology in broilers (42 d).

	Villus height (VH, μm)			Crypt depth (CD, μm)			VH:CD ratio		
Diet	Duodenum	Jejunum	Ileum	Duodenum	Jejunum	Ileum	Duodenum	Jejunum	Ileum
NC	1748^{e}	1582^{e}	936^{e}	248^{a}	218^{b}	136^{b}	7.06^{d}	7.27^{c}	6.9^{c}
PC	1694^{d}	1533^{d}	893^{d}	248^{a}	228^{a}	140^{a}	6.83^{c}	6.72^{d}	6.39^{d}
TB1	1795^{c}	1648^{c}	1040^{c}	218^{c}	209^{c}	133^{bc}	8.24^{b}	7.91^{b}	7.85^{b}
TB2	1806^{b}	1659^{b}	1068^{b}	216^{c}	209^{c}	127^{d}	8.35^{a}	7.94^{b}	8.38^{a}
TB3	1872^{a}	1705^{a}	1113^{a}	223^{b}	208^{c}	134^{bc}	8.42^{a}	8.19^{a}	8.31^{a}
P-value	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05	> 0.05	< 0.05	< 0.05	< 0.05

^{abcd} Means within column not sharing a common suffix are significantly different at $P < 0.05$.

IV. DISCUSSION

TB is a triglyceride containing 3 molecules of butyric acid, and its dietary supplementation has shown positive effects on gut barrier function and gut microbiota balance in broilers (Gong et al., 2021). The mechanism by which TB operates involves its hydrolysis in the small intestine, releasing butyric acid that serves as an important energy source for enterocytes, the absorptive cells lining the gut. In addition to nourishment of intestinal cells, butyric acid strengthens the gut barrier preventing the translocation of harmful bacteria and their toxins (El-Saadony et al., 2022).

The observed absence of TB cleavage in the upper GIT aligns with the understanding that the crop, proventriculus, and gizzard primarily serve by mechanical and preliminary enzymatic functions (Svihus, 2014). These compartments lack the specific lipases required to hydrolyze TB effectively, which are predominantly secreted by the pancreas into the duodenum. The gradual hydrolysis of TB into dibutyrin, monobutyrin, butyric acid, and glycerol in the duodenum/jejunum reflects the action of pancreatic lipases which are known to efficiently cleave triglycerides

(Lindberg, 2020). This step-wise breakdown process is consistent with previous studies (Liu et al., 2019), which reported similar patterns of triglyceride hydrolysis under the influence of pancreatic enzymes. The observed decrease in dibutyrin and monobutyrin with a concomitant increase in glycerol and butyric acid over time is indicative of the sequential hydrolysis of triglycerides. This finding is in line with the work of Deplancke and Gaskins (2001), who noted that intermediate products of triglyceride hydrolysis are transient and quickly further metabolized into free fatty acids and glycerol. The non-metabolism of butyric acid by the ileum microbiota was also observed in the study of den Besten et al. (2013), which highlighted that butyric acid is primarily absorbed in the small intestine rather than being extensively metabolized by the microbiota.

In this study, TB supplementation improved growth performance in broilers which agrees with Gong et al. (2021) who demonstrated that TB supplementation positively modulated gut microbiota and improved growth performance in broilers. There exists a link between improved gut morphology and disease prevention and improved growth performance in animals (Hu et al., 2012). Similarly, in this study, birds supplemented with different levels of TB had improved gut morphology (VH, VH:CD ratio) in different gut segments and growth performance.

In conclusion, the results of study 1 elucidate the digestion and metabolic fate of TB in a simulated GIT environment. The findings are consistent with existing literature, reinforcing the role of pancreatic enzymes in TB hydrolysis and the beneficial properties of TB on butyric acid release. These insights underscore the potential of TB as a dietary supplement to enhance gut health, nutrient utilization and performance in commercial broilers. Based on results of study 2, dietary TB supplementation at different inclusion levels improved gut morphology and production performance compared to control fed broilers. These findings suggest that SpeoCare™ T60 supplementation in AGP-free diets not only bolsters gut integrity but also contributes to overall better performance in broilers, highlighting its potential as an effective alternative to traditional antibiotic growth promoters in poultry production.

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EFFECT OF DIFFERENT IONOPHORES IN EXPERIMENTALLY INDUCED NECROTIC ENTERITIS IN BROILERS

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Summary

Necrotic enteritis is an acute to chronic enterotoxemia caused by *C. perfringens* mostly NetB positive strains. Though the presence of such strains is necessary, it is not alone sufficient for the pathogenesis of necrotic enteritis. It requires certain predisposing factors facilitating initial damage of the mucosa and altering the intestinal microbiome, coccidiosis, being the most common one. Ionophores – anticoccidial products produced by fermentation are commonly used to prevent coccidiosis, thus eliminating the major predisposing factor to necrotic enteritis, but also possess well demonstrated activity against Clostridia. Narasin possesses numerically the lowest MICs against *C. perfringens* among ionophores, thus there is an alleged perception that it has a superior effect in preventing necrotic enteritis in the field. The aim of this study was to investigate the efficacy of three different ionophores – narasin, salinomycin and semduramicin, in an *in-vivo* floor pen necrotic enteritis challenge model. All three ionophores were able to significantly reduce mortality and intestinal necrotic enteritis lesions. Furthermore, body weight gain and FCR were significantly improved in all treated groups compared to the infected untreated control. In conclusion, it can be stated that ionophore supplementation in feed is able to reduce severity necrotic enteritis in broiler chickens under floor pen conditions. Probably, this is not only due to a direct effect on Clostridia, but also through prevention of coccidiosis which is the major predisposing factor for the disease. Moreover, there is no significant difference in this effect between narasin, salinomycin and semduramicin with numerical advantage for the latter, indicating that the numerical differences between the *in-vitro* MIC values of the different ionophores against *C. perfringens* are not relevant for their efficacy *in-vivo*.

I. INTRODUCTION

Necrotic enteritis (NE) in poultry, caused mostly by NetB toxin producing strains of *C. perfringens* type G, but also some type A NetB-negative strains (Emami and Dalloul, 2021), is one of the major veterinary challenges in the poultry industry, causing annual losses of more than $6 billion worldwide (Wade and Keyburn, 2015). Though the above mentioned *C. perfringens* strains are specific etiological agent for NE, they are not sufficient pathogenetic factor on their own and require certain predisposing conditions, causing initial intestinal tissue damage and/or dysbacteriosis such as: imbalanced diets with abundant undigested protein in the rear segment of the intestinal tract; antinutritional factors or high buffering capacity of the diet compromising protein digestion and absorption; high viscosity of the intestinal content; different mycotoxins and especially deoxynivalenol (DON); management stress of the birds such as high stocking density, heat or cold stress, feed deprivation, etc.; and different viral or protozoal pathogens (Moore, 2015). Among the latter, the most important predisposing factor to NE is coccidiosis (Van Immerseel et al., 2008) and specifically *E. maxima* and *E. brunetti* infections (Nicholds et al., 2021). *Eimeria* spp. produce initial tissue damage, which leads to inflammation and respective inflammatory proteins leakage into the intestinal lumen and increased mucogenesis (Van Immerseel et al., 2008). All these can alter the microecology of the intestine and provide an abundant amount of protein for the growth of *C. perfringens* which is a very common inhabitant of the intestinal tract. In this environment, pathogenic *C. perfringens* flourish in the intestinal tract, attach to the mucosa of the jejuno-ileum and produce

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specific necrotizing toxins that further damage the mucosa (Moore, 2015). The importance of *Eimeria* infection as a triggering factor of NE has its indirect evidence in the incidence and prevalence of the disease. Often witnessed by field veterinarians in different regions, the incidence of NE is rather low in conventional production systems using in-feed anticoccidials that suppress *Eimeria* cycling, but it could be more common in systems applying coccidiosis vaccination (in broilers, but also in breeder or layer pullets), as coccidiosis vaccines provide a controlled coccidia challenge and respective tissue damage in the mucosa aiming at immunity development, thus predisposing to NE. Furthermore, cases of NE are not uncommon in conventional production systems when anticoccidial drugs are not rotated and resistance to field *Eimeria* strains develops.

NE is a typical enterotoxemia that could be present in a clinical or subclinical form. In clinical cases, the onset is sudden with rapidly increasing mortality (2-5%), which in severe non-treated cases could reach up to 50%. Clinical cases of NE typically respond well to treatment with narrow spectrum penicillins (e.g. phenoxypenicillin) or amoxicillin, as well as other antibiotics with gram-positive spectrum. Historically, in-feed antibiotics with gram-positive spectrum have been used to prevent NE. However, to reduce antibiotic use and respective risk of resistance development, prophylactic use of antibiotics is under scrutiny. Nevertheless, ionophores, in-feed anticoccidials used to control *Eimeria* infection can prevent coccidiosis effectively, thus eliminating the major predisposing factor to NE, while they also provide direct inhibitory effect on *Clostridia*, having low MICs. Among ionophores, narasin possess numerically the lowest MICs against *C. perfringens* (Lankriet et al., 2010), thus there is an alleged perception that it has a superior effect in preventing NE in the field. The current study aimed to assess comparative efficacy of three different ionophores – narasin, salinomycin and semduramicin, to control NE in a clinical NE challenge model *in-vivo*.

II. METHOD

NE, being multifactorial disease requiring certain predisposing factors could not be reproduced *in-vitro*, thus the study utilized an *in-vivo* challenge model. In total, 440 healthy Ross 308 male day-old broilers were set-up at day one in the Poulpharm research site at Buntelarestraat 19, 9880 Aalter, Belgium. From day 1 to day 12, all animals were supplemented with starter feed according to the breed standard and commercial practices. All birds were individually weighed, neck-tagged for individual identification with unique numbers, and 420 of them were allocated to five treatments with six replicates of 14 birds each, or 30 replicates (floor pens) in total, in a way that there was no significant difference between the groups in terms of body weight at day 12 – the start of the trial. From day 12 to day 17, the animals were fed a (un)supplemented starter diet as follows: T1, an uninfected untreated control group (UUC); T2, an infected untreated control group (IUC); T3, an infected treated group fed a diet supplemented with 70 ppm narasin (NAR); T4, an infected treated group fed a diet supplemented with 70 ppm salinomycin (SAL); and T5, an infected treated group fed a diet supplemented with 25 ppm semduramicin (SEM).

From day 17 to day 23, all animals were fed a (un)supplemented grower feed according to the five treatments with high protein (30% fish meal) as a part of the experimental model, utilizing high dietary protein as a predisposing factor to NE.

On day 14 and day 16, all birds except for the UUC received a 10-fold overdose of Paracox-8® by oral gavage as a source of coccidia being a mean to create an initial mucosal damage as a predisposing factor to NE. Starting from day 18 and up to day 21, on 4 consecutive days, all birds except for the UUC were inoculated by oral gavage with 1 mL of broth containing a NetB positive *C. perfringens* strain (targeted at $1x10^9$ CFU/mL) originating from Sweden.

To avoid the effects of post recovery compensatory growth, the experimental period for zootechnical parameters (body weight (BW), body weight gain (BWG), feed intake (FI), feed conversion ratio (FCR) and mortality) was from day 12 (day of birds' allocation) up to day 23 (end of study). Birds were individually weighed on days 12, 17, 22 and 23. Feed per pen was weighed on days 12, 17 and 23. General health observations were performed daily along the duration of the study. Any bird that died or needed to be removed on animal welfare grounds, was recorded, weighed and submitted to a necropsy as soon as possible after death. On day 22, half of the birds in each pen were

subjected to macroscopic NE lesion scoring according to the method described by Timbermont *et al.* (2010): score 0 – no gross lesions; score 1 - congested intestinal mucosa; score 2 - small focal necrosis or ulceration (1-5 foci); score 3 - focal necrosis or ulceration (6–15 foci); score 4 - focal necrosis or ulceration (16 or more foci); score 5 - patches of necrosis 2–3 cm long; and score 6 - diffuse necrosis typical of clinical field cases. Remaining birds followed the same protocol on day 23.

The experimental unit was the pen for all outcome variables. Data was analyzed with SAS, using generalised linear model (proc GLM) was used. Additional analyses with the bird as the unit of analyses was conducted for variables measured at individual level (NE lesion scores (LSs), body weights). The random effect of pen was included in the models where the bird was the unit of analyses, using linear mixed regression models with treatment as fixed effect and pen as random effect (proc MIXED). Multivariate analysis was used as appropriate controlling for the clustering of birds within pens, when the analysis was on bird basis. In all models, treatment groups were compared to the IUC group as reference. Statistical significance was assessed at $P \leq 0.05$. Residual plots were checked to evaluate model fit. Post-hoc pairwise comparisons were made with Tukey-Kramer test to assign statistical grouping.

Birds were humanely handled and reared according to the legal requirements and commercial practices in intensive broiler production in Europe in line with FOR-TEST-027 and were vaccinated in the hatchery with live ND and IB vaccines. The study was conducted according to Good Clinical Practice and approved by the Poulpharm Ethical Committee.

III. RESULTS

Two samples of each trial diet were tested for recovery of the respective ionophores, and results were in line with the target levels within the uncertainty range of the laboratory methods. In total, 62 dead birds were observed during the entire period of the study from day 12 to day 23, of which 8 were before the challenge on day 17 and were not related to NE, neither their number was significantly different between different treatments. In total, 54 dead birds were observed after challenge (day17 – day 23) as shown in Table 1. NE related mortality, confirmed by postmortem examination, after the challenge was 0% in the UUC and 48.8% in the IUC, illustrating the successful challenge model reproducing clinical NE. The mortality in the three supplemented groups was not significantly different from UUC, indicating that the three ionophores could successfully prevent clinical NE associated mortality in the same way.

Table 1 - Overview of mortality and zootechnical parameters per treatment group.

Treat-ment	Mortality % d 12-23	Mortality % d 17-23	BW (g) d 12	BW (g) d 17	BW (g) d 22	BW (g) d 23	ADWG (g) d 12-17	ADWG (g) d 17-22	ADWG (g) d 17-23	DFI (g/d) d 12-17	DFI (g/d) d 17-23	FCR 12-17	FCR 17-23
UUC	1.19^{b}	0.00^{b}	370^{a}	702^{a}	1164^{a}	1252^{a}	66.30^{a}	92.60^{a}	93.40^{a}	86.1^{a}	93.70^{a}	1.302^{a}	1.007^{b}
IUC	50.00^{a}	48.80^{a}	367^{a}	696^{a}	958^{b}	924^{b}	65.72^{a}	54.90^{c}	43.40^{c}	81.7^{a}	67.50^{c}	1.242^{a}	1.665^{a}
NAR	13.10^{b}	9.50^{b}	368^{a}	702^{a}	1126^{a}	1154^{a}	67.19^{a}	83.70^{b}	75.10^{b}	79.1^{a}	81.90^{b}	1.182^{a}	1.135^{b}
SAL	7.14^{b}	4.80^{b}	369^{a}	710^{a}	1149^{a}	1155^{a}	68.05^{a}	88.00^{ab}	77.90^{b}	86.2^{a}	85.00^{ab}	1.275^{a}	1.149^{b}
SEM	2.38^{b}	1.20^{b}	371^{a}	700^{a}	1135^{a}	1163^{a}	66.03^{a}	87.30^{ab}	82.00^{ab}	84.4^{a}	89.20^{ab}	1.313^{a}	1.047^{b}

Means with different superscripts are significantly different at $P < 0.05$

BW at day 12 and day 17, as well as ADWG, DFI and FCR for the period between day 12 to 17 were not different among all groups (Table 1). NE challenge at day 17, significantly reduced BW at day 22 and day 23, ADWG and DFI day 17-23 and increased FCR 17-23 in IUC compared to UUC illustrating the impact of NE on zootechnical performance. All the three ionophores were able to mitigate the effect of NE on performance and were not significantly different between each other, with SEM providing best numerical response and was the only treatment that provided same DWG 17-23 as the UUC group.

An overview of the NE mean LSs and frequency (%) is provided in Table 2. Lesions confirmed the successful challenge (mean LS of 5.8 in IUC) with 82.1% of the birds in this group having the

most severe lesion score 6 characteristic for clinical NE. The three ionophores successfully reduced NE LSs compared to IUC with SEM having the lowest frequency of LS 6 (most severe lesions characteristic for clinical NE) compared to NAR and SAL.

Table 2 - Mean NE lesions scores on day 22 and 23 and score frequency distribution per treatment group.

Treat-ment	Mean NE score	SD	P value	Score 0 (%)	Score 1 (%)	Score 2 (%)	Score 3 (%)	Score 4 (%)	Score 5 (%)	Score 6 (%)
UUC	0.1[c]	0.7	<0.001	97.6	0.0	0.0	1.2	0.0	0.0	1.2
IUC	5.8[a]	0.6	Ref.	1.2	0.0	0.0	1.2	6.0	9.5	82.1
NAR	3.5[b]	2.7	<0.001	31.3	0.0	5.0	5.0	6.3	6.3	46.3
SAL	3.0[b]	2.6	<0.001	37.0	0.0	8.6	4.9	11.1	4.9	33.3
SEM	3.3[b]	2.2	<0.001	24.1	0.0	13.3	8.4	22.9	6.0	25.3

Means with different superscripts are significantly different at $P < 0.05$.

IV. DISCUSSION

Induction of NE was successful as high NE-related mortality (48.8%) was induced, combined with high intestinal NE lesions and reduced zootechnical performance in the IUC compared to the UUC group. In-feed supplementation of the three different ionophores: narasin (70 ppm), salinomycin (70 ppm) and semduramicin (25 ppm) led to successful mitigation of NE challenge, demonstrated by mortality, BW and FCR in the respective treatments being significantly improved compared with IUC. Besides the above-mentioned parameters in the SEM group were not significantly different from UUC group. Furthermore, SAL and SEM effect was not statistically significant different from the effect of NAR, though SEM was the only product providing ADWG 17-23 not different from UUC. The above is in contrast with the numerically lower MIC values of NAR compared to other ionophores. Thus, we can make the conclusion that the ionophore supplementation in feed is able to prevent NE in broiler chickens under floor pen conditions. Most probably, this is not only due to a direct effect on Clostridia, but also preventing coccidiosis which is the major predisposing factor for the disease. Moreover, there is no significant difference in this effect between NAR, SAL and SEM with a numerical advantage for the latter, indicating that the numerical differences between the *in-vitro* MIC values of the different ionophores against *C. perfringens* are irrelevant in regard to their efficacy *in-vivo*.

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EFFECTS OF MULTI-STRAIN AND MONO-STRAIN PROBIOTICS ON INTESTINAL HEALTH, INTESTINAL MORPHOMETRICS, PERFORMANCE, AND PROCESSING CHARACTERISTICS OF BROILERS EXPOSED TO INTESTINAL CHALLENGE

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Summary

The current study aims to compare the effects of a four-strain probiotic containing $4x10^9$ CFU/g total viable spores of *B. subtilis*, *B. licheniformis*, *B. amyloliquefaciens* and *B. coagulans* (MicroLife®Prime) and single strain *B. licheniformis* and *B. subtilis* probiotics in a floor pen intestinal challenge model mimicking commercial conditions to infected untreated control (IUC) and uninfected untreated control (UUC). Each of the three probiotics was applied at 4 different levels: $5x10^8$, $10x10^8$, $15x10^8$, and $20x10^8$ CFU/kg feed respectively.
The model successfully mimicked natural intestinal challenge significantly increasing mortality, compromising performance and intestinal health in the IUC compared to UUC. Both mono-strain and multi-strain probiotics provided dose-dependent reduction of inflammation scores, *Salmonella* spp. incidence, oocyst shedding, and *C. perfringens* and *E. coli* counts in caeca as well as improvement in intestinal morphometrics and zootechnical performance. However, the four-strain probiotic provided superior effect for most of the parameters when compared to both mono-strain products, when applied at identical inclusion rate suggesting synergistic effect of the different strains in the multi-strain probiotic, in line with previous findings in different species.

I. INTRODUCTION

Numerous scientific publications and commercial use data indicate the positive effect of *Bacillus* spp.-based probiotics on broilers' intestinal health and performance when exposed to different challenge or non-challenge models. The current study aims to compare the effects of a four-strain probiotic containing $4x10^9$ CFU/g total viable spores of *B. subtilis* NRRL B65574, *B. licheniformis* ATCC PTA122188, *B. amyloliquefaciens* NITE BP01844 and *B. coagulans* ATCC 31284: MicroLife®Prime direct-fed microbial (PRM) and single strain *B. licheniformis* DSM 17236 (BL) and *B. subtilis* C-3102 DSM 15544 (BS) probiotics in a floor pen intestinal challenge model mimicking commercial conditions to infected untreated control (IUC) and uninfected untreated control (UUC). Each of the three probiotics was applied at 4 different levels: $5x10^8$, $10x10^8$, $15x10^8$, and $20x10^8$ CFU/kg feed respectively.

II. METHOD

A total of 8 736 as hatched day-old Ross708 broilers, vaccinated with live coccidiosis vaccine CoccivacB52® were allocated to 14 treatments as per table 1 with 12 replicates each and 52 birds/pen. Birds were placed on used litter known to contain coccidia, *C. perfringens*, *E. coli*, *Salmonella spp*., and other known avian pathogens. Additionally, before birds' placement, litter was supplemented with 100 000 oocysts/bird, primarily *E. acervulina* and *E. maxima*, to mimic natural field enteritis infection. Mortality, body weight (BW), body weight gain (BWG), feed

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conversion ratio (FCR), European Poultry Efficiency Factor (EPEF), and body weight coefficient of variation (BWCV) were measured at 42 days of age. Body weight was measured at 14 days as well. Processing characteristics (prechilled carcass yield, breast yield and. abdominal fat) were measured at 42 days. At 14 and 42 days of age four birds per pen, two male and two female respectively, were humanely sacrificed and scored visually for intestinal inflammation as follows: 0 – no lesions found; 1 – mild hyperemia, but no cell sloughing or mucous; 2- moderate hyperemia and /or mild cell sloughing; 3 – severe hyperemia and/or severe cell sloughing and 4 – actual necrosis or bleeding observed. The same birds were sampled for cecal *C. perfringens* and *E. coli* count and *Salmonella spp*. incidence, as well as for intestinal morphometrics measurements (villus height and crypt depth measurement from an intestinal area few cm anterior to the Meckel's diverticulum). All birds were processed at day 42 and carcass yield, breast yield and abdominal fat were measured.

Test animals were humanely handled. The study was conducted according to Good Clinical Practice and approved by the AHPharma Ethical Committee.

III. RESULTS

The model successfully mimicked natural intestinal challenge significantly increasing mortality, compromising performance and intestinal health in the IUC compared to UUC. An overview of intestinal health parameters is provided in Table 1. Both mono-strain and multi-strain probiotics provided dose-dependent reduction of inflammation scores, *Salmonella* spp. incidence, oocyst shedding, and *C. perfringens* and *E. coli* counts in ceca. However, the four-strain probiotic PRM provided superior effect for most of the parameters compared to both mono-strain BS and BL when applied at identical inclusion rate suggesting synergistic effect of the different strains in the multi-strain probiotic.

Table 1 - Overview of intestinal health parameters: intestinal inflammation lesion scores and pathogen loads.

Treatment		Intestinal inflammation		*C. perfringens* (CFU/g log10)		*E. coli* (CFU/g log10)		*Salmonella* incidence (%)		*Eimeria* spp. OPG log 10	
		d 14	d 42	d 14	d 42	d 14	d 42	d 14	d 42	d 14	d 42
UUC		0.188^{a}	0.283^{a}	2.325^{a}	2.356^{a}	5.312^{a}	5.293^{a}	6.250^{a}	35.833^{abc}	4.401^{a}	4.573^{a}
IUC		1.854^{g}	1.908^{f}	4.850^{e}	4.324^{f}	6.818^{f}	6.706^{e}	89.583^{f}	94.167^{g}	6.681^{i}	6.830^{h}
PRM	500 000	1.146^{cde}	1.350^{d}	3.279^{bc}	3.633^{cde}	6.039^{cde}	6.010^{cd}	39.583^{cd}	40.000^{bcde}	5.724^{def}	5.831^{cd}
PRM	1 000 000	1.083^{bcd}	1.242^{cd}	3.172^{b}	3.439^{cd}	5.748^{bc}	5.736^{b}	37.500^{bcd}	37.500^{bcd}	5.547^{cde}	5.664^{bc}
PRM	1 500 000	1.042^{bc}	1.142^{c}	2.981^{b}	3.088^{b}	5.425^{ab}	5.418^{a}	22.917^{abc}	31.667^{abc}	5.424^{cd}	5.395^{b}
PRM	2 000 000	0.813^{b}	0.850^{b}	2.914^{b}	3.081^{b}	5.315^{a}	5.336^{a}	16.667^{ab}	21.667^{a}	4.826^{b}	4.886^{a}
BL	500 000	1.458^{ef}	1.758^{ef}	3.876^{d}	3.874^{e}	6.310^{e}	6.167^{d}	64.583^{e}	53.333^{ef}	6.292^{h}	6.419^{fg}
BL	1 000 000	1.438^{ef}	1.608^{e}	3.337^{bc}	3.682^{de}	6.164^{de}	6.220^{d}	50.000^{de}	45.833^{cdef}	6.145^{gh}	5.940^{cde}
BL	1 500 000	1.271^{cdef}	1.333^{cd}	3.695^{cd}	3.499^{cd}	6.044^{cde}	6.025^{cd}	56.250^{de}	60.000^{f}	5.892^{efg}	6.129^{defg}
BL	2 000 000	1.250^{cdef}	1.183^{cd}	3.312^{bc}	3.359^{bc}	5.320^{a}	5.508^{a}	35.417^{bcd}	35.000^{abc}	5.295^{c}	5.361^{b}
BS	500 000	1.333^{cdef}	1.650^{e}	3.973^{d}	3.596^{cde}	6.016^{cde}	5.910^{bc}	56.250^{de}	52.500^{def}	5.889^{efg}	6.480^{gh}
BS	1 000 000	1.479^{f}	1.708^{e}	3.703^{cd}	3.596^{cde}	5.886^{cd}	6.084^{cd}	47.917^{de}	51.667^{def}	6.022^{fgh}	6.321^{efg}
BS	1 500 000	1.375^{def}	1.342^{d}	3.656^{cd}	3.600^{cde}	6.071^{cde}	6.067^{cd}	39.586^{cd}	42.500^{bcde}	6.122^{gh}	6.014^{cdef}
BS	2 000 000	1.167^{cdef}	1.308^{cd}	3.348^{bc}	3.331^{bc}	5.450^{ab}	5.475^{a}	22.917^{abc}	29.167^{ab}	5.395^{cd}	5.343^{b}

Means with different letters in the superscripts are significantly different ($P \leq 0.05$), as determined by Duncan's New Multiple Range Test

In line with the improved intestinal health the three probiotics provided significant improvements of the intestinal morphometrics, exhibiting the same pattern: dose-dependent improvement with superior effect from the multi-strain probiotic compared to the single strain ones (table 2).

Table 2 – Overview of intestinal morphometrics measurements.

Treatment	Villus height (µm) d 14	Crypt depth (µm) d 14	Villus / crypt ratio d 14
UUC	1102.6^{a}	268.9^{a}	4.262^{a}
IUC	834.0^{f}	416.0^{f}	2.034^{h}
PRM 500 000	972.8^{e}	391.5^{def}	2.543^{fg}
PRM 1 000 000	995.3^{de}	372.7^{cd}	2.733^{ef}
PRM 1 500 000	1039.0^{bc}	330.7^{b}	3.276^{c}
PRM 2 000 000	1079.2^{a}	290.6^{a}	3.865^{b}
BL 500 000	937.7^{e}	403.2^{ef}	2.454^{g}
BL 1 000 000	987.1^{de}	373.8^{cd}	2.709^{ef}
BL 1 500 000	1013.9^{cd}	352.8^{bc}	2.946^{de}
BL 2 000 000	1048.4^{b}	342.9^{b}	3.149^{cd}
BS 500 000	968.8^{e}	396.4^{def}	2.508^{fg}
BS 1 000 000	984.3^{de}	388.3^{de}	2.630^{fg}
BS 1 500 000	1007.2^{d}	374.2^{cd}	2.744^{ef}
BS 2 000 000	1037.5^{bc}	340.0^{b}	3.143^{cd}

Means with different letters in the superscripts are significantly different ($P \leq 0.05$), as determined by Duncan's New Multiple Range Test.

An overview of zootechnical parameters is provided in table 3.

Table 3 – Overview of zootechnical results and processing characteristics.

Treatment	Mortality (%)	BW (g)	BW (g)	BW CV	BWG (g)	FCR	EPEF	Carcass yield %	Breast yield %	Abdominal fat %
	d 0-42	d 14	d 42	d 42	d 0-42	d 0-42	d 0-42	d 42	d 42	d 42
UUC	1.563^{a}	482^{a}	2847^{a}	11.778^{a}	2790^{a}	1.771^{ab}	371^{ab}	72.45^{bcd}	27.72^{a}	1.75^{bcd}
IUC	9.375^{f}	430^{g}	2571^{g}	17.393^{d}	2516^{h}	1.885^{d}	286^{k}	69.97^{g}	25.14^{f}	1.66^{e}
PRM 500 000	2.257^{ab}	466^{cd}	2731^{def}	13.230^{bc}	2676^{defg}	1.847^{cd}	338^{fgh}	71.66^{def}	26.32^{cd}	1.72^{cde}
PRM 1 000 000	1.910^{ab}	469^{bc}	2771^{bcd}	13.445^{bc}	2715^{bcde}	1.801^{bc}	354^{cde}	72.81^{abc}	25.84^{e}	1.77^{abc}
PRM 1 500 000	1.389^{a}	475^{ab}	2795^{b}	11.902^{a}	2738^{b}	1.773^{ab}	364^{bc}	73.56^{a}	27.25^{b}	1.80^{ab}
PRM 2 000 000	0.868^{a}	481^{a}	2852^{a}	11.961^{a}	2795^{a}	1.741^{a}	381^{a}	73.28^{ab}	27.41^{ab}	1.82^{a}
BL 500 000	4.340^{cde}	444^{f}	2698^{ef}	13.687^{bc}	2642^{fg}	1.862^{d}	323^{ij}	71.39^{ef}	25.41^{f}	1.72^{cde}
BL 1 000 000	3.299^{bcd}	455^{e}	2724^{def}	13.221^{bc}	2668^{efg}	1.844^{cd}	334^{ghi}	70.83^{fg}	26.48^{c}	1.72^{cde}
BL 1 500 000	2.257^{ab}	461de	2751^{bcde}	13.767^{bc}	2695^{bcdef}	1.806^{bc}	349^{def}	72.01^{cde}	26.19^{cde}	1.74^{bcd}
BL 2 000 000	0.868^{a}	463^{cd}	2785^{bc}	11.773^{a}	2729^{bcd}	1.778^{ab}	364^{bc}	73.38^{ab}	26.44^{c}	1.76^{abc}
BS 500 000	4.861^{e}	446^{f}	2692^{f}	14.146^{c}	2636^{g}	1.863^{d}	320^{j}	71.58^{def}	25.36^{f}	1.68^{de}
BS 1 000 000	4.688^{de}	463^{cd}	2738^{cdef}	13.095^{b}	2682^{cdefg}	1.841^{cd}	331^{hij}	70.75^{fg}	26.56^{c}	1.74^{bcd}
BS 1 500 000	3.125^{bc}	469^{bc}	2753^{bcd}	13.512^{bc}	2698^{bcde}	1.804^{bc}	346^{efg}	72.21^{cde}	25.96^{de}	1.73^{bcde}
BS 2 000 000	1.563^{a}	470^{bc}	2789^{bc}	12.082^{a}	2733^{bc}	1.782^{ab}	361^{bcd}	73.02^{abc}	26.61^{c}	1.77^{abc}

Means with different letters in the superscripts are significantly different ($P \leq 0.05$), as determined by Duncan's New Multiple Range Test.

