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CHARACTERIZING ENDOCRINAL AND TRANSCRIPTIONAL DETERMINANTS OF PHOSPHORUS UTILIZATION MEDIATED BY THE ENVIRONMENT-HOST INTERACTION IN LAYING HENS AND BROILER CHICKENS

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I dedicate this work to my family for their love and support, which kept me motivated in the pursuit and attainment of this academic feat.

Contents

List of Abbreviation

ACTB	beta actin b
ATP	adenosine triphosphate
BAs	bile acids
BBM	brush border membrane
DEGs	differentially expressed genes
ECM	extracellular matrix
ELISA	enzyme-linked immunoassay assay
FAE	follicle-associated epithelium
FAO	food and agriculture organisation
FAOSTAT	food and agriculture organisation statistics
FC	fold change
FCR	feed conversion ratio
FTU	phytase unit
GALT	gut-associated lymphoid tissue
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
GIT	gastrointestinal tract
HH	birds fed high phosphorus in starter and grower periods
HHH	birds fed high phosphorus in starter through to finisher periods
HL	birds fed high phosphorus in starter but low at grower periods
HLL	birds fed high phosphorus in starter but low at grower and finisher periods
IECs	intestinal epithelial cells
IgA	immunoglobulin A
IL-4	interlukin 4
$InsP_6$	inositol hexakis phosphate
InsPx	inositol phosphates
IPA	ingenuity pathway analysis
IUPAC	international union of pure and applied chemistry
IUPAC-IUB	international union of pure and applied chemistry-[international union of biochemistry]
KEGG	kyoto encyclopedia of genes and genomes
LB	lohmann brown
LCa	low calcium diet applied to laying hens
LCaP	low phosphorus and calcium diet
LP	low phosphorus diet
LSL	lohmann selected leghorn
MALT	mucosa associated lymphoid tissues
MI	myo-inositol
ML	birds fed recommended phosphorus in starter but low at grower periods
MLL	birds fed recommended phosphorus in starter but low at grower and finisher periods
MM	birds fed recommended phosphorus in starter and grower periods
MMM	birds fed recommended phosphorus in starter through to finisher periods
nPP	non-phytate P
OTUs	operational taxonomic units
PTH	parathyroid hormone
PUE	phosphorus use efficiency
ROS	reactive oxygen species
u	unified atomic mass unit (dalton)

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Abstract

The optimal utilization of phosphorus (P) has been of particular interest in the productivity of monogastric farm animals in recent times owing to the non-renewability and rapid depletion of finite P reserves, as well as the environmental impact of the sub-optimal utilisation of P attributable to monogastric livestock farming. Efficient P utilization in laying hens and broiler chickens is crucial for driving several biological and physiological processes ranging from bone mineralization, cellular energy production (ATP), blood buffering processes, nucleotide formation, muscle and nerve maintenance, all of which culminate in the optimal growth, production and welfare of the organism. Since P is retained in plant-based diets as phytate (an anti-nutritional factor), stepwise enzymatic cleaving is required to release P and enable intestinal absorption. Because broiler chickens and laying hens have limited production of endogenous phosphatases required for phytate degradation, conventional farming systems use feed that is usually supplemented with phytase of microbial origin. However, phytase use is prohibited under organic farming systems in the European Union. Hence, the need arises to foster and explore the bird's innate/intrinsic mechanisms for efficient P utilization, homeostasis and resource allocation. The studies reported in this project sought to harness and exploit the bird's inherent P efficiency mechanisms, which span homeostatic endocrinal and transcriptional determinants recruited at distinctive developmental phases under various P and calcium (Ca) dietary regimens.

Studies 1 and 2 conducted a holistic transcriptomic profiling on the jejunum of two commercial layer strains, namely the Lohmann Brown (LB) and Lohmann Selected Leghorn (LSL). Study 1 entails the temporal assessment of holistic transcriptomic profiles across five developmental time points (weeks 10, 16, 24, 30 and 60 of life) for a total of hundred laying hens (LB: n = 50; LSL: n= 50). Study 2 considered the assessment of jejunal transcriptomic profiles in response to the varying dietary Ca and non-phytate P levels (standard vs. reduced) fed to eighty laying hens (LB: n = 40; LSL: n= 40). Alongside jejunal samples obtained from the two high-yielding layer strains for RNA sequencing in both studies, blood samples were also collected to estimate mineral homeostasis dynamics. Results from study 1 markedly distinguished between layers in the pre-laying and laying phase, as inferred from plasma levels of estradiol, calcitriol, Ca and triiodothyronine. Moreover, the expression patterns of the jejunal mucosa responded directly to the changing metabolic profiles at the onset of egg laying activity. Notably, significant changes in gene expression profiles were observed for pathways such as RANK/RANKL signaling and cellular senescence. Conclusively, adequate supply during sexual maturity of laying hens is

crucial given the observed endogenous mechanisms (endocrinal and transcriptional). Hence, further investigation is needed to unravel precise metabolic requirements for efficiency and production. In addition, a pronounced strain-specific metabolic pattern that suggests different Ca, P, and vitamin D requirements of the laying hen strains in defining their distinct phenotypes, as inferred from the different exhibitions of enriched molecular pathways.

In study 3, broiler chickens were subjected to a dietary P depletion strategy initiated at the starter phase and maintained throughout the productive lifespan, i.e., grower and finisher. The responses elicited following the dietary P depletion were investigated via a combination of intrinsic parameters, including endocrinal, bone parameters, and transcellular intestinal and renal regulation of P transport. The results revealed a marked response to P depletion at the earliest developmental phase, showing the most severe response to the depletion compared to grower and finisher developmental stages. However, with advancing ages, the birds activated an effective compensatory mechanism, including endocrine control mediated by calcitriol action, intestinal P uptake, renal P reabsorption and mineral mobilization from the bone. Thus, the application of dietary P depletion strategies in broiler production should be no earlier than the grower developmental stage, when mineral stores are established and physiological adaptation mechanisms can occur in broiler chickens. Using the sample material from the same animals, study 4 investigated the contributory role of the broiler's gut microbiota to the homeostatic compensatory mechanism following the dietary P depletion strategy. The contribution of the gut-microbiota of the jejunum to these compensatory mechanisms was subtle. Microbial taxa that proliferated under the higher P supply might serve as biomarkers for discerning excess dietary P supply in broiler farming. Adaptive responses to improve P efficiency are evident due to the pronounced low levels of phytate in feces after P depletion. Conclusively, reductions in P supply to broilers are possible, but precise timing, duration, and magnitude of a P depletion strategy in broiler chickens should be considered for optimized mineral utilization for production, welfare, and health.

ZUSAMMENFASSUNG

Die optimale Nutzung von Phosphor (P) ist aufgrund der Nicht-Erneuerbarkeit und der Erschöpfung der endlichen P-Reserven sowie der Umweltauswirkungen einer suboptimalen P-Nutzung durch die monogastrische Tierhaltung von aktuellem Interesse. Eine effiziente P-Verwertung bei Legehennen und Masthühnern ist für die Steuerung verschiedener biologischer und physiologischer Prozesse von entscheidender Bedeutung, die von der Knochenmineralisierung über die zelluläre Energieproduktion (ATP), die Blutpufferung, die Nukleotidsynthese bis hin zum Muskel- und Nervenerhalt reichen und zu optimalem Wachstum, Produktion und Wohlergehen des Organismus führen. Da P in pflanzlicher Nahrung als Phytat (ein antinutritiver Faktor) gebunden vorliegt, ist eine schrittweise enzymatische Spaltung erforderlich, um P freizusetzen und über die Darmschleimhaut absorbieren zu können. Masthühner und Legehennen können die für den Phytatabbau erforderlichen körpereigenen Phosphatasen nur in begrenztem Umfang produzieren, was zur Folge hat, dass in konventionellen Haltungssystemen üblicherweise Phytasen mikrobiellen Ursprungs mit dem Futter supplementiert werden. Allerdings ist die Verwendung von Phytase im ökologischen Landbau in der Europäischen Union verboten. Daher besteht der Bedarf, die angeborenen/intrinsischen Mechanismen für eine effiziente P-Nutzung, Homöostase und Ressourcenverteilung zu erforschen und gezielt zu fördern. Die im Rahmen dieses Projekts durchgeführten Studien zielten darauf ab, die Mechanismen der P-Effizienz von Geflügel zu erforschen. Dies umfasst die Darstellung der endokrinen und transkriptionellen Determinanten der P-Homöostase in verschiedenen Entwicklungsphasen unter Berücksichtigung verschiedener P- und Kalzium (Ca)-Diätregime.

