Investigations into biological factors associated with variation in growth performance of broiler chicks.

By

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Thesis submitted to the division of Animal Sciences, School of Bioscience, University of Nottingham, for the degree of Doctor of Philosophy in Animal Science

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May 2024

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List of Abbreviations

ADF Acid detergent fibre

ADWG Average daily weight gain

ANFs Anti-nutritional factors

BWC Bodyweight

BWC Bodyweight change

CCL17 CC chemokine ligand 17

cDNA Complementary DNA

CLDN20 Claudin-20

CV Coefficient of variation

D0 Day 0

D14 Day 14

D21 Day 21

D7 Day 7

DEG Differentially expressed genes

DNA Deoxyribonucleic acid

GIT Gastrointestinal tract

GSTA3 Glutathione S-transferase A3

HBW High bodyweight

HW Hardwood

ICPMS Inductively coupled plasma–mass spectrometer.

IgA Immunoglobulin A

IgM Immunoglobulin M

IgY Immunoglobulin Y

IL20RA Interleukin 20 receptor, alpha subunit

IL8L1 Interleukin 8-like 1

IL26 Interleukin 26

LBW Low bodyweight

mRNA Messenger RNA

NDF Neutral detergent fibre

NK Natural killer cells

PAMPs Pathogen-associated molecule patterns

PET Petroleum ether

PRRs Pattern recognition receptors

RNA Ribonucleic acid

RT-qPCR Quantitative reverse transcription polymerase chain reaction

SEM Standard error of mean

SOD3 Superoxide dismutase 3

SP Super performers UP

SW Softwood

TLRs Toll-like receptors

UP Under performers

Declaration

I declare that this thesis has not been submitted for the award of any degree at the University of Nottingham, United Kingdom or at any other university in the world. I also declare that the research work contained therein is originally mine.

Dedication

This work is dedicated to My Lord Jesus Christ, The Author and Finisher of my faith.

Acknowledgements

My profound gratitude goes to my wonderful supervisor, Dr. Cormac O'Shea, who has been a pillar of support and source of inspiration all through this academic journey. Dear Cormac, you have been an all-round wonder and more like a good friend, my heartfelt gratitude for all the useful advice and support. Thank you immensely Cormac, you made this journey smooth! My gratitude also goes to my second supervisor Dr. Gavin White for his useful contributions, advice and always ready to offer solutions to problems. Dear Gavin, you are amazing, and I can't thank you enough. I am very grateful to Professor Emily Burton and the entire team at the poultry research unit, Nottingham Trent University, Brackenhurst Campus. Thank you, wouldn't be enough! Dear Emily, you have been very helpful throughout this research. Your contributions paved the way to the success of this whole thesis, I appreciate all you have done and the important role you played towards the success of my PhD journey. Many thanks to Associate Professor Jean Margerison, for her valuable support, advice, and words of encouragement throughout my Nottingham journey. Dear Jean, I appreciate you a lot. My gratitude also goes to Professor Phil Garnsworthy, for his guidance and contributions as my first internal assessor. Many thanks to Tim Parr for his interest and involvement as my second internal assessor.

I am very grateful to all the laboratory technicians who have provided lots of support and assistance during this journey, I say a huge thank you to Dr. Kamila Derecka, Saul Vazquez Reina, Jennifer Hankin, Agnes Cerniauskaite, Neil Saunders, Doris Klisch, Dr. Marcos Castellanos Uribe, Kamal Swarup, Dr. Charlene Dambire and all those that contributed to the success of my PhD. You all made it happen, and I am so grateful for all your advice and support.

To my financial sponsors, The University of Nottingham, and Tertiary education trust fund (TETFUND), I truly appreciate all your support, which played major role towards the success of

this research. I also appreciate KTN UK, Researcher Academy, Sam Burton, and Lamming award sponsors for funding my conference attendance and travels.

To my beloved family, my husband, and daughters, thank you for being the main engine room of my success, for lighting up my moody days and hyping me to success. I truly and deeply appreciate you all and the support I have received during this journey and always. I appreciate my lovely parents for their immense support and prayers, I am very pleased to have been able to make you both proud as the first doctoral researcher in the family, thank you for always being there for me and encouraging me. Many thanks to my siblings for their support and encouragement. To my late father-in-law, I am very much convinced you would be proud of me wherever you are, thank you for always supporting my academic ambitions and all the encouragement. To my mother-in- law, I appreciate all your words of encouragement and support, I am truly grateful for all you have done and always do. To my brothers and sister's in-laws, I truly appreciate you all for your support towards the success of this journey, you all are the best!

To my friends and colleagues, you all made it happen, and I am saying a huge thank you to each of you for being there when I needed someone to talk to.

My highest and greatest gratitude goes to God Almighty, whose grace and mercy sustained me throughout this research program and always!

Publications and Research Presentations

Journal publications

Elvis-Chikwem, C.L., White, G.A., Burton, E. and O'Shea, C.J., 2024. Differences in broiler bone, gut, and tissue mineral parameters, as influenced by broilers grouped based on bodyweight. *Animal Production Science*, *64*(2). doi:10.1071/AN23270

Conference oral presentations

Elvis-Chikwem, C.L., White, G., Burton, E., and Cormac O'Shea (2022). Evaluating gut pH and gizzard fibre content of 21day old broiler chicks of varying bodyweight. *Book of Abstract Animal Science and Aquaculture- Challenges and Research, 1st International Scientific Conference for students, page 17*

Elvis-Chikwem, C.L., White, G., Burton. E., and Cormac O'Shea (2023). Differences in ileal transcriptomic profiles of 7days old broilers with distinct bodyweights. Book of Abstract, Animal Science and Aquaculture – *Challenges and Research, 2nd International Scientific Conference for student, page 18*

Conference poster presentations

Elvis-Chikwem, C.L., White, G., Burton, E., Derecka, K., Reina, S.V., Hankin, J. and O'Shea, C., (2021). 180 Evaluation of the tibial bone ash and mineral concentration of Ross 308 broilers with divergent bodyweights on D7. *Animal-science proceedings*, *12*(1), p.150.

Elvis-Chikwem, C.L., Burton, E., White, G. and O'Shea, C. (2022). Identifying Differences in Tibial Ash, Morphometric Parameters, and Mineral Profile in 21-day-old Broiler Chicks of Varying Bodyweights. In *Poultry Science Symposium Series* (p. 203).

Preliminary chapter

Thesis abstract

Compositional differences in high and low bodyweight chick groups in early life may provide useful insights into the underlying causes of variation in broiler chick growth performance. Due to the substantial improvement in broiler genetics, it is usually speculated that broilers kept under the same environment and diet management would have relatively comparable uniformity in bodyweight. However, studies have shown considerable variations in bodyweight (11-18% coefficient of variation) in broiler flocks. Thus, the present research study was designed to investigate the differences in the digestive organ weight, tissue mineral profile, behavioral activities and transcriptomic profile of broiler chicks categorized as low or high bodyweight in early weeks of life. The study utilized day old male Ross 308 broilers which were kept under common environmental and dietary management throughout the experimental period. Early life was studied as it is the most critical period in broiler chick growth and development, for this reason it may be speculated that any difference observed during this period may be influential in understanding the characteristics of broilers performing lower or higher than the target breed. This would thus narrow the search to design more precise interventions to optimise growth performance and uniformity in broiler production.

The first trial evaluated the differences in the digestive organ weights, bone mineral concentration and ileal transcriptomic of chicks categorized as super performing (SP) and underperforming (UP) chicks in the first week of life. Results obtained indicated that the SP had significantly higher bone sodium, phosphorus and rubidium concentrations relative to the UP group, in contrast the UP had significantly higher bone cadmium, caesium and lead compared to the SP group. Interestingly, the UP group had higher relative gizzard weight compared to the SP group. It was also revealed in the study that the SP group had higher ileal expression of important cytokine (IL26), and chemokine (IL8L1) compared to the UP group. In summary, it was observed that chicks of the same breed and of common environmental

and diet conditions exhibited considerable differences in digestive organ weights, tibial bone mineral concentrations and ileal gene expression.

In the second trial, further evaluation was conducted to better understand the differences observed in the first trial. Chicks were kept for up to 21days before sample collection, the study evaluated further differences in bone characteristics, liver mineral profile, and gut parameters of broilers categorized as low and high bodyweight to gain more insights into the differences in bone morphometry, liver and bone mineral profiles, gut pH and gizzard fibre content of the two group of chicks. Results indicated that the low weight group had higher concentration of bone manganese and strontium, and significantly higher liver manganese, cadmium and caesium compared to the high weight group. The low weight also had an interestingly low gizzard pH which corresponds to the significantly higher gizzard neutral detergent fiber (NDF) observed in the group. In summary, the results showed that broilers of the same breed, which were kept under the same diet and environmental conditions were different in bodyweights, tibial measurements, tibial bone mineral concentrations, liver mineral concentration, gut pH and gizzard fiber content.

The third trial was designed to evaluate the behavioral activity of the low, average, and high weight chick groups to confirm the speculations obtained in trial 2 about litter consumption due to the observed reduction in gizzard digesta pH and fiber content. The study characterised chicks based on the hatch weight into low weight, average weight, and high weight groups on day 0, chicks' behavioural activities such feeding, litter eating was monitored on day 0 and day 7. Bone, liver, and gizzard digesta samples were collected for mineral profile analysis, and the weight of the crop and gizzard were also recorded. In summary, results revealed higher feeding tendency of the high weight group on day 0, which may have influenced the higher bone mineral concentrations (Ca, P and Zn) in the high weight group, resulting in better performance than the low weight group.

Trial 4 was a 2 by 2 factorial arrangement that assessed D0 bodyweight (High versus low) and litter type

(Hardwood versus softwood). Results revealed that litter type did influence chicks' bodyweight, and led to downregulation of tight junction, solute carriers and antioxidant genes in the bursal of the experimental chicks. The results obtained from the research studies revealed some interesting physiological and transcriptional differences in broiler chicks of varying bodyweight in early weeks of life and may contribute to further research targeting improved uniformity in broiler production.

Keywords: Broiler chicks, bodyweight variation, growth performance, transcriptomic profile, tissue mineral profile.

CHAPTER ONE

1.0 Literature Review

1.1 Origin of domestic chicken

The domesticated chicken known as (*Gallus gallus domesticus*) is one of the most ubiquitous domesticated animals, which has been in domestication beginning at least 8,000 years ago (West and Zhou, 1988; Lawal, et al., 2020), they provide humans with a consistent source of good quality meat and egg (FAO, 2007). The global chicken population in 2017 was >22 billion (FAO, 2020) and 23.7 billion as of 2018 (Statista, 2020). Domestic chicken originated from the red jungle fowl native of the tropical Southeast Asia and Southwest China (Pitt, et al., 2016; Fumihito, et al., 1994). Aside from its production for food, it has a long anthropomorphic history in Southeast Asia where it has been bred for entertainment and ornamentation (MacDonald and Edwards, 1993). It has also been used as a non-mammalian research model in biomedical research (Wu and Kaiser, 2011). The domestic chicken specifically raised for meat production is referred to as broilers. They are typically bred to a uniform size, with high lean muscle deposition relevant to the chicken industry, when compared to laying hens. Chicken has been in greater demand by the increasing global population due to its health associated benefits such as low saturated fat content, and source of vitamins and minerals. Most of the broiler chickens are raised to slaughter age between four to six weeks (Bessei, 2006). The fast growth in broilers has been reported to impact negatively on welfare and disease resistance (Ghayas et al., 2020). The slow growing broiler production is currently gaining attention in the poultry industry to alleviate problems associated with the fast-growing broilers population. These slow growing broilers are reared for a longer period and are characterised with a significantly reduced growth rate when compared to the fast-growing strains (National Chicken Council, 2017).

1.2 Broiler chicken global consumption

Poultry production is one of the world's leaders among all livestock production. Among poultry production, broiler production has been reported to account for 88% of the total poultry meat output globally (Macleod et al., 2013). In the UK, chicken consumption accounts for a third of all The meat consumed is about 23kg per head annually (Sheppard, 2004), and the highest consumed meat in the US annually since 1993 (Havenstein, 2006). As the global population increases, which is predicted to reach 9.6 billion in 2050 (Malik, 2013; UN, 2015), so will the demand for broiler meat continue to increase which is predicted to be 61% higher between 2005 to 2030 (Macleod et al., 2013). As a result, chicken is predicted to become the world's most consumed type of animal protein (OECD/FAO, 2014). This is because, compared to other meat such as pork, there are no religious concerns associated with eating poultry meat and its products, which has led to its popularity globally.

It has been reported that 74 billion chickens are being slaughtered each year, and this is predicted to increase to 85 billion per year by the year 2032 (Figure 1.1 and 1.2). This figure is expected to rise due to the high preference of chicken by consumers compared to other animal protein sources (OECD/FAO 2023; Maharjan, et al., 2021). The expanding increase in the population in developing countries also drives the increase in chicken consumption (Boland, et al., 2013). Therefore, with regards to the increase in global population which has resulted in higher demand for broiler meat, ensuring food security becomes paramount. To tackle food security challenges, sustainable food production systems come into play. It is vital to venture into sustainable animal production practices to help reduce our carbon footprint. One of the strategies to achieve this is to raise modern broilers with fast growth and reduced amount of feed use in their production. Broiler chickens grow fast, high yielding and good feed efficiency (Siegel, 2014). Growing chicks with high feed efficiency means less environmental emission and carbon footprint in production system (Williams, et al., 2006).

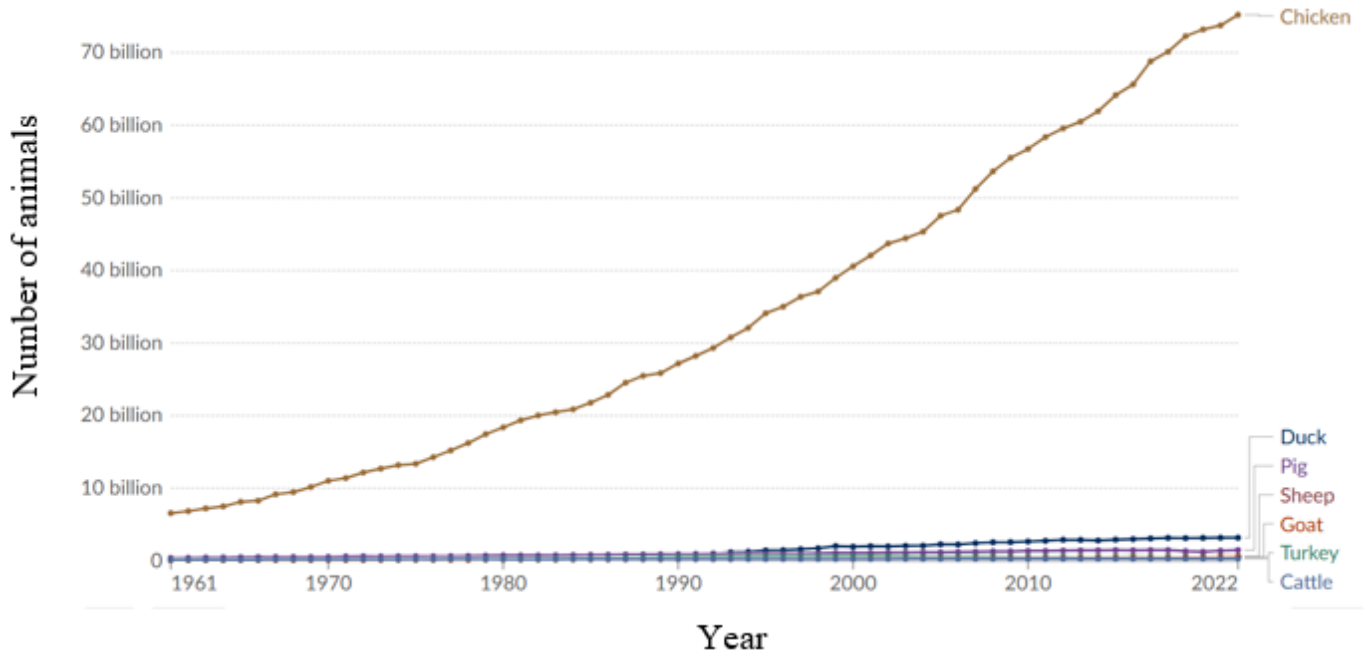


Figure 1.1 Annually number of animals slaughtered for meat from 1961-2022

Source : https://ourworldindata.org/meat-production

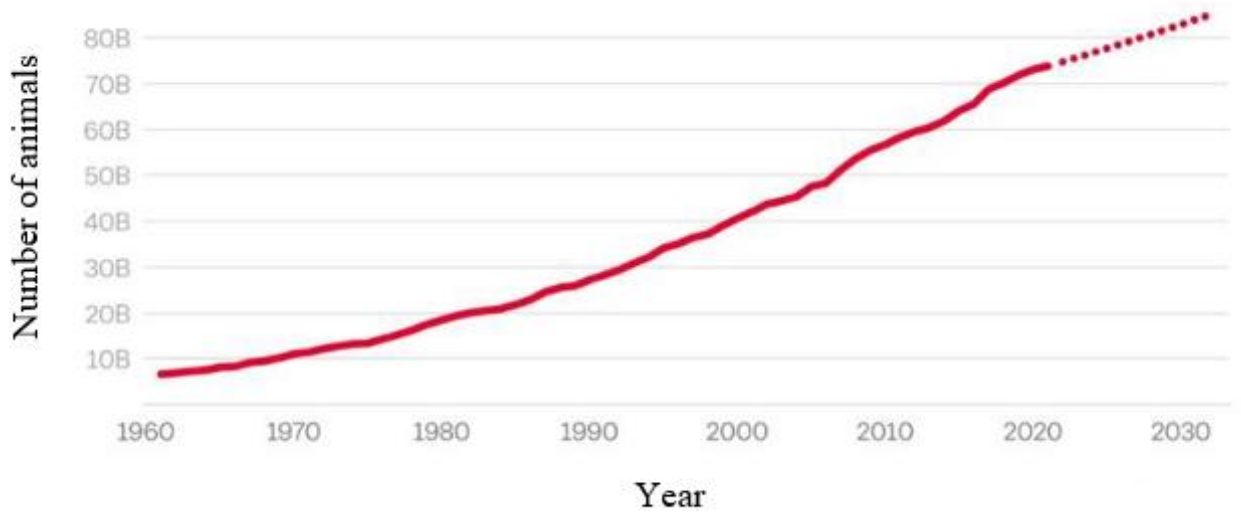


Figure 1.2 Annually number of chickens slaughtered and predicted to be slaughtered from 1960-2030.

Source : OECD/FAO, https://www.vox.com/future-perfect/2023/8/4/23818952/chicken-meat-forecast-predictions-beef-pork-oecd-fao

1.3 Broiler chicks' growth and development

1.3.1 Early life growth performance

Early life growth and development of broiler chicks starts from the pre-egg and incubation period. There are many factors affecting hatching egg quality and chick post hatch performance. These include genetics, nutrition of broiler breeders, egg handling, equipment, flock age, storage and transport (Sozcu and Aydin, 2013). To ensure high hatchability and post hatch performance, all the afore-mentioned factors should be considered and tightly managed. It has been reported that the requirements for developing embryos such as relative humidity, ventilation, temperature and turning vary during the embryonic development stages (Sozcu and Aydin, 2013). During the early embryonic stage, the incubation factors mentioned above affected organ development, yolk sack utilization and absorption, hatching quality, embryonic and post hatch mortalities (Sozcu and Aydin, 2013). The embryonic and post hatch periods represent a critical phase in the broiler growth cycle which substantially contributes to attaining quality broiler performance at slaughter age (Noy and Uni, 2010). Therefore, it is very vital to maintain efficient transition from late embryonic stage to immediate post hatch period to achieve good growth performance results in broiler production. In animals, growth and development must be accompanied by metabolic precursors, and the availability of these precursors is very vital (Sklan, 2001). There are several factors that influence early life growth and development which need to be taken into consideration in broiler production. These factors ranged from early feeding strategy (Henderson, et al., 2008; Bartov, 1987; Plavnik, 1986), physical chick characteristics such as length and weight (Petek, 2010), environmental factors such as brooding conditions, housing and diet management (Baarendse, et al., 2006) and genetic factors. Generally, it has been reported that variation of 1g in broiler chick hatching weight

results to slaughter weight as 50-100g losses. Therefore, the first stage of successful and profitable broiler production is highly associated with hatchery management and good practices.

1.3.2 Feed intake in broiler chicks

Feed intake in broiler chicks is one of the most important factors that determines the growth rate of chicks (Ferket and Gernat, 2006). Higher feed intake in broiler results in increased weight gain, possibly because of increase in nutrient intake, which reduces the birds' maintenance energy proportion in relation to gain (Svihus, et al., 2004a). It has been reported that feed intake strongly correlates with bodyweight in Ross 308 broiler chicks as shown in Figure 1.3 (Mohammadrezaei, et al., 2011). The relationship between feed intake and body weight is a quadratic relationship as intake becomes maximum and declines once an animal reaches its mature weight (Lewis and Emmans, 2020). Therefore, to ensure that the broiler chicks meet up with their genetic potential, feed intake should be monitored to make sure all factors inducing feed intake suppression are minimized (Abdollahi, et al., 2018). Several factors influence feed intake in broilers, they include management factor such as stocking density, temperature, lighting, stress and water access, feed form, dietary factor such as dietary energy, amino acids, vitamins and minerals, antinutritional factors, bird factor such as age, capacity of the GIT, genotype, sex, diseases and immunological stress (Sklan, 2001; Ferket and Garnat, 2006; Brickett et al., 2007; Latshaw and Moritz, 2009; Applegate, 2012 and Abdollahi et al., 2013c). Therefore, maintaining adequate feed intake in early life results in adequate growth rate and efficiency in nutrient utilization (Ferket and Gernat, 2006).

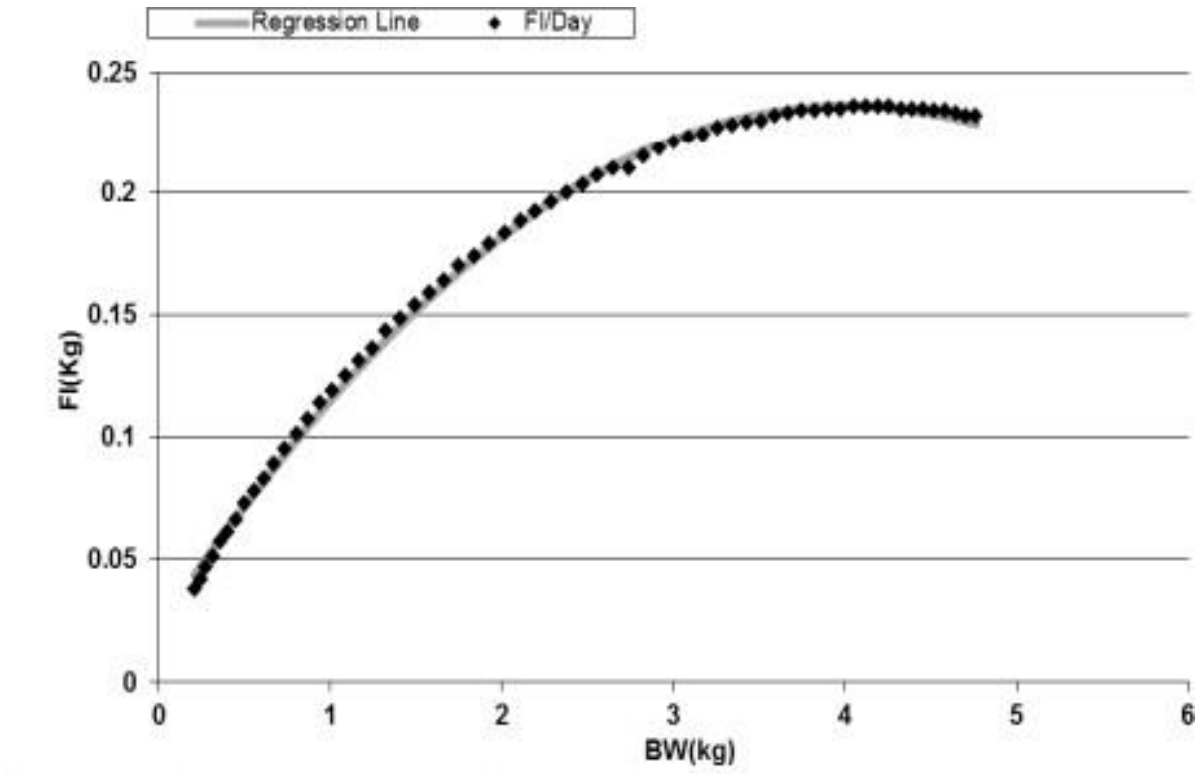


Figure 1.3 Relationship between feed intake (kg) and bodyweight (kg) in broiler chicks.

Source: Mohammadrezaei, et al., 2011

1.4 Overview of the avian gastrointestinal tract (GIT)

The avian gastrointestinal tract is uniquely designed to digest feed and absorb nutrients from the feed component. GIT also serves as a vital mediator of minerals and other nutrients via a highly mechanistic process (Villa, et al., 2017). It is made to accommodate a wide range of quantitative needs, and it is morphologically designed to adapt to the changes in nutritional needs of the avian species (Diamond, 1991; Klasing, 1998). The histological structure of the regions of the chicken GIT is shown in figure 1.4 below. The GIT comprises the oral cavity (mouth), oesophagus, crop, proventriculus, gizzard, small (duodenum, jejunum, and ileum), large (ceca and colon) intestines and cloaca (Figure 1.5). Feed goes in through the mouth and undergoes a sequence of processes in these organs, which includes grinding, hydrolysis, acidification, emulsification, and transportation of the end products to where needed (Klasing, 1999). The gastrointestinal tract of the chicken also poses metabolic demands for example it requires about 8% of the metabolised energy but only contributes to 1.5% of the body weight (Spratt, et al., 1990).

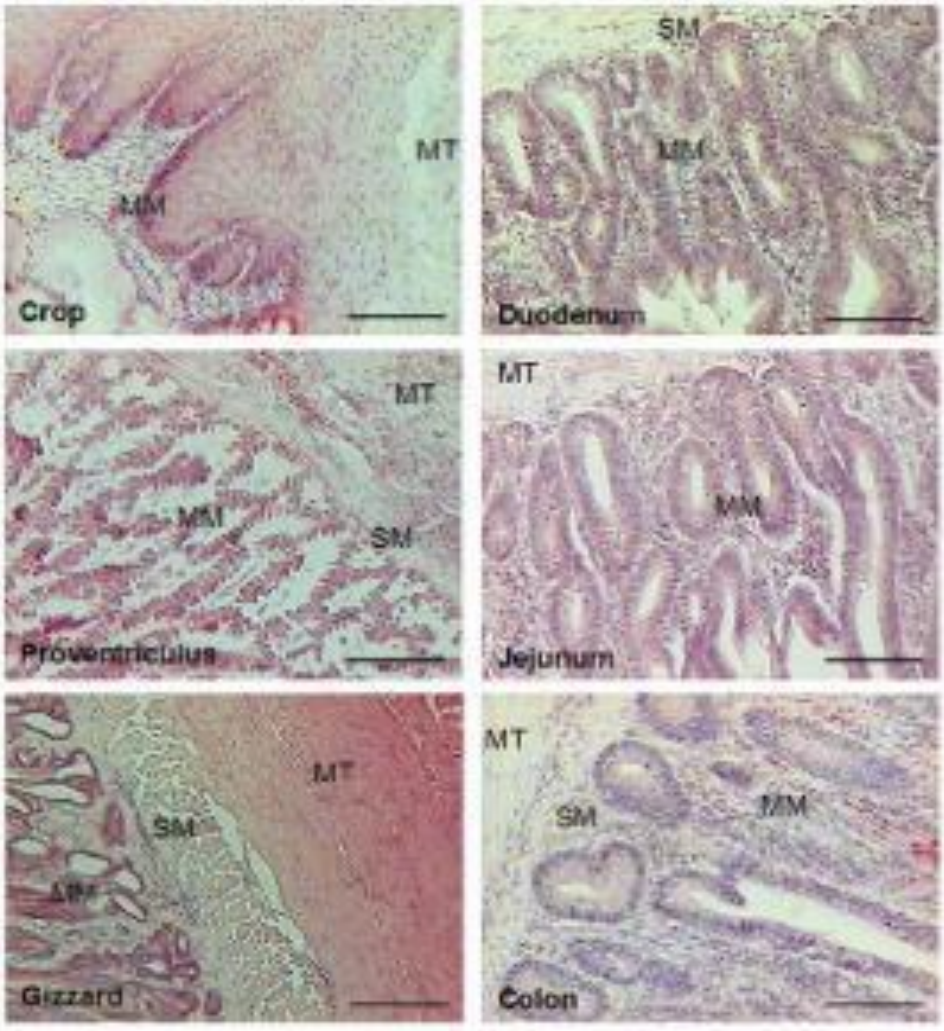


Figure 1.4 Histological structure of regions of the chicken gastrointestinal tract.

Footnotes: MM: mucous membrane, SM: submucosa, MT: muscular tunic

Haematoxylin and eosin staining, scale bar - 100µm.

Source: Scanes and Pierzchala-Koziec, 2014)

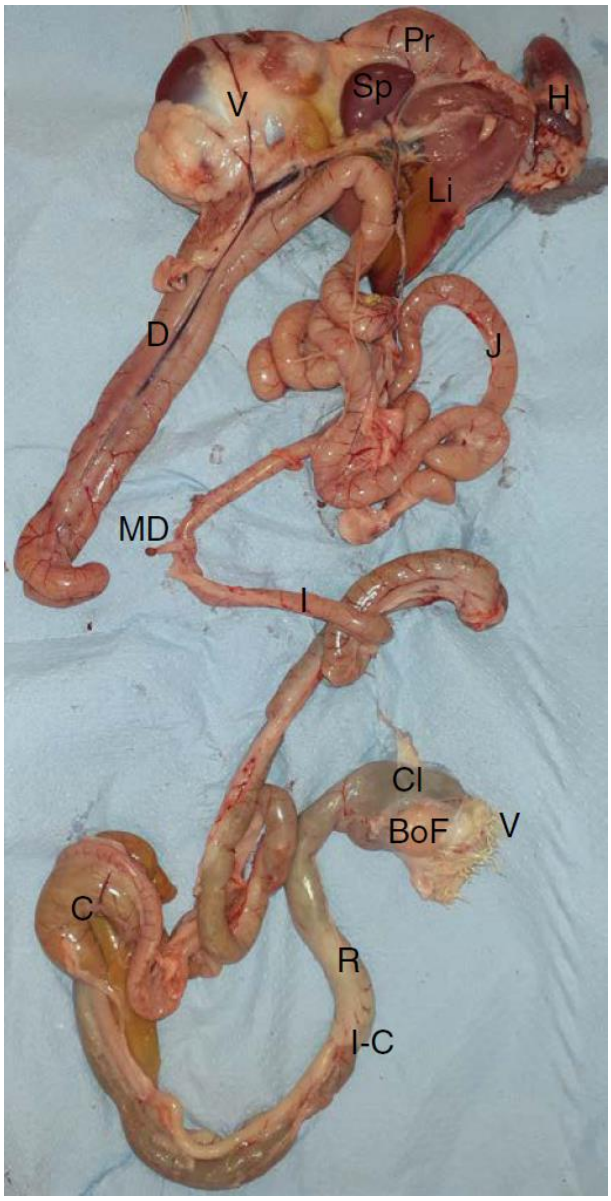


Figure 1.5 The gastrointestinal tract of a chicken.

Pr – proventriculus; V – ventriculus (gizzard); Sp – spleen; Li – liver; H – heart; D – duodenum; J – jejunum; MD – Merckel's diverticulum; I – ileum; C – caeca, I-C – ileo-caecal junction; R – rectum; Cl – cloaca; BoF – Bursa of Fabricius; V – vent

Source: Barrow, et al., 2021

1.4.1 Growth and development of the gastrointestinal tract

The initial growth and development of the gastrointestinal tract is reported to be directly associated with feed intake. Growth of the digestive tract in broiler chicks is allometrical with the components of the GIT reported to grow at different rates than the rest of the body (Katanbaf, et al., 1988). The gizzard is reported to be the largest organ associated with the gastrointestinal tract and even larger than the liver (Ravindran, et al., 2006). The organs that make up the GIT at hatch are immature, particularly the intestines and most of the nutrients absorbed by the hatchlings are targeted at intestinal development. The rate at which the GIT develops in individual chicks will determine its functional capacity and the ability of the chick to absorb nutrients from feed to achieve its genetic potential (Noy and Uni, 2010). Hatchlings during the early weeks of life must transition from utilizing the energy supplied by the yolk to the utilisation of exogenous feed. This process often initiates dramatic changes in the size, morphology, development, and function of the intestine (Uni, et al., 1996). The development of GIT is often associated with early access to nutrients via exogenous feed consumption. It has been reported that access to nutrient initiates growth 24hours after feed intake in young birds which mainly contributes to the development of the intestine during this critical period (Sklan, 2001). The growth and development of the GIT components have been studied extensively with maximal proportion in weight of the GIT achieved between 3 and 8 days of age (Uni, et al., 1998; Nitsan, et al., 1991; Nir, et al., 1993; Pinchasov, 1995; Nitsan, et al., 1991; Iji, et al., 2001; Murakami, et al., 1992 and Ravindra, et al., 2006; Wijtten, et al., 2012).

1.4.2 Gross development:

During the early phase, the gastrointestinal organs increased in weight more rapidly compared to the bodyweight, while other digestive organs such as the gizzard and pancreas do not show rapid increase in relative weights (Uni et al., 1999). The process of the relative increase in weight of the gastrointestinal organs were maximal at 6-10 days in chicks (Noy and Sklan,1998a). It is also interesting to note that this preferential relative growth of the small intestine occurred regardless of exogenous feed intake (Figure 1.6). However, it was reported that the chicks that had early access to feed had higher absolute and relative intestinal growth (Noy and Sklan, 1999).

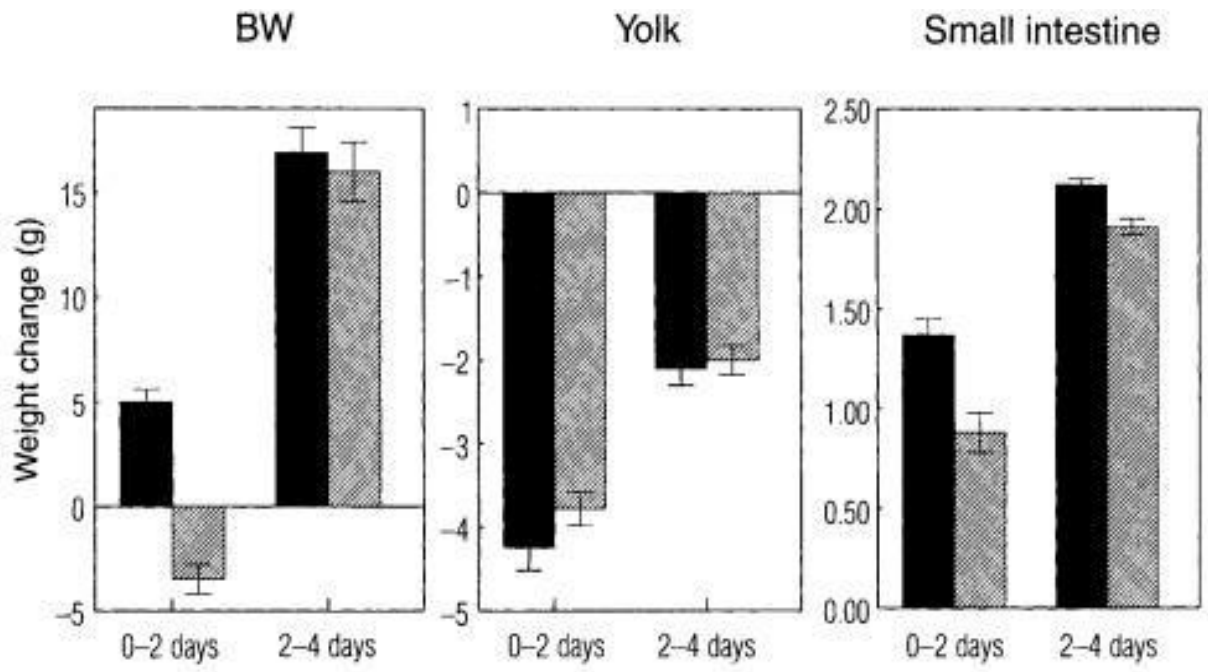


Figure 1.6 Changes in body weight, yolk weight and small intestine weight 0-2 and 2-4 days after hatching

The filled bars are the fed chicks, and the cross-hatched bars are held chicks. The results are mean values with standard deviations.

Source: Sklan, 2001

1.4.3 Morphological development

At hatch, the enterocytes of the small intestine increase rapidly both in length, pronounced polarity and defined brush border as shown in figure 1.7. The villi are also undeveloped and the crypts in the intervillous spaces are not detectable, but one hours post hatch the crypts begin to form, and they become well defined after 2-3days post hatch as shown in figure 1.8. The development of the intestinal segments follows a distinct pattern as the duodenum and jejunum continue to develop after the ileum has reached its constant number of crypts per villus (Sklan, 2001). The intestinal villus height increases twofold in 48 hours post hatch and reaches its limit from 6-8 days in the duodenum, and then after 10days in both jejunum and ileum (Sklan, 2001). As the villus increases during this period, the number of the enterocyte per villus also increases (Geyra, et al., 2001a).

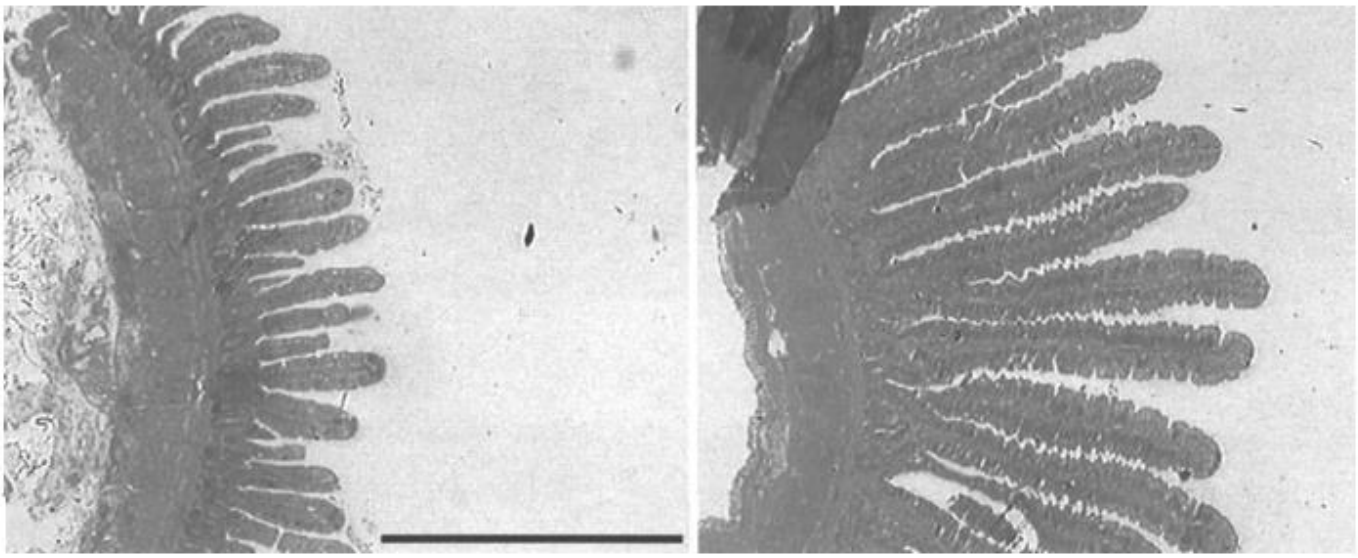


Figure 1.7 The stage of development in the jejunal mucosa in 1 day (left) and 21 days (right) broiler chicks.

Scale bar = 1000µm

Source : Iji, et al., 2001

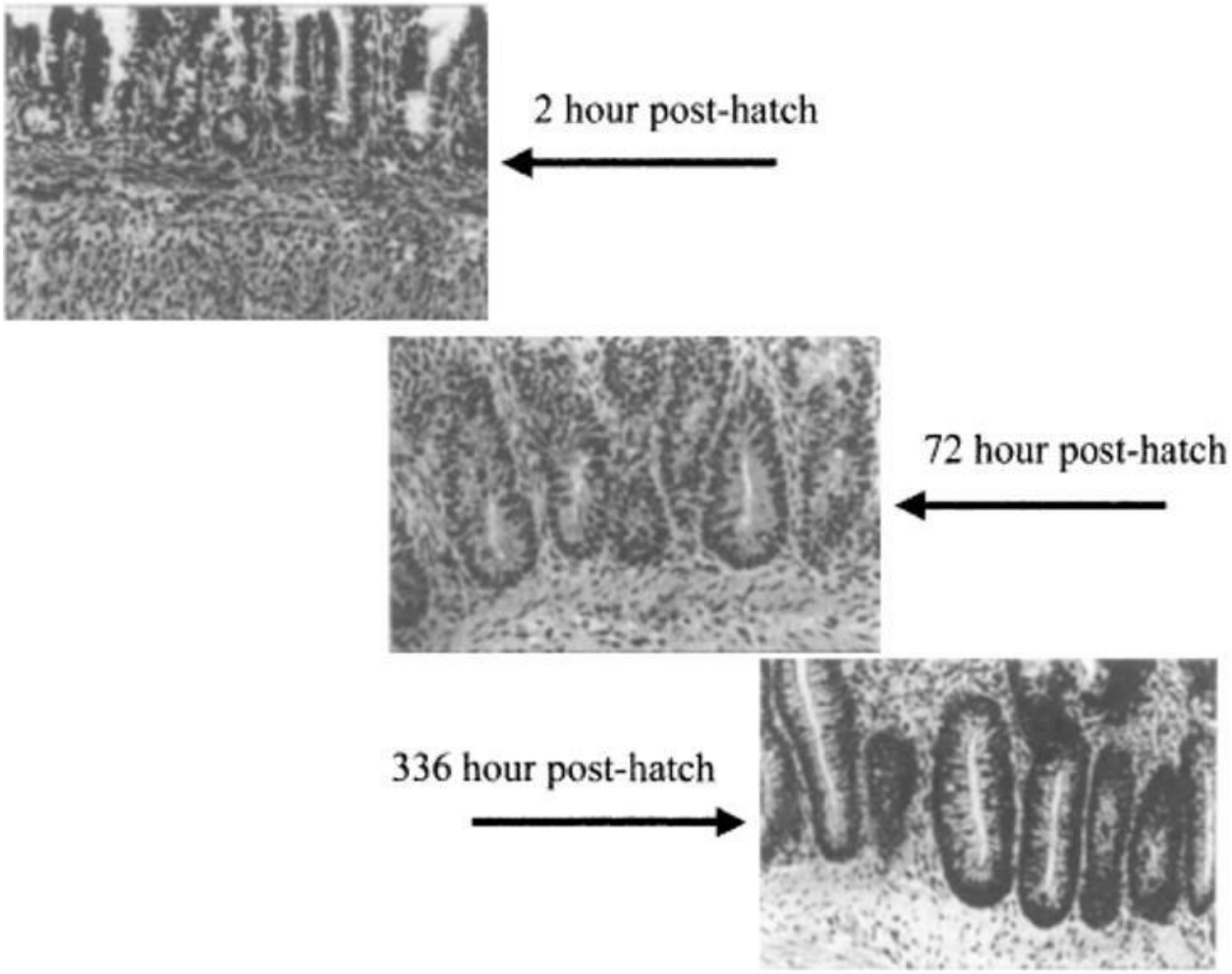


Figure 1.8 Light microscopy sections of Jejunum at 2, 72, and 336-hours post-hatch.

Source: Sklan, 2001

1.4.4 Functional development

One of the most important functions of the gastrointestinal tract of chicks during the early period of life is digestion of both the yolk and exogeneous feed from its macro units to smaller units. This process is stimulated by various enzymes secreted by the pancreas. These enzymes have been reported to be present in the small intestine from the late embryonic stage (Marchaim and Kulka, 1967). It has been reported that pancreatic enzymatic activities in the small intestine are associated with body weight and intestinal weight (Sklan and Noy, 2000). This established the report that the production of pancreatic enzymes is initiated by feed intake and are secreted relatively at steady amounts per feed intake as the chick ages (Sklan, 2001). Figure 1.9 shows the daily secretion of trypsin, amylase, lipase, bile acids and nitrogen into the duodenum in the first 2 weeks of life.

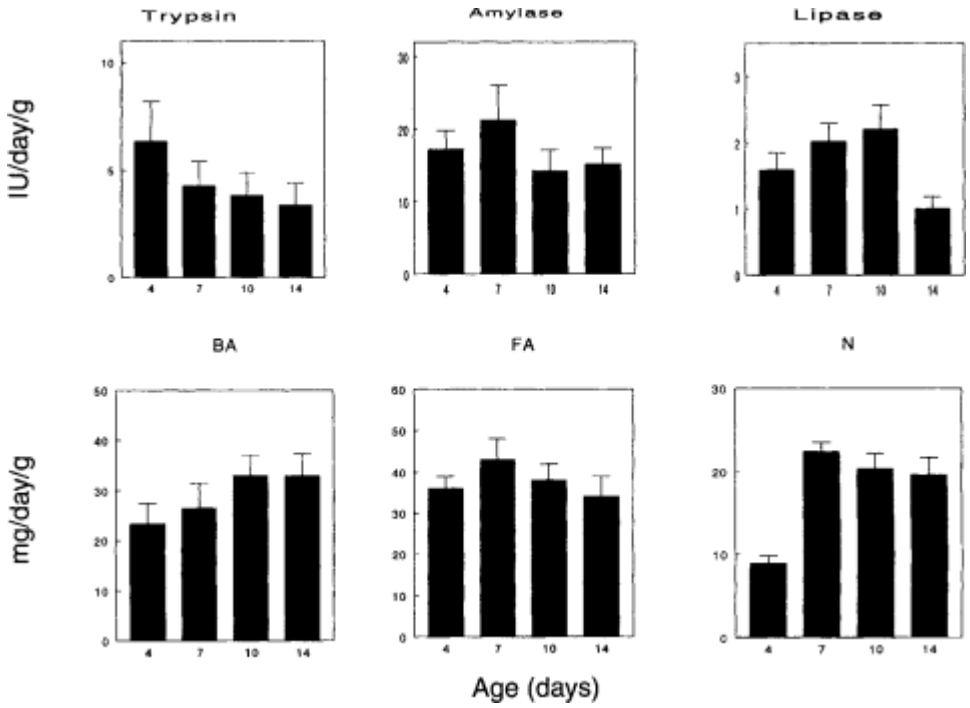


Figure 1.9 Net daily secretion to the duodenum per g feed intake of trypsin, amylase, lipase, bile acids (BA), fatty acids (FA) and nitrogen (N) from day 4 to 14 post hatch.

Values are means with standard deviations.

Source: Sklan, 2000

1.5 Gut pH and microbial community of broiler chicks

The pH of the chicken gut plays a role in determining the activity of the digestive enzyme, nutrient bioavailability, and microbial population, thereby affecting the digestive capacity of the broiler chick (Pang and Applegate, 2007; Hajati and Rezaei, 2010). The pH measurement of the different segments of the chicken gut and its associated impact on digestion and mineral absorption have received good attention and studied (Bohak, 1970; Mahagna and Nir, 1996; Bristol, 2003; Barua, et al., 2021). Figure 1.10 illustrates the pH and transit time of the different segments of the chicken digestive tract. Protein digestion had been reported to be influenced by gut pH, low digesta pH due to hydrochloric acid production initiates the conversion of pepsinogen to pepsin for protein digestion (Bohak, 1970). Several factors affect the gut pH of broiler chicks which includes particle size and amount of feed components such as limestone (Guinotte et al., 1995). Ensuring that the pH of the gut is maintained at an optimal level is crucial as trace changes in pH values outside the recommended range would impact digestion and absorption process in the gut. Normal pH range of the gizzard is reported to be between 1.2-4.0 and duodenum between 5.7-6.5 (Pang and Applegate, 2007; Jimenez-Moreno, et al, 2009; Walk et al., 2012).

The chicken gut is densely populated with microbes that coexist with the host (Fathima, et al., 2022). However, the digestive tract of the hatchling is sterile but rapidly colonized by microbiome through the feed and environment (Harrow, et al, 2007). They play a useful role in regulating digestion and metabolism by synthesizing bile acids, vitamins, and short chain fatty acids (Shang, et al., 2018). The composition of the gut microbiota is highly impacted by several factors such as diet, age, genetics, sex, environment, health or disease state and pharmacological agents (Holzapfel, et al., 1998; Faith, et al., 2013). In young hatchings, the commensal microbes play a crucial role in initiating the immune response and establishing an activated immune system (Amoroso, et al., 2020; Al Hakeem, et al.,2023). Microbial colonization in early life is very crucial in the development of a well-functioning immune

system, in this regard for the hatchling, feed plays major role in microbial colonization of the gastrointestinal tract and feed antigens seem to interact with the gut immune system (Simon, 2016). The gut microbial community also plays an important role in the maturation of the immune system in early life (Villa, et al., 2013). In addition, chicken gut microbiota has been reported to influence chick behaviour via gut-brain axes (Hu, et al., 2022) and mediates responses to stresses especially heat stress (Liu, et al., 2022). In recent research, it has been reported that cecal microbiome characteristics are associated with bodyweight in broiler chicks (Lundberg, et al., 2021).

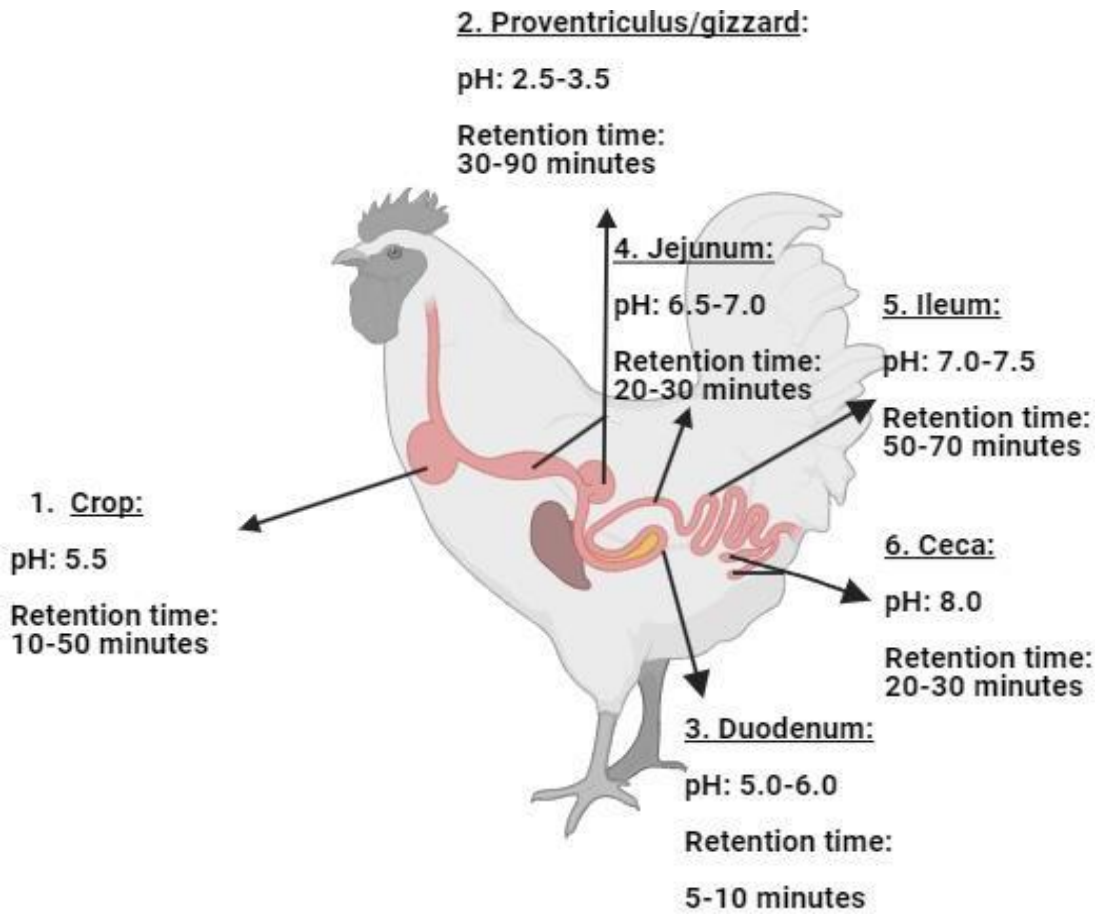


Figure 1.10 pH and average transit time in different segments of the digestive tract of broiler chicks.

Source: Adapted from Ravindran, 2013, created with http://biorender.com/

1.6 Skeletal development in young chicks

At hatch, the skeleton of the chick is poorly mineralized. However, its growth and mineralization occur rapidly during the first two weeks of age when adequate Ca and P are available in the diet (Angel, 2007; Ravindran, et al., 2021). In early life, the yolk sac has been reported to contribute greatly to the supply of Ca (Moran, 2007). The concentration of Ca in the tibia has been reported to be higher in broilers during the first week of age compared to those of other ages (Skinner and Waldroup, 1995). The age of the broiler chick has been an important factor influencing the tibial ash of broiler chicks, fat free tibia ash content increases rapidly within the first week of life and then reaches its peak on day 14 (Ravindran, et al., 2021) (Table 1.1). There is reportedly high demand of calcium during the first two weeks of life, which is coupled with efficient calcium absorption during this stage to meet the demand for bone formation in young chicks. It has been reported that tibia calcium concentration was greater during the first week post hatch compared to other ages up to 8 weeks (Skinner and Waldroup, 1995). The bone which is the major component of the avian skeleton is a living tissue that is subject to change and adaptation during growth and development by the cells of the bones via remodeling in response to external stimuli such as calcium demand, bodyweight and physical activity (Glimcher, 1998). Several factors have been indicated to affect avian bone development and properties including nutrition, genetic factor, sex, and environment (Rose et al., 1996; Leterrier et al., 1998; Rath et al., 2000; Fleming, 2008; Talaty et al., 2009). The changes in the bone mineralization and morphology of the bone during skeletal development in broiler chicks have been well documented (Williams et a., 2001, 2004; Applegate and Lilburn, 2002; Rawlinson, et al, 2009; Talaty et al., 2009; Shim et al., 2012). The tibial bone has been widely studied because it provides good indication of the overall skeletal mineralisation in broiler chicks (Skinner and Waldroup, 1995; Angel, 2007).

Table 1.1 Influence of age on the fat-free tibia ash content of broilers fed maize soyabean diets containing recommended concentrations of calcium and non-phytate phosphorus

Age (days)	Fat-free tibia ash (%)
1	28.7±3.90
7	42.3±2.67
14	51.3±2.69
21	50. 0±3.56
28	49.1±2.94
35	51.5 ±2.63
42	49.3±2.75 [1]

[1] Mean ± standard deviation of 6 replicates (Source : Ravindran, et al., 2021)

1.7 Early life nutrition

1.7.1 Residual yolk sac

Residual yolk sac is the internalized yolk sac during the parafetal stage of the embryo in broiler, this sac ensures the hatchlings have adequate nutrient reserve during the first 3-5 days post hatch. It also plays critical role in growth and development during the early days post hatch, as a supplier of nutrient up to 5 days after hatch (Turro et al., 1994; Lamot, 2017), the absorption of nutrient from the yolk by the chick initiates growth within 24 hours after hatch. It has been reported that the relative contribution of the residual yolk to the absorption of dietary energy and protein intake by chick's post hatch depends on the moment, amount, and the composition of the exogenous feed intake (Lamot, 2017). The physiological role of the residual yolk sac in early life of broiler chicks is well documented (Noy and Sklan, 2002; Murakami et al., 1992 and Thomas, et al., 2008).