Both multi-strain and mono-strain probiotics provided dose-dependent effect. However, PRM reduced mortality to a level not different from UUC already at $5x10^8$ CFU/kg feed, while BL and BS provided such effect at $15x10^8$, and $20x10^8$ CFU/kg feed respectively. The three probiotic products provided significant improvement of BW compared to IUC already with the lowest inclusion tested. However, at equal inclusion rates PRM provided superior effect compared to BL and BS, and only PRM at $20x10^8$ CFU/kg provided BW not statistically different from the clean control. Same applies for the EPEF. BWCV was also significantly improved by the three probiotics. However, PRM provided CV not different from UUC already at $15x10^8$ CFU/kg, while BL and BS had similar effect, but at a higher inclusion rate ($2.0x10^8$ CFU/kg). Similarly, the three products provided a significant and dose-dependent improvement of FCR in comparison to IUC. Same dose-dependent effect was measured regarding processing characteristics but is worth to mention that only PRM improved breast yield in comparison to IUC with the lowest inclusion of $5x10^8$ CFU/kg feed, while the single strain products provided such effect only at higher levels.

IV. DISCUSSION

As previously observed, multi-strain probiotics inhibit the adhesion and growth of pathogenic microorganisms better than mono-strain probiotics (Chapman et al., 2011). The advantage of multi-strain probiotics comes mainly from bacterial synergistic interactions, which enhance their positive effect on the host (Torres-Miranda et al., 2022). Monteagudo-Mera et al. (2019) reported dose-dependent effect of probiotics in human patients. A similar dose-dependent effect was observed by Alexopoulos et al., (2004) in pigs. The results of the current trial are in line with previous reports demonstrating *Bacillus* spp.-based probiotics dose-dependent improvement of performance, intestinal health and processing characteristics of broilers exposed to intestinal challenge. Besides the overall superior effect of the four-strain probiotic, PRM compared to mono-strain products BS and BL when compared at equal inclusion rates indicates the synergistic effect of multi-strain probiotics in poultry, which is in-line with previously reported data from other species.

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EVALUATION OF A TRIPLE-STRAIN BACILLUS-BASED PROBIOTIC FOR IMPROVING BROILER WELFARE AND PERFORMANCE UNDER HEAT STRESS CONDITIONS

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Summary

Heat stress (HS) is a major challenge in broiler production, significantly impairing welfare, gut health, and productivity. This trial aimed to investigate the potential of a triple-strain Bacillus-based probiotic to alleviate these negative effects in broilers exposed to HS conditions. Results revealed that the probiotic-supplemented birds showed significantly higher gut microbiota diversity and a higher abundance of beneficial SCFA-producing bacteria, contributing to improved nutrient absorption and immune function. Importantly, stress biomarkers were improved in the probiotic group with serum corticosterone significantly reduced by 20% and serotonin levels significantly increased. These improvements translated into superior zootechnical performance, with birds in the probiotic group showing 10% increase in body weight gain and 6% improvement in feed conversion ratio compared to the control groups.

I. INTRODUCTION

Heat stress (HS) is a critical concern in poultry production, particularly in tropical and subtropical regions where elevated ambient temperatures can severely compromise animal welfare and productivity. Broiler chickens, due to their rapid growth and high metabolic rates, are especially susceptible to HS, which leads to physiological disruptions such as oxidative stress, hypothalamic-pituitary-adrenal (HPA) axis dysregulation, and impaired gut integrity. These disturbances often result in reduced performance and elevated mortality rates (Lara and Rostagno, 2013).

A key consequence of HS is the disruption of the gut microbiota, which plays a central role in maintaining overall health and immune function. HS is associated with a reduction in microbial diversity, which is linked to an increase in pathogenic bacteria and a decline in beneficial gut flora such as short-chain fatty acid (SCFA) producers (Rimoldi et al., 2015). The gut-brain axis is also affected, as stress-induced microbial shifts increase corticosterone, a stress biomarker, and decrease serotonin, a key neurotransmitter for well-being and stress resilience (Sobotik et al., 2023).

Conventional methods to mitigate HS, such as antibiotics and feed supplements, are increasingly limited due to concerns over antimicrobial resistance (AMR) and regulatory restrictions. As a result, probiotics have gained attention as a promising alternative, known for their ability to support gut health, enhance immune response, and improve stress resilience. Bacillus-based probiotics, in particular, are favored for their spore-forming capacity, which allows them to survive harsh gastrointestinal conditions, suppress pathogenic bacteria, and boost nutrient absorption (Grant et al., 2018).

Bacillus probiotics also play a role in enhancing antioxidant defenses. Under HS, the production of reactive oxygen species (ROS) increases, leading to cellular damage. Bacillus-based probiotics can mitigate this by increasing antioxidant enzymes like superoxide dismutase (SOD) and catalase, contributing to improved resilience to HS and promoting both welfare and productivity (Yang et al., 2012).

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The current study aimed to evaluate the efficacy of a triple-strain Bacillus-based probiotic in improving welfare parameters in broiler chickens exposed to HS. Key indicators included gut health, stress biomarkers such as corticosterone and serotonin, and zootechnical performance, aiming to present a natural alternative to traditional antibiotic use in poultry production.

II. METHOD

The study was conducted using 720 Ross 308 broiler chickens randomly assigned to one of three treatment groups: (T1) Negative Control (NC), receiving a standard basal diet; (T2) Probiotic group, receiving a basal diet supplemented with a triple-strain Bacillus probiotic (*B. subtilis* DSM 32325, *B. subtilis* DSM 32324 and *B. amyloliquefaciens* DSM 25840, GalliPro® Fit) ($1.6x10^6$ CFU/g of feed); and (T3) Positive Control (PC), receiving a basal diet supplemented with lincomycin. Each treatment had eight replicates of 30 birds, housed in two separate houses (12 pens each). Environmental conditions were monitored and controlled, with feed and water provided ad libitum. HS was induced in one house (32°C for 8-10 hours/day, days 21-35), while the other was kept thermoneutral (NHS, <24°C). The trial followed a randomized factorial design.

Data were collected on days 21, 28, and 35, focusing on: a) Zootechnical Parameters: Body weight, feed intake, and feed conversion ratio (FCR); b) Gut Microbiome Analysis: Fecal samples from 10 birds per treatment on day 35 were analyzed via 16S rRNA sequencing for alpha-diversity (Shannon Index) and beta-diversity; c) Stress Biomarkers: Blood samples from 16 birds per treatment on days 28 and 35 were analyzed for serum corticosterone and serotonin levels using ELISA kits; d) Oxidative Stress Markers: Liver tissue was analyzed for superoxide dismutase (SOD) activity on days 28 and 35. Data were analyzed using ANOVA for zootechnical and biomarker measurements, with microbiome diversity evaluated through principal coordinate analysis (PCoA). Treatment means were compared using Tukey's post-hoc test ($P \leq 0.05$). Gene expression data were normalized using the $2^{-\Delta\Delta Ct}$ method (Livak et al., 2001).

III. RESULTS

a) Zootechnical Performance

The probiotic group (T2) showed a significant improvement in growth under heat stress (HS) compared to the negative control (T1) (Table 1). At day 35, probiotic-fed birds had 10% higher body weight gain (BWG) ($P \leq 0.05$) and better feed conversion ratio (FCR) (1.38 vs. 1.45, $P \leq 0.05$). Under thermoneutral (NHS) conditions, the probiotic group showed numerical improvements.

Table 1 - Body weight in gram, FCR of non-heat-stressed and heat-stressed broilers at day 35.

	NHS		HS	
Treatment group	Body Weight (g)	FCR	Body Weight (g)	FCR
Negative control	2308 [a]	1.44	2228[a]	1.45 [a]
Probiotic	2416 [b]	1.38	2304 [b]	1.38 [b]
Positive control	2352 [a]	1.43	2287 [a]	1.44 [a]

For different letter in the same column: $P \leq 0.05$.

b) Gut Microbiome Composition

HS significantly reduced gut microbiota alpha-diversity in the control group ($P \leq 0.05$) (Figure 1), while probiotic supplementation maintained a higher alpha-diversity index compared to both control groups ($P \leq 0.05$) (Figure 2). Probiotic-fed birds exhibited a higher abundance of beneficial SCFA-producing bacteria, like *Faecalibacterium* and *Turicibacter*, while reducing pathogenic bacteria, such as *Clostridium* and *Salmonella*. Beta-diversity analysis showed distinct microbiome clustering in the probiotic group under HS, indicating enhanced gut stability.

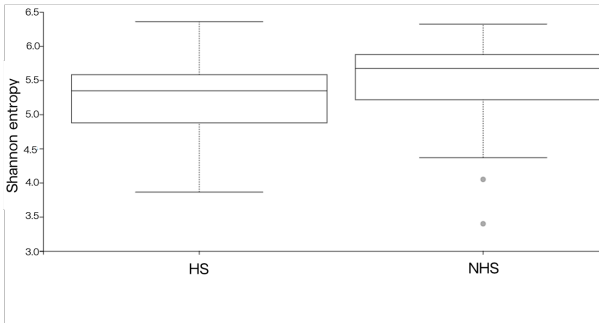

Figure 1 - Lower Alpha-diversity in heat-stressed broilers compared to broilers raised in thermoneutral conditions at day 35.

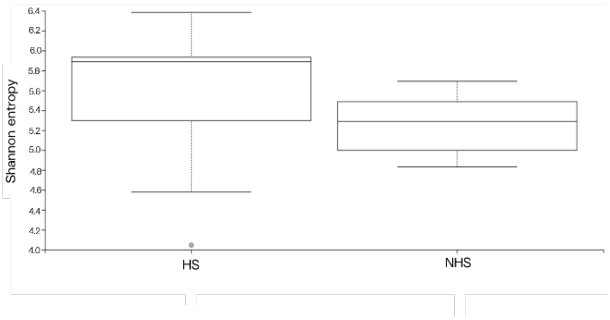

Figure 2 - Higher Alpha-diversity in heat-stressed broilers fed with the probiotic compared to the control group at day 35.

c) Stress Biomarkers

Serum corticosterone levels were significantly elevated in the negative control group under HS (Table 2), while the probiotic group showed reduced levels at days 28 and 35 ($P \leq 0.05$), indicating lower stress. Probiotic-fed birds also exhibited significantly higher serotonin levels (Table 3), reflecting improved well-being ($P \leq 0.05$).

Table 2 - Heat-stressed broilers fed the probiotic demonstrate lower levels of corticosterone (x10mg/ml) at day 28 compared to NC and at day 35 compared to NC and PC.

	NHS		HS	
Treatment group	Corticosterone (x10 mg/ml) at D28	Corticosterone (x10 mg/ml) at D35	Corticosterone (x10 mg/ml) at D28	Corticosterone (x10 mg/ml) at D35
Negative control	6.04[a]	7.96[a]	17.11[a]	15.45[a]
Probiotic	3.76[b]	2.13[b]	7.82[b]	9.91[b]
Positive control	3.26[b]	6.38[a]	12.79[a]	13.47[a]

For different letter in the same column: $P \leq 0.05$.

Table 3 - Daily feeding of probiotic results in higher concentrations of serotonin serum level (ng/ml) compared to NC and PC at day 35 under heat-stressed.

	NHS		HS	
Treatment group	Serotonin (ng/ml) at D28	Serotonin (ng/ml) at D35	Serotonin (ng/ml) at D28	Serotonin (ng/ml) at D35
Negative control	23.77	13.86[a]	19.80	11.82[a]
Probiotic	26.69	20.38[b]	19.84	19.98[b]
Positive control	24.11	16.21[a]	20.09	15.36[a]

For different letter in the same column: $P \leq 0.05$.

d) Oxidative Stress

Probiotic supplementation led to significantly higher superoxide dismutase (SOD) activity in liver tissue under HS ($P \leq 0.05$) (Table 4), suggesting enhanced antioxidant defenses against oxidative stress.

Table 4 - Probiotic fed-birds demonstrate a high antioxidant defense potential against heat stress (oxidative stress factor) with a higher SOD activity observed in liver at day 28 and day 35 compared to NC and PC.

	NHS		HS	
Treatment group	SOD activity (U/mg) at D28	SOD activity (U/mg) at D35	SOD activity (U/mg) at D28	SOD activity (U/mg) at D35
Negative control	1.08[a]	1.19[a]	0.49[a]	0.59[a]
Probiotic	1.34[b]	2.08[b]	1.10[b]	1.30[b]
Positive control	0.80[a]	1.24[a]	0.43[a]	0.64[a]

For different letter in the same column: $P \leq 0.05$.

e) Overall Welfare and Productivity

The trial demonstrated that this triple-strain Bacillus-based probiotic significantly improved broiler welfare and productivity under HS by maintaining gut health, enhancing antioxidant defenses, and reducing stress biomarkers. These benefits collectively resulted in better overall performance and well-being.

IV. DISCUSSION

This study highlights the efficacy of this triple-strain Bacillus-based probiotic in mitigating the negative impacts of HS on broiler welfare and performance. Probiotic supplementation resulted in significant improvements in gut microbiome diversity, stress biomarkers, and overall zootechnical performance.

The improved microbial diversity in the probiotic-fed birds supports the role of these probiotics in maintaining gut health under stress. Heat stress often causes gut dysbiosis, characterized by a reduction in beneficial bacteria and an increase in pathogenic species (Rimoldi et al., 2015). In this trial, probiotic supplementation promoted the abundance of SCFA-producing bacteria such as *Faecalibacterium* and *Turicibacter*, enhancing gut health and immune function (Yang et al., 2012). These changes likely contributed to the observed improvements in body weight gain and feed conversion ratio, as gut microbiota health is closely linked to nutrient absorption and energy utilization (Grant et al., 2018).

A critical outcome was the modulation of stress biomarkers. Lower corticosterone levels in the probiotic group indicate reduced physiological stress, while higher serotonin levels suggest enhanced mood and well-being. This supports findings that probiotics can influence the gut-brain axis, thereby modulating the body's response to stress (Sobotik et al., 2023). Probiotics have been shown to reduce stress-induced hormonal imbalances by stabilizing the gut microbiome, which plays a key role in neurotransmitter production, including serotonin (Cryan and Dinan, 2012).

Furthermore, the increased superoxide dismutase (SOD) activity in probiotic-fed birds highlights the enhancement of antioxidant defenses, crucial for mitigating oxidative stress under HS conditions. Heat stress elevates reactive oxygen species (ROS), leading to cellular damage and inflammation (Yang et al., 2012). The probiotic's ability to boost antioxidant enzyme activity, such as SOD, aligns with previous findings that Bacillus probiotics improve oxidative stress resistance in poultry (Chen et al., 2020). In conclusion, supplementation with this triple-strain Bacillus-based probiotic significantly mitigated the effects of HS in broilers, improving gut integrity, reducing oxidative stress, and modulating stress biomarkers. These physiological benefits translated into improved growth performance and welfare under heat stress conditions. The findings support the use of this Bacillus-based probiotic as a viable, natural alternative to antibiotics, enhancing both welfare and productivity in poultry production systems.

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ENTEROCOCCUS FAECIUM INSIGHTS INTO HOST-MICROBIOME EFFECTS AND POTENTIAL FOR CLINICAL DISEASE IN BROILERS

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Summary

The present abstract presents insights from a broiler trial unintentionally challenged with *Enterococcus* spp. in Belgium and a field isolate of *Enterococcus* from a Malaysian commercial broiler farm, experiencing clinical *E. faecium* disease. The Belgian study highlights the effect of *E. faecium* colonisation on intestinal health. The second study in this abstract used genomic and classic microbiology to elucidate how an *Enterococcus* species seen as typical more commensal can pose varied risks to commercial poultry flocks.

I. INTRODUCTION

As a result of the growing antimicrobial resistance threat, and regional usage bans, like in Europe for example, the interaction between microbiota and host is increasingly a focus of studies. Enterococci are commonly included as known (Werner, *et al.* 2008) vectors for spreading AMR genes within related species. The present two studies looked at separate incidences where *Enterococcus* presented clinical disease, as well as on characterisation of the causative pathogen.

The first study (BE1) was part of a group of trials which aimed to investigate potential markers of intestinal health "HEPPY Markers project" (VLAIO, 2024). Following a pre-trial, this broiler experiment was performed in Belgium, monitoring all male broilers for 5 weeks. All birds in the study BE1 were showing clinical signs of infection, ranging from poor weight gain, lameness, to wet litters and increased mortality. The birds were unintentionally infected with *Enterococcus spp*. Because of this infection, the trial was repeated (BE2) and provided the opportunity to compare naturally occurring *Enterococcus infection with uninfected* broiler birds in an otherwise similar setup.

The aim of the 2nd study (SG1) was to characterise the genomic and phenotypic properties of an *Enterococcus* sp., isolated from the gizzard of clinically diseased broiler breeders from a commercial farm with recurrent clinical disease, showing similar clinical signs to the Belgian trial BE1. Literature describes *E. faecium* both as a potential probiotic (Ghattargi *et al. 2018*), but also as a risk for harbouring and distributing antibiotic resistance (Makarov *et al.* 2022). Thus, the second (SG1) study emphasised its pathogenicity and putative antimicrobial resistance mechanisms. Whole-genome sequencing was performed to identify virulence factors, antimicrobial resistance genes (ARGs), and mobile genetic elements.

While both cases were unrelated, some relevant connections between both studies could be observed, prompting this combined abstract rather than two separate ones.

II. METHOD

For the Belgian (BE1) study, each group had 8 pens containing 20 broilers respectively, monitored until day 35. All birds were fed the same diets. Intestinal samples were taken from sacrificed individual birds at multiple timepoints and included ileal tissue and content. In *vivo*

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gut permeability was assessed using FITC-dextran-4kDa. Further analyses included performance measurements, microbiota (V1-V9 16S rRNA sequencing), gut morphology, and ileal gene expression (high-throughput qPCR analysis). The repeat study (BE2) had an identical set-up for all factors that could be controlled, such as using exact same location, with the same feed, bird genetics and hatchery origin, the only intentional difference in the trials being the absence of the accidental *Enterococcus* infection. Performance, permeability and morphology data were analysed using ANOVA in JMP Pro software (version 17, SAS Institute Inc, Cary, NV). Linear Discriminant Analysis (LDA) effect size (LEfSe) analysis was applied with a minimum LDA score of 4 to identify taxa and genes that were significantly differentially expressed between groups.

The second study (SG1) pathogen was isolated from a recurring field outbreak, suspected to be caused by a *Pasteurella* sp., which could not be resolved by antibiotic treatments. Initial colony morphology and later whole-genome sequencing identified it as *Enterococcus faecium* PM1. Virulome analysis was undertaken but of no further relevance to the present abstract. Mobilome analysis identified plasmids using PlasmidFinder (v2.1, CGE server) and PLSDB (v2023_11_03_v2), both under default parameters. The identification of antibiotic resistance genes (ARGs) and their locations were conducted using the Resistance Gene Identifier (RGI, v.6.0.3) and ResFinder (v4.4.3, CGE server), both under default parameters. For antibiotic susceptibility testing, a single colony of *E. faecium* PM1 was inoculated into a 15 ml tube containing 10 ml of Tryptic soy broth with 0.6% yeast extract (TSBYE; Oxoid Limited, England) and incubated at 37 ℃ for 18-20 h; then diluted to a final concentration of 10^5 to 10^6 colony forming unit (CFU) per ml into corresponding test wells of 96-well plate. Stocks of antibiotics were prepared, diluted serially and aliquoted into respective wells and the plates were incubated for 24 h at 37℃.

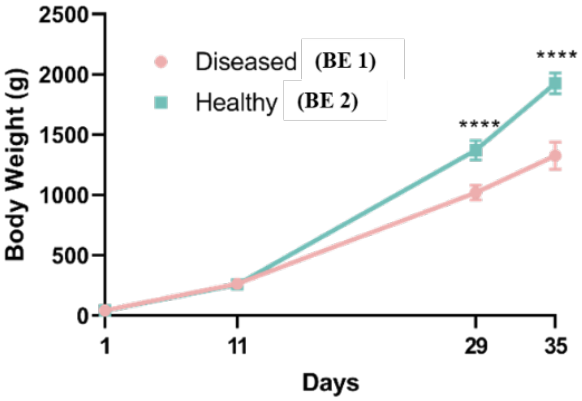
The mobilome analysis revealed the presence of four plasmids in *E. faecium* PM1. As for the resistome analysis, the PM1 chromosome was found to harbor four ARGs (i.e., *aac(6')-Ii*, *efmA*, *msr(C)* and *vanY-B*). In contrast, the plasmid pPM1_1 contained three ARGs (i.e., *ant(6)-Ia*, *lnu(B)* and *lsa(E)*) (Table 3). Notably, all the three ARGs on pPM1_1 were associated with transposable elements *cn_43879_ISEfa4* and *cn_32360_IS256*.

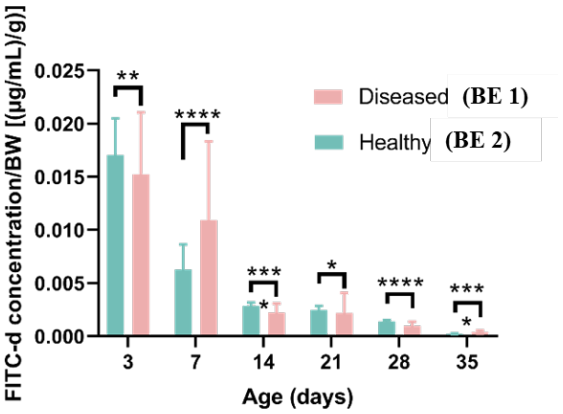
III. RESULTS

The infected trial (BE1) showed differences in metabolism and nutrient transport gene expression, when compared to the repeat trial (BE2), as well as severely diminished performance parameters, while no bird from study B1 appeared to be completely unaffected by the exposure to *E. faecium*.

Strains isolated from the knee joints and spinal fluid of infected birds were identified as *E. faecium*. Healthy birds, from study BE2 had higher body weights (Figure 1) and had a higher villi height to crypt depth ratio on days 21 to 35, compared to birds from study BE1.

Diseased birds, which includes all birds of study BE1, had a significantly higher gut permeability on day 7 relative to healthy birds (Figure 2). Furthermore, comparing healthy (BE1) with unaffected birds (BE2), there was a clear lower number of differentially abundant species, and a higher abundance of Enterobacteriaceae and Enterococcus species in the diseased animals. *E. faecium strain PM1* was found in study SG1 and genetically analysed for the presence of ARGs (table1).





P value <0.0001 = ****; 0.0001 to 0.001 = ***; 0.001 to 0.01 = **; 0.01 to 0.05 = *; 0.05 ≥ ns

Figure 1 - Comparative growth g/day for infected (BE1) und uninfected (BE2) trials.

Figure 2 - Intestinal integrity for infected (BE1) and uninfected (BE2) trial.

Table 1 - The MIC value and predicted antimicrobial resistance genes (ARGs) in *Enterococcus faecium* strain PM1.

Antibiotic/Antimicrobial	MIC (µg/ml)		Predicted AR	Resistance Mechanism
	EUCAST (v14.0)	PM1		
Amoxicillin	8	<1	No hit	
Ampicillin	4	8	*pbp5**	Chromosomal mutations
Bacitracin Zinc Salt	32	1024	No hit	
Chlortetracycline	4	<1	No hit	
Ciprofloxacin	8	N/A	*gyrA*, parC*	Chromosomal mutations
Clindamycin	-	N/A	*lnu(B)***	Antibiotic inactivation
	-	N/A	*lsa(E)***	Antibiotic target protection
Erythromycin	4	N/A	*msr(C)**	Antibiotic target protection
	4	N/A	*efmA**	Antibiotic efflux
Gentamicin	32	*N/A*	*aac(6')-Ii**	Antibiotic inactivation
Novobiocin Sodium Salt	N/A	<1	No hit	
Oxacillin	-	64	N/A	
Rifampicin	-	2	No hit	
Spectinomycin	-	>2048	No hit	
Streptomycin Sulfate	128	2048	*ant(6)-Ia**	Antibiotic inactivation
Tylosin Tartrate	-	2	No hit	
Vancomycin	4	2	*vanY* gene	Antibiotic target alteration

*The ARGs that predicted to locate in PM1 chromosome; **The ARGs that predicted to locate in pPM1_1; N/A - Data not available (not tested).

IV. DISCUSSION

E. faecium as found in the SG1 study is commonly found in the gastrointestinal tract of both humans and animals. Despite increasing number of reports of *Enterococcus*-associated disease in French poultry over the past 20 years according to Souillard *et al.* (2022), the incidence of *Enterococci* in poultry farming is not well described in literature. It has gained increasing attention as a significant opportunistic pathogen in both healthcare (Kim & Cha, 2021) and agriculture settings due to the potential to harbour antibiotic resistance (Makarov *et al.* 2022). SG1 revealed that full sequencing of field strains might lead to unanticipated species identifications.

Both studies showed *E. faecium* may have detrimental effects on broilers leading to clinical diseases. While this is anticipated in *E. cecorum* this is not usually the case in *E. faecium*. The presence of multiple AMR genes was found in the *E. faecium* isolated in SG1. Though it cannot explain the outbreak in this study, it confirms previously noted antibiotic treatment failures at the farm.

The occurrence of poultry pathogenic *E. faecium* in trial poultry farms in Belgium and Malaysia confirms the issue is globally present as suggested by Souillard *et al.* (2022). The Belgian study should ideally be repeated under identical conditions, to confirm how much of the differences between BE1 & BE2 were truly due to the *E. faecium* infection. The widespread antibiotic resistance in the Malaysian strain suggests new control or prevention might be needed for *E. faecium*, as current antibiotic strategies might not be sufficient to control the disease.

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EVALUATION OF BCO LAMENESS INCIDENCE IN *STAPHYLOCOCCUS AUREUS*-CHALLENGED BROILERS EARLY SPRAYED WITH AN *ENTEROCOCCUS FAECIUM*-BASED PROBIOTIC

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Summary

Bacterial chondronecrosis with osteomyelitis (BCO) lameness remains one of the biggest problems of today's broiler producers worldwide. Intense body weight gains associated with bacterial translocation from compromised gut functions favor the appearance of microfractures in the leg bones leading to lameness and eventual death. Carcass condemnations as well as compromised animal wellbeing result in heavy economic losses for the industry. Therefore, further research into preventative measures of BCO lameness in broiler chickens prevails. This study aims at assessing the impact of the spray of 2 different doses (low and high) of a commercial *Enterococcus faecium*-based probiotic (*E. faecium* - 669) on day-of-hatch on BCO lameness incidence, using the *Staphylococcus aureus* (*S. aureus*) drinking water challenge model ($1x10^5$ CFU/mL) at five days of age (d5). 720 Cobb 500 male chicks were allocated to 12 isolated chambers, making up four different treatments with three chambers each, at an initial density of 60 chicks/pen from d0-13 and reduced to 50 chicks/pen from d14 onward. Experimental treatments were as follows: T1 as Negative control (NC) – No probiotic spray, no challenge; T2 as Positive control (PC) – No probiotic spray + d5 *S. aureus* challenge; T3 as Probiotic Low dose (L) - d1 probiotic spray at $4x10^8$ CFU/chick + d5 *S.aureus* challenge; T4 as Probiotic High dose (H) - d1 probiotic spray at $2x10^9$ CFU/chick + d5 *S.aureus* challenge. Total cumulative lameness incidence at the end of the study on d57 shows that T2 (PC) had the highest percentage of lame birds at 58.00%, followed by T3 (L) with 36.00%, T4 (H) with 28.67%, and T1 (NC) with 25.33%, respectively, with a significant difference between T2 and T4 ($P < 0.0001$). As a result, early application of the *Enterococcus faecium*-based probiotic successfully mitigated lameness incidence in birds challenged with *S. aureus* compared to NC birds with the high product dosage further reducing an additional 7.33% in cumulative lameness compared to the low dosage. More research is needed to further understand the mode of action and effectiveness of this probiotic in addressing BCO-induced lameness. Nevertheless, the *S. aureus* challenge model utilized in this study demonstrates that early application of an effective probiotic in commercial broilers may support normal gut functions and therefore, sustainably improve animal welfare and performance through lowered incidence of lameness.

I. INTRODUCTION

Bacterial chondronecrosis with osteomyelitis (BCO) lameness is one of the major health challenges currently faced by the poultry industry, affecting approximately 3 to 15% of commercial broilers (Wideman et al., 2016). BCO lameness is thought to occur as bacterial translocation from the broiler's respiratory system and compromised gastrointestinal tract (GIT) through the blood is deposited in microfractures in the leg bones of the broiler as the animal grows and accumulates body weight, leading to bacterial colonization of the growth plate and eventual necrosis – which often results in the broiler's death due to an inability to

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access feed and water from the lameness that ensues (Wideman et al., 2016). The modern broiler is highly susceptible to this disease, with far-reaching body weight gain rates related to intensive genetic selection outstripping leg bone development and inducing severe shear stress on the latter. Several bacterial genera are associated with this disease, including Enterococcus and Staphylococcus commonly found within lameness lesions (Alrubaye et al., 2020). Interestingly, *Staphylococcus aureus* shares the same prevalence in association with osteomyelitis in humans (Wideman et al., 2012), which also suggests a potential translational model of high human medical importance. The use of *Enterococcus faecium* bacteria strain as a prophylactic probiotic with a wide range in its mode of action has been widely studied, with suggested positive outcomes relating to broiler performance and intestinal health of young chicks against pathogens (Huang et al. 2019). Additionally, administration routes of probiotic *E. faecium* strains in most studies (and in the industry) remain as either a feed component, in drinking water (El-Sawah et al., 2020), or via *in ovo* vaccination (Castañeda et al., 2020, Skjøt-Rasmussen et al., 2019). The aim of this study is to evaluate the impact of *two E. faecium* probiotic concentrations, administered via a controlled spraying system on newly hatched chicks, on cumulative BCO lameness incidence over 56 days using an isolated *S.aureus* challenge model.