In den Studien 1 und 2 wurden holistische Transkriptom-Profile des Jejunums von zwei kommerziellen Legehennenlinien, nämlich Lohmann Brown (LB) und Lohmann Selected Leghorn (LSL), erstellt. Studie 1 umfasste die Bewertung von Transkriptom-Profilen über fünf Entwicklungszeitpunkte (10, 16, 24, 30 und 60 Lebenswochen) für insgesamt hundert Legehennen (LB: n = 50; LSL: n= 50). Studie 2 befasste sich mit der Abbildung von Transkriptom-Profilen im Jejunum als Reaktion auf unterschiedliche Ca- und mineralische (non-phytate-P) P-Gehalte in den Futterrationen ("Standard" vs. "reduziert"), die an 80 Legehennen (LB: n = 40; LSL: n = 40) verfüttert wurden. Zusätzlich wurden Blutproben entnommen, um die Dynamik der Mineralstoffhomöostase abzuschätzen. Die Ergebnisse aus Studie 1 zeigten einen deutlichen Unterschied zwischen Legehennen in der Vorlege- und Legephase, wie aus den Plasmaspiegeln von Estradiol, Calcitriol, Ca und Triiodthyronin (T3) zu schließen ist. Darüber hinaus zeigten die Expressionsmuster der Dünndarmschleimhaut eine

direkte Reaktion auf die sich ändernden Stoffwechselanforderungen und die einhergehende Mineralversorgung zu Beginn der Legetätigkeit. Insbesondere wurden signifikante Veränderungen der Genexpressionsprofile für Signalwege wie RANK/RANKL und zelluläre Seneszenz beobachtet. Eine angemessene Versorgung während der Geschlechtsreife von Legehennen ist angesichts der beobachteten endogenen (endokrinen und transkriptionellen) Mechanismen von entscheidender Bedeutung. Daher sind weitere Untersuchungen erforderlich, um die genauen Stoffwechselbedürfnisse in dieser Lebensphase zu entschlüsseln. Darüber hinaus wurde aus den Ergebnissen der Studie 2 ein ausgeprägtes linienspezifisches Stoffwechselmuster abgeleitet, das auf einen unterschiedlichen Ca-, P- und Vitamin-D-Bedarf der Legehennenlinien LB und LSL hindeutet.

In Studie 3 wurden Masthühner einer zeitlich festgelegten P-Unterversorgung (P-Depletion) unterzogen. Dies umfasste verschiedene Lebensphasen der Masthühner, inklusive Aufzucht, Vormast und Endmast. Die durch die veränderte P-Versorgung ausgelösten molekularen Mechanismen wurden anhand intrinsischer Parameter untersucht. Dies schloss endokrine Regulatoren, Knochenparameter sowie transzelluläre intestinale und renale P-Transporter ein. Eine stark reduzierte P-Versorgung während der Aufzuchtphase führte zu gravierenden Entwicklungsverzögerungen, während in der anschließenden Vormast- und Endmastperiode eine entsprechend reduzierte P-Versorgung kompensiert werden konnte. Die Masthühner aktivierten einen wirksamen Kompensationsmechanismus, der die endokrine Kontrolle der intestinalen P-Aufnahme, die renale P-Rückresorption und die Mineralstoffmobilisierung aus den Knochen umfasste. Daher sollten Rationen mit stark reduzierter P-Versorgung in der Masthähnchenproduktion frühestens mit beginnender Vormast erfolgen, wenn die Mineralstoffspeicher angelegt sind und physiologische Anpassungsmechanismen bei Masthähnchen wirken können. Unter Verwendung des Probenmaterials derselben Tiere wurde in Studie 4 untersucht, welchen Beitrag die Dünndarmmikrobiota von Masthähnchen für die Mineralstoffhomöostase leistet. Der Beitrag der Darmmikrobiota des Jejunums zu diesen Ausgleichsmechanismen war unauffällig. Dagegen könnten mikrobielle Taxa, die sich unter einer über die aktuellen Empfehlungen hinausgehenden P-Versorgung vermehrten, als Biomarker für die Feststellung einer übermäßigen P-Versorgung in der Masthähnchenhaltung dienen. Intestinale Anpassungsmechanismen zur Verbesserung der P-Effizienz sind aufgrund der beobachteten ausgeprägten Phytat-P-Mobilisierung des Geflügels offensichtlich. Daraus folgt, dass Reduzierungen in der P-Versorgung bei Masthühnern möglich sind, jedoch Zeitpunkt, Dauer als auch Ausmaß im Hinblick auf eine optimale Nährstoffverwertung, Wohlbefinden und Gesundheit berücksichtigt werden sollten.

CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

1 Introduction and Literature Review

1.1 Phosphorus, as a naturally existing element

Phosphorus, a highly-reactive element classified as a non-metal and represented by the symbol (P), with an atomic number and mass of 15 and 30.97 u, respectively, on the chemical periodic table [IUPAC, 2022], was discovered by a German alchemist named Hennig Brand in 1669. Chemically, P exists mainly in two allotropic forms, namely the white (or yellow) waxy, solid, highly reactive, toxic and non-crystalline (P_4) form, and the solid red, less harmful and more stable form (P_n), which is produced by the combustion of white P [RSC, 2023; NCBI, 2023].

1.1.1 Phosphorus, its origin and global reserve

Natural sources of P are mainly in reserves of rock phosphate, including the apatite phosphate mineral [RSC, 2023], which is sparsely deposited geologically as igneous or sedimentary rock origin within a few countries across the continents yet utilized globally across agriculture and manufacturing industries [Brownlie, 2021; Desmidt, 2015]. Albeit apatite is the most abundant crystalline phosphate mineral from igneous rocks, serving probably as the primary origin of all other phosphates (mineral or organic), phosphate rock deposits from sedimentary sources account for about 70% of the globally mined phosphates utilised commercially [Jasinski, 2022; El Bamiki, 2021]. Phosphorites or phosphate rock is a term that classifies an ore excavated from the earth, graded applicable only on adequate P content, which usually is between 4-13% phosphorus pentoxides; P_2O_5 [Daneshgar, 2018]. Subsequently, phosphorites are subjected to an enrichment process termed beneficiation, which entails the purification and purging of the ore of sand and clay impurities typically associated with sedimentary phosphate, yielding as high as 30% P_2O_5 marketable phosphate rock commercially used in several sectors [Sajid, 2022].