The residual yolk sac serves not only as a nutrient backup source during the early life of broiler chicks, but they also contain maternal antibody mainly immunoglobulin Y (IgY) for passive immunity, phospholipids, choline, triglycerides for cell membrane development (Ravindra, et al., 2021) and other immunological components such as cytokine which are all relevant to the innate immune response and the establishment of the adaptive immunity (Lamot, 2017).

1.7.2 Nutrient digestion and utilization in early life

The digestive tract of the newly hatched chicks is immature and limited in terms of digesting and absorbing nutrients from feed (Ravindran and Abdollahi, 2021). Efficient digestion, absorption, and utilization of nutrient in the animal gut are important parameters involved in growth and development of broiler chicks. Several factors are associated with improved digestion and absorption of nutrients in broiler chicks, these include, increased secretion of digestive enzymes increase in the surface area of the gut absorptive region, and efficient nutrient transporters (Ravindra, et al., 2021). Nutrients are basically the major factor that determines the performance efficiency and immune competence of broilers

(Katanbaf et al., 1988; Klasing and Barnes, 1988). They also initiate magnitude of antibody responses in chicks' body (Latshaw, 1991), antibody responses are generated in regions of the gut, the cloacal bursa and the hindgut (Panda, et al., 2015).

The main driver of the antibody response both systemically and locally in the gut is feed (Bar- Shira, et al., 2005). The intestine plays a substantial role in nutrient digestion and its absorption into the blood stream for utilization by the cells. Table 1.2 below shows the effect of broiler age on the total fat digestibility of different fat sources. This report revealed that fat digestibility is low in the first week of life. However, the digestibility of fat with a high proportion of saturated fatty acid was lower compared to those with high proportion of unsaturated fatty acid, and the ability of the young hatchling to digest both saturated and unsaturated fats increases with age (Tancharoenrat, et al.,2013). Poor fat digestion during early life has been attributed to poor fat emulsification due to low bile secretion, poor recycling of bile salt and inadequate fatty acid binding protein (Ravindran, et al., 2016). Research reports from most digestibility studies have revealed that carbohydrate feed sources are more readily utilized than fat during the first weeks of life (Sulistiyanto et al., 1999; Batal and Parsons, 2002b; Batal and Parsons, 2004; Thomas, et al., 2008). At that stage, the lipids from the yolk sac are present in higher proportion compared to lipids introduced via the gastrointestinal tract (Sklan, 2003), while the glucose appearance in circulation is very low at hatch which increased more than 2-fold on day 3 post-hatch (Sklan, 2003). Basically, it appeared that lipoproteins synthesised maternally or in yolk facilitate lipid transportation at hatch, which decreases with age (Sklan, 2003), thereby reducing the utilization of the circulating lipids. Glucose has been reported to be the major source of energy after hatching due to the increase in the uptake of this nutrient during this period when the yolk lipids decrease (Sklan, 2003). The utilization of energy, lipids and amino acids in young chicks have received attention decades ago and are well documented in published articles (Zelenka, 1968; Carew, et al., 1972; Riesenfeld, et al., 1980; Zelenka and Fajmonová, 2000; Zelenka and Čerešňáková, 2005; Batal and Parsons, 2002, 2004; Svihus, 2014).

Table 1.2 Effect of broiler age on the total tract fat digestibility in three fat sources

Fat source	Age (days)	Fat digestibility (%)
Tallow	7	36.8
	14	65.3
	21	73.6
Soybean oil	7	59.1
	14	89.8
	21	96.5
Poultry fat	7	60.0
	14	84.5
	21	92.8

Source: (Tancharoenrat, et al., 2013

1.7.3 Mineral absorption and utilization

Mineral nutrition has been an important aspect of poultry production, ensuring adequate mineral nutrition in broiler chicks establish overall health and wellbeing of the chicks. The physiological importance of minerals in broiler nutrition has been extensively studied (Spears, 1999; Underwood and Suttle, 1999; Thomas and Ravindran, 2009). Minerals play a vital role in all aspects of metabolism, for example controlling free radicals in the body (Goff, 2018). Most of the minerals in broilers are reportedly stored in the skeleton, making skeletal integrity a critical issue in broiler growth and development (Kleyn and Ciacciariello, 2021). Mineral absorption in broilers is affected by several factors which should be noted when considering mineral requirement of broiler chick (Ashmead, 1993; Vieira, 2008; Linares, 2018 and Goff, 2018).

The chick's mineral metabolic system depends on homeostatic mechanism by which the uptake and excretion of essential mineral components are tightly regulated both at the cellular and systemic levels, this makes it possible for the chick to maintain mineral concentrations in tissues within narrow range (Kleyn and Ciacciariello, 2021). To maintain the efficiency of essential mineral uptake, its bioavailability in the diet becomes crucial which initiates the upregulation of mineral transporters (Hu, et al., 2018). Mineral absorption mainly takes place in the intestinal mucosa and its uptake mechanism is notably complex, with both paracellular and transcellular absorption (Goff, 2018). In a situation whereby the mineral concentration in the digesta adjacent to a tight junction exceeds its extracellular fluid, paracellular absorption occurs whereas when the mineral concentrations are low in the diet, transcellular absorption occurs (Goff, 2018). It has been reported that age influences mineral utilisation in broiler chicks during the first two weeks of life (Thomas and Ravindran 2009) as shown in table 1.3. There are several factors that influence mineral absorption in broiler chicks, these include antinutritional factors (ANFs) such as phytates, saponins, alkaloids, tannins and protease inhibitor, for example trypsin inhibitor has been reported to reduce mineral digestibility in broilers (Aderibigbe, et

al., 2021; Ali, et al., 2022 and Dey, et al., 2022). It has been reported also that these ANFs impact negatively on nutrient metabolism depending on the animal's age, species, concentration of the ANFs and interaction with other nutrients (Salim, et al., 2023). For example, the bioavailability of calcium is substantially reduced by phytates which negatively influence its absorption by the chick. Mineral absorption can also be affected by antagonistic interactions of nutrients within the chicks' gastrointestinal tract. Mineral interactions are basically due to the chemical and physical properties of the elements, which indicate that elements with similar ionic size are likely to interact competitively (Martin, et al., 2013). Calcium, one of the most important bone minerals, has a close interaction with phosphorus and vitamin D (Suttle, 2010). Several factors contribute to its absorption, distribution and excretion. The level of dietary phosphorus and vitamin D are the most important factors affecting Ca absorption. Calcium is mostly absorbed from the intestine by an active transport mechanism in the proximal duodenum requiring vitamin D, which also involves the calcium-binding protein calbindin (Martin, et al., 2013). Calcium is also absorbed by passive ionic diffusion which also may be vitamin D dependent and occurs in the other segments of the small intestine (Martin, et al., 2013) and it has been reported that excess Ca can cause the formation of insoluble Ca-phosphate salts, which leads to reduced phosphorus availability. Figure 1.11 illustrates the indispensable role of macro and micro minerals in broiler nutrition. Manganese, which is one of the essential trace minerals in broiler mineral nutrition, has been reported to be the fundamental for normal immune function, regulation of blood sugar, digestion, reproduction and bone development (Dominguez, 2013). This trace element is basically absorbed in the mucosa cells of small intestine, and its mainly excreted via bile and pancreatic juice (three main factors influence the manganese absorption in broiler chicks, and they include developmental status, dietary constituents and translocation of membrane (Kies, 1987). Manganese absorption is also affected by nutrient interaction, for example high levels of calcium and phosphorus decreases the bioavailability of manganese in broilers (Świątkiewicz et al., 2014).

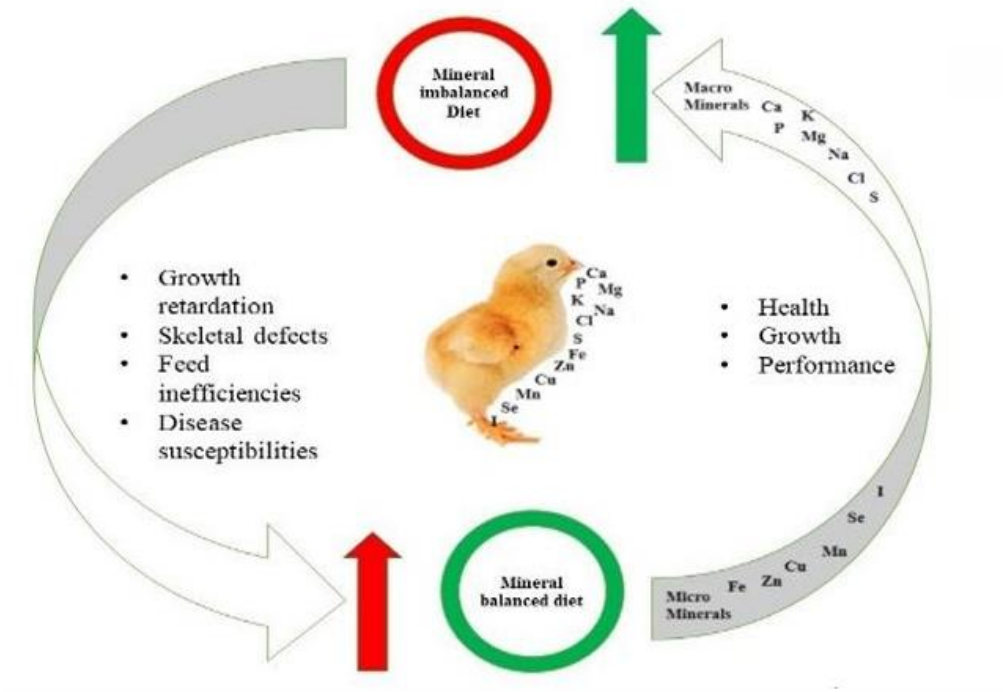


Figure: 1.11 The importance of macro and micro mineral elements in broiler nutrition

Source: WAQAS, et al., 2024

Table 1.3 Total tract retention (% of intake) of minerals in broilers fed maize-soybean meal- based diet during the first 14days post hatch.

Minerals	Age (Days)					
	3	5	7	9	14	P-value
Calcium	43	45	40	42	40	
Phosphorus	60	55	47	49	49	*
Potassium	49	38	34	35	30	*
Sodium	95	68	66	63	68	*
Magnesium	39	29	26	27	23	*
Iron	34	20	21	24	21	*
Manganese	25	13	11	17	11	*
Zinc	28	13	10	13	0	*
Copper	23	12	8	9	4	*

* Significant effect of age ($p<0.05$). Source: Thomas and Ravindran 2009

1.7.4 Rate of digesta passage in the gut

The rate of digesta passage in the chicken gut has major influence on the digestion of feed consumed and nutrient absorption. The slower the digesta passage along the gut, the better the digestion and nutrient absorption, because of the longer retention time in the GIT, more time is given for contact between the digestive enzymes, digesta components and intestinal mucosa (Ravindran, et al., 2021). The transit time of the digesta in early weeks of life in broiler chicks have been reported by several authors (Noy and Sklan, 1995; Uni, et al., 1996; Barua, et al., 2021; Tur, et al., 1986).

1.7.5 Digestive enzyme production and secretion

Enzymes play a vital role in the digestion of feed and transportation of the products across the intestinal epithelium (Ravindran, et a., 2021). The transport mechanisms for various nutrients during early life have received contradictory speculations by different researchers (Buddington, 1992; Tako, et al., 2005; Li, et al., 2008). It has been reported that nutrient transport mechanism in young chicks do not impede early growth, and therefore transport capacity of young chicks is regulated to meet or slightly exceed nutrient inputs (Obst and Diamond, 1992). The changes observed in the intestinal digestive enzyme and expression of genes involved in nutrient transport are strongly associated with the morphological changes reported during the late embryonic stage to the first day's post-hatch (Li, et a., 2008). To meet the increasing demand of the hatchling to digest and absorb nutrients for growth and metabolic support, activities of the brush border membrane, digestive enzymes and nutrient transporters increase during the early days of life in broilers (Nitsan et al., 1991; Uni et al., 1999, 2003b; Sklan and Noy, 2000; Gilbert et al., 2007). The intestine secretes increasing levels of several enzymes such as sucrose, isomaltase and aminopeptidase during the later stage of incubation from 17-21 (Uni, et al., 2003), however the young hatchling still has limited ability to digest proteins and lipids (Sulistiyanto, et al., 1999; Sklan, 2003).

1.8 Immune function and development in broilers

1.8.1 Intestinal mucosa maturation

Mucosal modulation and healthy gut function and development are valuable contributors to broiler growth and development. The chicks' intestinal mucosa structure is not fully developed at hatch, although they are present, they develop and mature rapidly with age (Schat and Myers, 1991). The rate of cell proliferation peaked at 7 days of age and rate of cell migration at 14 days of age (Geyr, al., 2001; Iji, et al., 2001).

This increase is attributed to the support of the crypt and villus growth at that age. It is also noteworthy that the changes observed in the intestinal mucosal structure including villus height, crypt depth and submucosal thickness influences nutrient uptake to meet the nutrient demand of the hatchlings (Ravindra, et al., 2006). Published articles on intestinal mucosa maturation in the first weeks of life in broiler chicks are available (Yamauchi and Isshiki, 1991; Uni, et al., 1995; Uni, et al., 1996; 1996; Noy and Sklan, 1997; Uni, et al., 1998; Uni, et al., 2000; Yamauchi, 2002).

1.8.2 Innate immune response in broiler chicks

The innate immune system is the first line of immune defense against a wide range of pathogens in hatchlings. The development of the chick's immune system is usually initiated during embryogenesis, though it does not become fully functional until a few weeks post hatch. It could be stunted when there is limited supply of essential nutrients because of feed and water deprivation after hatch (Panda, et al., 2015). In terms of broiler chicks' immune development, the ileum is generally known as the main site of immune activation during the first weeks of life (Bar-Shira et al., 2003). Young chicks depend entirely on their innate immune system and maternal antibodies from the breeder hen which are deposited into the egg (Bar-Shira and Friedman, 2006; Hamal, et al., 2006). The innate immune response in hatchling comprises cellular responses by macrophages, dendritic cells, heterophils and natural killer cells (NK), which recognizes pathogen-associated molecule patterns (PAMPs) and then

bind to pattern recognition receptors (PRRs), e.g. Toll-like receptors (TLRs) (Sebok et al., 2021). The functionality of the innate immune response by the time of hatch is well known, however its development continues during the first week of life. During the first 2days after hatch, the intestinal tract is already populated by matured heterophils which protects the tract through the secretion of antibacterial B-defensins (Bar-Shira and Friedman, 2006).

During this stage, the enterocytes from the gut epithelium of the chick lacks the ability to produce defensins, as a result the secretion of the B-defensins by the heterophils make up for this lack at that stage (Bar-Shira and Friedman, 2006). The young broiler relies totally on the maternal antibody for protection because the B cells which are responsible for antibody production and part of the acquired immune system become functional approximately 2-weeks of age (Bar-Shira et al., 2003). The maternal antibody system consists mainly of the IgY and limited amount of IgM and IgA (Hamal et al., 2006). The levels of maternal IgY, IgM and IgA decline within the first 2-3 weeks of age (Lammers et al., 2010; Sahin et al., 2001). The chicken IgM is linked with the primary immune response and its monomer is a B-cell receptor (Morgan, 2021). The innate immune response in broiler is very important for establishing disease resistance especially during early life (Song, et al., 2021). It has also been observed that in early, broiler chick's control of infection especially *Salmonella* depends entirely on proinflammatory and T helper (Th1)-type cytokines (Wigley, 2013). Therefore, manipulating the immune system of hatchling in early life through immunomodulatory nutritional interventions pre and probiotics could be beneficial (Song, et al., 2021). it has also been reported that the development of the innate immune system of broilers is stimulated by interaction with the host commensal microbiota (Levy, 2007; Jiao et al., 2009; Thaiss et al., 2016).

1.8.3 Mechanisms of immune development in broilers

The mechanisms of immune development in broiler chicks had been proposed by (Dibner et al., 1998). It stated that early nutrition makes substrates available that may otherwise be insufficient, secondly, early feeding may stimulate endogenous levels of hormones or other immunomodulatory molecules and thirdly the presence of feed antigens in the gastrointestinal tract is essential to modulate full differentiation of the primary immune cells. Another important mechanism proposed by other authors stated that early feeding leads to faster development of the mucosal immune system especially the hind gut GALT (gut-associated lymphoid tissue) in broilers (Bar-Shira, et al., 2005). Therefore, since gut developments occur in association with the development of the GALT, hence early feeding was reported to be involved with rapid functional and structural development of the gut together with the GALT (Thompson et al., 1996; Geyra et al., 2001).

1.8.4 Development of immune organ in broiler chicks

The organs of the avian immune system are classified into two primary (central) and secondary lymphoid organs as shown in figure 1.12. Early feeding has been reported to be strongly associated with immune organ development and the functioning of the immune system in early life (Panda, et al., 2015). The primary organs are mainly associated with the production and maturation of the adaptive immune cells while the secondary organs mainly control the immune responses in which they activate the immune effector cells such as lymphocytes (Boehm and Swann, 2014). The location, structure and functions of the immune organ (Table 1.4).

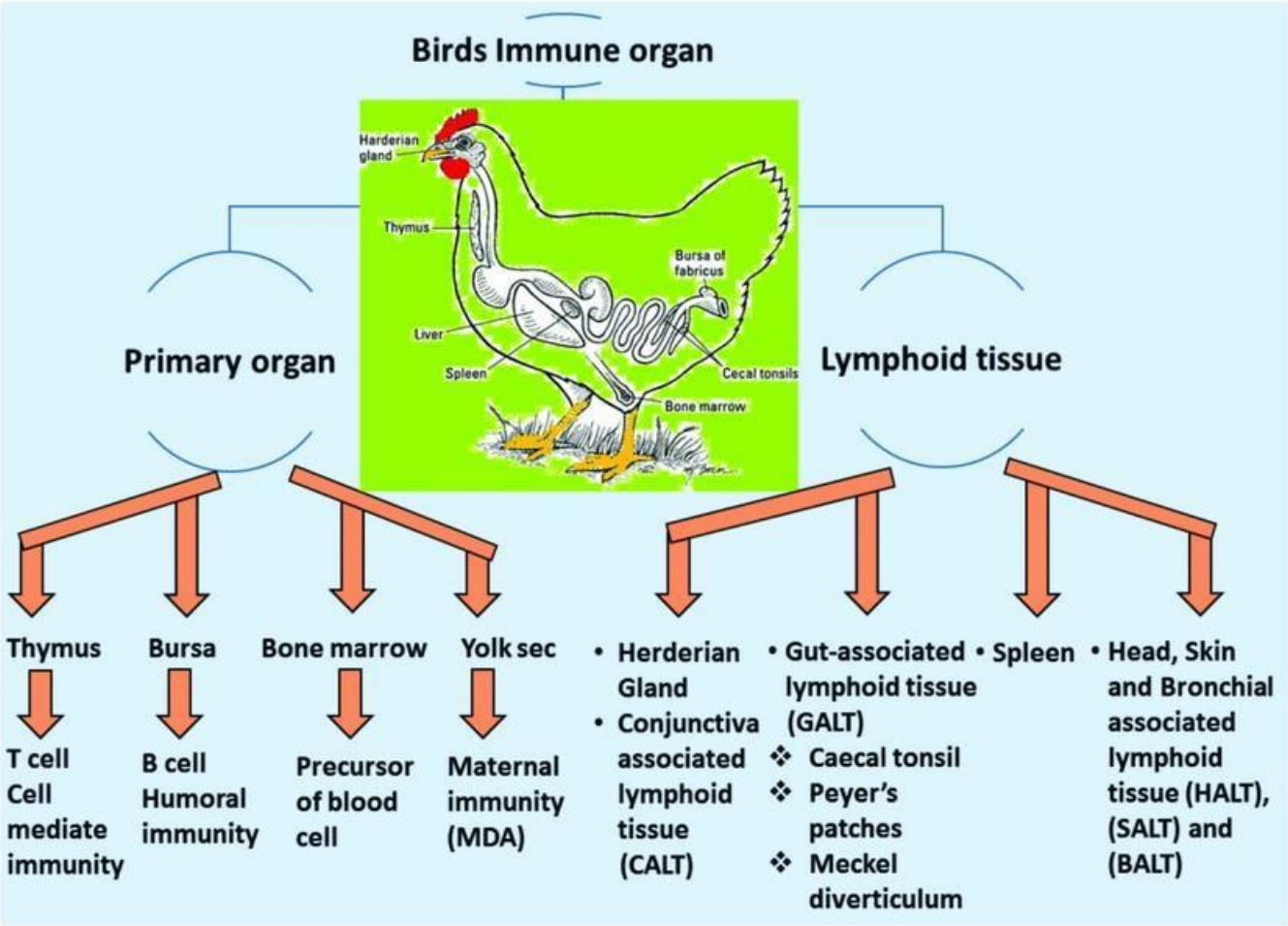


Figure 1.12 Overview of the broiler immune organ

Source: Bhuiyan, et al., 2021

Table 1.4: The location, structure, and function of the avian immune organs

Organ	Location	Structure	Function
Bursa of Fabricius	Dorsal to cloaca	Round/oval sac with lymphoid follicles	Production and development of B lymphocytes
Thymus	Along two sides of the neck	Multi lobular with follicles	Production and development of T lymphocytes
Spleen	Right side of proventriculus- gizzard junction	Variable shape	Systemic immunity
Meckle's diverticulum	Attached to the mid intestine	Loosely follicular	Local immunity
Caecal tonsils	Proximal end of caeca		Local immunity
Peyer's patches	Ileocecal junction		Local immunity

Source: (Panda and Reddy, 2007)

1.9 Measuring growth performance in broiler chicks

1.9.1 Bodyweight gain

Performance parameters such as liveweight, weight gain, feed conversion ratio, and mortality rate are very vital for broiler producers. Growth parameters, especially bodyweight gain, is an important parameter in broiler production. It is a major determinant of economic success in the production of broilers. The bodyweight of chicks is obtained by weighing them using a digital weighing scale. Poultry production standards have been substantially improved over the decades in terms of innovative technology which has resulted in improved broiler efficiency. Bodyweight gain is a result of the feed consumed by the chicks over time, the modern broiler chicken has the genetic

potential for significant weight gain within a very short period compared to other birds. Several factors have been reported to affect bodyweight gain in broilers, they include amount of feed consumed, environmental temperature, litter quality, disease agents and human factors and genetics (Billgilli, et al., 1999b; Ferket, et al., 2006). The performance of Ross 308 male broilers from 0-21 days is presented in appendix 1.

1.9.2 Feed intake as a measure of growth performance

Feed intake is the major determinant of both bodyweight and feed efficiency in broiler chicks, t is in fact the major driver of growth rate in broiler chicks (Ferket and Gernat, 2006; Abdollahi, et al., 2018). It can be described as feed consumed by the chicks over a given time. Feed intake of broilers is influenced by several factors such as environmental temperature, stocking density, lighting programme, stress, age, sex, digestive tract capacity, feed particle size, nutrient density, antinutritional factors (Brickett, et al., 2007; Latshaw and Moritz, 2009; Applegate, 2012; Abdollahi, et al., 2013c; Abdollahi, et al., 2018). Higher feed intake has been reported to increase weight gain, thus reducing maintenance energy in relation to gain (Svihus, et al., 2004a). It is very important to maintain adequate feed intake in broiler

chicks by possibly minimizing the feed intake stressors as this consequently leads to significant reduction in growth performance, thereby limiting modern broilers from achieving their full genetic potential. It has been reported that feeding diet high in protein from animal source increased feed palatability and feed intake in chicks (Abro, et al., 2012). In broilers, feed intake regulation is complex, the domestics chickens have been reported to have relatively well-developed mechanism for the regulation of feed intake at hatching (Leeson, et al., 1996; Furuse, 2002). Specific mechanisms regulate feed intake which involves sensing cellular and whole-body energy status (Richards, et al., 2010). These sensing mechanisms are linked to the unique signalling pathways that connects peripheral tissue (Richards, et al., 2010). Feed intake regulation in poultry has been studied and well documented (Denbow, 1994; Kuenzel, 1994; Kuenzel, et al., 1999; Furuse, 2002; Richards 2003; Richards and Proszkowiec-Weglarz, 2007; Furuse, et al., 2007). Feed intake and energy expenditure in broiler chicks must be tightly regulated to be able to maintain a constant bodyweight and energy balance (Richards and Proszkowiec-Weglarz, 2007). Broilers selected for growth and meat production traits may not be able to tightly regulate their intake and this often leads to overconsumption of feed resulting in overweight and predisposing chicks to skeletal abnormalities (Richards and Proszkowiec-Weglarz, 2007). Figure 1.13 shows the mechanisms involved in feed regulation and energy balance in poultry. Signals coming from the periphery include peptide hormones secreted by the GIT, adipose tissue, liver, pancreas, and vagal afferents. The brain contains the brainstem called satiety center, that receives and process signals from vagal afferent nerves and sends signal back to the GIT through vagal afferents that control the peripheral tissue functions and produce a sense of satiety.

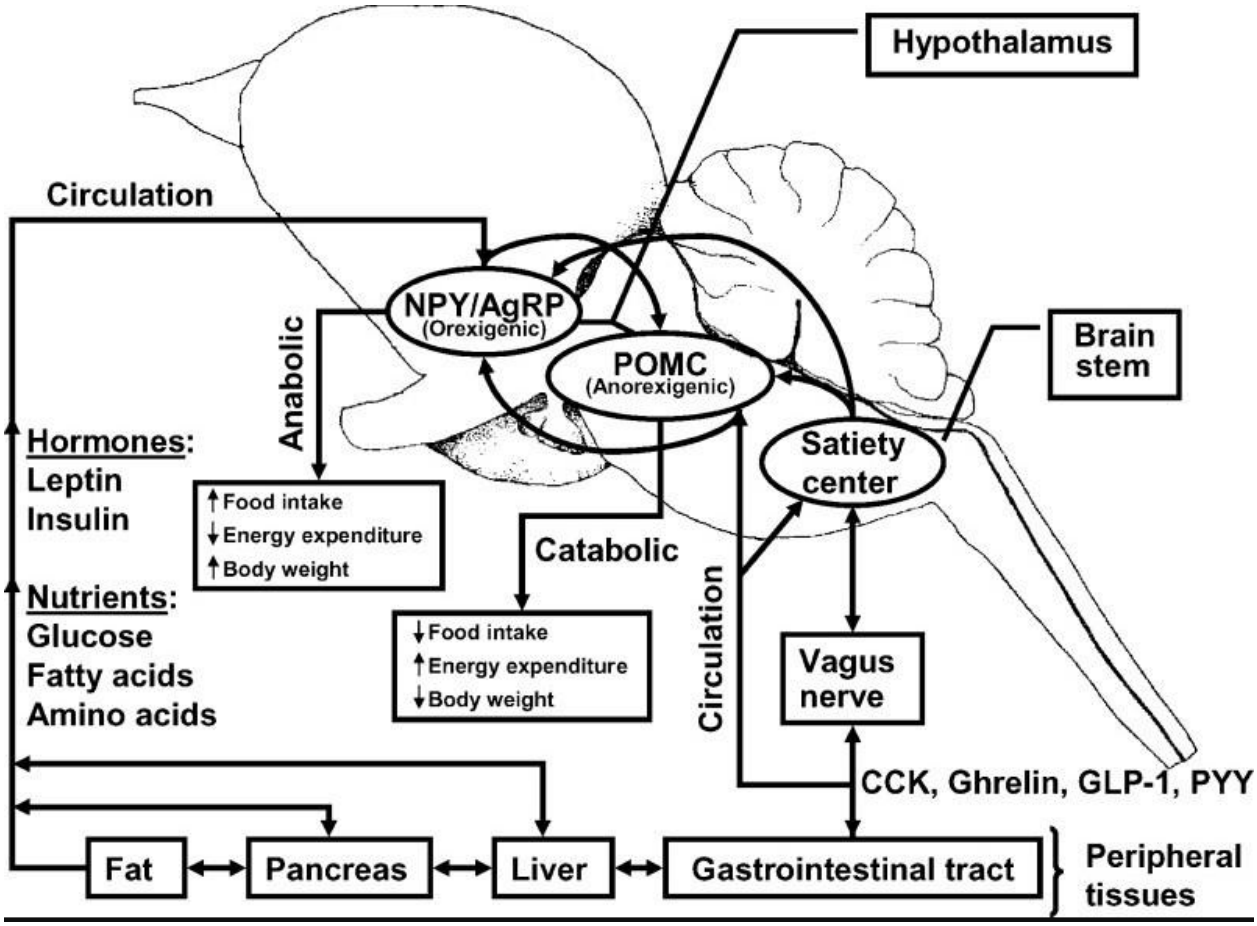


Figure 1.13: Illustration of mechanisms of feed regulation (appetite) and energy balance in poultry to achieve a stable bodyweight.

This model of appetite regulation and energy balance comprises of the peripheral tissues and the central nervous system controlled by hormonal, neural, neuroendocrine, and nutrient signalling mechanisms. NPY: neuropeptide Y; AgRP: agouti-related peptide; POMC proopiomelanocortin; CCK = cholecystokinin; GLP-1: glucagon-like peptide 1; PYY: peptide YY.

Source: Richards and Proszkowiec-Weglarz, 2007

1.9.3 Feed conversion ratio

Feed conversion ratio (FCR) a function of feed efficiency (FE) can be described as the capacity of broiler to convert kg of feed into kg of bodyweight gain (Mebratie, et al., 2019). The feed conversion ratio has

been reported to be moderately heritable in poultry (Havenstein et al., 1994, 2007; Emmerson, 1997). In broiler production, an improvement in feed conversion ratio is strongly associated with economic importance, decreased CH4 emission (Wang and Huang, 2005) and the amount of waste produced which enters the manure storage (Miles et., 2006; Hill and Azain, 2009).

1.10 Bodyweight variation in broiler chicks

The poultry industry has improved over the years in terms of growth rate and feed efficiency of the flock, however despite the significant improvement in production and management of poultry, there are still considerable variations in growth performance and feed efficiency within the commercial broiler strains (Hughes, et al., 2017). Bodyweight has been an important determinant of production efficiency and health status of the flock (Danni, et al., 2023). The daily bodyweight is basically used in common broiler production practice to evaluate feed consumed and body weight gained, as this illustrates if there is a deviation from the expected target, which may indicate vitality issues (Lott et al., 1982; Flood et al., 1992). Bodyweight variation in broiler chicks is being attributed to genetic and non-genetic maternal effects (Liu et al., 1993). Variation of 11-18% (CV) has been reported in mixed sex flocks and 8-10% have been reported for male only flock (Neto, et al., 2013; Vasdal, et al., 2019).

1.11 Environmental factors associated with bodyweight variation in broiler chicks.

1.11.1 Nutrition

Nutrient level, feed form and quality are contributory factors in performance variation of broilers. Energy and protein levels influence growth rate of broiler chicks. However, the major determinant of nutrient requirement of broiler is genetics (Mebratie, 2019). The impact of nutrient levels on bodyweight of broilers are reported by these authors (Plavnik and Hurwitz, 1990; Havenstein et al., 2003b). Feed forms such as pellets, mash and crumble also influence growth performance of broilers (Jones et al., 1995; Reece et al., 1986). The effect of feed form on broiler growth performance has been extensively explored by different researchers (Jones et al., 1995; Hamilton and Proudfoot, 1995; Calet,

1965; Nir et al., 1995; Savory, 1974).

1.11.2 Access to feed and water

Access to feed and water is one of the determinant factors associated with flock bodyweight variability. It is very important that the chicks have free access to feed and water from the day of hatch up to the slaughter age. Figure 1.14 shows a standard feed trough and nipple drinkers used in broiler production. In early life, access to feed and water is very important, as the chicks need energy derived from the feed to drive gut function and development. It has been reported that providing early access to feed and water promotes the development of intestinal microbiota, increased body weight, immune organ, and physiological development (Van de Ven et al., 2013; Proszkowiec-Weglarz et al., 2022). Sufficient feeders and drinkers if not in place may lead to the submissive birds within the flock population not consuming the required amount of feed *ad libitum* which consequently impacts nutrient intake and then growth performance. It has been reported that high flock bodyweight variability is an indication of inadequate feeder and drinkers, its placement within the barn and its access by the chicks without stress or maneuvering through other birds (Ferket and Garnat, 2006).

Figure 1.14: Standard feeders and drinkers used in broiler production.

Source : https://www.biomin.net/science-hub/essential-oils-in-drinking-water-using-flexibility-and-speed-to-help-poultry-during-gut-health-challenges/

1.11.3 Disease agents

Disease agents also contribute to variation in growth performance of the chicks, as they directly reduce feed intake which consequently affects growth performance. Some of the enteric diseases have a strong effect on feed intake reduction, thereby compromising the nutritional status of the bird. It has been reported that about 70% of reduced performance observed in a flock population during an infection challenge are attributed to reduced feed intake, while the remaining 30% emanate from nutrient malabsorption (Klasing, et al., 1987). Diseases play a substantial role in influencing feed intake and growth in broiler population. Kanno et al., (1996) reported that water, sodium, chloride, and glucose absorption were reduced significantly by sepsis which often resulted in diarrhea in birds, due to the bird's nutritional status mounting an immune response which is compromised by reduced absorption of specific nutrients.

1.11.4 Immunological challenge

Immunological stress has a massive effect on the chicks hormonal setting resulting to molecule cascades that may produce negative result on the chicks' performance. In young broiler chicks, the innate immune response is more nutritionally demanding compared to the acquired immune response (Koutsos and Klassing, 2002). Immunological stress often results in proinflammatory cytokine cascade which is associated with the innate immune response (Koutsos and Klassing, 2002). This influences the chick's behaviour and induces lethargy and anorexia, when may result in depressed intake and bodyweight gain (Koutsos and Klassing, 2002).

1.11.5 Litter type

Litter material and type also affects broiler growth rates. Litter material is used in broiler production as a source of insulation and a barrier to reduce birds direct contact with the floor, to promote efficient absorption of faecal moisture (Garcia et al., 2010). The type of litter material used in production determines the chick's skin integrity and may influence welfare conditions such as incidence of injuries,

occurrence of lesions mainly on chicken breast and footpad (Willis, et al., 1997; Godwin et al., 2000). There are various types of litter materials used in production of chicks globally, they include sawdust, wood shave, rice husk, sugarcane pulp, chopped straw, paper, sand, oat hulls, dried leaves, coffee husk (Monira, et al., 2003). Among all these, wood shave and saw dust are the most used globally (Rao, 1986). Litter type has been reported to influence bodyweight performance of chicks, litter consumption, litter bacteria and behaviour (Malone et al., 1983; Lien et al., 1992; Shields et al., 2004; Arnould, et al., 2004; Shield, et al., 2005; Toghyani, et al., 2010). Mondal, et al., (2020), reported that sawdust influenced growth performance of broiler compared to other litter type.

1.12 Strategies to improve growth and bodyweight uniformity in broiler chicks.

1.12.1 Breeder hen nutrition

Manipulating the nutrition of the breeder often enhances the development of the embryo and growth performance of the hatchlings. The manipulation of the broiler breeder nutrition was first explored and documented by (Spratt and Leeson, 1987), they reported that the content of protein and energy in the diet of the hen could highly influence the carcass fat and protein deposition in broilers during slaughter and processing. It has also been reported that increasing the levels of vitamin D3 in the breeder hens' diet improved broiler performance and bone characteristics (Atencio et al., 2005a, b, c). The impact of manipulating breeder hens' nutrition has been studied extensively and well documented in published articles (Peebles, et al., 1999, 2002; Surai and Sparks, 2001; Kidd, 2003; Kidd, et al., 2005; Calini and Sirri, 2007; Cherian, 2015; Surai, 2015).

1.12.2 In ovo nutrition

This is a practice whereby nutrients are injected into eggs (amnion) at the last phase of embryonic development. This practice ensures the embryo ingests the rich protein inoculated fluid which in turn facilitates the development of the gastrointestinal tract of the hatchling (Romanoff, 1960). In figure 1.15, the correct injection procedure is demonstrated whereby only the amnion fluid is stained. The

success and importance of this strategy have been well documented which includes increasing the embryo villus surface area during the third day post hatch (Smirnov, et al., 2006), enhancing dietary carbohydrate absorption (Foye, et al., 2007), increasing brush border nutrient transporter activity (Tako, et al., 2004, 2005), enhanced intestinal development (Uni and Ferket, 2004).

Although this practice had shown lots of positive results in terms of broiler performance, its limitations also need to be taken into consideration such as need for adequate and specialised technology, time and capital, its complexity in terms of commercial practice, and negative effects on hatchability due to inoculating eggs with certain nutrients (Ravindran, et al., 2021).

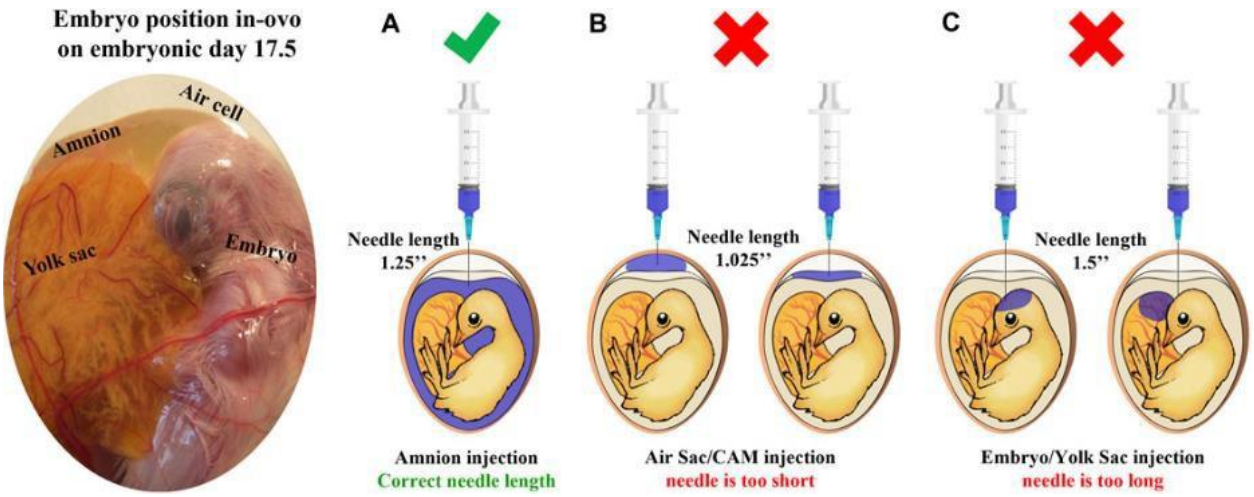


Figure 1.15: Illustration of the correct in ovo injection procedure

Source: Dayan, et al., 2023

1.12.3 Enhanced pre starter feeding

Early feeding of highly modified solutions and diet during the first week of life has proved promising in the aspect of enhancing broiler performance. These special early diets are formulated to consider the intestinal and digestibility limitations of hatchlings. Administration of highly digestible sugars and B-complex vitamins solution during the first 48 hours post hatch had been reported to alleviate stress in chicks (Ravindra, et al., 2021). Pre-starter diets are mainly based on quality and highly digestible ingredients to make nutrients more available for absorption.

1.12.4 Feed additives

Diet additives have proved useful over the years in young broiler nutrition, it has been in practice and plays a role in maintaining flock uniformity, production efficiency and health status of broiler chicks. In early broiler nutrition, several additives have practical importance such as the exogenous enzymes that stimulate the capacity of digestion (Bedford, et al., 2022), such as providing enzymes that alternate with limited enzyme capability such as lipase and protease. Carbohydrase and phytases have been widely used by the poultry industry as carbohydrase cleave the viscous fiber component, while phytase target the phytate complexes in plant-based feed ingredients (Selle and Ravindran, 2007; Woyengo and Nyachoti, 2011; Cowieson, et al., 2015). The use of feed additives in early nutrition of broiler chicks have been well documented (Cowieson and Roos, 2016; Ravindran, et al., 2016). Table 1.5 below illustrates the list of feed additives used in young broiler nutrition.

Table 1.5: List of feed additives in young broiler chick nutrition

Feed additive	Examples	Nutritional role
Enzymes	Protease, phytase, xylanases and B-glucanases	To overcome antinutritional factors present in feed ingredients such as arabinoxylans in wheat B-glucans in barley and phytate in all plant-based feedstuff. To improve feed digestibility and nutrient availability To enhance the feed value.
Emusifiers	Lysophosphatidyl choline	Aid in fat emulsification, thereby improving fat digestion.
Prebiotics	Fructo-oligosaccharide (FOS), mannan oligosaccharide (MOS)	Improves gut health by enhancing the proliferation of beneficial microbes in the gut.
Direct fed microbials	Probiotics	Improves the growth of beneficial bacterial species such as the lactobacilli and streptococci
Organic acids	Propionic acid, diformate	It encourages low gut pH, thereby eliminating the growth of pathogenic microbes in the gut
Botanicals	Herbs, spices, plant extracts, essential oils	Promotes healthy gut environment
Antimicrobial proteins	Lysozyme, lactacin F, lactoferrin, alpha lactalbumin	Prevents the growth of pathogenic microbes
Synthetic AA	DL-methionine, L-lysine, L-threonine	Provides adequate and digestible protein

Source: Ravindran, et al., 2021

1.13 Transcriptomic analysis in broiler research

Transcriptomic studies have been a vital tool in exploring differences in gene type and expression level under specific conditions (Cheng, et al., 2019). Several transcriptomic studies have been able to identify genes related to metabolic adaptations, apoptosis, cell proliferation, immune response and oxidative stress in meat typed chicken (Costa, et al., 2010; Zhang et al., 2017; Monson et al., 2019). Transcriptomic analysis has been used extensively by researchers to identify important mechanisms underlying physiological processes such as bodyweight variation, appetite regulation, relative feed intake, feed efficiency and other processes (Wang et al., 2009; Ozsolak and Milos, 2011; Cui et al., 2017; Ni et al., 2019; Peng, et al., 2019; Piórkowska, et al., 2020).

1.14 Conclusion

Broiler meat is predicted to become the world's most popular source of animal protein, due to its high consumer preference resulting from its economic and health benefits. As the world's population is increasing, the demand for broiler meat increases, therefore, to meet this increasing demand, sustainable broiler management and production should be prioritized. Several progresses have been achieved over the decades in terms of improving broiler production performance and efficiency. Genetic improvement in broiler production has resulted in broilers with higher production efficiency which can efficiently convert feed to muscle within a short period of time, thereby making cheap quality animal protein source readily available and alleviates protein malnutrition especially in the developing countries of the world.

A review of related literature has shown that growth and development of broiler chicks speeds up after hatching, and the first week of life is crucial and determines the subsequent progress in broiler production. Several mechanisms are involved in broiler growth development during the first few weeks of life, and feed intake or early access to feed is the main driver of growth and gut development at that age. Poor performance and uniformity indicate a decrease in profitability in the broiler business and thus contribute to economic loss in production. Therefore, it is desirable to achieve

the stipulated target by producing healthy and productive chicks reaching the targeted final body weight. Many factors have been attributed to trigger poor performance and uniformity in a broiler population which includes genetic variations, poor management practices and environmental factors.

Performance and uniformity improvement in broiler chicks have received good attention over the decades, research reports have indicated that growth performance and uniformity in broiler chicks can be improved through various management strategies such as genetic manipulation which has resulted in more efficient broiler chicks and secondly through nutritional manipulations such as feeding high quality diets and supplementation with feed additives. However, there is still gap in knowledge regarding the important biological factors associated with variation in growth performance of broilers in early life with more emphasis on the characteristics and physiological differences between the high and low performing broiler chicks in a broiler population. Therefore, this thesis was designed to identify those physiological and transcriptomic differences in broiler with high and low bodyweight in a flock of chicks during the starter phase. The answer from these trials contained in the thesis would be useful in narrowing the search into identifying precise intervention strategies to optimize broiler growth performance and improve flock uniformity.

The research hypothesis of this thesis was that broilers of the same breed, reared under common diet and environmental conditions, with contrasting body weight in the early weeks of life may exhibit differences in their physiological and genomic characteristics which may be contributing to the differences observed in their bodyweight.

1.15 Thesis aims and objectives.

The main aim of this thesis was to understand the physiological and transcriptomic characteristics of the low and high weight broiler chicks in early life and to identify physiological, genetic, and behavioral markers associated with bodyweight variation in broiler chicks in early life. Below is a brief overview and the objectives of each trial.

Trial 1: Day 7 bodyweight in broiler chicks is an important predictor of final bodyweight of chicks and therefore could be a vital target for improvement. Physiological and transcriptomic differences observed in this period could offer a better understanding of the important drivers of variation in bodyweight of chicks. This trial objective was to understand the differences in the digestive organ weight, bone mineral concentrations and transcriptomic characteristics of the super and underperforming chicks in the first week of life.

Trial 2: Poor BW gain in young broilers is a critical factor underpinning morbidity, mortality, and welfare. Compositional differences between high and low bodyweight chicks in bone parameters, gut pH and tissue mineral concentrations during the starter stage (0-21days) may provide insight into underlying causes of variation in bodyweight. The objective of this trial was to investigate the differences in bone morphometric, tissue mineral concentrations and gut parameters of Ross 308 male broilers reared under common environmental and diet conditions with distinct bodyweight on D21.

Trial 3: The behaviour of chicks especially feeding behaviour during the first week of life may have a direct influence on the growth rate of the broiler chicks. The differences observed in broiler growth rates have been attributed to the time and access to feed and water post hatch. The main objective of this trial was to evaluate the differences in the behavioural activities, and tissue mineral profile of chicks in the first week of life.

Trial 4: The litter material on which the chicks are reared also play an important role in intestinal morphology and microbial population, because litter type can significantly influence the efficiency of the chicken immune system and growth performance. This trial evaluated the effect of hardwood (Aspen) litter which has been reported in previous literature to contain higher levels of Xylo oligosaccharide (XOS) compared to the standard soft wood used in broilers on the bodyweight performance, gut pH, tissue mineral concentrations and bursa transcriptomic profile of chicks. The hypothesis was that the hardwood would influence growth performance of the chicks.

CHAPTER TWO

2.0 Materials and methods

2.1 Introduction

Trials were run to better assess the possible factors associated with bodyweight variations in broiler chicks in early life. Most of the protocols employed in the present study were common to all trials and some of these have been detailed in the individual results chapters.

2.2 Animal management and housing

All experimental animals used in the trials were given the same environmental management conditions and were all raised in a deep litter pen. The chicks used throughout the experiment were day old male Ross 308 chicks supplied by PD Hooks hatchery. The housing temperature and ventilation settings in all trials within the room were appropriate and based upon the age of the bird. There was lighting intensity of 20 lux during the lighting periods. During the experiment photoperiod cycles of 18h light and 6 h darkness was maintained. A relative humidity of 60-70% during the first 0-2 days and above 50% thereafter was allowed according to the Ross management manual 2018 (Aviagen, 2018)

2.3 Experimental diets and study locations

The diets used in trial 1 and 3 were commercial diets sourced from a commercial feed company (Heygates country feeds and GLW Shepshed) respectively. The details of all diets used in each of the trials were contained in each result chapter below. Animal trials were conducted in two different locations, Trial 1 was conducted at the University of Nottingham, Sutton Bonington Campus, whereas Trials 2, 3 and 4 were conducted at the poultry research unit of Nottingham Trent University, Brackenhurst Campus, Southwell.

2.4 Ethical approval

For all trials contained herein, ethical approvals were sought and obtained from the two institutions. The approval reference numbers were contained in each trial as detailed below.

2.5 Behaviour monitoring

The behavioural monitoring details are outlined in chapter 5 (trial 3) below.

2.6 Sample collection

2.6.1 Sample collection for analytical procedure

All samples collected for mineral analysis, ADF and NDF analyses including bones, liver, gizzard, were collected immediately after euthanisation (further details are provided in subsequent Chapters). Tissue samples were individually stored in well labelled bags and kept in -20 freezer until further analysis.

2.6.2 Sample collection for gene expression analysis

In Trial 1, the ileal tissue was collected immediately after euthanizing the chicks into 1ml Eppendorf tubes and immediately into a box of dry ice before transporting them to the laboratory and stored in -80^0C till RNA was extracted. In Trial 4, 200mg of bursal tissue were collected immediately with minimal handling into 1ml RNA later in a 1ml RNAase free Eppendorf tubes. During all collections, RNA Zap was used for frequent cleaning, to avoid sample contamination.

2.7 Analytical protocol

2.7.1 Bone Ash determination

In all trials, the left and right legs were allowed to thaw, bones were cleaned to extract the tibias and then allow them to dry in the oven (Griffin oven) for 24 hours at 105^0C before ashing overnight in a muffle furnace (Carbolite AAF 11/18) at 600^0C. Bone ash was determined afterwards.

2.7.2 Bone morphometric and strength analysis

Bone morphometric measurement and strength analysis protocol are detailed in chapter 4 (trial 2)

2.7.3 Determination of acid detergent fiber (ADF)

Acid detergent fibre determines the percentage of the plant cell wall that is made up of cellulose and lignin (Van Soest, et al., 1991). In the present study ADF was measured in the south laboratory of the University of Nottingham, Sutton Bonington campus. The standard procedure with method number AN-04-205 used is detailed below:

Fibre bags obtained from a commercial company (Gerhard, UK) were dried in an oven at 100- 105^{0}C for 30 minutes, then they were transferred to a desiccator to cool for 5 minutes. All fibre bags were weighed and labelled with waterproof markers. Approximately 1.0g of sample were weighed using a weighing boat and then transferred into the fibre bag. Bags with the sample were defatted by rinsing the bag into three portions of 40-60 petroleum ether, and then a spacer was inserted into each bag and placed into a holder when free from petroleum ether. The detergent solution was prepared by dissolving 20g of Cetyltrimethylammonium bromide (CTAB) in 1 litre of 0.5M sulphuric acid. CTAB was allowed to completely dissolve before commencing analysis. Total of 250mls of detergent solution was poured into the first extraction beaker, then the first holder containing the samples were lowered gently into the beaker of solution and mixed by rotating for one minute, then allowed to stand for 10 minutes. The carousel was raised from the liquid and a further 100mls of reagent was added and the carousel lowered and mixed for a further minute.

The beaker was placed on a cold hotplate, the condenser was fitted and brought to boil with full setting, while setting was reduced when 90^{0}C was reached. This was repeated for each set of tests at five minutes intervals. After 1 hour from the point of boiling, each beaker was removed from the heater and the carousel removed. All liquid and soluble were discarded and the bags and residues were washed in at least 4 portions (250mls each) of hot distilled water to ensure the detergent was completely washed out. A jet of distilled water from a wash bottle was directed onto each spacer and the spacer removed from the bag while rinsing. All bags were placed on tissue paper for a few minutes to completely drain.

The bags containing the residues were dried at 100-105^{0}C in the oven for four hours, then desiccated for 15minutes, cooled, and weighed. The weighed bags were placed into 50mls beakers. They were then placed in the muffle furnace at 600^{0}C and ashed for a period of four hours, desiccated and cooled for 15 minutes and reweighed. The ash was then removed using a balance brush and the beaker was reweighed.

ADF was calculated as: % ADF = ((Beaker + Residue weight – bag weight) -(Beaker + ash weight)) * 100/Sample weight

2.7.4 Determination of neutral detergent fiber (NDF)

Neutral detergent fibre (NDF) was determined using the laboratory standard procedure with the method number AN-04-204:

Fibre bags were dried in an oven at 100-105^{0}C for 30 minutes, bags were then transferred to the desiccator to cool for 5 minutes. Bags were weighed and labelled with a waterproof marker, approximately 0.5g of sample was weighed using a weighing boat and then transferred to the bag.

Bags with samples in were defatted by rinsing in three portions of 40-60 petroleum (PET) ether. Spacers were inserted into the bags and placed onto a holder when the PET ether had completely evaporated. Neutral detergent solution was measured out into the extraction beaker and placed on a cold hotplate. The condensers were fitted and brought to boil with full setting and then reduced just as they started to boil. Sodium sulphate (1%) solution measuring 250ml was placed in a spare extraction beaker, then the carousel was lowered into the solution and mixed, fibre bags were allowed to stand for 5 minutes. After 5 minutes they were spanned to remove excess sodium sulphate, and then lowered gently into the NDF solution, mixed by rotating the holder and then handling tool was removed. The beaker was placed on the hotplate, this procedure was repeated for each set of the tests at five minutes intervals, after 30 minutes from the point of boiling, each beaker was removed from the hotplate in turn. The hotplates were turned off, handling tool attached, then the holder was removed from the beaker and

bags were allowed to drain.

Hot detergent solution measuring 180ml was poured into a plastic cylinder, another 180ml cold detergent was then added with 12ml of amylase solution which was prepared by dissolving 2g of amylase in 90mls of distilled water and 10mls of 2-ethoxyethanol. The solution was then returned to the beaker, the carousel was lowered into the liquid. The extraction beaker was returned to the hotplate, condenser fitted and then brought to boil with full setting which was reduced when 900°C was reached. The procedure repeated for each set of the test at 5 minutes intervals, after 30 minutes from the boiling, each beaker was removed from the heater and carousel removed. All liquid and soluble were discarded and the bags and residues were washed in three portions of hot distilled water to ensure they were detergent free. Another 350ml of hot water and 25ml of amylase solution were added to the beaker, bags were lowered into the solution and mixed gently and allowed to stand for 15minutes, then the carousel was removed and washed with 3 x 400ml of hot distilled water. A jet of distilled water was directed onto each spacer from a wash bottle, spacers were removed from the bags whilst rinsing. All drained bags containing the residue were placed for a few minutes on tissue paper to drain completely. Bags with the residues were dried at 100- 105°C in the oven for four hours, then desiccated for 15 minutes, cooled, and then weighed. The dried bag with residues were placed into 50mls tall beakers. They were placed in a muffle furnace 600°C and ashed for a period of four hours desiccated, and cooled for another 15 minutes after ashing, then reweighed. The ash was then removed from the beaker using the balance brush and the beaker reweighed.

NDF was calculated as follows %NDF = ((Beaker + residue weight – bag weight) – (Beaker +ash weight)) *100 /sample weight

2.7.5 Defatting of bone

The tibias of the 21days old chicks were defatted following the protocol outlined in chapter 4 (trial 2).

pH determination

In Trial 2 and 4, a digital pH piercing probe (Apera Instruments PH60S spear pH tester) was inserted directly into the digesta in the crop lumen, proximal gizzard, and distal ileum and caecum of the sampled birds. The pH was measured and recorded in triplicate for each chick in both trials.

2.8 Mineral Analysis

2.8.1 Acid digestion of bone ash

The acid digestion protocols for each trial are detailed in the individual result chapters below.

2.8.2 Acid digestion of liver and gizzard digesta samples

Acid digestion for the liver and gizzard digesta samples were detailed in trials 2, 3 and 4. Postdigestion samples were submitted to laboratory B05 of the Gateway Building, Sutton Bonington Campus. Each digestion batch was run with 2 blanks to ensure there was no contamination in the samples.