II. METHOD

Cobb 500 male chicks were placed in pens in each of 12 completely isolated environmental chambers, on wood shavings, at a density of 60 chicks per pen on the first day of the experiment (d1) and culled down to 50 birds per pen on d14. Three pens, each representing a treatment replicate, were allocated to each of the four treatments involved in the study as follows: T1: Negative control (NC) – No probiotic spray, no challenge; T2: Positive control (PC) – No probiotic spray + d5 *S.aureus* challenge; T3: Probiotic Low dosage - d1 probiotic spray at $4x10^8$ CFU/chick + d5 *S.aureus* challenge (L); T4: Probiotic High dosage - d1 probiotic spray at $2x10^9$ CFU/chick + d5 *S.aureus* challenge (H). As all 12 chambers were completely isolated from one another, pen and block randomization were not conducted. All birds received industry standard formulated starter (crumbles) and grower (pellets) diets and had access to clean water and feed ad libitum. The probiotic containing an *E. faecium* strain (*E. faecium* - 669) was a commercially available product (GalliPro® Hatch, Chr. Hansen A/S, Denmark). Boxes of 60 chicks were manually spray-vaccinated with multiple passes until exhaustion of a set volume per concentration (Low dosage = 150 mL/60 chicks; High dosage = 75 mL/60 chicks). Non-toxic blue food dye was added to each prepared stock to aid in visualization of vaccine dispersion on chicks. On d5 of the study, except for three negative control (NC) chambers, birds in all remaining treatment chambers received a *S. aureus* challenge in their drinking water. Glycerol stock *S. aureus* strain used in the study was revived, incubated for 24 hours with viable CFU concentration determined, and diluted to a final concentration of 1.0×10^5 CFU/mL. All challenged pens received the bacterial water challenge on d5 until exhaustion of the carboys, after which the water source was switched back to clean water. Starting from d22 of the study, daily clinical lameness in each pen was evaluated by gently encouraging the birds to walk brief distances. Birds that were reluctant to walk or incapable of walking were diagnosed as clinically lame, euthanized, and necropsied to assess BCO lesions on femoral and tibial heads per Wideman et al. 2012. Data was entered and processed using Microsoft® Excel (Microsoft Corporation, Redmond, WA, US) from which cumulative lameness incidence over time and lesion categories per treatment (expressed in percentages) were calculated and plotted. Total cumulative lameness data was prepared separately, followed by binomial generalized regression (or generalized linear model) analysis using JMP® Pro v17.1 (SAS Institute Inc., Cary, NC). All statistical significance was determined at $\alpha < 0.05$.

III. RESULTS AND CONCLUSIONS

There were no apparent trends for lesion severity between right and left legs in both femoral and tibial lesions, nor was there a dominating trend between treatments in severity reduction. Exhibition of clinical lameness first appeared in the NC group on d26, but not in all treatments until d35 of the study. Cumulative lameness incidence per treatment (in percentage) from d35-57 of the study is presented in **Figure 1**.

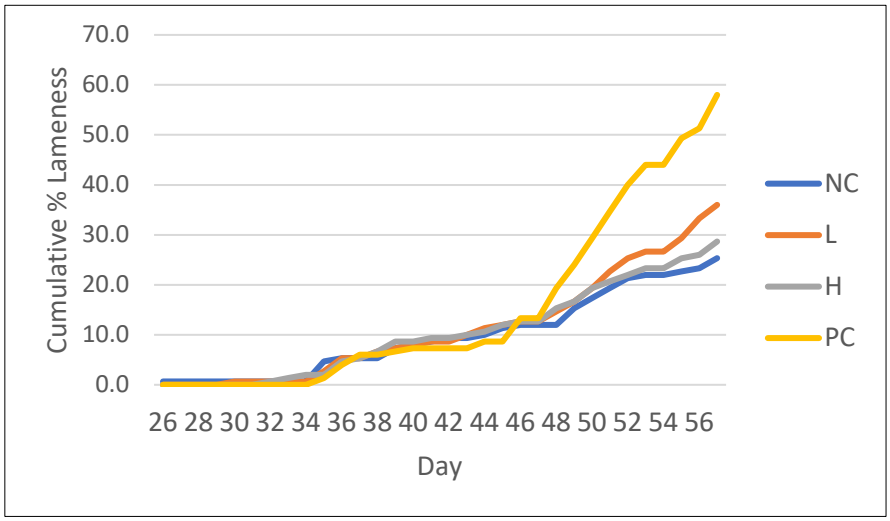

Figure 1 - Cumulative percent lameness by treatment groups from d35-57 of the study.

Cumulative lameness incidence trends between all treatments remained largely similar until d46-47 of the study, following which incidence rate in the positive control (PC) group increased sharply and continued to do so until the end of the study, peaking at 58% and followed by the LOW (L; 36%), HIGH (H; 28.67%), and negative control (NC; 25.33%) groups. In summary, this study was conducted to assess the impact of spraying a probiotic, composed of a specific *Enterococcus faecium* strain on day-old broiler chicks at hatch on the incidence of clinical BCO lameness using a *S. aureus* bacterial challenge model in drinking water. Cumulative lameness incidence at the end of the study was significantly higher in the challenged and untreated PC group compared to remaining treatments (58.00%; $P < 0.05$). Treated and challenged Low and High groups saw no significant differences compared to untreated and unchallenged NC group (36.00%, 28.67%, and 25.00%, respectively; $P \geq 0.05$), suggesting effective mitigation of BCO lameness incidence with application of the probiotic strain by establishing robust gut microbiome foundations and supporting normal intestinal functions and integrity, with a higher dosage able to further reduce cumulative lameness incidence by 7.33% compared to the low dosage. While further research is needed to further understand the mode of action and effectiveness of this *Enterococcus faecium*-based probiotic, the challenge model utilized in this study suggests that early application of an effective probiotic to day-old chicks could contribute to the attenuation of BCO lameness, and thus, to a more sustainable broiler production.

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EFFICACY OF BACILLUS SUBTILIS PB6 ON BROILER PERFORMANCE AND GUT HEALTH IN MITIGATING NECROTIC ENTERITIS

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Xylanase and *Bacillus subtilis* PB6 are promising feed additives in broiler nutrition. However, their effects, particularly the combined impact of *B. subtilis* PB6 and xylanase on necrotic enteritis (NE) incidence in corn-based diets, remain inadequately explored due to inconsistent results and limited data. This study assessed the effects of xylanase (Xy) (xylanase, 3000U/kg) and *Bacillus subtilis* PB6 (Pb) (*Bacillus subtilis*, 2. 2 x 10^8 CFU/g) on broiler performance and gut health with a corn-soybean diet. A total of 630 Cobb 500 mixed-sex broiler chicks were randomly assigned to a 2 × 2 + 1 factorial arrangement with 9 replicates of 14 birds each. Treatments included: NE challenge without additives (CC), NE challenge with Xy (0.03%) (CC+Xy), NE challenge with Pb (0.05%) (CC+Pb), NE challenge with Xy (0.03%) and Pb (0.05%) (CC+Xy+Pb), and non-challenge birds without additives (NC). Before the NE challenge (d0-8), NC and CC were collectively considered as a control group. Subclinical NE was induced by gavaging *Eimeria* species on day 9 and *Clostridium perfringens* on d 14 and 15. The body weight and feed intake were measured on d 8, 19, and 35 to calculate performance parameters. On d16, serum fluorescence isothiocyanate dextran (FITC-d) marker and jejunal histomorphology were evaluated. Data were analyzed using JMP 14.0 and significance was determined at $P < 0.05$ by the Tukey HSD test. Significant Xy × Pb interactions were observed in feed conversion ratio (FCR; $P < 0.05$) and body weight gain (BWG; $P < 0.01$) before challenge (d0-8). It was shown that the extent of Pb efficacy for reducing FCR was highest when Xy was not supplemented. However, Xy increased BWG ($P < 0.01$) without the presence of Pb, but did not make any difference when supplemented with Pb. During the NE challenge period, (d9-19), all challenged birds had lower BWG ($P < 0.05$) and higher FCR ($P < 0.05$) compared to the NC birds. In the finisher (d20-35) and overall study period (d0-35), the presence of Pb in the diet reduced ($P < 0.05$) feed intake and FCR without affecting BWG. A significant Xy×Pb interaction ($P < 0.05$) was observed for villus height (VH), and the VH to crypt depth (CD) ratio. Supplementation of Pb alone enhanced VH and VH:CD ($P < 0.05$), however, these improvements were not observed when Pb was supplemented with Xy. NE challenge significantly increased the serum FITC-d ($P < 0.05$) concentration in CC birds compared to the NC birds. Supplementing additives in the challenged broiler diets shifted the FITC-d level towards the NC birds from CC birds showing no difference from both NC and CC birds. This finding suggests that dietary Pb significantly enhances performance and gut health in NE-affected broilers, as evidenced by improved FCR, jejunal VH, VH:CD, and shifted gut permeability. Furthermore, the results indicate that the efficacy of probiotics on growth parameters and intestinal morphology might be influenced by the presence of xylanase. These observations underscore the need for further research to clarify the mechanisms behind these interactions and optimize dietary formulations.

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ESTABLISHING AN *IN VITRO* FERMENTATION MODEL FOR CHICKEN CAECAL INOCULA

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The caecum is the primary site of fermentation in chickens, where undigested dietary components are utilized by microorganisms, contributing to gut health and growth rate (Ali et al., 2022). However, there is limited knowledge on how these undigested components affect gut microbiota, fermentation products, and ultimately performance. *In vivo* trials involving gut microbiota are costly and may be influenced by host and external environments. To address this, the present study established an *in vitro* anaerobic fermentation model that could be used to study microbiota composition and metabolic products.

This model was used to evaluate the fermentation capacity of caecal samples from 7- or 42-day-old chickens using 10 carbohydrate substrates, including (in vitro) digested wheat and wheat bran with different particle sizes, FOS (fructoligosaccharides), wheat starch, maize starch, arabinoxylan, and beta-glucan, representing a range of expected fermentabilities. It was hypothesized that the model would show the affinities of caecal inocula for specific substrates and that these preferences would differ with bird age. Caecal samples from chickens fed a standard commercial starter or grower diets (for 7- or 42-day-old, respectively) were diluted and filtered. The inocula (2.5mL) were transferred to bottles containing different substrates and basal medium (43 mL). All procedures were performed anaerobically with 5 replicates for each substrate. *In vitro* fermentation was conducted at 40 ℃ for 48 h, using the Automated Trace Gas Recording System (AGRS-III, China Agricultural University, China), where gas production is reported as the cumulative volume per gram of dry matter (DMCV). The end-point pH and short chain fatty acid (SCFA) production was also analysed (Yao et al., 2023).

The results demonstrate that the *in vitro* method was suitable for the study of both ages of chickens. The DMCV values showed that although high levels of gas were produced for both 7- and 42-day-old chickens, the order of fermentability of the substrates was different. Arabinoxylan was the most fermented by the 42-day-old chicken inocula (97.59±5.84 mL/gDM) and wheat bran was the least fermented (17.91±5.21 mL/gDM). Whereas for 7-day-old chicks, arabinoxylan produced only 35.43±5.61 mL/gDM of gas, but maize starch produced the most gas with 105.92±11.91 mL/gDM. These differences in fermentability suggest variations in microbiome composition and abundance between age groups. The relative proportions of SCFA produced by each substrate also varied with inocula. This was particularly apparent for FOS, resulting in lower propionate than butyrate releases in 42-day-old chickens (0.68±0.04 mmol/g and 1.73±0.07 mmol/g, respectively). In contrast, 7-day-old, produced similar amounts of both metabolites (0.58±0.08 and 0.67±0.06 mmol/g, respectively). This also suggests that different bacteria in each inoculum were responsible for degrading the substrate.

In conclusion, an *in vitro* fermentation model has been developed, and is suitable for 7- or 42-day-old chickens. The model was able to show the different relative fermentation by caecal microbiota in both ages. The model reported was successful as a proof-of-concept for future studies assessing the influence of feed on microbiome, including its resilience and relative fermentation profiles.

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POSITIVE IMPACT OF A TRIPLE STRAIN BACILLUS-BASED PROBIOTIC ON INTESTINAL BIOMARKERS AT 7 DAYS OF AGE

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Summary

Broiler chicks undergo significant changes in their first week of life, impacting their long-term performance. Probiotics have shown to support chicks during this critical period. This study evaluated the comparative effects of feed supplements, specifically an antibiotic growth promoter (Lincomycin) and a triple-strain bacillus-based probiotic (Prob_3S), in the early life at 7 days of age. Three treatment groups of 200 male chicks (control-CTRL, lincomycin-LINCO, and Prob_3S) were observed to assess growth performance, intestinal morphology, and intestinal biomarkers. The 200 Ross 308 male broilers were evaluated over 7 days, focusing on growth, duodenal and jejunal tissue histology (Villi height/Crypt depth ratio), gene expression of intestinal integrity markers (MUC2, OCCUD, JAM), and TLR4 and IL-2 gene expression. Prob_3S significantly affected the expression of immunological and epithelial junctional proteins (TLR4, IL-2, IL-6, MUC2, OCCUD, VEGF, JAM) in the jejunum compared to CTRL and LINCO groups ($P<0.05$), suggesting a stronger interaction between Prob_3S and the gut environment in this area. Prob_3S also improved feed efficiency and numerically increased body weight gain at day 7, significantly reducing ($P<0.05$) FCR compared to CTRL and LINCO. These findings demonstrate that a triple-strain bacillus probiotic can positively affect key intestinal biomarkers, histology, and FCR by day 7, suggesting that Prob_3S could enhance chick quality, minimize losses, and support overall health and performance.

I. INTRODUCTION

The early development of young chickens is crucial for maximizing poultry production efficiency. During the initial life stages, the chick's digestive, immune, and other physiological systems mature rapidly. Support in this period lays a foundation for improved health, growth, and productivity, leading to better feed efficiency, faster growth rates, and stronger disease resistance. These early advantages are key to economic success in poultry farming, as healthier birds yield higher productivity and lower costs related to medical treatments and mortality.

First-week mortality (FWM) is widely recognized as a key performance and welfare indicator in poultry production (Yerpes et al., 2020). Mortality during this critical phase signifies a loss of revenue potential and reflects the quality of the neonatal chick batch, making it an important predictor of flock performance during the rearing period (Yassin et al., 2009). The administration of a three-strain Bacillus probiotic has shown promise in reducing FWM, thereby improving chick quality—a critical factor in subsequent flock performance (Fairchild, 2005; Yassin et al., 2009). Infectious and non-infectious factors are known contributors to early mortality in broiler chickens (Yerpes et al., 2020), and the intestinal tract, which undergoes

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rapid development within the first week post-hatch, plays a central role in balancing nutrient acquisition with protective barrier functions (Wijtten et al., 2012).

Therefore, early-life nutrition that includes high-quality, digestible ingredients, along with functional feed additives like probiotics, is crucial for optimizing gut health and protecting the gut epithelium. These practices are essential for ensuring long-term productivity and minimizing early mortality, thereby enhancing overall flock performance.

II. MATERIALS AND METHOD

a) Experimental Design

Ross 308 male day-old broilers (600 birds in total) were randomly allocated to one of 3 treatments equally in two houses (25 chicks per replicate, 8 replicate pens (1.5 m^2 floor space per pen)) and reared to 35 days of age. Three treatments were referred to hereafter as a control group (CTRL, neither antibiotics/probiotics), a treatment group fed daily with antibiotic growth promoter (4.4 g Lincomycin/T feed from Lincomix Zoetis, USA, LINCO), and a treatment group fed daily with a mix of triple strains probiotic (Prob_3S) consist of *B. subtili*s (DSM 32325), *B. subtilis* (DSM 32324) and *B. amyloliquefaciens* (DSM 25840) at 500 g/T (1.6 ×109 CFU/kg feed). During the 7d experimental period, all birds had ad libitum access to feed and water. The lighting program for Ross 308 birds was employed (1 – 3 days 18 hr and 4 – 7 days 23 hr). Biosecurity measures were applied including spraying phenyl on a regular daily basis through the entrance and exit passage for the whole experimental period (7 days). Performance data such as mortality (%), feed intake, and body weight at day 7 were recorded. Feed conversion ratio was calculated as the ratio between total feed intake and final body weight minus 42g (standard chick weight at day 0).

b) Sampling of Intestinal Tissue Samples

At the end of the experimental period, one bird per pen was randomly selected and euthanized by cervical dislocation. Duodenal and jejunal tissue samples (30mg) were obtained and immediately placed in liquid nitrogen and kept at −80 °C for total RNA extraction.

c) Gene Expression

The real-time RT-PCR was performed (qPCR). For it, total RNA was extracted from the duodenal and jejunal tissues using QIAzol (Qiagen, Germany) according to the manufacturer's instructions. The total concentration and quality of the extracted RNA was determined using NanoDrop® ND–1000 Spectrophotometer (NanoDrop Technologies, United States) and only samples with a A260/A280 ratio of between 1.8 and 2.0 were used in further analyses. Biological replicates were analyzed for each treatment group, in triplicate, per SI site (the replicates are pooled and expressed as single sample, so each group represented by 4 samples and was run in triplicates.

d) Statistical Analysis

Day 7 weights were analyzed by ANOVA using the GLM procedures of MINITAB® 19 software version. Treatment means were analyzed by Tukey's test for post hoc multiple comparisons. Mortality data were analyzed using Fisher's Exact Test. Differences were considered significant when $P \leq 0.05$.

III. RESULTS AND DISCUSSION

The relative gene expression of MUC2 in the duodenum (Figure 1(a)) and jejunum of the Prob_3S group was higher compared to the CTRL and LINCO treatment groups ($P \leq 0.05$). A significant difference in MUC2 expression was observed in the jejunum between the Prob_3S and Linco groups ($p \leq 0.05$). These results suggest that Prob_3S significantly enhances MUC2 gene expression in 7-day-old chicks. MUC2, the primary intestinal mucin in chickens, plays a key role in mucosal protection, and its depletion is associated with increased susceptibility to infections (Smith et al., 2022; Van der Sluis et al., 2006). The elevated MUC2 expression in probiotic-fed chicks is likely to strengthen barrier function by regulating interactions between microorganisms and the gastrointestinal tract epithelium.

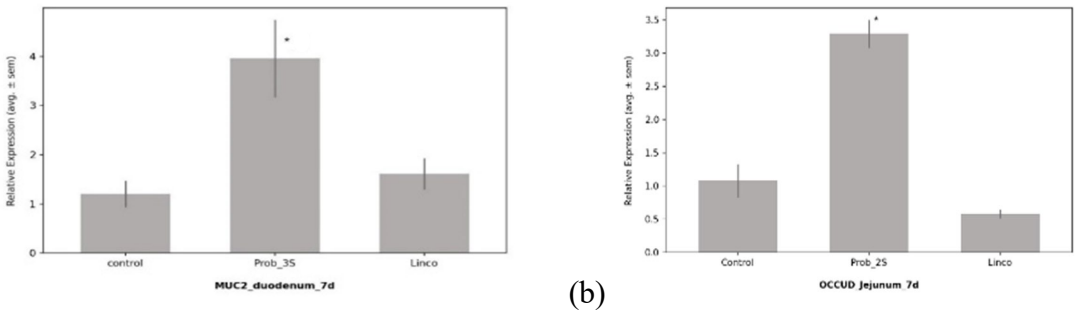
The gene expression of TLR4 in chicks from Prob_3S treatment group was significantly up-regulated ($p \leq 0,05$) in comparison to CTRL and LINCO treatment group, either in duodenum or in ileum. In both the duodenum and ileum of probiotic birds, there was an upregulation of the well-known TLR4, which recognizes bacterial lipopolysaccharide (LPS) and activates cellular pathways leading to production of cytokines (e.g., IL6) (Smith and Fiddaman, 2022). This suggests enhanced innate sensing and immune surveillance as well as potential preparedness of local immune responses.

The interleukin 2 (IL2) gene expression at 7 days old chicks in duodenum and jejunum showed that Prob_3S group had a down-regulated gene expression in duodenum in comparison with control and Linco group. On the other hand, LINCO group had a up-regulated IL2 gene expression in comparison to Prob_3S ($P \leq 0.05$) and CTRL ($P \leq 0.05$). However, this pattern changed in the jejunum, where the highest expression of IL2 was demonstrated in Prob_3S group ($P \leq 0.05$). Given that broiler chickens' digesta retention duration in the duodenum is only a few minutes, while it is estimated to be one hour in the jejunum (Svihus and Itani, 2019), any variation in these regional reactions may not be surprising. Comparably, variations in the expression of IL2 in the duodenum and jejunum (down- and up-regulation relative to control birds, respectively) may be explained by differential (opportunity for) interactions between the probiotic strains inside the GIT segments. It is intriguing to see how different regions react differently to probiotic administration, but since IL2 is essential to many biological processes, identifying the precise mechanisms or pathways promoting chick health can be difficult.

Interleukin 6 (IL6) gene expression were up regulated in Prob_3S and LINCO groups in comparison to CTRL, either in duodenum or in jejunum ($P \leq 0.05$). IL6, which is frequently considered a crucial pro-inflammatory cytokine, has been shown to have broad, context-dependent, immunological (including anti-inflammatory) and physiological effects (Hunter and Jones, 2015). In the probiotic and AGP-supplemented group (LINCO), gene expression of IL6 was found to be up-regulated in the duodenum and jejunum when compared to the control birds. The up-regulated expression of IL6 in these one-week-old birds may suggest enhanced intestinal defenses and their capacity to counteract potential treats to the gastrointestinal tract.

The gut integrity biomarkers, OCCUD (Occludin), JAM (junction adhesion molecule) and VEGF (vascular endothelial growth factor) for duodenum and jejunum revealed that chicks from Prob_3S group showed up-regulated OCCUD expression (Figure 1(b)) in comparison to other groups in jejunum ($P \leq 0,05$). Similarly, it could be also seen up-regulation of JAM in jejunum of Prob_3S chicks in comparison to LINCO ($P \leq 0,05$) and CTRL ($P \leq 0,05$). Finally, the VEGF expression was up regulated in Prob_3S group in comparison to CTRL group ($P \leq 0,05$) in jejunum. These results indicate that increased JAM and OCCUD expression after probiotic intervention may strengthen the GIT barrier and avert potentially harmful outcomes from failing. To further assist blood vessel creation and potentially tissue regeneration in the quickly expanding chick intestine, increased intestinal VEGF expression in the probiotic-

supplemented group is essential for supporting GIT activities and managing GIT difficulties (Gentile and King, 2018).



(a) (b)

*Figure 1 - Relative gene expression of (a) Mucin 2 (MUC2) in duodenum and (b) Occludin (OCCU) in jejunum at 7d. * P ≤0,05.*

At day 7, Prob_3S exhibited a significant decrease in FCR (P ≤0.05) when compared to the CTRL and LINCO groups, which showed similar results (P>0.05 Under the conditions of the study, birds had similar 7-day body weights across treatment groups, but supplementation with Prob_3S reduced FCR, indicating improved GIT function and more efficient acquisition and utilization of nutrients. FWM reduced 3% and 2,5% in PROB_3 and LINCO, respectively compared with CTRL. However, these differences were not significant (P>0,05).

IV. CONCLUSION

These different findings demonstrated that supplementation a combination of specific multi strains probiotic was able to change the gene expression of selected genes related to gut barrier as well as morphology of gut epithelium and the key zootechnical parameter, the FCR as early as 7 days of age.

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MITIGATING BACTERIAL LAMENESS IN BROILERS WITH PROBIOTIC BACILLUS LICHENIFORMIS DSM 28710

W. VAN DER VEKEN[1] and T. WAKEFORD[2]

A general decline in gut health and reduced efficiency of tight junctions has been linked to the re-emergence of bacterial lameness in broilers. Managing the challenge requires a multi-factorial approach, including feed interventions such as probiotics. A probiotic *Bacillus licheniformis* (DSM 28710) was tested in a bacterial lameness model, showing a significantly reduced lameness percentage compared to control. Results obtained were in line with the lameness percentage of an antibiotic treatment group in the same experimental set-up.

Animals in modern production systems often experience microfractures in the cartilage of rapidly developing bones such as femurs, as a result of their high growth rate combined with an increased stress force. These microfractures offer prime infection locations for bacteria that translocate from the gut, which occurs when general gut health declines and tight junctions are not as efficient as they should be. Probiotics can be used to mitigate the challenge, with two main principles to choose from: either add probiotics that focus on inhibiting specific bacteria (for example Enterococcus spp.) or opt for a probiotic that supports general gut health, thereby reducing translocation altogether. The latter option seems to make the most sense, as it has multiple benefits: an improved gut health will support the animals' feed utilization, whilst a generally reduced translocation curbs Enterococcus as well as any other bacterial species that might cause issues outside of the gastrointestinal tract. To test this hypothesis, broilers were supplemented with probiotic *B. licheniformis* and reared in a bacterial lameness model, to evaluate the probiotic's effect on gut health and thus the incidence of bacterial lameness.

A total of 120 Ross 308 broilers were divided into three treatment groups and housed in pens with an uneven surface (wire floor) for 53 days. The latter condition was added to increase the stress on cartilage and induce a higher number of microfractures. Simultaneously, all animals received a challenging diet (more fiber, less digestible) to induce dysbacteriosis and subsequent bacterial translocation. Animals then either received probiotic from start to finish (1.6×10^{12} CFU/ton of feed; B-Act®, Huvepharma NV, Belgium;), a treatment dose of enrofloxacin from day 38 to day 47 (10 mg enrofloxacin/kg body weight) or nothing at all (control group).

Figure 1 shows the final percentage of lameness for each group, indicating that the model to induce bacterial lameness was successful. Both the antibiotic treatment as well as the addition of probiotic *B. licheniformis* mitigated the level of lameness significantly ($P < 0.05$). The onset of the first cases of lameness were also 5 to 6 days later in the treatment groups compared to the control.

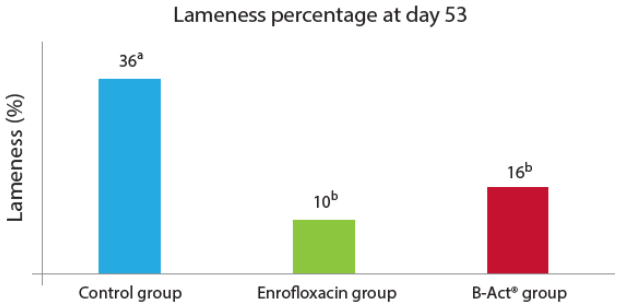

Figure 1 - Lameness percentages on day 53 of all three groups. Different superscripts indicate significant differences at $P < 0.05$.

Bacterial lameness is firmly rooted in general gut health. Managing dysbacteriosis is thus essential in the prevention and mitigation of bacterial lameness. Probiotics offer a tool to do so and within this group, those probiotics focusing on general gut integrity make the most sense. As shown here, *B. licheniformis* is a prime example thereof, with proven efficacy versus bacterial lameness to be added to its earlier described benefits on technical performance.

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SUPPLEMENTING MAIZE-BASED DIETS WITH WHEAT BRAN INCREASES BROILER FLOCK UNIFORMITY

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Wheat bran (WB), a by-product of milling wheat, is rich in fermentable fibre, containing approximately 30% total non-starch polysaccharides (NSP), primarily arabinoxylan (≈ 70%). Maize has low NSP content, meaning maize-based broiler diets contain approximately only 7-11% NSP. Supplementing low levels of NSP into NSP-poor diets can increase fermentation capacity of the microbiota, resulting in increased production of microbial enzymes and thus enhanced utilisation of dietary NSP, improving nutrient absorption. In view of this, the hypothesis of this study was that replacing 5% of the maize in a maize-based diet with WB would increase nutrient digestibility, improving flock body weight (BW) and ileal protein digestibility uniformity, and feed conversion ratio corrected for mortality (cFCR). The impact of WB on ileum and caeca pH, indicating influence on microbiota profile, was also examined. Ross 308 mixed sex broilers were fed either a commercial-type maize-soybean meal-based diet (measured gross energy (GE) 15.7 MJ/kg and crude protein (CP) 20.2%) (n = 28), or the same diet with 5% of the maize directly replaced with wheat bran (GE 15.9 MJ/kg; CP 20.0%) (n = 28), fed from day of hatch. Both diets were supplemented with 100g/t of a stimbiotic (Signis) and phytase (Quantum Blue 5G) (AB Vista, Marlborough, UK). On bird age day 28, individual bird body weight and ileum and caeca pH were measured. Ileal digesta samples were collected and analysed for protein and titanium dioxide concentration, to calculate ileal protein digestibility. Uniformity (%) was calculated using the equation: (1- (Standard Deviation / Mean)) x 100. Paired sample T-Tests, using IBM SPSS Statistics 29, were used to compare effects of the dietary treatments, with differences considered significant at $P < 0.05$.

Table 1 - Effect of replacing 5% of the maize in maize-based diets with wheat bran (WB) on mortality and cFCR at day 0-28, and BW, ileal protein digestibility and ileum and caeca pH at day 28.

Dietary Treatment		Maize	Maize + WB	*P*-value
Mortality (%)		17.24[a]	0.00[b]	<0.001
cFCR		1.39[a]	1.29[b]	0.004
BW	Mean (g)	1956	1986	0.671
	Uniformity (%)	86.99	89.18	
Ileal Protein Digestibility	Mean (%)	76.84[b]	86.73[a]	<0.001
	Uniformity (%)	85.08	96.31	
Ileum pH	Mean	7.40	7.49	0.255
	Uniformity (%)	95.14	96.80	
Caeca pH	Mean	6.75[a]	6.50[b]	0.026
	Uniformity (%)	92.89	95.39	

Supplementing maize-based diets with WB improved flock uniformity for all parameters measured, along with feed conversion, mortality and protein digestibility. It is speculated that the WB both improved gizzard function and established a caeca microbiota that was comparatively richer in NSP-degrading bacteria species. This susbequently resulted in more consistent nutrient absorption, including protein, and less variation between individuals because of improved caeca function, more reliable supply of short chain fatty acids (SCFA), as illustrated by observed reduced caeca pH, and improved fermentation of dietary NSP. In conclusion, providing low levels of a cheap source of NSP, such as WB, in maize-based diets could improve flock uniformity and performance, and thus profitability.

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PRECISION GLYCANS AS TOOLS FOR CONTROLLED GUT COLONISATION

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Chicken gut microbiota influences growth, digestion, nutrient absorption, feed efficiency, and product quality. They also contribute to disease resistance by influencing and supporting the immune system, stress response, behaviour, and animal welfare. The poultry industry separates the fertilised eggs from the hen and incubates them in a clean and controlled environment; thus, once they hatch, the chicks have no contact with maternal microbiota and are more susceptible to disease.

Glycans are oligosaccharides, which are major components of glycoprotein in the mucus layer. Precision Glycans (PG) are synthetic oligosaccharides synthesised for targeted action in GI microbiota function. PG modulates microbiome pathways and metabolic outcomes to improve chicken resilience to gastric stress and growth performance. Our preliminary and published data (Lobo et al. 2023, Petranyi et al. 2024) show that PG alter microbiota in the intestinal mucus layer to exclude pathogens and promote a beneficial community. Here, we hypothesised that supplementing PGs during initial gut colonisation can permanently affect poultry health and performance.

For this experiment, 320 Ross 308 day-old chicks were randomly assigned into control normal feed (CTR) PGN (Recommended dose of PG), PGH (half the recommended dose) and PGD (double the recommended dose) treatments with eight pens per treatment and ten birds per pen. The treatment was provided for 48 hours in the first feed; after 48 hours, the treatment was removed and replaced with the basal diet for the rest of the trial. In week four, the birds were challenged with dexamethasone to introduce a leaky gut (Horyanto et al. 2024). After week four, the birds were euthanised, and samples were taken from the ileum, ileum mucosa and cecum for microbiota study.