As of 2022, Northern Africa collective accounted for the largest global deposition of P, with Morocco having the highest global rock phosphate reserve, with approximately 50 billion metric tons, followed by Egypt, Tunisia, Algeria and China, with about 2.8, 2.5, 2.2, and 1.9 billion metric tons, respectively [Statista, 2023a]. China remains the highest global consumer and producer of rock phosphate with approximately 85 million metric tons, ahead of Morocco, the United States of America, Russia, and Jordan, which produced approximately 38, 22, 14 and 9.2 million metric tons, respectively [Statista, 2023b]. Recently, a massive deposit of phosphate rock was discovered in the south-western region of Norway [EURACTIV, 2023]. According to the Norge mining company, the mineral deposit is estimated to hold as much as 70 billion tonnes of the non-renewable resource, which would meet production demands for

fertilizers, solar, electric automobile battery use for the next 50 years [EURACTIV, 2023]. These metrics brings the world's phosphate rock resources to more than 300 billion metric tons without any imminent shortages of phosphate rock [Jasinski, 2021]. In addition, the global production forecast was projected to reach 261 million metric tons by 2024 [Jasinski, 2020]. In contrast, previous reports suggested that the current use of mined phosphate rock is rapidly depleting, with a peak P production between 2030 and 2050 and its exhaustion in the next century, depending on the recycling rate via sewage and agricultural runoff [Cordell and White, 2011]. Thus, based on these assertions, unless novel P recycling techniques are developed, or more ore deposits are discovered, an erratic supply of P will impede global food security [Wendling, 2013; Cordell, 2011], considering the continuously increasing human population and their corresponding demand for food. However, the current exploration potential for phosphatic rocks, mainly driven by the rising prices and the continental expansion of phosphatic rock mines, may offset the predicted threat much further into the future [Jasinski, 2023; Pufahl and Groat, 2017].

1.1.2 Global phosphorus use and allocation

The commercial supply of global rock phosphate is driven chiefly by demands from the agricultural and non-agricultural sectors, with the former accounting for the more considerable utility (between 80–90% of the total world demand) [Childers, 2011]. This is utilized principally in the production of inorganic phosphate fertilizer products such as ammonium phosphates, superphosphates and pesticides for agronomical purposes, as well as in the production of animal feed supplements in monogastric animal production [Cisse and Mrabet, 2004].

The non-agricultural utilization of rock phosphates cuts across several sub-sectors, including construction, e.g., in the production of flame-retardant materials used in insulation, wood products, and textiles [van der Wielen, 2006], or in medical applications for bone grafts procedures, and dental implants [Daneshgar, 2018], as well as for industrial purposes in the production of detergents, safety matches and food additives [Smit, 2009]. With the projected global population surpassing 9.6 billion by 2050 [UN, 2023], the demand for P mining and usage will inevitably increase to support agriculture and non-agricultural human activities. Moreover, there are growing concerns regarding the environmental impact of inefficient usage and the limited availability of this non-renewable resource.

The farming of monogastric livestock has been identified as a major contributor to this issue since its production accounts for a significant global yield of the needed animal protein sources

[FAO, 2007]. For example, the production and consumption of broiler meat and pork rank highest among the farmed animal genetic resources ("the big five"), with approximately 133 million metric tons and 110 million metric tons of annual global output, respectively [FAOSTAT, 2023], hence, validating the concerns associated with the efficient utilization of P in monogastric livestock production. However, the limitation on promising alternatives for P, coupled with the environmental impact of inefficient P use in monogastric animal farming [Shastak and Rodehutschord, 2015], poses significant challenges leading to fragmented global cycles and accumulation of P in arable land in several European Union countries and by extension the world.

1.2 Phosphorus as an indispensable macro-mineral within biological systems

P is a vital macro-mineral within biological systems, as it plays a plethora of crucial roles in maintaining optimal biological function at both cellular and physiological levels. P is a significant constituent of several biomolecules, including phospholipids, nucleotides, and ATP, in cellular energy metabolism [Lovio-Fragoso, 2021].

In humans, P is the second most abundant mineral after Ca, and it is mainly deposited in bones and teeth in complex forms of hydroxyapatite $Ca_{10}(PO_4)_6(OH)_2$ [Fukumoto, 2014]. P is essential for maintaining nerve function, blood buffering processes, and bone mineralisation [Fukumoto, 2014] in vertebrates generally, humans and the domestic fowl inclusive. However, in meat-type chicken (broilers) and egg-laying hens (layers), the utilization of P is comparatively less efficient due to its storage as phytate in plant-based feed sources.

1.2.1 Phytate and the utilization of phosphorus in the domestic fowl

Phytate is the salt form of phytic acid, also known as myo-inositol 1,2,3,4,5,6-hexakisphosphate; (dihydrogen phosphate) $InsP_6$ [IUPAC-IUB, 1978], resulting primarily from complexes with various metallic cations such as Ca^{2+}, iron (Fe^{2+}), zinc (Zn^{2+}), magnesium (Mg^{2+}), potassium (K^{+}), and manganese (Mn^{2+}) [Humer, 2014]. The affinity and strength of $InsP_6$ to form complexes with cations are listed in the following ranking order: $Cu^{2+} > Zn^{2+} > Co^{2+} > Mn^{2+} > Fe^{3+} > Ca^{2+}$ [Singh, 2008].

Phytic acid is an unstable free acid with six phosphate groups, esterified with hydroxyl groups of myo-inositol. [Pallauf and Rimbach, 1997]. Phytate is considered an anti-nutritional factor in monogastric livestock nutrition [Dersjant-Li, 2014], broilers and layers inclusive, because these animals have limited capacity to utilize phytate bound P due to the inadequate production of enteral phytase/phosphatases needed to hydrolyze phytate resulting in adverse effects on the bioavailability of essential dietary minerals as well as increasing the tendency for environmental

P losses [Panagos, 2022; Rama Rao, 1999]. As a result, poultry diets are supplemented exogenously with phytases of microbial origin to aid in degrading phytate to release P to meet the bird's metabolic demands.

However, the use of phytase is prohibited in organic farming practices in the European Union due to regulations disallowing the use of synthetic substances, including enzymes, in organic farming [Council of the European Union, 2007]. In addition, the limited availability of P in phytate-bound forms, its increased excretion and the non-renewability of the P in itself [Gilbert, 2009] culminate dire concerns about the sustainability of monogastric livestock production to meet the nutritional demands for humans in the future. Moreover, the concerns about P inefficiencies not only impact the birds' development, production, and welfare but also have significant environmental implications.

1.2.2 The environmental implication of sub-optimal mineral P utilization and efficiency in domestic fowl farming

Environmental concerns attributable to P inefficiencies from monogastric livestock production sources stem from P losses to the environment from poultry and pig farming and have been associated with an hazardous event termed eutrophication. Eutrophication is the excessive plant and algal bloom due to the increased deposition of growth factors such as sunlight, CO_2, and nutrients such as P required for photosynthesis [Chislock, 2013; Schindler, 2006] (Figure 1).