2.8.3 Multi-elemental analysis by ICPMS

Multi-element analysis of diluted solutions is undertaken by ICP-MS (Thermo-Fisher Scientific iCAP-Q; Thermo Fisher Scientific, Bremen, Germany). Samples were introduced (flow rate 1.2 mL min-1) from an autosampler (Cetac ASX-520) incorporating an ASXpress™ rapid uptake module through a perfluoroalkoxy (PFA) Microflow PFA-ST nebuliser (Thermo Fisher Scientific, Bremen, Germany). Sample processing was undertaken using Qtegra™ software (Thermo-Fisher Scientific) utilizing external cross-calibration between pulse-counting and analogue detector modes when required. The instruments were run by employing several operational modes. The iCAP-Q employs in-sample switching between two modes using a collision cell (i) charged with He gas with kinetic energy discrimination (KED) to remove polyatomic interferences and (ii) using H2 gas as the cell gas. The latter was used only for Se determination. Typically, in-sample switching was used to measure Se in H2-cell mode and all other elements in He-cell mode. Peak dwell times are 100 mS for most elements with 150 scans per sample.

Internal standards, used to correct for instrumental drift, were introduced to the sample stream on a separate line (equal flow rate) via the ASXpress unit or are added directly to calibration standards and samples and introduced on a single line. Internal standards typically include combinations of Sc (10 µg L-1), Ge (10 µg L-1), Rh (5 µg L-1), Re (5 µg L-1) and Ir (5 µg L-1). The matrices used for internal standards, calibration standards and sample diluents were typically 2% Primar grade HNO3 (Fisher Scientific, UK) with 4% methanol (to enhance ionization of some elements). Calibration standards typically include (i) a multi-element solution with Ag, Al, As, Ba, Be, Cd, Ca, Co, Cr, Cs, Cu, Fe, K, Li, Mg, Mn, Mo, Na, Ni, P, Pb, Rb, S, Se, Sr, Ti, Tl, U, V and Zn, in the range of 0 – 100 µg L-1 (0, 20, 40, 100 µg L-1) (Claritas-PPT grade CLMS-2 from SPEX Certiprep Inc., Metuchen, NJ, USA); (ii) a bespoke external multi-element calibration solution (PlasmaCAL, SCP Science, France) with Ca, Mg, Na and K in the range 0-30 mg L-1 and (iii) a mixed phosphorus, boron and sulphur standard made in-house from salt solutions (KH2PO4, K2SO4 and H3BO3).

CHAPTER THREE

Trial 1

3.0 Identification of differences in digestive organ weight, bone mineral concentration, and ileal transcriptomic profiles of low and high weight broiler chicks

Submitted to Journal of Agricultural Science, Cambridge (AGS-2024-0073)

3.1 Abstract

A growth monitoring study (0-7 day of age) was conducted involving 87, one-day old Ross 308 male broilers to evaluate organ weights, bone parameters and ileal transcriptomic profile of broiler chicks influenced by day 7 bodyweight (BW) grouping. The chicks were raised in a deep litter house under common controlled environmental conditions and commercial starter diet. Chicks were grouped according to their body weight on day 7 into two distinct BW, super performer (SP) and under performer (UP) with bodyweights >260g, and <200g respectively. Results obtained revealed that the SP chicks had significantly higher bone ash, Sodium (Na), Phosphorus (P) and Rubidium (Rb) concentrations when compared to the UP chicks on D7. In contrast, the UP chicks had significantly higher bone Cadmium (Cd), Cesium (Cs) and Lead (Pb) compared to the SP group, also the UP chicks had proportionally heavier gizzard than the SP chicks. The ileal transcriptomic data revealed differentially expressed genes between the two groups of chicks, with 150 upregulated and 83 down regulated genes with a fold change of ≥1.25 or ≤ 1.25 in the SP chicks relative to the UP chicks. Furthermore, functional annotation and pathway analysis revealed that some of these differentially expressed genes were involved in various pathways including calcium signaling, Wnt signaling, cytokine-cytokine receptor interaction and mucin type O-glycan biosynthesis. This study revealed that chicks of the same breed and of uniform environmental and diet management, exhibited differences in digestive organ weights, tibial bone characteristics and ileal gene expression that may be related to differential bodyweights.

Keywords: Transcriptomics, ileum, bodyweight, variation, bone mineral concentration

3.2 Introduction

Chicken is one of the most preferred animal protein sources globally due to its comparatively lower cost, nutritional content and perceived health values. Despite improved genetic modification and stringent management practices in broiler production, there have been reports of considerable bodyweight variation which results in varying slaughter weight (Piórkowska, et al., 2020; Lundberg, et al., 2021). About 8-10% coefficient of variation in male broilers raised under similar management and environmental conditions had been reported (Neto, et al., 2013). There are many reasons underpinning variation in broiler growth such as broiler breeder age, incubation factors, genetics, disease, nutrient malabsorption, and poor feed intake (Tegeda, et al., 2021). Day 7 bodyweight has been reported to have strong correlation with important parameters such as slaughter weight and carcass composition compared to hatch weight (Ribeiro, et al., 2004 and Tona et al., 2004b). Bodyweight increases two to threefold during the first week and considerable changes occur in the gut and muscle weight (Jin et al., 1998). The first week of life is a very critical period for the broiler, as the chicks are exposed to more varied conditions on the farm following a controlled environment during the incubation period (Yerpes, et al., 2020). During this period, important changes in gastrointestinal development occur (Iji, et al., 2001; Willemsen et al., 2008), which continue to develop during the starter phase. These developmental changes can be categorized into morphological, functional and immunological development (Schokker, et al., 2009). The development of the chicken intestine as digestive and absorptive system is closely related to the development of the gut-associated lymphoid tissue (Shira, et al., 2005). It has also been reported that the immunological development of the chicken intestine occurs within the first two weeks of life. This was further reported to be associated with early nutrition which makes essential substrates available, leading to the stimulation of endogenous hormone levels or other immunomodulatory molecules (Dibner et al., 1998). Research has reported that the expression of proinflammatory cytokine and chemokine (IL-1β, IL-8, K203) during the first week of

life in broiler are initiated by the exposure of the hatchlings to exogenous feed and the environment (Bar-Shira et al., 2006). This unique development of the chicken intestine with a coinciding succession of microbiota and changes in microbial community during the early life can influence the host physiological and metabolic functions (Tang, et al., 2020).

Mineral metabolism is an important aspect in broiler nutrition and growth as minerals play useful roles as catalyst in most enzyme and hormone activities (Suttle, 2010). Bone mineral concentrations especially calcium and phosphorus affect skeletal integrity (Underwood and Suttle, 1999) and determine the extent of mineralization. They are also actively involved in many physiological and metabolic roles in the body such as cell signaling and nerve impulse transmission (Underwood and Suttle, 1999). Study has reported bone mineral concentration as a vital tool in assessing mineral bioavailability, utilization and storage in broiler chicks (Yair and Uni, et al., 2011), for example calcium concentration in the tibia serves as a reservoir for maintaining serum calcium levels (Weaver, et al., 2016). Therefore, evaluating bone mineral concentration in broiler chicks in early life could be a more potential valuable biomarker to determine the mineral status of chick's post hatch. Generally, mineral absorption in broilers is uniquely governed by the activation of important pathways, for example Wnt signaling that comprises of several ligands, when activated by Wnt proteins, which when secreted binds to the frizzled transmembrane receptors to initiate intracellular signaling cascade that modulates gene expression (Mohammed, et al., 2016), resulting in specific mineral absorption such as Ca and P (Wang, et al., 2022).

The small intestine plays a vital role in the regulatory, endocrine, and immune function, which can thus affect birds' health, feeding behavior and energy homeostasis (Scanes and Pierzchala-Koziec, 2014; Sugiharto, 2016 and Honda, et al., 2017). Svihus (2014) reported that the functionality of the digestive tract is pivotal to optimal performance of broiler chicks. The present study evaluated differences in digestive organ weight, ileal transcriptomic profile, and bone mineral concentrations of 7days old broiler

chicks. Birds were characterized based on their day 7 body weight because several research have proven that day 7 bodyweight in broiler chicks is an important predictor of final bodyweight of chicks and therefore could be a vital target for improvement. Therefore, the hypothesis of the current trial was that broiler chicks of same breed raised under common conditions with varying D7 bodyweight would exhibit differences in digestive organ weights, bone mineral profile and ileal gene expression.

3.3 Materials and Methods

3.3.1 Ethical approval, Experimental Design and Animal Management

All the experimental protocols and animal ethics used in the study were approved by the University of Nottingham ethical review committee (Reference code: 223). A total number of 87-day old male Ross 308 chicks were used for the study and all chicks were housed in the same deep litter pen with wood shaving as bedding, and under the same common environmental and diet conditions. The chicks were reared from day 0 to day 7 and were characterized based on the day 7 bodyweight, before sample collection. Chicks were fed commercial Hygates baby chick crumbs (containing 19% crude protein, 4.5% crude fiber and 3.5% oil) that met the nutritional requirement of the Ross 306 breed.

Bodyweight of chicks was recorded individually on day 0 and day 7. Chicks were ranked and those in the first and fifth quintiles were categorized as under performers (UP) and super performers (SP) respectively. SP chicks had an average bodyweight of 260g and UP; 200g, bodyweight thresholds were selected based on the performance target outlined for male Ross 308 chicks on day 7 (Aviagen, 2019). On day 7, ten chicks from each group SP and UP (n=10/bodyweight group) were randomly selected and euthanized. Bodyweight uniformity was calculated using the formula below.

Uniformity % = Number of birds within range ±10% of mean weight ÷ Total number of birds weighed × 100. The liver, gizzard and full intestine were excised and weighed using a precision balance of 0.0001g while the legs were collected and stored at -20^{0}C until further bone mineral analysis. The ileal segment was excised, and snap frozen immediately with dry ice before being stored at -80°C until RNA

extraction.

3.3.2 Crude ash and mineral analysis

The legs collected were thawed and defleshed to extract the tibial bones. Adequate care was taken to make sure all the flesh was removed and immediately froze at -20^{0}C until drying the next day. The tibial bones were oven-dried at 105^{0}C for 24hrs and ashed at 600^{0}C overnight to determine the tibial ash, then the ash weight of individual tibial bone was expressed as a percentage of dry weight. The tibial bone ash was acid digested using the hot plate method following internal laboratory procedure for sample preparation. A maximum of 0.2g of each sample was digested with 10ml of nitric acid and heated for 2 hours at 95^{0}C, 50ml MilliQ water was added to each and 8ml taken from the top into 8ml tubes and samples were diluted to 1/10 and mineral concentration analyzed using the ICP-MS methods (Thermo-Fisher Scientific iCAP-Q; Thermo Fisher Scientific, Bremen, Germany).

3.3.3 RNA extraction and microarray analysis

RNA was extracted from the ileal tissue of 7-day old broiler chicks using the Direct-zol™ RNA MiniPrep Kit (Cambridge Bioscience, UK). RNA integrity was confirmed using an Agilent 2100 Bioanalyzer with the RNA 6000 Nano Kit (Agilent Technologies, Palo Alto, CA). The RNA integrity numbers (RIN) were ≥8.7 for all samples. Whole-genome transcriptome analysis was conducted by hybridising three biological samples of total RNA per group to GeneChip^{TM} Chicken Gene 1.0 ST arrays (Affymetrix, Santa Clara, CA, USA). The first strand cDNA was produced by reverse transcription followed by second strand synthesis. Double stranded cDNA was then used to synthesise biotinylated complementary RNA *in vitro*, which was purified and fragmented in different sizes (200-2000 bp). These fragments were hybridised onto GeneChip^{TM} Chicken Gene 1.0 ST arrays using the GeneChip System 3000 instrument platform (Affymetrix, Santa Clara, CA, USA). All steps were taken at the Nottingham Arabidopsis Stock Centre. Gene expression profile data was generated as CEL files and analysed using Partek Genomics Suite 6.6 (Partek Incorporated, St. Louis, MO, USA). The raw

CEL files were normalised using the RMA background correction with quantile normalisation, log base 2 transformation and mean probe-set summarisation with adjustment for GC content.

Quantitative real-time polymerase chain reaction (qRT-PCR) confirmation of the microarray data

To verify the reliability of the microarray data, three immune related genes (IL20RA, IL8L1 and CCL17) and one gene related to detoxification (GSTA3) were selected for further validation using the RT-qPCR technology. The immune-related genes were selected to verify the fact that the SP chicks had better innate immune activation compared to the UP group. Four genes from the microarray data GAPDH, GALNS, FABP5 and FAM133B were chosen as housekeeping genes for qRT-PCR because there was no change in their expressions between the two groups. The primer pairs used for the quantitative PCR of these genes are contained in appendix 1.

Total RNA (250ng) was reverse transcribed using the cDNA reverse transcription kits according to the manufacturers' protocol UltraScript 2.0 cDNA synthesis kit (PCR Biosystems, London UK). The real time PCR reactions were performed using the Bio-Rad CFX Maestro, the reaction

contained 1ul of cDNA as a template in a 10ul reaction, the master mix contained 0.4ul of the reverse and forward primers from a 10uM stocks, 5ul of the Syber green master mix 2X qPCRBIO SyGreen Blue Mix Hi-Rox (PCR Biosystems, London UK), and 3.6ul of RNase free water. The PCR reaction conditions were set at 95^0C for 20 seconds, followed by 40 cycles of 95^0C for 3seconds and 60^0C for 30 seconds. A melting temperature curve for every PCR reaction was determined at the end of each run for amplification specificity, and all the 4 samples were performed in triplicate. Relative expression of each mRNA was determined using the $2^{-\Delta\Delta Ct.}$ method using the Bio-Rad software.

3.4 Functional annotation and pathway analysis

The Database for Annotation, Visualization, and Integrated Discovery (DAVID) (https://david.ncifcrf.gov/tools.jsp) and Ingenuity Pathway Analysis (IPA) were used to determine

the biological functions of the differentially expressed genes based on the *Gallus gallus* reference. Pathway analysis was carried out using the KEGG database as utilized through the DAVID online database.

3.5 Statistical Analysis

The individual chick served as the experimental unit. Day 0 bodyweight, digestive organ weights and other data derived from the three experimental BW groups were compared using ANOVA and experimental group means were separated using the Tukey test (SPSS software ver. 21, IBM Corp. Released 2012. IBM SPSS Statistics for Windows, Version 21.0. Armonk, NY: IBM Corp). The student t-test (Prism version 8.0.0 for Windows, GraphPad Software, San Diego, California USA, www.graphpad.com)) was used for contrasts between the SB and UP groups for selected variables. Significant differences were observed at $p<0.05$. Differentially expressed genes (DEG) were identified by one-way ANOVA. DEG comprised genes upregulated or downregulated by at least 1.25-fold with an un-adjusted p-value ≤ 0.05. Statistical analysis for the qPCR data was performed using ANOVA statistical package of the Bio-Rad CFX Maestro analysis software.

3.4 Results

3.4.1 Day 7 bodyweight and Digestive Organ Weights

The simple statistics of day 7 body weight were represented using the histogram in figure 3.1 below. The mean bodyweight of the bird population on day 7 was 231.2±34.2g, CV of 14.8% and uniformity of 56%. The organ characteristics of the chicks in the BW groups are presented in Table 1. The SP chicks had significantly heavier liver (SP = 12g; UP = 8g; $P < 0.0001$), gizzard (SP = 14g; UP = 10g; $P < 0.0001$), intestine weight (SP = 23g; UP = 15g; $P < 0.0001$) and intestinal length (SP = 110cm; UP = 94cm; $P = 0.0001$). It was interesting to note that the UP group had a proportionally heavier gizzard compared to the SP groups.

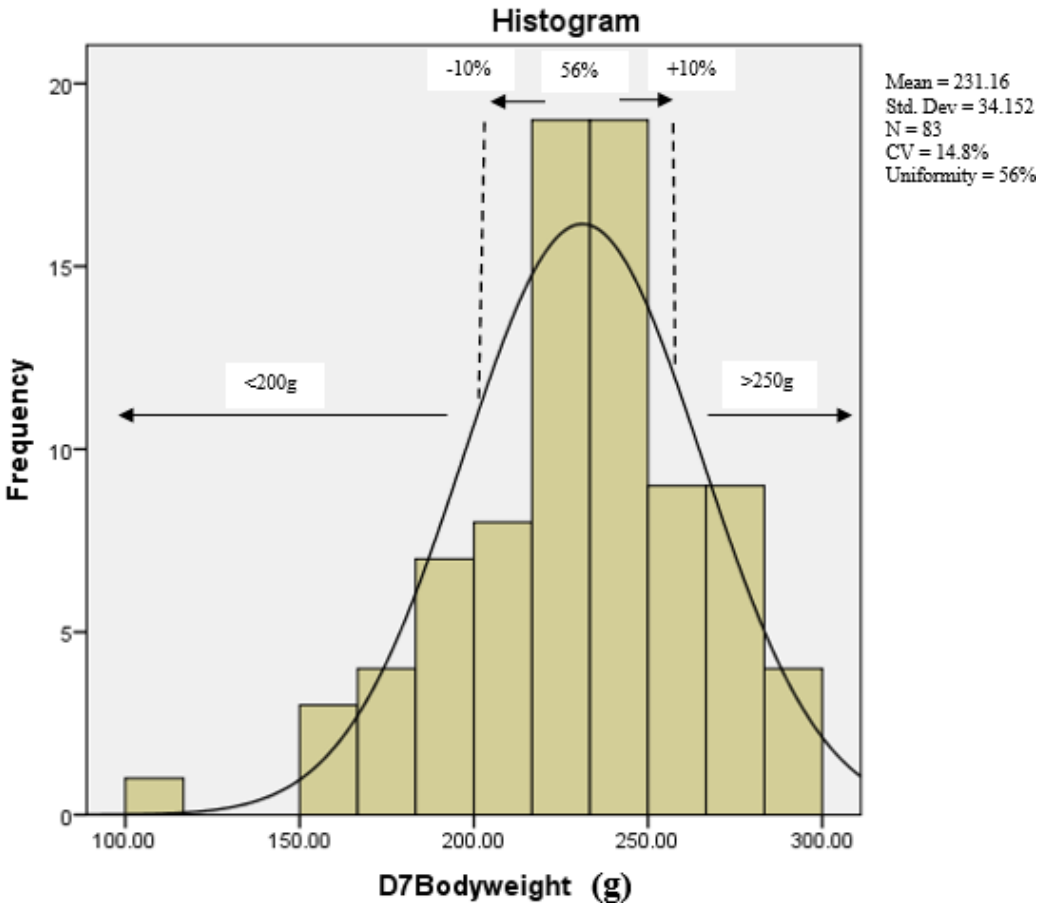


Figure 3.1: The histogram of day 7 bodyweight of male Ross 308 broiler chicks fed the same diet and environmental condition (n = 83 chicks)

Table 3.1: Digestive tract and ancillary organ weight of ROSS 308 chicks at 7days of age (n= 10 per BW group)

Parameters	SP	UP	SEM	P-value
D0 BW (g)	61	52	±2.3	0.001
D7 BW (g)	276	174	±6.4	<.0001
LW (g)	12	8	±0.7	<.0001
*Relative LW (g/kg)	44.27	43	±0.3	0.921
Gizzard wt (g)	14	10	±0.6	<.0001
*Relative gizzard wt	52	58	±0.2	0.015
Intestinal wt (g)	23	15	±1.1	<.0001
*Relative intestinal wt (g/kg)	86	83	±0.4	0.463
Intestinal length (cm)	110	94	±4.5	0.003

UP- Under-performers, and SP- Super-performers chicks, D0 BW – Day 0 bodyweight, D7 BW- Day 7 body weight, ADWG- Average daily weight gain, LW – liver weight. SEM – Standard error of the mean (for both groups). * Values are relative to body weight

Tibia bone ash and mineral concentration

The tibial bone ash and macro mineral concentration of the UP and SP chicks on D7 is shown in table 3.2, while the trace mineral concentration is presented in table 3.3. The SP group had higher bone ash when compared with the UP group (SP = 470g/kg; UP = 440g/kg; P = 0.014). The UP group had significantly higher Cs (UP = 0.04mg/kg; SP = 0.03mg/kg; P = 0.023), Cd (UP = 0.02mg/kg; SP = 0.01mg/kg; P = 0.048) and Pb (UP = 0.34mg/kg; SP = 0.20mg/kg; P = 0.014) when compared with the SP group. While the SP chicks had significantly higher tibial Na (SP = 12.7g/kg; UP = 11g/kg; P = 0.014), P (SP = 19.57g/kg; UP 18.62g/kg; P = 0.018), and Rb (SP = 0.009, UP = 0.008; P = 0.033) concentrations compared to the UP group.

Table 3.2 Tibial ash and macro mineral concentrations of the UP and SP chicks at D7 of age, (n = 10 chicks per BW group)

Ash and mineral concentrations (g/kg)	SP	UP	SEM	P-value
Ash	470.0	440.0	±1.20	0.014
Ca	363.0	352.0	±7.00	0.143
P	195.0	186.0	±3.56	0.018
Na	12.7	11.0	±0.56	0.014
S	4.0	3.0	±0.31	0.066
K	9.0	10.0	±0.49	0.215
Mg	8.0	7.0	±0.26	0.506

UP denotes Under performers group, SP denotes Super performers group; Minerals are expressed on a crude ash basis. (n= 10 per BW group), SEM Standard error of the mean (for both groups).

Table 3.3 Trace mineral concentrations of the UP and SP chicks at D7 of age (n = 10 chicks per BW group)

Trace mineral concentrations (mg/kg)	SP	UP	SEM	P-value
Cd	0.01	0.02	±0.002	0.048
Cs	0.03	0.04	±4e-3	0.023
Rb	0.01	0.01	±7e-3	0.034
Pb	0.20	0.34	±0.04	0.014
Mn	14.0	16.0	±1.06	0.097
Se	0.23	0.23	±0.02	0.765
Sr	225	208	±8.86	0.062
Cr	1.22	0.98	±0.19	0.230
Fe	308	318.	±38.04	0.789
Cu	3.17	3.09	±0.20	0.709
Zn	466	467	±19.41	0.970

UP denotes Under performers group, SP denotes Super performers group. (n= 10 per BW group), SEM Standard error of the mean (for both groups)

3.4.3 Ileal transcriptomic profile and differentially expressed genes.

The transcriptomic profile analysis revealed 233 genes that were differentially expressed with a $P< 0.05$ and fold change cutoff of ≥1.25 between the SP and UP groups. The biological details of the DEGs mapped in the IPA database are provided in appendix 3, while the details of the top 29 most conspicuous DEGs with foldchange (≥+1.5 and ≥-1.5) are shown in table 3.2. All the DEGs including the up-regulated (150 genes with low stringent cutoff ≥+1.25) and down-regulated (83 genes with cutoff ≥-1.25) expressed in the ileum of 7-day old chicks of distinct bodyweight were categorized into 3 main functions of biological process, molecular function, and cellular component according to GO analysis using DAVID online tool. Each of the GO categories were further divided into subcategories, and the DEGs were all annotated in all the three GO terms as shown in figure 3.3.

The biological process comprises of 26 terms, including prostaglandin biosynthesis, positive regulation of cell proliferation, superoxide metabolic process, tissue development, inflammatory response etc. Molecular function was divided into 12 terms, including heparin binding, frizzled binding, growth factor activity etc. The cellular component comprises of 8 terms which includes extracellular space, integral component of plasma membrane, extracellular region, photoreceptor outer segment, brush border etc. (Figure 3.2). Functional annotation clustering was performed using DAVID tool on the GO terms and 2 clusters were obtained. The first cluster relates to Wnt protein binding, and the second cluster relates to polymerase II core promoter proximal region sequence-specific DNA binding. The enriched pathways annotated include calcium signaling, Wnt signaling, cytokine-cytokine receptor interaction, cardiac muscle contraction, mucin type O glycan and other mucin type O glycan (Table 3.3).

Table 3.4: Differentially expressed genes (foldchange from +1.50 or -1.50) in the ileum of 7-day old Ross 308 male chicks in SP group compared to the UP group.

Gene symbol	Entrez Gene Name	Location	Type of molecule	Expr Fold Change	*P*-value
IL22RA2	interleukin 22 receptor subunit alpha 2	Plasma Membrane	transmembrane receptor	+2.77	0.010
CDHR1	cadherin related family member 1	Plasma Membrane	other	+2.34	0.029
TTLL2	tubulin tyrosine ligase like 2	Other	other	+2.16	0.039
ATP8B1	ATPase phospholipid transporting 8B1	Plasma Membrane	transporter	+2.12	3.35E-5
IL20RA	interleukin 20 receptor subunit alpha	Plasma Membrane	transmembrane receptor	+1.92	0.034
ODF2L	outer dense fiber of sperm tails 2 like	Cytoplasm	other	+1.86	0.036
NOXO1	NADPH oxidase organizer 1	Plasma Membrane	other	+1.85	0.023
mir-27	microRNA 27a	Cytoplasm	microRNA	+1.81	0.004
IL26	interleukin 26	Extracellular Space	cytokine	+1.77	0.019
ITGBL1	integrin subunit beta like 1	Extracellular Space	other	+1.74	0.042

mir-23	microRNA 23a	Cytoplasm	microRNA	+1.69	0.029
ME1	malic enzyme 1	Cytoplasm	enzyme	+1.65	0.008
CCL17	C-C motif chemokine ligand 17	Extracellular Space	cytokine	+1.63	0.026
PCNX2	Pecanex 2	Other	other	+1.63	0.002
ZPLD1	zona pellucida like domain containing 1	Other	other	+1.59	0.022
SMOC2	SPARC related modular calcium binding 2	Extracellular Space	other	+1.58	0.015
MFAP5	microfibril associated protein 5	Extracellular Space	other	+1.58	0.039
HPGDS	hematopoietic prostaglandin D synthase	Cytoplasm	enzyme	+1.54	0.026
SHISAL1	shisa like 1	Other	other	+1.54	0.016

SLC38A4	solute carrier family 38-member 4	Plasma Membrane	transporter	+1.52	0.017
GSTA3	glutathione S-transferase alpha 3	Cytoplasm	enzyme	+1.51	0.002
WNT7B	Wnt family member 7B	Extracellular Space	other	+1.50	0.036
DDX60	DExD/H-box helicase 60	Cytoplasm	enzyme	-1.57	0.040
COL17A1	collagen type XVII alpha 1 chain	Extracellular Space	other	-1.65	0.044
WASF1	WASP family member 1	Nucleus	other	-1.88	0.003
LRFN5	leucine rich repeat and fibronectin type III domain containing 5	Nucleus	other	-1.92	0.006
CPO	carboxypeptidase O	Plasma Membrane	enzyme	-2.13	0.024
CA7	carbonic anhydrase 7	Cytoplasm	enzyme	-2.42	0.047
SLC34A2	solute carrier family 34-member 2	Plasma Membrane	transporter	-3.62	0.002

Table 3.5: Enriched Pathway implicated by bodyweight differences in SP and UP chicks.

Pathways	No of genes	%	P- value	DEGs involved
Calcium signalling pathway	9	4.6	5.9E-3	HTR2A, ADCY1, CACNA1C, CCKAR, GDNF, NOS2, PPIF, RET, TACR2
Wnt Signalling pathway	6	3.1	3.6E-2	CTBP2, WNT7B, FZD1, ROR2, SFRP1, SERPINF1
Cytokine-cytokine receptor interaction	7	3.6	1.5E-2	LOC418668, IL1RAP, IL20RA, IL4R, IL8L1, TNFRSF1B
Cardiac muscle contraction	4	2.1	4.5E-2	CACNB4, CACNA1C, SLC9A7, UQCR10
Mucin type O-Glycan biosynthesis	3	1.5	6.0E-2	ST3GAL1, GALNT15, WBSCR17
Other types of O-glycan biosynthesis	3	1.5	1.0E-1	WBSCR17, GALNT15, POGLUT1

SP: Super performers, UP: Under performers, DEG: Differentially expressed genes.

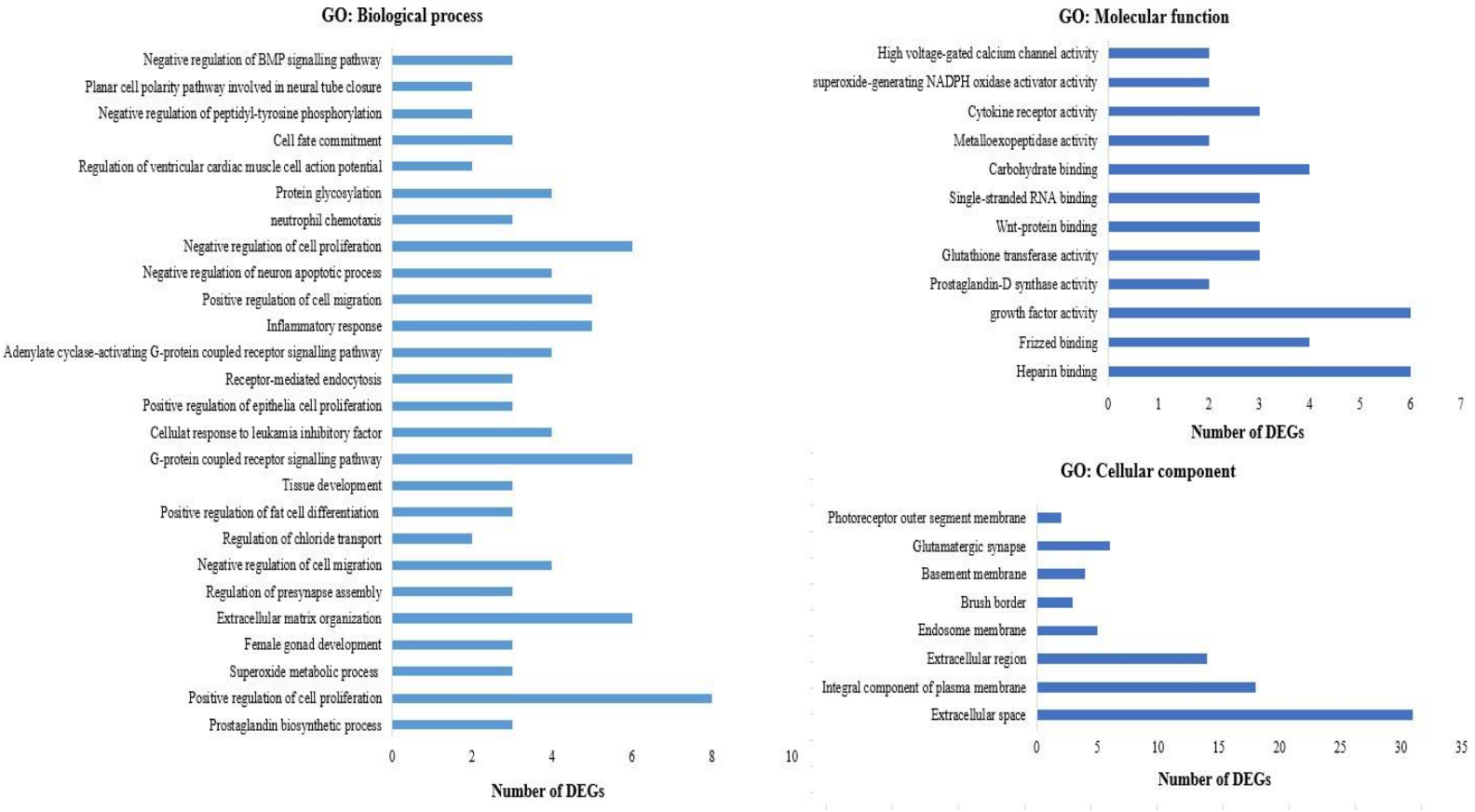


Figure 3.2: Functional annotation of the ileal DEGs in 7days old Ross 308 chicks (SP relative to UP

SP denotes Super performer and UP denotes Under performers. The higher the number of DEGs in each process, the more implicated the process will be in the SP group relative to the UP group.

3.4.4 Validation of the microarray result using qRT-PCR

To validate the ileal DEGs of between the SP and UP chicks, the relative expression levels of 4 selected DEGs were quantified using the qRT-PCR method. The microarray result showed that the four selected genes were all upregulated in the SP chicks relative to the UP. Three out of the four genes had been confirmed by qRT-PCR, showing a similar trend of being upregulated in the SP group relative to the UP (Table 3.6). Thereby confirming the reliability of the microarray data and supporting the observation that the SP chicks exhibited higher expression level of the IL8L1, CCL17, and GSTA3 genes.

Table 3.6: Normalised relative expression of the target genes from the list of DEGs

Target genes	Normalized relative expression		*P-value*
-	SP	UP	-
CCL17	1.35	1.00	0.586
GSTA3	1.06	1.00	0.912
IL20RA	0.16	1.00	0.234
IL8L1	3.06	0.44	0.024

Table 3.7: Standard curve analysis for housekeeping genes and gene of interest

Housekeeping Genes	**Efficiency value (%)**
FAB5	101.0
FAM1	108.8
GALN5	88.3
GAPDH	100.0
Gene of interest	-
CCL17	91.9
GSTA3	82.3
IL20RA	96.5
IL8L1	95.1

3.5 Discussion

Broiler chicks exhibit considerable variation in bodyweight (BW) performance despite the successive selective inbreeding and stringent management practices, impacting flock uniformity. While there is an abundance of literature investigating improvement in growth performance, the basis for variation in bodyweight has received less attention. Therefore, the present study explored various physiological and transcriptomic aspects in understanding the important drivers of variation in bodyweight in the early life of the broiler chick. As expected, the SP chicks had heavier organs when compared to the UP group. Published research reported that the weight contribution of internal organs to bodyweight reflects the health condition of the animals (Smith et al., 2011). It was also reported that the size of the visceral organs may influence energy requirements for basal metabolism as it relates to feed intake (Fitzsimons et al., 2014). Thus, in the present study, the SP chicks exhibited heavier liver, and intestinal weight with longer intestines compared to the UP chicks, indicating that these observed differences in the digestive organ, are related to BW and possibly feed intake. The significant difference observed in this study in gizzard weight relative to body weight of the UP chicks disagreed with the report of (Ribeiro, et al., 2004) who reported no significant effect of body weight on the relative weight of the gizzard of Ross 308 chicks on day 7. The gizzard acts as a pacemaker of normal gut motility (Ravindra, et al 2021), stimulating the mixing of digesta with enzymes and nutrient digestion. In the present study, it may be suggested that heavier gizzard in proportion to bodyweight observed in the UP chicks may not be associated with increased gizzard stimulation and functionality.

Bone ash has been used to assess the skeletal mineralization in poultry production (Hall et al., 2003), The percentage of bone ash in poultry is a general indicator of bone mineralization (Thorp and Waddington, 1997). High bone ash and mineralization correlates to stronger bone and ability of the skeleton to withstand gravity and additional loading (Shim, et al., 2012). One of the primary bone minerals (calcium) showed no significant difference between the two groups, although the calcium concentration was

numerically higher in the SP compared to the UP group. Interestingly, there was a significant increase in bone phosphorous concentration in the SP chicks compared to the UP group, this significant increase in bone phosphorus concentration in the SP chicks may be linked to the wnt signaling pathway which was enriched in the SP relative to the UP group.

Wnt signaling had been reported to be associated with calcium and phosphorus absorption in broilers (Wang, et al., 2022). Wnt signaling cascade had also been reported to play a central part in regulating the development of calcium signaling pathway (Lu and Carson, 2009). It is also noteworthy that the calcium signaling pathway which was one of the most enriched pathways was identified in the SP group relative to the UP. Taken together, these pathways identified in the SP group could be linked to the higher concentration of bone phosphorus and calcium in the SP group, thus having impact on absorption of these two important minerals in the SP group relative to the UP group.

Minerals of physiological importance including toxic metals can bioaccumulate in calcified tissues such as teeth and bones (Rasmusson and Eriksson 2001), and 80% of the bioaccumulation results from dietary intake (Baykov et al., 1996; Orzechowska et al., 2010). The UP group had significantly higher concentration of cadmium (Cd), caesium (Cs) and lead (Pb) in their bones compared to the SP group. The increase in the concentration of these minerals in the UP group is interesting and merits further mechanistic investigation. For example, the higher bone Cd concentration may be linked to the decrease in phosphorus concentration in this group, as it was reported that when cadmium accumulates in the body, it causes damage to the kidney which in turns inhibits the activity of vitamin D, thus preventing the calcination and storage of phosphorus in the bone (Youness, et al., 2012).

The exploratory ileal transcriptomic profiling of 7days old Ross 308 chicks was aimed at identifying the potential candidate genes and pathways associated with variability in growth performance of 7-day old chicks. The concept of the present study benefited from the sampling of chicks from the same breed population maintained under the same environmental and diet conditions. The functional annotation of

the differentially expressed genes (DEGs) performed to elucidate the biological implication of these genes reported interesting observations which may be associated with the differences in the growth rate of these chicks. In the current study, it was noticed an upregulation of the IGF gene (IGF-1) in the SP group relative to the UP which modulates the growth-promoting effect of growth hormones (Wang, et al., 2003). IGF-1 is among the members of the insulin-like growth factor family which regulates cell growth, and proliferation and plays a distinct role in lean meat content during the growth of dairy cattle (Mullen, et al., 2011). IGF-1 is an important gene controlling body size (Wang, et al., 2004). It has been reported that the signal transduction commenced from the binding of GH to its receptor which leads to the activation of specific gene coding insulin like growth factor 1 (IGF-1) and is released into circulation to bind to its specific receptor known as the IGF type-1 receptor which then stimulates cell proliferation (Okumura and Kita, 1999). The up-regulation of the IGF-1 gene in the SP chicks relative to UP chicks could be associated with their higher in body weight, as this gene is wholly involved in growth and controlling body size (Wang, et al., 2004).

There was an up-regulation in the expression of genes acting as immune mediators including pro-inflammatory cytokines and chemokines such as Interleukin 8 like 1 (IL8L1) in the SP compared to the UP group. Interleukin 8 Like 1 (IL8L1) has been reported to be involved in the recruitment of heterophils to the site of infection in the chicken intestine (Kogut., 1994 & 2002) and these heterophils are pivotal in activating the innate immune response (Genovese, 2000). Based on the reported literature (Swaggerty, et al., 2005., Bar-Shira, and Fridman., 2006., Terada, et al., 2018).

It may be speculated that the upregulations of these proinflammatory and chemokine genes in the ileum of the experimental chicks may play distinct roles in innate host defense triggered by exposure to feed and microorganism during the first week of life. It has been reported that young hatchlings respond to environmental stimuli by gradual development of pro inflammatory functions (Withanage, et al., 2004; Bar-Shira and Friedman, 2006). The immune protection of hatchlings

could emanate from maternal antibodies which are active systemically and in the gut cavity and innate effector mechanisms which are active alongside all mucosa linings (Bar-Shira and Fridman, 2006).

Another interesting cytokine that was upregulated in the SP chicks in the present study is Interleukin 26 (IL26). Interleukin 26 is a member of the IL-10 cytokine family which plays a role in the local mechanism of mucosal immunity and induces the expression of IL8 (Ouyang and O'Garra, et al., 2019). It has also been reported that the IL26 activates the immune-related pathways such as JAK/STAT, NF-kB, and MAPK signalling pathways, crosstalk between these pathways may modulate the expression of chemokines and cytokines in chicken cell lines (Truong, et al., 2017). Also, the JAK/STAT pathway is crucial to T cell differentiation, B cell maturation, and development, secretion of SIgA, mucus, and antibody production which are pivotal to maintaining antiviral and anti-bacterial defense at the mucosal surface (Heneghan, et al.,2013). Based on this report, the up regulation of IL26 and chemokine (IL8L1), may suggest that the SP chicks could be more advantaged in terms of innate preparedness of the gut for development and strong defense against enteric pathogens.

In addition to the increased expression of important pro-inflammatory cytokines genes involved in immune response, in the SP group, we observed an increase in the expression of glutathione S-transferase alpha (GSTA3), which is an antioxidant enzyme specifically involved in the clearance of various peroxidation products (Anyia and Imaizumi, 2011). The increase in the expression of the GSTs (GSTA3) and their activities in the SP chicks compared to UP chicks may positively affect glutathione metabolism and metabolism of xenobiotics by cytochrome P450. The chicken intestine is known to be the primary site of exposure to dietary xenobiotics, which are potential toxins and may promote the proliferation of cellular free radicals (Wang, et al., 2019). Thus, it may be speculated that the observed increase in expression of the GSTs genes in the SP group may play a strong role in the detoxification of xenobiotic toxins and reduction in oxidative stress compared to the UP chicks. This may also be attributed to the speculated higher feed intake in the SP chicks, as a result, SP

group may be exposed to higher rate of xenobiotics, thus higher expression of the GST genes to combat this. It is also noteworthy that in the present study there was an upregulation of micro RNAs such as MicroRNA 23, 25, 27 and 7 (Mir-23, Mir-25, Mir-27, and Mir-7), in the SP relative to UP group. MicroRNAs (MiRNAs) are a class of endogenous non-coding RNA, comprising about 22 nucleotides (Bartel, 2004) which are known to play a crucial role in the regulation of gene expression at the post-transcriptional level. They act by binding complementary sequences on messenger RNA target genes, thereby causing cleavage or repressing translation (Bartel, 2004). Mir-27 is known to regulate the expression of NFE2L2 (a transcriptional factor that modulates gene transcription of an antioxidant response element), and an increase in the expression level of NFE2L2 is associated with oxidative stress (Zaccaria, et al., 2017). An increase in the expression level of Mir-27 has been reported to downregulate mRNAs coding for NFE2L2 and in turn reduce oxidative stress markers in an in-vitro study involving Human keratinocyte cell lines (HaCat cells) (Zaccaria, et al 2017). There was an upregulation of Mir-27 and downregulation of the NFE2L2 gene in the SP group relative to the UP group, this may agree with the study of Zaccaria, et al. (2017), who reported an increased expression level of Mir-27 which consequently led to a decrease in the expression level of NFE2L2 in an in-vitro experiment.

The enriched pathways annotated by DAVID from the DEGs reported in the SP and UP chicks revealed 6 pathways that could be associated with the differences in bodyweight performance of these chicks, and they involved calcium signalling, Wnt signalling, cytokine-cytokine receptor interaction, cardiac muscle contraction, mucin-type O-glycan biosynthesis, and other O-glycan biosynthesis. Genes involved in the calcium signalling pathway were mostly upregulated in the SP chicks which include HTR2A, ADCY1, CACNA1C, CCKAR, and NOS2. Calcium signalling has been noted to be one of the highly versatile intracellular signals that participates in cell signalling for a wide range of cell processes such as death, cell cycle, division, migration, invasion, metabolism, differentiation, transcription etc. (Pratt, et al., 2020). It also governs intracellular regulation and contributes to long term

physiological response regulation (Pratt, et al., 2020). This important pathway enriched in the SP chicks may be playing a vital role in growth and contributing to the differences observed in the SP and UP groups. Importantly, further studies may be conducted to understand if circulatory levels of calcium serve as a better biomarker in assessing differences in growth rates in broiler chicks.

The second most enriched pathway reported in this study is the Wnt signalling pathway. This pathway had been reported to play a vital role in self-renewal of most tissue in mammals, particularly the development and renewal of small intestinal epithelial tissue and stimulates the differentiation of crypts Paneth cells (Liu, et al., 2022). It is also reported to be linked to liver development, haematopoietic system development and osteoblast maturation (Clevers, 2006: Perugorria, et al., 2019). Wnt signalling also facilitates Ca and P metabolism in broilers (Wang, et al., 2022), thus the enrichment of wnt pathway in the SP group in this study may be linked to the increase in the concentration of bone P in the SP compared to the UP group, as higher concentration of mineral in animal tissues are valuable biomarker of its bioavailability (Wang, 2007).

The significance of the wnt signalling and its implication in the SP chicks in the present study may provide insight into the undying factors contributing to growth and body size differences in these groups of chicks studied. Most of the genes involved in Wnt signalling, cytokine-cytokine receptor interaction, and mucin- type O-glycan biosynthesis was up-regulated in the SP chicks' group. Notably, all genes related to mucin-type O-glycan biosynthesis were upregulated in the SP group, which includes ST3GAL1, GALNT15, and WBSCR17. It has been demonstrated that mucin-type O-glycans are pivotal in establishing whether host diseases will be averted or promoted concerning interactions with microbes present in the environment (Bergstrom and Xia, 2013). Mucins are the main component of mucus which are secreted by the goblet cells and form a protective homeostatic barrier between resident microbiota and the underlying immune cells (Johansson, et al., 2008; Struwe, et al., 2015). It has been reported that homeostasis of gut bacteria in chicken can be implicated by mucin types, O-glycan composition, i.e., the

extent of glycosylation and oligomerization of mucin and mucus layer characteristics (Derrien, et al., 2010). Having the mucin type O-glycan pathway activated in the SP group may suggest implications which include a higher level of mucin glycosylation which may enable mucins to function as a protective barrier. The SP chicks exhibited superior bodyweight which correlates to high feed intake. As a result, this may prompt us to speculate that the SP group consumed more feed post-hatch compared to the UP group well enough to support the development of the intestinal epithelium including enterocytes and goblet cells which may drive the gut barrier function. Immediate access to feed by hatchlings has been reported to support intestinal epithelium development including goblet cells and enterocytes for more efficient barrier function (Duangnumsawang, et al., 2021). Mucus production is very important in young chicks for gut protection as they still have developing immune system (Duangnumsawang, et al., 2021), and for assimilation of metal ions in their available form in the intestine (Powell, et al., 1999).

3.6 Conclusion

The present study revealed differences in the digestive organ weights, bone ash and mineral concentrations in 7-day old Ross 308 chicks with distinct bodyweights. The SP chicks had higher bone ash and bone P concentration which may be linked to the enriched Wnt signalling pathway in this group relative to the UP group. The increase in bone Cd, Pb and Cs in the UP group, merits further mechanistic investigation, to ascertain the possible drivers of the accumulation. The transcriptomic analysis revealed the up regulation of cytokines and chemokine genes, GSTs, and Mir genes, together with the implication of Ca signalling and Wnt signalling pathways in the SP group relative to the UP group, which may be influenced by bodyweight differences. From the observed heavier relative gizzard weight, higher concentration of Cd, Pb and Cs in the UP group, the next study was conducted to evaluate the mineral profiles of the bone and another metabolic tissue (liver), gut pH and bone morphometric parameters in the low and high weight groups for more insights.

CHAPTER FOUR

Trial 2

4.0 Differences in broiler bone, gut, and tissue mineral parameters, as influenced by broilers grouped based on bodyweight.

Published in Animal Production Science 64 (https://doi.org/10.1071/AN23270)

4.1 Abstract

Variation in bodyweight is an undesirable feature in broiler production. Compositional differences between high- and low-bodyweight (BW) chicks in bone parameters and tissue mineral concentrations may provide insight into underlying causes of variation in BW. This study aimed to investigate differences in bone measurements, tissue mineral concentrations, and gut parameters of Ross 308 male broiler chicks with identical diet and environmental conditions, but with distinct BW on Day 21 (D21). A 3-week growth study was conducted involving 40 male, day-old chicks from the Ross 308 line. Chicks were reared in a deep-litter house with a controlled environment and the same commercial diet. On D21, BW data collected from chicks were used as a criterion to rank them into high- and low-BW groups (n = 11/group). Retrospective BW measurements were compared between groups. Birds were selected for assessing bone parameters, liver mineral profile, gut pH, gizzard neutral detergent fibre (NDF) and acid detergent fibre (ADF) contents**.** Retrospective BW measurements among the high- and low- BW groups showed a consistent difference in BW between the two groups in early life. Tibial concentrations of manganese and strontium were significantly ($P < 0.05$) higher in the low-weight (LW) group relative to the high-weight (HW) group. Concentrations of manganese, cadmium and caesium in the liver tissue showed significant differences, with the LW group having higher concentration of these trace elements. The LW chicks had lower gizzard digesta pH, higher gizzard NDF and a statistical tendency for higher ADF concentrations compared to the HW group. In summary, broilers ranked based on D21 BW showed differences in tibial bone, gut, and tissue mineral parameters. The LW group had lower gizzard pH and higher gizzard fibre content than did the HW group, which may be attributed to factors such

as behavioral activities relating to more litter consumption among the LW group than the HW group.

Keywords: animal production, bone strength, broilers, growth, minerals, uniformity, variation.

4.2 Introduction

Bodyweight (BW) uniformity is an important consideration in broiler production (Molenaar et al. 2008; Jin et al. 2019) as poor BW gain in young broilers is a critical factor underpinning morbidity, mortality, and welfare (Noy and Pinchasov 1993; Noy and Sklan 1997; Chen et al. 2013). The growth of contemporary meat-type chickens has been improved over decades through genetic selection, improved husbandry, and nutritional and health management (Zuidhof et al. 2017). However, significant variation in BW has been reported (Chen et al. 2013; Montanhini Neto et al. 2013; Vasdal et al. 2019; Lundberg et al. 2021), which may lead to carcass devaluation and economic loss at the end of broiler production (Lundberg et al. 2021). Different strategies and feeding managements to improve the growth performance and uniformity of broilers have been investigated and excellent reviews are available (Ipek et al. 2009; Islam et al. 2012; Wang et al. 2015; Zuidhof et al. 2017; Gous 2018; Jha et al. 2019). However, such dietary and management strategies have investigated the physiological and genomic responses of treatments on groups of broiler chicks, without accounting for their individual differences and responses.

The heterogenicity of the experimental test population may limit the success of nutritional interventions and hinder an understanding of the underlying mechanism. Many factors affect broiler growth in early life, including genetics, age of parent breeders, environmental conditions, nutrition, and management practices, which, consequently, may contribute to variation in BW of broilers (Lundberg et al. 2021).

In a previous study, some of these factors such as diet, sex, breeder flock age and environmental conditions were controlled to understand the inherent variation that arises in a flock of Ross 308 male chicks (Elvis-Chikwem et al. 2020). In that study under highly controlled experimental conditions, a uniformity score of 56% was noted, which was considerably lower than the recommended uniformity score of 80–85% in broilers (Toudic 2007).

Mineral metabolism is a critical factor in broiler growth performance because some minerals play major roles as catalysts in many enzyme and hormone systems (Suttle 2010). Minerals support growth, skeletal integrity, and other physiological processes, with mineral homeostasis being regulated by the balance between tissue mineral storage and excretion. Tissue mineral concentration has been a tool reported by researchers (Sunder et al. 2006: Wang et al. 2007; Yair and Uni 2011) to assess bioavailability, physiological mineral utilization, and storage, especially in the bone and liver tissues. The rapid absorption of minerals, especially Ca, in hatchlings (Skinner and Waldroup 1995; Ravindran and Abdollahi 2021), to meet the developing skeletal need, makes it a dynamic and potentially valuable biomarker to ascertain the mineral status of chicks' post- hatch. Gut pH has been used to assess gut health functionality and the solubility of minerals such as Cu and Ca (Shafey et al. 1991; Maenz et al. 1999; Dibner and Buttin 2002; Ferket 2004; Loddi et al. 2004; Pang and Applegate 2007; Morgan et al. 2014). A healthy gut is vital in enhancing the productivity of broiler chicks and facilitates nutrient uptake by absorptive cells, greater nutrient digestibility, and its bioavailability (Lewis et al. 2003; Niewold 2007). This study aimed at understanding the differences in selected bone parameters, liver mineral profile and gut parameters of low BW and high-BW chicks in the early weeks of life. Therefore, the hypothesis of the current trial was that chicks of same breed with varying day 21 bodyweight that exhibited differences in bone mineral profile and ileal gene expression may be characterised with variable bone morphometrics, gut pH, bone and liver mineral concentrations.

4.3 Materials and methods

4.3.1 Ethical approval and location of the experiment

The current study was conducted at the poultry research unit of the Nottingham Trent University, Brackenhurst campus, and the laboratory analysis was conducted at the University of Nottingham, Sutton Bonington Campus. All experimental protocols used in the animal study were approved by Nottingham Trent University's Animal Ethics Review Committee (Code: ARE192024), and the University of Nottingham's Animal Welfare and Ethical Review Body (Approval reference number: 255).

4.3.2 Birds, housing, and diets

In total, 40-day-old male Ross 308 broiler chickens were used in this experiment. Chicks were individually weighed then randomly allocated to one of four deep-litter pens, each containing 10 chicks. Each pen was equipped with feeders and nipple drinkers until Day 21 (D21), the lighting protocol started at 23 h on D1, with darkness increasing by 1 h/day until 6 h of darkness was established. The chicks were fed a common commercial starter mash diet from D1 till D14 and were fed a grower diet until 21 days of age *ad libitum*. The starter and grower diets used in the present study are presented in Tables 4.1A and 4.1B below; water was also provided *ad libitum* via nipple drinkers. The temperature of the deep litter house was set to 31°C on D0 and gradually decreased over the course of a 21-day period to 22°C, while pine shavings were used as litter substrate. Birds were individually weighed each week up to D21.

Table 4.1A: Ingredient composition and calculated nutrient composition of commercial starter diet (Fed from D0-D14)

Ingredients	%
Wheat	40.77
Soyabean meal	29.00
Oats	15.00
Maize	10.00
Limestone flour	0.88
Dicalcium phosphate (18%)	1.17
Soya oil	0.80
Salt	0.23
Sodium bicarbonate	0.10
Liquid lysine 50(T)	0.61
Methionine H-A liquid	0.51
Soya oil spray	0.40
Vitamin premix[1]	0.35
L-Threonine	0.15
Ronozyme liquid 35.7%[2]	0.03
Total	100
Calculated components	
Metabolisable energy MJ/Kg	12.56
Dry matter	87.72
Moisture	12.28
Crude protein	21.81
Crude Ash	5.12
Crude fat	3.74
Crude fiber	3.92
Total Calcium (g/kg)	0.78
Available Phosphorus (g/kg)	0.58

[1] Vitamin/mineral premix supplied per kg diet: Selenium: 0.25mg, Iron: 50mg, Manganese: 120mg, Molybdenum: 1mg, Vitamin A: 12,00iu, Vitamin D: 2500iu, HyD: 2500iu, Vitamin E: 100iu, Vitamin K: 5mg, Vitamin B1: 3mg, Vitamin B2: 8mg, Vitamin B6: 6mg, Vitamin B12: 30ug, Iodine: 2mg, Folic: 2mg, Nicotinic: 70mg, Cal-D-Pant: 18mg, Biotin: 0.3mg, Choline: 250mg, Copper: 20mg, Zinc: 100mg

[2] Ronozyme WX: 100mg, Ronozyme HiPhos: 100mg, Ronozyme ProAct: 200mg, Maxiban: 625mg, CRINA poultry plus: 300mg, Aresto: 25mg.

Table 4.1B: Ingredient composition and calculated nutrient composition of commercial grower diet (Fed from D15-D21)

Composition	Quantity	Units
Wheat	46.50	%
Soya bean meal (Dehulled)	34.50	%
Rapeseed (Whole)	6.60	%
Maize distillers' grain	4.76	%
Soya bean oil	3.45	%
Calcium Carbonate	0.85	%
Mono Dicalcium Phosphate	1.85	%
Sodium Chloride	0.30	%
Lysine-HCL	0.39	%
Methionine-DL	0.30	%
Premix	0.50	%
Total	100	%
Analytical Constituents		Units
Crude fat	5.02	%
Crude protein	21.27	%
Crude fibre	2.78	%
Crude ash	4.74	%
Methionine	0.65	%
Sodium	0.13	%
Lysine	1.32	%
Total phosphate	0.44	%
Calcium	0.64	%
Vitamin A	8	MIU/T
Vitamin D3	5	MIU/T

4.3.3 Bird grouping and sample collection

On D21, all chicks were weighed individually and then the heaviest (n = 11) and lightest (n = 11) chicks from across all pens were selected for euthanasia as high-BW (HW) and low-BW (LW) chicks respectively. Birds were euthanized by cervical dislocation and immediately after euthanasia, the crop, gizzard, and small intestine were dissected and collected from individual

chick of each BW group, and liver, gizzard, and tibial bones were collected for further analysis and stored at −80°C.