Although PG did not affect microbiota richness or alpha diversity, it significantly altered microbial beta diversity in the ileum mucosa, ileum, and cecum, indicating its potential influence on gut colonisation. Specifically, in the ileum mucosa, unweighted UniFrac analysis revealed significant differences between PGN vs. CTR and PGD vs. CTR ($P < 0.05$), and weighted UniFrac analysis showed differences between PGH vs. CTR ($P < 0.05$). In the ileum, significant differences in community structure were observed between PGD vs. CTR ($P < 0.05$). In the cecum, unweighted Unifrac based community structure differed significantly between PGN vs CTR and PGD vs CTR ($P < 0.05$), while weighted UniFrac distances showed significant differences between PGD vs CTR and PGH vs CTR ($P < 0.05$).

The reproducible significant alterations in different gut sections suggest that PG influences the microbial community structure, impacting the gut colonisation process.

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PRECISION GLYCAN SUPPLEMENTATION ENHANCES GUT MICROBIOTA DIVERSITY IN BROILERS

E. LOBO[1], Y.S. BAJAGAI[1] and D. STANLEY[1]

Summary

The intensive poultry production systems face challenges like poor performance, infections and welfare concerns. With reduced antibiotic use due to regulations and increased antimicrobial resistance, precision glycans offer an alternative by modulating gut microbiome functions. Here we compare the effects of broilers fed precision glycan-supplemented and non-supplemented diets in a commercial broiler farm.

I. INTRODUCTION

The poultry industry plays a crucial role in global meat production with the United States, China and Brazil being the leading producers (The Business Research Company, 2024). Australian poultry meat production is also forecasted to increase to AUD 3.9 billion in 2024-25 (AgriFutures Australia, 2024). While the industry is growing, it faces challenges like high-density housing, welfare issues and increased disease susceptibility due to widespread antimicrobial resistance, which affects production (Economou and Gousia, 2015).

Newcastle disease, caused by the avian paramyxovirus is a highly contagious virus that significantly impacts the poultry industry. It causes symptoms similar to influenza A, with distinct pathological symptoms like haemorrhagic lesions in the gut-associated lymphoid tissue (GALT) and the respiratory tract. (Regmi et al., 2024).

To address these issues, research has shifted focus towards antibiotic alternatives like precision glycans, which are specialised biological molecules like glycans, peptides etc, designed to control specific microbiome metabolic pathways (Deehan et al., 2020).

Precision biotics, specifically precision glycans have shown promising results in influencing gut microbiota composition by serving as substrates for beneficial bacteria. A study conducted over multiple broiler trials indicates that different formulations of precision glycans alter other aspects of bird performance by positively modulating the gut microbiome metabolic pathways (Walsh et al., 2021). Another research group demonstrated that supplementing feed with precision glycan enhanced broiler productivity, reduced footpad lesions due to decreased ammonia and improved litter quality (Jacquier et al., 2022). A recent study evaluated the effect of precision glycan-fed birds under enteric stress and observed a marked improvement in broiler performance and intestinal health (Blokker et al., 2022). These studies indicate precision glycans' potential to boost poultry performance and sustainability while enhancing welfare.

This study investigated the effects of precision glycan treatment on pathogen load and intestinal microbial communities in broilers under commercial conditions. Our results showed an improvement in intestinal health and disease resistance with a reduction in mortality (Lobo et al., 2024).

II. METHOD

The trial was conducted in a commercial farm and involved 32,400 male Ross 308 broilers vaccinated in-ovo against Newcastle disease (ND) and Infectious Bursal Disease (IBD), and vaccinated against Infectious Bronchitis (IB), ND and IBQX at day old. Additionally, all birds were vaccinated against ND and IBD on day ten and against IB on day 16. The birds were provided with feed and water ad libitum and housed in sheds controlled by an evaporative cooling system and

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tunnel ventilation. This was an unbalanced study where the sheds were divided into two groups: a control shed with 10,800 birds and two treatment sheds, each with 10,800 birds that received precision glycan (PG) supplemented feed at 900 grams per ton. Each shed consisted of 12 replicate pens with 900 birds per pen. The feeding phases included Starter, Grower, Finisher and Withdrawal over 38 days, with performance parameters measured after each feeding phase. At the processing facility, final bird weights for the entire flock were collected automatically and feed conversion ratio (FCR) was calculated by dividing feed intake by body weight over 38 days.

On day 38, samples were collected from caecum, ileum and ileum mucosa of 72 random birds for DNA extraction and sequencing of the 16S rRNA gene. A total of 199 samples were successfully sequenced and analysed using bioinformatic tools like QIIME2. R packages like Phyloseq, Phylosmith and Microeco were used for downstream analysis and visualisation. For histology, 20 ileum samples from the control and treatment groups were collected, processed and stained with Hematoxylin and Eosin (H&E) for microscopic analysis of gut morphology.

III. RESULTS

The alpha diversity was investigated to compare the effect of precision glycan on the microbial population of the different gut sections. The Chao and Shannon Entropy indices were used to compare richness and diversity. PG reduced richness in caecum and ileum mucosa but did not affect ileum richness (Figure 1A). In caecum diversity was reduced, while both ileum and ileum mucosa showed increased microbial diversity in the PG treated group (Figure 1B). Microbiota analysis indicated that precision glycan increased the relative abundance of *Firmicutes* while reducing the abundance of *Proteobacteria* in the caecum (Figure 2A&B).

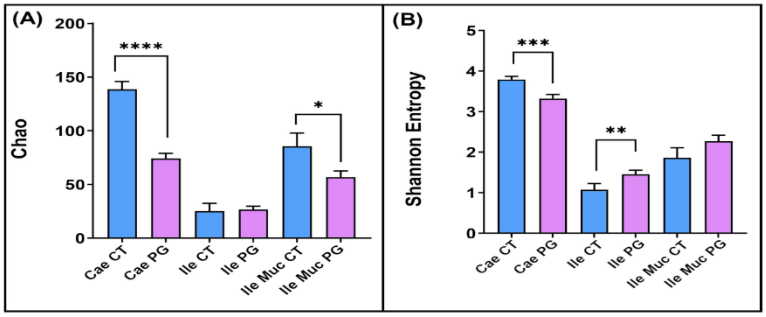

Figure 1 - Alpha diversity of gut sections where CT is control group and PG is the precision glycan treated group. Chao index indicates microbial richness (A). Shannon entropy index indicates microbial diversity (B). Cae = Caecum, Ile = Ileum, Ile Muc = Ileum mucosa.

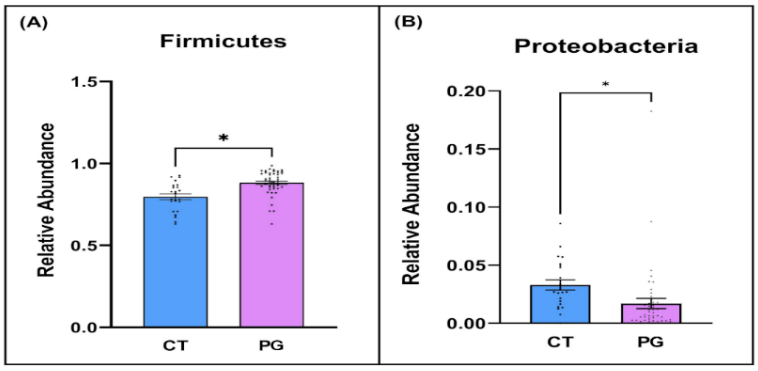

Figure 2 - Relative abundance of biomarkers. Phylum *Firmicutes* increased abundance (A), and phylum *Proteobacteria* reduced abundance (B) in caecum.

The ileal mucosa morphology was well preserved in both groups. However villus surface area was significantly larger in the PG group ($P < 0.0001$) (Figure 3A). No pathological changes

in liver tissue were observed during the analysis. However the control group showed a notably larger area of congested sinusoids compared to the precision glycan group ($P < 0.046$) (Figure 3B).

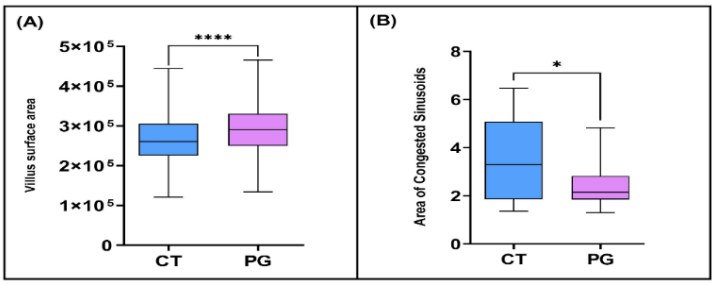

Figure 3 - Histological analysis of ileum and liver. PG increased villus surface area in ileum mucosa (A). PG reduced the area of congested sinusoids in the liver compared to CT (B*)*.

IV. DISCUSSION

The precision glycan treatment influenced the alpha diversity of different gut sections. In caecum, both species richness and diversity decreased. In the ileum, microbial diversity increased without a significant change in species richness, indicating an alteration in species interactions. In the ileum mucosa, the Chao index shows a reduction in richness, while the Shannon index implies an increase in diversity. This indicates that fewer species might have a greater influence on the ileum mucosa. This trend in alpha diversity may promote gut health as increased diversity is generally considered beneficial. However, a sudden increase in the richness of a balanced microbial community could result in adverse outcomes like pathogen overgrowth and habitat fragmentation (Ma et al., 2019, Chetcuti et al., 2020). Hence, a reduction in species richness of ileum mucosa could stabilise the microbial community by reducing pathogen overgrowth and reducing species interaction with mucosa and epithelium.

PG significantly reduced the abundance of *Proteobacteria* in caeca (Figure 2B). The phylum *Proteobacteria* is a member of the class *Gammaproteobacteria* which includes harmful human and animal pathogenic genera like *Salmonella, E. coli, Pseudomonas, Vibrio, Yersinia* etc. and historically significant pathogens like *Vibrio cholerae* and *Yersinia pestis* (Williams et al., 2010). *Salmonella* is considered a food safety risk as it is pathogenic to both humans and birds, while *E.coli* causes colibacillosis that affects multiple organ systems (Ehuwa et al., 2021, Panth, 2019). Our datasets showed a reduction in *Gammaproteobacteria* without differential abundance of individual genera indicating that PG-driven reduction of this class in caeca is likely due to marginal decreases of multiple genera within this class. This suggests that PG might target common functions associated with *Gammoproteobacteria* which could improve bird health and deserved further investigation.

PG also significantly increased the abundance of *Firmicutes* in the caecum (Figure 2A). Phylum *Firmicutes* contributes towards short-chain fatty acid (SCFA) production in the gut (Louis and Flint, 2017). SCFAs like butyrate, propionate and acetate help regulate immune response and several other functions while supporting gut health and maintenance (Liu et al., 2021, den Besten et al., 2013). This effect of PG may be direct by providing nutrients for SCFA production or indirect through beneficial modulation of the gut microbiome.

Histological analysis showed PG-treated birds had larger villus surface area in the ileum (Figure 3A), which aids in enhanced nutrient absorption and feed utilisation (Sobolewska et al., 2017). While both groups showed no significant pathological changes in the liver, the control group exhibited significantly larger areas of congested sinusoids, indicative of impaired blood flow (Petranyi et al., 2024, Gonzalez et al., 2016) (Figure 3B). The reduction of congested sinusoids in PG-treated group suggests that treatment may have alleviated congestion, improved blood flow or potentially liver health (Volin et al., 2016). The improvement in blood flow could support liver functions like detoxification and immune response (Ozougwu, 2017). These histomorphological

improvements of both the ileum and liver may indicate improved nutrient absorption, reduced intestinal inflammation and enhanced immune response, which would help in reducing the severity and mortality during disease outbreaks.

V. CONCLUSION

This study demonstrated the necessity of research into gut microbiota manipulation and modulation to improve poultry health on a large scale. Precision glycans positively influenced gut health by increasing the abundance of beneficial bacteria like *Firmicutes* while reducing the presence of harmful *Gammaproteobacteria* like *E.coli* and *Salmonella.* The observed histomorphology also suggests PG may have beneficial effects on liver health and functions. However, further research on precision glycans is required to understand their interactions with the gut microbiome.

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PREBIOTIC XYLO-OLIGOSACCHARIDES MODULATE EXPRESSION OF GENES ASSOCIATED WITH CELL MOBILISATION IN THE BROILER CHICKEN JEJUNUM

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Xylo-oligosaccharides (XOS) when supplemented at prebiotic doses (kg/t) together with arabinoxylan can improve performance by reducing cell renewal and energy of maintenance in the chicken's small intestine (Castro et al., 2024). Low doses (g/t) of XOS have shown to stimulate fibre-degrading bacteria, referred to as a stimbiotic effect. This study evaluated the effect of stimbiotic, moderate, and prebiotic XOS doses on gut health in non-challenged chickens. It was hypothesised that stimbiotic and prebiotic doses would improve intestinal health indicators.

Four levels of XOS were tested in a corn/soybean meal-based diet (0, 0.005% (stimbiotic), 0.05% (moderate), and 0.5% (prebiotic)). Each treatment was randomly distributed across 12 pens (n=12; three Ross 308 broiler chickens/pen). Feed was provided in three phases of two weeks each (starter, grower and finisher). On day 42, jejunum samples from one chicken per pen were collected for gene expression analysis. The analysis targeted tight junction proteins, and immune-related and cell mobilisation genes. Relative expression level was calculated using the $2^{-\Delta\Delta CT}$ method. Gene expression and performance data were statistically analysed in R using one-way ANOVA and ANCOVA, respectively. The results showed that the prebiotic dose significantly increased BW (472 ± 16.2 vs 413 ± 16.3; P = 0.042) and ADG (30.7 ± 1.16 vs 26.5 ± 1.16; P = 0.042) when compared to the control in the starter period. Whereas the stimbiotic dose significantly increased ADFI (121 ± 3.80; P = 0.023) when compared to the control (110 ± 3.65), moderate (104 ± 3.66), and prebiotic dose (109 ± 3.63) in the grower phase. No differences were found in the finisher period (P > 0.05). As for gene expression, the prebiotic dose downregulated Destrin (DSTN) compared to the control and stimbiotic dose (P = 0.04; Fig. 1). In addition, the moderate dose upregulated actin related protein 2/3 complex subunit 2 (ARPC2) expression when compared to the control and prebiotic group (P = 0.04).

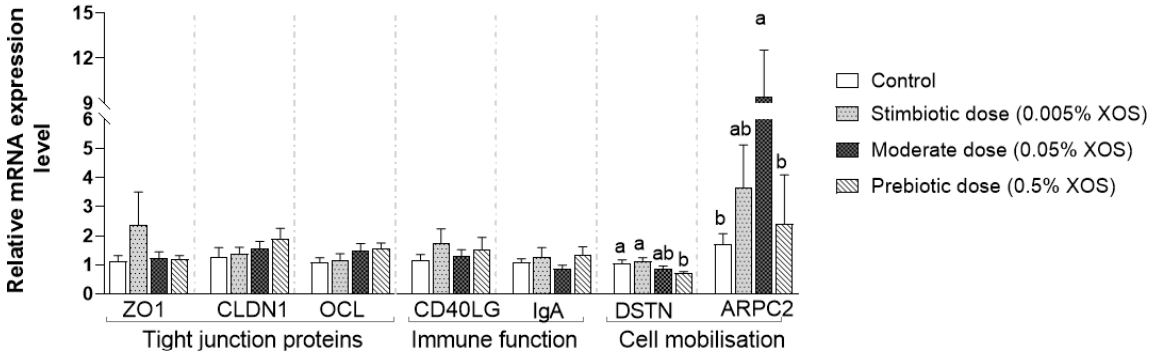

Figure 1 - Effect of xylo-oligosaccharides at different doses on gene expression. [a,b] Bars with different letters differ significantly (P < 0.05).

In conclusion, prebiotic and stimbiotic XOS doses improved BW and FI at an early age, respectively. The prebiotic and moderate doses downregulated DSTN and upregulated ARPC2, both relevant to epithelial cell mobilisation, respectively. This study shows that the effect of XOS on gut health can change depending on the dose applied.

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IN OVO DELIVERY OF ESSENTIAL OILS MODULATES THE EXPRESSION OF NUTRIENT SENSING GENES IN THE JEJUNUM OF BROILER HATCHLINGS

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The first week post-hatch is critical for the development of physiological and metabolic systems in broiler chickens. At this stage the availability and efficient absorption of essential nutrients in the gut can become critical (Gaweł et al., 2022). Essential oils (EOs) are secondary plant metabolites with functional activities such as stimulating nutrient digestibility (Brenes & Roura, 2010). Some of the main mechanisms involved in nutrient digestion and absorption include specific sensing receptors present in the intestinal mucosa responding to amino acids (T1R1, T1R3 and CaSR), fatty acids (FFAR2L, FFAR2L7 and FFAR4), and glucose (GLUT1, GLUT4 and SGLT1), which improve absorption by stimulating hormonal and transporter-mediated function. This study investigated the impact of *in ovo* injection of selected EOs on nutrient sensing genes at hatch. We hypothesized that *in ovo* injection of EOs can modulate the capacity of the small intestine to sense and absorb nutrients post-hatch in broiler chickens.

The study included 4 treatments, saline (control) and 3 potential EOs: black pepper (BP), clary sage (CS) and ylang ylang (YY) selected based on their effects on gut functionality (Unpublished data). The eggs (220/treatment) were incubated under standard conditions. On embryonic day 17.5, 1 mL EO solution (5 µL EO + 5 µL polysorbate-80 + 990 µL saline) was injected into the amnion. At hatch, jejunum samples from six hatchlings per treatment were collected for testing the expression of sensing receptor and transporter genes described above for amino acids, fatty acids, and carbohydrate. The RNAs were reverse transcribed to cDNA using the ABI High-Capacity Reverse transcription Kit and qPCR was run using Power SYBR Green Master mix, both by Applied Biosystems™ Waltham, Massachusetts, USA. The General Linear Model procedure in SPSS 27 was used to compare the EO treatments with the control saline-injected group. Pairwise comparisons were applied using Tukey's test when the multiple comparison analysis showed a significant P value ($P<0.05$). CS and YY significantly ($P<0.05$) decreased the expression of the genes of interest (Table 1) when compared to the saline group. No significant differences were observed when comparing the BP to the saline treatment.

Table 1 - Impact of *in ovo* delivery of essential oils on expression of nutrient sensing genes in the jejunum of broiler chicken hatchlings (n=6).

Treatments	FFAR2L	FFAR2L7	FFAR4	GLUT1	GLUT4	SGLT1	T1R1	T1R3	CaSR
Saline	1.11^{a}	1.29^{a}	0.78^{a}	0.82^{a}	0.71	0.92^{a}	0.83^{a}	0.90^{a}	0.86^{a}
Black pepper	1.06^{a}	1.21^{a}	0.77^{a}	0.81^{a}	0.65	0.97^{a}	0.91^{a}	0.95^{a}	0.83^{a}
Clary sage	0.64^{b}	0.75^{b}	0.64^{b}	0.68^{b}	0.64	0.62^{b}	0.63^{b}	0.66^{b}	0.60^{b}
Ylang ylang	0.66^{b}	0.78^{b}	0.63^{b}	0.72^{ab}	0.60	0.64^{b}	0.64^{b}	0.69^{b}	0.61^{b}
P value	<0.001	<0.001	0.001	0.001	0.103	<0.001	<0.001	<0.001	<0.001

It was concluded that *in ovo* delivery of CS and YY EOs downregulated the sensing capacity and may decrease the uptake of amino acids, fatty acids and glucose in jejunum of broiler hatchlings.

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NETWORK ANALYSIS AND MOLECULAR DOCKING OF PHYTOBIOTICS AND THEIR SYNERGISTIC MECHANISM ON GROWTH AND IMMUNITY IN POULTRY

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Summary

Phytogenic feed additives include numerous herbs, spices, and plant-derived products. They are used as alternatives to antibiotics due to their antimicrobial, immunomodulating, gut health enhancing and growth promoting effects. Their usage gains importance as indiscriminate conventional antibiotic use leads to resistant strains and the breakdown of host-flora symbiosis. The phytogenic feed additives like rhizomes of *C. longa*, *Z. officinale*, *A. galanga* and leaves of *O. tenuiflorum, M. oleifera*, *S. androgynus*, *A. panniculata* have been considered in poultry nutrition because of their potentially beneficial impact on gut flora, immune response, growth and production. Screening for biomarkers in these local cultivars of phytogenic feed additives through different qualitative and quantitative methods was done to formulate the experimental diet in combinations and study the synergistic combinations. The UV-Vis absorbance spectra of *O. tenuiflorum, M. oleifera*, *S. androgynus*, *A. panniculata* found at a wavelength of 1100 nm to 190 nm were characteristic of flavonoids and aromatic compounds like eugenol having antioxidant and growth-promoting activities at 282 nm by *S. androgynus* and 284.90 nm by *M. oleifera*. The results of the FTIR analysis of leaves of *O. tenuiflorum, M. oleifera*, *S. androgynus* powder confirmed the presence of alcohol, alkanes, amides, nitro compounds, phenols, epoxides, anhydrides. Various phytocompounds were found through GCMS analysis with anti-inflammatory, antioxidant effects and antimicrobial effects. Then the pathways associated with these active components and their targets in poultry and their synergistic action were computed with network analysis and confirmed by molecular docking. In-feed supplementation of these phytogenic feed additives positively influenced growth, feed intake, immune response, haematology, and gut health of slow-growing dual-purpose CARI-Debendra variety.

I. INTRODUCTION

The quest for non-antibiotic growth promoter(s) in poultry feed has been intensified after the ban of in-feed antibiotics. Antibiotics have mostly been used as growth promoters at prophylactic doses in animals than for treatment of diseases (Walia et al., 2019). The demand of growing population with preference for protein rich foods has put pressure on food production systems. The demand for eggs and poultry meat has exponentially increased by around 76% globally, especially for local/desi chicken products almost doubling in recent decades (Henchion et al., 2017). The determinants of profit in poultry production include feeding practices, management and health care. Feed is a major input cost in the poultry industry, accounting for 70% of total recurring expenditures. This has created a multifaceted challenge for providing sustainable animal protein sources to the ever-increasing population without compromising food quality. Recently the focus on natural/phytogenic feed additives as antibiotic alternatives for chicken performance and productivity has been evaluated (Kamau et al., 2023).

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Phytogenic feed additives or phytobiotics include various plants and their secondary metabolites (Windisch et al., 2008). Study on diverse phytochemicals as antibiotic alternatives, as well as their modes of action in main agricultural animals (poultry, swine and ruminants) is increasingly important for sustainability of production and quality of food. Commercial application of promising antibiotic-alternative phytochemicals to minimise in feed antibiotics will help to build a sustainable animal production system without antibiotics (Lillehoj et al., 2018). These natural, antibiotic alternatives as feed supplements can be rhizomes such as *Curcuma longa* Linn. (turmeric), *Zingiber officinale* Roscoe (ginger), *Alpinea galanga* (L.) Sw. (alpinea) and leaves of *Ocimum tenuiflorum* L. (tulsi), *Moringa oleifera* Lam (drumstick tree), *Sauropus androgynus* (L.) Merr. (chekurmanis), *Andrographis paniculata* (Burma f.) (kalmegh). The aim of this study was to assess the phytogenic constituents of potential herbs through various methods, analyzing their nutritive value following standard procedure (AOAC, 2000), exploring the network and associated pathways, and then to elucidate their effect on various production performances and immune responses in poultry.

II. METHODS

The leaves of Tulsi (*Ocimum sanctum*), Chekurmanis (*Sauropus androgynus*) and Drumstick tree (*Moringa oleifera*) were harvested, cleaned, oven-dried and pulverised to fine powder. Spectroscopic wavelength scan was done by UV-Vis spectrum analyser and Fourier transform infrared analyzer (FTIR) to assess the presence of phytoconstituents. Quantification of these phytoconstituents were done by GC-MS. The pathways associated with these active components and their targets in poultry and their synergistic action were computed with network analysis and confirmed by molecular docking. The SMILE file of compounds identified in studied herbs by GCMS were obtained by PubChem database (https://pubchem.ncbi.nlm.nih.gov/) and are used as input files in BindingDB database (https://www.bindingdb.org/rwd/bind/index.jsp) with threshold of 0.8 in order to identify binding targets and their associated functions or pathways of the compounds of different herbs. Cytoscape v3.9.0 (Shannon et al., 2003) was used to build networks among identified compounds and their targets. A total of 90 (30X3) CARI-Debendra, dual-purpose chicken were used in this experiment as control and treatment groups for 12 weeks respectively. Phase 1 and 2 feeding of dual-purpose birds were followed for 0-6 & 7-12 weeks as per recommendations of nutritional requirements of ICAR (2013). Ingredients and feed formulae of basal ration was as per standards for dual purpose birds. Water was provided ad lib. during the experiment. Based on the active components and nutritional value, tulsi, chekurmanis and drumstick leaves were superimposed in poultry feed at 2% level. Various zootechnical parameters, immune response and intestinal architecture were compared in control and treatment groups.

III. RESULTS

FTIR methanolic extract showed the presence of functional groups like hydroxyl. Carbonyl, aromatic and organosulfur in the range of 589.39 cm-1 -2917.82 cm-1. On GC-MS profiling, the phytocompounds found in *O. tenuiflorum* contain 2-nonen-1-ol, (Z)- with anti-inflammatory effects, 3-ethyl heptanoic acid with antioxidant effects and 4-amino cyclohexanone, n-acetyl- with antimicrobial effects. *M. oleifera* contains acetic acid n-octadecyl ester as an antioxidant agent acting as free radical terminator, hexacosyl acetate as antimicrobial agent and 1-docosanol, acetate as antioxidant agent. *S. androgynus* contains 1-hepten-4-ol, 4-propyl with antimicrobial activity, cyclohexanol, 3,5-dimethyl - with antibacterial and antifungal activity. For Tulsi, compound 2-NONEN-1-OL, (Z)- found to interact with Fatty acid-binding protein 1, while compound 3-ETHYLHEPTANOIC ACID found to interact with Aldo-keto reductase family 1 member B. For Chekurmane compound

(1S,2R,4R,7R)-4-ISOPROPYL-7-METHYL-3,8-DIOXATRICYCLO [5.1.0.02,4] OCTANE was found to interact with Lysosomal acid glucosylceramidase, while compounds CYCLOHEXANOL, 3,5-DIMETHYL- and 3,4-DIMETHYLCYCLOHEXANOL were found to interact with Carbonic anhydrase 2 (Figure 1). Lysosomal acid glucosylceramidase found to be involved in a glycan degradation pathway (hsa00511), Sphingolipid metabolism pathway (hsa00600), Metabolic pathways (hsa01100) and lysosome pathway (hsa04142). Carbonic anhydrase 2 is involved in Nitrogen metabolism (hsa00910), Metabolic pathways (hsa01100), Proximal tubule bicarbonate reclamation (hsa04964), Collecting duct acid secretion (hsa04966), Gastric acid secretion (hsa04971), Pancreatic secretion (hsa04972), Bile secretion (hsa04976). A significant difference (P<0.05) was observed in body weights of CARI-Debendra variety in week 8 (507.26±6.85/ 550.69±6.72) and week 12 (1176.04±11.33/ 1240.30±11.47) between the control and treatment groups, respectively. Actual water: feed intake was 0.26 for treatment and 0.29 for control at 12 weeks. The cell-mediated immune response was higher in the treatment group with FI of 0.25±0.15 and 0.14±0.03 for control. Total RBC count and Hb concentration were higher but lymphocyte % was less in the treatment group. Jejunal villi length was significantly higher (P<0.05) while ileal crypt depth was lower in the treatment group than control.

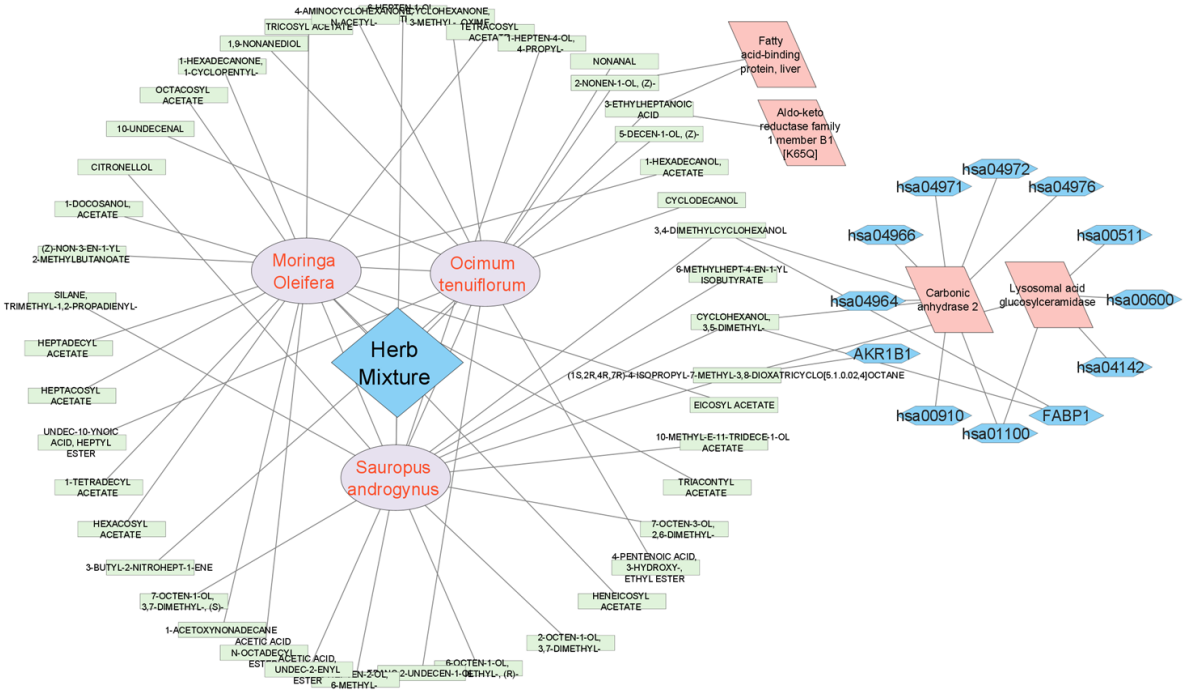

Figure 1 - Network of compounds and their interaction between binding targets. The compounds in the given figure are indicated in light green rectangular shapes and their interacting targets are indicated in light pink parallelograms. Different pathways associated with targets are indicated in hexagons.

IV. DISCUSSION

The functional groups found in FTIR analysis like hydroxyl, Carbonyl, aromatic and organosulfur are part of flavonoids and polyphenols that extended antioxidant and growth promoting activities. The peaks seen in UV Vis analysis coincided with polyphenols like catechins, eugenol, tannins, etc. The presence of eugenol in *O. tenuiflorum* increased feed consumption that may be related to enhanced hunger arising from their antibacterial activity, boosted secretion of digestive enzymes, increased digestive function, and intestinal absorbability. The presence of flavonoids like eugenol, saponins, anthraquinones, tannins, sterols, terpenoids in *M. oleifera* leaves may be responsible for their effects on chick growth

performance. The phytocompounds found GCMS analysis of *O. tenuiflorum* contain 2-nonen-1-ol, (Z)- with anti-inflammatory effects, 3-ethyl heptanoic acid with antioxidant effects and 4-amino cyclohexanone, n-acetyl- with antimicrobial effects. *M. oleifera* contains acetic acid n-octadecyl ester as an antioxidant agent acting as free radical terminator, hexacosyl acetate as antimicrobial agent and 1-docosanol, acetate as antioxidant agent. *S. androgynus* contains 1-hepten-4-ol, 4-propyl with antimicrobial activity, cyclohexanol, 3,5-dimethyl - with antibacterial and antifungal activity. High protein, fibre and micro mineral contents of these phytobiotics contributed for growth, gut health and blood profiling. In conclusion, feeding of phytogenic feed additives at 2% inclusion level acted synergistically and improved the growth, immune-response, feed conversion ratio and gut health in backyard poultry.