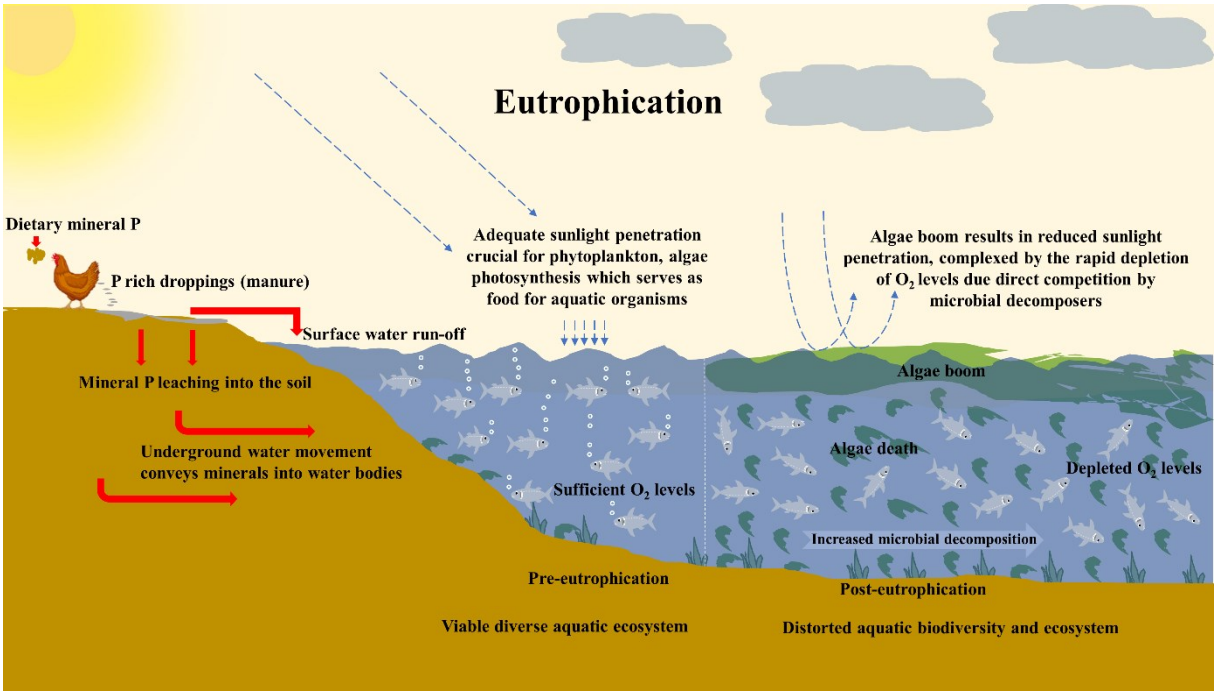

Figure 1. Schematic representation of the environmental footprint of excess dietary P input in poultry farming.

The detrimental impact of human activities including the deposition of mineral P to the earth either through point-source discharges (e.g., phosphate fertilization, organic manures) or non-point discharges, and its eventual transition into aquatic ecosystems, results in severe repercussions for the aquatic biodiversity and availability of useable water sources [Carpenter, 1998]. Mainly, because deposited P accelerate rate of eutrophication in aquatic ecosystems than it would have occurred due to chance or naturally (Figure 1). Non-point eutrophication of the lakes, rivers, estuaries and coastal oceans has been a recurrent hazard over the years due to the excessive inputs of P and N [Carpenter, 1998]. On a broader outlook, the advent of climate change and the increasing global human population threaten the sustainability of global staple food production for the sustenance of humankind [Naheed, 2023; Kim, 2017]. Thus, environmental legislations already enacted needs to be strictly implemented with strong cooperation with the research institutions to re-orientate agro-allied industries, commercial and subsistence animal producers alike on the perils of the current environmental trends, towards facilitating the adoption of greener and environment-friendly modus operandi in the various sectors.

Opportunities to drastically reduce the environmental impact of P from monogastric livestock farming, particularly the poultry, present themselves in several scientific approaches broadly categorized into two [Kebreab, 2012], namely: the review of the P levels provided to the poultry species in ways that focus on fitting the dietary needs to the age-specific need of broilers and layers [Adeola, 2011; Pomar, 2011].

Secondly, there is a need to improve the poultry stocks to be efficient P and Ca utilizers with minimal loading of the macro-mineral to the environment. Based on these categories, different nutritional and animal-inclined strategies have been suggested, e.g., cognizance of the Ca:P ratios in poultry feed formulation [Zampiga, 2021], the exploitation of early-life programming/conditioning of the bird [Valable, 2020; Omotoso, 2023], improvement of P use efficiency (PUE) of the crops used as feed materials [Khanal, 2016], proffering viable alternative P sources in monogastric livestock nutrition [Oster, 2021], and the administration of nutritional interventions such as prebiotic supplements to foster proliferation of beneficial microbiota and possibly phytate degrading microbes within the GIT of the birds [Askelson, 2013].

However, prior to either of the suggested strategies, emphasis on a preliminary characterization of existing variability within and between populations needs to be harnessed and continuously

geared towards exploiting the existing intrinsic capabilities of monogastric livestock, broilers and layers inclusive.

1.3 Fowl-specific physiological P and Ca demand for optimal performance and welfare

Broiler chickens and laying hens possess an immense genetic potential for performance, welfare, and health, which is only fully realized when the birds are provided with optimal environmental conditions. One crucial aspect of this is the supply of a comprehensive nutritional program that prioritizes the availability of essential macro-minerals, such as P and Ca, meticulously tailored to meet the needs of the birds at specific developmental stages to ensure the attainment of their maximum potential [Alagawany, 2020].

Broiler chickens and laying hens adopt distinct mechanisms to attain homeostasis for minerals P and Ca, which is principally dependent on their different physiological demand and adaptive features over their developmental stages throughout the productive life [Omotoso, 2023; Reyer, 2021a; Omotoso, 2021; Sommerfeld, 2020a]. Layers and broilers share a similar gastrointestinal tract (GIT) and associative endogenous homeostatic mechanisms crucial for the absorption of nutrients to define their productive phenotypes, which occurs regardless of the difference in productive objective for which they are raised.

Moreover, the dietary P and Ca requirements for the meat-type broiler and egg-laying hen are distinct due to the different physiological and productive lifespans. On the one hand, a high-yielding layer strain has a productive lifespan of 60 weeks or more from the pullet developmental stage till the cessation of egg production, requiring less P in their diets but more dietary Ca [Ahmadi and Rodehutscord, 2012] to meet the metabolic demands for eggshell calcification. This results in the complex interaction between macro-minerals (P and Ca) to maintain homeostasis for body growth and bone mineralization processes while simultaneously compensating for egg production throughout their productive life span [Sommerfeld, 2020b]. On the other hand, the meat-type broiler has a higher sensitivity to P at the expense of Ca to meet its metabolic needs for physiological growth, which ultimately translates into production in its relatively shorter productive life span (~8 weeks from the starter to the finisher developmental stage).

In addition, high dietary Ca concentration in broiler diet significantly reduces P utilization due to 1 of 3 reasons [Tamim, 2004; Tamim, 2003; Sebastian, 1996]; first is the possible bonding of phytate with Ca to form Ca-phytate complex which inhibits the absorption of P [Wise, 1983], secondly, is the competitive interaction of Ca and the enzyme phytase [McGuaig, 1972] and thirdly, is the increased enteral pH caused by oversupplied Ca, which in turn reduces mineral P

solubility, and thus, its availability correlating to poorer growth rate [Walk, 2012; Guinotte, 1995; Shafey, 1991]. Furthermore, insight into the mechanisms recruited by broiler chickens and laying hens to utilize dietary nutrients to attain production potential and sustain health and welfare encompasses the synergistic association of the small intestine, kidney and bone modulated by endogenous endocrinal and transcriptional determinants as well as the gut-microbiota [Matuszewski, 2020; Blau and Collins, 2015] (Figure 2).

1.4 Endocrinal and transcriptional determinants synergy within the gut–renal–bone complex for mineral homeostasis in the domestic fowl

The maintenance of mineral homeostasis within the domestic fowl is a complex and multifarious process, which encompasses different determinants including endocrinal transcriptional and microbial, majorly niched within gut, kidney and bone axis (Figure 2).

1.4.1 Role of the small intestine in mineral P and Ca homeostasis in the domestic fowl

The small intestine is a part of the chickens' digestive tract situated before the large intestine, after the stomach. It is partitioned into three distinct parts, including the duodenum, jejunum and ileum, facilitating the digestion and absorption of ingested dietary nutrients. Following the immediate post-hatch period, when the chick has transited from its dependence on nourishment from the embryonic yolk sac to exogenously supplemented diet, the small intestine undergoes a rapid developmental process compared to the whole-body mass and other organs within the digestive system, e.g., gizzard [Sklan, 2001; Uni, 1999]. The rapidity in the relative development of the small intestine, which comprises the increased villus proportion, length, vascularity, polarity, and crypt depth, is maximum from 4th-day post-hatch in the chicken [Sklan, 2001; Uni, 1998] in preparedness for optimal absorption and utilization of nutrients pivotal for growth, development, productivity and general welfare. Regarding the mineral absorption capacities of the different sections (duodenum, jejunum and ileum) for P of phytate origin, previous studies have reported on the complex interactions and influence of different factors, which are either dietary or animal-related [Dersjant-Li, 2014].