4.3.4 Bone morphometric measurements and bone breaking analysis.

Tibial weights were recorded using a precision balance (Ohaus Spu6001) and the tibial length and width were measured using a digital caliper as shown in Figure 4.1. Bone breaking strength was analyzed using a texture analyzer TA.XT Plus 100 (Stable Microsystems, Guildford, UK), with a 50 kg load cell set up and 3-point bend fixture (Alkhtib et al. 2020), which generated the maximum force (N) value for each sample.

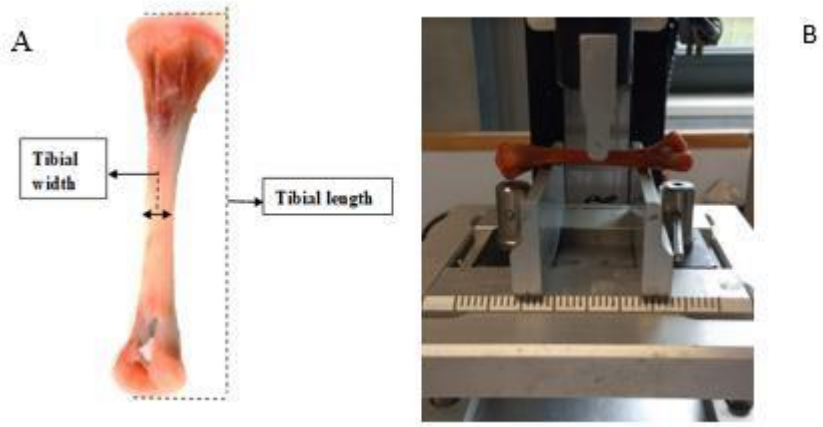


Figure 4.1: (A) Tibia bone showing points of morphometric criteria, width, and length measurements (B) Bone breaking test using TA. XT plus 100 machine

Tibia bone was placed horizontally on two support holders and submitted to a vertical force from above.

4.3.4 Crude ash and mineral analysis

The legs collected and stored at −80°C were allowed to thaw at room temperature and were subsequently defleshed to extract the tibial bones. The extracted tibias of each bird were collected for estimation of percentage ash on a fat-free dry basis. Tibias were defatted by soaking in petroleum ether for 2 h and then allowed to dry in a fume cupboard to expel petroleum ether residues. The defatted tibial bones were oven-dried at 105°C for 24 h to achieve a constant weight and ashed at 600°C overnight to determine the tibial ash concentration. Tibial bone ash was acid digested using the following hot plate method for sample preparation. A maximum of 0.2 g of each sample was digested with 10 mL of hydrogen peroxide and heated for 2 h at 95°C in the fume cupboard. A solution of 50 mL of MilliQ water was added to each tube after digestion and 8 mL were taken from the top into 8 mL tubes. Digested samples were diluted to 1/10, and mineral concentration was analysed using an inductively coupled plasma–mass spectrometer (ICP–MS, Thermo-Fisher Scientific iCAP-Q, Thermo Fisher Scientific, Bremen, Germany).

4.3.5 Liver sample digestion and mineral analysis

The liver mineral concentration was determined using an ICP–MS method. The liver samples were freeze-dried using a freeze drier (Thermo Savant SuperModulyo) at the temperature setting of −45°C for 1 week prior to digestion. Approximately 0.2 g of the freeze-dried samples were weighed into the digestion vessels and the weight was recorded. Each of the samples was digested using 3 mL of nitric acid, 3 mL MilliQ water and 2 mL of hydrogen peroxide in the fume cupboard. The digestion tubes containing the samples were positioned in the microwave rotor for 45 min to obtain complete digestion. The liquid was decanted into universal containers and digestion tubes were rinsed with 7 mL of MilliQ water, which was decanted back to the labelled universal tubes ready for ICP–MS analysis. Samples were diluted to 1/10 into the ICP tubes and analysed using the ICP–MS (Thermo-Fisher Scientific iCAP-Q; Thermo Fisher Scientific, Bremen, Germany) for mineral concentrations.

4.3.6 Statistical analyses

Birds were grouped based on 21-day BW. The individual broiler served as the experimental unit. Descriptive statistics of the BW data were analysed using SPSS software ver. 21 (IBM Corp. Released 2012. IBM SPSS Statistics for Windows, Version 21.0. Armonk, NY: IBM Corp). The Shapiro–Wilk test of the GraphPad Prism 9.0 software was used to assess the normality of data and then the bone mineral profile, liver mineral and gut pH data were analysed using Student t-test of the GraphPad Prism 9.0 software (GraphPad Prism version 9.0.0 for Windows, GraphPad Software, San Diego, California USA, www.graphpad.com), with BW as the main factor. Differences were considered statistically significant at 0.05 level of probability.

4.4 Results

4.4.1 Growth performance

Descriptive statistics of the 39 broiler chicks is shown in Table 4.2, while the histogram graph showing bodyweight distribution of all chicks is presented in Fig. 4.2. The experimental chicks had a low BW from D0 till D21, compared with the suggested breed BW target on D0, D7, D14 and D21. However, their low BW was not an indication of apparent poor health status as they were all healthy and thrived with only one mortality recorded throughout the experimental period. The BW of chicks was noted on D0, with mean BW of 37.9 (±6.22 g; percentage uniformity), D7 with mean BW of 138 (±20.32), D14 with mean BW of 369 (±49.08) and D21 with mean BW of 887 (±118.16). The associations between chick BWs on D0 and D7, D14 and D21 are presented in Fig. 5.3. The BW performance and BW changes during the experimental periods between the high- and low-BW groups are shown in Table 4.3 and Fig. 4.4 respectively. The mean BWs of the high- and low-weight chicks on D7 and D21 were 155g versus 122g and 1020g versus 746g respectively.

Table 4.2: Descriptive statistics of weekly bodyweight performance of 39 male Ross 305 broiler chicks

Age	Mean bodyweight (g)	Min	Max	SD	CV (%)
D0	37.9	29.5	49.6	6.2	16.4
D7	138	100	188	20.3	14.7
D14	369	273	499	49.1	13.3
D21	887	655	1159	118	13.3

D0: Day 0, D7: day 7, D14: day 14, D21: day 21, Min: minimum, Max: maximum, SD: standard deviation, CV: coefficient of variation

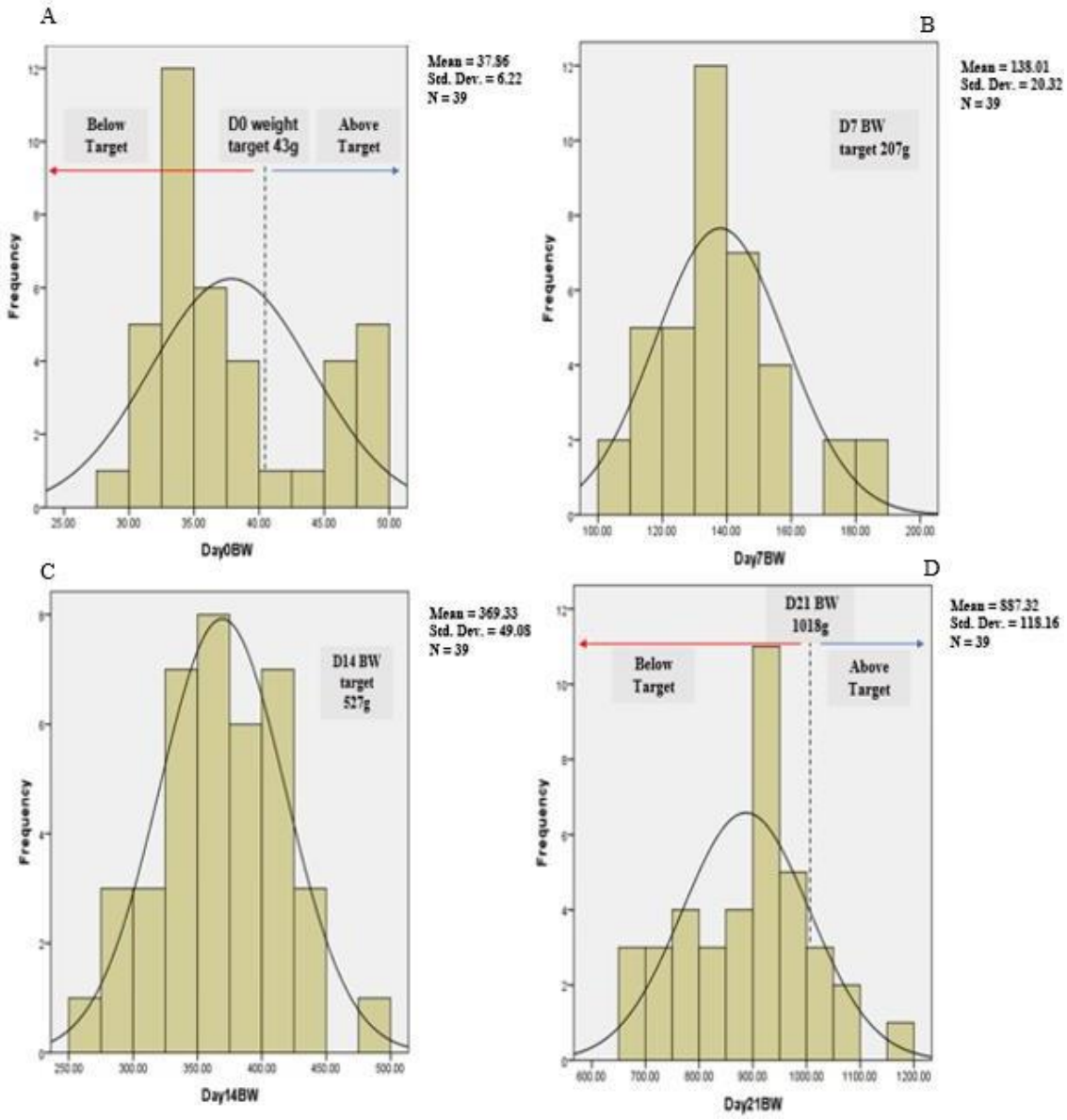


Figure 4.2: Histograms showing bodyweight distribution of 39 Ross 308 male broiler chicks on (A) D0, (B) D 7 (C) D14 and (D) D21, (n= 39)

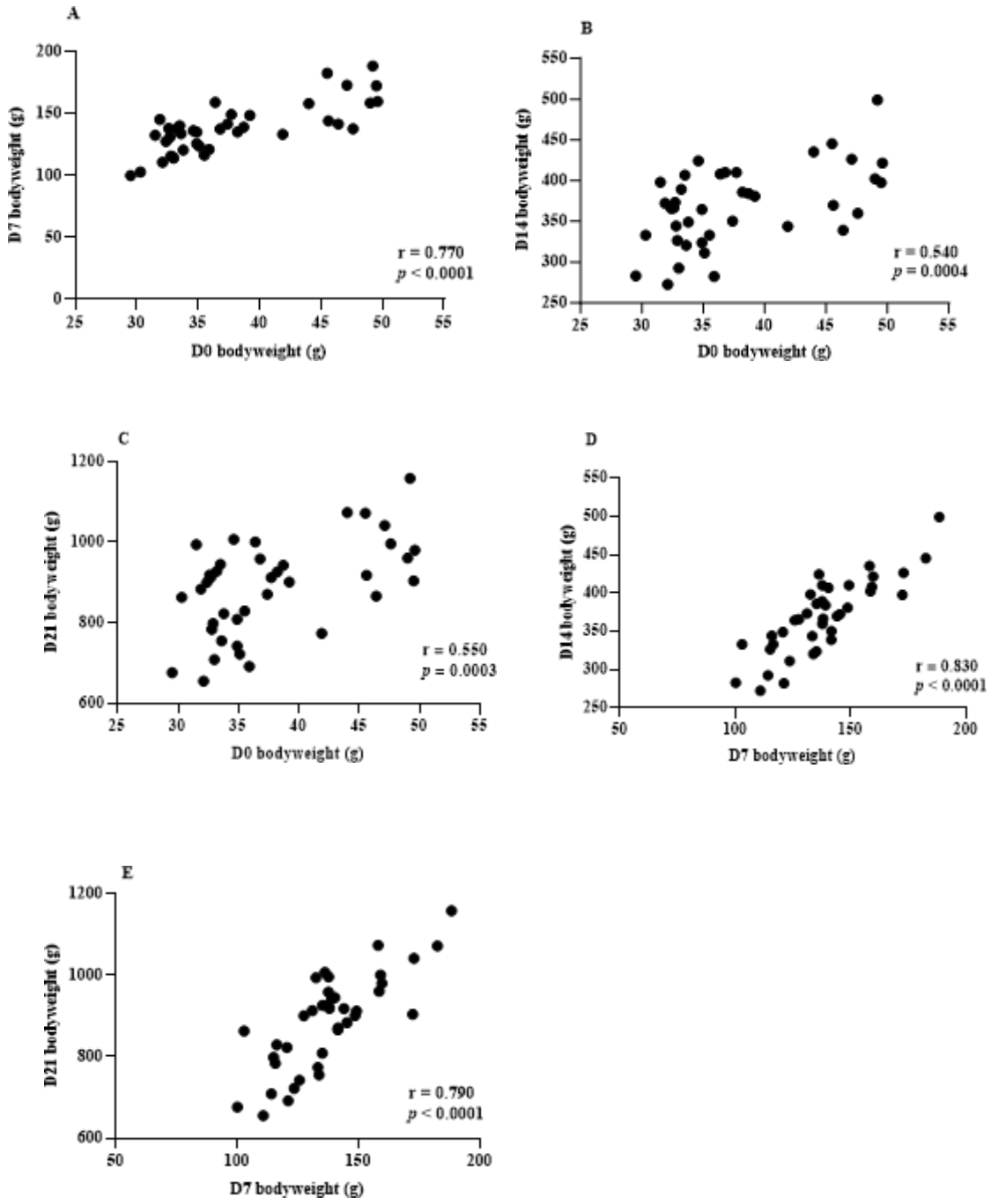


Figure 4.3: Association between bodyweight performance of the experimental chicks.

(A) Day 0 and D7 BW, (B) D0 and D 14 (C) D0 and D21, (D) D 7 and D14, (E) D7 and D21, (n=39).

Table 4.3: Average bodyweights of HW and LW male Ross 308 Chicks at different stages in starter phase

Age (Days)	HW BW (g)	LW BW (g)	SEM	P value
D0	41	35	2.52	0.025
D7	155	122	6.96	.0001
D14	421	318	13.8	<.0001
D21	1021	743	26.19	<.0001
ADWG (0-7)	16	12	0.8	<.0001
ADWG (7-14)	38	28	1.5	<.0001
ADWG (14-21)	85	61	2.5	<.0001

Footnote: HW: High weight; LW: low weight; BW: bodyweight; ADWG: Average daily weight gain; TWG: total weight gain, (n=11/group)

4.4.2 Gastrointestinal pH and gizzard fibre content

The gastrointestinal pH values of the crop, gizzard and ileum and the gizzard fibre content are presented in Fig. 4.5. The result of this study showed a significant ($P < 0.05$) difference between the HW and LW chicks in the gizzard digesta pH; the mean gizzard pH of the low- and high-BW chicks was 2.88 and 3.21 respectively. The ileal and crop pH was not different between the BW groups. There was also a greater ($P < 0.05$) gizzard NDF content of the LW group than the HW group. There was a non-significant trend for a greater ADF concentration ($P < 0.1$) in the low-BW group when compared with the high-BW group.

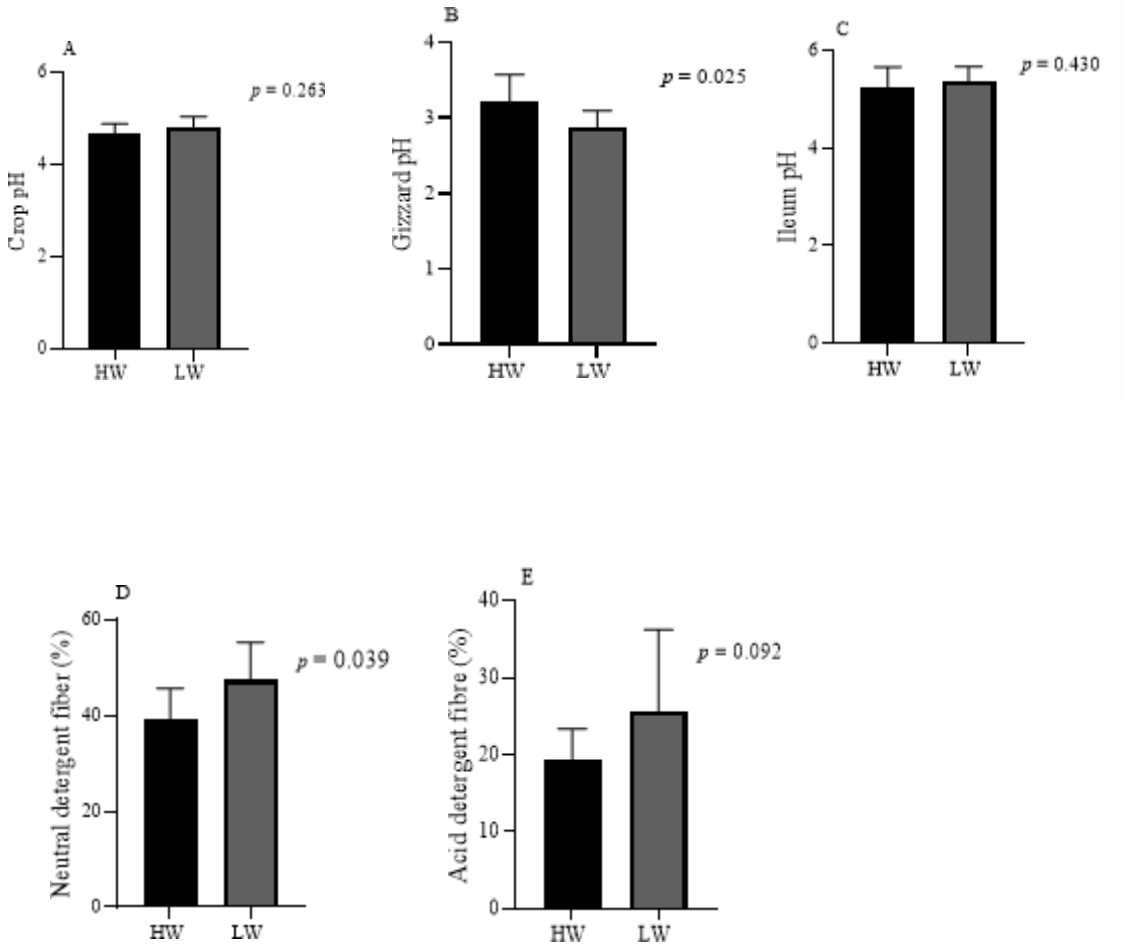


Figure 4.4: pH values of (A) crop, (B) gizzard, (C) ileum, (D) gizzard NDF and (E) gizzard ADF contents of 21d broilers in high or low weight group.

Footnote: HW: High weight; LW: low weight; NDF: neutral detergent fibre from gizzard digesta; ADF: acid detergent fibre (% of freeze-dried material) from gizzard digesta, (n = 11/group)

4.4.3 Liver mineral profile

The macro and trace liver mineral profile of the HW and LW chicks are presented in Figure 4.6 and Table 4.4. The concentrations of manganese (Mn) ($P = 0.018$), caesium (Cs) and cadmium (Cd) were significantly ($P < 0.05$) greater in the low-weight group than in the high-weight group. There was a non-significant tendency for higher liver cobalt (Co) concentrations ($P = 0.052$) in the HW group than in the LW group.

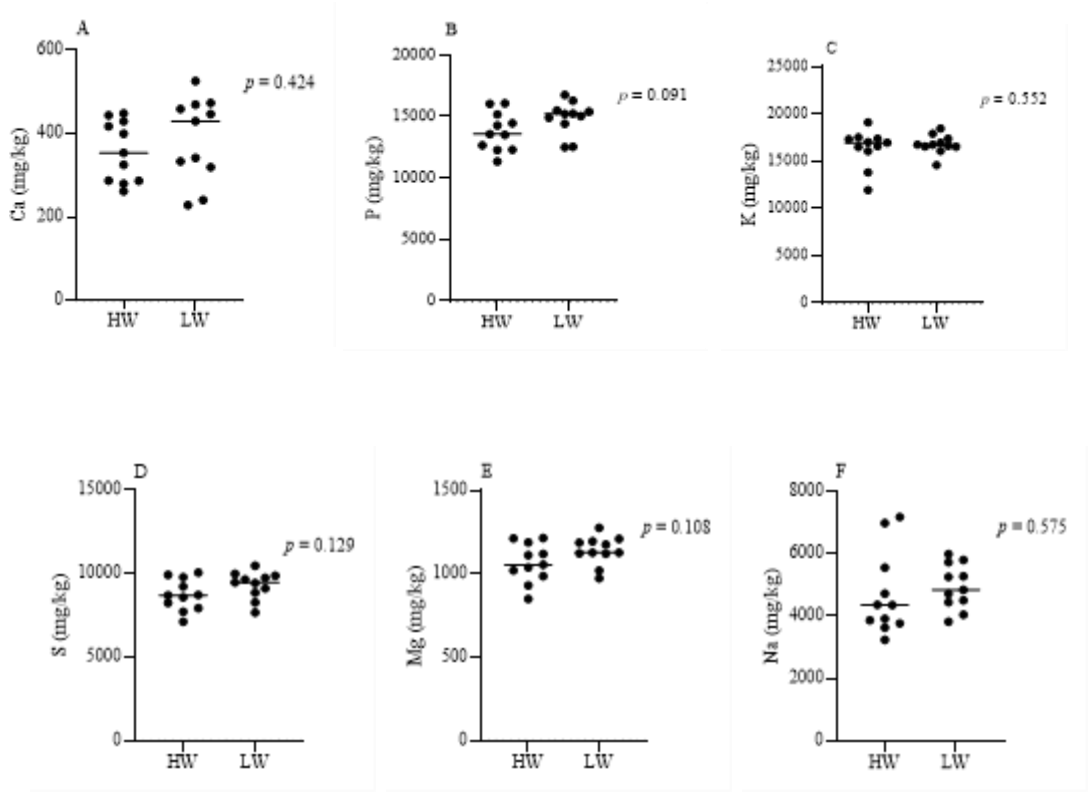


Figure 4.5: Liver macro mineral concentration of high and low bodyweight broiler chicks on D21 in mg/kg ash

(A) calcium, (B) phosphorus (C) potassium, (D) sulphur, (E) magnesium, (F) sodium, HW: High weight;

LW: low weight**,** (n=11/group)

Table 4.4: Liver trace mineral concentration of high and low bodyweight broiler chicks on D21

Minerals (mg/kg)	HW	LW	SEM	P-value
Zn	102	93	±4.95	0.369
Cu	15.1	14.5	±0.81	0.499
Fe	558	614	±97.00	0.570
Mn	15.9	18.1	±0.871	0.018*
Sr	0.3	0.3	±0.030	0.413
Cr	0.1	0.2	±0.073	0.419
Mo	2.6	2.4	±0.162	0.224
Pb	0.0	0.0	±0.003	0.433
Cs	0.1	0.11	±0.005	0.004*
Cd	0.05	0.07	±0.010	0.005*
Co	0.11	0.07	±0.021	0.052
Se	2.63	2.82	±0.134	0.176

(Zn) zinc, (Cu) copper, (Fe) iron, (Mn) manganese, (Sr) strontium, (Cr) chromium, (Mo) molybdenum, (Pb) lead, (Cs) caesium, (Cd) cadmium, (Co) cobalt, (Se) selenium, HW: High weight; LW: low weight. * Denotes significant difference at ≤ 0.05**,** (n=11/group)

4.4.4 Bone morphology and breaking strength

The bone morphometric measurements and breaking strength results are presented in Table 4.5 and the association between the tibial length and weight and width and length are presented in figure 4.7. The HW chicks had a significantly greater bone-breaking strength than did those in the LW group (219 N versus 156 N; $P < 0.05$). Tibial width, length, and weight (g) showed significant ($P < 0.05$) differences between the LW and HW chicks. The HW group had higher values of tibial width, length and weight than did the LW group.

Table 4.5: Bone morphometric parameters of high and low bodyweight broiler chicks on D21

Bone parameters	HW	LW	P-value
Bone strength	219	156	<.0001
Tibial weight	4.7	3.4	<.0001
Tibial length	68	62	<.0001
Tibial width	5.8	5.1	<.0001

HW: High weight; LW: low weight, (n=11/group)

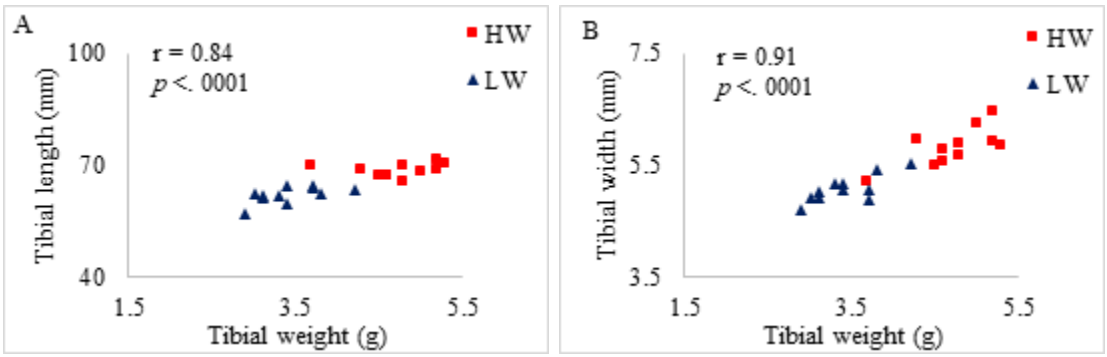


Figure 4.6: Association between tibial length and weight and tibial width and weight

(A) Tibial weight and tibial length, (B) tibial weight and tibial width, HW: High weight; LW: low weight, (n=11/group)

4.4.5 Bone mineral profile

The tibial ash, and macro-mineral concentrations of the HW and LW chicks are presented in Fig. 4.8, and the tibial trace mineral concentrations are shown in Table 4.6, while the heatmap showing the correlation among mineral concentrations in the tibia of the broiler chicks is shown in figure 4.9. The LW group showed significantly ($P = 0.037$) higher Mn and Sr concentrations than the HW group, with the Mn concentration of 24.5 mg/kg versus 20.3 mg/kg, $P = 0.019$, and Sr concentration of 293 mg/kg versus 266 mg/kg respectively. There was no significant ($P > 0.05$) difference in other trace-mineral concentrations between the groups, and no significant ($P > 0.05$) difference in the bone ash and macro-mineral concentrations between the two groups. There was a strong positive correlation between bone Ca and P, Na, Mg, Mn, and Cs. There was also a weak negative correlation between bone strength and Mn, Cd and Cs, and positive strong correlation between bone and D7 and D21 BW.

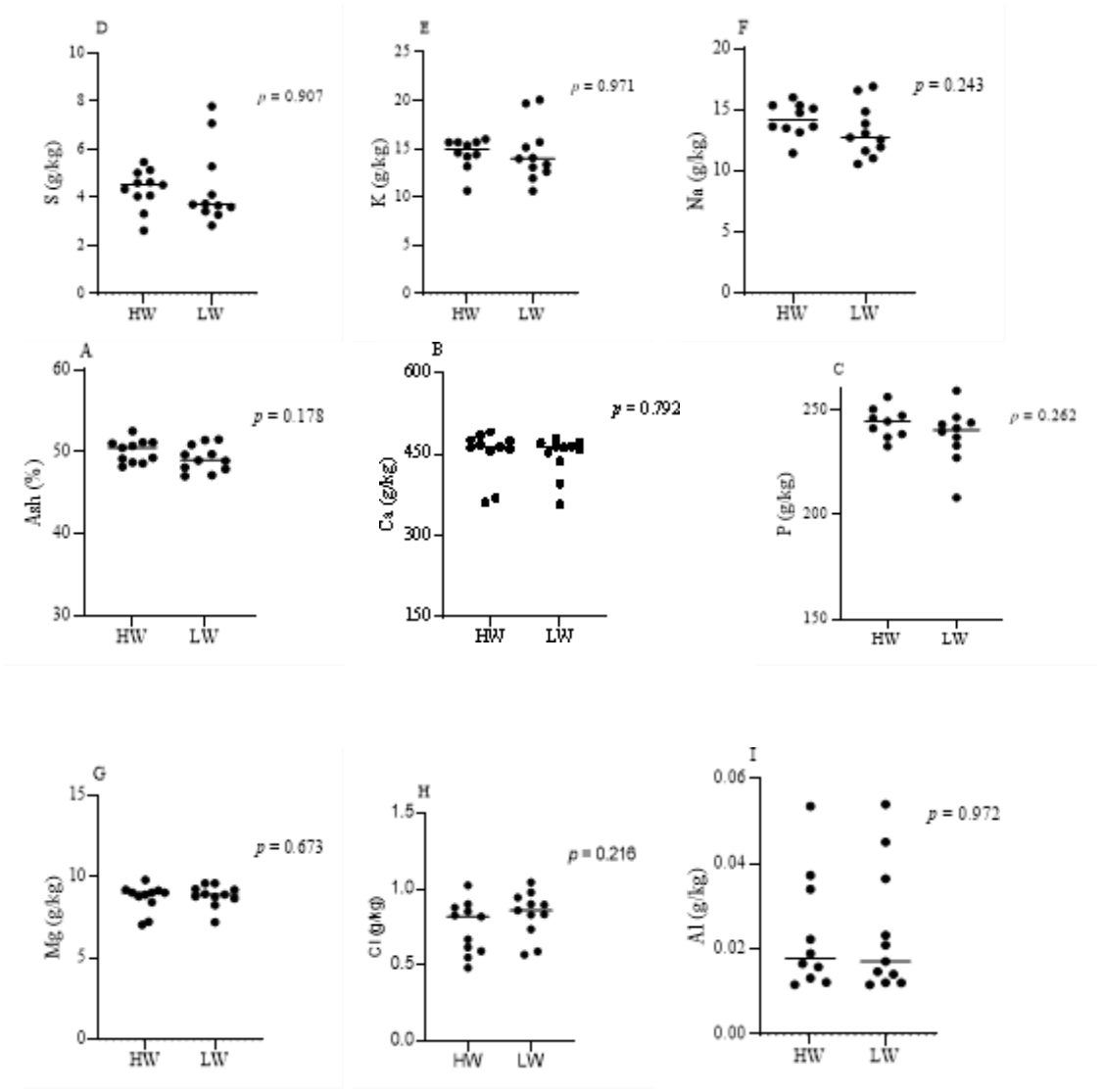


Figure 4.7: Tibial ash and macro mineral concentration of high and low bodyweight broiler chicks on D21. (A) ash content, (B) calcium, (C) phosphorus, (D) sulphur, E) potassium, (F) sodium, (G) magnesium, (H) chlorine, (I) aluminum. HW: High weight; LW: low weight, (n= 11/group)

Table 4.6: Tibial trace mineral concentration of high and low bodyweight broiler chicks on D21

Minerals (mg/kg)	HW	LW	SEM	P-value
Zn	501.10	491.50	±21.700	0.664
Cu	2.99	3.20	±0.315	0.528
Fe	312.40	308.9	±20.580	0.868
Mn	20.31	24.53	±1.653	0.019*
Sr	266.10	292.90	±11.990	0.037*
Cr	0.46	0.41	±0.080	0.503
Mo	0.95	0.92	±0.060	0.602
Pb	0.33	0.28	±0.040	0.298
Cs	0.10	0.12	±0.010	0.109
Cd	0.02	0.02	±0.003	0.163
Co	0.12	0.11	±0.020	0.881
Se	0.14	0.15	±0.010	0.118

(Zn) zinc, (Cu) copper, (Fe) iron, (Mn) manganese, (Sr) strontium, (Cr) chromium, (Mo) molybdenum, (Pb) lead, (Cs) caesium, (Cd) cadmium, (Co) cobalt, (Se) selenium, HW: High weight; LW: low weight.

* Denotes significant difference at ≤ 0.05, (n=11/group)

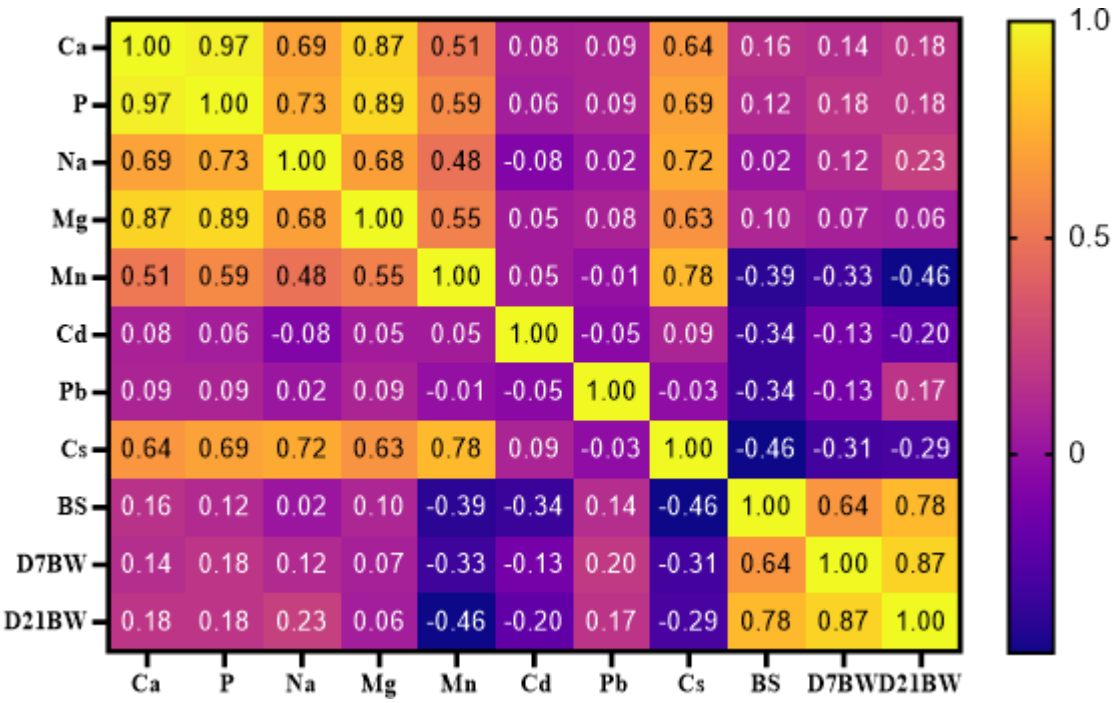


Figure 4.8: Heatmap showing the correlation (r) among mineral concentrations in the tibial bone of 21-day old broiler chicks.

Correlation (r) between 0.5-1 indicates strong positive correlation while -0.5 – (-1) indicates strong negative correlation, 0 indicates no correlation, (n=11/group), BS: bone strength, D7BW: day 7 bodyweight, D21BW: day 21 bodyweight.

4.5 Discussion

This study evaluated BWs and relevant metabolic organs and tissues to pinpoint essential physiological differences between low- and high-BW broiler chicks during the early weeks of life. The retrospective BW performance of the chicks investigated in this study showed higher variations as reflected in the CV values of 13–16%, which exceeded the recommended range of 8–10% in broiler chicks (Feddes et al. 2002; Toudic 2007). Generally, chicks were observed to exhibit low BWs when compared with the Ross 308/Ross 308 FF male broiler performance objectives (2019).

Despite the low BWs exhibited by the experimental chicks, they appeared healthy throughout the period of the experiment, with 2.5% mortality. The very low mortality observed in this study suggested that the experimental chicks were not health challenged. While hatch weight is an important consideration to ensure broilers have a strong start to life, BW after the first week of life is often used in the industry as a measure of early performance and future growth potential. There was a positive strong association between D0 and D7 BWs ($r = 0.77$), and a positive weaker association was also found to exist between D0 and D14 ($r = 0.54$) and D0 and D21 ($r = 0.55$). The D7 BW was observed to have a stronger positive association with D14 ($r = 0.83$) and D21 ($r = 0.79$) than with D0 BW. This result also agreed with the findings of Tona et al. (2004) who reported no relationship between day-old chick weight and slaughter weight but indicated that a stronger relationship exists between weight on D7 and slaughter-age weights in broiler chicks.

The chicken gut provides a means for acquiring nutrients and energy but is also a route for disease entry, which can directly affect overall growth performance. The pH of the digesta contents along the gastrointestinal tract has been reported to influence mineral solubility in the gizzard and its absorption in the small intestine (Lee et al. 2021). The pH of the gut has a direct influence on Ca solubility and its availability for absorption; thus, an acidic gut environment has been shown to promote the dissolution of CaCO3 (Walk et al. 2012). Low gizzard digesta pH has been suggested to increase pepsin activity and the

solubility and absorption of mineral salts (Bucław 2016). Gizzard digesta pH in broiler chicks has been reported to be influenced by diet type, wholly or coarsely ground grains, and fibre, which decreases the pH of the gizzard by 0.2–1.2 units (Svihus et al. 2013; Jiménez-Moreno et al. 2019; Aziz-Aliabadi et al. 2023). This could be attributed to increased gizzard volume and more retention time, which allows more fermentation to produce various acids (Svihus 2014).

In our previous study, it was reported that low-BW chicks had proportionally heavier gizzards than did the high-BW group. In the present study, the low-BW group had a low gizzard digesta pH compared with the high-BW group, which was very interesting, and it may suggest greater gizzard functionality in the low-BW group, which may seem more like a compensatory strategy. Alternatively, it could reflect a lower quantity of gizzard contents, and the various acid buffering components such as Ca, which would be present. It was speculated that the low gizzard pH observed in the low-BW group in the present study could also be linked to the increased intake of fibrous litter substrate compared with the high-BW group which influenced acid production in the gut, thus resulting in a lower gizzard digesta pH. During the study, some feeding behaviors were observed, such as, for example, the tendency for chicks to habitually consume litter and, at necropsy, litter was found in the gizzards. It was speculated that HW chicks that displayed dominance may have led to greater consumption of litter in the LW group (Estevez et al. 1997; Bokkers and Koene 2004). This observed tendency for litter consumption in the LW group could be the reason for the higher NDF and ADF content found in the gizzard digesta of these chicks compared to the HW chicks. The low gizzard pH observed in the LW group would correspond with the increase gizzard digesta fiber content in the same group of chicks. The crop pH obtained in the present study ranged from 4.7 to 4.8, which fell within the range of 4.5–5.9 reported by other researchers (Svihus 2014) and was lower than the range of 4.8–4.9 in low- and high-BW broiler starters respectively, reported by (Dono et al. 2014). The digesta pH of the gizzard in a healthy chick was summarized in a report by Svihus (2011) and it ranged from 1.9 to 4.5, with an average of 3.5; the present study reported gizzard

digesta pH within this range in both groups. It has been reported that most of the pathogenic microbes have been reported to thrive in pH close to 7 and above (Rahmani et al. 2005), but in this study, the pH values recorded in both high- and low-BW chicks for the crop, gizzard, and ileum were below 7 and could suggest healthy gut environment in those gut segments measured in this study across the two groups. Liver mineral concentrations are an important indication of its status, storage, and bioavailability of ingested minerals (Wang et al. 2007). The liver plays an important role as a storage facility, it has an important role in the metabolism of minerals and detoxification, and it is also specifically the main storage site of Co and fat-soluble vitamins (Zaefarian et al. 2019). It is the target organ for the accumulation of Cd, which exerts several negative effects such as cellular changes, acceleration of lipid peroxidation, DNA-chain breakages and impact on mitochondrial function (Toman et al. 2005; Berzina et al. 2007; Hu et al. 2018). In the present study, there was an interestingly higher concentration of Cd in the LW group. Cd is one of the heavy metals that has a deleterious effect on growth performance; it has high water solubility and toxicity even at low concentrations (Tkalec et al. 2008). In the present study, liver Cd concentration in the LW group exceeded the permissible limit of 0.05 μg/g in broiler chicken liver (FAO/WHO 2002; Korish and Attia 2020) compared with the HW group, which may have detrimental implications on growth performance and gut function. Bioaccumulation of Cd in the liver of chicken has been reported to be associated with poor weight in chickens (Akyolcu et al. 2003).

Higher liver Cd concentration in the low-weight group observed in this study may possibly lead to distinct pathological changes in the liver, even at very low concentrations, thus influencing the mitochondrial function, and may induce hepatotoxicity in the low-weight group compared to the high-weight group (Casalino et al. 2002; Arnold et al. 2006; Berzina et al. 2007; Li et al. 2013). The significant increase in Mn, and Cs in the low-weight group may be attributed to various factors, such as differences in individual intake, nutrient requirement, and its retention in the hepatic tissue.

It is noteworthy that Cd and Cs concentrations were significantly higher in the tibial bones of the low BW chicks in our previous study with a similar experimental design (Elvis-Chikwem et al. 2021). This interesting trend in bioaccumulation of Cd in both liver and bone of the low-BW chicks requires further investigation. Bone ash and mineral concentrations such as calcium and phosphorus were not significantly different between the BW groups in this study. These constituents are important markers of overall bone composition and integrity. The bone concentrations of Cd, Pb and Cs were not affected by BW differences in the present study, as was observed in previous work, albeit in 7-day-old chicks (Elvis-Chikwem, C.L. et al. 2021), where these heavy metals where higher in the LW group than in the HW group.

Manganese is an essential trace element that acts as a cofactor to many enzymes such as Mn superoxide dismutase, arginase, and pyruvate carboxylase, which aid reactive oxygen scavenging, bone formation and immune response (Shahnazari et al. 2007). In contrast, Sr has been reported to have a positive effect on bone formation and strength in broiler chicks (Shahnazari et al. 2007). Both minerals Mn and Sr are associated with bone-health performance. The reasons forthe higher concentrations of these minerals, especially Mn, in the tibias of the LW chicks than in those of the HW chicks is unclear and may be partly attributed to variation in Mn requirement for individual chicks influenced by BW differences. It has been reported that Mn concentration in the tibial bone has a linear relationship with Mn intake by the chicks and is an indication of its bioavailability (Sunders et al. 2006, 2007). This might not be the case in the present study because the high- and low-BW chicks were fed the same diet without additional dietary supplementation of Mn or Sr. It is also noteworthy to mention that there was no environmental source of Mn and Sr accessible to the chicks in the present study, as the mineral profile of the diet and wood shave were done (see supplementary file) to verify this. Basically, the higher concentration of Mn seen in both the bones and the liver of the LW group suggests a more systemic accumulation of this mineral in the lighter chicks.

4.6 Conclusion

This study evaluated differences in bone, gut parameters, and liver mineral profile of male Ross 308 broilers with varying BW on D21, that were exposed to the same environmental and management conditions. Chicks with a high and low BW showed differences in bone characteristics, liver trace mineral concentrations of Mn, Cd and Cs, gizzard digesta pH and gizzard fibre content. The significant reduction in gizzard digesta pH, and high gizzard fibre content of the LW group relative to the HW group could be linked to fibrous litter consumption of the low-BW group, which may be contributing to a shift in acid production in the chicken gut. This study also indicated that broilers with a low BW on D21 had a higher concentration of Mn in both bone and liver tissues, which may have physiological implications; hence, more research is needed to understand Mn requirements, and tissue retentions as influenced by BW in early life of broiler chicks.

Due to the reduced gizzard digesta pH and high gizzard fiber content in the low weight group relative to the high weight, an increase in litter consumption in this group was speculated. The next study (Trial 3) was conducted to evaluate the behavioral activities of low and high weight chicks during the first week of life and its association with growth performance and tissue mineral profile.

CHAPTER FIVE

Trial 3

5.0 Effect of hatch weight on early life behavioral activities and tissue mineral profile of Ross 308 broiler chicks

Paper in preparation to be submitted to Animals.

5.1 Abstract

Growth performance is an important parameter in broiler production, influenced by nutritional, environmental and disease agents. Chick feeding behavior in the first week of life has been reported to have direct influence on growth performance. The behavioral activity, body and organ weight, and tissue mineral concentration of Ross 308 male day-old chicks were investigated to understand the influence of hatch weight on the mentioned parameters. A behavioral and growth monitoring study was conducted involving 60 1-day old male Ross 308 broiler chicks. The chicks were divided into 3 groups including low weight (LW), medium weight (MW) and high weight (HW) groups based on hatch weight and then equally distributed across 5 pens, with 4 chicks from each group in every pen (12 chicks per pen). Results showed bodyweight variations among the individual chicks (CV > 10%). The LW group had higher relative crop weight compared to the HW group, but there was otherwise no difference in relative organ weight. In the present study, hatch weight differences showed little influence on behavioral activities of the HW and LW groups Bone Ca, P and Zn concentrations were influenced by hatch weight differences as the HW had higher concentrations of these minerals in their bones compared to the LW group. This study revealed that chicks from the same breed, and common diet and environmental conditions showed differences in relative crop weight and tibial mineral concentrations, with the HW group have higher concentrations of the Ca, P and Zn relative to the LW group, this may be associated with their higher tendency to feeding on D0 in the HW compared to the LW group.

Keywords: Variation, growth, early life, behavior, mineral profile

5.2 Introduction

The broiler industry is one of the most important sectors contributing to sustainable animal protein sources globally. Poultry meat consumption has risen globally, due to its preference by consumers because of lower prices, product consistency, adaptability, higher protein, and lower fat content (OECD-FAO 2022-2023). This exponential increase in the demand of poultry products requires a corresponding improvement in broiler production efficiency particularly in broiler health, welfare, and nutritional management.

The genetics of the broiler chicken is geared toward fast growth and efficient feed conversation ratios. Bodyweight is an important parameter for assessing growth performance in broiler population, and thus it is important to have a uniform body weight while maintaining specific bodyweight targets at key stages in the production cycle of broiler chicks (Sacranie, 2019). Hatch weight, which can be related to egg weight (Pinchasov, 1991), is an important initial starting point that is related to subsequent performance, particularly in early life (Wilson, 1991; Traldi, et al., 2011). This highlights the importance then for why we chose to rank the birds based on hatch weight. Variation in bodyweight among chicks have been reported to be associated with feed and water access within the early weeks post hatch (Nielsen, et al., 2010). It has also been reported that the variation in body weight of birds of the same genotype and management is directly related to their feed intake differences (Bottje and Carstens, 2009).

Broiler behavioural activities such as feeding, walking, and pecking are all influenced by environmental conditions and increasing age (Bokkers and Koene, 2003). The previous study (Chapter 3) revealed that the low weight broiler chicks had higher fibre content and a reduced gizzard digesta pH compared to their high weight group (Elvis-Chikwem, et al., 2024). This was

speculated to be the impact of their weight differences and behavioural activities, especially feeding behaviour. As birds were fed a common diet in the previous study, it was speculated that low weight chicks may habituate to litter consumption which may be associated with their low bodyweight (Malone, et al., 1983). Several behavioural studies have showed differences in the behaviour of fast and slow growing broiler chicks, and they reported that fast growing broilers showed a lower activity level than the slow growing population, this could be certainly attributed to bodyweight differences (Lewis, et al., 1997; Siegel, et al., 1997). It has been noted that differences observed in growth rates may be associated with the time and access to feed and water post hatch (Nielsen, et al., 2010). Research study also reported that 74% of the total activity of broiler chicks within the first week of life was linked to feeding activity (Bizeray, et al., 2000). While the behaviour of the modern broiler chicks from day 0 up to 6 weeks of age has been explored extensively both commercially and experimentally (Murphy and Preston, 1988; Newberry et al., 1988; Blokhuis and Van der Haar, 1990; Bessei, 1992; Weeks et al., 2000; Hall, 2001), the comparative behaviour of high and low weights broiler in the first weeks of life and its influence on growth performance parameters, tissue mineral and digesta mineral concentration still requires attention.

Mineral nutrition is a vital aspect in broiler nutrition, especially early life nutrition. Both macro and trace minerals play vital roles in several physiological and metabolic activities in broilers. Therefore, to safeguard the functional and structural integrity of animal tissues, growth, health and productivity, the concentration of essential minerals must be tightly maintained within narrow limits (Underwood and Suttle, 1999). Tissue mineral concentration is an important indication of its bioavailability at the tissue level and not dietary level (Nesbit and Elmslie, 1960; Bunch et al., 1961; Watson, eta l., 1970; Black, et al., 1984a, b). Bone and kidney mineral concentration have

been reported to be more sensitive for bioassay than other tissues such as muscle. (Black, et al., 1984a) Therefore, an increase in tissue mineral concentration in broiler chicks translates to more readily available mineral in the tissue and reduction of its excretion as waste (Miles, 2000).

Hence this study aimed at understanding the behavioural activities of chicks with LW, MW and HW groups in early life, and association of bodyweight with tissue mineral concentration during the first week of life. Therefore, the hypothesis of the current trial was that chicks with varying hatch weight would exhibit differences in their behavioural activities especially feeding behaviour, which in turn may influence tissue mineral profile

5.3 Materials and methods

5.3.1 Ethical Approval and location of the experiment

The animal trial was carried out at the Nottingham Trent University poultry research unit, Brackenhurst campus. All the experimental protocols used in the study were approved by the University of Nottingham (Reference code: 258) and Nottingham Trent University Ethical Committee (Reference code: ARE202170).

5.3.2 Animal housing and Diet Management

A total number of 60-day old Ross 308 chicks were used for the trial, all chicks were weighed on D0 to obtain the initial bodyweight and then separate into three different bodyweight groups using quintile method. The bodyweight groups were as follows; low weight group (BW ranged from 33- 36g), medium weight (39-42g) and high weight (BW, 45-48g), chicks were allotted to 5 pens containing 4 chicks per bodyweight group, totaling 12 chicks per pen and 60 chicks in total for the observation study, these chicks were marked with visibly non-toxic marker for easy identification. All chicks in each pen were given the same management and were fed *ad libitum* with GLW super chick starter crumb (GLW, Shepshed), that met the nutritional requirements of the Ross 308 breed. The diet contained crude protein (21.96%), crude ash (5.37%), crude fats and oil (3.25%), crude fibre (3.72%), calcium (0.79%),

phosphorus (0.66%), sodium (0.16%), methionine (0.53%) and lysine (1.18%). The trial lasted for 7days.

5.3.3 Behaviour monitoring

The behaviour study was conducted at the poultry unit of the Nottingham Trent University, Brackenhurst Campus. Behaviour monitoring protocol followed the standard procedure of the poultry unit of Nottingham Trent University. All the behaviour parameters used in the study are described in table 5.1. Prior to observation an adjustment time of 5mins was given to allow chicks familiarize with the observer's presence. On D0, 4 chicks per bodyweight group were marked using non-toxic marker on the back of the head and wings with different color markers for easy identification. The behaviours of these chicks were monitored on D0 and D7, the chicks were monitored by 5 trained observers for the 5 pens, chicks were monitored in the morning and timing was 10 minutes for each bodyweight group per pen. The total number of chicks per body weight group in each pen, performing each behavioral activity were recorded for every 30 seconds for 10 minutes, which was done sequentially by each observer.

Table 5.1: Behaviour parameters investigated in the study (recorded as number of occurrence/hour)1

Behaviour parameters	Description
Feeding	Pecking at all parts of the feed trough including food
Drinking	Pecking at the drinking nipple
Wooden furniture peck	Pecking at the wooden furniture only and not feeder, nipple drinker or litter
Feather peck	Pecking the feather of another bird only, different from an aggressive act.
Litter eating	Pecking the litter material only
Sit	Sitting without doing anything else
Stand	Standing only without doing anything else
Walk	Walking around for a distance, without doing anything else
Rest	Immobility
Aggression	Pecking aggressively on anotherchick particularly on the areas of the head and neck different from feather pecking

[1] Behaviour descriptions curated from the behavior ethogram (de Jong et al., 2005 and Fortomaris, et al., 2007)

5.3.4 Sample Collection

On Day 7, all chicks were weighed to obtain the D7 bodyweight, 2 chicks were randomly selected from the HW and LW groups in each pen for sample collection. The selected birds were euthanized by cervical dislocation. Then the crop, gizzard, liver, and tibial bones were collected and stored at -20°C for further analysis.

5.3.5 Tibial bone ash and mineral analysis

The legs collected which were stored at -80°C, were allowed to thaw at room temperature and were subsequently cleaned manually to extract the tibial bones. The cleaned tibial bones were oven-dried at 105^0C for 24hr and ashed at 600^0C overnight to determine the tibial ash. Tibial bone ash was acid digested using the hot plate method for sample preparation. A maximum of 0.2g of each sample was digested with 10ml of hydrogen peroxide and heated for 2 hours at 95^0C in the fume cupboard. A solution of 50ml MilliQ water was added to each tube after digestion and 8ml taken from the top into 8ml tubes. Digested samples were diluted to 1/10 and mineral concentration analyzed using the ICP-MS methods (Thermo-Fisher Scientific iCAP-Q; Thermo Fisher Scientific, Bremen, Germany).

5.3.6 Liver and gizzard digesta samples digestion and mineral analysis:

The liver and gizzard digesta mineral concentration were determined using the ICP-MS method, the liver and gizzard digesta samples were freeze dried using the freeze drier (Thermo Savant SuperModulyo) at the temperature setting of -45^0C for one week prior to digestion. Approximately 0.2g of the freeze-dried samples were weighed into the digestion vessels and the weight recorded. Each of the samples were digested using 3ml of nitric acid, 3ml MilliQ water and 2ml of hydrogen peroxide in the fume cupboard. The digestion tubes containing the samples were positioned in the microwave rotor for 45 min to obtain complete digestion. The liquid was decanted into universal containers and digestion tubes rinsed with 7ml of MilliQ water which was decanted back to the labelled universal tubes ready for ICP-MS analysis. Samples were diluted to 1/10 into the ICP tubes and analyzed

using the ICP-MS (Thermo-Fisher Scientific iCAP-Q; Thermo Fisher Scientific, Bremen, Germany) for mineral concentrations.

5.3.7 Statistical analysis

Descriptive statistics of the bodyweight weight data were analyzed using the SPSS software tool version 21 (IBM Corp. Released 2012. IBM SPSS Statistics for Windows, Version 21.0. Armonk, NY: IBM Corp). The behaviour data were analyzed using the Microsoft excel software tool, while the bone mineral profile, liver mineral and gizzard digesta mineral concentration data were analyzed using the student t-test of the GraphPad Prism 9.0 software (GraphPad Prism version 9.0.0 for Windows, GraphPad Software, San Diego, California USA, www.graphpad.com), with body weight as the main factor.

5.4 Results

5.4.1 Bodyweight performance

The descriptive statistics of the experimental chicks (n=60) is represented in table 5.2 below, the average bodyweight performance of the LW and HW bodyweights chicks are shown in table 5.3. The mean bodyweights of the experimental chicks on D0 were 41g (±4.5), D7: 174g (±19.3), ADWG: 19g (±2.5) and BWC: 133 (±17.2). The mean D0 bodyweight of the LW group was 35.6g versus 46.1g in the HW group.