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BILE ACIDS IMPROVE GROWTH PERFORMANCE OF BROILERS FED A LOW LEVEL OF AFLATOXIN B1 – CONTAMINATED DIET

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Summary

Aflatoxin B1 (AFB1) is a highly toxic mycotoxin produced by *Aspergillus flavus* and *Aspergillus parasiticus*, commonly found in feedstuffs, posing serious health risks to poultry by impairing growth performance, inducing hepatotoxicity, and suppressing immunity. This study aimed to investigate the effects of supplemental dietary bile acids (BAs) on liver immune function and antioxidant capacity in broiler chickens exposed to AFB1. A total of 640 broilers were randomly assigned to four dietary groups: Control (basal diet), BA (basal diet + 60 mg/kg bile acids), AFB1 (AFB1-contaminated diet, 2.65ppb), and AFBAs (AFB1-contaminated diet + 60 mg/kg bile acids). Results showed that AFB1 significantly increased plasma alanine aminotransferase, aspartate aminotransferase, total protein, urea, glucose, and lactate dehydrogenase levels, indicating that AFB1-induced liver damage. The inclusion of BA (AFBAs group) significantly reduced the parameters and modified plasma lipid profiles by increasing total bile acids and lowering low-density lipoprotein cholesterol ($P < 0.05$). In liver tissue, BAs significantly increased total antioxidant capacity and decreased malondialdehyde, indicating enhanced antioxidant capacity. Moreover, AFB1 exposure significantly upregulated pro-inflammatory cytokines (*IL1B*, *IL-6*, *IL-8*, *TNFA*) in the liver, while BA supplementation to the AFB1 diet notably increased the expression of the anti-inflammatory cytokine *IL-10* ($P < 0.05$). The findings suggest that BAs can partially mitigate low levels of AFB1-induced metabolic profile, improve antioxidant defense, and modulate immune responses. In conclusion, dietary BAs could improve the growth performance of broilers. A lot more work is required to elucidate the efficacy of BA against mycotoxins in broiler chicken diets.

I. INTRODUCTION

Aflatoxin B1, produced primarily by fungi such as *Aspergillus flavus* and *Aspergillus parasiticus*, is a highly toxic mycotoxin that contaminates agricultural products, particularly grains and feed (Qiu et al., 2024). AFB1 poses serious health risks to poultry by negatively affecting growth performance and inducing various health problems, including hepatotoxicity, genotoxicity, and immunosuppression (Qiao et al., 2023). These issues not only threaten animal health but also pose significant risks to human health due to residual toxins in animal products. Therefore, effective strategies to reduce AFB1 toxicity are of great interest. Bile acids are important biological molecules with multiple functions, such as promoting fat digestion, regulating metabolism, and exhibiting anti-inflammatory and antioxidant properties (Geng et al., 2022). Recent studies have suggested that BA may play a crucial role in reducing the toxicity of exogenous toxins like AFB1 (Yu et al., 2024). BAs can modulate the expression of detoxifying enzymes and transporters in the liver, promoting the biotransformation and excretion of AFB1 (Chen et al., 2023; Pożarska et al., 2024). Given the potential of BAs to mitigate AFB1 toxicity, this study aimed to investigate the effects of dietary BA supplementation on liver immunity and antioxidant capacity in broiler chickens fed with AFB1-contaminated diets. Through examining the growth performance of broilers, as well as the potential mechanisms by which BAs facilitate AFB1 detoxification, we aim to provide new insights and strategies for managing AFB1 contamination in poultry production, thereby improving animal health and production efficiency.

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II. METHOD

a) Animals and Experimental Design

A total of 640 male AA broiler chickens (1-day-old) were randomly divided into four groups, with each group consisting of 8 pens and 20 birds per pen. The experimental groups included: (1) Control (basal diet), (2) BAs (basal diet + 60 mg/kg bile acids), (3) AFB1 (AFB1-contaminated diet, 2.65ppb), and (4) AFBAs (AFB1-contaminated diet + 60 mg/kg bile acids). The basal diet was formulated according to the nutritional requirements set by the National Research Council (NRC, 1994). The birds were housed in an environmentally controlled facility with ad libitum access to feed and water. The experimental period lasted for 42 days.

b) Sample Collection and Analysis

At the end of the experiment, plasma and liver samples were collected from six birds randomly selected from each group. Plasma biochemical parameters were measured using an automatic biochemical analyzer, and liver tissues were analyzed for total antioxidant capacity, malondialdehyde, and bile acid content. The gene expression of inflammatory markers (IL1B, IL6, IL8, TNFA, TLR4, IL10, TGFB, IFNR, NFKB, MYD88) in liver tissue was analyzed by quantitative real-time PCR (qPCR).

III. RESULTS

a) Effects of BA on Plasma Parameters in AFB1-Fed Broilers

Compared to the Control group, the AFB1 diet significantly increased plasma levels of alanine aminotransferase, aspartate aminotransferase, total protein, urea, glucose, and lactate dehydrogenase ($P < 0.05$, Table 1). Supplementation of BAs in the AFB1 diet (AFBAs group) significantly increased plasma total bile acids and decreased low-density lipoprotein cholesterol ($P < 0.05$, Table 1). BA supplementation alone also increased TBA and decreased triglyceride levels in plasma ($P < 0.05$, Table 1).

Table 1 - Effects of AFB1 Diet and BA Supplementation on Plasma Indices in Broilers.

Parameters	BAs	Diet			Factor	*P* values
		Control	AFB1	Means		
Total Bile Acids, μmol/L	Non-BAs	25.87 ^{b}	25.86 ^{b}	25.86 ^{y}	Diet	NS
	BAs	26.39 ^{b}	37.51 ^{a}	31.02 ^{x}	BAs	0.046
	Means	26.13	31.15		Diet×BAs	0.041
Alanine aminotransferase, U/L	Non-BAs	7.57	9.43	8.50	Diet	0.004
	BAs	5.83	8.86	7.46	BAs	NS
	Means	6.77 ^{n}	9.14 ^{m}		Diet×BAs	NS
Aspartate aminotransferase, U/L	Non-BAs	233.3	201.3	217.3	Diet	0.0001
	BAs	252.0	178.4	212.7	BAs	NS
	Means	242.6 ^{m}	189.1 ^{n}		Diet×BAs	NS
Total protein, g/L	Non-BAs	31.56	29.74	30.65	Diet	0.016
	BAs	32.96	26.69	29.83	BAs	NS
	Means	32.26 ^{m}	28.21 ^{n}		Diet×BAs	NS
Albumin, g/L	Non-BAs	7.50	7.45	7.48	Diet	0.067
	BAs	7.65	6.29	7.01	BAs	NS
	Means	7.58	6.91		Diet×BAs	NS
Urea, mmol/L	Non-BAs	0.65	0.51	0.58	Diet	0.001
	BAs	0.57	0.51	0.54	BAs	NS
	Means	0.61 ^{m}	0.51 ^{n}		Diet×BAs	NS
Uric acid, μmol/L	Non-BAs	308.5	259.1	283.8	Diet	0.051
	BAs	314.5	241.38	277.9	BAs	NS
	Means	311.5	250.3		Diet×BAs	NS
	Non-BAs	13.16	12.48	12.82	Diet	0.010

Glucose, mmol/L	BAs	13.17	11.82	12.49	BAs	NS
	Means	13.16 ^{m}	12.15 ^{n}		Diet×BAs	NS
Triglycerides,	Non-	0.63	0.49	0.57 ^{x}	Diet	NS
mmol/L	BAs	0.40	0.41	0.41 ^{y}	BAs	0.004
	Means	0.52	0.44		Diet×BAs	NS
Low-density	Non-	0.34 ^{b}	0.46 ^{a}	0.40	Diet	NS
lipoprotein	BAs	0.46 ^{a}	0.30 ^{b}	0.38	BAs	NS
cholesterol, mmol/L	Means	0.40	0.38		Diet×BAs	0.003
Lactate	Non-	1153	560.4	856.6	Diet	0.001
dehydrogenase, U/L	BAs	1336	696.1	1016	BAs	NS
	Means	1244 ^{m}	628.3 ^{n}		Diet×BAs	NS

Data are presented as Mean ± SE (n = 8). $^{a, b}$ There was a significant (P < 0.05) interaction between diet and BAs supplementation; $^{m, n}$ Means in the same row differ significantly, P < 0.05; $^{x, y}$ Means in the same column differ significantly, P < 0.05.

b) Effects of BA on Liver Antioxidant and Immune Function

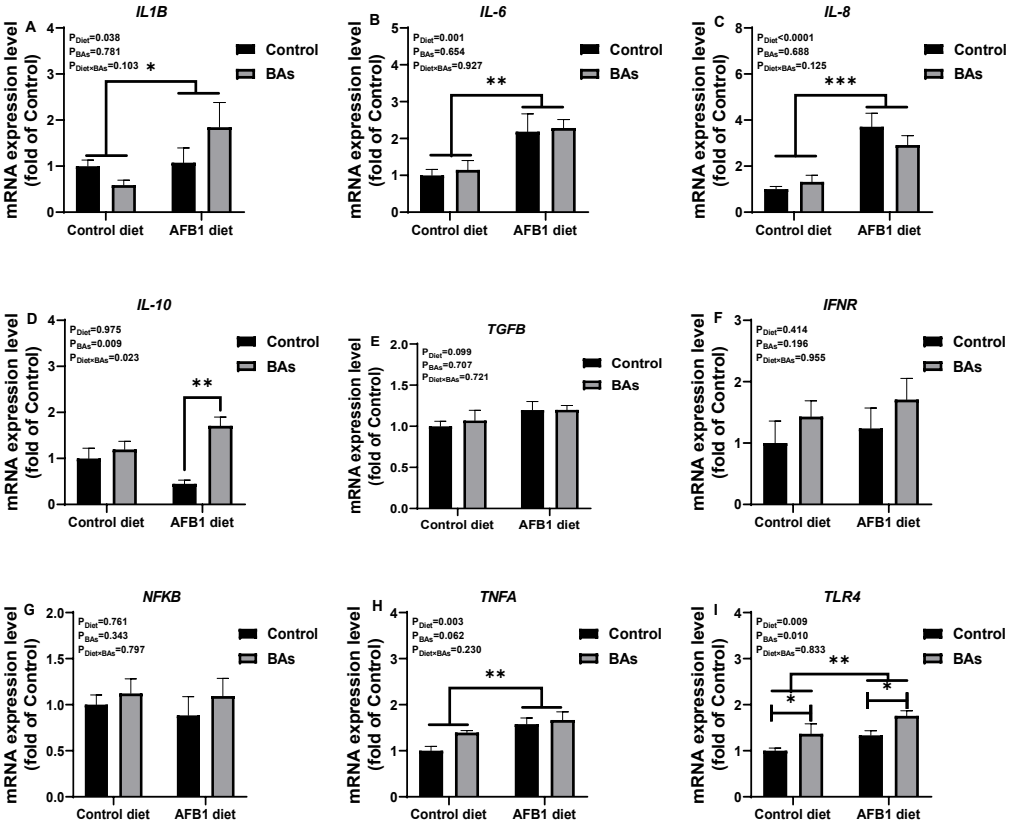
BA supplementation did not significantly affect total BA content in liver tissue (P > 0.05). However, the AFB1 group exhibited significantly higher total cholesterol levels in the liver compared to the control group (P < 0.05, Table 2). BAs significantly reduced triglycerides levels in liver tissue (P < 0.05, Table 2). In terms of antioxidant capacity, BAs significantly increased total antioxidant capacity and reduced malondialdehyde levels in liver tissue (P < 0.05, Table 2), indicating enhanced antioxidant function. Furthermore, AFB1 treatment significantly upregulated the expression of inflammatory genes *IL1B*, *IL-6*, *IL-8*, *TNFA*, and *TLR4* in liver tissue (P < 0.05, Figure 1). BA supplementation to the AFB1 significantly increased *IL-10* expression (P < 0.05, Figure 1), suggesting a potential anti-inflammatory effect of BA. Other inflammatory markers such as *TGFB*, *IFNR*, *NFKB*, and *MYD88* were not significantly affected by dietary treatments (P > 0.05, Figure 1).

Table 2 - Effects of AFB1 Diet and BAs Supplementation on Liver Indices in Broilers

Parameters	BAs	Diet			Factor	*P* values
		Control	AFB1	Means		
Total bile acids, μmol/g prot	Non-BAs	0.25	0.26	0.25	Diet	NS
	BAs	0.26	0.28	0.27	BAs	NS
	Means	0.25	0.27		Diet×BAs	NS
Total cholesterol, mmol/g prot	Non-BAs	0.45	0.67	0.56	Diet	0.017
	BAs	0.36	0.61	0.51	BAs	NS
	Means	0.41 ^{n}	0.64 ^{m}		Diet×BAs	NS
Triglycerides, mmol/g prot	Non-BAs	3.26	2.71	3.01 ^{x}	Diet	NS
	BAs	2.06	2.39	2.22 ^{y}	BAs	NS
	Means	2.70	2.55		Diet×BAs	0.025
Total antioxidant capacity, mmol/g prot	Non-BAs	0.10	0.11	0.11 ^{x}	Diet	NS
	BAs	0.11	0.12	0.12 ^{y}	BAs	0.025
	Means	0.11	0.12		Diet×BAs	NS
Malondialdehyde, mmol/g prot	Non-BAs	0.71	0.68	0.70 ^{x}	Diet	NS
	BAs	0.46	0.46	0.46 ^{y}	BAs	0.001
	Means	0.59	0.57		Diet×BAs	NS
Glutathione, μmol/g prot	Non-BAs	13.89	14.69	14.29	Diet	NS
	BAs	15.36	14.85	15.11	BAs	NS
	Means	14.63	14.77		Diet×BAs	NS
Catalase, U/mg prot	Non-BAs	1.39	1.29	1.34	Diet	NS
	BAs	1.26	1.37	1.32	BAs	NS
	Means	1.33	1.33		Diet×BAs	NS
Superoxide dismutase, U/mg prot	Non-BAs	56.1 ^{c}	66.4 ^{a}	66.27	Diet	NS
	BAs	68.6 ^{a}	62.8 ^{b}	65.73	BAs	NS
	Means	62.4	64.6		Diet×BAs	0.015
Glutathione peroxidase, U/mg prot	Non-BAs	705.2	673.5	689.4	Diet	NS
	BAs	516.0	533.0	524.5	BAs	NS
	Means	610.6	603.3		Diet×BAs	NS

Data are presented as Mean ± SE (n = 8). $^{a, b}$ There was a significant interaction between diet and BAs supplementation, P < 0.05; $^{x, y}$ Means in the same column differ significantly, P < 0.05.

Figure 1 - Effects of AFB1 Diet and BAs on Gene Expression of Inflammation-Related Genes in Broiler Liver. Levels of mRNA for IL1β (A), IL-6 (B), IL-8 (C), IL-10 (D), TGFB (E), IFNR (F), NFKB (G), TNFA (H), and TLR4 (I) were assessed in broiler liver. Data are presented as Mean ± SE (n = 8). * P < 0.05, ** P < 0.01, *** P < 0.001.



IV. DISCUSSION

The current study demonstrated that dietary supplementation of BAs had the potential to mitigate the adverse effects of AFB1 on broiler chickens by improving liver function, enhancing antioxidant capacity, and modulating immune responses. AFB1 is known to induce liver damage, as evidenced by elevated levels of alanine aminotransferase, aspartate aminotransferase, and other plasma parameters related to liver injury (Xu et al., 2023). In the present study, the AFB1-contaminated diet significantly increased these markers, indicating hepatotoxicity. However, the addition of BAs to the AFB1 diet reduced liver damage markers, suggesting that BAs may help alleviate AFB1-induced liver injury. This protective effect may be due to the ability of BAs to enhance bile flow and promote the excretion of AFB1 metabolites, thereby reducing their accumulation in the liver (Pożarska et al., 2024). In terms of antioxidant function, BA supplementation increased total antioxidant capacity and reduced malondialdehyde levels in liver tissue, indicating improved antioxidant defense. Oxidative stress is a major consequence of AFB1 exposure, leading to lipid peroxidation and cellular damage (Muhammad et al., 2018). The ability of BAs to reduce oxidative stress may be attributed to their role in activating nuclear receptors, such as the farnesoid X receptor , which regulates the expression of antioxidant enzymes (Liu et al., 2022). The upregulation of inflammatory cytokines (*IL1B*, *IL-6*, *IL-8*, *TNFA*) in the AFB1 group suggests that AFB1 induces a pro-inflammatory response in the liver, which is consistent with previous studies (Chen et al., 2023). BAs supplementation, particularly in the AFBAs group, led to increased expression of the anti-inflammatory cytokine *IL-10*, which may contribute to the attenuation of AFB1-induced inflammation. *IL-10* is known for its ability to suppress the production of pro-inflammatory cytokines and regulate immune responses, suggesting that BAs may exert an immunomodulatory effect in AFB1-exposed broilers (Islam et al., 2021).

In conclusion, the findings of this study suggest that dietary supplementation of BAs can enhance the capacity of antioxidant defense and modulate immune responses. BAs may partially alleviate the negative effect induced by low-level AFB1. However, the potential use of BAs as a nutritional strategy to mitigate AFB1 contamination in poultry production needs further work.

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A BLEND OF ORGANIC ACIDS ADMINISTERED IN WATER IMPROVES THE GROWTH PERFORMANCE AND ECONOMICS OF BROILERS

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The ban and restrictions on antibiotic use have accelerated the search for alternatives to maintain the health and productivity of animals. Organic acids (OA) are seen as a potential replacement due to their antibacterial properties and possible effects on gut health and animal performance (Dai et al, 2021). OA can compensate for gastric acidification, enhance nutrient utilization, and suppress pathogenic bacteria in the gut (Nguyen and Kim, 2020). In this study, the effect of a water acidifier based on a synergistic blend of free and buffered short-chain fatty acids (Selko-pH, Trouw Nutrition, The Netherlands) was investigated on the growth performance and economics of broilers. Cobb male broiler chicks (n=720) were allocated into two treatments including a basal diet (Control) and a basal diet plus 1 L/1000 L Selko-pH (SPH). Each treatment was replicated 18 times with 20 birds per pen. The birds were fed a common corn-soy basal diet without antibiotics in three feeding phases over 35 days. Performance parameters were recorded throughout the experiment and the return on investment (ROI) was calculated at the end of the production period. Data were analyzed using the MIXED procedure in SAS and Tukey's range test was used to determine the significance between treatment means ($P < 0.05$).

Over the entire production period (d1-35), birds supplemented with SPH had significantly higher feed intake (+3.7 %) and average daily gain (+3.0 %) compared to those on Control diets (Table 1). On day 35, the SPH-supplemented birds were significantly heavier (+3.1 %) than the Control birds (Table 1). The FCR and mortality rate did not differ significantly between treatments. However, SPH supplementation remarkably increased the ROI (an additional 5.9, or an extra 0.12 USD per broiler), demonstrating the economic benefits of using SPH in broiler production. In conclusion, these findings suggest that supplementing SPH in an antibiotic-free production system is a cost-effective approach that can improve the growth performance of broilers.

Table 1 - Performance of broilers from day 1 to 35.

Treatment	BW, kg	ADG, g	ADFI, g	FCR	Mortality rate, %
Control	2.27[b]	63.69[b]	86.26[b]	1.450	10.00
SPH	2.34[a]	65.61[a]	89.48[a]	1.444	7.78
SEM	0.022	0.624	0.708	0.019	1.571
P-value	0.04	0.04	0.001	0.80	0.32

[a,b] means in a column with no common superscripts differ significantly ($P < 0.05$). BW, final body weight; ADG, average daily gain; ADFI, average daily feed intake; FCR, feed conversion ratio.

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EVALUATION OF SUPPLEMENTED ZINC-COMPLEXED MINERAL ON PERFORMANCE OF BROILERS FED DIETS WITH HIGH DOSE OF PHYTASE

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Summary

This study investigated the performance of broilers fed diets formulated to optimize phytate hydrolysis in the upper gut and supplemented with reduced levels of zinc (Zn) in the form of Zn-amino acid complexed mineral (ZnAA). A total of 3,600 d-old Ross 308 broilers were distributed across 72 floor pens. Birds received 1 of 6 diets (corn-soybean meal-sunflower meal based, 30 ppm intrinsic Zn) from 0 to 37 days (d) with 12 replicate pens per treatment, in a randomized block design. The control diet (T1) followed a 3-phase feeding program with total Ca (0.95-0.75-0.65%) and available P (0.50-0.43-0.36%) as per Aviagen 2022 broiler recommendations and supplemented with 90 ppm Zn from $ZnSO_4$. The other 5 dietary treatments followed a 5-phase feeding program (d 1-10; 11-16; 17-24; 25-30; 31-37) as per University of Maryland (UMD) total Ca (0.94-0.80-0.69-0.69-0.69%) and digestible P recommendations (0.53-0.40-0.32-0.32-0.18%) allowing no inorganic P (Pi) supplementation after 17d. The UMD diets were supplemented with 90 ppm Zn from $ZnSO_4$ (T2) or 20 (T3), 40 (T4), 60 (T5), and 80 ppm Zn (T6) as ZnAA. Phytase at 2000 FTU/kg feed was added in all diets. Variables were analyzed by one-way ANOVA and $P < 0.05$ accepted as significant. At d37, T1 and T2 birds had similar average body weight (BW); feed conversion ratio adjusted to mortality (mFCR) was negatively affected in T1 compared to all UMD diets, which were similar to each other. Severe carcass back scratches were higher in T1 and T2 and supplementing 40 ppm ZnAA and above was the most effective to reduce these lesions ($P < 0.05$).There was a stepwise improvement ($P < 0.05$) with increasing Zn for BW and mFCR at 20, 40, 60, and 80 ppm ZnAA. Breast yield was highest in birds fed 40, 60, and 80 ppm ZnAA compared to the lowest breast yield at 20 ppm ZnAA. There were no differences in tibia ash among treatments, and tibia Zn was affected by Zn levels ($P < 0.05$). All UMD diets reduced P excretion per kg broiler produced (by 30%) vs T1. The 5-phase feeding program with no Pi supplementation from 17d onwards sustained broiler performance with positive impact on the environment and supplementing this feeding program with Zn as ZnAA improved breast yield broiler carcasses along with better skin integrity and reduction on Zn excretion as well.

I. INTRODUCTION

Macro and micro minerals are essential for a wide variety of metabolic and physiological functions, such as bone and protein metabolism. Phytate is a strong chelator of macro and microminerals in the gastrointestinal tract. Under an environment in which phytate is rapidly hydrolyzed, absorption and digestion of zinc (Zn) from raw materials could be maximized (Sandberg, 1991). Poultry diets are commonly supplemented with exogenous phytase aiming to maximize dephosphorylation of phytic acid. This results in less need for supplementation of inorganic phosphates (Pi), which are expensive and when excreted can have negative impacts on the environment.

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High solubility limestone results in an excessive amount of Ca^{2+} ions in the upper part of the intestine that quickly bind to the phytate molecule. This phytate-Ca complex hinders phytase efficiency by reducing phytate hydrolysis, then reducing the availability not only of Ca and P, but also of other minerals, like Zn^{2+}. Zinc plays a pivotal nutritional role such as growth and maintenance of the musculoskeletal system, metalloenzyme function, immunity, signaling cascades, enzymatic functions, DNA synthesis, and feathering among others (Galdes and Vallee, 1983; O'Dell, 1992).

This study investigated the performance, bone mineralization and mineral balance of broilers fed diets formulated to minimize phytate mineral interactions by using high dose of phytase on different micromineral programs and supplemented with reduced Zn levels in the form of Zn-amino acid complexed mineral (ZnAA).

II. MATERIALS AND METHODS

A total of 3,600 d-old Ross 308 broilers were distributed across 72 floor pens. Birds received 1 of 6 diets (corn-soybean meal-sunflower meal based, 30 ppm intrinsic Zn) from 0 to 37 days (d) with 12 replicate pens per treatment, in a randomized block design. The control diet (T1) followed a 3-phase feeding program with total Ca (0.95-0.75-0.65%) and available P (0.50-0.43-0.36%) as per Aviagen 2022 broiler recommendations and supplemented with 90 ppm Zn from $ZnSO_4$ ($ZnSO_4$). The other 5 dietary treatments followed a 5-phase feeding program (d 1-10; 11-16; 17-24; 25-30; 31-37) as per University of Maryland (UMD) recommendations for total Ca (0.94-0.80-0.69-0.69-0.69%) and digestible P (dig P) (0.53-0.40-0.32-0.32-0.18%) with no inclusion of supplementation of inorganic P after 17d. The UMD diets were supplemented with 90 ppm Zn from $ZnSO_4$ (T2) or 20 (T3), 40 (T4), 60 (T5), and 80 ppm Zn (T6) as ZnAA. All diets were supplemented with 2000 FTU/kg feed of phytase. Feed and water were available *ad libitum*.

a) Variables measured.

Body weight (BW) was measured at d1 and at the end of each dietary phase (10, 16, 24, 30 and 37d of age). Feed intake was calculated at the end of each phase to determine the feed conversion ratio (FCR), which was corrected for mortality weights (mFCR). At 37d, 2 broilers/pen close to the mean BW were selected and weighed, euthanized by cervical dislocation, slaughter weight determined and carcass and parts yield calculated. Right tibias of these birds were collected to determine ash and Zn content. Tibias of 2 birds/pen were also collected at d10. On d 37, 20 broilers per pen were randomly selected and scored for skin back scratches, based on a 4-point scoring system as score of 3 as the most severe (scratch/wound larger than 1.5 cm), and score of 0 as no scratches present. At d 37, litter from each pen was collected dried and then analyzed for minerals.

b) Statistical Analysis

Data were analyzed in JMP 16.0 (JMP, 2022) as a one-way ANOVA at a significance accepted at $P < 0.05$. A Rao-Scott chi-square analysis was used for categorical data.

III. RESULTS

At d 37, T1 and T2 birds had similar average BW but mFCR was negatively affected in T1 fed birds as compared to those fed any of the UMD formulated diets. There was a stepwise improvement ($P < 0.05$) with increasing Zn from ZnAA for BW and mFCR (Table 1). Breast yield

was highest in birds fed 40 ppm ZnAA and above compared to the lowest breast yield at 20 ppm ZnAA. Severe carcass back scratches were higher in T1 and T2 but supplementing more than 40 ppm ZnAA decreased these lesions ($P < 0.05$) (Table 1). There were no differences in tibia ash among treatments at d10 or d37, but tibia Zn was affected by Zn levels ($P < 0.05$) at 37d. All UMD diets reduced P excretion per kg broiler produced (by 30%) versus T1, and lowering Zn levels in diets led to lower Zn excretion ($P < 0.05$) (Table 2).

Table 1 - Performance and carcass quality of broilers fed different micromineral programs and sources and levels of zinc to 37 days of age.

Diets	Ca & P program	Zn source	Zn, ppm	BW, g	mFCR, g/g	Carcass yield, %	Breast yield, %	Scratches severe, %
T1	Control	$ZnSO_4$	90	2681^{xy}	1.410^{z}	79.65	29.48^{yz}	12.1^{yz}
T2	UMD	$ZnSO_4$	90	2688^{xy}	1.377^{xy}	79.60	29.32^{y}	18.8^{z}
T3	UMD	ZnAA	20	2651^{x}	1.385^{y}	79.70	29.22^{y}	12.7^{yz}
T4	UMD	ZnAA	40	2671^{xy}	1.375^{xy}	79.17	30.29^{z}	7.1^{y}
T5	UMD	ZnAA	60	2720^{yz}	1.365^{x}	79.71	30.25^{z}	5.4^{y}
T6	UMD	ZnAA	80	2737^{z}	1.370^{xy}	80.17	30.12^{yz}	9.0^{y}
SEM				15.7	0.006	0.39	9.84	3.4
p value				0.001	0.001	0.412	0.001	0.001

UMD, University of Maryland, USA; BW, average body weight; FI, average feed intake; mFCR, feed conversion ratio corrected to mortality.
^{xyz}Means within a column with different superscript letters differ ($P < 0.05$).

Table 2 - Bone parameters (10 and 37 days of age) and phosphorus and zinc excretion (to 37 days of age) of broilers fed different micromineral programs and sources and levels of zinc.

Diets	T1	T2	T3	T4	T5	T6	SEM	*p* value
d10 Tibia ash, %	50.39	50.41	50.81	50.62	50.59	51.26	0.14	0.257
d37 Tibia ash, %	50.47	49.99	50.02	50.50	50.46	50.30	0.10	0.204
d37 Tibia Zn, ppm	332^{yz}	338^{z}	312^{x}	322^{xy}	328^{yz}	327^{yz}	4.02	<0.05
P excreted, mg/kg BW	10.12^{z}	7.05^{y}	6.65^{y}	7.18^{y}	7.00^{y}	7.53^{y}	0.04	<0.001
Zn excreted, mg/kg BW	89.0^{yz}	101.8^{z}	38.6^{v}	54.0^{w}	72.0^{x}	87.9^{y}	1.55	<0.001

^{vxyz}Means within a column with different superscript letters differ ($P < 0.05$).

IV. DISCUSSION

In this study, broilers fed a diet following the UMD recommendations for Ca and P with 90 ppm $ZnSO_4$ exhibited a lower mFCR compared to the control. This may be partly due to the differences in early-phase nutrition, where the UMD diets contain a higher P content in the form of monocalcium phosphate (MCP) in the earlier dietary phases (1 to 10d) compared to the breeder line control diet (0.62 vs 0.53%, respectively). It has been reported that an elevated dig P content in the starter diet improves growth and mineralization in the young broiler (Jiménez-Moreno *et al*., 2013). A higher inclusion of MCP results in the treatment diets containing a smaller amount of limestone due to the relative Ca contribution from MCP. This is hypothesized to result in more effective growth as the anti-nutritional qualities of limestone, increasing buffer capacity and making the gizzard environment less acidic, are reduced (Paiva *et al*., 2013).