In practice, the use of phytate P stored in plant-based feed materials is usually supplemented with phytases of microbial origin to enhance adequate absorption of the macro-mineral by the bird. However, the efficacy of phytase in the gut is influenced by different factors, namely, the animal's age, intestinal pH, dietary Ca:P ratio, resistance to endogenous proteases, temperature, and species variation [Dersjant-Li, 2014], as well as strain variation within the species [Sommerfeld, 2020b].

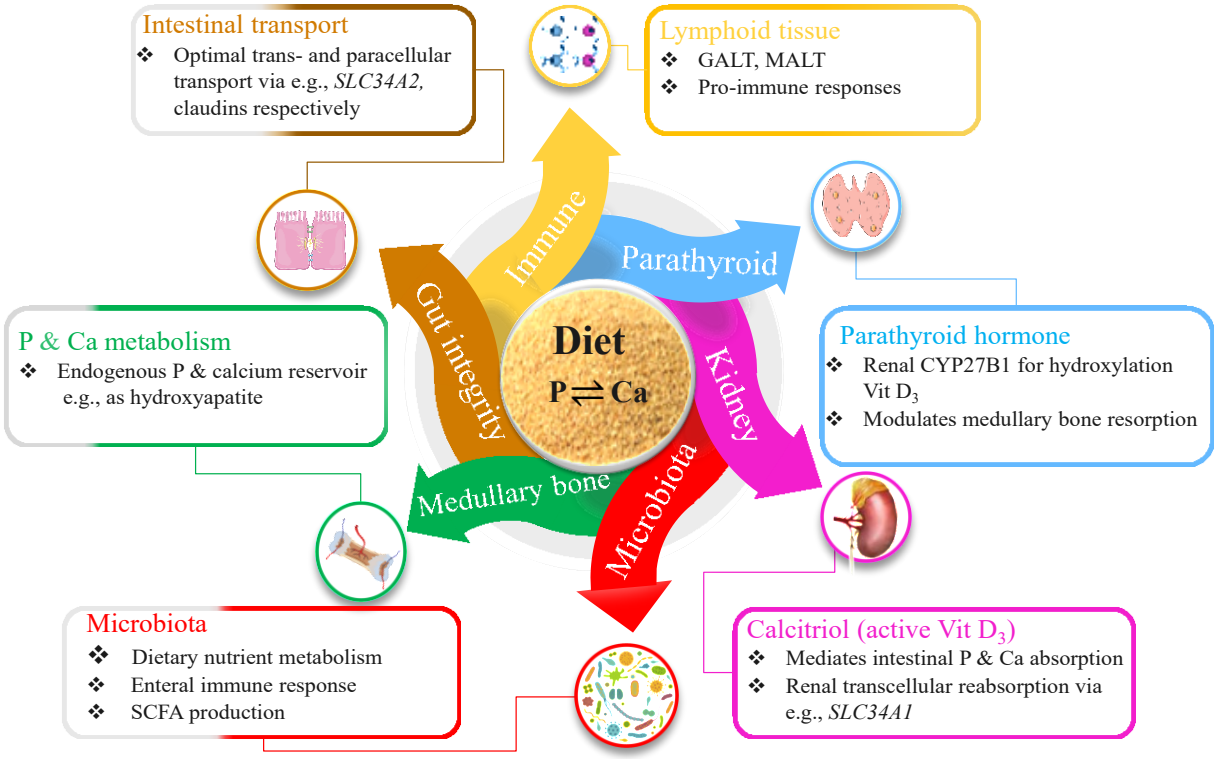

Figure 2. Synergistic association of the small intestine with endocrinal, bone, transcriptional and microbiota determinants to mediate mineral P homeostasis in broiler chickens and laying hens (PTH: parathyroid hormone; GALT: gut-associated lymphoid tissues; MALT: mucosa-associated lymphoid tissues

Higher total endogenous phytase activity was reported in the duodenal and the proximal jejunal brush-border membrane (BBM) vesicles of laying hens than those of broilers [Maenz and Classen, 1998]. More so, a significant within-species difference between two laying hen strains for the total tract $InsP_6$ degradation was reported elsewhere [Abudabos, 2012]. The dietary Ca:P ratios have also been shown to influence the resultant absorptive capacity of the duodenum. Hu *et al.* (2021) reported an increased duodenal mRNA expression of mineral transporters and tight-junction protein (claudins), in broiler fed low Ca diets, suggesting that the birds attempted to fortify their absorption capacity to cope with the mineral deficit, however insuffient it was to prevent a compromise on growth performance. Recent studies on broiler chickens and turkey (*Meleagris gallopavo*), which measured jejunal and ileal mucosal phosphatase activity, phytate degradation, and nutrient digestibility, reported that phytase supplementation increased jejunal mucosal phosphatase activity, prececal $InsP_6$ disappearance, and prececal P and Ca digestibility in both species [Novotny, 2023]. Furthermore, the average mucosal phosphatase activity in the jejunum was higher in 6-week-old birds than in 3-week-old birds, validating the influence of age in phytase degradation kinetics in poultry nutrition [Novotny, 2023].

1.4.2 Role of the kidney in mineral P and Ca homeostasis

The kidney plays a pivotal role in maintaining homeostasis by engaging in a dual function. It reabsorbs minerals through specialized renal *SLC34A1* co-transporters located at the proximal convoluted tubule brush border membrane (BBM) when these minerals are significantly depleted. Simultaneously, it aids in the excretion of macro-minerals when plasma/serum levels surpass the upper threshold, orchestrated by a feedback mechanism mediated by calcitriol and parathyroid hormone (PTH) [Khundmiri, 2016].

PTH modulates renal functions within the homeostatic axis by increasing Ca re-absorption in renal tubules, elevating serum Ca levels. Moreover, PTH, often called a “calciostat”, is a hormone secreted by parathyroid glands, usually when serum Ca levels are low within the biological system. Additionally, in the kidney PTH mediates the hydroxylation of $25(OH)_2D_3$ (calcidiol), synthesizing the active form of $1,25(OH)_2$ vit.D_3 (calcitriol), which is crucial for optimal intestinal absorption of P and Ca. [Matuszewski, 2020] (Figure 2).

1.4.3 Role of the medullary bone in mineral P and Ca homeostasis

The bone is a compact calcified tissue comprising all vertebrates' skeletal systems, including poultry. It comprises approximately 30% organic protein matrix, such as collagen fibres, 60% inorganic hydroxyapatite crystals, and 10% water [Feng, 2009]. The bone provides vital roles within the organism, including structural and postural support, facilitates locomotion, protects vital organs such as the lungs, heart, and liver, regulates mineral homeostasis and serves as a site for immune cell development [Feng, 2009].

In poultry, precisely the laying hen, the medullary bone, a non-structural specialized type of bone tissue formed at sexual maturity within the haematopoietic medullary cavities of bone under the influence of synergistic action of androgenic and oestrogenic hormones, alongside the maturation of the ovarian follicles [Prondvai and Stein, 2014; Dacke, 1993]. The medullary bone possesses no mechanical function compared to the cancellous and cortical bone.

Regarding its role in the dynamics of mineral homeostasis, the medullary bone essentially serves as an endogenous macro-mineral reservoir containing accessible hydroxylapatite crystals distributed randomly throughout its matrix [Nys and Le Roy, 2018; Ascenzi, 1963], from which the bird access mineral (Ca) intrinsically for egg-shell calcification processes usually when the needed mineral is inadequately supplied exogenously through diet [Dacke, 1993]. More so, during hypocalcemic conditions, a complex PTH-driven homeostatic action on the medullary bone is activated to elevate serum Ca levels.