Table 5.2: Descriptive statistics of the bodyweight performance of Ross 308 Broiler chicks from D0 to D7 (n= 60)

Parameters	Mean	MIN	MAX	SD	CV (%)
D0 BW (g)	41	33	49	4.5	11.1
D7 BW (g)	174	120	212	19.3	11.1
ADWG (g)	19	12.3	24	2.5	13
BWC (g)	133.4	86.3	165.3	17.2	13

D0 BW: Day 0 bodyweight, D7BW: Day 7 bodyweight, ADWG: Average daily weight gain, BWC: Bodyweight change.

Table 5.3: Average bodyweights of LW and HW broiler chicks during the first week of life (n=40)

Age (days)	LW BW (g)	HW BW (g)	SEM	P value
D0	35.6	46.1	±0.41	.0001
D7	162.0	185.3	±7.79	.007
ADWG	18.1	20.0	±1.09	.115
BWC	126.4	139.1	±7.64	.114

LW: Low weight; HW: High weight; BW: Bodyweight; ADWG; Average bodyweight gain; BWC: Bodyweight change.

5.4.2 Organ weights of the experimental chicks

The weights of the crop and gizzard of the LW and HW groups are shown in figure 5.2, the LW group showed significantly higher relative crop weight compared to the HW group (LW = 4.8, HW = 3.5, P = 0.036).

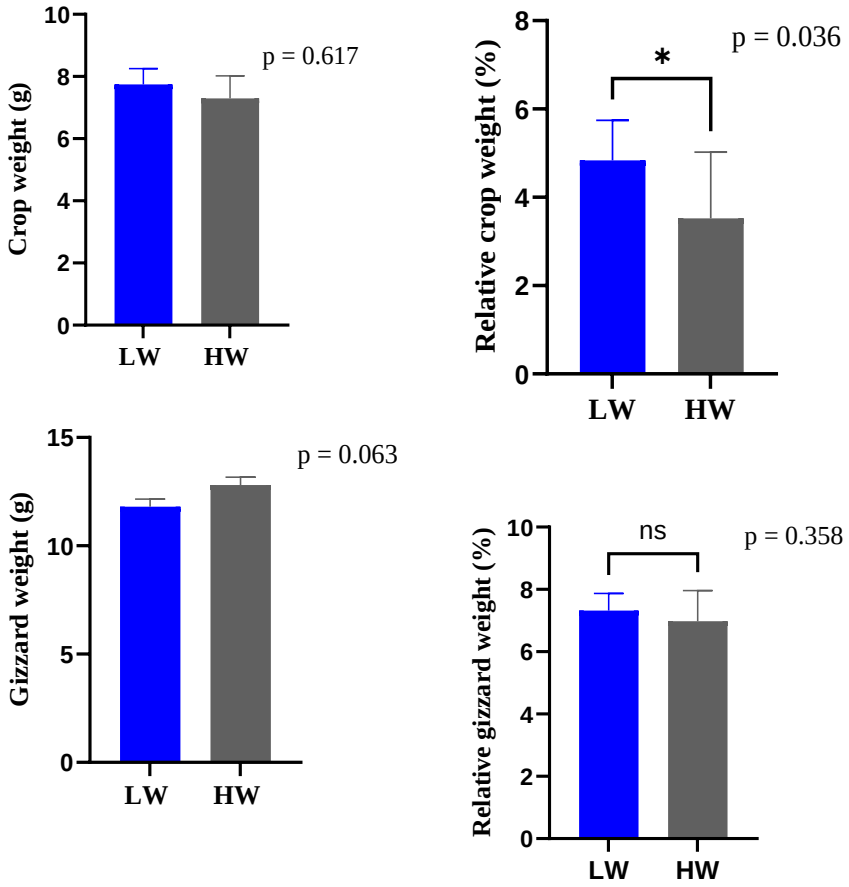


Figure 5.2: Crop and gizzard absolute and relative weights of the low weight (LW) and high weight (HW) chick groups on day 7 (n=10 per group)

Footnotes: LW: light weight, HW: heavy weight.

5.4.3 Behavioral parameter

The percentage of chick engaged in each behavioral activity for each group, LW, MW and HW on D0 and D7 are shown in figure 5.3 and 5.4 below, while the percentage of chicks engaged in litter eating in the LW, MW and HW groups are presented in figure 5.5. The feeding, liter and other behaviors recorded for each bodyweight group on D0 and D7 are presented in appendix 8 and 9 below. The percentage of chicks engaged in feeding behavior was higher in the HW group on D0 compared to the MW and LW groups. In contrast, litter eating had the highest percentage of chicks from the LW group on D0 compared to the MW and HW groups. Sitting behavior had the highest percentage of chicks on D7 in the LW group compared to the MW and HW groups.

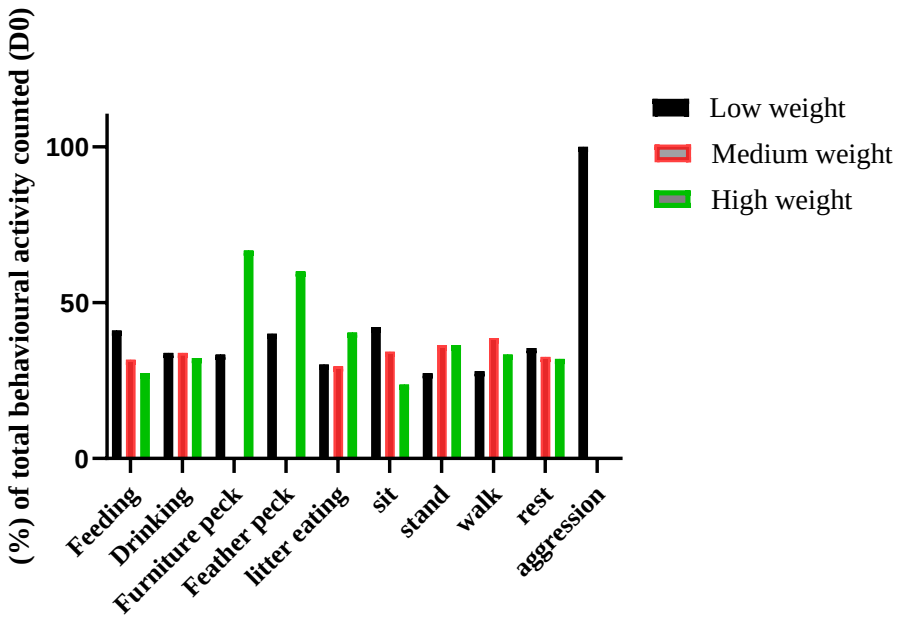


Figure 5.3 behavioural activity of broiler chicks with distinct bodyweight on D0 (n=20 per group)

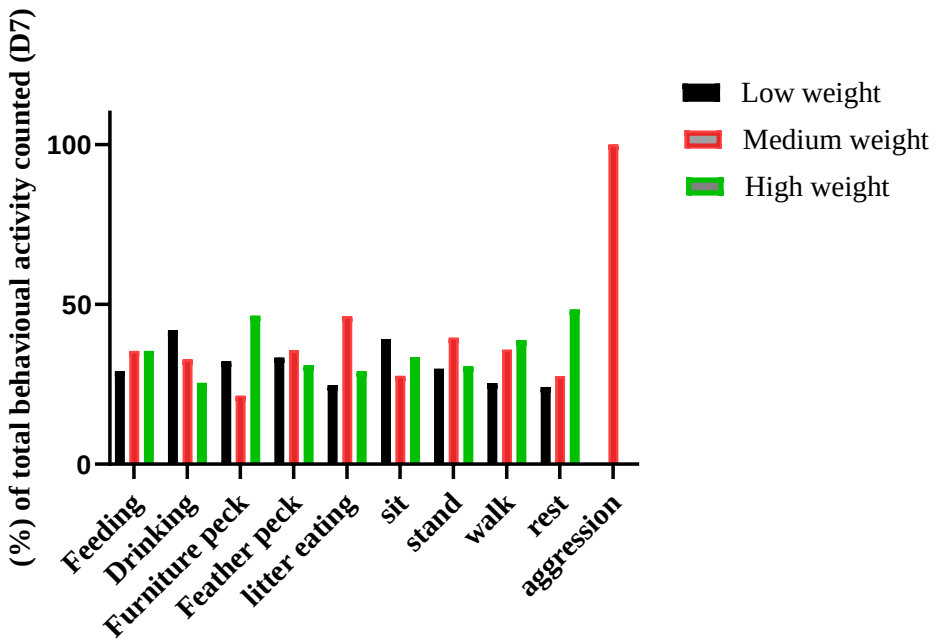
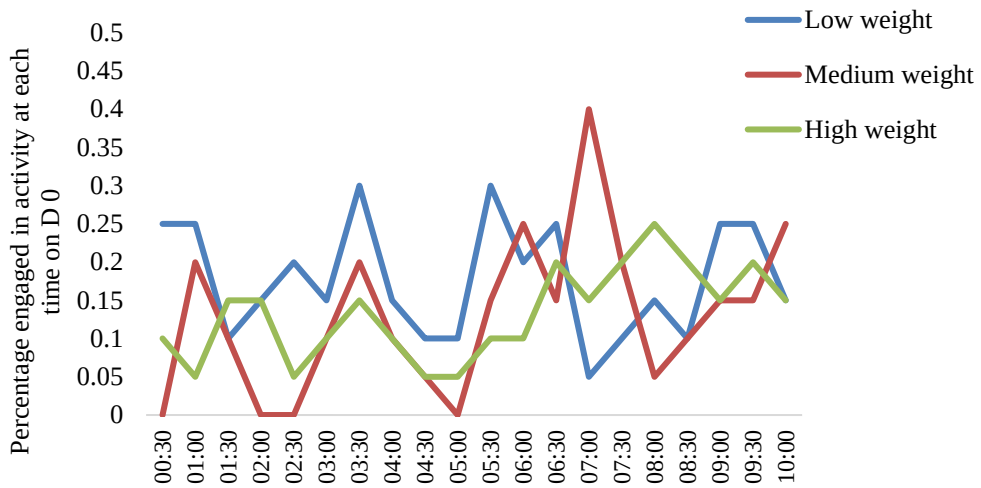


Figure 5.4 behavioral activity of broiler chicks with distinct bodyweight on D7 (n=20 per group



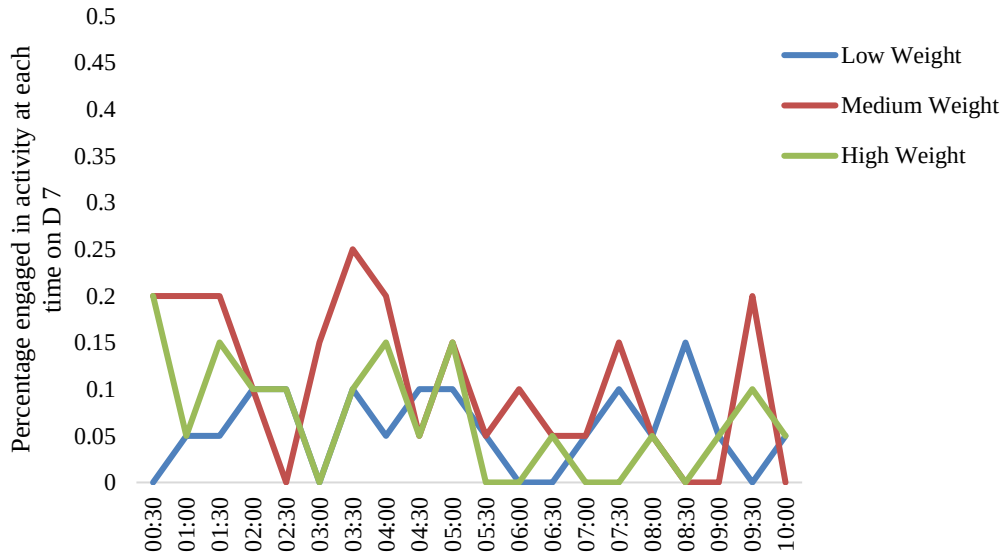


Figure 5.5: Proportion of chicks engaged in eating litter from the low, medium, and high weight groups on D0 and D7 (n=20 per group)

5.4.4 Tibial mineral profile

The tibial macro and trace mineral profile of the LW and HW groups are presented in figure 5.6, table 5.4 and 5.5. There were significantly higher Ca and P concentrations in the bone of the HW group relative to the LW group, Ca (P = 0.049) and P (P = 0.033). Apart from the two major bone minerals, all other macro mineral measured showed no significant difference between the two groups. Among all the trace mineral elements measured in the bone of the experimental chicks, only zinc showed significant higher concentration in the HW group compared to the LW group (P= 0.021). Then the heatmap showing the correlation between bone minerals and bodyweights is shown in figure 5.5 below.

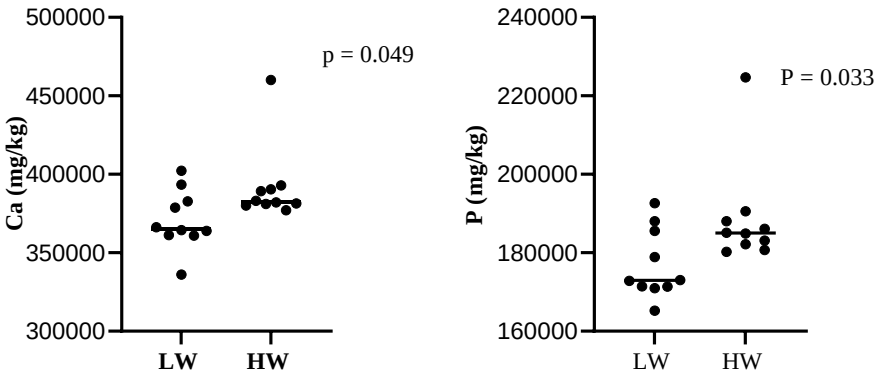


Figure 5.6: Tibial Ca and P concentration of the low and high weight chicks.

Table 5.4: Tibial macro mineral concentration of the low and high weight chicks

Minerals (mg/kg)	LW	HW	SEM	P value
Ash (%)	46.47	46.90	±0.75	0.576
Na	12956.0	13324.0	±793.9	0.650
Mg	7170.0	7880.0	±444.0	0.127
K	12392.0	13132.0	±815.0	0.376
S	4388.0	4508.0	±514.0	0.819

(Na) sodium, (Mg) magnesium, (K) potassium, (S) sulphur, LW: light weight; HW: Heavy weight, SEM, standard error of the mean (both groups), (n=10/group).

Table 5.5: Tibial trace mineral concentration of the low and high weight chicks

Minerals (mg/kg)	LW	HW	SEM	P value
Cr	1.25	2.09	±0.71	0.256
Mn	14.21	14.41	±0.83	0.816
Cu	3.53	5.77	±1.76	0.219
Co	0.06	0.08	±0.02	0.468
Fe	284.10	287.01	±55.46	0.960
Zn	418.6	470.8	±20.54	0.021*
Mo	2.17	2.72	±0.37	0.155
Cs	0.03	0.03	±0.002	0.944
Pb	0.35	0.37	±0.04	0.581
Cd	0.01	0.02	±0.003	0.085
Rb	11.77	12.71	±0.73	0.213
Sr	91.69	95.12	±2.12	0.123

(Cr) chromium, (Mn) manganese, (Cu) copper, (Co) cobalt, (Fe) iron, (Zn) zinc, (Mo) molybdenum, (Cs) caesium, (Pb) lead, (Cd) cadmium, (Rb) Rubidium, (Sr) strontium, LW: light weight; HW: Heavy weight, SEM Standard error of mean (both groups), * Denotes significant difference at ≤ 0.05**,** (n=10/group).

5.4.5 Liver mineral profile

The liver macro and trace mineral elements of the LW and HW groups are presented in tables 5.6 and 5.7. There was no significant difference ($p>0.05$) observed in all macros and trace mineral concentrations measured between the LW and HW groups in the current study.

Table 5.6: Liver macro mineral concentration of the low and high weight chicks

Minerals (mg/kg)	LW	HW	SEM	P value
P	12710.0	12581.0	±683.0	0.852
Ca	318.3	315.20	±38.75	0.938
Na	6221.0	5439.0	±680.50	0.266
Mg	849.6	829.9	±42.44	0.648
K	15689.0	15418.0	±864.20	0.758
S	8171.0	8289.0	±432.00	0.780

(P) phosphorus, (Ca) calcium, (Na) sodium, (Mg) magnesium, (K) potassium, (S) sulphur, LW: light weight; HW: Heavy weight, SEM, standard error of the mean (both groups), (n=10/group).

Table 5.7: Liver trace mineral concentration of the low and high weight chicks

Minerals (mg/kg)	LW	HW	SEM	P value
Cr	0.15	0.06	±0.08	0.227
Mn	10.12	9.67	±0.67	0.513
Cu	12.21	12.18	±0.97	0.973
Co	0.05	0.05	±0.004	0.301
Fe	382.5	359.50	±63.23	0.721
Zn	79.45	83.20	±5.84	0.529
Mo	2.27	2.34	±0.152	0.668
Cs	0.03	0.03	±0.002	0.858
Pb	0.01	0.01	±0.01	0.931
Cd	0.01	0.01	±0.001	0.458
Rb	20.46	20.22	±1.29	0.858
Sr	0.12	0.11	±0.02	0.628

(Cr) chromium, (Mn) manganese, (Cu) copper, (Co) cobalt, (Fe) iron, (Zn) zinc, (Mo) molybdenum, (Cs) caesium, (Pb) lead, (Cd) cadmium, (Rb) Rubidium, (Sr) strontium, LW: light weight; HW: Heavy weight. SEM: standard error of the mean (both groups), *Denotes significant difference at

≤ 0.05**,** (n=10/group).

5.4.6 Gizzard digesta mineral profile

All mineral elements measured, both macro and trace showed no significant difference ($p>0.05$) between the LW and HW groups in this study, as shown in table 5.8 and 5.9 below.

Table 5.8: Gizzard digesta macro mineral concentration of the low and high weight chicks

Minerals (mg/kg)	LW	HW	SEM	P value
P	2977.0	3297.0	±190.4	0.107
Ca	6288.0	7364.0	±683.2	0.133
Na	3243.0	3262	±126.4	0.885
Mg	822.1	837.5	±57.58	0.792
K	4896.0	5021.0	±321.2	0.702
S	2781.0	2803.0	±112.1	0.844

(P) phosphorus, (Ca) calcium, (Na) sodium, (Mg) magnesium, (K) potassium, (S) sulphur, LW: light weight; HW: Heavy weight, SEM, standard error of the mean (both groups), (n=10/group).

Table 5.9: Gizzard digesta trace mineral concentration of the low and high weight chicks.

Minerals (mg/kg)	LW	HW	SEM	P value
Cr	2.74	2.66	±0.22	0.720
Mn	76.29	55.48	±15.87	0.206
Cu	23.17	27.06	±2.24	0.099
Co	0.19	0.20	±0.01	0.073
Fe	232.7	253.4	±42.96	0.636
Zn	72.86	87.50	±9.53	0.142
Mo	5.05	5.57	±0.43	0.246
Cs	0.03	0.03	±0.002	0.838
Pb	0.91	1.02	±0.09	0.282
Cd	0.05	0.05	±0.003	0.477
Rb	4.37	4.49	±0.24	0.604
Sr	4.55	4.97	±0.39	0.302

(Cr) chromium, (Mn) manganese, (Cu) copper, (Co) cobalt, (Fe) iron, (Zn) zinc, (Mo) molybdenum, (Cs) caesium, (Pb) lead, (Cd) cadmium, (Rb) Rubidium, (Sr) strontium, LW: light weight; HW: Heavy weight. SEM: standard error of the mean (both groups), * Denotes significant difference at ≤ 0.05**,** (n=10/group).

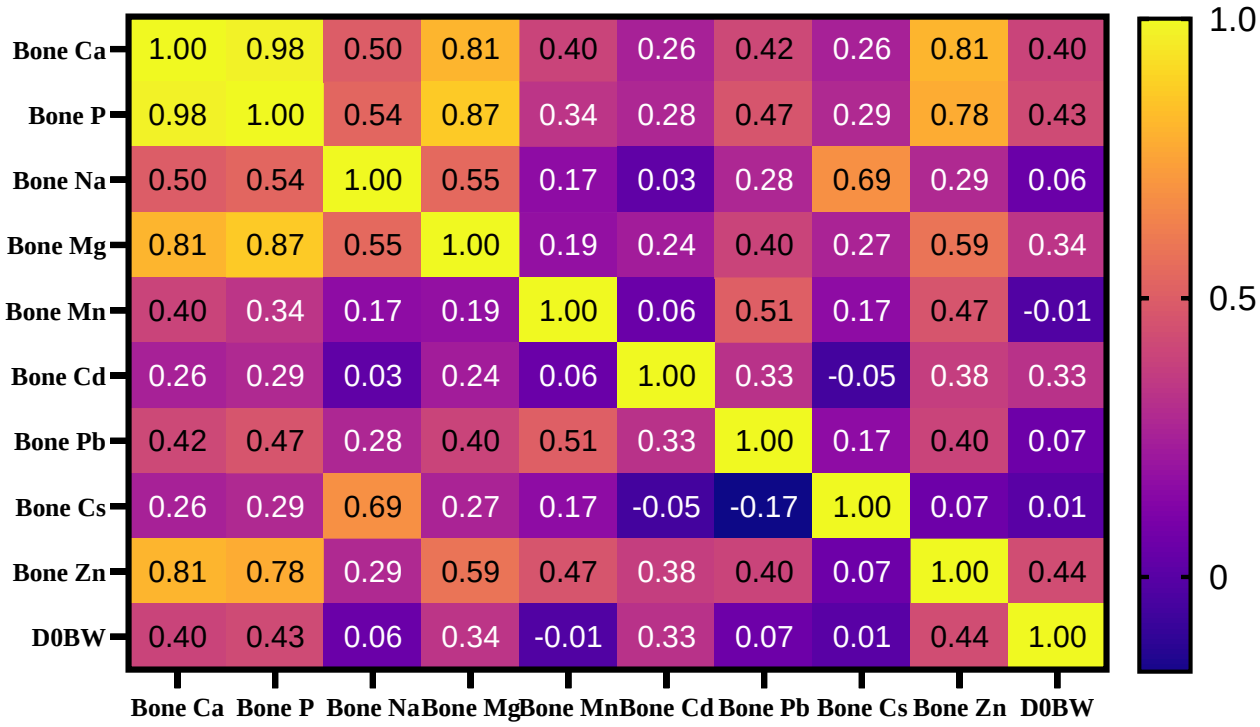


Figure 5.7: Heatmap showing the correlation (r) among tibial bone mineral concentrations of 7days old broiler chicks. Correlation (r) between 0.5-1 indicates strong positive correlation while-0.5 – (-1) indicates strong negative correlation, 0 indicates no correlation

5.5 Discussion

This study was conducted to better understand the effect of hatch weight differences on early life behavioral activities, organ weight and tissue mineral concentration of high and low weight broilers during the first week of life. In the present study, growth performance of the experimental chicks showed a degree of variability as indicated by bodyweight coefficient of variation of >10% which is higher than the recommended CV of <8% in a productive broiler population (Toudic 2007). The relative weight of the crop in the low weight group is significantly heavier than those from the high group. The relative gizzard weight also followed the same trend and were heavier in the low weight birds, although not significantly heavier. This result corresponds with recent unpublished work, and other published studies (Nir and Nitsan, 1979; Plavnik and Hurwitz, 1983; Dunnington, 1995) who reported heavier gizzard weight in light weight broiler strain compared to their heavy weight counterparts regardless of the greater amount of feed intake consumed by the heavy group. Heavier relative gizzard weight had been reported in laying hen with lighter bodyweight compared to heavier broiler strain (Shires, et al., 1987).

The present study recorded higher percentage of chicks performing feeding behavior in the high weight group compared to the medium and low weight groups on D0. It was also interesting to record greater percentage of chicks engaged in sit and rest behavior in the high weight chicks compared to other groups on D0, this could be attributed to their heavier weight, as heavier broilers then to exhibit low activity behaviors than the lighter ones (Siegel, et al., 1997). In broilers, rest behavior is reportedly associated with energy conservation, tissue restoration and growth (Blokhuis, 1983, 1984). It may be speculated that the high weight group that spent more time feeding, could possibly rest more than the low weight group. Conversely, the percentage of chicks engaged in litter eating, stand and walk were higher in the low weight group compared to the heavy weight group on D0. Behavioral differences in broilers have been attributed to differences in body weight (Bokkers and Koene, 2003), it was also reported that litter eating behavior in

broilers has an age-related effect and other unknown stimuli encouraging litter consumption during the first week of life (Malone and Chaloupka, 1983). At this stage of life, feeding behavior could be deemed important as chicks required feed consumption to drive gut function and growth. However, the higher frequency for litter feeding in the lighter chicks may be the reason for their light weight and consequently heavier relative crop weight. The high frequency for litter eating in the low weight group may agree with previously reported low gizzard pH in the low weight chicks (Elvis-Chikwem, et al., 2024) relative to the high weight group.

The two important bone minerals Ca and P were higher in concentration in the high weight group compared to the low weight group, although there was no significant difference in the bone ash content between the two groups. Ca and P are important minerals involved in bone mineralization and played vital role in maintaining skeletal integrity (Underwood and Suttle, 1999; Rath, et al., 2000). Ca and P not only combine in the bone to provide strength and rigidity while protecting soft tissues in the body, but they are wholly involved in many physiological and metabolic roles for example, Ca plays major role in intracellular cell signaling and transmitting nerve impulses at the extracellular level (Underwood and Suttle, 1999). Phosphorus on the other hand plays a vital role in cell energy exchange including breaking of high energy phosphate bonds (Underwood and Suttle, 1999). In the present study, the high weight chick group had higher concentration of both Ca and P in their bones relative to the low weight group, this may indicate better mineral storage depot which can be readily mobilized for nutritional and metabolic emergencies, thereby contributing to their better performance compared to the low weight group. This study also recorded higher concentration of zinc in the bone of the heavy chicks compared to the lightweight group. Zn has been reported as one of the trace minerals used as a growth promoter in broiler diet (Kwiecień, et al., 2016), it has been reported to mediate correct course of ossification and bone mineralization in broilers (Scrimgeour et al., 2007; Salim, et al., 2008; El- Husseiiny et al., 2012). Zn also stimulates the synthesis of DNA in osteoblasts resulting in an increase in bone weight and Ca ion

concentration (Ma and Yamaguchi, 2000). Zinc has also been reported to control appetite in animals and increase in feed intake and growth have been noted in broilers chicks (Park et al., 2004; Bao et al., 2007; Gheisari et al., 2010; Ao et al., 2011).

The increase in the concentration of zinc in the bone of the high weight chicks may be related to the antagonist influence of zinc with other minerals or may be attributed to the increase in feed intake as the more this mineral has been consumed, the more they are deposited in the animal tissue (Mohanna and Nys 1999; Kwiecień, et al., 2016). Increase in the concentration of both Ca, P and Zn in the tibia of the high weight chick group may be associated with their better performance compared to the low weight group, due to higher feeding behavior activity in the high weight group on D0 which may have led to more nutrient intake and deposition of essential minerals in their bones.

5.6 Conclusion

Results revealed bodyweight variations among individual chicks used in the present study (CV>10%). High weight chicks had significantly lower relative crop weight compared to the low weight group. Behavioral activities revealed that the high weight group of chicks showed higher feeding tendency on D0 compared to the low weight group. Behavior data also indicated that the low weight group showed a high percentage engaged in litter consumption on D0 compared to other groups, which may be contributing to the differences observed in their bodyweight. Tissue mineral concentration indicated that the high weight chick groups are characterised by higher concentration of Ca, P and Zn in their bones compared to the low weight group. Taken together, the observed higher tendency for litter eating on day 0 in the LW group compared to the HW may be associated with their poor growth as feeding is very essential after hatch to facilitate nutrient utilisation from the yolk sack and gut function. Due to the litter eating habit of the low weight group, the next study was designed to evaluate the effect of two litter sources, hardwood (aspen) and softwood (pine) on chicks' growth performance.

Hardwood has been reported to be a potential source of xylo oligosaccharide (Fang, et al., 2022) which when consumed may be beneficial to the gut health and improve chicks' growth performance.

CHAPTER SIX

Trial 4

6.0 Bodyweight performance, gut digesta pH, tissue mineral profile and bursal transcriptomic profile of broiler chicks with varying bodyweights raised on two different litter materials in early weeks of life.

Preparing to be sent to Animals.

6.1 Abstract

The present study evaluated hardwood (Aspen) litter when compared with conventional softwood (pine wood) on bodyweight performance, gut pH, mineral and bursa transcriptomic profile of broiler chicks with low and high bodyweights on D0 to gain insights into the comparative effect of both wood sources. A total of 72-day old Ross 308 male chicks were distributed into 2 by 2 factorial arrangement with D0 body weight (High bodyweight – 'HBW', or Low bodyweight – 'LBW' and litter type (Hardwood - 'HW' or soft wood – 'SW' as factors). Chicks were raised in deep litter pens under controlled environmental management and commercial diet were fed ad libitum. Analyzed result used D0 bodyweight as a covariate and it revealed significant effect of bodyweight on day 7 bodyweight (<.0001) and day 14 bodyweight (0.03). However, there was significant effect of litter type on D14 (P = 0.03), D21(P = 0.03) BW, ADWG (P = 0.03) and BWC (0.03). Bodyweights were observed to be greater in hardwood litter treatment compared to softwood treatment. The result also revealed significant interaction between the effect of bodyweight and wood type on gizzard digesta pH, bone cesium and liver strontium. Litter type influenced bone manganese and had no effect on other bone and liver macro and trace minerals, with LBW group having higher concentration of bone manganese relative to the HBW group. The pH of the crop, gizzard and caecum were affected neither by bodyweight nor by litter type, the litter type influenced the gizzard ADF, whereby gizzard ADF was greater in softwood treatment relative to the hardwood treatment. The transcriptomic analysis revealed candidate genes expressed in the chicken bursa. HBW chicks on softwood relative to HBW chicks on hard wood had down regulation of CLDN20 and solute carrier family gene expressions in the bursal of Fabricius of experimental chicks, while LBW chicks on softwood relative to LBW on hard wood, had downregulation of the antioxidant gene (SOD3) expression which may have implication on the chicks' physiological functions

This study revealed that bodyweight weight differences and litter type both influenced chicks' bodyweights, bone manganese concentration, gizzard ADF and the expression of tight junction protein, antioxidant, and transporter genes.

Keywords: Bodyweight; gut pH; broilers; early life; litter; transcriptomic, bursal

6.2 Introduction

The profitability and success in broiler production depends on several contributory factors which include growth performance, gut health, environmental and nutritional factors, and gene expression. The previous study indicated that low weight broiler chicks consumed more litter material than feed on day of hatch compared to the high weight group which further impacted their weight performance when compared to their high weight counterparts.

Litter material in broiler production is an important criterion of the deep litter broiler production system to meet the health and welfare standards. It can influence production efficiency, health, and environmental impacts of broiler production (Dunlop et al., 2016), and the type of litter material has significant effect on chicks' performance (Malone, et all., 1982) Litter source and type also impact chicks' intestinal morphology and microbial population (Taherparvar, et al., 2016). It has been reported that bodyweight of chicks decreased when litter consumption was above 4% of the diet. It was speculated that hard wood and soft wood with varying compositional and technical attributes may affect the immune status of broiler chicks and therefore growth performance in the early life.

Transcriptomic analysis has been used as a tool in measuring the expression of an organism's genes in different tissue to know how genes are regulated (Lowe, et al., 2017). It also determines the function of previously unannotated genes (Lowe, et al., 2017), in understanding how gene expression changes in different organisms, and how it affects physiological functions and diseases

(Khodadadian, et al., 2020). In the present study, it was speculated that hard wood (aspen) would influence the expression of genes associated with immune response in chick when compared with the standard wood shave (pine). In the present study, the bursal of Fabricius was investigated because of its association with the immune system of broilers. The bursa of Fabricius is a lymphoid organ that specifically plays a vital role in developing immunity against several diseases in chickens especially gumboro disease and differentiation of B-lymphocytes (Cazaban, et al., 2015; Raji, et al., 2017).

In the previous study (Chapter 5), it was reported that the low weight chicks consumed more litter materials (pine shave) on D0 post hatch compared to their high weight counterparts, which may have played a role in their slow growth performance. Chicks normally peck at and consume litter material throughout their rearing stage, and this could affect the intestinal function through modulating the gut microbiota, thereby impacting growth performance depending on the type of litter material used (Malone and Chaloupka, 1983; Taherparvar, et al., 2016). Several intervention approaches have been investigated by researchers to evaluate the efficacy of feed additives, such as prebiotics, on broiler performance and gut health. The use of prebiotics such as such as mannan and xylo oligosaccharides which are fermented by the hind gut microbes have been reported to improve the feed conversion ratio, villus length and increases the number of beneficial microbes in chicken gut (Ferket, 2004; Baurhoo, et al., 2007; De Maesschalck, et al., 2015).

Both softwood (pine) and hardwood (aspen) are commonly used in broiler production, however, aspens and silver birch hardwoods are high in oligosaccharides; a fibre fraction that is usually added to broiler diets to enhance the development of a healthy gut (Fang, et al., 2022). Thus, the current study evaluated the efficacy of hardwood (Aspen) and softwood (pine) to improve the bodyweight performance of chicks and other physiological traits, especially those of a low weight group, through its beneficial action in the gut. Due to the speculation from previous trial that the contributing factor to the higher concentration of heavy metals (Cadmium, lead and caesium) in the UP chicks maybe arising from the

increased consumption of softwood litter substrates, the hypothesis of the current study was that hardwood litter would influence the growth performance, gut pH, tissue mineral concentrations and bursal gene expression especially in the low weight chicks group.

6.3 Materials and methods

6.3.1 Ethical Approval and location of experiment

The animal trial was conducted at the Nottingham Trent University poultry research unit, Brackenhurst campus, while all laboratory analysis was carried out at the University of Nottingham, Sutton Bonington Campus. All the experimental protocols used in the study were approved by the University of Nottingham (Reference code: UON44) and Nottingham Trent University Ethical Committee (Reference code: ARE1736110).

6.3.2 Birds, Housing and Diets

A total of 72 one day old Ross 308 male chicks were used for the trial and were distributed into 2 by 2 factorial arrangement with D0 body weight (High bodyweight– 'HBW', or Low bodyweight– 'LBW' and litter type (Hardwood - 'HW' or soft wood – 'SW' as factors. Broiler chicks were fed starter crumbs formulated at the Nottingham Trent University, Poultry Research Unit, Brackenhurst Campus. The formulated diet met the nutrient specification of 7days old broilers, the diet composition is shown in appendix 12. During the experimental period, chicks were allowed *ad libitum* access to feed and water. Water was offered *ad libitum* using nipple drinkers, and environmental conditions kept at standard throughout the experimental period according to the EU Council Directive 2007/43/EC. Lighting with an intensity of 20 lux during the lighting period was allowed, measured at bird eye level, and illuminating at 80% of the usable area. The lighting protocol followed 24 hours rhythm, including periods of darkness which lasted six hours in total. The temperature of the rearing house was maintained at 30^0C on the day of placement and gradually reduced over the course of a 27-day period by 20^0C, according to the Ross management manual 2018. The relative humidity was maintained at 60-70% from days 0-2 and

above 50% thereafter as recommended in Ross management manual, 2018 (Avigen, 2018).

6.3.3 Bird grouping and sample collection protocol

Birds were grouped into four treatments namely, treatment A: high weight on hardwood, treatment B: high weight on softwood, treatment C: low weight on hardwood and treatment D: low weight on softwood. Bodyweight performance was recorded once weekly from D7 to D21, and the average daily weight gain and bodyweight change were calculated at the end of the experiment. On day 21, postmortem pH readings were taken in situ from the proximal crop and gizzard, and distal caecum of each sampled chick using a digital pH piercing probe (Apera instruments PH60S spear pH tester).

6.3.4 Bone ash and mineral analysis

The legs collected from the sampled chicks were cleaned to extract the tibial bones. The tibias from each group were defatted by soaking bones in petroleum ether for two hours and then allowed to dry in the fume cupboard to expel PET ether residues. The percentage ash content and mineral analysis were determined using the methods described previously (Elvis-Chikwem, C.L., et al., 2024).

6.3.5 Liver sample digestion and mineral analysis

Liver samples from each sampled chick were freeze dried using a freeze drier (Thermo Savant SuperModulyo) at the temperature setting of -45°C for one week prior to digestion. The sample digestion and mineral analysis followed the same protocol described previously (Elvis-Chikwem et al., 2024).

6.3.6 RNA extraction and microarray analysis

RNA was extracted from the bursa of 21-day old broiler chicks using the Direct-zol™ RNA MiniPrep Kit (Cambridge Bioscience, UK), following the manufacturers' instructions. Extracted RNAs were stored in a suitable condition before being transported to the Nottingham Arabidopsis Stock Centre for microarray analysis. Microarray analysis was performed following the same protocol in chapter 3 (trail 1).

6.3.7 Statistical analysis

Data from this study were analyzed using the GLM procedure of SAS, D0 bodyweight were used as a covariate for the litter type to account for the differences in the initial bodyweight of the chicks. The student t-test (Prism version 9.0.0 for Windows, GraphPad Software, San Diego, California USA, www.graphpad.com) was used for contrasts between the HW and LW groups for liver and bone mineral profile data, while significant differences were observed at $p<0.05$. Functional annotation analysis for the bursal transcriptomic data was carried out using DAVID online database https://david.ncifcrf.gov/conversion.jsp?VFROM=NA

6.4 Results

6.4.1 Bodyweight performance and gut pH parameters

The body weight parameters and gut pH results from this study are presented in table 6.1 and 6.2 below. D0 bodyweight was used as covariate in data analysis, and the results revealed significant effect of bodyweight on day 7 ($P <.0001$) and 14 ($P = 0.03$) BW. There was no significant effect ($P > 0.05$) of bodyweight on day 21 bodyweight (D21BW), average daily weight gain (ADWG) bodyweight change (BWC), crop digesta pH, gizzard digesta pH and cecal digesta pH. Similarly, there was a significant effect of litter type on D14 ($p = 0.03$), D21 ($p = 0.03$) bodyweights, ADWG (0.03) and BWC (0.03). Significant interaction ($p =0.02$) between bodyweight and litter type on the gizzard pH was observed.

Table 6.1: Simple statistics for weekly bodyweight performance of 72 male Ross 308 broiler chicks.

Age	Mean bodyweight (g)	Min	Max	SD	CV (%)
D0	38.07	31.8	48	4.84	12.71
D7	109.76	62.6	155.2	20.51	18.69
D14	269.41	117.3	407.2	59.96	22.25
D21	638.98	286	962	142.39	22.28

Min: Minimum. Max: maximum, SD: standard deviation, CV: coefficient of variation

Table 6.2: Bodyweight Performance and Gut digesta pH of broiler chicks with varying bodyweights raised on different litter materials (n =9 per treatment group)

Parameters	Bodyweight			Litter			P-value		Interaction
	HBW	LBW	SE	HW	SW	SE	Bodyweight	Litter	BW × Litter
Initial weight (g)	40.9	35.3	-	33.8	36.8	-	-	-	-
D7 BW (g)	121.9	99.9	±3.5	115.4	106.5	±3.5	***	0.09	-
D14 BW (g)	288.2	248	±12.4	287.2	248.9	±11.7	*	*	-
D21 BW (g)	681.6	597.7	±32.9	689.1	590.2	±29.2	0.08	*	-
ADWG (g)	30.5	26.8	±1.5	31	26.3	±1.4	0.09	*	-
BWC (g)	640.7	562.5	±32.5	651	552.1	±29.2	0.09	*	-
Crop pH	5.2	5.2	±0.1	5.3	5.1	±0.1	0.61	0.32	-
Gizzard pH	3.4	3.5	±0.1	3.5	3.4	±0.1	0.79	0.16	*
Cecal pH	6.2	6.10	±0.1	6.1	6.1	±0.1	0.39	0.25	-
Gizzard ADF (%)	26.07	25.86	±2.2	23.29	28.64	±2.2	0.923	*	-

BW: bodyweight, HBW: high bodyweight, LBW: low bodyweight, HW: hardwood, SW: softwood, SE: standard error, ADWG: average daily weight gain, BWC: bodyweight change, * denotes p-value ≤ 0.05, *** denotes p-value ≤0.0001.

6.4.2 Liver mineral profile

The liver macro and trace mineral profiles of the treatment groups are shown in table 6.3 and 6.4 below. There were no significant effect ($P > 0.05$) of bodyweight and litter type on the liver mineral profile of high and low weight chicks reared on hardwood and softwood litter material.

Table 6.3: Liver macro mineral concentration of the high and low bodyweight chicks in hard and softwood litter material (n= 9 per treatment group)

Parameters	Bodyweight			Litter			P-value	
Mineral concentration. (g/kg)	HBW	LBW	SE	HW	SW	SE	Bodyweight	Litter
Ca	0.2	0.2	±0.02	0.16	0.18	±0.02	0.478	0.421
P	14.5	14.8	±0.44	14.7	14.6	±0.44	0.517	0.776
Na	4.4	4.6	±0.24	4.4	4.6	±0.24	0.372	0.591
Mg	9.6	9.7	±0.31	9.6	9.6	± 0.31	0.767	0.602
K	16.0	15.9	±4.90	15.2	15.8	±4.90	0.862	0.528
S	9.3	9.8	±5.19	9.9	9.2	±5.19	0.362	0.169

BW: bodyweight, HBWG: high bodyweight group, LBWG: low bodyweight group, HW: hardwood, SW: softwood, SE: standard error, * denotes p-value ≤ 0.05, ** denotes p-value ≤ 0.001.

Table 6.4: Liver trace mineral concentration of the high and low bodyweight chicks in hard and softwood litter material (n = 9 per treatment group)

Parameters	Bodyweight			Litter			P-value		Interaction
Mineral concentration (g/kg)	HBW	LBW	SE	HW	SW	SE	Bodyweight	Litter	BW × Litter
Zn	91.34	92.23	±4.11	92.91	90.66	±4.11	0.832	0.589	-
Fe	646.6	584.7	±105.0	584.0	647.4	±105.0	0.56	0.55	-
Cu	15.44	16.33	±1.07	16.46	15.31	±1.07	0.410	0.293	-
Mn	13.33	12.99	±0.79	13.87	12.45	±0.79	0.672	0.082	-
Cd	0.08	0.08	±0.007	0.08	0.07	±0.007	0.543	0.219	-
Pb	0.02	0.03	±0.02	0.04	0.02	±0.02	0.790	0.169	-
Cs	0.015	0.016	±0.001	0.015	0.017	±0.001	0.353	0.211	-
Co	0.04	0.04	±0.03	0.04	0.04	±0.03	0.073	0.934	-
Mo	2.64	2.70	±0.14	2.63	2.68	±0.14	0.797	0.689	-
Sr	0.02	0.02	±0.04	0.01	0.03	±0.04	0.937	0.542	*

BW: bodyweight, HBWG: high bodyweight group, LBWG: low bodyweight group, HW: hardwood, SW: softwood, SE: standard error, * denotes p-value ≤ 0.05, ** denotes p-value ≤0.001.

6.4.3 Bone mineral profile

The bone macro and trace mineral profiles of the treatment groups are shown in table 6.5 and 6.6 below. Bodyweight showed significant ($P<0.05$) effect on bone manganese with the LW group having higher concentration of this mineral in their bone compared to the HW group. There were no significant effect ($P > 0.05$) of bodyweight and litter type on the macro and other trace minerals tested in the high and low weight chicks reared on hardwood and softwood litter material.

Table 6.5: Bone macro mineral concentration of the high and low bodyweight chicks in hard and softwood litter material (n = 18 per treatment group)

Parameters	Bodyweight			Litter			P-value	
Mineral concentration. (g/kg)	HBW	LBW	SE	HW	SW	SE	Bodyweight	Litter
Ash (%)	43.4	44.1	±2.0	44.7	42.8	±2.0	0.72	0.36
Ca	338.7	344.7	±13.1	345.7	337.7	±13.1	0.65	0.54
P	182.3	188.0	±7.0	187.0	183.3	±7.0	0.42	0.60
Na	101.9	103.9	±0.4	103.7	102.2	± 0.4	0.67	0.73
Mg	7.7	7.9	±0.3	7.9	7.8	±0.3	0.35	0.78
K	8.6	8.9	±3.6	8.6	8.9	±3.6	0.54	0.45
S	2.5	2.7	±2.1	2.5	2.7	±2.1	0.31	0.28

BW: bodyweight, HBWG: high bodyweight group, LBWG: low bodyweight group, HW: hardwood, SW: softwood, SE: standard error, * denotes p-value ≤ 0.05, ** denotes p-value ≤ 0.001.

Table 6.6: Bone trace mineral concentration of the high and low bodyweight chicks in hard and softwood litter material (n= 18 per treatment group)

Parameters	Bodyweight			Litter			P-value		Interaction
Mineral concentration(mg/kg)	HBW	LBW	SE	HW	SW	SE	Bodyweight	Litter	BW × Litter
Zn	386.5	398.4	±15.56	392.5	392.4	±15.56	0.448	0.993	-
Fe	264.0	282.3	±17.52	273.6	272.7	±17.52	0.299	0.958	-
Mn	15.19	22.24	±2.22	17.70	19.72	±2.22	**	0.368	-
Cd	0.03	0.03	±0.01	0.032	0.03	±0.01	0.760	0.690	-
Pb	0.23	0.34	±0.06	0.26	0.33	±0.06	0.059	0.226	-
Cs	0.039	0.050	±0.01	0.045	0.05	±0.01	0.064	0.951	*
Co	0.04	0.05	±0.00	0.05	0.04	±0.00	0.638	0.456	-
Cu	2.15	2.49	±0.19	2.39	2.25	±0.19	0.089	0.485	-
Mo	0.92	0.93	±0.06	0.95	0.89	±0.06	0.927	0.317	-
Sr	185.70	191.60	±10.02	192.60	184.60	±10.02	0.559	0.429	-

BW: bodyweight, HBWG: high bodyweight group, LBWG: low bodyweight group, HW: hardwood, SW: softwood, SE: standard error, * denotes p-value ≤ 0.05, **denotes p-value ≤0.001.

6.4.4 Bursal transcriptomic profile of 21days old broiler chicks

In this study, the bursal transcriptomic profiles of broiler chicks raised in two different litter substrates identified total number of 138 DEGs (differentially expressed genes) with a foldchange of ≥1.50 and P<0.05 in the high weight group raised on softwood and hardwood. The low weight group on softwood versus low weight on hardwood revealed total of 21 DEGs with foldchange of ≥ 1.5 and P- value of P<0.05 in the softwood and hardwood group. The list of the DEGs obtained in the high weight group raised on softwood versus hardwood material are presented in table 6.7, while table 6.8 presented the DEGs obtained from the low weight group on softwood versus hardwood with the same fold change. Functional annotation revealed two main annotation clusters and gene ontology related to biological function, cellular component, and molecular function. For the high weight group on softwood and hardwood, two clusters were indicated with an enrichment score of ≥ 2.0. Cluster 1 (enrichment score of 2.17) relates to axon, voltage-gated sodium, neuronal action, and sodium ion transmembrane. Cluster 2 with an enrichment score (0.03) relates to RNA polymerase 11 core promoter proximal region sequence-specific DNA binding, RNA polymerase 11 transcription factor activity, sequence-specific DNA binding, DNA binding and nucleus.

Table 6.7: Differential expressed genes (DEGs) in the bursal of 21 days old broiler chicks ranked as high body weight raised on softwood versus hardwood litter material (Foldchange values ≥1.5 and ≤ -1.5)

Gene Symbol	**p-value (HW + softwood vs. HW + hardwood)**	**Fold-Change (HW + softwood vs. HW + hardwood)**	**Fold-Change (HW + softwood vs. HW + hardwood) (Description)**
LOC428754	0.001085	-2.01192	HW + softwood down vs HW + hardwood
GKN1	0.001574	-1.63503	HW + softwood down vs HW + hardwood
DYTN	0.001605	-1.54394	HW + softwood down vs HW + hardwood
PGRMC1	0.002555	1.62115	HW + softwood up vs HW + hardwood
ARFGAP3	0.002766	1.73118	HW + softwood up vs HW + hardwood
AGTR1	0.003032	-1.56467	HW + softwood down vs HW + hardwood
NDUFB6	0.003433	1.52123	HW + softwood up vs HW + hardwood
SLC5A7	0.003884	-1.6894	HW + softwood down vs HW + hardwood
FAM155A	0.004002	-1.59896	HW + softwood down vs HW + hardwood
TMEM27	0.00576	-1.71605	HW + softwood down vs HW + hardwood
MIR1800	0.00672	-1.51689	HW + softwood down vs HW + hardwood
HNMT	0.007823	-1.52682	HW + softwood down vs HW + hardwood
PLA2G4E	0.008474	-1.60493	HW + softwood down vs HW + hardwood

OSCP1	0.008476	2.57668	HW + softwood up vs HW + hardwood
FAM20C	0.00885	1.51537	HW + softwood up vs HW + hardwood
MAPK11	0.00898	2.1445	HW + softwood up vs HW + hardwood
KCNA4	0.009765	-1.65128	HW + softwood down vs HW + hardwood
LRRC3B	0.00998	-1.96111	HW + softwood down vs HW + hardwood
LOC101747717	0.010208	-1.66856	HW + softwood down vs HW + hardwood
CLDN20	0.01033	-1.6476	HW + softwood down vs HW + hardwood
MIR3538-1	0.010544	-1.8932	HW + softwood down vs HW + hardwood
IFI27L2	0.010745	1.93054	HW + softwood up vs HW + hardwood
F7	0.011191	-1.87265	HW + softwood down vs HW + hardwood
LOC770890	0.011462	-1.85137	HW + softwood down vs HW + hardwood
IL1RAPL2	0.011905	-1.76217	HW + softwood down vs HW + hardwood
LOC415844	0.01195	-1.71104	HW + softwood down vs HW + hardwood
CCDC96	0.012155	-1.93577	HW + softwood down vs HW + hardwood
LOC770271	0.012434	-1.70853	HW + softwood down vs HW + hardwood
CNR1	0.012467	-1.65916	HW + softwood down vs HW + hardwood
KCNQ1	0.01265	1.89293	HW + softwood up vs HW + hardwood
PLEKHG1	0.013405	1.55678	HW + softwood up vs HW + hardwood

RFESD	0.013648	-1.50935	HW + softwood down vs HW + hardwood
PNOC	0.013791	-1.50849	HW + softwood down vs HW + hardwood
MIR1710	0.013798	-2.14496	HW + softwood down vs HW + hardwood
LOC423786	0.013932	-1.78996	HW + softwood down vs HW + hardwood
KRT15	0.014534	1.63033	HW + softwood up vs HW + hardwood
SCN3A	0.015001	-1.99209	HW + softwood down vs HW + hardwood
PKD2L1	0.015337	-1.6216	HW + softwood down vs HW + hardwood
MIR1760	0.015608	-1.60133	HW + softwood down vs HW + hardwood
FAM19A2	0.015639	-2.39609	HW + softwood down vs HW + hardwood
MIR15B	0.016231	-1.75606	HW + softwood down vs HW + hardwood
LOC101749877	0.016524	-1.58909	HW + softwood down vs HW + hardwood
DCT	0.016886	-1.62336	HW + softwood down vs HW + hardwood
LOC421690	0.01814	-1.52421	HW + softwood down vs HW + hardwood
RNF128	0.018162	1.87843	HW + softwood up vs HW + hardwood
OCA2	0.01827	-1.60735	HW + softwood down vs HW + hardwood
LOC421285	0.018379	-1.55193	HW + softwood down vs HW + hardwood

IAH1	0.019064	1.54157	HW + softwood up vs HW + hardwood
LOC427595	0.019728	-1.60434	HW + softwood down vs HW + hardwood
LMO4	0.019978	1.72245	HW + softwood up vs HW + hardwood
IL13RA2	0.020027	-1.78799	HW + softwood down vs HW + hardwood
SERPINA9	0.02011	-1.62463	HW + softwood down vs HW + hardwood
WASF3	0.02025	-1.5672	HW + softwood down vs HW + hardwood
C6ORF165	0.020756	-1.62311	HW + softwood down vs HW + hardwood
EXOC3L4	0.020804	1.5115	HW + softwood up vs HW + hardwood
ABCA13	0.021056	-1.5436	HW + softwood down vs HW + hardwood
ACOT9	0.021202	1.89459	HW + softwood up vs HW + hardwood
ZSWIM8	0.021388	1.50916	HW + softwood up vs HW + hardwood
SEC23A	0.021811	1.53028	HW + softwood up vs HW + hardwood
KCNS3	0.021889	-1.80816	HW + softwood down vs HW + hardwood

MIR1462	0.022136	-1.81556	HW + softwood down vs HW + hardwood
STYK1	0.022648	1.58006	HW + softwood up vs HW + hardwood
MOV10L1	0.022765	-1.67881	HW + softwood down vs HW + hardwood
GSC2	0.023064	-1.50068	HW + softwood down vs HW + hardwood
ATP10A	0.023222	1.57945	HW + softwood up vs HW + hardwood
MIR1574	0.023366	1.51696	HW + softwood up vs HW + hardwood
TSSK3	0.023379	-1.51601	HW + softwood down vs HW + hardwood
TRAPPC2	0.023797	1.51576	HW + softwood up vs HW + hardwood
HCN1	0.023914	-1.66476	HW + softwood down vs HW + hardwood
LRRN3	0.023953	-1.75289	HW + softwood down vs HW + hardwood
MIR1622	0.024034	-1.88817	HW + softwood down vs HW + hardwood
BG8	0.025206	2.46218	HW + softwood up vs HW + hardwood
MSX1	0.025427	-1.74518	HW + softwood down vs HW + hardwood
MIR128-2	0.02575	-1.54952	HW + softwood down vs HW + hardwood
IL17REL	0.026222	1.93664	HW + softwood up vs HW + hardwood
LOC101748302	0.02636	-1.62263	HW + softwood down vs HW + hardwood

RABIF	0.026506	-1.52357	HW + softwood down vs HW + hardwood
TMC3	0.026697	-1.65684	HW + softwood down vs HW + hardwood
MBOAT2	0.026766	2.02594	HW + softwood up vs HW + hardwood
CERKL	0.027173	-1.50242	HW + softwood down vs HW + hardwood
RPL36	0.027192	1.54271	HW + softwood up vs HW + hardwood
MIR18A	0.027327	-1.97124	HW + softwood down vs HW + hardwood
FAM102B	0.029252	1.83031	HW + softwood up vs HW + hardwood
AGXT2	0.029852	-1.73838	HW + softwood down vs HW + hardwood
LOC101748693	0.029987	-1.63912	HW + softwood down vs HW + hardwood
CASR	0.030005	-1.64782	HW + softwood down vs HW + hardwood
CCDC149	0.030706	1.51954	HW + softwood up vs HW + hardwood
RANBP3L	0.030752	1.97449	HW + softwood up vs HW + hardwood
CAMK1G	0.030837	-1.65574	HW + softwood down vs HW + hardwood
MIR1692	0.031172	1.54529	HW + softwood up vs HW + hardwood
NRAP	0.031413	-1.52098	HW + softwood down vs HW + hardwood
GALNT12	0.031692	1.83296	HW + softwood up vs HW + hardwood

GAL3ST1	0.031992	-1.52845	HW + softwood down vs HW + hardwood
SLC25A21	0.032221	-1.51396	HW + softwood down vs HW + hardwood
LOC396380	0.032879	1.58805	HW + softwood up vs HW + hardwood
SLC13A5	0.032982	-1.57144	HW + softwood down vs HW + hardwood
PPDPF	0.033054	1.75076	HW + softwood up vs HW + hardwood
F2	0.033306	-1.57644	HW + softwood down vs HW + hardwood
LAG3	0.033587	-1.50166	HW + softwood down vs HW + hardwood
LOC770271	0.033594	-1.67121	HW + softwood down vs HW + hardwood
LOC423943	0.034319	-1.64497	HW + softwood down vs HW + hardwood
SRD5A2	0.034975	1.51195	HW + softwood up vs HW + hardwood
LOC418667	0.036133	1.55753	HW + softwood up vs HW + hardwood
ERMN	0.036767	-1.66166	HW + softwood down vs HW + hardwood
ACKR4	0.038269	1.53504	HW + softwood up vs HW + hardwood
TLDC2	0.038357	-1.55113	HW + softwood down vs HW + hardwood
FSHB	0.0385	-1.53514	HW + softwood down vs HW + hardwood
TTC6	0.038602	-1.72248	HW + softwood down vs HW + hardwood

SCN4A	0.039084	-1.71675	HW + softwood down vs HW + hardwood
AXDND1	0.039162	-1.6046	HW + softwood down vs HW + hardwood
C21H1ORF174	0.039303	1.58809	HW + softwood up vs HW + hardwood
TOMM6	0.039495	1.56065	HW + softwood up vs HW + hardwood
SLC43A2	0.039787	1.52742	HW + softwood up vs HW + hardwood
MAP2	0.0401	1.50398	HW + softwood up vs HW + hardwood
MIR133A1	0.040387	-1.52769	HW + softwood down vs HW + hardwood
LOC424334	0.040774	-1.59242	HW + softwood down vs HW + hardwood
LOC424511	0.040901	-1.60769	HW + softwood down vs HW + hardwood
FAM179A	0.041011	-1.55919	HW + softwood down vs HW + hardwood
OSTN	0.041379	-1.61104	HW + softwood down vs HW + hardwood
LINC00954	0.041805	-1.59704	HW + softwood down vs HW + hardwood
NUP210L	0.041812	-1.59154	HW + softwood down vs HW + hardwood
VAMP2	0.04193	-1.50239	HW + softwood down vs HW + hardwood
ACTR10L	0.042869	-1.54225	HW + softwood down vs HW + hardwood
LOC428831	0.043049	-1.99806	HW + softwood down vs HW + hardwood
TM4SF4	0.043658	-1.62003	HW + softwood down vs HW + hardwood

PSMG4	0.044373	1.61725	HW + softwood up vs HW + hardwood
C9H2orf72	0.045125	-1.78051	HW + softwood down vs HW + hardwood
TCEANC	0.045374	1.51883	HW + softwood up vs HW + hardwood
TM4SF1a	0.045705	-1.62179	HW + softwood down vs HW + hardwood
GRHL2	0.046176	1.50101	HW + softwood up vs HW + hardwood
A4GNT	0.046672	-1.55826	HW + softwood down vs HW + hardwood
FBXO16	0.04849	-1.53391	HW + softwood down vs HW + hardwood
FUT9	0.048631	1.8562	HW + softwood up vs HW + hardwood
HOXA9	0.048838	1.53379	HW + softwood up vs HW + hardwood
RP5-864K19.6	0.049238	1.79031	HW + softwood up vs HW + hardwood
MIR1679	0.049249	-1.59914	HW + softwood down vs HW + hardwood
FGF23	0.049486	-1.5854	HW + softwood down vs HW + hardwood
SLC7A4	0.049905	-1.50444	HW + softwood down vs HW + hardwood

Table 6.8: Differential expressed genes (DEGs) in the bursal of 21 days old broiler chicks ranked as low body weight raised on softwood versus hardwood litter material (Foldchange values ≥1.5 and ≤ -1.5)

Gene Symbol	p-value (LW + softwood vs. LW + hardwood)	Fold-Change (LW + softwood vs. LW + hardwood)	Fold-Change (LW + softwood vs. LW + hardwood) (Description)
MIR103A1	0.000759	1.75306	LW + softwood up vs LW + hardwood
ENPP1	0.009489	1.78838	LW + softwood up vs LW + hardwood
MOXD1	0.011126	1.54752	LW + softwood up vs LW + hardwood
LOC100858187	0.012214	-1.5104	LW + softwood down vs LW + hardwood
MIR1729	0.014129	-1.64784	LW + softwood down vs LW + hardwood
LOC101751764	0.015007	-1.8231	LW + softwood down vs LW + hardwood
LOC418667	0.01504	-1.57389	LW + softwood down vs LW + hardwood
MIR15B	0.01562	-1.59048	LW + softwood down vs LW + hardwood
PRLR	0.017175	1.71747	LW + softwood up vs LW + hardwood
THEMIS	0.017264	1.82936	LW + softwood up vs LW + hardwood
KBTBD11	0.02175	-1.55016	LW + softwood down vs LW + hardwood
EFEMP1	0.022909	-1.51426	LW + softwood down vs LW + hardwood
VWA2	0.023701	1.98073	LW + softwood up vs LW + hardwood
FAM179A	0.024458	1.51812	LW + softwood up vs LW + hardwood

C7	0.027656	-1.73527	LW + softwood down vs LW + hardwood
MIR1580	0.030301	-1.50594	LW + softwood down vs LW + hardwood
NOV	0.030399	-1.88593	LW + softwood down vs LW + hardwood
SOD3	0.03441	-1.66505	LW + softwood down vs LW + hardwood
MTNR1B	0.035628	-1.51091	LW + softwood down vs LW + hardwood
MTHFS	0.038368	-1.64992	LW + softwood down vs LW + hardwood
C4ORF33	0.045851	-2.02724	LW + softwood down vs LW + hardwood

Table 6.9: The main functional annotation clusters of gene ontology (GO) implicated by the differentially expressed genes in 21days old broilers ranked as high bodyweight raised on softwood and hardwood litter material.