Supplemented Zn recommendations in diets for Ross 308 are 120 ppm Zn (Aviagen, 2022). Through high dosage of phytase, there is the potential to reduce the supplemental levels of Zn in diets. Bello *et al*. (2023) reported that reduced levels of inorganic Zn can be used in the presence of a high phytase dose. The role of Ca and P on Zn absorption is well documented, where higher

levels of Ca and phytate decrease Zn bioavailability (Oberleas *et al*., 1966; Xu *et al*., 1992). The solubility of Ca-Zn-inositol phosphate complexes are higher at a pH of 3 to 4 but drastically decreased at a pH of 5 to 6 and are near completely insoluble at a pH of 7 (Xu *et al*., 1992). This is important as the majority of nutrient digestion and Zn absorption occurs in the small intestine, at pH between 6.5-7.0 (Ravindran, 2013), emphasizing the importance of phytase supplementation in diets. Work on comparing bioavailability of ZnAA to an inorganic Zn source has shown higher availability for ZnAA (Star *et al*., 2012; de Grande *et al*., 2020), allowing Zn recommendation to be reduced, particularly if used with a high dose of phytase. This study evidenced this effect, where broilers fed ZnAA treatments performed equal to (20 and 40 ppm) or better than (60 and 80 ppm) those on $ZnSO_4$ treatments, with no differences in bone mineralization with increasing of Zn in the tibia as Zn levels in the diets increase. Broilers fed 20 ppm ZnAA had decreased growth performance to 37d compared to higher levels of ZnAA, but similar compared to Control and UMD both with 90 ppm Zn as $ZnSO_4$ with consistent bone mineralization, as seen at d10 and 37. Supplementing 60 ppm ZnAA would be the optimum inclusion rate for the heaviest BW and lowest mFCR at 37d.

There are other essential roles that Zn plays a fundamental part in, including that of wound healing and maintenance of the integumentary system through the promotion of keratinocytes and modulation of inflammatory cytokine proliferation (Ogawa *et al*., 2018). This was evidenced in this study where a decreased incidence of severe back scratches was seen when broilers were fed above 40 ppm ZnAA.

Given the increased bioavailability of ZnAA compared to $ZnSO_4$, one may be able to provide levels of Zn in the broiler diet well below industry standards whilst maintaining growth and production performance, if used in conjunction with high dose of phytase. Based on the results of this study, 60 ppm ZnAA can be used with 2000 FTU/kg phytase in a diet that follows UMD recommendations for improved growth performance, carcass characteristics, bone and skin integrity as well as decreased P and Zn excretion in the modern broiler.

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COPPER NANOPARTICLES: ENHANCING BROILER GROWTH AND IMMUNITY WHILE ENSURING LOW TOXICITY IN A DOSE-DEPENDENT MANNER

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Copper (Cu) is essential for maximum growth in broiler chickens, and its nanoparticles (Cu-NP) incorporated into feed improve growth, immunity, and gut microbiota (Safwan H. AL- Ruwad *at el*., 2024). However, the excess usage of Cu in feed causes economic losses and human health hazards after consumption. This study aims to determine overall production performance, gut health, immunological parameters, and toxicity level by determining residual concentrations of Cu in different organs, excreta and histopathological examination in broiler. Hence, it was assumed that Cu-NP could be used for boosting growth and immunity along with ensuring low toxicity in broilers. 160 broiler chicks were randomly divided into five treatment groups: T0, T1, T2, T3, and T4, with four replications for each group. The T0 group received basal diet; the T1, T2, and T3 groups received 5, 10, and 15 mg/L Cu-NP (<100 nm) powder, respectively, while the T4 group received 10 mg/L coarse Cu powder. Similar iso-nitrogenous and iso-caloric starter (1 to 14 days) and grower (15 to 28 days) diets were used having 3000 kcal/kg, 22% CP and 3100 kcal/kg and 21% CP, respectively. Weekly live weight, weekly feed consumption, feed conversion ratio (FCR), mortality and accessory organs like liver, bursa, thymus weight were recorded. Caecal swab was collected at 4th week of age to count *Salmonella sp.* and *E coli sp*. Data were analyzed in One way ANOVA with SPSS statistical method version 26. Results showed a significant improvement in production parameters (P<0.05), including body weight gain, FCR, dressing percentage, and meat yield in the T3 group compared with control and other treatment groups. Moreover, Cu-NP significantly (P<0.05) improved the liver and bursa of fabricius weight and the villi length and width. Importantly, Cu-NP residual concentration was significantly (P<0.05) lower, while coarse Cu was found in excreta. The E. coli and *Salmonella spp*. count was significantly (P<0.05) lower in T3 compared to others. The number of immune cells were the highest (P<0.05) in T4. The results of the study demonstrated the beneficial effects of supplementing Cu-NP in a dose-dependent manner, and 15 mg Cu-NP/L drinking water showed the most promising outcome for growth performance and gut microbiota, with minimal toxicity. Therefore, supplementations of Cu-NP with specific doses can be a useful source to promote growth and gut health in broiler chicken.

Table 1 - Effects of Copper nanoparticle and copper coarse particle on growth parameters of different treatments groups.

Parameter	Treatment					Mean±SD	Level of significance
	T0	T1	T2	T3	T4		
FI/bird(g)	2682.5^{b}	2512.5^{b}	2540.0^{b}	2582.5^{a}	2702.5^{a}	2584±103.07	*
BWG/bird(g)	1717.5^{bc}	1707.5^{bc}	1677.5^{c}	1775.0^{a}	1732.5^{bc}	1722±54.73	*
FCR	1.56^{a}	1.46^{b}	1.51^{ab}	1.39^{c}	1.56^{a}	1.49±0.072	*
Dressing %	62.00^{b}	62.50^{ab}	63.25^{ab}	68.25^{ab}	68.25^{ab}	64.00±4.02	*
Liver(g)	38.45^{b}	48.22^{a}	45.75^{ab}	52.42^{a}	38.90^{b}	44.75±1.20	*
Thymus(g)	0.20	0.80	0.40	0.37	0.10	0.38±0.05	NS
BF (g)	1.45^{b}	2.22^{a}	1.47^{b}	0.92^{c}	0.37^{d}	1.29±0.146	*
No. of im. cell/100 m.a.	42.25^{a}	62.75^{ab}	76.75^{b}	67.75^{ab}	86.5^{b}	67.23±21.00	*
Sal. sp. (CFU/ml)	7.45 x10^6^{b}	5.52 x10^6^{a}	5.40 x10^6^{b}	4.92 x10^6^{b}	7.40 x10^6^{a}	6.14±1.27	*
E. Coli (CFU/ml)	8.12 x10^6^{a}	7.32 x10^6^{a}	7.25 x10^6^{a}	5.10 x10^6^{b}	7.65 x10^6^{a}	7.09±1.29	*

T0 = Control, T1 = 5mg/L Cu-NP, T2 = 10mg/L Cu-NP, T3 = 15mg/L Cu-NP, T4 = 10mg/L coarse Cu Particle, (*) indicates significance, NS= Non-significance, a, b, c: means in the same row with different letters show significant differences among groups. (P<0.05), Mean ± SD. BF=bursa of Fabricius, im.=immune, m.a.=micrometer area. Sal. Salmonella.

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COMPARISON OF THE IMPACT OF FIVE SORGHUM HYBRIDS ON GROWTH PERFORMANCE AND NUTRIENT UTILIZATION IN BROILER CHICKENS

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In Australia, sorghum is the second most utilized feed grain in broiler diets after wheat, serving primarily as an energy source. Efficient energy utilisation from sorghum is critical for broiler production, yet its inherent antinutritional factors such as kafirin protein, phenolic compounds, and phytate can negatively impact energy utilization in broiler chickens (Liu et al., 2013; Truong et al., 2017). For the 2022-2023 cultivation period, estimated sorghum production in Australia reached 2.45 million MT (ABARES, 2022-23). While hybrid sorghum cultivation began in the 1960s in Australia, contemporary Australian hybrids are tannin-free, though nutrient profiles vary among hybrids and between seasons. Consequently, the use of different hybrids in broiler diets may result in altered growth performance outcomes. Few studies have explored the effects of different hybrids in broiler diets in the Australian context. This study compared the impact of six dietary treatments formulated with different commercially available sorghum hybrids (Liberty, Cracka, Buster, Resolute, and A66) on growth performance and nutrient utilization in broiler chickens. The hybrids used in this study were from the same season and produced with non-stressed cropping conditions. The hybrids were analysed for crude protein, starch, and amino acid concentrations prior to diet formulation. The mean crude protein concentration of five sorghums was 115±5.3 g/kg ranged from 109 g/kg (Resolute) to 121 g/kg (Buster). Starch content ranged from 624 g/kg (Cracka) to 667 g/kg (Liberty), with a mean value of 638±32.5 g/kg. The most variable amino acids were tyrosine (10.2% CV), glutamic acid (8.34% CV), and leucine (8.28% CV). Kafirin indices ranged from 6.33 (Cracka) to 9.53 (Buster) with 7.24±1.324 mean value. Each diet included 380 g/kg of a specific sorghum variety, with consistent inclusion of wheat, soybean meal, canola seed, and meat and bone meal across treatments. All diets were formulated to meet the nutrient requirements specified by breeder guidelines. A total of 200 mixed-sex Ross 308 broilers were randomly allocated to 50 bio-assay cages, with 10 replicates per treatment (4 birds/cage). Birds were fed with dietary treatments from 14 to 30 days post-hatch. Mixed-sex birds were used in this study while previous evaluations were in male birds only. Data were analysed using one-way ANCOVA, with the male birds ratio as a covariate. Growth performance across all treatments exceeded breeder objectives, with no significant differences in weight gain ($P = 0.162$), feed intake ($P = 0.620$), FCR ($P = 0.052$). Diets formulated with Resolute showed significantly lower apparent metabolizable energy (AME) and nitrogen-corrected AME compared to other hybrids. Notably, Resolute sorghum required significantly less AME per 1 kg of body weight gain (8.10 MJ) than Cracka (8.65 MJ) and A66 (8.79 MJ) ($P = 0.022$). Interestingly, relative fat-pad weights were not significantly different between treatments ($P > 0.150$). These findings suggest that while growth performance is not substantially impacted by the choice of current sorghum hybrids, energy utilisation does vary, with certain hybrids offering advantages for formulating low-energy broiler diets, which could enhance the sustainability of chicken meat production in Australia.

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STERCULIA QUADRIFIDA STEM EXTRACT AS A DRINKING WATER SUPPLEMENT ON GROWTH AND BLOOD BIOCHEMICAL PARAMETRES OF BROILER CHICKENS

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Summary

The study was conducted to evaluate the effect of *Sterculia quadrifida* (SQ) stem extract as a drinking water supplement on growth and antioxidant capacity of broiler chickens. A total of 100 day old chicken were randomly allocated to 4 treatments with 5 replicates per treatment and 5 chickens per replicate. The four treatments were : control provided drinking water without addition of SQ; water containing 1ml SQ/L drinking water (SQ1), SQ2 containing 2ml/L and SQ3 containing 3ml/L drinking water. The results showed that SQ supplementation in drinking water did not affect growth performance and carcass yields (P>0,05). Drinking supplementation with SQ improved total antioxidant capacity in the serum (P<0,05). In conclusion, supplemental SQ increased antioxidant capacity in the serum

I. INTRODUCTION

Indonesia has a wealth of herbs that can be utilized as phytobiotics in broiler production. Previous studies have explored the use of various herbs like turmeric, ginger, nutmeg leaves, and cloves as alternatives to antibiotics in broiler feed (Irwani *et al*., 2022; Sapsuha *et al*., 2019; Widodo, 2022). Incorporating these herbs in feed or drinking water can enhance broiler growth performance, reduce production costs, and promote the availability of organic broiler meat. These findings suggest that herbal supplements can be effective in enhancing broiler production while addressing concerns about antibiotic use in the poultry industry. Another natural herbs form Indonesia particularly in east Nusa Tenggara province is where its bark has been traditionally utilized for various medicinal purposes for human. The *Sterculia quadrifida* (**SQ**) bark extract showed potent antioxidant effects in vitro, with an IC50 value of 4.86 ppm (Tenda et al., 2019). The use of SQ as a natural antioxidant can be a viable alternative to synthetic antioxidants, which are often scrutinized for potential health risks. Synthetic antioxidants have shown adverse effects on consumers, prompting a shift towards natural sources (Sithole et al., 2023). SQ as a natural herbs has not yet been explored on their effect for broiler chickens. Herbs used in human medicine can also be used in veterinary medicine, but dosage conversion is essential for efficacy and safety (Deolekar et al., 2023). Therefore, the objective of this study was to analyse the effect of SQ stem extract as a drinking water supplement on growth, meat quality and antioxidant capacity of broiler chickens.

II. METHOD

A total of 100 one-day-old broiler chickens (35.00 ± 0.52 g) obtained from a commercial hatchery were selected for the experiment. Broilers were vaccinated for Newcastle disease at 3rd d. Chickens were raised in 20 wire cages (5 chickens/cage). The room temperature was maintained at 30°C to 33°C for the first wk and then gradually reduced to a constant temperature of 25°C. All chickens were randomly assigned into 4 treatments, 4 replicates/treatment, 5 chickens/replicate. The four treatments were : control provided drinking water without addition of SQ; water containing 1ml SQ/L drinking water (SQ1), SQ2 containing 2ml/L and SQ3 containing 3ml/L drinking water. All chickens were fed commercial

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diet with ad libitum access including the starter (d 1−21) and the finisher (d 22−35) phases. The nutrient composition were formulated according to the nutrient requirements for broilers recommended by national Indonesia standard (SNI, 2017) as shown in Table 1.

Table 1 - Nutrient composition of starter and finisher diets.

	Starter diet (1-21d)	Finisher diet (21-35d
Energy (kkal/kg)	3100	3100
Protein (%)	21	19
Fat (%)	3-7	3-8
Calcium (%)	0,9-1,1	0,9-1,1
Phospor (%)	0,6-0,9	0,6-0,9

Dry powder SQ used in this study was obtained from a company that specialized in selling traditional herbs in Kupang NTT. Dry powder SQ was extracted by using Decoction method which is a traditional method of extracting medicinal components from herbs by boiling them in water (*BPOM, 2019*).

Feed intake, body weight gain and FCR of broilers per cage was recorded weekly. At day 35, after a 12 h fasting period, 2 broilers from each duplicate cage with body weight close to average were chosen, weighed, and manually slaughtered for the measurement of blood parameter. The blood samples were centrifuged at 2,500 x g at 4°C for 10 min in order to obtain the serum and stored under controlled refrigerated conditions ($4^oC \pm 2^oC$) until they were analyzed.

III. RESULTS AND DISCUSSION

The effect of supplementation of SQ extract in drinking water on growth performance of broiler and antioxidant capacity was shown in Table 2.

Table 2 - Effect of supplementation of SQ extract in drinking water on growth (1-35 d), carcass traits and blood parametres of broiler.

Item	Control	SQ1	SQ2	SQ3	SEM	P-value
Growth performance (1-35d) :						
Average feed intake (g)	63.34	64.42	63.11	60.01	1.07	0.749
Average daily gain (g)	36.01	36.28	36.16	37.1	0.53	0.365
FCR (g:g)	1.76[a]	1.78[a]	1.74[a]	1.62[b]	0.04	0.002
Carcass traits (35d):						
Dressing percentage (%)	78.0	79.1	78.5	79.02	0.517	0.527
Breast percentage (%)	15.1	16.0	15.9	15.77	0.211	0.478
Thigh percentage 9%)	20.0	19.8	21.0	21.12	0.301	0.302
Abdominal fat (%)	3.9	3.10	2.98	2.87	0.104	0.153
Blood parametres (35d):						
Total cholesterol	3.78[a]	3.74[a]	2.40[b]	2.10[b]	0.091	0.001
Tryglyceride (mmol/L)	0.85[a]	0.80[a]	0.54[b]	0.51[b]	0.017	0.002
HDL	1.78	2.01	1.89	1.96	0.034	0.144
LDL	0.546	0.477	0.428	0.400	0.017	0.130

SEM = standar error means (n=8); dressing percentage = carcass weight/pre-slaughter weight x 100; [a-b]means within a row with different superscripts differ significantly ($P < 0.05$)

As shown in Table 2, there was significant ($P < 0.05$) improvement in FCR of birds supplemented with SQ. This could be associated with improving the digestibility of dietary protein in the intestine. Several metabolites in SQ, such as flavonoid, and phenols also play an important role in the chemical function of digestive tract. SQ is a medicinal plant with various bioactive compounds and potential health benefits. Extracts of SQ bark exhibit strong

antioxidant activity due to high concentrations of flavonoids, phenols, and tannins (Dillak et al., 2019). Extraction methods significantly influence the yield and quality of SQ extracts. Differences in the efficiency of the application of herbs may result from incorrect improper dose (too low or too high doses, which may have toxic effects), and method of application as well as the different taste and smell of SQ.

This present study found that there were no significant differences in dressing percentage, breast percentage, thigh percentage and abdominal fat of broilers among treatment. These were consistent with the previous study conducted providing herbs in drinking water.

This study revealed that an addition of SQ to water decreased cholesterol and triglycerides, as compared with the control group. This could be due to that SQ have an inhibitory effect on the enzyme responsible for the synthesis of cholesterol in the liver, thereby reducing its content in the blood of birds.

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THE APPARENT METABOLIZABLE ENERGY AND NITROGEN-CORRECTED APPARENT METABOLIZABLE ENERGY OF BLACK SOLDIER FLY LARVAE IN BROILER CHICKEN NUTRITION

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Broiler meat, being the most consumed meat globally and with the highest annual consumption growth, faces sustainability challenges due to the reliance on soybean meal, which has fluctuating prices and environmental concerns. To address these issues, sustainable feeding practices are crucial, and the use of insects like black soldier fly larvae (BSFL) has emerged as a promising alternative due to their excellent nutritional profile ,with crude protein ranging from 26-31.46% DM, crude fat 38.58- 40.33% DM, gross energy 25.68-26.91 MJ/kg, and ability to convert organic waste into high-quality biomass. The aim of this study was to determine the apparent metabolizable energy (AME) and nitrogen-corrected apparent metabolizable energy (AMEn) of black soldier fly larvae (BSFL) (*Hermetia illucens*) produced at three different waste stream facilities as a protein-rich ingredient for broiler chickens. The larvae were raised on pre-consumer or post-consumer recycled food waste (e.g vegetables, fruit, bread, coffee and meat) collected from the kindergarten, airport, and supermarket facilities.

A total of 144 one-day-old Ross 308 male broiler chickens were fed a commercial starter feed for the first 13 days and fed either a basal diet or one of three experimental diets with BSFL from the three different waste streams, substituting 30% of the basal diet from day 14 to day 21(6 birds per cage, 6 replicate cages per diet). Total excreta was collected for 7 days, and feed intake and excreta output recorded during the final 3 days. The AME of the diet was calculated as described by De Marco et al. (2015). Then, the AME of the BSFL was calculated by equation below:

$$\text{AME}_{\text{BSF larvae}}(\text{MJ/kg}) = \frac{\text{AME of BSF larvae diet} - (\text{AME of basal diet} \times 0.70)}{0.30}$$

The AME was corrected for N retention, assuming a value of 36.54 KJ per gram of N retained in the body to provide AMEn. Data were analyzed using one-way ANOVA using SPSS. The AME values of the three BSFLs ranged from 22.52 to 24.54 MJ/kg DM, with an average of 23.07 MJ/kg DM. and AMEn ranging from 22.35 to 24.34, with an average of 23.21. The AME data obtained in this study was higher than those reported by De Marco et al. (2015) and Mahmoud et al. (2023), who found values of 17.38 and 16.60 MJ/kg DM, and 19.1 and 18 MJ/kg DM, respectively. This increase can be attributed to the high crude protein and fat content of the BSFL used in this study, influenced by the substrates the larvae were raised on as well as their age at harvesting. The results indicate that full-fat BSFL is a rich source of nutrients for broiler chickens in sustainable feed formulations.

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BLACK SOLDIER FLY LARVAE RAISED ON FOOD WASTE HAVE HIGHER DIGESTIBILITY THAN SOYBEAN MEAL IN A CHICKEN *IN VITRO* MODEL

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The poultry industry has traditionally relied on soybean meal (SBM) as the main protein source for poultry diets. However, the majority of SBM used in Australia is imported (Copley et al., 2023), resulting in a major threat to the Australian poultry industry. Using alternative local protein sources may reduce SBM dependence while improving sustainability. The study was to assess the protein digestibility of locally produced black soldier fly larvae (BSFL) raised on food waste in comparison with SBM. It was hypothesised that BSFL has higher protein digestibility than SBM and could potentially be an alternative protein source in poultry diets.

Four black soldier fly (*Hermetia illucens*) larvae products were evaluated based on the sources of food waste streams from Lendlease (BSFL1), Brisbane (BSFL2), Hume (BSFL3) and Albury (BSFL4). The larvae were fed with organic waste, including pre- or post-consumer foods, such as bread, vegetables, fruit, and meat collected from supermarkets, kindergartens, or airports. In addition, a SBM sample was evaluated as a control. The ingredient digestibility was analysed using an in vitro model based on the INFOGEST protocol 2.0 with amendments relevant to chicken (*Gallus gallus domesticus*) physiology consisting of three phases: oral (2 min), gastric (1h), and intestinal phase (2h). Each ingredient was analysed in triplicate without or with one of four different exogenous enzymes xylanase (Econase XT 25P; 16,000 BXU/kg feed), phytase (Quantum Bue10G, 500 FTU/kg feed), beta-glucanase (Econase GT P, 20,000 BU/kg feed), or mannanase (Econase MP 1000, 100,000 MNU/kg feed). In vitro digesta samples were collected at the 2h mark of the intestinal digestion phase for protein concentration analysis. A two-way ANOVA was conducted to examine the effect of ingredients and individual exogenous enzymes on protein digestibility.

The four BSFL products showed significantly higher protein digestibility ($P < 0.05$) than SBM. In addition, in the xylanase test BSFL2 and BSFL3 showed higher protein digestion rates than BSFL1, BSFL4 and SBM (93.07% and 94.64% vs. 85.47%, 86.95%, and 72.37%; $P < 0.001$, respectively). There was no ingredient and xylanase interaction ($P > 0.05$). Similarly, the phytase test, BSFL2 and BSFL3 exhibited greater protein digestion levels compared to BSFL1, BSFL4 or SBM (93.52% and 94.36% vs. 85.95%, 87.33%, and 72.59%; $P < 0.001$, respectively) but no interaction was observed ($P > 0.05$). The study revealed an interaction between beta-glucanase and ingredient type ($P = 0.036$), showing the presence of beta-glucanase increased protein digestibility of BSFL2, BSFL3, and BSFL4 but did not influence other ingredients. Manannase improved protein digestibility of BSFL2 and BSFL4 but did not influence the protein digestibility of BSFL1, BSFL3 or SBM ($P = 0.017$).

In conclusion, protein from BSFL2 (Brisbane) or BSFL3 (Hume) had higher digestibility in vitro than SBM. The use of beta-glucanase and mannanase has the potential of increasing protein digestibility of BSFL2, BSFL3 and BSFL 4; BSFL2 and BSFL4, respectively. The adoption of these alternative protein sources by industry will depend on feed ingredient cost relative to nutritional value and sustainability policies.

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UPGRADE OF WHEAT BY-PRODUCTS PROXIMATE AND TAA PROFILE FOR POULTRY

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Wheat by-products such as wheat pollard, wheat bran, and wheat middlings are commonly used in poultry nutrition due to their availability and cost-effectiveness. These by-products are derived from the wheat milling process and provide a valuable source of fiber, protein, and energy, making them suitable for poultry diets. Wheat pollard, which consists of fine particles of bran, germ, and flour, offers higher protein and energy content compared to wheat bran, which is more fibrous. Wheat middlings, a blend of fine particles of bran, germ, and starchy endosperm, serve as a balanced energy and protein source. Although these ingredients can reduce feed costs, their high fiber content can limit digestibility, necessitating the use of feed additives to optimize nutrient absorption and bird performance in commercial poultry production (Boros et al., 2004).

Table 1 - Proximate and total amino acids values from current study and selected datasets.

	DM	CP	EE	CF	ASH	Lys	Met	Sample size
Wheat Pollard-CS[1]	88.17	17.02	4.64	8.19	4.25	0.75	0.25	50
Wheat Bran-CS	90.40	14.85	4.44	12.40	5.39	0.61	0.20	50
Wheat Middling-CS	89.03	15.69	3.51	2.58	2.50	0.56	0.25	50
Wheat Pollard – BT[2]	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
Wheat Bran- BT	88.50	15.20	3.40	9.07	N/A	0.60	0.24	6-29
Wheat Middling-BT	88.20	13.60	2.11	6.55	N/A	0.46	0.21	1
Wheat Pollard-FT[3]	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
Wheat Bran-FT	86.90	17.60	3.80	10.50	5.60	0.70	0.27	N/A
Wheat Middling-FT	88.00	17.90	7.90	4.40	4.70	0.71	0.27	N/A

DM: dry matter, CP: crude protein, EE: ether extract, CF: crude fiber, ASH: ash content. [1]CS: current study; [2]BT: Brazilian Table (2024), wheat middling was represented by wheat screening; [3]FT=Feedtable (INRA). Complete AA profiles not shown, taking Lys and Met as representatives.

Current available databases for these ingredients have some deficiencies, including relatively small sample size, delayed update, and missing for some ingredients. For example, nutritional profiles of wheat bran and wheat screening (middllings) from Brazilian Table (2024) were generated from up to 29 samples. And wheat pollard profiles could not be found from both Brazilian Table (2024) and Feedtable (INRA). In this case, 50 samples for wheat pollard, wheat bran and wheat middlings were provided by Malayan Flour Mills Berhad (Malaysia) for proximate and total amino acids profile update/establishment (Table 1. Only Lys and Met values were given due to limited space) in Adisseo Research and Innovation Center (China). Obvious differences for a few parameters such as CP, EE, CF and total lysine were observed among the results from current study and values from selected database (Table 1), likely due to the divergent processing methods of these feedstuffs and sample size disparities. We believe current study can provide a more comprehensive dataset for wheat by-products utilization in broiler feed.

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NOVEL PROTEIN-RICH INGREDIENTS TO PARTIALLY REPLACE SOYBEAN MEAL AND IMPROVE SUSTAINABILITY OF BROILER CHICKEN PRODUCTION

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Summary

A 42-d trial was conducted to study the nutritional potential of atypical feed ingredients including microalgae and duckweed to partially replace soybean meal (SBM), in broiler chicken feed. The inclusion of microalgae and duckweed, each at two inclusion rates on growth performance, feed utilisation, gut morphology, and meat quality in broiler chickens was studied. The results showed that both duckweed and microalgae can be added to the chicken feed up to 2.5% replacing 10% of SBM with no detrimental effects on growth performance and feed efficiency. However, 5% inclusion of either duckweed or microalgae reduced growth performance and feed efficiency during the finisher phase and the total period. The findings of the current trial suggest that these novel ingredients provide an opportunity for long-term sustainable solutions for the chicken feed industry where the imported SBM inclusion in the diet can be reduced by locally and sustainably produced novel ingredients.

I. INTRODUCTION

Australian poultry industry is highly dependent on soybean meal (SBM) as a protein source, however, due to the low productivity of this crop in the Asia-Pacific region, the provision of soybean co-product, SBM, for animal and poultry feed is of high concern (Ravindran and Blair, 1993; Willis, 2003). Australia is a net importer of SBM, sourcing over 1M tons from overseas annually, with a cost of 650 – 750 AUD per ton. The reliability on international markets and constant price change introduces a high uncertainty and potential risk factor for livestock producers in Australia where the feed formula is highly dependent on SBM inclusion. As a result, the Australian poultry industry faces the challenge of ensuring a sufficient and more sustainable protein supply to feed meat-producing chickens. Some novel protein-rich ingredients such as microalgae, macroalgae, duckweed, yeast protein concentrate, bacterial protein meal, leaf protein concentrate, and insect meal have been introduced as potential long-term solutions as protein supply for future application in poultry diets (Tallentire, Mackenzie, & Kyriazakis, 2018). Microalgae and duckweed, small aquatic plants, developed over or below the water surface, don't need arable land to be cultivated. They are found practically everywhere, except for the Polar Regions and deserts. The aim of this project was to investigate the potential nutritive value and the impact of incorporating novel ingredients including microalgae (*Nannochloropsis Oculata*) and duckweed (*Lemnoideae*), into future chicken diet formulations to serve as Australian sourced alternatives to imported SBM.

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II. METHOD

A 42-day trial was conducted to investigate the nutritive value and the potential of atypical protein-rich feed ingredients to be incorporated into the feed formulation substituting SBM in the diet. A total of three hundred and twenty-day-old male broiler chickens (Ross 308) were purchased from Aviagen and transferred to the Queensland Animal Science Precinct (QASP) Facility at Gatton Campus, University of Queensland. All chickens were weighed at arrival and randomly allocated to 40 floor pens with 8 birds in each (n=64 chicks). The experimental diets were formulated to meet the nutrient recommendations for the three growth phases (starter, 1 to 10 days of age; grower, 11 to 28 days of age, and finisher, 29 to 42 days of age). The birds had *ad libitum* access to feed and water for the total experimental period and were fed a basal corn-wheat-soybean meal diet during the starter phase. The experimental diets were formulated to be isocaloric and isonitrogenous and were fed during the grower and finisher periods. Duckweed was cultivated at the Pinjarra Hills campus of the University of Queensland, and microalgae was sourced from Algae Pharm (Goondiwindi 4390, QLD, Australia). The experimental diets included two inclusion rates of either duckweed or microalgae substituting SBM in the basal diet (control 0%, 2.5% and 5%). Feed intake and body weight gain were recorded for each phase and feed conversion rate (FCR), average daily feed intake (ADFI), average daily gain (ADG) were calculated and presented for total period (1-42 d). On day 42, after recording final body weight (FBW) and feed residue, five bird per pen were euthanized and various samples were collected for further analyses.

III. RESULTS

The effects of duckweed inclusion on chicken growth production and feed efficiency for the entire production period is presented in Table 1. Duckweed inclusion at 2.5% rate did not have any negative impact on production performance and FCR, where 5% inclusion impaired the production performance, when compared to birds fed the control diet.

Table 1 - The effect of dietary inclusion of duckweed substituting soybean meals on the production performance of broiler chickens.

Experimental diets[1]	FBW (g)	ADG (g)	ADFI (g)	FCR
	d42	d1-42	d1-42	d1-42
Control diet	3197.30[a]	75.25[a]	109.52	1.46[b]
Duckweed, 2.5%	3101.53[ab]	72.97[ab]	106.78	1.46[b]
Duckweed, 5%	3036.04[b]	71.41[b]	107.93	1.51[a]
SEM	31.54	0.75	1.12	0.01
P Value	<0.01	<0.01	0.25	<0.01

[1] Duckweed substituting soybean meal during the growing and finishing phases.
[2] Means with different superscripts differ (P≤0.05)

Table 2 - The effect of dietary inclusion of microalgae substituting soybean meals on the production performance of broiler chickens.

Experimental diets[1]	FBW (g)	ADG (g)	ADFI (g)	FCR
	d42	Total	Total	Total
Control diet	3197.30[a]	75.25[a]	109.52	1.46[b]
Microalgae, 2.5%	3068.62[ab]	72.15[ab]	106.00	1.46[b]
Microalgae, 5%	2977.75[b]	70.00[b]	106.00	1.51[a]
SEM	39.96	0.95	1.52	0.01
P Value	<0.01	<0.01	0.89	<0.01

[1] Microalgea substituting soybean meal during the growing and finishing phases.
[2] Means with different superscripts differ (P≤0.05)

Similar to duckweed inclusion, chickens fed with 2.5% microalgae substituting SBM had a similar growth performance and feed efficiency to the control diet, however, 5% inclusion reduced FBW and ADG, and increased FCR ($P<0.01$). Feed intake was not different among experimental groups (Table 2).