This process involves PTH binding to its receptor (PTH1R) located on bone-forming cells, osteoblasts, activating the RANK/RANKL pathway. RANKL binds to its receptor (RANK) located on precursors of bone resorbing cells, the osteoclasts, stimulating them to mature osteoclasts, thereby resorbing the bone to release Ca for physiological use [Khundmiri, 2016]. Moreover, balanced levels of P are crucial for the bone to drive the apoptotic process of matured chondrocytes in the epiphyseal regions of the bone. This process results in the cellular differentiation and re-modelling of bone cells, translating into growth [Penido, 2012]. Hence, over the developmental period of the bird, sufficiently balanced levels of P and Ca are needed to drive primary and secondary ossification of soft tissues and their storage in complex forms of hydroxyapatite [Shao, 2019; Taylor, 2013].

1.5 Mode of mineral transport absorption and re-absorption within the domestic fowl

In the presence of bioavailable minerals, e.g., P and Ca, the birds' intestinal absorption capacity of the minerals is dependent on either the transcellular (active) or paracellular (passive) transport mechanisms [Proszkowiec-Weglarz, 2019; Bar, 2009]. Furthermore, it is noteworthy that the kidney also plays an active role in the re-absorption of minerals through active transport mechanisms.

1.5.1 Paracellular (passive) mode of mineral transport

The passive paracellular absorption of P and Ca involves a selective movement of ions through tight-junction protein (TJP) enabled by passive diffusion. TJP facilitates the restriction or movement of ions/molecules via its alternatively sealing or pore-forming attributes, which depend on the concentration gradients across the selective permeability gradients of intercellular spaces [Marks, 2019; Knöpfel, 2019] (Figure 3). TJPs are specialized membrane structures located in the apical region of adjacent enterocytes forming intercellular structures producing proteins such as occludin (OCLD) and Claudin (CLD) [Hoenderop, 2005; Itoh, 1999] (Figure 3).

1.5.2 Transcellular (active) mode of mineral transport

The transcellular transport mechanism of minerals, e.g., P and Ca, is an energy-dependent, sodium-phosphate co-transporter at the brush border membrane that modulate uptake through the cell [Eto, 2006] (Figure 3). The synergistic mediation of the transcellular transport mechanism by endocrinal and transcription factors suggests it to be the birds' preferential route for mineral absorption under severe conditions (e.g. dietary mineral restrictions), requiring rapid P uptake.

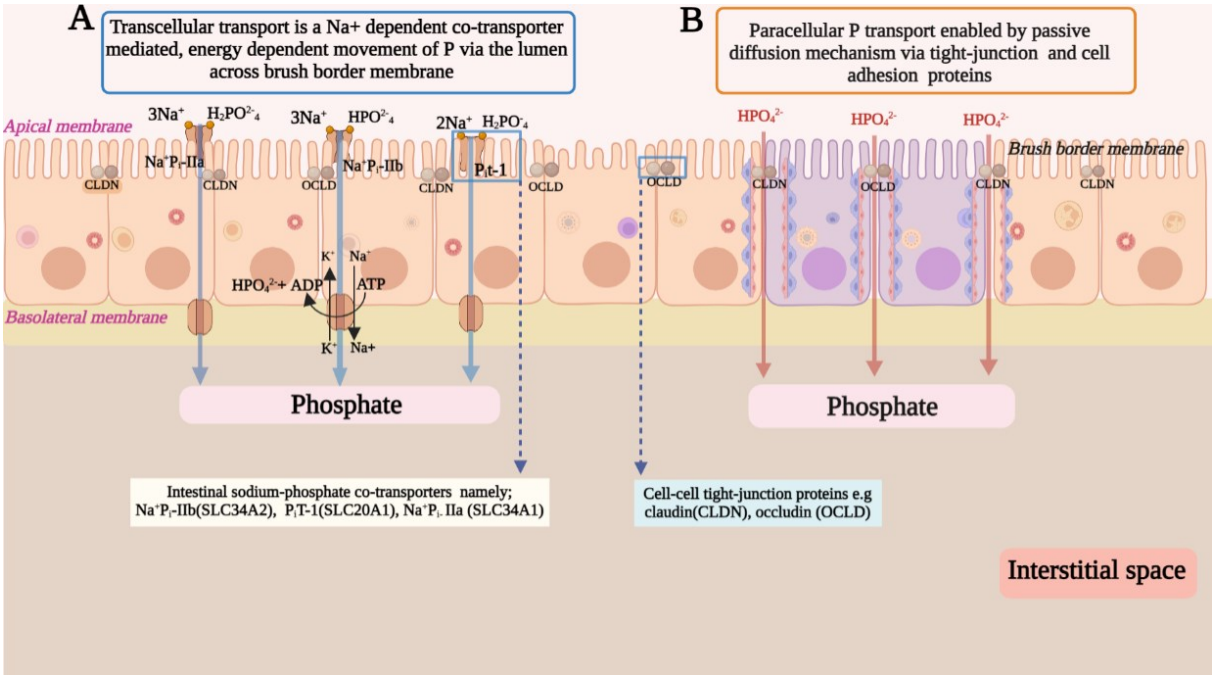

Figure 3. Intestinal phosphate transport detailing: **(A)** Transcellular sodium-dependent phosphate co-transporters (NaPi-IIb) present at the luminal surface of brush border membrane at the apical region inwards/downwards towards the basolateral membrane enabled via cellular energy dispensation via a Na+/K+ ATPase co-transporter, **(B)** Paracellular passive diffusional movement of P across the intercellular spaces in the intestine. HPO2-4 as NaPi2b substrate; H2PO-4 as PiT1/PiT2 substrate [Candeal, 2017].

For example, the adaptive mechanisms exhibited by birds exposed to depleted dietary minerals, resulting in lower serum P, increased the synthesis of $1,25(OH)_2D_3$ (calcitriol), which in turn facilitates intestinal uptake of P [Berndt and Kumar, 2009]. This is coupled with the transcriptional expression of intestinal sodium-dependent phosphate cotransporters (*SLC34A2*) alongside renal expression of *SLC20A1* and *SLC20A2* to facilitate renal re-absorptive processes [Omotoso, 2023; Marks, 2019].

1.5.2.1 Sodium-dependent phosphate co-transporters in the domestic fowl

The sodium-dependent phosphate co-transporters are generally classified into the solute carrier family type I, II and III. Four sodium-dependent phosphate co-transporters, including *SLC20A1, SLC20A2, SLC34A1 and SLC34A2,* have been identified in the *Gallus gallus domesticus* based on current knowledge. Members of the type II transporters include *Na^+Pi-IIa* (*SLC34A1*), *Na^+Pi-IIb* (*SLC34A2*), and *Na^+Pi-IIc* (*SLC34A3*) [Murer, 2004]. In comparison, the type III transporter members included the *Pi-T1* (*SLC20A1*) and *Pi-T2* (*SLC20A2*). Moreover, 2 of 3 members of the type II transporter (*SLC34*) have been identified within the brush border membrane of the intestinal lumen as well as the kidney, where they facilitate the luminal transcellular phosphate uptake or renal P re-absorption, respectively [Omotoso, 2023; Marks,

2010]. Both type II and III members of the solute carrier family exhibit distinct characteristics relating to the preferences for phosphate valency and its stoichiometric regulation [Marks, 2010]. Regarding the phosphate valency preferences, the type II transporters (*SLC34A1*, *SLC34A2* and *SLC34A3*) have an affinity for divalent phosphate (HPO_4^{2-}) [Marks, 2010]. More so, both *SLC34A1* and *SLC34A2* are electrogenic and transport sodium/phosphate at a stoichiometric ratio of 3:1, i.e., $3Na^+$:HPO_4^{2-} (Figure 3), while, (*SLC34A3*) is electroneutral, preferring a 2:1, i.e., $2Na^+$:HPO_4^{2-} stoichiometric ratio [Marks, 2010].