Annotation	Enrichment score	GO term	Gene count	P-value
Cluster 1	2.17	GO: 0030424 - Axon	6	2.2E-3
		Voltage-gated sodium channel activity	3	3.7E-3
		Neuronal action potential	3	6.6E-3
		Sodium ion transmembrane transport	3	3.7E-2
Cluster 2	0.03	RNA polymerase 11 core promoter proximal region region sequence-specific DNA binding	3	9.1E-1
		Regulation of transcription from RNA polymerase 11 promoter	4	9.2E-1
		RNA polymerase 11 transcription factor activity, sequence-specific DNA binding	3	9.3E-1
		DNA binding	3	9.6E-1
		Nucleus	8	1.0E0

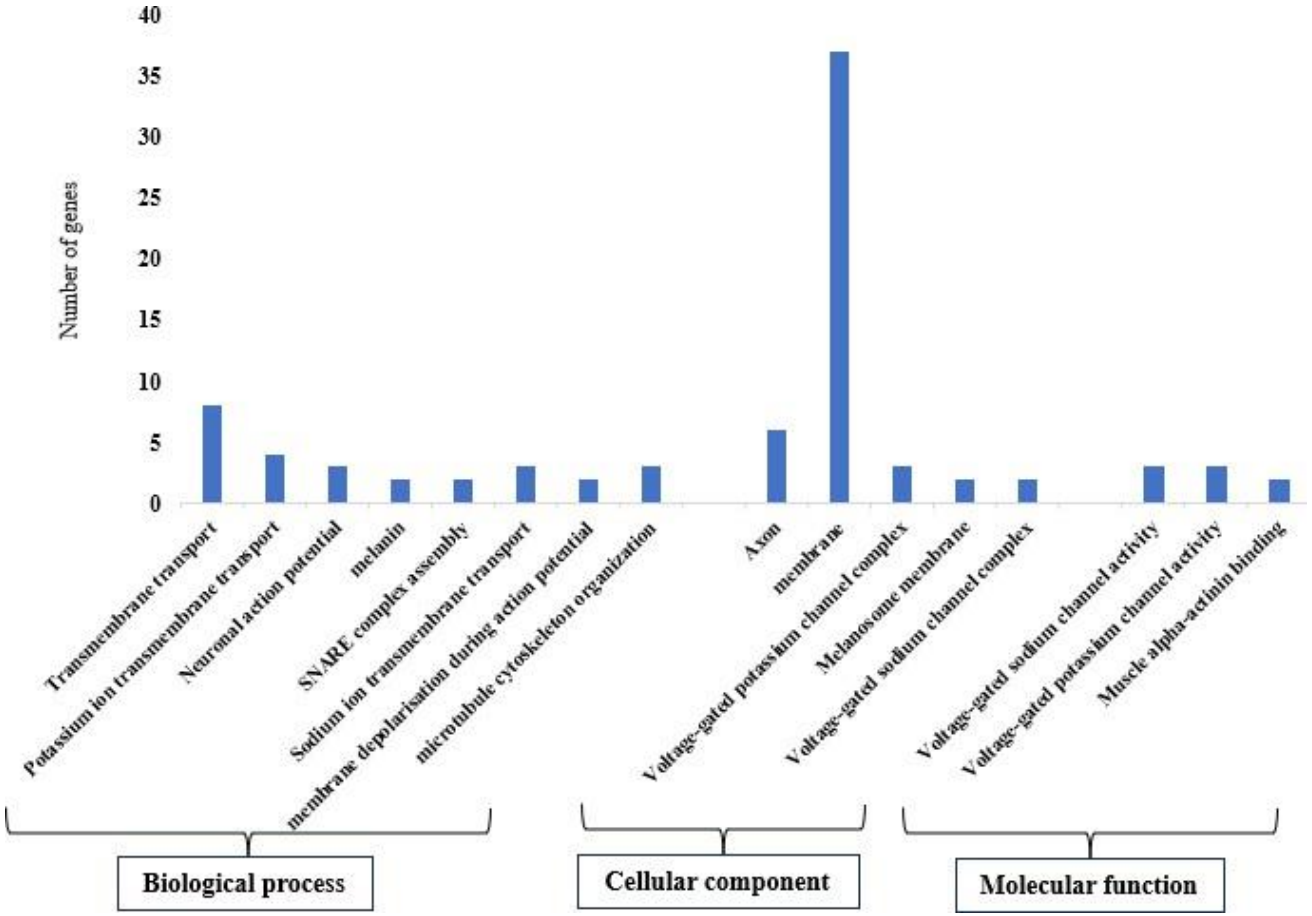


Figure 6.1: Functional annotation of the bursal differential expressed genes (DEGs) in 21 days broiler chicks ranked as high body weight raised on softwood and hardwood litter material.

Table 6.10: Enriched pathway impacted by softwood and hardwood litter material in the high weight chicks' group.

Pathway	No of genes	%	P-value	Differential expressed genes involved
Neuroactive ligand-receptor interaction	6	5.0	4.6E-2	Angiotensin 11 receptor type 1 (AGTR1), cannabinoid receptor 1 (CNR1), coagulation factor 11, thrombin (F2), follicle stimulating hormone beta subunit (FSHB), prepronociceptin (PNOC), progesterone receptor membrane component 1 (PGRMC1)

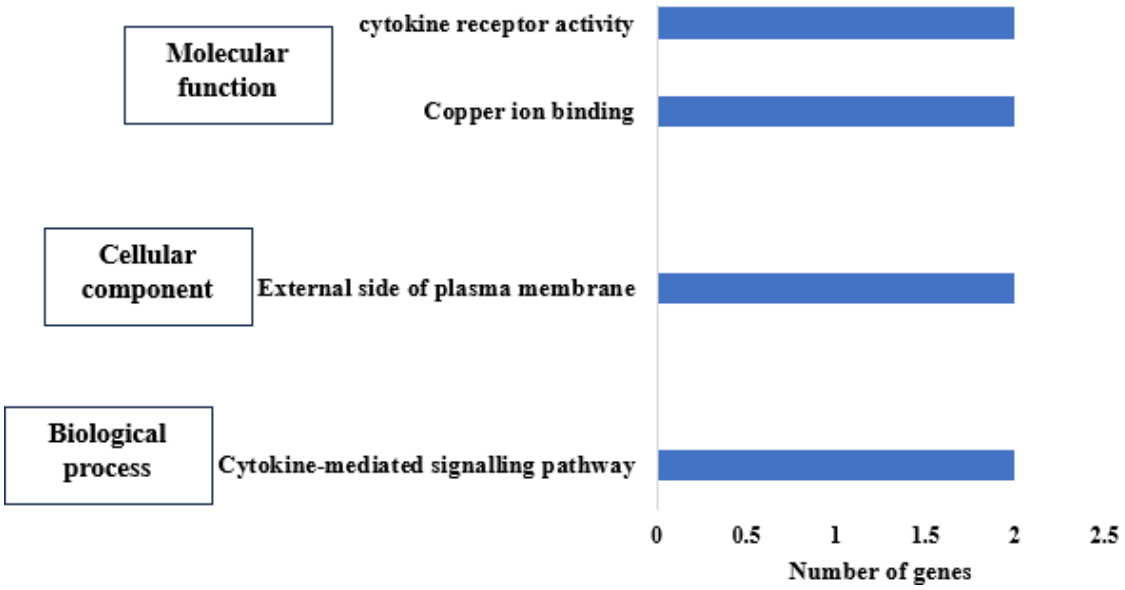


Figure 6.2: Functional annotation of the bursal differential expressed genes (DEGs) in 21 days broiler chicks ranked as low body weight raised on softwood and hardwood litter material.

6.5 Discussion

Hardwood litter was associated with an increase in BW, the significant effect of litter type on D14 observed in the present study agreed with the reports of (Ali, et al., 2009 and Torok et al., 2009) who reported a significant effect of litter material on the growth of broiler chicks on D14 post hatch. Bodyweight performance on D14 was greater (287.9g versus 248.9g) with hardwood treatment compared to softwood litter treatment.

The tissue mineral concentration results indicated that body weight and litter type used in broiler chick production have little impact on the concentration of mineral stored in the liver and bone. In the present study, litter type significantly influenced manganese (Mn) concentration in the bones of the experimental chicks with low body weight. This observation is in line with our previous report on light weight broilers having a higher concentration of manganese in their bones. The increase in bone manganese concentration in the low weight group could be attributed to the differences in individual chick intake and absorption of this mineral from the diet or the environment. Bone mineral concentrations were not significantly affected by bodyweight and litter treatments which agreed with the report of (Khan et al., 2023), who reported that broilers raised on different litter material shown no significant difference on their tibial Ca, Mg, K and P concentrations. The bursal transcriptomic analysis revealed several candidate genes expressed in the bursa of the experimental chicks. The functional annotation of these DEGs expressed in the bursa of the high weight chicks raised on softwood relative to high weight group raised on hard wood revealed they are mostly involved in transmembrane transport and involved in neuroactive ligand-receptor interaction pathway. Functional annotation of the DEGs expressed in the bursa of the low weight raised on softwood relative to low weight raised on hardwood indicated that these DEGs are mostly involved in cytokine activity and no pathway enriched in the comparison.

It is interesting to note that the tight junction gene (CLDN20) was down regulated by -1.64-fold change in high weight chicks on softwood when compared with those HW chicks bedded on softwood. CLDN20 is a protein coding gene, and a member of the claudins family. It plays a very crucial role in

signal transduction and serves as a barrier to prevent solute and water from passing freely through the paracellular space, due to its tight junction component (Niessen, 2007). The down regulation of CLDN20 in the high weight chicks raised on softwood could be associated with leaky gut problems (Braekatain, et al., 2018), compared to high weight group raised on hardwood, thus indicating the positive influence of hardwood on gut health in broiler chicks. Most of the important solute carrier family such as SLC5A7, SLC13A5, SLC74A were all down regulated in the high weight chicks raised on a softwood relative to the high weight group raised on hardwood. In this aspect, transport of molecules within and around cells may be affected by the downregulation of these solute carrier family members, in the high weight group raised on softwood litter.

In low weight chicks raised on softwood had the antioxidant gene (SOD3) downregulated. SODs are a group of molecules known to be involved in protecting cells against oxidation (Tikhonov, et al., 2016). It has been reported that SOD disproportionate superoxide free radicals both inside and outside the cells to protect the DNA and cell membrane from damage by free radicals (Tikhonov, et al., 2016). The downregulation of this gene in the low weight group may suggest less protection of DNA and cell membrane against free radicals in the low weight group which may be impacting its general growth performance.

6.6 Conclusion

Hardwood litter treatment influenced bodyweights on D14, and D21, as well as ADWG and BWC. The bone and liver mineral concentration were affected neither by bodyweight nor by litter treatments, except tibial manganese concentration which was higher in the low weight group relative to the high weight. The transcriptomic analysis revealed candidate genes expressed in the bursa of Fabricius of the experimental chicks. In particular, the low weight chicks on softwood treatment relative to the hardwood group had down regulation of SOD3 gene, which is an

important antioxidant genes in broilers. Also, the high weight group raised on softwood showed downregulation of important nutrient transporter genes from the solute carrier family, and the downregulation of CLDN20 an important tight junction protein coding gene. In addition, wood type influenced the expression of genes involved in gut integrity. Therefore, the choice of litter material in broiler production may play an important role in influencing growth performance in the early stage of broiler life.

CHAPTER SEVEN

7.0 General discussion

7.1 Thesis conclusion

The research hypothesis of this thesis was that broilers of common breed, diet and management with contrasting bodyweight in early life may have differences in their physiological and transcriptomic characteristics associated with the differences in their bodyweights. The main aim was to identify the physiological and transcriptomic differences in the high weight and low weight broilers raised under common environmental and diet conditions. Bodyweight of broiler chicks is an important parameter in determining the production and economic progress in broiler business. In a population of broilers, considerable variation in growth rate has been observed which often results to bodyweight variation and poor uniformity, impacting the economic potential and profitability in broiler business (Szőllősi, et al 2014). There has been a reportedly considerable variation between animal to animal in both growth, intestinal and nutrient utilization (Dono, 2012). Studies have indicated that that variation in growth performance of individual birds strongly relates to the differences observed in nutrient retention (Punna and Roland Sr, 1999) and altered differences in gene expression analysis especially the myogenic genes (Xiao, et al., 2017). Despite the high efficiency and rapid growth of modern broilers, there still exist low bodyweight uniformity and variations which are already visible in the first week of life, and up to 1.5kg in six weeks in one population (Piórkowska, et al., 2020). High variation in bodyweight in broilers could result in a significant economic loss in broiler business (Hughes, 2017; Gous, 2018).

In general, the body weight results from all trials indicated that the chicks with higher bodyweight on D0 consistently were heavier throughout each study period compared to the low weight group, indicating that chicks with higher bodyweight tend to eat more to meet up with growth and maintenance requirements and to sustain a more superior bodyweight than the lightweight chicks (Dono, 2012). There was a positive correlation between day 7 bodyweight with day 14 and 21 bodyweights compared to day

0. This may confirm the importance of day 7 bodyweight as the ultimate predictor of later weights in broiler chicks, and an indication that hatch weight is not as important as the day 7 bodyweight as previously reported by other researchers (Tona, et al., 2004b). The report of the current study contradicted the report of (Mendes, et al., 2011) who revealed that bird size and its weight on the first day of life are the most important factors of broiler performance. The results of this thesis showed that the low weight chicks had significantly heavier relative gizzard weight (trial 1) and crop weight (trial 3), the differences observed in the relative gizzard weight in the low weight chicks compare to the high weight group agreed with the findings of (Alshamy, et al., 2018; Mohammadigheisar, et al., 2020; Singh, et al., 2021; Gorenz, et al., 2024), who reported significantly heavier gizzard in the slow growing birds compared to the fast growing cobb. It has been reported that reduction in the visceral organ weight relative to bodyweight was associated with a higher growth rate in modern broiler breeds (Havenstein, et al., 2003). This was further attributed to a reduction in maintenance energy required and therefore an overall increase in energy utilization and efficiency (Tallentire, et al., 2016).

The low weight also showed significantly lower gizzard pH and NDF contents compared to the high weight group. The low gizzard digesta pH value obtained in the research study in the low weight broilers disagrees with the report of (Dono. 2012) who reported reduced digesta pH in high weight broiler chicks. However, (Angel et al., 2010) reported that intestinal pH changes with gastrointestinal section and age, which may explain the difference observed in the current study with that of (Dono, 2012). Taken together this may be an indication that the low weight group may have been consuming higher amount of litter substrate compared to the high weigh group which may have influenced the gizzard pH and resulted in higher gizzard fibre content. This speculation agrees with the reported literature which revealed that chicks with high consumption of litter during the first week of life had increased gizzard weight compared to the control (Malone and Chaloupka 1983). Secondly, relatively heavier gizzard, crop weight and reduced gizzard digesta pH in the low weight group could be

attributed to compensatory mechanism to make up for the poor intake and nutrient absorption. Low digesta pH has been reported to act as a defense mechanism against the growth of pathogenic microbes in the chicken gut (Hinton Jr, et al., 1990). The heavier and longer intestines observed in the high weight group compared to the low weight group could be attributed to differences in the absolute feed intake level and nutrient intake levels (Lamot, et al., 2019).

Mineral nutrition is an important aspect of broiler nutrition, they play a vital role in several physiological and metabolic activities in animals. Tissue mineral concentrations have been studied extensively and used as a tool to indicate mineral bioavailability at the tissue level (Black et al., 1984a, b). Therefore, higher tissue mineral concentration in broiler chicks may be an indication of its more readily availability in the animal tissue, which in turns reduces its excretion as waste (Miles,2000). The SP (high weight) chicks had higher concentration of bone ash, phosphorus, and sodium compared to the UP group. While the UP group had a higher concentration of heavy metals (cadmium, lead and cesium) in their tibias. The higher concentration of these heavy metals in the UP group compared to the SP group may be associated with their decreased rate of growth, as accumulation of cadmium in the tissue of broiler chicks are reported to cause decrease in growth rate of chicks (Akyolcu, et al., 2003). The higher concentration of heavy metals (Cadmium, lead and caesium) in the tibial of the UP group was speculated to result from increased litter consumption, this hypothesis was later tested in trial 3 as the low weight chicks showed higher tendency of litter consumption compared to the high weight group on D0. Interestingly, in trial 2 the low weight chicks' group also had higher concentration of manganese, cadmium and cesium in the liver tissue compared to the high weight group. In the bone tissue, the low weight had higher concentration of manganese and strontium compared to the high weight group. The significant higher concentration of cadmium in the liver tissue of the low weight chicks compared to the high weight group may further confirm that accumulation of this heavy metal in broiler tissues, especially cadmium may be associated with impaired growth leading to poor growth performance (Youness, et al.,

2012). The observed high concentration of bone and liver manganese in the low weight group in the present study was interesting and may be attributed to differences in the utilization and absorption of this mineral by individual chick as influenced by body size. This interesting finding in manganese accumulation in liver and bone tissues of the low weight group requires further mechanistic study to clearly understudy if this mineral accumulation in the tissue could serve as a better biomarker in understanding differences in chicks growth rates.

The consistent higher tibial concentration of phosphorus across trials 1 and 3, may further confirm the importance of this mineral and its influence on several physiological and metabolic processes in chicks, thus impacting growth rate. The significantly higher concentration of calcium, phosphorus and zinc in the high weight group in trial 3, may further prove that these minerals were associated with growth rates in broilers as they play important roles in the body metabolic and physiological processes such as bone metabolism, cell signaling and immune health (Proszkowiec-Weglarz and Angel, 2013).

Manganese concentration in the tibia was affected by bodyweight in trial 4, this followed the existing trend in the previous reports and was higher in the low weight group compared to the high weight chicks. This also confirmed from this thesis that the low weight chick group evaluated were characterized with higher concentration of manganese in their tissue compared to the high weight group which requires further research attention. Certainly, these results have opened a research pathway for further studies to better understand the reasons behind the consistent increase in tissue manganese and heavy metals in low weight chicks.

Transcriptomic analysis is vital in understanding and measuring the gene expression of an organism in tissues to understand how genes are regulated and how they affect physiological functions and diseases.

The high weight group showed higher expression of important cytokine, chemokine and antioxidant genes such as IL26, IL8L1, CCL17 and GSTA3. The increase in the expression of IL8L1, IL26 and CCL17 may be attributed to a more activated immune system in the SP group compared to the UP

group. The increase in expression of IL8L1 agreed with the report of (Bar-Shira, et al., 2006), who reported increase expression of IL8 gene in broiler chick during the first week of life and had been attributed to be triggered by exposure of the chicks to feed and the environment post hatch. The SP chicks may have been able to access feed and water more readily than the UP group on D0 which may have triggered the upregulation of the cytokines and chemokines, thus facilitating more active immune response in the SP group. The upregulation of the GSTA3 gene in the SP also may be associated with more active clearance of toxins compared to the UP group. From this study it has been revealed that chicks categorized as SP were characterized with upregulation of immune associated genes and gene involved in antioxidation. These may be contributing to a more immune system activation in the SP group compared to the UP group, thus influencing their growth rates. The important pathways such as calcium signaling, wnt signaling, cytokine-cytokine receptor interaction, and mucin type O-glycan, which were enriched in the SP chicks relative to UP may be associated with growth performance. Calcium signaling and wnt signaling pathways which were implicated in the SP group may be associated with their superior growth as these two pathways are involved in many signaling cascades and cell proliferation associated with growth and cell maintenance (Wang, et al, 2022).

It was interesting to observe the downregulation of transporter genes from the solute carrier family in low weight group raised on softwood compared to those raised on hardwood (trial 4). On the other hand, there was a downregulation of CLDN20, a tight junction protein in the high weight group raised on softwood compared to those raised on hardwood. These results confirm that softwood influenced the expression of these important solute transporters and an important tight junction protein. This may indicate that softwood litter may not be efficient in enhancing gut health of the chicks due to its downregulation of the important tight junction protein, which present hardwood substrate as a promising and more beneficial litter source in broiler production.

The behavioral result revealed that HW group had higher frequency of feeding on D0 compared to the

LW group. Early access to feed post hatch is very important for the new hatchlings. The HW group showed a higher tendency of feeding on D0 which could be an important factor contributing to their superior bodyweight when compared with their LW counterparts. In contrast, the low weight group had higher litter eating frequency on D0 compared to the high weight group. Litter consumption on D0 may lead to poor utilization of the yolk sac nutrient and poor gut function leading to detrimental effect on growth performance (Malone and Chaloupka, 1983). Reported literature revealed that litter eating may be a learned behavioural trait developed at the early stage of life (Malone and Chaloupka, 1982; Caldwell and McDaniel, 1982). This may be an indication that young hatchlings may be prone to litter eating during this period which often declines from day 14 upwards (Malone and Chaloupka, 1983). Based on the report from the present study it could be deduced that part of the contributory factors in chick's bodyweight differences may be associated with the consumption of wood shave substrates in early weeks of life.

7.2 Thesis recommendation

The results contained in this thesis demonstrated variable differences in digestive organ weight, tissue mineral profile and gene expression of broilers from common breed, diet and environmental conditions with contrasting bodyweights in early weeks of life and agreed with the proposed research hypothesis. It was also interesting to observe from this work that the high weight group of chicks were characterized with stronger immune activation compared to the low weight group and distinct tissue mineral profile. This research work has provided valuable knowledge on some physiological characteristics of chicks of the same breed reared under common environmental and diet conditions with variable bodyweights in early life. This thesis contributes to the knowledge and narrow the search into the important drivers of bodyweight variations in broiler chicks in the early weeks of life. It provided useful insights into the differences in digestive organ characteristics, tissue mineral concentrations, bone morphometry, gut pH, ileal and bursa transcriptomic profile of the low and high weight chicks in early weeks of life. This

thesis also provided some novel findings which may be relevant in designing more precise intervention strategies to optimize growth performance and uniformity in broilers in early life, which may have significant impact on the latter performance. It also revealed that hardwood litter substrate could serve as a more sustainable bedding source in broiler production.

7.3 Future research

The systemic accumulation of manganese, cadmium, and cesium in the low weight broiler population requires further mechanistic investigations to better understand the important drivers of the difference in concentrations of these minerals in their tissue and its association with growth performance. Further research is required to investigate the association between bone calcium and molecular calcium in the high and low weight chicks to understand its influence on growth performance. This study made use of human observers for monitoring chicks' behavioral activities which may be prone to human error, more research is needed to study the behavioral activity of the low and high weight groups using more accurate method such as digital scans to monitor behavioural activities.

7.4 Research limitations

The present study collected data from chicks raised in one pen which may be a potential source of limitation in the study, replication and larger sample size are recommended in further research to get more indebt knowledge of the wider chick population. This behaviour data collected from this research made use of human trained observers which may bring some bias into the data, it would be recommended to used digital and more accurate techniques to study the behaviour of the high and low weight broiler chicks.

CHAPTER EIGHT

8.0 References

"Number of chickens worldwide from 1990 to 2018". *Statista*. Archived *from the original on November 27, 2020*. Retrieved February 23, 2020

Abdollahi, M.R., Ravindran, V. and Svihus, B., (2013). Influence of grain type and feed form on performance, apparent metabolisable energy and ileal digestibility of nitrogen, starch, fat, calcium and phosphorus in broiler starters. *Animal Feed Science and Technology*, *186*(3- 4), pp.193-203.

Abdollahi, M.R., Zaefarian, F. and Ravindran, V., (2018). Feed intake response of broilers: Impact of feed processing. *Animal Feed Science and Technology*, *237*, pp.154-165.

Abro, M.R., Sahito, H.A., Memon, A., Soomro, R.N., Soomro, H. and Ujjan, N.A., (2012). Effect of various protein source feed ingredients on the growth performance of broiler. *Journal of Medicinal Plants Research*, *1*(4), pp.038-044.

Aderibigbe, A.S., Cowieson, A.J., Ajuwon, K.M. and Adeola, O., (2021). Contribution of purified soybean trypsin inhibitor and exogenous protease to endogenous amino acid losses and mineral digestibility. *Poultry Science*, *100*(12), p.101486.

Akyolcu, M.C., Ozcelik, D., Dursun, S., Toplan, S. and Kahraman, R., (2003). Accumulation of cadmium in tissue and its effect on live performance. In *Journal de Physique IV (Proceedings)* (Vol. 107, pp. 33-36). EDP sciences.

Al Hakeem, W.G., Acevedo Villanueva, K.Y. and Selvaraj, R.K., (2023). The Development of Gut Microbiota and Its Changes Following C. jejuni Infection in Broilers. *Vaccines*, *11*(3), p.595.

Ali, A., Devarajan, S., Manickavasagan, A. and Ata, A., (2022). Antinutritional factors and biological

constraints in the utilization of plant protein foods. In *Plant protein foods* (pp. 407-438). Cham: Springer International Publishing

Ali, M.A., Iji, P.A., MacAlpine, R. and Mikkelsen, L.L., (2009). The effect of litter material on productivity and health of broiler chickens. in *20th Annual Australian Poultry Science Symposium* (p. 77)

Alkhtib, A., Scholey, D., Carter, N., Cave, G.W., Hanafy, B.I., Kempster, S.R., Mekapothula, S., Roxborough, E.T. and Burton, E.J., (2020) Bioavailability of methionine-coated zinc nanoparticles as a dietary supplement leads to improved performance and bone strength in broiler chicken production. *Animals*, *10(9), p.1482.*

Alkhtib, A., Scholey, D., Carter, N., Cave, G.W., Hanafy, B.I., Kempster, S.R., Mekapothula, S., Roxborough, E.T. and Burton, E.J., (2020). Bioavailability of methionine-coated zinc nanoparticles as a dietary supplement leads to improved performance and bone strength in broiler chicken production. *Animals*, *10(9), p.1482.*

Alshamy, Z., Richardson, K.C., Hünigen, H., Hafez, H.M., Plendl, J. and Al Masri, S., (2018). Comparison of the gastrointestinal tract of a dual-purpose to a broiler chicken line: A qualitative and quantitative macroscopic and microscopic study. *PloS one*, *13*(10), p.e0204921.

Amoroso, C., Perillo, F., Strati, F., Fantini, M., Caprioli, F. and Facciotti, F., (2020). The role of gut microbiota biomodulators on mucosal immunity and intestinal inflammation. *Cells*, *9*(5), p.1234.

Angel R, Humphrey B, and Saylor W (2010). Age changes in gastrointestinal pH in broilers. *Journal of Dairy Science 93, 650.*

Angel R. (2007). Metabolic disorders: Limitations to growth of and mineral deposition into the broiler skeleton after hatch and potential implications for leg problems. *J. Appl. Poult. Res., 16: 138–149.*

Aniya, Y. and Imaizumi, N., (2011) Mitochondrial glutathione transferases involving a new function for

membrane permeability transition pore regulation. *Drug metabolism reviews*, 43(2), pp.292-299.

Ao, T., Pierce, J.L., Pescatore, A.J., Cantor, A.H., Dawson, K.A., Ford, M.J., Paul, M., (2011). Effects of feeding different concentration and forms of zinc on the performance and tissue mineral status of broiler chicks. *Br. Poult. Sci. 52, 466–471*.

Applegate, T.J. and Lilburn, M.S., (2002). Growth of the femur and tibia of a commercial broiler line. *Poultry science*, *81*(9), pp.1289-1294.

Applegate, T.J., 2012. Factors affecting feed intake–What do we know. In Proceeding of Arkansas. Nutrition Conference. *The Poultry Federation; Rogers, Arkansas, USA.*

Arnold, S.M., Zarnke, R.L., Lynn, T.V., Chimonas, M.A.R. and Frank, A., (2006). Public health evaluation of cadmium concentrations in liver and kidney of moose (Alces alces) from four areas of Alaska. *Science of the total environment*, *357*(1-3), pp.103-111

Arnould, C., Bizeray, D., Faure, J. M., & Leterrier, C. (2004). Effects of the addition of sand and string to pens on use of space, activity, tarsal angulations and bone composition in broiler chickens. *Animal welfare*, *13(1), 87-94*.

Ashmead, H.D. (1993). Factors which affect the intestinal absorption of minerals. In Ashmead H.D. (Ed.). The Roles of Amino Acid Chelates in Animal Nutrition. Westwood, NJ: Noyes Publications

Atencio A, Edwards HMJr, Pesti GM (2005a). Effects of vitamin D3 dietary supplementation of broiler breeder hens on the performance and bone abnormalities of the progeny. *Poultry Science 84:10581068*

Atencio A, Pesti GM, Edwards HM (2005b). Twenty-five hydroxycholecalciferols as a cholecalciferol substitute in broiler breeder hen diets and its effect on the performance and general health of the progeny. *Poultry Science 84:1277-1285*

Atencio A, Edwards HMJr, Pesti GM (2005c). Effects of the level of cholecalciferol supplementation of broiler breeder hen diets on the performance and bone abnormalities of the progeny fed diets containing various levels of calcium or 25-hydroxycholecalciferol. *Poultry Science 84:1593-1603*

Aviagen. (2018). Ross broiler management handbook. *https://aviagen.com/assets/Tech_Center/Ross_Broiler/Ross-BroilerHandbook2018-EN.pdf*

Aviagen. (2019). Ross 308 Ross 308 FF Performance Objectives. pp. 4

Aziz-Aliabadi, F., Hassanabadi, A., Golian, A., Zerehdaran, S. and Noruzi, H., (2023). Evaluation of the Effect of Different Levels of Fiber and Fat on Young Broilers' Performance, pH, and Viscosity of Digesta Using Response Surface Methodology. *Iranian Journal of Applied Animal Science*, *13*(2), pp.333-343.

Baarendse, P.J.J., Kemp, B. and Van Den Brand, H., (2006). Early-age housing temperature affects subsequent broiler chicken performance. *British Poultry Science, 47*(2), pp.125-130.

Bao, Y.M., Choct, M., Iji, P.A., Bruerton, K., (2010). Trace mineral interactions in broiler chicken diets. *Br. Poult. Sci. 51, 109–117.*

Bar Shira, E., Sklan, D. and Friedman, A. (2005) Impaired immune response in broiler hatchling following delayed access to feed. *Veterinary Immunology and Immunopathology105: 33- 45*

Barekatain, R., Chrystal, P.V., Howarth, G.S., McLaughlan, C.J., Gilani, S. and Nattrass, G.S., (2019). Performance, intestinal permeability, and gene expression of selected tight junction proteins in broiler chickens fed reduced protein diets supplemented with arginine, glutamine, and glycine subjected to a leaky gut model. *Poultry Science*, *98*(12), pp.6761- 6771.

Bar-Shira, E., D. Sklan, and A. Friedman. (2003). Establishment of immune competence in the avian galt during the immediate post-hatch period. *Dev. Comp. Immunol. 27:147- 157.* doi:10.1016/S0145-305X (02)00076-9

Bartel, D.P., (2004) MicroRNAs: genomics, biogenesis, mechanism, and function. *cell*, 116(2), pp.281-297.

Bartov, I., (1987). Effect of early nutrition on fattening and growth of broiler chicks at 7 weeks of age. *British poultry science*, *28*(3), pp.507-518.

Barua, M., Abdollahi, M.R., Zaefarian, F., Wester, T.J., Girish, C.K., Chrystal, P.V. and Ravindran, V., (2021). An investigation into the influence of age on the standardized amino acid digestibility of wheat and

sorghum in broilers. *Poultry Science*, *100*(11), p.101466.

Barua, M., Abdollahi, M.R., Zaefarian, F., Wester, T.J., Girish, C.K., Chrystal, P.V. and Ravindran, V., (2021). An investigation into the influence of age on the standardized amino acid digestibility of wheat and sorghum in broilers. *Poultry Science*, *100*(11), p.101466.

Barrow, P., Nair, V., Baigent, S., Atterbury, R. and Clark, M. eds., (2021). *Poultry health: a guide for professionals*. Cabi.

Batal, A. B. and C. M. Parsons. (2002a). Effects of age on nutrient digestibility in chicks fed different diets. Poult. Sci. 81:400- 407.

Batal, A. B. and C. M. Parsons. (2004). Utilization of various carbohydrate sources as affected by age in the chick. Poult. Sci. 83:1140-1147.

Batal, A. B., and C. M. Parsons. (2002b). Effects of age on nutrient digestibility in chicks fed different diets. Poult. Sci. 81:400-407. doi:10.1093/ps/81.3.400

Batal, A. B., and C. M. Parsons. (2004). Utilization of various carbohydrate sources as affected by age in the chick. Poult. Sci. 83:1140-1147. doi:10.1093/ps/83.7.1140

Batal, A.B. and Parsons, C.M., (2002b). Effect of fasting versus feeding oasis after hatching on nutrient utilization in chicks. *Poultry Science*, *81*(6), pp.853-859.

Baurhoo, B., Phillip, L. and Ruiz-Feria, C.A., (2007). Effects of purified lignin and mannan oligosaccharides on intestinal integrity and microbial populations in the ceca and litter of broiler chickens. *Poultry science*, *86*(6), pp.1070-1078.

Baykov, B.D., Stoyanov, M.P. and Gugova, M.L., (1996) Cadmium and lead bioaccumulation in male chickens for high food concentrations. *Toxicological & Environmental Chemistry*, 54(1-4), pp.155 159.

Bedford, M.R., Partridge, G.G., Hruby, M. and Walk, C.L. eds., (2022). *Enzymes in farm animal nutrition*.Cabi.

Bergstrom, K.S. and Xia, L., (2013) Mucin-type O-glycans and their roles in intestinal homeostasis. *Glycobiology*, 23(9), pp.1026-1037.

Berzina, N., Markovs, J., Isajevs, S., Apsite, M. and Smirnova, G., (2007). Cadmium-Induced Enteropathy in Domestic Cocks: A Biochemical and Histological Study after Subchronic Exposure. *Basic & clinical pharmacology & toxicology*, *101*(1), pp.29-34.

Bessei, W., (2006). Welfare of broilers: a review. *World's Poultry Science Journal*, *62*(3), pp.455- 466.

Bhuiyan, M.S.A., Amin, Z., Rodrigues, K.F., Saallah, S., Shaarani, S.M., Sarker, S. and Siddiquee, S., (2021). Infectious bronchitis virus (gammacoronavirus) in poultry farming: vaccination, immune response and measures for mitigation. *Veterinary sciences*, *8*(11), p.273.

Biggs, P., C. M. Parsons, and G. C. Fahey. (2007). The effects of several oligosaccharides on growth performance, nutrient digestibilities, and cecal microbial populations in young chicks. *Poult. Sci. 86:2327-2336.* doi:10.3382/ps.2007-00427

Bilgili, S. F., Montenegro, G. I., Hess, J. B., & Eckman, M. K. (1999). Live performance, carcass quality, and deboning yields of broilers reared on sand as a litter source. *Journal of Applied Poultry Research*, *8*(3), 352-361.

Bizeray, D., Leterrier, C., Constantin, P., Picard, M. and Faure, J.M., (2000). Early locomotor behaviour in genetic stocks of chickens with different growth rates. *Applied Animal Behaviour Science*, *68*(3), pp.231-242.

Black, J. R., C. B. Ammerman, P. R. Henry and R.D. Miles. (1984a). Tissue manganese uptake as ameasure of manganese bioavailability. Nutr. Rep.Inter. 29: 807-814.Black, J. R., C. B. Ammerman, P. R. Henry and R.D. Miles. 1984b. Biological availability ofmanganese sources and effects of high dietary manganese on tissue mineral composition of broiler-type chicks. Poultry Sci. 63: 1999-2006.

Blokhuis, H.J. and Haar, J.V.D., (1990). The effect of the stocking density on the behaviour of broilers.

Blokhuis, H.J., (1983). The relevance of sleep-in poultry. *World's Poultry Science Journal*, *39*(1), pp.33-37.

Blokhuis, H.J., (1984). Rest in poultry. *Applied Animal Behaviour Science*, *12*(3), pp.289-303.

Boehm, T. and Swann, J.B., (2014). Origin and evolution of adaptive immunity. *Annu. Rev. Anim. Biosci.*, *2*(1), pp.259-283.

Bohak, Z., (1970). Chicken pepsinogen and chicken pepsin. In *Methods in Enzymology* (Vol. 19, pp. 347-358). Academic Press.

Bokkers, E.A. and Koene, P., (2004). Motivation and ability to walk for a food reward in fast-and slow-growing broilers to 12 weeks of age. *Behavioural Processes*, *67*(2), pp.121, 130

Bokkers, E.A. and Koene, P., (2003). Behavior of fast-and slow growing broilers to 12 weeks of age and the physical consequences. *Applied Animal Behaviour Science*, *81*(1), pp.59-72.

Bokkers, E.A. and Koene, P., (2003). Behaviour of fast-and slow growing broilers to 12 weeks of age and the physical consequences. *Applied Animal Behaviour Science*, *81*(1), pp.59-72.

Boland, M.J., Rae, A.N., Vereijken, J.M., Meuwissen, M.P., Fischer, A.R., van Boekel, M.A., Rutherfurd, S.M., Gruppen, H., Moughan, P.J. and Hendriks, W.H., (2013). The future supply of animal-derived protein for human consumption. *Trends in food science & technology*, *29*(1), pp.62-73.

Bottje WG, Carstens GE, (2009). Association of mitochondrial function and feed efficiency in poultry and livestock species. *Journal of Animal Science. 87 (E. Suppl.): 48–63.*

Brickett, K.E., Dahiya, J.P., Classen, H.L. and Gomis, S., (2007). Influence of dietary nutrient density, feed form, and lighting on growth and meat yield of broiler chickens. *Poultry Science*, *86*(10), pp.2172-2181.

Bucław, M., (2016). The use of inulin in poultry feeding: a review. *Journal of animal physiology and animal nutrition*, *100*(6), pp.1015-1022

Buddington, R.K., (1992). Intestinal nutrient transport during ontogeny of vertebrates. *American Journal of Physiology-Regulatory, Integrative and Comparative Physiology*, *263*(3), pp. R503-R509.

Bunch, R. J., V. C. Speer, V. W. Hays, J. H. Hawbaker and D. V. Catron. (1961). Effects of copper sulfate, copper oxide and chlortetracyclineon baby pig performance. *J. Anim. Sci. 20: 723- 726*

Caldwell, D.K. and McDaniel, G.R., (1982). Performance of broilers reared on a plastic-coated metal floor and in a conventional environment. *Poult. Sci*, *61*, p.1371.

Calet C. (1965). The relative value of pellets versus mash and grain in poultry nutrition. *World Poult. Sci.J. 21: 23-52.*

Calini, F. and Sirri, F., (2007). Breeder nutrition and offspring performance. *Brazilian Journal of Poultry Science*, *9*, pp.77-83.

Carew Jr, L.B., Machemer Jr, R.H., Sharp, R.W. and Foss, D.C., (1972). Fat absorption by the very young chick. *Poultry Science*, *51*(3), pp.738-742.

Casalino, E., Calzaretti, G., Sblano, C. and Landriscina, C., (2002). Molecular inhibitory mechanisms of antioxidant enzymes in rat liver and kidney by cadmium. *Toxicology*, *179*(1-2), pp.3750.

Cazaban C, Masferrer NM, Pascual RD, Espadamala MN, Costa T, Gardin Y (2015). Proposed bursa of fabricius weight to body weight ratio standard in commercial broilers. Poult. Sci. 94(9):2088-2093.

Chaloupka, G.W., Malone, G.W. and Ritter, W.F., (1981). Processed newspaper litter particle size evaluation.

Chen, F., Wu, P., Shen, M., He, M., Chen, L., Qiu, C., Shi, H., Zhang, T., Wang, J., Xie, K. and Dai, G., (2019). Transcriptome analysis of differentially expressed genes related to the growth and development of the jinghai yellow chicken. *Genes*, *10*(7), p.539.

Chen, Y.P., Chen, X., Zhang, H. and Zhou, Y.M., (2013). Effects of dietary concentrations of methionine on growth performance and oxidative status of broiler chickens with different hatching weights. *British poultry science*, *54*(4), pp.531-537.

Cherian, G., (2015). Nutrition and metabolism in poultry: role of lipids in early diet. *Journal of animal science and biotechnology*, *6*, pp.1-9.

Clevers, H., (2006) Wnt/β-catenin signaling in development and disease. *Cell*, 127(3), pp.469-480.

Costa, V., Angelini, C., De Feis, I. and Ciccodicola, A., (2010). Uncovering the complexity of transcriptomes

with RNA-Seq. *BioMed Research International*, *2010*.

Cowieson, A.J. and Roos, F.F., (2016). Toward optimal value creation through the application of exogenous mono-component protease in the diets of non-ruminants. *Animal Feed Science and Technology*, *221*, pp.331-340.

Cowieson, A.J., Schliffka, W., Knap, I., Roos, F.F., Schoop, R. and Wilson, J.W., (2015). Meta- analysis of effect of a mono-component xylanase on the nutritional value of wheat supplemented with exogenous phytase for broiler chickens. *Animal Production Science*, *56*(12), pp.2014-2022.

Cui, X., Marshall, B., Shi, N., Chen, S.Y., Rekaya, R. and Liu, H.X., (2017). RNA-Seq analysis on chicken taste sensory organs: An ideal system to study organogenesis. *Scientific Reports*, *7*(1), p.9131.

Clavijo, V. and Flórez, M.J.V., 2018. The gastrointestinal microbiome and its association with the control of pathogens in broiler chicken production: A review. *Poultry science*, *97*(3), pp.1006-1021.

Danni, Z.H.O.U., Yi, Z.H.O.U., Pengguang, H.E., Lin, Y.U., Jinming, P.A.N., Lilong, C.H.A.I. and Hongjian, L.I.N., (2023). Development of an automatic weighing platform for monitoring bodyweight of broiler chickens in commercial production. *Frontiers of Agricultural Science & Engineering*, *10*(3).

de Jong, I.C., Fillerup, M. and Blokhuis, H.J., (2005). Effect of scattered feeding and feeding twice a day during rearing on indicators of hunger and frustration in broiler breeders. *Applied Animal Behaviour Science*, *92*(1-2), pp.61-76.

De Maesschalck, C., Eeckhaut, V., Maertens, L., De Lange, L., Marchal, L., Nezer, C., De Baere, S., Croubels, S., Daube, G., Dewulf, J. and Haesebrouck, F., (2015). Effects of xylo- oligosaccharides on broiler chicken performance and microbiota. *Applied and Environmental Microbiology*, *81*(17), pp.5880-5888.

Denbow, D. M. (2015). Gastrointestinal Anatomy and Physiology - Scanes, Colin G. Chapter 14 - Pages 337-366 in Sturkie's Avian Physiology (Sixth Edition) Academic Press, San Diego.

Denbow, D.M., (1994). Peripheral regulation of food intake in poultry. *The Journal of nutrition*, *124*, pp.1349S-1354S.

Derrien M, van Passel MWJ, van de Bovenkamp JHB, Schipper RG, de Vos WM, Dekker J. (2010) Mucin-Bacterial Interactions in the Human Oral Cavity and Digestive Tract. Gut Microbes 1:254–68. doi: 10.4161/gmic.1.4.12778

Dey, S., Saxena, A., Kumar, Y., Maity, T. and Tarafdar, A., 2022. Understanding the antinutritional factors and bioactive compounds of kodo millet (Paspalum scrobiculatum) and little millet (Panicum sumatrense). *Journal of Food Quality*, *2022*(1), p.1578448.

Diamond, J., (1991). Evolutionary design of intestinal nutrient absorption: enough but not too much. *Physiology*, *6*(2), pp.92-96.

Dibner JJ and Buttin P. (2002). Use of organic acids as a model to study the impact of gut microflora on nutrition and metabolism. Journal of Applied Poultry Research, 11: 453- 463.

Dibner, J.J., Knight, C.D., Kitchel, M.L., Atwell, C.A., Downs, A.C. and Ivey, F.J. (1998) Early feeding and development of the immune system in neonatal poultry. Journal of Applied PoultryResearch7: 425-436

Dono, N.D., (2012). Nutritional strategies to improve enteric health and growth performance of poultry in the post antibiotic era (Doctoral dissertation, University of Glasgow).

Dono, N.D., Sparks, N.H. and Olukosi, O.A., (2014). Association between digesta pH, body weight, and nutrient utilization in chickens of different body weights and at different ages. The Journal of Poultry Science, 51(2), pp.180-184.

Duangnumsawang, Y., Zentek, J. and Boroojeni, F.G., (2021) Development and Functional Properties of Intestinal Mucus Layer in Poultry. *Frontiers in Immunology*, 12, p. 745849

Dunlop, M.W., McAuley, J., Blackall, P.J. and Stuetz, R.M., (2016). Water activity of poultry litter: Relationship to moisture content during a grow-out. *Journal of Environmental Management*, *172*, pp.201-206.

Dunnington, E.A. and Siegel, P.B., (1995). Enzyme activity and organ development in newly hatched chicks selected for high or low eight-week body weight. *Poultry Science*, *74*(5), pp.761-770.

El-Husseiny, O.M., Hashish, S.M., Ali, R.A., Arafa, S.A., Abd El-Samee, L.D., Olemy, A. A., (2012). Effects of feeding organic zinc, manganese and copper on broiler growth, carcass characteristics, bone quality and mineral content in bone, liver and excreta. *Int. J. Poult. Sci. 11, 368–377.*

Elvis-Chikwem, C.L. E. Burton, G. White, K. Derecka, C.J. O'Shea (2020) Evaluating the Causes of Variation in Performance of Broiler Chicks at first week of life *Proceedings of the British Society of Animal Science Conference*, Nottingham, UK, page 35

Elvis-Chikwem, C.L., White, G., Burton, E., Derecka, K., Reina, S.V., Hankin, J. and O'Shea, C., (2021). 180 Evaluation of the tibial bone ash and mineral concentration of Ross 308 broilers with divergent bodyweights on D7. *Animal-science proceedings, 12(1), p.150.*

Elvis-Chikwem, C.L., White, G.A., Burton, E. and O'Shea, C.J., (2024). Differences in broiler bone, gut, and tissue mineral parameters, as influenced by broilers grouped based on bodyweight. *Animal Production Science*, *64*(2).

Estevez, I., Newberry, R.C. and De Reyna, L.A., (1997). Broiler chickens: a tolerant social system. *Etologia*, *5*(1), pp.19-29.

Faith, J.J., Guruge, J.L., Charbonneau, M., Subramanian, S., Seedorf, H., Goodman, A.L., Clemente, J.C., Knight, R., Heath, A.C., Leibel, R.L. and Rosenbaum, M., (2013). The long-term stability of the human gut microbiota. *Science*, *341*(6141), p.1237439.

Fang, L., Su, Y., Wang, P., Lai, C., Huang, C., Ling, Z. and Yong, Q., (2022). Co-production of xylooligosaccharides and glucose from birch sawdust by hot water pretreatment and enzymatic hydrolysis. *Bioresource Technology*, *348*, p.126795.

FAO (2007). The State of the World's Animal Genetic Resources for Food and Agriculture, FAO Rome.

FAO/WHO (2002). *Codex Alimentarius*; Schedule 1 of the Proposed Draft Codex General Standards for Contaminants and Toxins in Food. Joint FAO/WHO, Food Standards Programme; Reference CX/FAC 02/16; *Codex Committee: Rotterdam, The Netherlands*

Fathima, S., Shanmugasundaram, R., Adams, D. and Selvaraj, R.K., (2022). Gastrointestinal microbiota and their manipulation for improved growth and performance in chickens. *Foods*, *11*(10), p.1401.

Feddes, J.J., Emmanuel, E.J. and Zuidhoft, M.J., (2002). Broiler performance, body weight variance, feed and water intake, and carcass quality at different stocking densities. *Poultry Science*, *81*(6), pp.774-779.

Ferket, P.R. and Gernat, A.G., (2006). Factors that affect feed intake of meat birds: A review. *Int. J. Poult. Sci*, *5*(10), pp.905-911.

Ferket, P.R., (2004). Alternatives to antibiotics in poultry production: responses, practical experience and recommendations. In *Nutritional biotechnology in the feed and food industries. Proceedings of Alltech's 20th Annual Symposium: re-imagining the feed industry, Lexington, Kentucky, USA, 23-26 May 2004* (pp. 57-67). Alltech UK.

Fitzsimons, C, Kenny, D.A and McGee, M (2014) Visceral organ weights, digestion and carcass characteristics of beef bulls differing in residual feed intake offered a high concentrate diet. Animal 8, 949–959.

Fleming, R.H., (2008). Nutritional factors affecting poultry bone health: Symposium on 'Diet and bone health'. *Proceedings of the Nutrition Society*, *67*(2), pp.177-183.

Flood, C.A., Koon, J.L., Trumbull, R.D. and Brewer, R.N., (1992). Broiler growth data: 1986- 1991. *Transactions of the ASAE*, *35*(2), pp.703-709.

Fortomaris, P., Arsenos, G., Tserveni-Gousi, A. and Yannakopoulos, A., (2007). Performance and behaviour of broiler chickens as affected by the housing system. *Archiv Fur Geflugelkunde*, *71*(3), p.97.

Foye, O.T., Ferket, P.R. and Uni, Z., (2007). The effects of in ovo feeding arginine, β-hydroxy-β- methyl-butyrate, and protein on jejunal digestive and absorptive activity in embryonic and neonatal turkey poults. *Poultry Science*, *86*(11), pp.2343-2349.

Fumihito, A., Miyake, T., Sumi, S.I., Takada, M., Ohno, S. and Kondo, N., (1994). One subspecies of the red junglefowl (Gallus gallus gallus) suffices as the matriarchic ancestor of all domestic breeds. *Proceedings of the National Academy of Sciences*, *91*(26), pp.12505- 12509.

Furuse, M., (2002). Central regulation of food intake in the neonatal chick. *Animal Science Journal*, *73*(2), pp.83-94.

Furuse, M., Yamane, H., Tomonaga, S., Tsuneyoshi, Y. and Denbow, D.M., (2007). Neuropeptidergic regulation of food intake in the neonatal chick: a review. *The journal of poultry science*, *44*(4), pp.349-356.