IV. DISCUSSION

The current trial investigated the potential of novel protein-rich feed ingredients including duckweed and microalgae as long-term solutions for future application in broiler chicken feed. Duckweed and microalgae are photosynthetic aquatic microorganisms, considered to be the fastest-growing flowering plant with almost exponential growth (Bog et al., 2019) and the ability to thrive in a range of extreme environmental conditions (Lee, Y., 2001). Microalgae and duckweed do not need arable land to be cultivated, grow in saline or wastewater using solar energy, and they have a high carbon dioxide fixation rate and nutrient uptake, contributing to carbon sequestration (Borowitzka M., 2013). This positions them as sustainable alternatives to traditional agricultural practices for feed production. Our results indicated that duckweed and microalgae can be included at an inclusion rate of 2.5% (replacing 10% of the SBM) in the chicken feed with no negative impact on growth performance and feed utilisation. This is due to high fibre contents of unprocessed (harvested and dried) duckweed and microalgae, which limits the inclusion rate up to 2.5%. Indeed, our results indicate that 5% inclusion impaired growth performance. Similar to our results, Zaffer et al., (2021) reported a decline in the FBW of chickens fed with 5% and 10% duckweed without enzyme addition, however, enzyme supplementation restored the body weight when duckweed was included at 5% but not 10%. The optimal inclusion rate of microalgae in feed varies according to the type of microalgae and of course the animal species. A recent study has reported that 3% inclusion of microalgae meal (*Arthrospira spp.*) in broiler chickens' diet did not show any differences to the control diet, where 6% inclusion impaired FCR (Zampiga et al., 2024). In a review paper, it was concluded that microalgae could be added to a diet up to 20 g/kg without compromising birds' performance (Coudert et al., 2020). Tissue, blood and gut tissue samples taken from our studies are being analysed to evaluate the effects of these ingredients on meat quality, immune response, and gut morphology.

In conclusion, duckweed and microalgae can be added up to 2.5% replacing 10% of SBM in broiler chickens' diet. The findings provide promising results for future applications in chicken feed industry to enhance the sustainability of chicken meat production and utilize ingredients that are not competing with human food supply. The SBM that is imported from regions employing land use and land use change, has a high sustainability concern and the use of the novel ingredients reduces the dependance on the imported SBM. At this stage it is difficult to do a complete economic evaluation of the novel ingredients, as these ingredients are not produced in commercial scale. However, they show promising results to be produced locally and replace imported ingredients in livestock feed.

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A NOVEL PHYTASE IMPROVED THE GROWTH PERFORMANCE, TIBIA MINERALIZATION AND PHOSPHORIUS UTILIZATION OF MEAT DUCK

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Phosphorus (P) is essential to animal growth and skeletal development (NRC, 1994) but is mostly present in feed in the form of phytate and is therefore poorly utilized by poultry, due to insufficient production of endogenous phytase in their gut (Angel et al., 2002). To compensate for this, and thereby decrease the need for inorganic P and excretion of P into the environment, it is now common practice to add an exogenous phytase to poultry diets. Among the poultry industry, duck production only accounts for 4.91% of the world's poultry meat production, with Asian countries alone contributing to 90.6% of this (FAOSTAT, 2022). This under-representation of the duck industry explains why so few studies exist describing the effect of phytase in ducks. A novel phytase has been developed (a fourth generation), encoded by a 6-phytase gene from *Citrobacter braakii*. This new phytase is believed to be more thermal and pH stable than other phytases on the market. The efficacy of this phytase must be validated in animal systems. Therefore, the objective of this study was to explore the effect of this novel phytase on growth performance, nutrient digestibility, and tibia mineralization in meat ducks.

A total of 480 eight-day-old Cherry-Valley ducklings were allocated to six dietary treatments, with eight replicate cages per treatment and ten ducks per replicate. The treatments included a positive control diet (PC, 0.9% total Ca, 0.8% total P, 0.48% nPP), a negative control diet (NC, 0.7% total Ca, 0.5% total P, 0.18% nPP), and 4 experimental diets (NC supplemented with either 500, 1000, 2000 or 4000 phytase units (FYT)/kg feed HiPhorius^{TM} dsm-firmenich, Switzerland). All the experimental diets were formulated to contain 0.32% phytate P, and corn, SBM, wheat, rice bran and rapeseed meal were the main dietary ingredients. The trial was run for 10 days (bird age 8 to18 days).

Compared with PC, the ducks fed NC diet significantly decreased body weight gain (BWG) and increased feed conversion ratio (FI/BWG) ($P < 0.05$), as well as decreased Ca and P digestibility ($P < 0.05$) and tibia ash weight, Ca and P content and strength ($P < 0.05$). This indicates that P was a limiting nutrient for the ducks. Supplementation of NC with phytase improved the growth parameters ($P < 0.05$), tibia ash weight, Ca and P content and strength ($P < 0.01$). Compared to the PC fed birds, providing 500 FYT/kg phytase achieved similar growth parameters, and 2000 FYT/kg phytase resulted in similar tibia mineralization. Phytase also increased Ca and P digestibility and ileum myo-inositol concentration ($P < 0.01$), and decreased ileum phytate P concentration ($P < 0.01$). These results clearly demonstrated that the new 4th generation phytase could not only increase P digestion and utilization, but also fully alleviated negative effects associated with P deficiency. In summary, feeding the novel phytase improved the growth performance, tibia quality and P utilization in meat ducks, by successfully releasing P and myo-inositol from the diet, thereby could decreasing the use of inorganic P in duck industry and providing economic and environment benefit to the farmer.

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UNLOCKING THE FULL MATRIX POTENTIAL OF A NOVEL CONSENSUS BACTERIAL 6-PHYTASE VARIANT IN ENHANCING THE GROWTH PERFORMANCE, CARCASS YIELD, AND TIBIA ASH OF TURKEYS

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Monogastric animals cannot efficiently utilise plant phosphorus because it is bound with phytic acid and forms a phytate bond. A phytase enzyme is needed to hydrolyse phytate bonds, but monogastric animals have limited phytase activity. Phytase supplementation not only improves P digestibility but also the digestibility of amino acids and energy. The improved production performance and bone mineralization of phytase supplementation in broiler and turkey diets have been reported by Sens, 2020; Bassi, 2021.

This study aimed to evaluate the application of a full nutrient matrix value of a novel consensus bacterial six phytase variant (PhyG) on production benefits in turkeys. A study was conducted on 300-day-old Nichola's turkeys to investigate the effect of PhyG in a corn soy-based diet with reduced energy and nutrients on production performance, carcass yield, and tibia ash. Two basal experimental diets were formulated: 1) Positive Control (PC) diet, which provides all the nutrients required for different growing phases as recommended by the breeder. 2) Negative Control (NC) diet had reduced available P, Ca, ME, dig AA, and Na. The reductions were 0.18%, 0.21%, 74.54 kcal/kg, 0.02-0.06%, and 0.05%, respectively, compared to the PC diet. 3) NC diet was supplemented with 2000 FTU/kg phytase (PhyG). The study had three experimental diets, each with ten replications with ten birds in each replication. Experimental crumbled and pelleted diets were fed *ad libitum* in six phases from day one until 120 days. Production performance data for all birds and tibia samples (2 birds per pen) were collected at 21, 63, and 120 days. Carcass yield from two birds per replication was recorded at the end. The data was analysed using one-way ANOVA (SAS 9.4), and the means were compared using the Tukey test at a significance level of $P < 0.05$. The results are presented in Table 1.

Table 1 - Efficacy of Phy G supplementation on growth performance and carcass yield in Turkeys.

Treatment Details	Body weight (kg)	Weight Gain (kg)	Feed intake (kg)	FCR	Dressed weight (kgs)	Feed Cost Cents/kg Live weight	Brest Meat (Kg)
Positive Control	17.611[a]	17.550[a]	39.373	2.259[b]	14.457[a]	200.7	4.25[a]
Negative Control (NC)	16.353[b]	16.292[b]	38.421	2.382[a]	13.266[b]	202.9	3.88[b]
NC+ Phytase 2,000 FTU/kg	17.240[a]	17.179[a]	39.390	2.294[b]	14.370[a]	198.8	4.32[a]
P	0.0005	0.0005	0.35	0.0001	0.0001	0.17	0.001

[ab] Means in columns with no common superscripts are significantly different (P<0.05).

Turkey production performance parameters, including body weight, weight gain, dressed weight and breast meat yield, were significantly reduced in the NC diet-fed group versus the PC group. Supplementation of PhyG at 2000 FTU/kg improved body weight, weight gain, dressed weight and breast meat weight by 0.887, 0.887, 1.111 and 0.440 kg, respectively, versus the unsupplemented NC group. PhyG completely counteracted the negative effects of reducing dietary energy and nutrients in the NC diet and maintained body weight gain, feed conversion ratio, tibia ash and carcass yield similar to the PC group.

Compared to PC, the feed cost per kilogram of live weight was reduced by 1.9 cents when the NC diet was supplemented with 2000 FTU of PhyG/kg of feed, representing a significant cost saving for a farm with one million birds. The current study demonstrates the production value of using a full nutrient matrix of a novel phytase in Turkey rearing.

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SUPPLEMENTAL EFFECT OF DIETARY PHYTASE AND EMULSIFIER ON GROWTH PERFORMANCE, NUTRIENT DIGESTIBILITY, ORGAN WEIGHT, AND FECAL SCORES IN BROILERS

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Animal fats and vegetable oils are usually added to increase the energy concentrations of broiler diets. Fat is mainly composed of triglycerides. Although fats are not water-soluble, their digestion takes place in an aqueous environment in the gastrointestinal tract. Such fats need to be emulsified by the detergent action of bile salts before they can be hydrolyzed by lipase into fatty acids and mono- and diglycerides. Research in poultry has indicated that newly hatched chicks have low fat digestion, primarily due to inadequate emulsification rather than a lack of lipase activity. Previously, Polin (1980) reported increased digestibility of fat when lecithin was added to chick diets. Zaefarian et al. (2013) reported the positive effect of microbial phytase (500 FTU/kg diet) on fat and fatty acid digestibility. Despite the potential interactive effects between emulsifiers and microbial phytase, no research has yet been conducted to explore this relationship in broiler chicks. So, we aimed at evaluating the effects of microbial phytase and an emulsifier on growth performance, nutrient digestibility, organ weight, and fecal scores in broilers.

A total of 1134 one-day-old mixed sex Ross 308 broilers chicks with an initial average body weight (BW) of 44.59±0.42g g were allocated into three treatment groups with 21 replicates of 18 birds/pen. The test treatments were: TRT1, basal diet + 1000FTU Phytase; TRT 2, basal diet + 1500FTU Phytase; TRT 3, basal diet + 1000FTU Phytase + 0.05% emulsifier. Birds had *ad libitum* access to water and a three-phase: starter: (d 0-8), grower (d 9-20) and finisher (d 21-35). Growth performance [BW, average daily feed intake (ADFI), average daily gain (ADG), and feed conversion ratio (FCR)] were recorded from d1-35. At d35 nutrient digestibility, fecal score, and meat quality was assessed.

Table 1 - The effect of dietary phytase and emulsifier on growth performance in brolier[1].

Items	TRT1	TRT2	TRT3	SEM[2]	p-value
Finisher phase					
ADG, g	78	76	79	0.52	0.054
BWG, g	1097^{ab}	1069^{b}	1109^{a}	6.77	0.045
FCR	1.535^{ab}	1.577^{a}	1.524^{b}	0.03	0.039
Overall					
ADG, g	56	55	57	0.35	0.059
BWG, g	1977	1929	1996	12.25	0.052
FCR	1.389^{ab}	1.421^{a}	1.382^{b}	0.02	0.037

During starter and grower phase there was no difference observed on the broiler's growth performance whereas, during finisher and the overall experimental period, compared to Trt 1 and 2, Trt 3 group birds showed significantly higher BW, ADG, and reduced FCR with no adverse effect on nutrient digestibility, fecal score, and meat quality. In conclusion, the addition of 1000FTU phytase with 0.05% emulsifier could be beneficial to enhance growth performance of broiler.

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HIGH DOSE OF PROTEASE AND A PROTECTED BLEND OF ORGANIC ACIDS AND ESSENTIAL OILS SUPPORTS THE GROWTH PERFORMANCE OF BROILER CHICKENS DURING HEAT STRESS

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Heat stress can negatively reduce broiler growth performance (Liu et al., 2020). Feed additives such as protease and blends of organic acids and essential oils improve protein digestibility and control pathogenic bacteria (Lu et al., 2020; Yang et al., 2019). In addition, protease can be used to reduce feed cost and, when supplemented in high dose, may also have extra-proteinaceous effects that support intestinal integrity and health, alleviating the negative effects of heat stress. Thus, this study aimed to evaluate the effect of a high dose of protease and a protected blend of organic acids and essential oils on growth performance of broiler chickens during heat stress.

At hatch, 480 one-day old broilers (Ross 708 males) were used in a 28-day study to determine body weight (BW), feed intake (FI), and feed conversion ratio (FCR). Broilers were housed in cages and received *ad libitum* access to feed and water. Treatments were randomly assigned to 48 cages resulting in 16 replicates per treatment with 10 birds per pen. Broilers were fed one of three diets: T1) Control, T2) Protected organic acid and essential oils - P(OA+EO) at 500 ppm, T3) P(OA+EO) at 500 ppm + Protease at 350 ppm. The P(OA+EO) consists of fumaric, sorbic, malic, and citric acids and thymol, vanillin, and eugenol. Treatment containing protease was credited with a crude protein and amino acid matrix. Body weight and FI were measured on days 0, 7, 14, 21 and 28 to calculate FCR. Broilers were introduced to heat stress from days 14-17 (temperature at 35°C and average humidity at 62%). Data were analyzed using JMP 16, and means were compared with Tukey's LSD. A $P \leq 0.05$ was used to indicate statistical significance. On day 7, broilers fed the high dose of protease with P(OA+EO) had 7.4% higher ($P = 0.010$) BW than birds fed only P(OA+EO), with the control treatment serving as intermediate. Feed conversion ratio was not different between treatments at day 7. On days 14, 21 and 28, birds fed the high dose of protease with P(OA+EO) had 8.1%, 4.0% and 3.5% higher BW than birds fed control diets, while birds fed P(OA+EO) only was not different than birds in the control treatment ($P \leq 0.05$). On days 7 and 28, there was no difference for FCR between treatments, but on 14 and 21 days birds fed the combination of high dose of protease and P(OA+EO) had 9 and 5 points reduced FCR compared to birds fed control diet ($P \leq 0.05$), respectively. Birds fed P(OA+EO) alone were not different than birds fed control diet on 7 and 28 days. Feed intake was not different between treatments in any age. In conclusion, the supplementation of a high dose of protease and a blend of protected organic acids and essential oils improved the growth performance of broiler chickens despite being subjected to heat stress. This may be due to the enhanced utilization of proteins as well as improved intestinal function and health. These additives are a good strategy to alleviate the negative effects of heat stress on broiler performance along with the benefit of having saves on feed cost with the use of the protease matrix in the feed.

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EFFICACY OF A NEW BIOSYNTHETIC BACTERIAL 6-PHYTASE ON GROWTH PERFORMANCE, CARCASS CHARACTERISTICS AND BONE TRAITS IN BROILERS FED INORGANIC PHOSPHATE-FREE DIETS

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Summary

An experiment was conducted to investigate the efficacy of a new biosynthetic bacterial 6-phytase on growth performance, carcass characteristics and bone mineralization in broilers fed inorganic P-free diets from 1 to 35 days of age. A total of 390-day-old male Ross 308 chicks were randomly allocated to three experimental dietary treatments using 10 replicate pens per treatment and each pen contained 13 birds. The experimental dietary treatments consisted of a nutritionally adequate positive control (PC) diet containing monocalcium phosphate (MCP) and two diets formulated without MCP and supplemented with phytase at 1000 and 1500 FTU/kg diet, respectively (PHY1000 and PHY1500). Body weight (BW) and feed intake (FI) were measured at 10, 23 and 35 days of age, and BW gain, feed conversion ratio (FCR) and European production efficiency factor (EPEF) were calculated for the whole experimental period. In addition, 10 birds per treatment (1 bird per replicate) were randomly selected for carcass composition and tibia traits determination. Phytase supplementation either at 1000 FTU/kg diet or 1500 FTU/kg diet maintained or improved final BW, overall BW gain, EPEF, carcass characteristics and tibia traits compared with PC treatment. In addition, FCR was improved by 6.0 and 5.7% in birds fed PHY1000 and PHY1500, respectively, compared with birds fed the PC treatment. The results of the present study demonstrated that the novel biosynthetic bacterial 6-phytase could be an effective replacement for fully inorganic P to improve feed efficiency, carcass characteristics, bone traits and sustainability in poultry production.

I. INTRODUCTION

Phosphorus (P) is involved in several chemical reactions in the body and acts on the growth, development and maintenance of skeleton and muscle. However, in plant-based feed ingredients commonly used in poultry diets such as wheat, corn and soybean meal, up to 85% of dietary total P is bound to phytate (Li et al., 2016; Aureli et al., 2017). Phytate is known to contain bound P that is unavailable to the broilers, resulting in the use of inorganic-P sources such as monocalcium phosphate and dicalcium phosphate to fulfill the broiler P requirements. However, the use of inorganic-P sources in broilers not only increases the feed cost but results also in an increase of P released resulting in environmental issues. So, one of the most efficient strategies to optimize P utilization in poultry is through the supplementation of poultry diets with exogenous phytases. This in turn allows for an increase of phytate-P utilization and a reduction in the use of expensive inorganic P. However, the efficacy and consistency of such phytase on these former parameters can vary depending on several factors such as the intrinsic characteristics of phytase (source, dose, optima pH and its affinity to phytate) and diet (composition and the content of phytate, calcium and available P levels). A novel biosynthetic bacterial 6-phytase produced by *Trichoderma reesei* was recently developed. It is characterized by a wide pH profile, a high affinity for myo-inositol hexakisphosphate (IP6) and other myo-inositol phosphate (IP) esters, and a high intrinsic thermostability (Jlali et al., 2024). Therefore, the aim of this study was to investigate the efficacy of a new biosynthetic bacterial 6-phytase on growth performance, carcass characteristics and bone traits in broilers fed inorganic P-free diets from 1 to 35 days of age.

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II. METHOD

A total of 390-day-old male Ross 308 chicks were obtained from a local hatchery. They were randomly allocated to 3 dietary treatments in a completely randomized design. Each treatment consisted of 10 replicate floor pens with 13 birds per pen. Each pen (1.2 m^2) contained rice hulls as litter, with manual feeders (one feeder/pen), and an automatic drinking nipple line system was used. The experimental conditions were automatically controlled and appropriate for the age of the broilers. Temperature for the chicks was initially set at 32 °C for the first week and gradually decreased to 25 °C at d 35. Birds were typically given 23 h of light at day old and stepped down to 18 h light and 6 h dark by 4 days. All birds were fed crumbs during the starter phase (1 to 10 days), and pellets during the grower (11 to 23 days) and finisher (24 to 35 days) phases. The experimental dietary treatments consisted of a nutritionally adequate positive control (PC) diet containing monocalcium phosphate (MCP) and two diets (PHY1000 and PHY1500) formulated without MCP and with a reduction in calcium (of 0.17-0.24% points) and supplemented with a new biosynthetic 6-phytase expressed in *Trichoderma reesei* at 1000 and 1500 FTU/kg diet, respectively.

Table 1 - Ingredient and calculated nutrient content of the 3 treatment diets in starter and grower phases.

Treatments	Starter (day 0 – 10)		Grower (day 11 - 23)		Finisher (day 24 - 35)	
	PC	PHY1000/ PHY1500	PC	PHY1000/ PHY1500	PC	PHY1000/ PHY1500
Ingredient (g/kg, as fed basis)						
Corn	200	200	200	200	200	200
Broken rice	200.4	200.4	221.2	221.2	245	245
Wheat	50.0	50.0	50.0	50.0	50.0	50.0
Soybean meal, 48% CP	204.4	204.4	202.5	202.5	200.3	200.3
Full fat soybean	150	150	100	100	50.0	50.0
Rice bran, extracted	60.0	60.0	60.0	60.0	80.0	80.0
Rapeseed meal	50.0	50.0	60.0	60.0	60.0	60.0
Palm oil	37.5	37.5	62.7	62.7	77.3	77.3
Monocalcium phosphate 22	14.5	-	13.2	-	10.3	-
Limestone	13.2	13.2	11.6	11.6	10.3	10.3
Salt	5.60	5.60	4.60	4.60	2.80	2.80
Sodium bicarbonate	-	-	1.50	1.50	3.00	3.00
DL-Methionine	3.80	3.80	3.20	3.20	2.80	2.80
L-Lysine	3.40	3.40	2.70	2.70	2.30	2.30
L-Threonine	1.60	1.60	1.20	1.20	0.80	0.80
Vitamin and premix	5.30	5.30	5.30	5.30	4.80	4.80
Phytase	-	0.2/0.3	-	0.2/0.3	-	0.2/0.3
Corn cob	0.3	14.6/14.5	0.3	13.3/13.2	0.3	10.4/10.3
Nutrient composition, g/kg						
ME, MJ/kg	12.34	12.34	12.76	12.76	12.97	12.97
Crude protein	220	220	205	205	190	190
Calcium	9.20	6.80	8.30	6.10	7.20	5.50
Total phosphorus	7.80	4.50	7.40	4.50	6.80	4.50
Available phosphorus	4.30	1.50	4.00	1.50	3.40	1.40
Fiber	43.7	43.7	42.2	42.2	42.1	42.1
Methionine	7.20	7.20	6.50	6.50	5.90	5.90
Methionine + Cysteine	10.8	10.8	9.90	9.90	9.10	9.10
Lysine	14.4	14.4	12.9	12.9	11.6	11.6
Threonine	9.70	9.70	8.80	8.80	7.80	7.80
Phytate phosphorus	3.10	3.10	3.10	3.10	3.20	3.20

Experimental diets were based on corn-soybean meal including broken rice, wheat, full-fat soybean meal, rice bran and rapeseed meal. The PHY1000 and PHY1500 diets were formulated without inorganic phosphate (MCP). Diets and water were *ad libitum* provided throughout the experimental period. Body weight (BW) and feed intake (FI) were measured at 10, 23 and 35 days of age, and BW gain, feed conversion ratio (FCR) and European production efficiency factor (EPEF) were calculated for the whole experimental period. On day 35, 10 birds per treatment (1 bird per replicate) were randomly selected and then slaughtered. All carcasses, breast muscles (*Pectoralis major* and *Pectoralis minor*), legs and wings were removed and weighed, and their respective yields were calculated as a percentage of BW at slaughter. In addition, right tibias were also collected from 10 birds per treatment

All data were analyzed using the GLM procedure of SAS software. Dietary treatment was considered as a fixed effect, and block was considered as a random effect. Pen was used as the experimental unit. The differences among means were compared using Duncan's New Multiple Range Test. Statistical significance was set at $P < 0.05$, and $0.05 < P < 0.10$ was considered a trend.

III. RESULTS

The main effects of treatment on growth performance, carcass composition and bone traits are shown in Table 1. No significant differences were observed among treatments on final BW, BW gain, EPEF, carcass characteristics and tibia traits. Birds fed the diets supplemented with phytase at 1000 FTU/kg (PHY1000) and 1500 FTU/kg (PHY1500) exhibited lower ($P < 0.001$) FCR by 6.0 and 5.7%, respectively, in comparison to birds fed the PC diet. Birds fed the inorganic P-free diet supplemented with phytase had lower ($P < 0.05$) feed intake compared with birds fed the PC diet.

Table 2 - Main effects of treatment on growth performance, carcass characteristics and tibia traits in broilers at 35 days of age.

Parameters	Treatments[1]			*SEM*	*P*-value
	PC	PHY1000	PHY1500		
Performances					
BW at 35d, g/bird	2481	2506	2532	18.61	0.55
BW gain 1-35d, g/bird	2438	2463	2489	18.62	0.55
FI 1-35d, g/bird	3541[a]	3363[b]	3408[b]	21.45	0.0005
FCR 1-35d	1.455[a]	1.367[b]	1.372[b]	0.01	0.0009
EPEF 1-35d	473	504	509	8.30	0.17
Carcass characteristics					
Carcass yield, %	93.45	93.62	93.34	0.14	0.72
Breast yield, %	25.82	26.57	25.89	0.34	0.64
Leg yield, %	24.93	24.40	24.60	0.24	0.68
Wing yield, %	7.94	8.11	8.13	0.07	0.55
Tibia traits					
Tibia ash, %	14.95	14.86	15.22	0.23	0.83
Tibia Ca, %	5.16	5.44	5.22	0.15	0.73
Tibia P, %	2.43	2.5	2.64	0.09	0.68

[a-c]Means in the same row with no common superscripts are significantly different at $P \leq 0.05$.

[1]Positive control (PC) represents an adequate-nutrient diet formulated to meet or exceed the requirements of birds and containing monocalcium phosphate, PHY1000 and PHY1500 represent diets formulated without MCP and with a reduction in calcium (of 0.17-0.24% points) and supplemented with a new biosynthetic 6-phytase expressed in *Trichoderma Reesei* at 1000 and 1500 FTU/kg diet, respectively.

BW: body weight; FI: feed intake; FCR: feed conversion ratio; EPEF: european production feed efficiency; Ca: calcium; P: phosphorus; SEM: standard error of the mean.

VI. DISCUSSION

Phytase is commonly used as a feed additive in diets to release phytate-bound P, thereby improving growth performance, carcass characteristics, bone mineralization and sustainability in poultry production and reducing the need for mineral phosphate to meet the P requirement of birds. In the present study, inorganic P-free diets supplemented with phytase either 1000 or 1500 FTU/kg diet presented equivalent final BW, BW gain, EPEF, carcass characteristics and tibia traits to nutrient-adequate PC diet. These results indicate that the new biosynthetic bacterial 6-phytase was efficient in hydrolyzing phytate and releasing enough P and Ca to support and maintain growth performance and bone mineralization of broilers in the absence of inorganic P during the entire cycle of production. As far as we know, only a few previous studies have tested the efficacy of exogenous phytase supplementation in broiler diets in the absence of inorganic P in all feeding phases (Marchal et al., 2021; Bello et al., 2022). These authors indicated the capability of the new generation of phytases added at 1000 FTU/kg diet or more to fully replace inorganic P in broilers fed diets containing high dietary levels of phytate-P. In addition to supporting and maintaining performance, phytase addition to an inorganic P-free diet was able to further improve feed efficiency compared to a nutrient-adequate diet. This improvement in feed efficiency could be due to the release of other nutrients such as amino acids and minerals and the improvement of energy utilization in addition to P and calcium. Previous studies have reported that phytase can exert an extra-phosphoric effect, especially at high doses (≥ 1000 FTU/kg diet) in broilers through the rapid breakdown of phytate principally at the upper part of the gastrointestinal tract which in turn limits the chelation of phytate with nutrients (Walk et al., 2013; Beeson et al., 2017). Further studies are needed to elucidate the benefits of the new phytase on other parameters beyond the performance and bioavailability of nutrients in monogastric animals. In conclusion, the present study demonstrated that the novel biosynthetic bacterial 6-phytase could be an effective replacement for fully inorganic P to improve feed efficiency, carcass composition, bone and sustainability in poultry production. It also highlights the ability of the new phytase to improve feed efficiency even better than the adequate-nutrient diet.

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GROWTH PERFORMANCE AND ENERGY UTILISATION OF BROILERS FED A HIGH LEVEL PALM KERNEL MEAL SUPPLEMENTED WITH BACILLUS-BASED PROBIOTICS AND NSPASE

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Summary

High-fiber poultry diets incorporating ingredients such as palm kernel meal (PKM) have been developed to mitigate rising feed costs. Although PKM is a viable feed option, its inclusion is limited due to anti-nutritive factors. Studies show that while broilers can tolerate up to 20% PKM without adverse effects, higher levels significantly reduce weight gain. Enhancing PKM nutrient utilisation through feed enzymes or probiotics has been proposed. This study investigated the impact of non-starch polysaccharide-degrading enzymes (NSPase) and a combination with Bacillus-based probiotics (PRO) on growth performance and nitrogen-corrected apparent metabolisable energy (AMEn) in broilers fed a high-PKM diet. A total of 3,360 Ross 308 chicks were divided into eight dietary treatments (2×4 factorial design), including control, PRO, NSPase, and their combination, across two PKM levels (0% and 20%). The experiment consisted of three phases: starter (0-10 days), grower (11-21 days), and finisher (22-35 days). AME was assessed from male chicks at age 21-27 days using the classical total excreta collection method, with feed intake and excreta collected and analysed to determine the diet's metabolisable energy content. Results showed that the inclusion of probiotics and NSPase improved body weight (BW), feed intake (FI), and apparent metabolisable energy (AME). The highest BW was observed in the PC+NSPase group, while the NC+NSPase group showed the highest AME and nitrogen-corrected AME (AMEn). However, a 20% PKM inclusion slightly reduced these energy values. The study concludes that Bacillus-based probiotics and NSPase can enhance broiler performance and nutrient digestibility, but higher PKM level reduce feed efficiency due to its fibrous nature.

I. INTRODUCTION

Diets that incorporate high-fiber components have been developed in response to the rising cost and volatility of poultry feed constituents (Tejeda & Kim, 2020; Wealleans *et al*., 2017; Yaophakdee *et al*., 2018). Palm kernel meal (PKM) is a promising byproduct for poultry feed, but its use is constrained by certain anti-nutritive factors, including 78% linear mannan, 12% cellulose, 3% glucoronoxylans, and 3% arabinoxylans (Abdollahi et al., 2016; Yaophakdee et al., 2018). *Abdollahi et al*. (2016) reported the weight gain of broilers fed 8% and 16% PKM increased, but severely reduced by 24% PKM compared to control. Moreover, Yaophakdee *et al.* (2018) stated that PKM can be incorporated at 20% in the broiler diet without any negative effects on the performance of the broilers. Enhancing the nutrient utilisation of PKM may be achieved through the inclusion of feed enzymes or probiotics in the diet (Mahmoud *et al.*, 2017; Shastak *et al*., 2019). Enzyme supplementation, such as mannanase and xylanase, has been shown to improve nutrient absorption and growth by breaking down non-starch polysaccharides (NSP) in high-fiber feeds like Palm Kernel Meal (PKM) (Lin et al., 2023; Navidshad et al., 2016; Wang et al., 2021). The efficacy of Bacillus-based probiotic (PRO) and a combination of two exogenous non-starch polysaccharide degrading enzymes, mannanase

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and xylanase (NSPase), on growth performance and nitrogen-corrected apparent metabolisable energy (AMEn) was investigated in broilers fed a high level of PKM.

II. METHOD

The experimental animal protocol was approved by The Committee of Ethical Clearance for Preclinical Research of The Integrated Laboratory of Research and Testing, Gadjah Mada University, Yogyakarta, Indonesia (Ref. 00036/VIII/UNI/LPPT/EC/2024). The experiment utilised a 2×4 factorial with CRD design, comprising two levels of PKM inclusion: 0% (NC) and 20% (PC), and four additives: 1) control (no additive), 2) PRO with 500 g/ton feed inclusion of *Bacillus licheniformis* DSM 28710 (B-Act®, Huvepharma), 3) NSPase, with combination inclusion of 100 g/ton feed Xylanase (Hostazym® X, Huvepharma) and 500 g/ton feed of Mannanase (Hemicell^{TM} HT, Elanco Animal Health), and 4) a combination of PRO and NSPase (PRO+NSPase), resulting in 8 dietary treatments.