In contrast, the type III transporters (*SLC20A1* and *SLC20A2*) have an affinity for monovalent phosphate ($H_2PO_4^-$); they are both electrogenic with a preferred stoichiometric ratio of sodium/phosphate at 2:1, i.e., $2Na^+$:$H_2PO_4^-$ [Marks, 2010]. Moreover, experiments on murine models previously established that *PiT2* (*SLC20A2*) is present at the renal brush border membrane (BBM) to enhance the re-absorption [Breusegem, 2009; Villa-Bellosta, 2009], and *PiT1* (*SLC20A1*) is at the intestinal BBM [Giral, 2009] with the expression of these candidates influenced by the level of dietary P present within the organismal biosystem [Breusegem, 2009; Giral, 2009; Villa-Bellosta, 2009]. However, similar tissue-specific expression was identified in the broiler intestine and kidneys fed varied levels of dietary P [Omotoso, 2023].

Considering the specific physiological P requirement of the laying hen and broilers, other hormones such as oestradiol might influence the actions of calcitriol and intestinal sodium/phosphate co-transporter type II (*SLC34A2*) for P absorption in the laying hen. In layers and broiler breeder hens, three oestrogenic precursory hormones have been identified, including oestrone (E1), oestradiol-17β (E2) and oestriol (E3). Of these, oestradiol-17β (E2) is classified as the primary female reproductive hormone mediating sexual maturation (i.e., the onset of lay) in the domestic fowl [Hanlon, 2022], indirectly stimulating a deterministic developmental phase for increased metabolic Ca demand for the egg-shell calcification process. Moreover, studies on the murine species have identified estrogen's regulatory roles on Ca absorption and homeostasis, intestinal NaPi-IIb (*SLC34A2*) gene expression [Xu, 2003; Guerreiro, 2002], and its modulation of $1,25(OH)_2$ vitamin D_3 synthesis [Xu, 2003; Van Abel, 2002; Schwartz, 2000].

1.6 Importance of the domestic fowl's gut microbiota

The gut microbiota represents an active constituent of the gastrointestinal tract (GIT), containing a complex community of hundreds of diverse microorganisms that are colonized after hatching and defined by several factors that can be broadly divided into (i) host characteristics, e.g., bird age, strain, sex, GIT section, and (ii) the environmental factors, e.g., husbandry system, feed, geographic location, and biosecurity [Ngunjiri, 2019; Kers, 2018]. In

fact, the microbiota contributes to the dynamics of complex structural, metabolic and immunological processes in the gut that define the host's health, welfare and age-appropriate development [Yu, 2021; Rubio, 2019; Onrust, 2015].

1.6.1 Role of the gut –microbiota in P and Ca homeostasis of the domestic fowl

Concerning the maintenance of homeostasis and efficiently utilizing plant-bound P, the diversity and functional contribution of the intestinal microbiota is a promising target. The intestinal microbiota contains specific phosphatase-secreting microbes such as the *Bifidobacteria* [Haros, 2005] and isolates of *Lactobacillus* [Kim, 2007], which are capable of hydrolyzing phytate and release inorganic P to the host for absorption. Moreover, the dietary supply of macro-minerals such as P and Ca to the broiler has been reported to modulate the gut microbiota [Ptak, 2015], thus indicating the microbiota as a potent, functional entity driving nutrient metabolism [Grice, 2012].

Furthermore, accumulating scientific studies on the chicken microbiome focused on the distal ileocecal region of the GIT, e.g., the caeca or colon, due to the high diversity of the microbial community in this GIT section crucial to mediate the final fermentation processes of ingested P which determines the corresponding levels of P and inositol phosphates excreted to the environment [Yan, 2017; Witzig, 2015]. However, the homeostasis and metabolism of P are initiated in the proximal small intestine, specifically, the jejunum, where co-transporter-enabled P uptake facilitates increased absorption and utilization after enzymatic phytate degradation [Hurwitz, 1970].

Hence, investigating the enzymatic role of the broiler's jejunal microbiota might be informative. Moreover, previous studies in pigs provided varied dietary P levels indicated significant differential abundances of the intestinal microbiota, suggesting the possibility of focused manipulation of the enteral microbiota through dietary interventions for optimal utilization enteral P and phytate [Reyer, 2021a].

1.6.2 Immunomodulatory role of the domestic fowl's gut –microbiota

The enteral microbiota contributes vital roles in maintaining gut health, modulating immune responses, and aiding in the digestion of feed materials to release nutrients, all culminating in the optimal performance and welfare of the domestic fowl [Khan, 2020]. The immune modulatory effects of the gut microbiota depend on the complex probiotic interactions to produce metabolites from within their diverse community or sourced from the host molecules or diet [Agus, 2018; Han, 2016]. Microbial metabolites emanating from the host-microbiota interaction result in the synthesis of short-chain fatty acids (SCFAs), tryptophan metabolites,

and secondary bile acids (BAs) [Kayama, 2020; Agus, 2018]. The probiotic effect of the microbiota within the immune response context has been reported to directly and competitively exclude pathogenic microbes proliferation via different actions of the microbiota or its interaction with the host intestinal epithelial cells (IECs), including the lowering the luminal pH in favour of beneficial microbes production, alongside the production of anti-microbial compounds, e.g. SCFAs and bacteriocins [Rhayat, 2017].

The production of SCFA also aids in the maintenance of energy metabolism crucial for the production of mucins by goblet cells for the fortification of the luminal epithelia barrier, which in turn increases the beneficial microbiota adhesion and reduction of pathogenic microbe adhesion [Broom, 2018]. It is imperative to note that the dysbiosis of gut microbiota can deleteriously impact the intestinal morphology and activities of chickens, resulting in heightened permeability and dysfunctional intestinal barrier, ultimately rendering them more vulnerable to bacterial infections, sepsis, inflammation compromising digestion [Shang, 2018].

In addition, the direct interaction between the microfold cells (M cells) and gut microbiota within the intestinal tract, enhances the mucosal immune surveillance via mechanisms that aid in the monitoring the shifts in resident intestinal microbiome [Dillon, 2019]. The intestinal mucosal sentinel micro fold cells (M cells) are present in the follicle-associated epithelium (FAE) embedded within the gut-associated lymphoid tissue (GALT). M cells are primarily composed of the intestinal epithelial and dendritic cells [Bai, 2013]. They play a crucial role in eliciting immune responses via antigen/pathogen sampling and presentation serving as gatekeepers within the mucosal immune system [Corr, 2008].

1.7 Research Aims and Objectives

The studies reported in this dissertation comprise two projects clustered based on the fowl-type used. Project 1 consists of studies 1 and 2 conducted on two strains of laying hens (Lohmann Brown LB and Lohmann selected leghorn LSL), while Project 2 consists of studies 3 and 4 conducted on broiler chickens. The broad research objectives of studies 1, 2, 3 and 4 are as follows;

Studies 1 and 2 adopted a holistic jejunal transcriptomic profiling of the LB and LSL hens, considering the influence of the different production periods (weeks 10, 16, 24, 30 and 60) (1) and varied dietary minerals, P and Ca (PCa, LPCa, LP, LCa) (2), coupled with the measurement of hormones and metabolites to approximate the dynamics of mineral homeostasis. The objective was to gain insights into the molecular mechanisms underlying mineral efficiency for productivity and physiological processes in layer chickens.

Research objectives of studies 3 and 4 was to identify the temporal physiological responses of broiler chickens exhibited through different endogenous adaptive mechanisms, including endocrinal, transcriptional, osseous and microbial processes, following a depleted dietary P regimen. The objective rests on the hypothesis that the depletion of dietary P supply and its timing contribute to endogenous adaptive responses for P efficiency during the productive life of the broiler and its age-specific requirements.

Collectively, studies 1-4 focused on gaining deeper insights into P utilization, its resource allocation and efficiency in the *Gallus gallus domesticus* species by exploring various endogenous responses elicited by the birds via plasma/serum hormones, metabolites, and bone mirrored at the transcriptional level as mRNA transcripts. The hypothesis is that phenotypic and genetic variation exists in the extent to which different regulatory pathways are recruited in response to the direct effects of dietary and digestive P and Ca availability and the corresponding indirect effects on microbiota composition to maintain P homeostasis. This variation could be used to improve utilization of P from various feed sources and reduce excretion of surplus P.