Garcia, R., Almeida, P. I., Caldara, F. R., Nääs, I. A., Pereira, D. F., Freitas, L. W., ... & Graciano, J. (2010). Effect of the litter material on drinking water quality in broiler production. *Brazilian Journal of Poultry Science*, *12*, 165-169.

Genovese, L.L., Lowry, V.K., Genovese, K.J. and Kogut, M.H., (2000) Longevity of augmented phagocytic activity of heterophils in neonatal chickens following administration of Salmonella enteritidis-immune lymphokines to chickens. *Avian Pathology*, 29(2), pp.117- 122.

Geyra, A., Uni, Z. and Sklan, D. (2001) The effect of fasting at different ages on growth and tissue dynamics in the small intestine of the young chick. *British Journal of Nutrition 86: 53-61*

Geyra, A., Uni, Z. and Sklan, D. (2001a) Enterocyte dynamics and mucosal development in the post hatch chick. Poultry Science 80: 776-782

Gheisari, A.A., Rahimi-fathkoohi, A., Toghyani, M., Gheisari, M.M., (2010). Effects of organic chelates of zinc, manganese and copper in comparison to their inorganic sources on performance of broiler chickens. J. Anim. Plant Sci. 6, 630–636

Gilbert, E.R., Li, H., Emmerson, D.A., Webb Jr, K.E. and Wong, E.A., (2007). Developmental regulation of nutrient transporter and enzyme mRNA abundance in the small intestine of broilers. *Poultry science*, *86*(8), pp.1739-1753.

Glimcher, M.J., Coe, F.L. and Favus, M.J., (1998). Metabolic bone disease and clinically related disorders. *Academic Press: New York*, *23*.

Godwin, J. L., Carter, T. A., & Grimes, J. L. (2000). The use of litter plus® as a bedding material for broilers.

Goff, J.P. (2018). Invited review: mineral absorption mechanisms, mineral interactions that affect acid-base and antioxidant status, and diet considerations to improve mineral status. J. Dairy Sci., 101:2763-2813.

Gorenz, B., Oelschlager, M.L., Jespersen, J.C., Cao, C., Smith, A.H., Mackie, R.I. and Dilger, R.N., (2024). Organ growth and fermentation profiles of broilers differ in body growth rate. *Poultry science*, *103*(5), p.103628.

Gous, R.M., (2018). Nutritional and environmental effects on broiler uniformity. *World's Poultry Science Journal*, *74*(1), pp.21-34.

Guinotte, F., Gautron, J., Nys, Y. and Soumarmon, A., (1995). Calcium solubilization and retention in the gastrointestinal tract in chicks (Gallus domesticus) as a function of gastric acid secretion inhibition and of calcium carbonate particle size. *British Journal of Nutrition*, *73*(1), pp.125-139.

Hajati, H. and M. Rezaei. (2010). The application of prebiotics in poultry production. Int. J. Poult. Sci. 9:298-304.

Hall, A.L., (2001). The effect of stocking density on the welfare and behaviour of broiler chickens reared commercially. *Animal welfare*, *10*(1), pp.23-40.

Hall, L.E., Shirley, R.B., Bakalli, R.I., Aggrey, S.E., Pesti, G.M. and Edwards Jr, H.M., (2003) Power of two methods for the estimation of bone ash of broilers. *Poultry Science*, *82(3), pp.414-418.*

Hamal, K. R., S. C. Burgess, I. Y. Pevzner, and G. F. Erf. (2006). Maternal antibody transfer from dams to their egg yolks, egg whites, and chicks in meat lines of chickens. *Poult. Sci. 85:1364-1372*. doi:10.1093/ps/85.8.1364

Hamilton RMG, Proudfoot FG. (1995). Ingredient particle size and feed texture: effects on the performance of broiler chickens. *Anim. Feed Sci. Technol. 51: 203 210*

Harrow, S.A., Ravindran, V., Butler, R.C., Marshall, J. and Tannock, G.W., (2007). The influence of farming practices on ileal Lactobacillus salivarius populations of broiler chickens measured by real-time quantitative PCR. *Appl. Environ. Microbiol*, *73*, pp.7123-7127.

Havenstein GB, Ferket PR, and Qureshi MA. (2003). Carcass composition and yield of 1957 vs 2001 broilers when fed representative 1957 and 2001 broiler diets. Poultry Sci. 82(10): 1509-1518.

Havenstein GB. (2006). Performance changes in poultry and livestock following 50 years of genetic selection. Lohman information. 41: 30-41.

Havenstein, G.B., Ferket, P.R., Scheideler, S.E. and Larson, B.T., (1994). Growth, livability, and feed conversion of 1957 vs 1991 broilers when fed "typical" 1957 and 1991 broiler diets. *Poultry science*, *73*(12), pp.1785-1794.

Henderson, S.N., Vicente, J.L., Pixley, C.M., Hargis, B.M. and Tellez, G., (2008). Effect of an early nutritional supplement on broiler performance. *International Journal of Poultry Science*, *7*(3), pp.211-214.

Heneghan, A.F., Pierre, J.F. and Kudsk, K.A., (2013) JAK-STAT and intestinal mucosal immunology. *Jak-stat*, 2(4), p.e25530.

Hinton Jr A, Corrier DE, Spates GE, Norman JO, Ziprin RL, Beier RC, and Deloach JR (1990). Biological control of Salmonella typhimurium in young chickens. *Avian Diseases 34, 626- 633*

Holzapfel, W.H., Haberer, P., Snel, J., Schillinger, U. and in't Veld, J.H.H., (1998). Overview of gut flora and probiotics. *International journal of food microbiology*, *41*(2), pp.85-101.

Honda, K., Saneyasu, T., & Kamisoyama, H. (2017) Gut hormones and regulation of food intake in birds. The *Journal of Poultry Science, 54, 103–110*. https://doi.org/10.2141/jpsa.0160100

Hu, Y., X. Liao, Q. Wen, Z. Lu, and X Luo. (2018). Phosphorus absorption and gene expression levels of related transporters in the small intestine of broilers. *Br J Nutr 119:1346-1354*

Hu, Y., Zhang, W., Chen, G., Cheng, H. and Tao, S., (2018). Public health risk of trace metals in fresh chicken meat products on the food markets of a major production region in southern China. *Environmental pollution*, *234*, pp.667-676.

Hu, J., Johnson, T.A., Zhang, H. and Cheng, H.W., (2022). The microbiota–gut–brain axis: gut microbiota modulates conspecific aggression in diversely selected laying hens. *Microorganisms*, *10*(6), p.1081.

Hughes R, Heberle N, Barekatin R, Edwards N, Hynd P (2017). Flock uniformity is it important and how it is assessed. Sydney, Australia: Conference: *Australian Poultry Science Symposium. pp 28.*

Iji, P.A, Saki, A and Tivey, D.R (2001) Body and intestinal growth of broiler chicks on a commercial starter diet. 1. Intestinal weight and mucosal development, *British Poultry Science, 42:4, 505-513,* DOI: 10.1080/00071660120073151

Ipek, A.Y.D.I.N., Karabulut, A., Sahan, U., Canbolat, O. and Yilmaz-Dikmen, B.İ.L.G.E.H.A.N., (2009). The effects of different feeding management systems on performance of a slow- growing broiler genotype. *British poultry science*, *50*(2), pp.213-217.

Islam, K.M.S., Schaeublin, H., Wenk, C., Wanner, M. and Liesegang, A., (2012). Effect of dietary citric acid on the performance and mineral metabolism of broiler. *Journal of Animal Physiology and Animal Nutrition*, *96*(5), pp.808-817.

Jha, R., Singh, A.K., Yadav, S., Berrocoso, J.F.D. and Mishra, B., (2019). Early nutrition programming (in ovo and post-hatch feeding) as a strategy to modulate gut health of poultry. *Frontiers in Veterinary Science*, *6*, p.82.

Jiao, L., Han, X., Wang, S., Fan, Y., Yang, M., Qiu, H. & Yang,X. (2009). Imprinted DC mediates the immune-educatingeffect of early-life microbial exposure. *European Journal of Immunology, 39, 469–480*

Jiménez-Moreno, E., González-Alvarado, J.M., de Coca-Sinova, A., Lázaro, R.P., Cámara, L. and Mateos, G.G., (2019). Insoluble fiber sources in mash or pellets diets for young broilers. 2. Effects on gastrointestinal tract development and nutrient digestibility. *Poultry science*, *98*(6), pp.2531-2547.

Jiménez-Moreno, E., J. M. González-Alvarado, R. Lázaro and G. G. Mateos. (2009). Effects of type of cereal, heat processing of the cereal, and fiber inclusion in the diet on gizzard pH and nutrient utilization in broilers at different ages. *Poult. Sci. 88: 1925-1933.*

Johansson, M.E., Phillipson, M., Petersson, J., Velcich, A., Holm, L. and Hansson, G.C., (2008) The inner of the two Muc2 mucin-dependent mucus layers in colon is devoid of bacteria. *Proceedings of the*

National Academy of sciences, 105(39), pp.15064-15069.

Jones FT, Anderson KE, Ferket PR. (1995). Effect of extrusion on feed characteristics and broiler chicken performance. *J. Appl. Poult. Res. 4:300-309.*

Julian RJ. (2005) Production and growth-related disorders and other metabolic diseases of poultry - A review. *Vet J. 169:350–69.*

Kanno, S., Emil, S., Kosi, M., Monforte-Munoz, H. and Atkinson, J., (1996). Small intestinal absorption during endotoxemia in swine. *The American surgeon*, *62*(10), pp.793-799.

Katanbaf, M.N., Dunnington, E.A. and Siegel, P.B. (1988) Allomorphic relationships from hatching to 56 days in parental lines and F1 crosses of chickens selected over 27 generations for high or low body weight. *Growth Development and Aging 52: 11-22*

Khan, K., Kaya, M., Fidan, E.D. and Kilimci, F.S., (2023). Impact of litter on femur and tibial morphology, bone biomechanics, and leg health parameters in broiler chickens. *Animal Bioscience*, *36*(9), p.1393.

Khodadadian, A., Darzi, S., Haghi-Daredeh, S., Sadat Eshaghi, F., Babakhanzadeh, E., Mirabutalebi, S.H. and Nazari, M., (2020). Genomics and transcriptomics: the powerful technologies in precision medicine. *International Journal of General Medicine*, pp.627- 640.

Kidd MT, McDaniel CD, Peebles ED, Barber SJ, Corzo A, Branton SL, Woodworth JC (2005). Breeder hen dietary L-carnitine affects progeny carcass traits. *British Poultry Science; 46:97-103*

Kidd, M.T., (2003). A treatise on chicken dam nutrition that impacts on progeny1, 2. *World's Poultry Science Journal*, *59*(4), pp.475-494.

Kies, C., 1987. Manganese bioavailability overview.

Kikenny, C., Browne, W.J., Cuthill, I.N., Emerson, M. and Altman, D.S., (2010). The ARRIVE Guidelines. *Animal Research: Reporting of In Vivo Experiments, National Centre for the Replacement Refinement and Reduction of Animals Research*.

Klasing, K.C., Laurin, D.E., Peng, R.K. and Fry, D.M., (1987). Immunologically mediated growth depression in

chicks: influence of feed intake, corticosterone and interleukin-1. *The Journal of nutrition*, *117*(9), *pp.1629-1637.*

Klasing, K.C. and Barnes, D.M. (1988) Decreased amino acid requirements of growing chick due to immunological stress. Journal of Nutrition118: 1158-1164.

Klasing, K.C., (1998). Comparative avian nutrition. *Cab International.*

Kleyn R.J. and Ciacciariello, M., (2021). Mineral nutrition in broilers: Where are we at? In *Proceedings of the Arkansas Nutrition Conference* (Vol. 2021, No. 1, p. 1).

Koenen ME, Boonstra-Blom AG, SHM J. (2002) Immunological differences between layer- and broiler-type chickens. *Vet Immunol Immunopathol. 89:47–56.*

Kogut, M.H., (2002) Dynamics of a protective avian inflammatory response: the role of an IL-8- like cytokine in the recruitment of heterophils to the site of organ invasion by Salmonella enteritidis. *Comparative immunology, microbiology and infectious diseases*, 25(3), pp.159-172.

Kogut, M.H., Tellez, G.I., McGruder, E.D., Hargis, B.M., Williams, J.D., Corrier, D.E. and DeLoach, J.R. (1994) Heterophils are decisive components in the early responses of chickens to Salmonella enteritidis infections. *Microb. Pathogenesis 16, 141–151*

Korish, M.A. and Attia, Y.A., (2020). Evaluation of heavy metal content in feed, litter, meat, meat products, liver, and table eggs of chickens. *Animals*, *10*(4), p.727.

Koutsos, E. and Klasing, K., (2002). Modulation of nutritional status by the immune response. In *Proc. Australian Poultry Science Symposium Sydney, Australia* (pp. 18-23).

Kuenzel, W.J., (1994). Central neuroanatomical systems involved in the regulation of food intake in birds and mammals. *The Journal of nutrition*, *124*, pp.1355S-1370S.

Kuenzel, W.J., Beck, M.M. and Teruyama, R., (1999). Neural sites and pathways regulating food intake in birds: a comparative analysis of mammalian systems. *Journal of Experimental Zoology Part A:*

Ecological Genetics and Physiology, *283*(4-5), pp.348-364.

Kwiecień, M., Winiarska-Mieczan, A., Milczarek, A., Tomaszewska, E. and Matras, J., (2016). Effects of zinc glycine chelate on growth performance, carcass characteristics, bone quality, and mineral content in bone of broiler chicken. *Livestock Science*, *191*, pp.43-50.

Lammers, A., W. H. Wieland, L. Kruijt, A. Jansma, T. Straetemans, A. Schots, G. den Hartog, and H. K. Parmentier. (2010). Successive immunoglobulin and cytokine expression in the small intestine of juvenile chicken. *Dev. Comp. Immunol. 34:1254-1262*. doi:10.1016/j. dci.2010.07.001

Lamot, D., (2017). First week nutrition for broiler chickens: effects on growth, metabolic status, organ development, and carcass composition (*Doctoral dissertation, Wageningen University and Research).*

Lamot, D.M., Sapkota, D., Wijtten, P.J.A., Van Den Anker, I., Heetkamp, M.J.W., Kemp, B. and Van Den Brand, H., (2019). Diet density during the first week of life: Effects on growth performance, digestive organ weight, and nutrient digestion of broiler chickens. *Poultry Science*, *98*(2), pp.789-795.

Latshaw, J.D. (1991) Nutrition - mechanisms of immune-suppression. *Veterinary Immunology and Immunopathology 30: 111-120.*

Latshaw, J.D. and Moritz, J.S., (2009). The partitioning of metabolizable energy by broiler chickens. *Poultry science*, *88*(1), pp.98-105.

Lawal, Raman Akinyanju; Martin, Simon H.; Vanmechelen, Koen; Vereijken, Addie; Silva, Pradeepa; Al-Atiyat, Raed Mahmoud; Aljumaah, Riyadh Salah; Mwacharo, Joram M.; Wu, Dong-Dong; Zhang, Ya-Ping; Hocking, Paul M.; Smith, Jacqueline; Wragg, David; Hanotte, Olivier (2020). *"The wild species genome ancestry of domestic chickens"*. *BMC Biology. 18 (1):* 13. *doi*:*10.1186/s12915-020-0738-1*. *PMC 7014787*. *PMID 32050971*

Lee, S.A., Febery, E., Mottram, T. and Bedford, M.R., (2021). Growth performance, real-time gizzard pH and calcium solubility in the gut of broiler chickens is dependent on the interaction between dietary calcium concentration and limestone particle size. *British Poultry Science*, *62*(6), pp.827-834.

Leeson, S., Caston, L. and Summers, J.D., (1996). Broiler response to diet energy. *Poultry science*, *75*(4), pp.529-535.

Leterrier, C., Rose, N., Constantin, P. and Nys, Y., (1998). Reducing growth rate of broiler chickens with a low energy diet does not improve cortical bone quality. *British Poultry Science, 39*(1), pp.24-30.

Levy, O. (2007). Innate immunity of the newborn: basic mechanisms and clinical correlates. *Nature Reviews Immunology, 7, 379–390*

Lewis, M.R., Rose, S.P., Mackenzie, A.M. and Tucker, L.A., (2003). Effects of dietary inclusion of plant extracts on the growth performance of male broiler chickens. *British Poultry Science, 44*(S1), pp.43-44.

Lewis, P.D., Perry, G.C., Farmer, L.J. and Patterson, R.L.S., (1997). Responses of two genotypes of chicken to diets and stocking densities typical of UK and 'Label Rouge'production systems: I. Performance, behaviour and carcass composition. *Meat Science, 45*(4), pp.501- 516.

Lewis, R.M. and Emmans, G.C., (2020). The relationship between feed intake and liveweight in domestic animals. *Journal of Animal Science, 98*(4), p.skaa087.

Li, H., Gilbert, E.R., Zhang, Y., Crasta, O., Emmerson, D., Webb Jr, K.E. and Wong, E.A., (2008). Expression profiling of the solute carrier gene family in chicken intestine from the late embryonic to early post-hatch stages. *Animal genetics*, *39*(4), pp.407-424.

Li, J.L., Jiang, C.Y., Li, S. and Xu, S.W., (2013). Cadmium induced hepatotoxicity in chickens (Gallus domesticus) and ameliorative effect by selenium. *Ecotoxicology and environmental safety*, *96*, pp.103-109.

Lien, R. J., Conner, D. E., & Bilgili, S. F. (1992). The use of recycled paper chips as litter material for rearing broiler chickens. *Poultry Science, 71*(1), 81-87.

Liu G, Dunnington EA and Siegel PB. (1993). Maternal effects and heterosis for growth in reciprocal cross populations of chickens. Journal of Animal Breeding and Genetics 110: 423-428

Liu, J., Xiao, Q., Xiao, J., Niu, C., Li, Y., Zhang, X., Zhou, Z., Shu, G. and Yin, G., (2022) Wnt/β- catenin

signalling: function, biological mechanisms, and therapeutic opportunities. *Signal transduction and targeted therapy*, 7(1), p.3.

Liu, W.C., Huang, M.Y., Balasubramanian, B. and Jha, R., (2022). Heat stress affects jejunal immunity of yellow-feathered broilers and is potentially mediated by the microbiome. *Frontiers in Physiology*, *13*, p.913696.

Loddi MM, Moraes VMB, Nakaghi LSO, Tuca FM, Hannas MI and Ariki J. (2004). Mannan oligosaccharide and organic acids on performance and intestinal morphometric characteristics of broiler chickens. Proceeding of World's Poultry Congress, Istanbul, Turkey.

Lott, B.D., Reece, F.N. and McNaughton, J.L., (1982). An automated weighing systemdc for use in poultry research. *Poultry Science*, *61*(2), pp.236-238.

Lowe, R., Shirley, N., Bleackley, M., Dolan, S. and Shafee, T., (2017). Transcriptomics technologies. *PLoS computational biology*, *13*(5), p.e1005457.

Lu, D. and Carson, D.A., (2009) Spiperone enhances intracellular calcium level and inhibits the Wnt signaling pathway. *BMC pharmacology*, 9, pp.1-8.

Lundberg, R., Scharch, C. and Sandvang, D., (2021). The link between broiler flock heterogeneity and cecal microbiome composition. *Animal microbiome*, *3*(1), pp.1-14.

Ma, Z.J., Yamaguchi, M., (2000). Alternation in bone components with increasing age of newborn rats: role of zinc in bone growth. *J. Bone Miner. Metab. 18, 264–270.*

MacDonald, K.C. and Edwards, D.N., (1993). Chickens in Africa: the importance of Qasr Ibrim. *Antiquity*, *67*(256), pp.584-590.

MacLeod M, Gerber P, Mottet A, Tempio G, Falcucci A, Opio C, Vellinga T, Henderson B & Steinfeld H. (2013). Greenhouse gas emissions from pig and chicken supply chains - A global life cycle assessment. Food and Agriculture Organization of the United Nations (FAO), Rome.

Maenz, D.D., Engele-Schaan, C.M., Newkirk, R.W. and Classen, H.L., (1999). The effect of minerals and

mineral chelators on the formation of phytase-resistant and phytase- susceptible forms of phytic acid in solution and in a slurry of canola meal. *Animal Feed Science and Technology*, *81*(3-4), pp.177-192.

Mahagna, M. and Nir, I., (1996). Comparative development of digestive organs, intestinal disaccharidases and some blood metabolites in broiler and layer-type chicks after hatching. *British Poultry Science*, *37*(2), pp.359-371.

Maharjan, P., Martinez, D.A., Weil, J., Suesuttajit, N., Umberson, C., Mullenix, G., Hilton, K.M., Beitia, A. and Coon, C.N., (2021). Physiological growth trend of current meat broilers and dietary protein and energy management approaches for sustainable broiler production. *Animal*, *15*, p.100284.

Malik, K., 2013. Human development report (2013). The rise of the South: Human progress in a diverse world. *The Rise of the South: Human Progress in a Diverse World (March 15, 2013). UNDP-HDRO Human Development Reports*.

Malone, G.W. and Chaloupka, G.W., (1982), January. Evaluation of shredded newspaper litter materials under various broiler management programs. In *Poultry Science* (Vol. 61, No. 7, pp. 1385-1385).

Malone, G.W., Chaloupka, G.W. and Saylor, W.W., (1983). Influence of litter type and size on broiler performance: 1. Factors affecting litter consumption. *Poultry Science*, *62*(9), pp.1741-1746.

Matin, H.H., Dashtbin, F. and Salari, J., 2013. Absorption and macromineral interactions in broiler production: an overview. *Global Veternaria*, *11*, pp.49-54.

Marchaim, U. and Kulka, R.G. (1967) The non-parallel increase of amylase, chymotrypsinogen and procarboxypeptidase in the developing chick pancreas. Biochimia Biophysica Acta 146: 553-559

Mebratie, W., Madsen, P., Hawken, R., Romé, H., Marois, D., Henshall, J., Bovenhuis, H. and Jensen, J., (2019). Genetic parameters for body weight and different definitions of residual feed intake in broiler chickens. *Genetics Selection Evolution*, *51*, pp.1-12.

Mebratie, W.S., (2019). *The genetics of body weight and feed efficiency in broiler chickens*. Wageningen University and Research.

Mendes, A.S., Paixão, S.J., Restelatto, R., Reffatti, R., Possenti, J.C., De Moura, D.J., Morello, G.M.Z. and de Carvalho, T.M.R., 2011. Effects of initial body weight and litter material on broiler production. *Brazilian journal of poultry science*, *13*, *pp.165-170.*

Miles, D., Owens, P. and Rowe, D. (2006) Spatial variability of litter gaseous flux within a commercial broiler house: ammonia, nitrous oxide, carbon dioxide, and methane. *Poultry Science85: 167-172*

Miles, R.D. and Henry, P.R., (2000). Relative trace mineral bioavailability. *Ciência Animal Brasileira/Brazilian Animal Science*, *1*(2), pp.73-93.

Mohammadrezaei, M., Gheisari, A., Toghyani, M. and Toghyani, M., (2011). Modeling daily feed intake of broiler chicks. In *4th International Conference in Animal Nutrition, Malaysia*.

Mohammadigheisar, M., Shouldice, V.L., Torrey, S., Widowski, T. and Kiarie, E.G., (2020). Research Note: Comparative gastrointestinal, tibia, and plasma attributes in 48-day-old fast-and slow-growing broiler chicken strains. *Poultry science*, *99*(6), pp.3086-3091.

Mohammed, M.K., Shao, C., Wang, J., Wei, Q., Wang, X., Collier, Z., Tang, S., Liu, H., Zhang, F., Huang, J. and Guo, D., (2016) Wnt/β-catenin signaling plays an ever-expanding role in stem cell self-renewal, tumorigenesis and cancer chemoresistance. *Genes & diseases*, 3(1), pp.11-40.

Molenaar, R., Reijrink, I.A.M., Meijerhof, R. and Van den Brand, H., (2008). Relationship between hatchling length and weight on later productive performance in broilers. *World's Poultry Science Journal*, *64*(4), pp.599-604.

Mondal, K., Chatterjee, J. K., Samanta, A. K., Bera, S., Roy, M., Biswas, S., ... & Patra, G. (2020). Effect of different litter materials on growth performance and carcass characteristics of broiler chicken. *Indian Journal of Animal Health*, *59*(2), 194-199.

Monira, K. N., Islam, M. A., Alam, M. J., & Wahid, M. A. (2003). Effect of litter materials on broiler performance and evaluation of manureal value of used litter in late autumn. *Asian- australasian journal of animal sciences*, *16*(4), 555-557.

Moran Jr, E.T., (2007). Nutrition of the developing embryo and hatchling. *Poultry science*, *86*(5), pp.1043-1049.

Morgan, N.K., Walk, C.L., Bedford, M.R. and Burton, E.J., (2014). The effect of dietary calcium inclusion on broiler gastrointestinal pH: Quantification and method optimization. *Poultry Science*, *93*(2), pp.354-363.

Morgan, P.M., (2021). Immune Response in Mammals and Chickens. In *IgY-Technology: Production and Application of Egg Yolk Antibodies: Basic Knowledge for Successful Practice* (pp. 31-47). Cham: Springer International Publishing.

Mullen, M.P., Berry, D.P., Howard, D.J., Diskin, M.G., Lynch, C.O., Giblin, L., Kenny, D.A., Magee, D.A., Meade, K.G. and Waters, S.M., (2011) Single nucleotide polymorphisms in the insulin-like growth factor 1 (IGF-1) gene are associated with performance in Holstein- Friesian dairy cattle. *Frontiers in genetics*, 2, p.3.

Murakami, H., Akiba, Y. and Horiguchi, M., (1992). Growth and utilization of nutrients in newly hatched chick with or without removal of residual yolk. *Growth, development, and aging: GDA*, *56*(2), pp.75-84.

Murphy, L.B. and Preston, A.P., (1988). Time-budgeting in meat chickens grown commercially. *British Poultry Science*, *29*(3), pp.571-580.

Nathan, B., Heller, E.D. and Perek, M. (1977) The effect of starvation on antibody production of chicks. *Poultry Science56: 1468-1473.*

Nesbit, A. H. and W. P. Elmslie. (1960). Biological availability to the rat of iron and copper from various compounds. *Transactions of the Illinois State Academy of Science, 53: 101-105*

Neto, R.M., Surek, D., da Rocha, C., Dahlke, F. and Maiorka, A., (2013) The effect of grouping one-day-old chicks by body weight on the uniformity of broilers. *Journal of Applied Poultry Research*, 22(2), pp.245-250.

Newberry, R.C., Hunt, J.R. and Gardiner, E.E., (1988). Influence of light intensity on behavior and performance of broiler chickens. *Poultry Science*, *67*(7), pp.1020-1025.

Ni, L., Song, C., Wu, X., Zhao, X., Wang, X., Li, B. and Gan, Y., (2019). RNA-seq transcriptome profiling of

porcine lung from two pig breeds in response to Mycoplasma hyopneumoniae infection. *PeerJ*, *7*, *p.e7900.*

Nielsen, B.L., Juul-Madsen, H.R., Steenfeldt, S. and Kjaer, J.B., (2010). Feeding activity in groups of newly hatched broiler chicks: effects of strain and hatching time. *Poultry Science*, *89*(7), pp.1336-1344.

Niessen, C.M., (2007). Tight junctions/adherens junctions: basic structure and function. *Journal of investigative dermatology*, *127*(11*), pp.2525-2532.*

Niewold, T.A., (2007). The nonantibiotic anti-inflammatory effect of antimicrobial growth promoters, the real mode of action? A hypothesis. *Poultry science*, *86*(4), *pp.605-609.*

Nir I, Hillel R, Ptichi I, Shefet G. (1995). Effect of particle size on performance. 3. Grinding pelleting interactions. Poult. Sci. 74: 771-78.

Nir, I., I. Petihi, and Z. Nitsan, (1979). Body composition, food utilization, intestinal adaptation and lipogenesis in meal-fed chicks. *Food Intake Regulation in Poultry. Pages 391- 403*

K. N. Boorman and B. M. Freeman, ed. Br. Poult. Sci. Ltd., Edinburgh, Scotland.

Nitsan, Z., Ben-Avraham, G., Zoref, Z. and Nir, I., (1991). Growth and development of the digestive organs and some enzymes in broiler chicks after hatching. *British poultry science*, *32*(3*), pp.515-523.*

Nitsan, Z., Dunnington, E.A. and Siegel, P.B., (1991). Organ growth and digestive enzyme levels to fifteen days of age in lines of chickens differing in body weight. *Poultry Science*, *70*(10), *pp.2040-2048.*

Noy, Y. and Pinchasov, Y., (1993). Effect of a single posthatch intubation of nutrients on subsequent early performance of broiler chicks and turkey poults. *Poultry Science*, *72*(10), *pp.1861-1866.*

Noy, Y. and Sklan, D. (1998b) Metabolic responses to early nutrition. *Journal of Applied Poultry Research 7:437-451*

Noy, Y. and Sklan, D. (1999) Energy utilization in newly hatched chicks. *Poultry Science 78:1750- 1756*

Noy, Y. and Sklan, D., (1997). Posthatch development in poultry. *Journal of Applied Poultry Research*, *6*(3), pp.344-354.

Noy, Y. and Sklan, D., (1995). Digestion and absorption in the young chick. *Poultry science*, *74*(2), pp.366-373.

Noy, Y. and Sklan, D., (2002). Nutrient use in chicks during the first week post hatch. *Poultry Science*, *81(3), pp.391-399.*

Noy, Y. and Uni, Z., (2010). Early nutritional strategies. *World's Poultry Science Journal*, *66*(4), pp.639-646.

Noy, Y., and D. Sklan. (1995). Digestion and absorption in the young chick. *Poult. Sci. 74:366-373*. doi:10.3382/ps.0740366

Obst, B.S. and Diamond, J., (1992). Ontogenesis of intestinal nutrient transport in domestic chickens (Gallus gallus) and its relation to growth. *The Auk*, *109*(3), pp.451-464.

OECD, F.A.O., (2022). OECD-FAO agricultural outlook 2022-2031.

OECD/FAO (2023), 'OECD-FAO Agricultural Outlook', OECD Agriculture statistics (database), http://dx.doi.org/10.1787/agr-outl-dataen.

OECD/FAO, (2014). OECD-FAO Agricultural Outlook 2014-2023.

Orzechowska-Wylęgała, B., Obuchowicz, A., Malara, P., Fischer, A. and Kalita, B., (2011) Cadmium and lead accumulate in the deciduous teeth of children with celiac disease or food allergies. *international journal of stomatology & occlusion medicine*, 4, pp.28-31.

Ouyang, W. and O'Garra, A., (2019). IL-10 family cytokines IL-10 and IL-22: from basic science to clinical translation. *Immunity*, 50(4), pp.871-891.

Ozsolak, F. and Milos, P.M., (2011). RNA sequencing: advances, challenges, and opportunities. *Nature reviews genetics*, *12*(2), pp.87-98.

Panda, A.K. and Reddy, M.R. (2007) Boosting the chick's immune system through early nutrition. *Poultry International 46: 22-26.*

Panda, A.K., Bhanja, S.K. and Sunder, G.S., (2015). Early post hatch nutrition on immune system development and function in broiler chickens. *World's Poultry Science Journal*, *71*(2), pp.285-296.

Panda, A.K., Bhanja, S.K. and Sunder, G.S., (2015). Early post hatch nutrition on immune system development

and function in broiler chickens. *World's Poultry Science Journal*, *71*(2), pp.285-296.

Panda, A.K., Rama R, S.V., Raju, M.V.L.N., Reddy, M.R. and Shyam S, G. (2008) Early Gut Development: Its importance in broiler production. Proceedings of the Seminar on Sustainable Poultry Production: *Rural and commercial approach, Hyderabad, pp. 79-83.*

Pang, Y. and Applegate, T.J., (2007). Effects of dietary copper supplementation and copper source on digesta pH, calcium, zinc, and copper complex size in the gastrointestinal tract of the broiler chicken. *Poultry Science*, *86*(3), pp.531-537.

Pang. Y. and T. J. Applegate. (2007). Effects of dietary copper supplementation and copper source on digesta pH, calcium, zinc, and copper complex size in the gastrointestinal tract of the broiler chicken. *Poult. Sci. 86:531-537.*

Park, S.Y., Birkhold, S.G., Kubena, L.F., Nisbet, D.J., Ricke, S.C., (2004). Review on the role of dietary zinc in poultry nutrition, immunity, and reproduction. *Biol. Trace Elem. Res. 101, 147–163.*

Peebles ED, Doyle SM, Pansky T, Gerard PD, Latour MA, Boyle CR, Smith TW (1999). Effects of breeder age and dietary fat on subsequent broiler performance (2. Slaughter yield). *Poultry Science 78: 512-515*

Peebles ED, Zumwalt CD, Gerard P.D, Latour MA, Smith TW (2002). Market age live weight, carcass yield and liver characteristics of broiler offspring from breeder hens fed diets differing in fat and energy contents. *Poultry Science 81:23-29.*

Peng, L.Y., Cui, Z.Q., Wu, Z.M., Fu, B.D., Yi, P.F. and Shen, H.Q., (2019). RNA-seq profiles of chicken type II pneumocyte in response to Escherichia coli infection. *PLoS One*, *14*(6), p.e0217438.

Perugorria, M.J., Olaizola, P., Labiano, I., Esparza-Baquer, A., Marzioni, M., Marin, J.J., Bujanda, L. and Banales, J.M., (2019). Wnt–β-catenin signalling in liver development, health and disease. *Nature reviews Gastroenterology & hepatology*, *16*(2), pp.121-136.

Petek, M., Orman, A., Dikmen, S. and Alpay, F., (2010). Physical chick parameters and effects on growth performance in broiler. *Archives Animal Breeding*, *53*(1*), pp.108-115.*

Pinchasov Y. (1991). Relationship between the weight of hatching eggs and subsequent early performance of broiler chicks. *British Poultry Science 32:109-11.*

Pinchasov, Y., (1995). Early transition of the digestive system to exogenous nutrition in domestic post-hatch birds. *British journal of nutrition, 73(3), pp.471-478.*

Piórkowska, K., Żukowski, K., Połtowicz, K., Nowak, J., Ropka-Molik, K., Derebecka, N., Wesoły, J. and Wojtysiak, D. (2020) Identification of candidate genes and regulatory factors related to growth rate through hypothalamus transcriptome analyses in broiler chickens. *BMC genomics*, 21(1), pp.1-12.

Pitt, J., Gillingham, P.K., Maltby, M. and Stewart, J.R., (2016). New perspectives on the ecology of early domestic fowl: an interdisciplinary approach. *Journal of Archaeological Science, 74*, pp.1-10.

Plavnik I, Hurwitz S. (1990). Performance of broiler chickens and turkeys' poults subjected to feed restriction or to feeding of low sodium diets at an early age. *Pout. Sci. 69(4): 945-952*.

Plavnik, I. and Hurwitz, S., (1983). Organ weights and body composition in chickens as related to the energy and amino acid requirements: effects of strain, sex, and age. *Poultry Science, 62*(1), *pp.152-163.*

Plavnik, I., McMurtry, J.P. and Rosebrough, R.W., (1986). Effects of early feed restriction in broilers. I. Growth performance and carcass composition. *Growth*, *50*(1), *pp.68-76*.

Powell, J.J., Jugdaohsingh, R. and Thompson, R.P.H., (1999). The regulation of mineral absorption in the gastrointestinal tract. *Proceedings of the Nutrition Society*, 58(1), *pp.147- 153.*

Pratt, S.J., Hernández-Ochoa, E. & Martin, S.S. (2020). Calcium signaling breast cancer's approach to manipulation of cellular circuitry. *Biophys Rev* 12, 1343–1359. https://doi.org/10.1007/s12551-020-00771-9

Proszkowiec-Weglarz, M. and Angel, R., (2013). Calcium and phosphorus metabolism in broilers: Effect of homeostatic mechanism on calcium and phosphorus digestibility. *Journal of Applied Poultry Research, 22*(3), pp.609-627.

Proszkowiec-Weglarz, M., Miska, K.B., Ellestad, L.E., Schreier, L.L., Kahl, S., Darwish, N., Campos, P. and

Shao, J., (2022). Delayed access to feed early post-hatch affects the development and maturation of gastrointestinal tract microbiota in broiler chickens. *BMC microbiology*, *22*(1), p.206.

Punna S and Roland Sr DA (1999). Variation in phytate phosphorus utilization within the same broiler strain. *The Journal of Applied Poultry Research 8, 10-15.*

Rahmani, H.R., Speer, W. and Modirsanei, M., (2005). The effect of intestinal pH on broiler performance and immunity. In *Proceedings of the 15th European Symposium on poultry nutrition, Balatonfüred, Hungary, 25-29 September 2005* (pp. 338-340). World's Poultry Science Association (WPSA).

Raji, A.A., Mohammed, B., Oladele, S.B., Saidu, L., Jibril, A.H. and Cazaban, C., (2017). Bursa body index as a visual indicator for the assessment of bursa of Fabricius. *Journal of veterinary medicine and animal health*, *9*(2), pp.32-38.

Rao, V. S. R. (1986). Litter-Its management and utility in broiler. *Poult. Advis*, *19*(7-12), 31-40.

Rasmusson, C.G. and Eriksson, M.A. (2001) Celiac disease and 228ineralization disturbances of permanent teeth. *International Journal of Paediatric Dentistry*, 11(3), pp.179-183.

Rath, N.C., Huff, G.R., Huff, W.E. and Balog, J.M., (2000). Factors regulating bone maturity and strength in poultry. *Poultry science*, *79*(7), pp.1024-1032.

Ravindran, V. and Abdollahi, M.R., (2021) Nutrition and digestive physiology of the broiler chick: State of the art and outlook. *Animals*, 11(10), p.2795.

Ravindran, V., Tancharoenrat, P., Zaefarian, F. and Ravindran, G., (2016). Fats in poultry nutrition: Digestive physiology and factors influencing their utilization. *Animal Feed Science and Technology*, *213*, pp.1-21.

Ravindran, V., Wu, Y.B., Thomas, D.G. and Morel, P.C.H., (2006). Influence of whole wheat feeding on the development of gastrointestinal tract and performance of broiler chickens. *Australian Journal of Agricultural Research*, *57*(1), pp.21-26.

Rawlinson, S.C., Murray, D.H., Mosley, J.R., Wright, C.D., Bredl, J.C., Saxon, L.K., Loveridge, N., Leterrier, C., Constantin, P., Farquharson, C. and Pitsillides, A.A., (2009). Genetic selection for fast growth

generates bone architecture characterised by enhanced periosteal expansion and limited consolidation of the cortices but a diminution in the early responses to mechanical loading. *Bone*, *45*(2), pp.357-366.

Reece FN, Lolt BD, Deaton JW, Branbn SL. (1986). Meal feeding and broiler performance. *Poult. Sci. 65:1497-1501*.

Reddy, C.V. (2000) Maintaining growth and production. *Poult. Inter. 39(2): 36- 40*

Ribeiro, A.M.L., Ribeiro, A.M.L., Krabbe, E.L., AM, P.J., Renz, S.V. and Gomes, H.A., (2004) Effect of Chick Weight, Geometric Mean Diameter and Sodium Level in Pre-starter Diets (1 to 7 Days) o n Broiler Performance up to 21 Days of Age. *Revista Brasileira de Ciência Avícola, 6(4/225), p.230.*

Richards, M.P. and Proszkowiec-Weglarz, M., (2007). Mechanisms regulating feed intake, energy expenditure, and body weight in poultry. *Poultry science*, *86*(7), pp.1478-1490.

Richards, M.P., (2003). Genetic regulation of feed intake and energy balance in poultry. *Poultry science*, *82*(6), *pp.907-916.*

Richards, M.P., Rosebrough, R.W., Coon, C.N. and McMurtry, J.P., (2010). Feed intake regulation for the female broiler breeder: In theory and in practice. *Journal of applied poultry research*, *19*(2), *pp.182-193.*

Riesenfeld, G., Sklan, D., Bar, A., Eisner, U. and Hurwitz, S., (1980). Glucose absorption and starch digestion in the intestine of the chicken. *The Journal of Nutrition*, *110*(1), *pp.117- 121.*

Romanoff, A.L., (1960). The avian embryo: structural and functional development. *The avian embryo: structural and functional development.*

Rose, N., Constantin, P. and Leterrier, C., (1996). Sex differences in bone growth of broiler chickens. *Growth, development, and aging: GDA*, *60*(2*), pp.49-59.*

Sacranie, A., (2019). Broiler performance: the importance of the first seven days. *International Poultry Production volume 27 Number 4*

Salim, R., Nehvi, I.B., Mir, R.A., Tyagi, A., Ali, S. and Bhat, O.M., (2023). A review on anti-nutritional

factors: Unraveling the natural gateways to human health. *Frontiers in Nutrition, 10,* p.1215873.

Salim, H.M., Jo, C., Lee, B.D., (2008). Zinc in broiler feeding and nutrition. *Avian Biol. Res. 1, 5–18.*

Savory CJ. (1974). Growth and behavior of chickens fed on pellets or mash. *Br. Poult. Sci. 15:281- 286.*

Scanes, C. G., & Pierzchala-Koziec, K. (2014). Biology of the gastro-intestinal tract in poultry. Avian Biology Research, 7.193222.https://doi.Org/10.3184/175815514X14162292284822

Schat, K.A. and Myers, T.J., (1991). Avian intestinal immunity.

Schokker, D., Hoekman, A.J., Smits, M.A. and Rebel, J.M., (2009) Gene expression patterns associated with chicken jejunal development. *Developmental & Comparative Immunology*, 33(11), pp.1156-1164.

Scrimgeour, A.G., Stahl, C.H.H., McClung, J.P., Marchitelli, L.J., Young, A.J., (2007). Moderate zinc deficiency negatively affects biomechanical properties of tibiae independently of body composition. *J. Nutr. Biochem. 18 (12), 813–819.*

Sebők, C., Tráj, P., Vörösházi, J., Mackei, M., Papp, M., Gálfi, P., Neogrády, Z. and Mátis, G., (2021). Two sides to every question: attempts to activate chicken innate immunity in 2D and 3D hepatic cell cultures. *Cells*, *10*(8), p.1910.

Selle, P.H. and Ravindran, V., (2007). Microbial phytase in poultry nutrition. *Animal feed science and technology*, *135*(1-2), pp.1-41.

Sevane, N., Bialade, F., Velasco, S., Rebolé, A., Rodríguez, M.L., Ortiz, L.T., Cañón, J. and Dunner, S., (2014) Dietary inulin supplementation significantly modifies the liver transcriptomic profile of broiler chickens. *PloS one*, 9(6*), p.e98942.*

Shafey, T.M., McDonald, M.W. and Dingle, J.G., (1991). Effects of dietary calcium and available phosphorus concentration on digesta pH and on the availability of calcium, iron, magnesium and zinc from the intestinal contents of meat chickens. *British Poultry Science*, *32*(1), *pp.185-194*

Shahnazari, M., Lang, D.H., Fosmire, G.J., Sharkey, N.A., Mitchell, A.D. and Leach, R.M., (2007). Strontium administration in young chickens improves bone volume and architecture but does not enhance bone

structural and material strength. *Calcified tissue international*, *80*, pp.160-166.

Shang, Y., Kumar, S., Oakley, B. and Kim, W.K., (2018). Chicken gut microbiota: importance and detection technology. *Frontiers in veterinary science*, *5*, *p.254.*

Sheppard A. (2004). The structure and economics of broiler production in England. *Special studies in agricultural economics. No. 59*

Shields, S. J., Garner, J. P., & Mench, J. A. (2004). Dustbathing by broiler chickens: a comparison of preferences for four different substrates. *Applied Animal Behaviour Science*, *87*(1-2*), 69- 82.*

Shields, S. J., Garner, J. P., & Mench, J. A. (2005). Effect of sand and wood-shavings bedding on the behavior of broiler chickens. *Poultry science*, *84(12), 1816-1824.*

Shim, M.Y., Karnuah, A.B., Mitchell, A.D., Anthony, N.B., Pesti, G.M. and Aggrey, S.E., (2012). The effects of growth rate on leg morphology and tibia breaking strength, mineral density, mineral content, and bone ash in broilers. *Poultry Science*, *91*(8), *pp.1790-1795.*

Shira, E.B., Sklan, D. and Friedman, A., (2005) Impaired immune responses in broiler hatchling hindgut following delayed access to feed. *Veterinary immunology and immunopathology*, *105(1-2), pp.33-45*.

Shires, A., Thompson, J.R., Turner, B.V., Kennedy, P.M. and Goh, Y.K., (1987). Rate of passage of corn-canola meal and corn-soybean meal diets through the gastrointestinal tract of broiler and white leghorn chickens. *Poultry Science*, *66*(2), pp.289-298.

Siegel, P.B., (2014). Evolution of the modern broiler and feed efficiency. *Annu. Rev. Anim. Biosci.*, *2*(1), *pp.375-385.*

Siegel, P.B., Picard, M., Nir, I., Dunnington, E.A., Willemsen, M.H. and Williams, P.E., (1997). Responses of meat-type chickens to choose feeding of diets differing in protein and energy from hatch to market weight. *Poultry Science*, *76*(9), *pp.1183-1192.*

Singh, M., Lim, A.J., Muir, W.I. and Groves, P.J., (2021). Comparison of performance and carcass composition of a novel slow-growing crossbred broiler with fast-growing broiler for chicken meat in

Australia. *Poultry Science*, *100*(3), *p.100966.*

Simon, K., (2016). Effects of early life conditions on immunity in broilers and layers *(Doctoral dissertation, Wageningen University and Research).*

Skinner, J.T. and Waldroup, P.W., (1995). Allometric bone development in floor-reared broilers. *Journal of Applied Poultry Research*, *4*(3), pp.265-270.

Sklan, D. and Noy, Y., (2000). Hydrolysis and absorption in the small intestines of posthatch chicks. *Poultry science*, *79*(9), *pp.1306-1310.*

Sklan, D., (2001). Development of the digestive tract of poultry. *World's Poultry Science Journal*, *57*(4), pp.415-428.

Sklan, D., (2003). Fat and carbohydrate use in posthatch chicks. *Poultry Science*, *82*(1), pp.117- 122.

Smirnov, A., Tako, E., Ferket, P.R. and Uni, Z., (2006). Mucin gene expression and mucin content in the chicken intestinal goblet cells are affected by in ovo feeding of carbohydrates. *Poultry science*, *85*(4), *pp.669-673.*

Smith, R.M., Gabler, N.K., Young, J.M., Cai, W., Boddicker, N.J., Anderson, M.J., Huff-Lonergan, E., Dekkers, J.C.M. and Lonergan, S.M., (2011). Effects of selection for decreased residual feed intake on composition and quality of fresh pork. *Journal of animal science*, *89(1), pp.192-200.*

Song, B., Tang, D., Yan, S., Fan, H., Li, G., Shahid, M.S., Mahmood, T. and Guo, Y., (2021). Effects of age on immune function in broiler chickens. *Journal of animal science and biotechnology*, *12*, pp.1-12.

Sözcü, A. and İpek, A., (2013). Incubation conditions affect chick quality and broiler performance.

Spears, J. W. (1999). Re-evaluation of the metabolic essentiality of minerals (A review). *Asian- Aust. J. Anim. Sci. 12:1002- 1008.*

Spratt RS, Leeson S (1987). Effect of protein and energy intake of broiler breeder hens on performance of broiler chicken offspring. *Poultry Science 66:1489-1494.*

Struwe, W.B., Gough, R., Gallagher, M.E., Kenny, D.T., Carrington, S.D., Karlsson, N.G. and Rudd, P.M.,

(2015) Identification of O-glycan Structures from Chicken Intestinal Mucins Provides Insight into Campylobactor jejuni Pathogenicity. *Molecular & Cellular Proteomics, 14(6), pp.1464-1477.*

Sugiharto, S. (2016). Role of nutraceuticals in gut health and growth performance of poultry. *Journal of the Saudi Society of Agricultural Sciences, 15, 99–111*. https://doi.org/10.1016/j.jssas.2014.06.001

Sulistiyanto, B., Akiba, Y. and Sato, K., (1999). Energy utilisation of carbohydrate, fat and protein sources in newly hatched broiler chicks. *British Poultry Science*, *40*(5), *pp.653-659.*

Sunder, G.S., Panda, A.K., Gopinath, N.C., Raju, M.V., Rao, S.V.R. and Kumar, C.V., (2006). Effect of supplemental manganese on mineral uptake by tissues and immune response in broiler chickens. *The Journal of Poultry Science*, *43*(4), pp.371-377.

Sunder, G.S., Panda, A.K., Gopinath, N.C.S., Rao, S.V.R., Raju, M.V.L.N. and Kumar, C.H.V., (2007). Bioavailability of manganese in broiler chickens as influenced by the levels of supplementation and age during juvenile period. *Indian Journal of Animal Sciences, 77*(3), p.269.

Surai, P.F. and Sparks, N.H.C., (2001). Designer eggs: from improvement of egg composition to functional food. *Trends in food science & Technology*, *12*(1), pp.7-16.

Surai, P.F., (2018). *Selenium in poultry nutrition and health*. Wageningen Academic Publishers.

Suttle, N.F, (2010) The mineral nutrition of Livestock, 4th Edition., *CABI Publishing, Oxfordshire, UK*

Svihus, B., (2014). Function of the digestive system. *Journal of Applied Poultry Research, 23*(2*), pp.306-314*

Svihus, B., (2014). Starch digestion capacity of poultry. *Poultry science*, *93*(9), *pp.2394-2399.*

Svihus, B., Kløvstad, K.H., Perez, V., Zimonja, O., Sahlström, S., Schüller, R.B., Jeksrud, W.K. and Prestløkken, E., (2004a). Physical and nutritional effects of pelleting of broiler chicken diets made from wheat ground to different coarsenesses by the physical roller mill and hammer mill. *Animal feed science and technology*, *117*(3-4), *pp.281-293.*

Svihus, B., Kløvstad, K.H., Perez, V., Zimonja, O., Sahlström, S., Schüller, R.B., Jeksrud, W.K. and Prestløkken, E., (2004). Physical and nutritional effects of pelleting of broiler chicken diets made from

wheat ground to different coarsenesses by the use of roller mill and hammer mill. *Animal feed science and technology*, *117*(3-4), *pp.281-293*.

Svihus, B., V. B. Lund, B. Borjgen, M. R. Bedford, and M. Bakken. (2013). Effect of intermittent feeding, struc-tural components and phytase on performance and behaviour of broiler chickens. *Br. Poult. Sci. 54:222–230*

Svihus, B.J.W.S.P.S.J., (2011). The gizzard: function, influence of diet structure and effects on nutrient availability. *World's Poultry Science Journal*, *67*(2), *pp.207-24.*

Swaggerty, C.L., Ferro, P.J., Pevzner, I.Y. and Kogut, M.H., (2005) Heterophils are associated with resistance to systemic Salmonella enteritidis infections in genetically distinct chicken lines. *FEMS Immunology and Medical Microbiology*, 43, *pp.149-154.*

Świątkiewicz, S., Arczewska-Włosek, A. and Jozefiak, D., 2014. The efficacy of organic minerals in poultry nutrition: review and implications of recent studies. *World's Poultry Science Journal*, *70*(3), *pp.475-486.*

Szőllősi, L., Szűcs, I. and Nábrádi, A., (2014). Economic issues of broiler production length. *Економика пољопривреде*, *61*(3), pp.633-646.

Taherparvar, G., Seidavi, A., Asadpour, L., Payan-Carreira, R., Laudadio, V. and Tufarelli, V., (2016). Effect of litter treatment on growth performance, intestinal development, and selected cecum microbiota in broiler chickens. *Revista Brasileira de Zootecnia, 45, pp.257- 264.*

Tako, E., Ferket, P.R. and Uni, Z., (2004). Effects of in ovo feeding of carbohydrates and beta- hydroxy-beta-methylbutyrate on the development of chicken intestine. *Poultry Science, 83*(12), *pp.2023-2028*.

Tako, E., Ferket, P.R. and Uni, Z., (2005). Changes in chicken intestinal zinc exporter mRNA expression and small intestinal functionality following intra-amniotic zinc-methionine administration. *The Journal of nutritional biochemistry*, *16*(6), pp.339-346.

Talaty, P.N., Katanbaf, M.N. and Hester, P.Y., (2009). The life cycle changes in bone mineralization and bone

size traits of commercial broilers. *Poultry science*, *88*(5), pp.1070-1077.

Tallentire, C.W., Leinonen, I. and Kyriazakis, I., (2016). Breeding for efficiency in the broiler chicken: A review. *Agronomy for Sustainable Development*, *36*, pp.1-16.

Tancharoenrat, P., Ravindran, V., Zaefarian, F. and Ravindran, G., (2013). Influence of age on the apparent metabolizable energy and total tract apparent fat digestibility of different fat sources for broiler chickens. *Animal Feed Science and Technology*, *186*(3-4), *pp.186-192.*

Tang, D., Li, Z., Mahmood, T., Liu, D., Hu, Y. and Guo, Y. (2020) The association between microbial community and ileal gene expression on intestinal wall thickness alterations in chickens. *Poultry science*, *99*(4), pp.1847-1861.

Terada, T., Nii, T., Isobe, N. and Yoshimura, Y., (2018) Changes in the expression of avian β- defensins (AvBDs) and proinflammatory cytokines and localization of AvBD2 in the intestine of broiler embryos and chicks during growth. *The Journal of Poultry Science*, 55(4), pp.280-287.Tejeda, O.J., Meloche, K.J. and Starkey, J.D., (2021) Effect of incubator tray location on broiler chicken growth performance, carcass part yields, and the meat quality defects wooden breast and white striping. *Poultry Science*, 100(2), *pp.654- 662.*

Thaiss, C.A., Zmora, N., Levy, M. & Elinav, E. (2016). The microbiome and innate immunity. *Nature, 535, 65–74*

Thomas, D.V. and Ravindran, V., (2009). Mineral retention in young broiler chicks fed diets based on wheat, sorghum or maize. *Asian-Australasian journal of animal sciences*, *23*(1*), pp.68- 73.*

Thomas, D.V., Ravindran, V. and Ravindran, G., (2008). Nutrient digestibility and energy utilisation of diets based on wheat, sorghum or maize by the newly hatched broiler chick. *British poultry science*, *49*(4*), pp.429-435.*

Thompson, F.M., Mayrhofer, G. and Cummins, A.G. (1996) Dependence of epithelial growth of the small intestine on T-cell activation during weaning in the rat. *Gastroenterology111: 37- 44*

Thorp, B. H., and D. Waddington. (1997). Relationships between the bone pathologies, ash, and mineral content of long bones in 35-day old broiler chickens. *Res. Vet. Sci. 62:67–73.*

Tikhonov, I.V., Pliss, E.M., Borodin, L.I. and Sen', V.D., 2016. Effect of superoxide dismutase on the oxidation of methyl linoleate in micelles inhibited by nitroxyl radicals. *Russian Chemical Bulletin, 65, pp.2985-2987.*

Tkalec, M., Prebeg, T., Roje, V., Pevalek-Kozlina, B. and Ljubešić, N., (2008). Cadmium-induced responses in duckweed Lemna minor L. *Acta Physiologiae Plantarum, 30, pp.881-890.*

Toghyani, M., Gheisari, A., Modaresi, M., Tabeidian, S. A., & Toghyani, M. (2010). Effect of different litter material on performance and behavior of broiler chickens. *Applied Animal Behaviour Science*, *122*(1), *48-52.*

Toman, R., Massanyi, P., Lukáč, N., Ducsay, L. and Golian, J., (2005). Fertility and content of cadmium in pheasant (Phasianus colchicus) following cadmium intake in drinking water. *Ecotoxicology and Environmental Safety*, *62*(1), *pp.112-117.*

Tona, K., Onagbesan, O., De Ketelaere, B., Decuypere, E, abd Bruggeman, V. (2004b) Effect of Age of Broiler breeders and Egg Storage on egg quality, hatchability, chick quality, chick weight and chick post hatch growth to 42 days. *Journal of Applied Poultry Research*., 13, 10-18

Torok, V.A., Hughes, R.J., Ophel-Keller, K., Ali, M. and MacAlpine, R., (2009). Influence of different litter materials on cecal microbiota colonization in broiler chickens. *Poultry science*, *88*(12), pp.2474-2481.