A total of 3360 one-day-old Ross 308 as hatched chicks, were assigned to 56 pens, with 60 birds per/pen, and 7 pens/treatment. There were 3 phases in the experiment: the starter phase (0-10 d), grower phase (11-21 d), and finisher phase (22-35d). AME evaluation phase (21-27 d) divided into adaptive phase (21-23 d) and test phase (24-27 d). During the rearing phases, birds were placed on floor-pen (4 x 1.5 m) with 5 cm of rice-hull as bedding material for 35 days with *ad libitum* supply of feed and water. On 21 d of age, 5 male birds per pen were selected based on their mean bodyweight and moved into a cage-pen (60 x 60 x 60 cm). The AME assay was conducted using the classical total excreta collection method. The diets were fed for 7 days (21-27 d), with the first 3 days served as the adaptation phase. During the last 4 days, feed intake was monitored, and the excreta was collected daily, weighed, and pooled within a cage. Pooled excreta were mixed, and representative samples were obtained and oven-dried. Dried excreta samples were ground to pass through a 0.5-mm sieve and stored in airtight plastic containers at 4°C for laboratory analyses. The dry matter (DM), gross energy (GE) and nitrogen (N) of the diet and excreta samples were determined.

The AME of diet was calculated using the formula as described by (Wang *et al*., 2022):

AME (kcal/kg DM) = ((Feed intake × GE diet) – (Excreta output × GE excreta)) / (Feed intake)

AMEn (kcal/kg DM) = AME – (Retained Nitrogen × 8.22) / (Feed Intake)

The pen was the experimental unit for statistical analysis of all data. The data were analysed using the General Linear Model procedure in SAS® 9.4 version (SAS® Institute Inc., Cary, NC, USA) in a completely randomised design with 2 x 4 factorial arrangement (PKM inclusion and additive supplementation). The differences among treatments were determined using post-hoc Tukey's HSD test. The significance level was set at $p < 0.05$.

III. RESULTS

This study evaluated the effects of Bacillus-based probiotics and NSPase on the growth performance and nutrient digestibility in broilers from 0 to 21 days. Two tables summarised the key outcomes across multiple treatment groups. Table 1 presents the effect of Bacillus-based probiotics and NSPase on growth performance. The growth performance of broilers from 0 to 21 days was significantly influenced by the addition of probiotics and NSPase, however PRO+NSPase group did not significantly enhance overall growth performance until the grower phase, prior to the digestibility study. Among the groups, the PC+NSPase resulted in the highest BW (2247 g) and FI (3849 g/bird), while NC+NSPase exhibited the lowest FCR (1.61) highest European Efficiency Factor (EEF) (374). The inclusion of 20% PKM slightly increased BW but had minimal effect on feed intake and FCR.

Table 2 presents the effects of Bacillus-based probiotics and NSPase on Apparent Metabolisable Energy (AME) and Apparent Metabolisable Energy corrected for nitrogen (AMEn). In terms of energy utilisation, the combination of probiotics and NSPase improved AME and AMEn values, with the NC+NSPase group yielding the highest AME (3247 kcal) and AMEn (3228 kcal). The use of PKM slightly reduced these values, with 20% PKM showing lower energy efficiency.

Table 1 - Effect of Bacillus-based Probiotics and NSPase on growth performance (0 – 35 d) of broilers.

Treatment group	BW (g)	FI (g/bird)	FCR	EEF
NC	2165^{ab} ±51.4	3511^{bc} ±72.1	1.60^{bc} ±0.02	371^{a} ±9.9
NC+PRO	2034^{b} ±42.6	3400^{c} ±50.9	1.67^{abc} ±0.01	331^{c} ±7.9
NC+NSPase	2222^{ab} ±33.3	3563^{bc} ±36.8	1.61^{c} ±0.01	374^{a} ±9.8
NC+PRO+NSPase	2059^{ab} ±82.3	3470^{bc} ±87.5	1.69^{abc} ±0.03	333 ^{bc}±19.5
PC	2177^{ab} ±67.7	3731^{ab} ±50.9	1.72^{a} ±0.03	350^{abc} ±16.3
PC+PRO	2226^{ab} ±44.3	3843^{a} ±59.9	1.71^{ab} ±0.01	366^{ab} ±8.2
PC+NSPase	2247^{a} ±26.5	3849^{a} ±51.3	1.71^{ab} ±0.01	364^{abc} ±5.1
PC+PRO+NSPase	2190^{ab} ±33.8	3658^{abc}±42.5	1.67^{abc} ±0.03	365^{abc} ±11.3
PKM 0%	2126^{b}	3491^{b}	1.65^{b}	362^{a}
PKM 20%	2210^{a}	3769^{a}	1.70^{a}	354^{b}
Without Additive	2170	3621	1.67	361
PRO	2139	3642	1.69	350
NSPase	2235	3717	1.66	369
PRO+NSPase	2136	3580	1.68	352
ANOVA	0.024	<0.0001	0.001	0.027
PKM	0.012	<0.0001	0.004	0.024
Additive	0.097	0.1045	0.823	0.150
PKM × Additive	0.219	0.1507	0.826	0.035

Means in the same column with different superscripts differ, $p < 0.05$
Statistically Tukey's Studentized Range (HSD) Test

Table 2. Effect of Bacillus-based Probiotics and NSPase on AME and AMEn (as 100%DM basis) of broilers.

Treatment group	AME	AMEn
NC	3145^{abc} ±56.8	3127^{abc} ±56.4
NC+PRO	3220^{ab} ±63.5	3202^{ab} ±63.1
NC+NSPase	3247^{a} ±32.1	3228^{a} ±31.7
NC+PRO+NSPase	3175^{abc} ±40.8	3157^{abc} ±40.4
PC	3036^{c} ±53.8	3019^{c} ±53.4
PC+PRO	3082^{bc} ±28.8	3064^{bc} ±28.5
PC+NSPase	3060^{bc} ±66.3	3043^{bc} ±65.8
PC+PRO+NSPase	3040^{c} ±51.1	3022^{c} ±50.5
PKM 0%	3197^{a}	3178^{a}
PKM 20%	3054^{b}	3037^{b}
Without Additive	3154	3135
PRO	3151	3133
NSPase	3108	3090
PRO+NSPase	3090	3073
ANOVA	0.021	0.021
PKM	0.001	0.001
Additive	0.520	0.525
PKM × Additive	0.890	0.895

Means in the same column with different superscripts differ, $p < 0.05$.
Statistically Tukey's Studentized Range (HSD) Test

IV. DISCUSSION

The results suggest that the use of Bacillus-based probiotics and NSPase can positively impact the growth performance of broilers. The highest BW and FI in the PC+NSPase group indicates improved nutrient utilisation, although this came with a trade-off in FCR, suggesting reduced feed efficiency. Yaophakdee *et al*. (2018) reported that FCR was found higher in birds fed the high PKM diet, which could relate to the physical characteristic of PKM and its contribution to the overall nutrients in the diet, in particular amino acids utilisation, as PKM contains low levels of key essential amino acids such as lysine and methionine.

The data from Table 2 further support the role of probiotics and NSPase in improving energy utilisation, as seen in the elevated AME and AMEn values. These findings align with previous studies suggested that enzyme supplementation can enhance nutrient digestibility (Shastak *et al*., 2019). However, the decline in energy efficiency with higher PKM levels suggests a limit to the benefits of PKM as a replacement feed ingredient, likely due to its fibrous nature and lower digestibility.

In conclusion, the study demonstrated that the use of Bacillus-based probiotics and NSPase could improve broiler growth performance and energy utilisation. The NSPase supplementation, particularly in the NC+NSPase group, resulted in the highest AME and AMEn values, indicating better nutrient digestibility. However, higher levels of PKM reduced these benefits, suggesting that PKM inclusion should be carefully managed to optimise feed efficiency.

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FACTORS AFFECTING UPLIFT IN INDIVIDUAL ILEAL AMINO ACID DIGESTIBILITY FOLLOWING EXOGENOUS PROTEASE SUPPLEMENTATION: A META-ANALYSIS

A. WEALLEANS[1] and S. COURT[2]

Summary

A meta-analysis of the effect of supplemental protease supplementation on the apparent ileal digestibility of amino acids in pig and poultry diets was conducted to assess the relationship between basal digestibility and protease effects, as well as average responses and differences between species. The mean response to protease was 3.21% (SE 0.11, P<0.001), and this ranged from 1.2% for tryptophan (Trp) to 4.33% for alanine (Ala). Background phytase presence significantly reduced the uplift in amino acid digestibility due to protease supplementation across the whole dataset, though only for proline (Pro) (+4.17 without, +1.26% with, P<0.05). Basal digestibility explained around 57% of the variance in response to protease (P<0.0001). This study contributes to the wider understanding of the mode of action of proteases, and indicates that consistent practical application would benefit from flexibility in dose and matrix, taking into consideration basal diet digestibility.

I. INTRODUCTION

It is well known that the basal digestibility of dietary nutrients is a key determining factor in the response to enzyme supplementation in pigs and poultry (Cowieson and Roos, 2014). For proteases, this may be particularly relevant as the substrate for protease is relatively ill-defined compared to that of other feed enzymes, but is largely regarded as the undigested fraction of dietary protein. A previous meta-analysis of the relationship between inherent digestibility and protease response found a curvilinear relationship, which explained approximately 47% of the variance in response to protease supplementation (Cowieson and Roos, 2014). However, this analysis focused narrowly on one commercial protease, and did not assess the effect of other dietary factors on the amino acid response to protease, including the presence or absence of background phytase supplementation.

Therefore, the aim of the present work was to assess the wider relationship between basal amino acid digestibility and the response to supplemental proteases. The database was wider than used by Cowieson and Roos (2014) or Lee et al. (2018), comprising data from a range of non-commercial and commercially available protease molecules. It aimed to assess the interaction between phytase presence and protease response.

II. METHOD

A meta-analysis of the effect of supplemental protease supplementation on the apparent ileal digestibility of amino acids in pig and poultry diets was conducted to assess the relationship between basal digestibility and protease effects, as well as average responses and differences between species. A total of 43 independent experiments were included, comprising a total of 2699 paired datapoints (within-study control and protease); 76.6% of data referred to protease supplementation in broilers, with 10.5 and 12.9% reporting results from turkeys and pigs, respectively. The ingredient profile of the contributing diets varied, but generally the major

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protein source was soybean meal (SBM); 39% of the control diets used SBM as the only protein meal in the formulation.

Extracted data were analysed in the Fit Model platform of JMP 17 (SAS Inst. Inc, Cary, NC). To account for the random effect of study, average digestibilities were assessed across species using the REML method, with trial considered the random effect. Means separation was conducted using Tukey's HSD, and significance was determined at P<0.05. To model the response of protease as a function of digestibility in the control, the digestibility of the control group and the square of the digestibility of the control group were used as the fixed effect, with study as a random effect.

III. RESULTS

There were significant differences in the basal digestibility of amino acids between species, with the highest levels seen in broilers, then turkeys, then pigs (78.97% broilers, 74.34% turkeys, 72.59% pigs; P<0.0001). Protease supplementation significantly improved the ileal digestibility of all amino acids (P<0.05) except for Trp (P=0.2239). While the average uplift was 3.21 ± 0.11% across all amino acids (AAs), the size of uplift ranged from 4.33 and 4.35% for Ala and cysteine (Cys), respectively, to 2.3, 2.44, 2.57 and 2.25% for arginine (Arg), aspartic acid (Asp), glutamic acid (Glu), histidine (His) and lysine (Lys), respectively. There were also significant differences in average uplift with protease between species, with turkeys benefiting most from protease supplementation (+9.86% turkeys, +4.48% pigs, +3.32% broilers, compared to control, P<0.001).

The digestibility of AAs in the within-study control diet was found to be highly predictive of protease ability to improve AA digestibility. The following quadratic model explains this relationship:

$$Uplift, \% = 18.054 - (0.192 \times C) + (0.005 \times (C - 79.563)^2)$$

(where C is the digestibility of amino acids in the control diet in percent. This model explained 56.81% of the variance in response to protease supplementation.)

1060 datapoints (39.72% of total datapoints) indicated the effect of protease on AA digestibility in the presence of phytase (average 1000 FTU/kg). Across all measured AAs, phytase presence had a significant negative effect on the ability of protease to improve amino acid digestibility (+4.52% without phytase, +2.26% with phytase, compared to control, P=0.0210), however the only individual AA to be significantly decreased with phytase was Pro (4.17 vs 1.26%, P<0.05). Regression models developed in the absence of phytase predicted an uplift in AA digestibility of 4.56% when control diet digestibility was at 70%, versus a predicted uplift of 2.85% at control diet digestibility of 90%. The same model in the presence of phytase predicted an uplift in AA digestibility of 5.20% when control diet digestibility was at 70%, versus a predicted change in digestibility of -1.02% at control diet digestibility of 90%.

IV. DISCUSSION

The present analysis confirms the importance of basal diet digestibility as a predictor of protease effect (Cowieson and Roos, 2014) and provides a foundation for rationalising and optimising protease use in animal feeds based on the inherent digestibility and availability of amino acids within the raw materials. Across the dataset, average amino acid digestibility of the control diets were 78.97 and 74.34% for broilers and turkeys, respectively. Applying the model, this would equate to estimated uplifts with protease supplementation of 2.89 and 3.75%, and subsequent digestibility of 81.25 and 77.13%, respectively. By contrast, the average uplift by species across the original dataset was 9.87% for turkeys and 3.32% for broilers, suggesting that the model is under-predicting the response in turkeys but broadly accurate for broilers.

The effect of phytase presence on the ability of protease to improve AA digestibility and subsequently growth performance is much discussed. Lee et al. (2018) found that when supplemented alone protease improved AA digestibility by 1.6%, but saw no significant effect when background phytase was present. This agrees with the present study, which identified a significant effect of phytase on the ability of protease to improve AA digestibility. For all amino acids, the effect of protease in diets without phytase was 4.52%, but with phytase this was reduced to 2.26% ($P<0.05$). The only individual AA to be significantly decreased with phytase was Pro (4.17 vs 1.26%, $P<0.05$).

Using the interquartile range to represent the 'average' diet, control digestibility measurements were between 75.46 and 86.90% (mean 79.57, median 82.06%). Within this range, the two models predict the differing uplifts for diets with/without phytase, as shown in Table 1. The predicted uplifts are very similar for diets up to the median, with divergence in predicted protease efficacy at a basal digestibility above 80%.

Table 1 - Effect of phytase on uplift in AA digestibility following protease supplementation at a range of control digestibility's.

Control digestibility, %	Uplift without phytase, %	Uplift with phytase, %
75.46	3.61	3.31
79.57 (mean)	2.76	2.35
82.06 (median)	2.53	1.57
86.90	2.34	0.02

In conclusion, the data provided in this paper indicates the size of uplift in individual amino acid digestibility following supplementation with exogenous proteases is driven by basal AA digestibility. Species differences in response to protease supplementation may be linked to basal digestibility, with lower mean digestibility in pigs potentially providing a greater opportunity for protease use. The impact of background phytase on the performance response to protease, given its ability to limit potential AA digestibility improvements in highly digestible diets, needs to be further investigated in practical conditions. The relationship between basal digestibility and protease effectiveness means that benchmarking of diet and ingredient digestibility would help nutritionists improve the flexibility and reliability of protease supplementation in commercial applications, potentially increasing the use of less digestible raw materials.

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IMPACT OF LYSOLECITHIN, MONOGLYCERIDE AND EMULSIFIER COMBINATIONS ON EMULSION STABILITY AND RATE OF LIPID HYDROLYSIS

A. WEALLEANS[1], R. SPAEPEN[1] and S. COURT[1]

Lysolecithin (LPL) is an effective tool to improve dietary nutrient availability– especially when supplemented in combination with other potentiating compounds (Wealleans et al., 2020), though little research has assessed the effect of dose. Therefore, the impacts of increasing LPL concentration and synthetic emulsifier and monoglyceride (ME) inclusion was assessed *in vitro*. It was hypothesized that addition of ME would ameliorate higher LPL inclusion.

Products used: A dose series of lysolecithin/oil mixes was created (0, 25, 35, 45, 55, and 65% LPL). LPL dilutions were either tested alone (LPL-) or with the inclusion of ME at 8.85% (LPL+). *Emulsion stability:* 1g LPL was added to 49g rapeseed oil and 50mL brine solution, mixed for 30s at 25,000 RPM, and stability was followed for 120 min. *Hydrolysis:* 1g LPL, 49g rapeseed oil and 150g simulated intestinal fluid were mixed for 30s at 25,000 RPM, then placed in a 40°C water bath and stirred at 700 RPM. 4g of pancreatin was added to start hydrolysis. For analysis, a 2mL sample was pipetted into 40mL 80°C neutralized ethanol solution and titrated with 0.1M KOH till equivalence was reached. The level of free fatty acids (expressed in % oleic acid) was calculated. *Statistics:* Data were analysed in the Fit Model platform of JMP17, with LPL dose and ME addition considered fixed factors in the model; $P<0.05$ was considered statistically significant.

Emulsion stability declined over time, though there were significant interactions with LPL dose and ME addition ($P<0.01$) – increasing LPL reduced separation, as shown in Figure 1, with LPL+ formulations significantly more effective than the corresponding LPL-. LPL level also significantly increased release of FFA ($P<0.0001$), as shown in Figure 2, with the difference in release between LPL concentrations increasing with time ($P=0.0009$). LPL level affected the release of DAG, MAG and FFA levels and degradation of triglycerides and production of FFA plateaued between 25 and 55% LPL addition. The results showed that supplemental ME was effective at increasing emulsion stability; further research is required to elucidate the effect of ME on rates of hydrolysis.

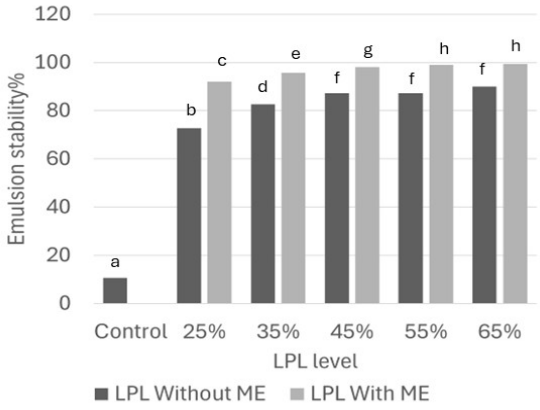

Figure 1 - Emulsion stability of various LPL mixtures with and without ME after 120 minutes.

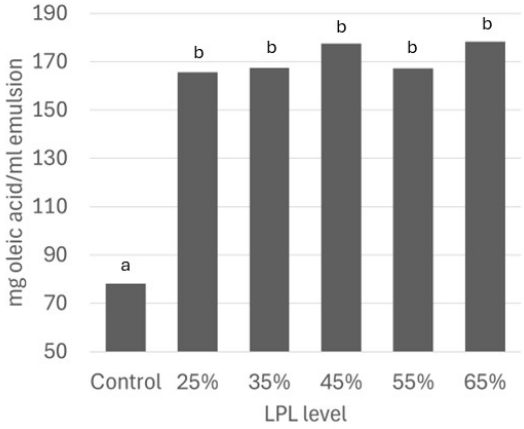

Figure 2 - Extent of lipid hydrolysis of different LPL mixtures after 120 minutes.

*Values that differ significantly $P<0.05$ are indicated by different letters. Control samples did not contain any LPL.

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EFFECT OF A LYSOLECITHIN PRODUCT ON BROILER PEFORMANCE AND NUTRIENT DIGESTIBILITY

B. GUO[1], C. DE WILDT[2] and Y.X. YANG[3]

Summary

In this trial, the effects of a commercial lysolecithin supplement were evaluated in broilers fed maize-soy based diets with high and reduced energy levels. As a result, we found that this lysolecithin product increased nutrient availability and utilisation in the presence of a multi-enzyme complex and phytase. Also, this product could well compensate for energy reduction of up to 85 kcal/ kg feed without affecting the broiler performance.

I. INTRODUCTION

Incorporating fat into broiler diets serves as a concentrated energy source, promoting faster growth and improved feed efficiency (Pesti et al., 2002) while enhancing feed milling processes. However, young birds have limited fat digestion capabilities due to underdeveloped lipase activity and insufficient bile salt production (Maisonnier et al., 2003). As birds age, fat utilisation naturally improves, but it can also be enhanced through the inclusion of biosurfactants such as lecithins, and lysolecithins (Oketch et al., 2023). Lysolecithins, in comparison to lecithins, exhibit stronger hydrophilic properties and more effective oil-in-water emulsification (Oketch et al., 2023), which makes them superior in aiding fat and oil digestion in broilers. Studies on broiler diets supplemented with lysolecithin have shown improvements in weight gain and feed conversion ratio (FCR) (Allahyari-Bake et al., 2017), while others have reported increases in apparent metabolisable energy (AME) (Boontiam et al., 2019). These enhancements in broiler performance are partly due to lysolecithin's ability to improve fat emulsification (Joshi et al., 2006), creating smaller fat droplets that facilitate more efficient lipase activity. Lysolecithins also interact with the phospholipid bilayers of intestinal cells, inducing local curvature in the bilayers (Wendel, 1995; Mandalari et al., 2009) and promoting alterations in protein channel formation, which increases ion exchange (Lundbaek & Andersen, 1994; Maingret et al., 2000), thereby enhancing membrane fluidity and permeability. Finally, lysolecithin can also improve broiler's gut health conditions by re-balancing microbiome (Liu et al., 2020).

Although there is existing research on the effects of lysolecithin on broiler performance, most of these studies focus on "on-top" supplementation, where performance improvements are anticipated, although it is not always the case. In contrast, commercial practices often reduce dietary energy content when adding fat sources derived from both animals and plants, like poultry tallow, palm oil and soy oil, to lower feed costs while maintaining performance. However, few studies have directly compared the effects of lysolecithin in standard broiler diets with those in reformulated diets where fats and oils are reduced or replaced. Therefore, the purpose of this study was to assess the efficacy and consistency of a commercial lysolecithin additive (FRA® LeciMax Dry, Lec as abbreviation in below) in enhancing performance across standard and reformulated broiler diets. The carcass properties and nutrient utilisation were also evaluated.

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II. METHOD

This trial was conducted in Thailand and lasted 35 days. For the study 400 male day-old birds (Ross 308) were used. The experimental design was a 2x2 factorial design studying the effect of metabolisable energy level (ME) and the supplementation of a lysolecithin additive resulting in four treatments with 10 replicates of 10 birds each. The four diet groups were formulated and tested as follows: Positive Control (PC): Basal maize-soybean meal starter (0-10 days), grower (11-24 days), and finisher (25-35 days) diets were formulated with high crude fat, using palm oil as the main fat source. The palm oil content of starter, grower and finisher phases, was 3.6%, 4.7% and 5.4%, respectively. The energy content of these diets was 3000, 3100, and 3200 kcal/kg ME for the starter, grower, and finisher phases, respectively (nutrients met or exceeded the Ross broiler recommendations by Aviagen). Negative Control (NC): These diets were derived from the PC diets by reducing the energy levels by 75 kcal ME/kg in the starter, 80 kcal ME/kg in the grower, and 85 kcal ME/kg in the finisher, mainly by removing palm oil. PC+Lec: This group received the PC diets supplemented with 500 g/MT Lec. NC+Lec: This group received the NC diets supplemented with 500 g/MT Lec. All diets were supplemented with a commercial multi-enzyme preparation (Rovabio® Advance T, containing1250 vu/g xylanase and 860 vu/g beta-glucanase) at 100 g/MT level. During the starter phase, diets were provided in crumble form, while grower and finisher phases were fed as pellets. Feed and water were available ad libitum. Birds were vaccinated on day 7 against ND and IB, and on day 14 against IBD.

During the trial, body weight, feed intake, body weight gain, and FCR were measured per pen. Mortality and culling were recorded daily. On day 35, two birds per pen were slaughtered for carcass trait measurements (dressing percentage, breast yield, thigh yield, drumstick yield, and abdominal fat).

For the digestibility study, excreta samples were collected from days 25 to 28. Growth performance, feed intake, FCR, and mortality were monitored. The AME corrected for nitrogen (AMEn) and digestibility of crude protein and crude fat were measured. Data were analysed using ANOVA in a randomised complete block design (RCBD) with SAS software, and differences among treatments were evaluated using Duncan's multiple range tests at a 5% significance level.

III. RESULTS AND DISCUSSION

During the trial, none of the treatments affected growth performance or survival rates (Table 1). When energy levels were reduced, feed intake ($P<0.0001$) and FCR ($P<0.0001$) significantly increased in the NC group compared to PC. As anticipated, adding Lec to the PC diet (PC+Lec) offered no additional benefit. However, supplementing the NC diet with Lec significantly lowered the FCR ($P<0.0001$) compared to the NC alone and resulted in performance comparable to the PC, suggesting that the energy reduction was offset by Lec. Moreover, there was an interaction (P=0.0282) between diet energy level (ME) and Lec on FCR, e.g. supplementation with Lec reduced FCR when ME was lowered but had no effect at the higher ME level. This may be because that a lower ME diet restricts energy availability and performance more than a higher ME diet, which increases the potential for Lec to enhance nutrient utilisation in energy-restricted conditions. Previous studies also revealed that lysolecithin inclusion can resort the performance when the broilers were offered an energy reduced diet, likely due to the improved gut morphology and nutrient transporters of small intestine (Zhang et. al., 2022; Khonyoung et al., 2015).

Table 1 - Effect of the supplementation of FRA® LeciMax Dry on broiler performance (day 0-35).

Treatment	Initial BW (g)	Final BW (g)	BWG (g)	Feed intake (g)	FCR[5]	Livability (%)
PC[1]	43	2556	2512	3302	1.314	98.00
NC[2]	43	2598	2555	3479	1.362	100.00
PC + Lec[3]	43	2590	2547	3322	1.305	99.00
NC + Lec[4]	43	2614	2570	3410	1.327	98.00
Pooled SEM		*23.865*	*23.824*	*28.576*	*0.005*	*1.134*
C.V., %		*2.91*	*2.96*	*2.67*	*1.29*	*3.63*
Source			*P*-value			
Dietary treatment		0.3874	0.3854	0.0005	<0.0001	0.5532
Factor A (diet)		0.1800	0.1799	<0.0001	<0.0001	0.6629
Factor B (Lec)		0.3032	0.3014	0.4074	0.0004	0.6629
Diet x Lec		0.7044	0.6993	0.1298	0.0282	0.1972

BW: body weight; BWG: body weight gain; FCR: feed conversion ratio
[1]PC: positive control; [2]NC: negative control; [3]PC + Lec: positive control + 500 g/MT FRA® LeciMax Dry; [4]NC + Lec: negative control + 500 g/MT FRA® LeciMax Dry
[5]Feed conversion ratio corrected for mortality and culls

A reduction in abdominal fat was observed in both the NC and NC+Lec groups compared to the PC group (*P*=0.0446), with the NC+Lec group showing the lowest abdominal fat percentage, likely due to improved energy utilisation (Figure 1). No other carcass characteristics were influenced by the treatments.

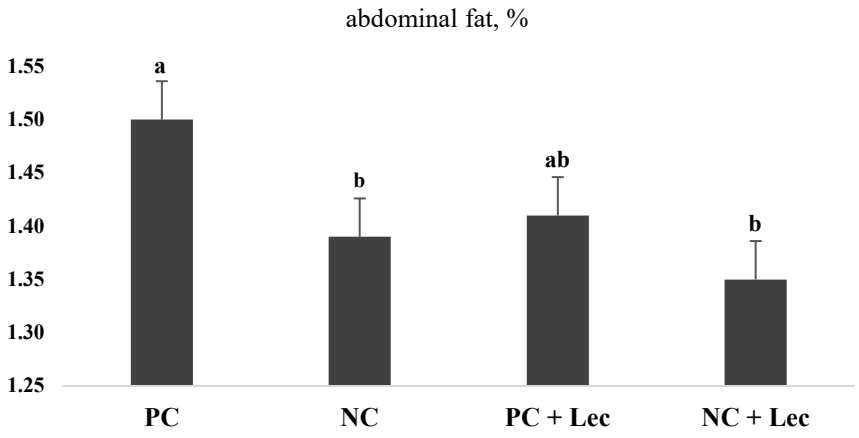

Figure 1 - Effect of supplementation of FRA® LeciMax Dry on broiler abdominal fat percentage (day 35). PC: positive control; NC: negative control; PC + Lec: positive control + 500 g/MT FRA® LeciMax Dry; NC + Lec: negative control + 500 g/MT FRA® LeciMax Dry.

In comparison to the PC, lowering energy levels in NC led to reduced AME (*P*=0.0127) and AMEn (*P*=0.0177) values, while crude protein digestibility (*P*<0.0001) increased (Table 2). Adding Lec to the PC diet did not impact AME levels or nutrient digestibility. However, the NC+Lec group showed significantly higher AME (*P*=0.0127) and AMEn (*P*=0.0177) than the NC, with performance similar to both the PC and PC+Lec groups. Additionally, the NC+Lec group surpassed all other treatments in crude protein (*P*<0.0001) and crude fat digestibility (*P*=0.0060). Besides the improved fat utilisation mentioned above, the enhanced AME and AMEn in both Lec treated groups may also result from the increased circulating glucose level and high-density lipoprotein as previously reported (Boontiam et al., 2019).

Table 2 - Effect of the supplementation of FRA® lecimax Dry on AME, amen, crude protein and crude fat digestibility.

Treatment	AME as fed (kcal/kg)	AMEn as fed (kcal/kg)	ATTD of CP, %	ATTD of CF, %
PC[1]	3111[a]	2899[a]	59.68[c]	70.55[b]
NC[2]	3054[b]	2843[b]	61.53[b]	70.82[b]
PC + Lec[3]	3119[a]	2903[a]	60.08[bc]	69.30[b]
NC + Lec[4]	3108[a]	2891[a]	63.97[a]	73.16[a]
Pooled SEM	13.696	*13.532*	*0.529*	*0.687*
C.V., %	1.25	*1.33*	*2.44*	*2.74*
Source		P-value		
Dietary treatment	0.0127	0.0177	<0.0001	0.0060
Factor A (diet)	0.0247	0.0194	<0.0001	0.0067
Factor B (Lec)	0.0356	0.0684	0.0199	0.4337
Diet x Lec	0.1044	0.1270	0.0932	0.0161

AME: apparent metabolisable energy; AMEn: apparent metabolisable energy corrected for nitrogen; ATTD: apparent total tract digestibility; CP: crude protein; CF: crude fat
[1]PC: positive control; [2]NC: negative control; [3]PC + Lec: positive control + 500 g/MT FRA® LeciMax Dry; [4]NC + Lec: negative control + 500 g/MT FRA® LeciMax Dry

IV. CONCLUSION

The study showed that Lec improves nutrient availability and utilisation when combined with a multi-enzyme complex and phytase, while also compensating for energy reductions of up to 85 kcal/kg in the finisher phase without negatively impacting animal performance. The findings from this study offer a practical example for the feed industry to optimise oil use in feed formulations, providing greater flexibility and efficiency under these conditions.

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