Toudic, C., (2007). Evaluating uniformity in broilers-factors affecting variation. *Recuperado el*, *23*.

Traldi, A.B., Menten, J.F.M., Silva, C.S., Rizzo, P.V., Pereira, P.W.Z. and Santarosa, J., (2011). What determines hatchling weight: breeder age or incubated egg weight? *Brazilian Journal of Poultry Science, 13, pp.283-285.*

Truong, A.D., Hong, Y., Hoang, C.T., Lee, J. and Hong, Y.H., (2017) Chicken IL-26 regulates immune responses through the JAK/STAT and NF-κB signaling pathways. *Developmental & Comparative*

Immunology, 73, *pp.10-20.*

Tur, J.A., Rial, R.V., Esteban, S. and Rayo, J.M., (1986). Ontogeny of gastrointestinal motility in young broilers. *Comparative Biochemistry and Physiology Part A: Physiology*, *83*(1), pp.61-65.

UN United Nations, (2015). World population projected to reach 9.7 billion by 2050 https://www.un.org/en/desa/world-population-projected-reach-98-billion-2050-and-112-billion2100#:~:text=Calendar-,World%20population%20projected%20to%20reach%209.8%20billion%20in%202050%2C%20and,Nations%20report%20being%20launched%20t oday

Underwood, E.J., Suttle, N.F., (1999). The mineral nutrition of livestock, 3rd edition. *CABI Publishing, Biddles Ltd., Guilford and King'S Lynn, UK, pp. 477–512.*

Uni, Z. and Ferket, R.P., (2004). Methods for early nutrition and their potential. *World's Poultry Science Journal*, *60*(1), *pp.101-111.*

Uni, Z., NOY, Y. and Sklan, D. (1999) Posthatch development of small intestinal function in the poult. *Poultry Science 78 215-222*

Uni, Z., Noy, Y. and Sklan, D. (1995). Posthatch changes in morphology and function of the small intestines in heavy– and light–strain chicks. *Poultry Science 74, 1622–1629.*

Uni, Z., Noy, Y. and Sklan, D., (1996). Development of the small intestine in heavy and light strain chicks before and after hatching. *British Poultry Science*, *37*(1*), pp.63-71.*

Uni, Z., Tako, E., Gal-Garber, O. and Sklan, D., (2003). Morphological, molecular, and functional changes in the chicken small intestine of the late-term embryo. *Poultry science*, *82*(11), *pp.1747-1754.*

Uni, Z., Tako, E., Gal-Garber, O. and Sklan, D., (2003b). Morphological, molecular, and functional changes in the chicken small intestine of the late-term embryo. *Poultry science*, *82*(11), *pp.1747-1754.*

Uni, Z.E.H.A.V.A., Ganot, S.A.H.A.R. and Sklan, D.A.V.I.D., (1998). Posthatch development of mucosal function in the broiler small intestine. *Poultry Science*, *77*(1), *pp.75-82.*

Van de Ven, L.J.F., Van Wagenberg, A.V., Decuypere, E., Kemp, B. and Van den Brand, H., (2013). Perinatal

broiler physiology between hatching and chick collection in 2 hatching systems. *Poultry science*, *92*(4), *pp.1050-1061*.

Van Soest, P.V., Robertson, J.B. and Lewis, B.A., (1991). Methods for dietary fiber, neutral detergent fiber, and nonstarch polysaccharides in relation to animal nutrition. *Journal of dairy science*, *74*(10), *pp.3583-3597*.

Vasdal, G., Granquist, E.G., Skjerve, E., De Jong, I.C., Berg, C., Michel, V. and Moe, R.O., (2019). Associations between carcass weight uniformity and production measures on farm and at slaughter in commercial broiler flocks. *Poultry Science*, *98*(10), *pp.4261-4268*.

Vieira, S.L. (2008). Chelated minerals for poultry. *Braz. J. Poultry Sci., 10: 73–79.*

Villa, C.R., Ward, W.E. and Comelli, E.M., (2017). Gut microbiota-bone axis. *Critical reviews in food science and nutrition*, *57*(8), *pp.1664-1672.*

Walk, C. L., M. R. Bedford, and A. P. McElroy. (2012). Influence of limestone and phytase on broiler performance, gastrointestinal pH, and apparent ileal nutrient digestibility. *Poult. Sci. 91:1371-1378*

Walk, C.L., Bedford, M.R. and McElroy, A.P., (2012). In vitro evaluation of limestone, dicalcium phosphate, and phytase on calcium and phosphorus solubility of corn and soybean meal. *Poultry science*, *91*(3), *pp.674-682.*

Wang, B., Wang, S., Ding, M., Lu, H., Wu, H. and Li, Y., (2022) Quercetin regulates calcium and phosphorus metabolism through the Wnt signaling pathway in broilers. *Frontiers in Veterinary Science*, 8, *p.786519.*

Wang, D., Huang, H., Zhou, L., Li, W., Zhou, H., Hou, G., Liu, J. and Hu, L., (2015). Effects of dietary supplementation with turmeric rhizome extract on growth performance, carcass characteristics, antioxidant capability, and meat quality of Wenchang broiler chickens. *Italian Journal of Animal Science*, *14*(3*), p.3870.*

Wang, S.Y. and Huang, D.J. (2005) Assessment of greenhouse gas emissions from poultry enteric fermentation.

Asian-Autralasian Journal of Animal Science18: 873-878.

Wang, W., Ouyang, K., Ouyang, J., Li, H., Lin, S. and Sun, H., (2004) Polymorphism of insulin- like growth factor I gene in six chicken breeds and its relationship with growth traits. *Asian-australasian journal of animal sciences*, 17(3), *pp.301-304.*

Wang, W.W., Wang, J., Zhang, H.J., Wu, S.G. and Qi, G.H., (2019) Transcriptome analysis reveals mechanism underlying the differential intestinal functionality of laying hens in the late phase and peak phase of production. *BMC genomics*, 20(1*), pp.1-14.*

Wang, Z., Cerrate, S., Coto, C., Yan, F. and Waldroup, P.W., (2007) Evaluation of Mintrex copper as a source of copper in broiler diets. *Int J Poult Sci*, 6(5), *pp.308-313.*

Wang, Z., Gerstein, M. and Snyder, M., (2009). RNA-Seq: a revolutionary tool for transcriptomics. *Nature reviews genetics*, *10*(1), pp.57-63.

Watson, L. T., C. B. Ammerman, S. M. Miller and R. R. Harms. (1970). Biological assay of inorganic manganese for chicks. *Poultry Sci. 49: 1548-1554.*

WAQAS, M.M., NASTOH, M.N.A. And SALMAN, M., (2024). Minerals, and vitamins in poultry, *P.169*

Weeks, C.A., Danbury, T.D., Davies, H.C., Hunt, P. and Kestin, S.C., (2000). The behaviour of broiler chickens and its modification by lameness. *Applied animal behaviour science*, *67*(1-2), *pp.111-125.*

West, B. and Zhou, B.X., (1988). Did chickens go north? New evidence for domestication. *Journal of archaeological science*, *15*(5), *pp.515-533.*

Wigley, P., (2013). Immunity to bacterial infection in the chicken. *Developmental & Comparative Immunology*, *41*(3), *pp.413-417.*

Wijtten, P.J.A., Langhout, D.J. and Verstegen, M.W.A., (2012). Small intestine development in chicks after hatch and in pigs around the time of weaning and its relation with nutrition: A review. *Acta Agriculturae Scandinavica, Section A–Animal Science*, *62*(1), *pp.1-12.*

Willemsen, H., Everaert, N., Witters, A., De Smit, L., Debonne, M., Verschuere, F., Garain, P., Berckmans, D., Decuypere, E. and Bruggeman, V., (2008). Critical assessment of chick quality measurements as an indicator of posthatch performance. *Poultry science*, 87(11*), pp.2358-2366*

Williams, B., Solomon, S., Waddington, D., Thorp, B. and Farquharson, C., (2000). Skeletal development in the meat-type chicken. *British Poultry Science*, *41*(2), *pp.141-149.*

Williams, B., Waddington, D., Murray, D.H. and Farquharson, C., (2004). Bone strength during growth: influence of growth rate on cortical porosity and mineralization. *Calcified Tissue International*, *74*, *pp.236-245.*

Wilson HR. (1991). Interrelationships of egg size, chick size, posthatching growth and hatchability. *World's Poultry Science Journal 1(47):5-20.*

Willis, W. L., Murray, C., & Talbott, C. (1997). Evaluation of leaves as a litter material. *Poultry Science*, *76*(8), *1138-1140.*

Withanage, G.S.K., Kaiser, P., Wigley, P., Powers, C., Mastroeni, P., Brooks, H., Barrow, P., Smith, A., Maskell, D. and McConnell, I., (2004) Rapid expression of chemokines and proinflammatory cytokines in newly hatched chickens infected with Salmonella enterica serovar typhimurium. *Infection and immunity*, 72(4), *pp.2152-2159.*

Woyengo, T.A. and Nyachoti, C.M., (2011). Supplementation of phytase and carbohydrases to diets for poultry. *Canadian Journal of Animal Science*, *91*(2), *pp.177-192.*

Wu, Z. and Kaiser, P., (2011). Antigen presenting cells in a non-mammalian model system, the chicken. *Immunobiology*, *216*(11), *pp.1177-1183.*

Xiao, Y., Wu, C., Li, K., Gui, G., Zhang, G. and Yang, H., (2017). Association of growth rate with hormone levels and myogenic gene expression profile in broilers. *Journal of animal science and biotechnology*, *8*, *pp.1-7.*

Yair, R. and Uni, Z., (2011). Content and uptake of minerals in the yolk of broiler embryos during incubation

and effect of nutrient enrichment. *Poultry Science, 90(7), pp.1523-1531*

Yair, R. and Uni, Z., (2011). Content and uptake of minerals in the yolk of broiler embryos during incubation and effect of nutrient enrichment. *Poultry Science*, *90*(7), *pp.1523-1531.*

Yamauchi, K.E. and Isshiki, Y., (1991). Scanning electron microscopic observations on the intestinal villi in growing White Leghorn and broiler chickens from 1 to 30 days of age. *British Poultry Science*, *32*(1), *pp.67-78.*

Yamauchi, K.E., (2002). Review on chicken intestinal villus histological alterations related with intestinal function. *The Journal of Poultry Science, 39*(4), *pp.229-242.*

Yerpes, M., Llonch, P. and Manteca, X. (2020). Factors associated with cumulative first week mortality in broiler chicks. *Animals, 10, 310*; doi:10.3390/ani10020310

Youness, E.R., Mohammed, N.A. and Morsy, F.A., (2012) Cadmium impact and osteoporosis: mechanism of action. *Toxicology Mechanisms and Methods*, 22(7), *pp.560-567.*

Zaccaria, V., Curti, V., Di Lorenzo, A., Baldi, A., Maccario, C., Sommatis, S., Mocchi, R. and Daglia, M., (2017) The effect of green and brown propolis extracts on the expression levels of microRNAs, mRNAs and proteins, related to oxidative stress and inflammation. *Nutrients*, 9(10*), p.1090.*

Zaefarian, F., Abdollahi, M.R., Cowieson, A. and Ravindran, V., (2019). Avian liver: the forgotten organ. *Animals, 9*(2), *p.6*

Zampiga, M., Bertocchi, M., Bosi, P., Trevisi, P., Meluzzi, A. and Sirri, F., (2019) Differences in productive performance and intestinal transcriptomic profile in two modern fast-growing chicken hybrids. *Journal of animal physiology and animal nutrition*, *103(1), pp.125-134.*

Zelenka, J. (1968). Influence of the age of chickens on the metabolisable energy values of poultry diets. *Br. Poult. Sci. 9: 135-142.*

Zelenka, J. and Ceresnakova, Z., (2005). Effect of age on digestibility of starch in chickens with different growth rate. *Czech J. Anim. Sci*, *50, pp.411-415.*

Zelenka, J., Fajmonová, E. and Blažková, E., (2000). Apparent digestibility of fat and nitrogen retention in young chicks. *Czech Journal of Animal Science*, *45*(10), *pp.457-462.*

Zuidhof, M. J., Schneider, B. L., Carney, V. L., Korver, D. R., & Robinson, F. E. (2014). Growth, efficiency, and yield of commercial broilers from 1957, 1978, and 2005. *Poultry Science, 93, 2970–2982.* https://doi. org/10.3382/ps.2014-0429

Zuidhof, M.J., Fedorak, M.V., Ouellette, C.A. and Wenger, I.I., (2017). Precision feeding Innovative management of broiler breeder feed intake and flock uniformity. *Poultry science*, *96*(7), pp.2254-2263.

Appendix

Appendix 1: Ross 308 male broiler chicks' performance from 0-21days

Day	Bodyweight (g)	Daily weight gain (g)	Daily intake (g)	FCR
0	43	-	-	-
1	60	17	-	-
2	78	18	16	0.346
3	98	20	20	0.474
4	121	23	23	0.579
5	147	26	28	0.666
6	175	29	32	0.738
7	207	32	36	0.799
8	242	35	41	0.851
9	281	39	45	0.896
10	323	42	50	0.934
11	369	46	55	0.969
12	418	49	60	0.999
13	471	53	66	1.027
14	527	56	71	1.053
15	587	60	77	1.076
16	650	63	83	1.099
17	717	67	89	1.120
18	788	70	95	1.141
19	861	74	101	1.161
20	938	77	108	1.181
21	1018	80	114	1.200

Source: Aviagen, 2019

Appendix 2: Macro and trace mineral concentration of the commercial diet (Trial 1)

Elements	Mean concentrations (mg/kg) ash basis	Concentration in fresh basis
Ash %	6.6	-
B	140.3	9.2
Na	29989.6	1976.3
Mg	27476.9	1810.7
P	139778.1	9211.4
S	7247.6	477.6
K	158700.2	10458.3
Ca	127773.3	8420.3
TI (semi-quant)	597795.1	39394.7
Li	0.7	0.0
Be	0.7	0.0
Al	1086.8	71.6
V	32.2	2.1
Chromium (Cr)	20.5	1.4
Manganese (Mn)	1727.3	113.8
Iron (Fe)	1231.7	81.2
Cobalt (Co)	3.8	0.3
Nickel (Ni)	9.7	0.6
Copper (Cu)	251.9	16.6
Zinc (Zn)	1397.1	92.1
As	1.7	0.1
Selenium (Se)	0.2	0.0
Rubidium (Rb)	73.4	4.8
Strontium (Sr)	121.1	8.0
Mo	22.5	1.5
Ag	0.1	0.0
Cadmium (Cd)	0.7	0.0
Cesium (Cs)	0.3	0.0
Ba	26.3	1.7
Ti	0.0	0.0
Pb	1.8	0.1
U	24.7	1.6

Appendix 3: Macro and trace mineral concentrations of the wood shavings (Trial 1)

Mineral elements	concentrations (mg/kg) ash basis	Concentration in fresh basis
%Ash on dry basis (ash/dry sample) *100	0.3	-
B	433.5	1.4
Na	1783.9	5.7
Mg	39042.7	124.9
P	8591.9	27.5
S	12607.8	40.3
K	76413.5	244.5
Ca	240616.9	770.0
TI (semi-quant)	220332.2	705.1
Li	1.8	0.0
Be	0.2	0.0
Al	719.5	2.3
V	1.2	0.0
Chromium (Cr)	12.0	0.0
Manganese (Mn)	28415.3	90.9
Iron (Fe)	664.8	2.1
Cobalt (Co)	8.6	0.0
Nickel (Ni)	13.5	0.0
Copper (Cu)	154.3	0.5
Zinc (Zn)	1777.4	5.7
As	0.7	0.0
Selenium (Se)	1.6	0.0
Rubidium (Rb)	144.5	0.5
Strontium (Sr)	1342.5	4.3
Mo	0.7	0.0
Ag	5.6	0.0
Cadmium (Cd)	20.0	0.1
Cesium (Cs)	0.5	0.0
Ba	5553.3	17.8
Ti	0.1	0.0
Pb	29.0	0.1
U	0.1	0.0

Appendix 4: Primer pairs used for the qRT-PCR analysis (Trial 1)

S/No	Gene	Accession number	Primer pair sequence (5' ->3')	Product length
1	IL8L1	NM_205018.2	Forward AATGGCTGGAGCAAAAGGTATG Reverse AGTGGGATCCAGACACACTTC	196
2	CCL17	NM_001293309.2	Forward CAGCCCCATATGCACCTTCA Reverse TTGAACAGACCTTGGTCCCG	150
3	GSTA3	NM_001001777.2	Forward AGAGTCGAAGCCTGATGCAC Reverse CACTCCACTTATCAGCAAACAGA	220
4	IL20RA	XM_046915188.1	Forward TGGAGGAGAAGTCCTGATGGA Reverse CCACTGTGTAGAAGTGGCGT	209
	Housekeeping genes			
1	GAPDH	NM_204305.2	Forward CCCCCATGTTTGTGATGGGT Reverse TGATGGCATGGACAGTGGTC	162
3	GALNS	NM_001389560.2	Forward ATACCTGCAGGAAGCGTCAG Reverse AGCCTTGTAAAGCCCGACTC	213
4	FABP5	NM_001006346.2	Forward GAGCAGGAATGGGATGGCAA Reverse GCACTCAGCTACTCAGTCCA	242
4	FAM133B	NM_001030975.2	Forward TGAGTCAGACAGCAAGGACAG Reverse GATAAAGGCCCATCACCACGA	123

Appendix 5: Basic information of the DEGs in the ileum of SP and UP 7days old chicks (190 mapped gene ids out of 233 genes submitted) performed by IPA.

Gene symbol	Entrez Gene Name	Location	Type of molecule	Expr Fold Change (SP vs. UP)	Expr p-value
ABCC2	ATP binding cassette subfamily C member 2	Plasma Membrane	transporter	-1.402	0.0109
ADAM5	ADAM metallopeptidase domain 5 (pseudogene)	Other	other	1.342	0.045
ADCY1	adenylate cyclase 1	Plasma Membrane	enzyme	1.267	0.000793
AGO1	argonaute RISC component 1	Cytoplasm	translation regulator	-1.271	0.0221
AGPAT5	1-acylglycerol-3-phosphate O-acyltransferase 5	Cytoplasm	enzyme	-1.256	0.047
AKAP12	A-kinase anchoring protein 12	Cytoplasm	transporter	1.375	0.0101
AKAP12	A-kinase anchoring protein 12	Cytoplasm	transporter	1.304	0.0132
AMD1	adenosylmethionine decarboxylase 1	Cytoplasm	enzyme	-1.446	0.00557
AMN	amnion associated transmembrane protein	Plasma Membrane	other	-1.376	0.0102
APOLD1	apolipoprotein L domain containing 1	Other	other	1.343	0.0304
ASIC5	acid sensing ion channel subunit family member 5	Plasma Membrane	ion channel	1.355	0.0246
ATP8B1	ATPase phospholipid transporting 8B1	Plasma Membrane	transporter	2.121	3.35E-05
ATRNL1	attractin like 1	Other	other	1.253	0.0419
BDH1	3-hydroxybutyrate dehydrogenase 1	Cytoplasm	enzyme	-1.31	0.011
BEST1	bestrophin 1	Plasma Membrane	ion channel	1.251	0.0334
BTBD6	BTB domain containing 6	Cytoplasm	other	1.467	0.0484
C1QC	complement C1q C chain	Extracellular Space	other	1.272	0.0316
CA7	carbonic anhydrase 7	Cytoplasm	enzyme	-2.419	0.0466
CACNA1C	calcium voltage-gated channel subunit alpha1 C	Plasma Membrane	ion channel	1.259	0.0215
CACNB4	calcium voltage-gated channel auxiliary subunit beta 4	Plasma Membrane	ion channel	-1.341	0.00464
CCDC141	coiled-coil domain containing 141	Cytoplasm	other	1.263	0.0417
CCKAR	cholecystokinin A receptor	Plasma Membrane	G-protein coupled receptor	1.382	0.000864
CCL17	C-C motif chemokine ligand 17	Extracellular Space	cytokine	1.634	0.0257
CDH13	cadherin 13	Plasma	other	1.261	0.0167

CDHR1	cadherin related family member 1	Plasma Membrane	other	2.344	0.029
CDX2	caudal type homeobox 2	Nucleus	transcription regulator	-1.389	0.0332
CEBPB	CCAAT enhancer binding protein beta	Nucleus	transcription regulator	1.297	0.00986
CERS4	ceramide synthase 4	Cytoplasm	transcription regulator	1.257	0.00538
CHRDL1	chordin like 1	Extracellular Space	other	1.414	0.0206
CLCN4	chloride voltage-gated channel 4	Plasma Membrane	ion channel	1.281	0.00503
CLEC3B	C-type lectin domain family 3 member B	Extracellular Space	other	1.373	0.00666
CNRIP1	cannabinoid receptor interacting protein 1	Extracellular Space	other	1.257	0.0105
COG7	component of oligomeric golgi complex 7	Cytoplasm	transporter	-1.385	0.00203
COL17A1	collagen type XVII alpha 1 chain	Extracellular Space	other	-1.645	0.0437
COL20A1	collagen type XX alpha 1 chain	Extracellular Space	other	1.367	0.00137
COQ3	coenzyme Q3, methyltransferase	Cytoplasm	enzyme	-1.265	0.0179
CPO	carboxypeptidase O	Plasma Membrane	enzyme	-2.13	0.0235
CRISPLD2	cysteine rich secretory protein LCCL domain containing 2	Cytoplasm	other	1.283	0.0487
CRY1	cryptochrome circadian regulator 1	Nucleus	enzyme	-1.283	0.00886
CTBP2	C-terminal binding protein 2	Nucleus	transcription regulator	1.261	0.028
DBF4	DBF4 zinc finger	Nucleus	kinase	-1.347	0.0195
DCX	doublecortin	Cytoplasm	other	1.493	0.0115
DDX60	DExD/H-box helicase 60	Cytoplasm	enzyme	-1.569	0.0405
DOT1L	DOT1 like histone lysine methyltransferase	Nucleus	phosphatase	-1.313	0.0352
DTNA	dystrobrevin alpha	Plasma Membrane	other	1.335	0.00404
DUSP6	dual specificity phosphatase 6	Cytoplasm	phosphatase	1.268	0.019
EDN2	endothelin 2	Extracellular Space	growth factor	-1.386	0.0231
ENPP1	ectonucleotide pyrophosphatase/phosphodiesterase 1	Plasma Membrane	enzyme	-1.37	0.0269
EPGN	epithelial mitogen	Extracellular Space	growth factor	1.266	0.000551
ETNK1	ethanolamine kinase 1	Cytoplasm	kinase	-1.255	0.0249
EVA1A	eva-1 homolog A, regulator of	Plasma	other	1.286	0.0371

	programmed cell death	Membrane			
EVA1C	eva-1 homolog C	Other	other	1.337	0.0232
FAM162A	family with sequence similarity 162 member A	Cytoplasm	other	-1.314	0.00433
FAM184A	family with sequence similarity 184 member A	Extracellular Space	other	1.256	0.0398
FAM81A	family with sequence similarity 81 member A	Other	other	-1.41	0.0401
FAP	fibroblast activation protein alpha	Cytoplasm	peptidase	1.326	0.0411
FBXL22	F-box and leucine rich repeat protein 22	Other	enzyme	1.279	0.0384
FBXL7	F-box and leucine rich repeat protein 7	Cytoplasm	enzyme	1.269	0.00434
FEN1	flap structure-specific endonuclease 1	Nucleus	enzyme	-1.279	0.0447
FGF14	fibroblast growth factor 14	Extracellular Space	growth factor	1.28	0.028
FITM2	fat storage inducing transmembrane protein 2	Cytoplasm	other	-1.41	0.0109
FZD1	frizzled class receptor 1	Plasma Membrane	G-protein coupled receptor	1.333	0.0476
G6PC2	glucose-6-phosphatase catalytic subunit 2	Cytoplasm	phosphatase	1.396	0.0284
GABRG3	gamma-aminobutyric acid type A receptor subunit gamma3	Plasma Membrane	ion channel	1.274	0.00637
GALNT15	polypeptide N-acetylgalactosaminyltransferase 15	Cytoplasm	enzyme	1.394	0.00613
GALNT17	polypeptide N-acetylgalactosaminyltransferase 17	Cytoplasm	enzyme	1.306	0.0309
GDF9	growth differentiation factor 9	Extracellular Space	growth factor	-1.289	0.046
GDNF	glial cell derived neurotrophic factor	Extracellular Space	growth factor	-1.348	0.0221
GLDC	glycine decarboxylase	Cytoplasm	enzyme	1.255	0.0487
GPR6	G protein-coupled receptor 6	Plasma Membrane	G-protein coupled receptor	1.299	0.000756
GPR85	G protein-coupled receptor 85	Plasma Membrane	G-protein coupled receptor	1.337	0.0274
GSTA3	glutathione S-transferase alpha 3	Cytoplasm	enzyme	1.513	0.0017
GYPC	glycophorin C (Gerbich blood group)	Plasma Membrane	other	1.303	0.0345
HPDL	4-hydroxyphenylpyruvate dioxygenase like	Other	other	1.285	0.046
HPGDS	hematopoietic prostaglandin D synthase	Cytoplasm	enzyme	1.54	0.0263
HRH3	histamine receptor H3	Plasma Membrane	G-protein coupled receptor	1.328	0.04
HTR2A	5-hydroxytryptamine receptor 2A	Plasma	G-protein	1.354	0.0445

		Membrane	coupled receptor		
IGF1		Extracellular			
	insulin like growth factor 1	Space	growth factor	1.454	0.00602
IGFBP2	insulin like growth factor binding	Extracellular			
	protein 2	Space	other	1.442	0.00182
IL17D		Extracellular			
	interleukin 17D	Space	cytokine	1.301	0.0184
IL1RAP	interleukin 1 receptor accessory	Plasma	transmembrane		
	protein	Membrane	receptor	1.391	0.0205
IL20RA		Plasma	transmembrane		
	interleukin 20 receptor subunit alpha	Membrane	receptor	1.921	0.0339
IL22RA2	interleukin 22 receptor subunit alpha	Plasma	transmembrane		
	2	Membrane	receptor	2.771	0.0101
IL26		Extracellular			
	interleukin 26	Space	cytokine	1.766	0.0195
IL4R		Plasma	transmembrane		
	interleukin 4 receptor	Membrane	receptor	1.274	0.00645
ITGBL1		Extracellular			
	integrin subunit beta like 1	Space	other	1.738	0.0422
ITIH5	inter-alpha-trypsin inhibitor heavy	Plasma			
	chain 5	Membrane	other	1.278	0.00443
KCNMB1	potassium calcium-activated channel				
	subfamily M regulatory beta subunit	Plasma			
	1	Membrane	ion channel	1.26	0.0118
let-7	microRNA let-7i	Cytoplasm	microRNA	1.284	0.0382
LHX5			transcription		
	LIM homeobox 5	Nucleus	regulator	1.265	0.00859
LPCAT1	lysophosphatidylcholine				
	acyltransferase 1	Cytoplasm	enzyme	-1.288	0.032
LRFN5	leucine rich repeat and fibronectin				
	type III domain containing 5	Nucleus	other	-1.921	0.00559
LRRN4		Plasma			
	leucine rich repeat neuronal 4	Membrane	other	1.269	0.0398
MAFF			transcription		
	MAF bZIP transcription factor F	Nucleus	regulator	1.39	0.0265
MATN3		Extracellular			
	matrilin 3	Space	other	1.268	0.0187
ME1	malic enzyme 1	Cytoplasm	enzyme	1.65	0.00816
MFAP5		Extracellular			
	microfibril associated protein 5	Space	other	1.58	0.0394
mir-23	microRNA 23a	Cytoplasm	microRNA	1.698	0.029
mir-25	microRNA 25	Cytoplasm	microRNA	1.27	0.0347
mir-27	microRNA 27a	Cytoplasm	microRNA	1.81	0.00436
mir-7	microRNA 7-1	Cytoplasm	microRNA	1.348	0.0469
MRPS22	mitochondrial ribosomal protein S22	Cytoplasm	other	-1.314	0.00346
MSC			transcription		
	musculin	Cytoplasm	regulator	1.319	0.0369
MYO1B	myosin IB	Cytoplasm	enzyme	-1.279	0.039

MYOCD	myocardin	Nucleus	transcription regulator	1.433	0.0404
NAALAD2	N-acetylated alpha-linked acidic dipeptidase 2	Plasma Membrane	peptidase	1.284	0.0446
NAV3	neuron navigator 3	Nucleus	other	1.261	0.0245
NINJ2	ninjurin 2	Plasma Membrane	other	1.334	0.0113
NMI	N-myc and STAT interactor	Cytoplasm	transcription regulator	-1.446	0.0246
NOS2	nitric oxide synthase 2	Cytoplasm	enzyme	1.361	0.0146
NOXA1	NADPH oxidase activator 1	Plasma Membrane	other	1.275	0.0441
NOXO1	NADPH oxidase organizer 1	Plasma Membrane	other	1.845	0.0229
NTF3	neurotrophin 3	Extracellular Space	growth factor	1.257	0.0258
NUBPL	nucleotide binding protein like	Cytoplasm	other	-1.327	0.013
ODF2L	outer dense fiber of sperm tails 2 like	Cytoplasm	other	1.86	0.0363
OGFRL1	opioid growth factor receptor like 1	Other	other	-1.332	0.0402
PAK5	p21 (RAC1) activated kinase 5	Nucleus	kinase	1.257	0.0482
PCBP3	poly(rC) binding protein 3	Nucleus	transcription regulator	1.279	0.00956
PCNX2	pecanex 2	Other	other	1.632	0.00177
PEX14	peroxisomal biogenesis factor 14	Cytoplasm	transcription regulator	-1.289	0.00506
PKP2	plakophilin 2	Plasma Membrane	other	-1.423	0.000936
PLXDC2	plexin domain containing 2	Extracellular Space	other	1.266	0.0123
PODN	podocan	Cytoplasm	other	1.328	0.00848
POGLUT1	protein O-glucosyltransferase 1	Extracellular Space	enzyme	-1.366	0.0326
POSTN	periostin	Extracellular Space	other	1.393	0.0308
PPIF	peptidylprolyl isomerase F	Cytoplasm	enzyme	-1.323	0.0215
PPL	periplakin	Cytoplasm	other	1.306	0.0192
PPP1R3B	protein phosphatase 1 regulatory subunit 3B	Cytoplasm	phosphatase	-1.35	0.00173
PRDM1	PR/SET domain 1	Nucleus	transcription regulator	1.252	0.000328
PRIMA1	proline rich membrane anchor 1	Plasma Membrane	other	1.318	0.00642
PRLH	prolactin releasing hormone	Extracellular Space	cytokine	1.358	0.0132
PTGES	prostaglandin E synthase	Cytoplasm	enzyme	1.468	0.0388
QPCT	glutaminyl-peptide cyclotransferase	Cytoplasm	enzyme	1.312	0.0381
RAMP1	receptor activity modifying protein 1	Plasma	G-protein	1.258	0.0448

		Membrane	coupled receptor		
RBM11	RNA binding motif protein 11	Nucleus	other	-1.331	0.0149
RCAN3	RCAN family member 3	Other	other	-1.286	0.0326
RET	ret proto-oncogene	Plasma Membrane	kinase	1.342	0.0365
RIMS1	regulating synaptic membrane exocytosis 1	Plasma Membrane	other	1.263	0.0139
RMDN1	regulator of microtubule dynamics 1	Cytoplasm	other	-1.279	0.00241
ROMO1	reactive oxygen species modulator 1	Cytoplasm	other	1.275	0.0133
ROR2	receptor tyrosine kinase like orphan receptor 2	Plasma Membrane	kinase	1.284	0.028
RRAGD	Ras related GTP binding D	Cytoplasm	enzyme	-1.284	0.0408
S100B	S100 calcium binding protein B	Cytoplasm	other	-1.407	0.0474
SAMM50	SAMM50 sorting and assembly machinery component	Cytoplasm	other	-1.258	0.0222
SCARNA15	small Cajal body-specific RNA 15	Other	other	1.266	0.0107
SDR42E2	short chain dehydrogenase/reductase family 42E, member 2	Other	other	-1.372	0.0339
SELENOO	selenoprotein O	Extracellular Space	enzyme	-1.333	0.0112
SEMA3D	semaphorin 3D	Extracellular Space	other	1.336	0.00604
SERPINF1	serpin family F member 1	Extracellular Space	other	1.275	0.0411
SFRP1	secreted frizzled related protein 1	Plasma Membrane	transmembrane receptor	-1.29	0.0292
SHISAL1	shisa like 1	Other	other	1.537	0.0157
SHQ1	SHQ1, H/ACA ribonucleoprotein assembly factor	Nucleus	other	1.345	0.0359
SLC15A1	solute carrier family 15 member 1	Plasma Membrane	transporter	-1.386	0.0144
SLC34A2	solute carrier family 34 member 2	Plasma Membrane	transporter	-3.615	0.00156
SLC38A4	solute carrier family 38 member 4	Plasma Membrane	transporter	1.522	0.0174
SLC7A6	solute carrier family 7 member 6	Plasma Membrane	transporter	-1.304	0.0349
SLC9A7	solute carrier family 9 member A7	Cytoplasm	transporter	-1.262	0.0363
SMAD9	SMAD family member 9	Nucleus	transcription regulator	1.259	0.0293
SMOC2	SPARC related modular calcium binding 2	Extracellular Space	other	1.583	0.015
SNX12	sorting nexin 12	Cytoplasm	transporter	-1.289	0.0246
SOX3	SRY-box transcription factor 3	Nucleus	transcription regulator	1.318	0.00388
SRGAP3	SLIT-ROBO Rho GTPase activating protein 3	Cytoplasm	other	-1.265	0.00798
ST3GAL1	ST3 beta-galactoside alpha-2,3-	Cytoplasm	enzyme	1.407	0.00685

	sialyltransferase 1				
STAC	SH3 and cysteine rich domain	Cytoplasm	other	1.38	0.0476
STEAP2	STEAP2 metalloreductase	Plasma Membrane	enzyme	1.43	0.00518
SULT4A1	sulfotransferase family 4A member 1	Cytoplasm	enzyme	1.266	0.0215
SWI5	SWI5 homologous recombination repair protein	Nucleus	other	-1.369	0.0184
SYT6	synaptotagmin 6	Cytoplasm	transporter	1.258	0.00642
TACR2	tachykinin receptor 2	Plasma Membrane	G-protein coupled receptor	-1.464	0.0169
TACR2	tachykinin receptor 2	Plasma Membrane	G-protein coupled receptor	1.351	0.028
TAF1D	TATA-box binding protein associated factor, RNA polymerase I subunit D	Nucleus	other	-1.271	0.0393
TAPT1	transmembrane anterior posterior transformation 1	Plasma Membrane	G-protein coupled receptor	-1.259	0.0364
TERB1	telomere repeat binding bouquet formation protein 1	Nucleus	peptidase	-1.29	0.00916
TIPARP	TCDD inducible poly (ADP-ribose) polymerase	Nucleus	enzyme	-1.289	0.00282
TMEM123	transmembrane protein 123	Plasma Membrane	other	-1.282	0.0437
TMEM47	transmembrane protein 47	Plasma Membrane	other	1.271	0.026
TNFRSF1B	TNF receptor superfamily member 1B	Plasma Membrane	transmembrane receptor	1.424	0.0351
TSHR	thyroid stimulating hormone receptor	Plasma Membrane	G-protein coupled receptor	1.264	0.00466
TSHZ2	teashirt zinc finger homeobox 2	Other	transcription regulator	1.402	0.0189
TTLL2	tubulin tyrosine ligase like 2	Other	other	1.401	0.00383
TTLL2	tubulin tyrosine ligase like 2	Other	other	2.16	0.0393
TYRO3	TYRO3 protein tyrosine kinase	Plasma Membrane	kinase	-1.368	0.0153
UQCR10	ubiquinol-cytochrome c reductase, complex III subunit X	Cytoplasm	enzyme	1.283	0.0224
WASF1	WASP family member 1	Nucleus	other	-1.878	0.00271
WDR4	WD repeat domain 4	Nucleus	other	-1.355	0.0344
WNT7B	Wnt family member 7B	Extracellular Space	other	1.502	0.0356
WWC2	WW and C2 domain containing 2	Cytoplasm	other	-1.312	0.0169
YWHAZ	tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein zeta	Cytoplasm	enzyme	-1.295	0.00702
ZNFX1	zinc finger NFX1-type containing 1	Nucleus	transcription	-1.364	0.0173

ZPLD1	zona pellucida like domain containing 1	Other	regulator other	1.598	0.0219

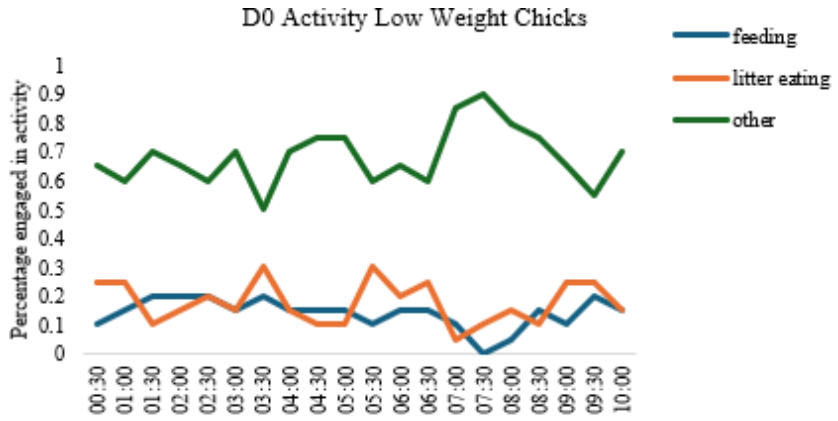
Appendix 6: Macro and trace mineral concentration of the experimental diet (Trial 2)

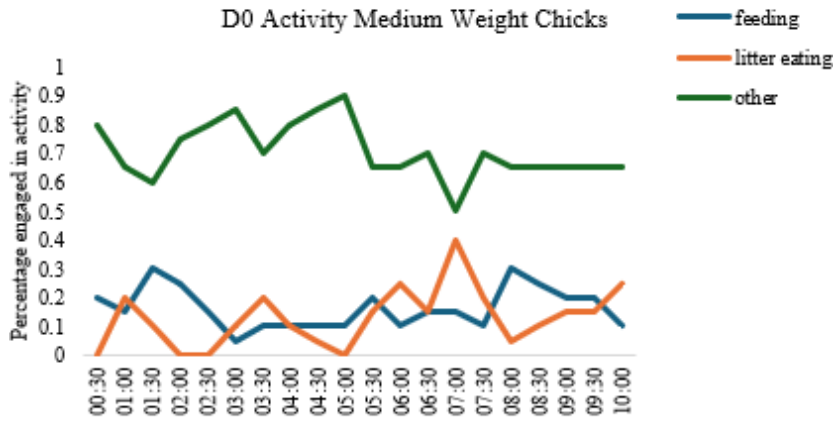
Elements	Concentration, ashed basis (mg/kg)	Standard deviation	Concentration, fresh basis
Ash %	5.1	-	-
B	224	8.3	11.3
Na	20465	1605	1035
Mg	29189	801	1477
P	128200	4486	6486
S	10409	274	526
K	178858	5221	9050
Ca	116306	5182	5885
TI (semi-quant)	415895	32111	21044
Li	0.6	0.1	0.0
Be	0.3	0.1	0.0
Al	557.4	17.5	28.2
V	26.8	1.7	1.4
Chromium (Cr)	22.4	1.2	1.1
Manganese (Mn)	1986	137	100
Iron (Fe)	907	32.9	45.9
Cobalt (Co)	1.4	0.1	0.1
Nickel (Ni)	27.4	2.4	1.4
Copper (Cu)	246	1.9	12.5
Zinc (Zn)	1899	108	96.1
As	1.2	0.0	0.1
Selenium (Se)	0.3	0.0	0.0
Rubidium (Rb)	107	3.1	5.4
Strontium (Sr)	230	9.0	11.7
Mo	29.8	1.1	1.5
Ag	0.0	0.0	0.0
Cadmium (Cd)	2.6	0.2	0.1
Cesium (Cs)	0.3	0.0	0.0
Ba	203	6.1	10.3
Ti	0.0	0.0	0.0
Pb	1.8	0.2	0.1
U	28.4	1.6	1.4

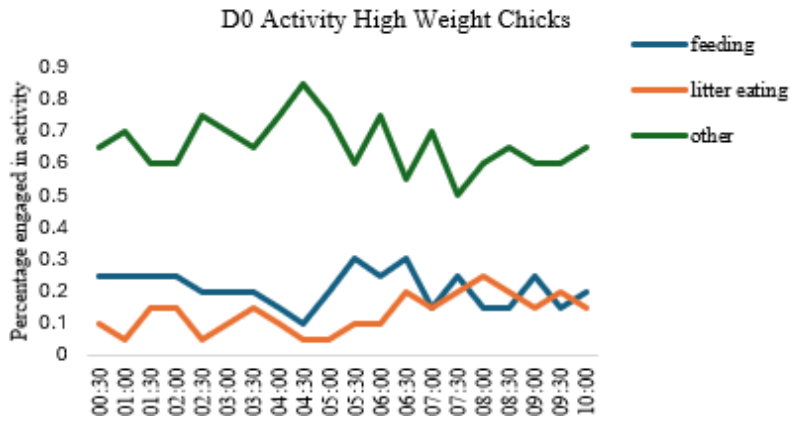
Appendix 7: Macro and trace mineral concentration of the wood shavings (Trial 2)

Elements	Concentrations (mg/kg)	Concentration in fresh basis
Ash %	0.22	-
DM %	93.35	-
B	666.1	1.5
Na	10600	23
Mg	47800	105
P	22235	48.
S	22436	49.
K	132144	290
Ca	214809	472
TI (semi-quant)	446212	981.7
Li	5.0	0.0
Be	0.0	0.0
Al	2349	5.2
V	3.9	0.0
Chromium (Cr)	116	0.3
Manganese (Mn)	23405	51
Iron (Fe)	2222.	4.9
Cobalt (Co)	43.6	0.1
Nickel (Ni)	55.6	0.1
Copper (Cu)	348.7	0.8
Zinc (Zn)	2587	5.7
As	1.4	0.0
Selenium (Se)	2.1	0.0
Rubidium (Rb)	300	0.7
Strontium (Sr)	1744	3.8
Mo	3.2	0.0
Ag	9.6	0.0
Cadmium (Cd)	20.9	0.0
Cesium (Cs)	1.6	0.0
Ba	4323	9.5
Ti	0.0	0.0
Pb	54.3	0.1
U	1.2	0.0

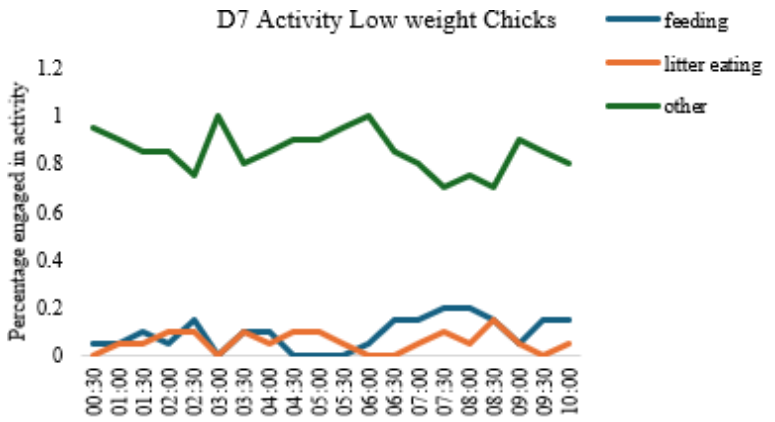
Appendix 8: Chicks' Behavioural activities in each bodyweight group on D0 (Trial 3)

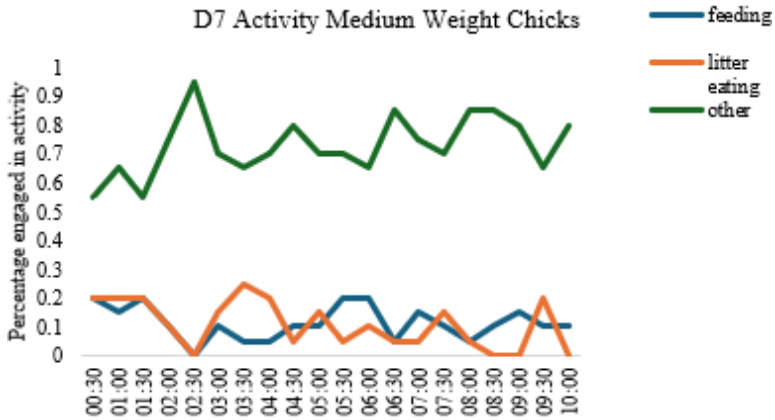


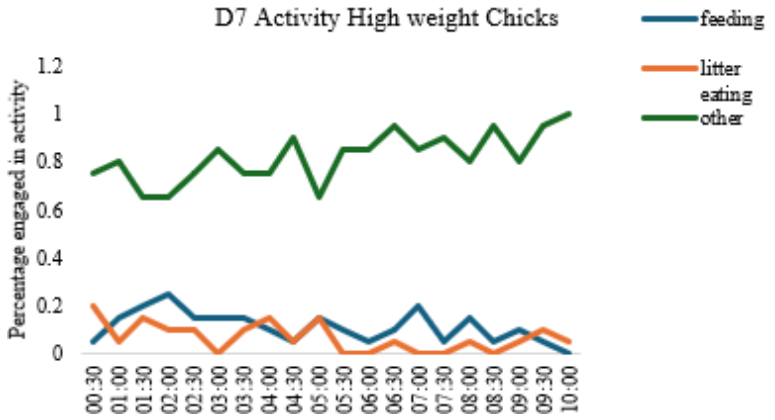




Appendix 9: Chicks' Behavioural activities in each bodyweight group on D7 (Trial 3)







Appendix 10: Macro and trace mineral concentration of the experimental diet (Trial 3)

Ash % on dry basis (ash/dry sample) *100	6.6	-
Elements	Mean concentration in ash basis	Concentration in fresh basis (calculation is mineral x 100 / ash %)
B	154.8	10.2
Na	24558.9	1620.9
Mg	29607.9	1954.1
P	109510.3	7227.7
S	6304.4	416.1
K	146694.2	9681.8
Ca	191183.2	12618.1
Ti semi-quant	31.8	2.1
Li	2.9	0.2
Be	0.1	0.0
Al	1561.6	103.1
V	5.4	0.4
Cr	27.4	1.8
Mn	1524.2	100.6
Fe	2573.2	169.8
Co	3.2	0.2
Ni	23.0	1.5
Cu	170.5	11.3
Zn	1613.9	106.5
As	0.7	0.0
Se	0.5	0.0
Rb	86.1	5.7
Sr	113.5	7.5
Mo	32.0	2.1
Ag	0.0	0.0
Cd	1.2	0.1
Cs	0.4	0.0
Ba	139.0	9.2
Tl	0.0	0.0
Pb	5.1	0.3
U	0.4	0.0

Appendix 11: Macro and trace mineral concentration of the wood shave (Trial 3)

Ash % on dry basis (ash/dry sample) *100	0.122	-
Mineral elements	Mean concentration in ash basis	Concentration in fresh basis
B	859.01	1.55
Na	7366.86	13.26
Mg	66107.79	118.99
P	22748.60	40.95
S	19022.16	34.24
K	102510.11	184.52
Ca	282094.66	507.77
Ti semi-quant	81.44	0.15
Li	2.25	0.00
Be	0.05	0.00
Al	1937.75	3.49
V	2.09	0.00
Cr	72.84	0.13
Mn	27539.29	49.57
Fe	2326.61	4.19
Co	9.00	0.02
Ni	76.18	0.14
Cu	242.06	0.44
Zn	1882.64	3.39
As	1.09	0.00
Se	5.49	0.01
Rb	133.24	0.24
Sr	2998.99	5.40
Mo	3.19	0.01
Ag	9.01	0.02
Cd	5.07	0.01
Cs	0.41	0.00
Ba	3807.80	6.85
Tl	0.01	0.00
Pb	33.56	0.06
U	0.06	0.00

Appendix 12: Composition of the experimental diet (trial 4)

Composition	Quantity	Units
Wheat	61.5	kg
Soya inclusion	32.44	kg
Soya oil	1.58	kg
Salt	0.38	kg
Limestone	0.16	kg
Dicalcium Phos, 18%P	2.17	kg
Lysine HCL	0.28	kg
DL-Methionine	0.32	kg
Threonine	0.16	kg
Vitamine and mineral premix	0.50	kg
Phytase (QB)	0.01	kg
Titanium	0.50	kg
Total	100	kg

Appendix 13: Macro and trace mineral concentration of the diet (Trial 4)

Ash (%)	7.08	-
Elements	Mean conc. In ash basis (mg/kg)	Conc. in fresh basis
B	194.13	13.74
Na	9086.27	643.31
Mg	25709.08	1820.20
P	107881.64	7638.02
S	7388.69	523.12
K	151729.54	10742.45
Ca	89545.99	6339.86
Ti semi-quant	212.60	15.05
Li	1.16	0.08
Be	0.34	0.02
Al	319.46	22.62
V	21.34	1.51
Cr	19.46	1.38
Mn	482.54	34.16
Fe	677.63	47.98
Co	0.70	0.05
Ni	26.25	1.86
Cu	140.64	9.96
Zn	611.98	43.33
As	1.05	0.07
Se	0.23	0.02
Rb	97.93	6.93
Sr	110.39	7.82
Mo	21.31	1.51
Ag	0.00	0.00
Cd	2.00	0.14
Cs	0.12	0.01
Ba	92.39	6.54
Tl	0.00	0.00
Pb	2.96	0.21
U	18.64	1.32

Appendix 14: Macro and trace mineral concentration of the hardwood shave (Trial 4)

Ash % on dry basis (ash/dry sample) *100	0.40	-
Elements	Mean conc. In ash basis (mg/kg)	Conc. In fresh basis
B	151.60	0.61
Na	26314.54	105.26
Mg	33751.50	135.01
P	4075.80	16.30
S	18061.61	72.25
K	118903.55	475.61
Ca	296399.46	1185.60
Ti semi-quant	54.32	0.22
Li	11.63	0.05
Be	0.06	0.00
Al	672.31	2.69
V	1.93	0.01
Cr	32.22	0.13
Mn	240.93	0.96
Fe	808.19	3.23
Co	7.65	0.03
Ni	105.74	0.42
Cu	56.15	0.22
Zn	1256.96	5.03
As	1.37	0.01
Se	0.99	0.00
Rb	42.30	0.17
Sr	1956.32	7.83
Mo	0.96	0.00
Ag	0.06	0.00
Cd	0.60	0.00
Cs	0.16	0.00
Ba	675.20	2.70
Tl	0.04	0.00
Pb	6.69	0.03
U	0.24	0.00

Appendix 15: Macro and trace mineral concentration of the softwood shave (Trial 4)

Ash % on dry basis (ash/dry sample) *100	0.42	-
Elements	Mean conc in ash basis (mg/kg)	Conc. In fresh basis
B	615.99	2.53
Na	11272.99	46.22
Mg	37200.58	152.52
P	9207.49	37.75
S	10357.67	42.47
K	92128.43	377.73
Ca	201124.37	824.61
Ti semi-quant	47.21	0.19
Li	75.48	0.31
Be	0.06	0.00
Al	2388.48	9.79
V	3.59	0.01
Cr	340.58	1.40
Mn	24460.00	100.29
Fe	2486.79	10.20
Co	14.92	0.06
Ni	161.49	0.66
Cu	295.75	1.21
Zn	1870.60	7.67
As	1.18	0.00
Se	1.18	0.00
Rb	115.35	0.47
Sr	1880.71	7.71
Mo	1.85	0.01
Ag	7.74	0.03
Cd	12.14	0.05
Cs	0.32	0.00
Ba	4062.47	16.66
Tl	0.08	0.00
Pb	72.88	0.30
U	0.09	0.00

Appendix 16: Differential expressed genes (DEGs) in the bursal of 21 days old broiler chicks ranked as low weight versus high weight group.

Gene Symbol	p-value (High weight vs. Low weight)	Fold-Change (High weight vs. Low weight)	Fold-Change (High weight vs. Low weight) (Description)
MIR1694	0.0008	-1.72149	High weight down vs Low weight
LOC395617	0.0033	-1.58399	High weight down vs Low weight
OR10A7	0.0045	-1.79864	High weight down vs Low weight
RP4-539M6.19	0.0045	-1.52141	High weight down vs Low weight
RRBP1	0.0046	-5.5553	High weight down vs Low weight
MIR106A	0.0046	-1.56456	High weight down vs Low weight
MIR199A2	0.0046	-1.62172	High weight down vs Low weight
KLHL1	0.0084	-1.50899	High weight down vs Low weight
RIMBP2	0.0086	1.50787	High weight up vs Low weight
MIR3538-1	0.0110	-1.85695	High weight down vs Low weight
ANO1	0.0195	-1.51249	High weight down vs Low weight
KIF20B	0.0222	1.55588	High weight up vs Low weight
SIRT4	0.0255	1.64939	High weight up vs Low weight
MIR1698-1	0.0256	1.65792	High weight up vs Low weight
MIR7-1	0.0302	-1.52162	High weight down vs Low weight
MIR1648	0.0307	-1.78244	High weight down vs Low weight
MIR1721	0.0314	-1.84961	High weight down vs Low weight
MIR1682	0.0319	-1.82587	High weight down vs Low weight

TAC1	0.0371	-1.54179	High weight down vs Low weight
MIR1699	0.0373	1.53013	High weight up vs Low weight
LOC101747539	0.0381	-1.50944	High weight down vs Low weight
ODF2L	0.0485	-1.59954	High weight down vs Low weight

Appendix 17: Differential expressed genes (DEGs) in the bursal of 21 days old broiler chicks raised in softwood litter versus hardwood litter.

Gene symbol	p-value (Soft vs. Hard)	Fold-Change (Soft vs. Hard)	Fold-Change description
MIR15B	0.001	-1.655	Soft down vs Hard
THEMIS	0.004	1.682	Soft up vs Hard
DSG4	0.014	1.581	Soft up vs Hard
MBOAT2	0.020	1.607	Soft up vs Hard
LOC101748511	0.024	-1.538	Soft down vs Hard
MIR215	0.024	-1.510	Soft down vs Hard
TAAR2	0.034	-1.713	Soft down vs Hard
NOV	0.035	-1.584	Soft down vs Hard
ITGBL1	0.035	-1.518	Soft down vs Hard
BG8	0.047	1.648	Soft up vs Hard