The specific aims of each study are documented as follows:

- Study 1 aimed to identify differentially expressed genes (DEGs) and molecular pathways in the jejunum of two-layer strains (LB and LSL) related to development, growth and the onset of laying, which might contribute to further improvements in nutrient efficiency and productivity as the hens mature across different productive stages.

- Study 2 aimed to elucidate the jejunal contribution to the complex regulation of mineral homeostasis in individual hens at the peak of egg production by identifying strain-specific transcriptional responses via differentially expressed genes (DEGs) and enriched molecular pathways in response to the varied dietary P and Ca intake.

- Study 3 aimed to decipher the endogenous adaptive responses for P efficiency during the productive life of the broiler as well as their age-specific requirements elicited by the broiler in response to depletion of dietary P supply and its timing. Consequently, the effects of a variable P supply throughout the entire production phases were evaluated by measuring growth performance, endocrine control, transcellular P transport, bone

mineralization, and health aspects in an array of tissues such as blood, jejunum, kidney, and bone.

- Study 4 aimed to identify the possible synergy of the gut microbiota with the endogenous mechanisms adopted by the bird to maintain P homeostasis. This entailed the comprehensive profiling of the jejunal microbiota composition of broilers subjected to P depletion throughout the grower and finisher stages via high-throughput 16S rRNA gene amplicon sequencing, coupled with the measurement of the corresponding levels of total fecal mineral P and Ca and phytate to approximate the unutilized mineral fractions.

CHAPTER 2

PUBLICATIONS AND SUMMARY

2 Summary of Publications

In order to characterize the endocrinal and transcriptional determinants of P utilization in laying hens and broiler chickens, the results of three experimental trials have been published in 4 interlinked studies. The experiments elaborately documented in studies 1 - 4 provide a robust, in-depth analysis of the transcriptional, endocrinal, and microbial determinants in domestic fowl, e.g., laying hens and broiler chickens (Figure 4).

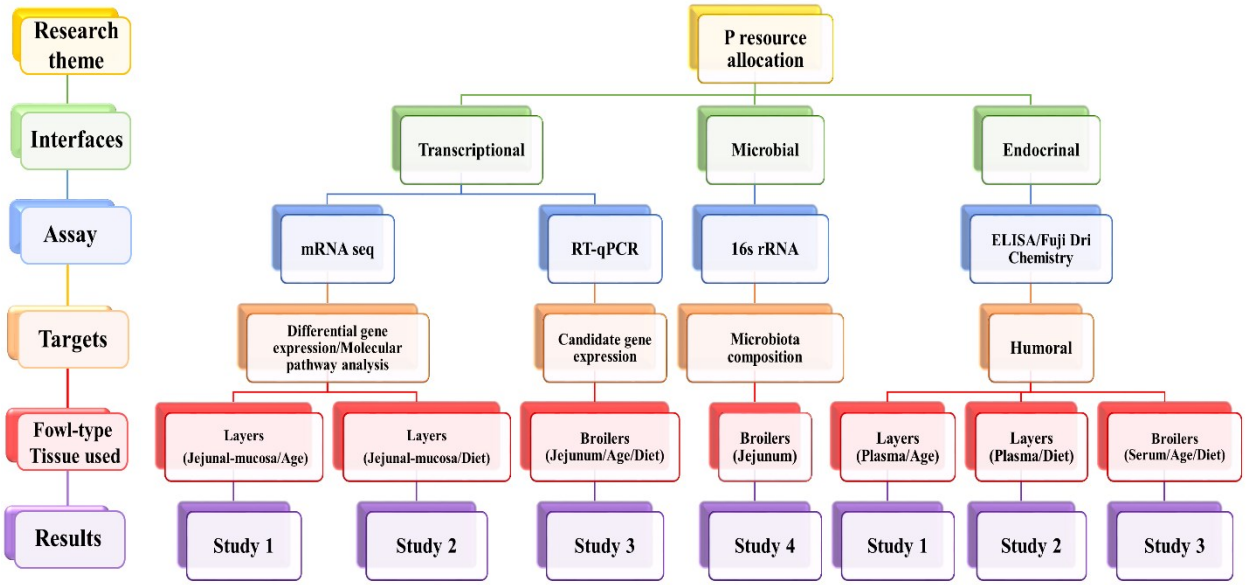

Figure 4. Schematic pipeline representation of the interrelatedness of the research interfaces, biological assays, fowl-type, tissues and treatment effect investigated in experiments reported.

In the first study, samples of jejunum mucosa from the two lines of laying hens were obtained at five different developmental stages, including weeks 10, 16, 24, 30 and 60, to represent the pullet, pre-lay, onset of lay, the peak of lay and senescence production time points, respectively. Samples of jejunum mucosa were used to determine the abundance of transcripts by mRNAseq in the longitudinal experiment designed to consider the temporal changes between time points within and between the two-layer strains. Plasma hormones and metabolites of the jejunal mucosa were measured to approximate the dynamics of mineral P and Ca homeostasis. It was found that the onset of laying was the most significant time point as several levels of physiological measurements shifted to ensure mineral utilization to the uterus and laying performance. At this transitional stage, the differences between the strains were also most striking, suggesting that both strains cope with altered metabolic needs and adapted nutrient supply to achieve comparable egg production performance, in part by recruiting different pathways in the intestinal mucosa.

In the second study, four diets (recommended vs low Ca and P) were fed to two lines of laying hens. Samples of jejunal mucosa were used to determine the abundance of transcripts by mRNAseq in the holistic transcriptomic response to dietary mineral supplements within and between the two-layer strains. Plasma hormones, metabolites, and the transcriptome of the jejunal mucosa were measured to approximate the dynamics of mineral P and Ca homeostasis. The study's findings showed that endogenous mechanisms to maintain mineral homeostasis in response to variations in the supply of Ca and P were effective in laying hen strains. However, the LSL and LB birds appeared to adopt different molecular pathways, as shown by circulating vitamin D levels and strain-specific transcriptome patterns.

The third study evaluated the effects of dietary P depletion on growth performance, endocrine control, and transcellular P transport and bone mineralisation in various tissues such as serum, jejunum, kidney, and bone at different developmental phases in the broiler chicken. Dietary P depletion was introduced at the early developmental phase of life and continued at advanced growth phases with the expectation that the intrinsic interplay of different tissues would avail insights into the regulation and dynamics of mineral P homeostasis and efficiency that confers adaptation and optimal resource allocation on the organism. Based on the study's findings, the threshold for P deprivation for environmental concerns should be set no earlier than the late start/early growth phase, as physiological adaptation mechanisms to P deficiency seem more effective than in the early growth phase.

The fourth study was a continuum of the third, involving the sampling of the broiler chicken jejunal digesta and faeces at the grower and finisher phases of development as proxies for the examination of the jejunal microbiota-associated response to the P depletion strategy and the estimation of corresponding inaccessible levels of mineral P and Ca and undegraded phytate in the excrement. Expression, mineral intake, and excrement data were integrated and analyzed by the open-sourced R software (statistics; visualization) to identify operational taxonomic units, relative abundance and the functional potentials of the microbiota affecting P efficiency regarding the ages and dietary P group of the broilers. Findings on the mineral P and Ca intake and corresponding fecal mineral (P, Ca) and phytate levels showed that the diets applied to the depleted and non-depleted cohorts were effective, with the depleted groups almost maximizing the phytate degradation. Overall, broilers allotted to the non-depleted group exhibited more significant relative microbial abundance of taxa than those fed the depleted P. Broiler chickens assigned to the dietary P depletion groups only showed significant relative abundances for *Facklamia, Lachnospiraceae, and Ruminococcaceae*, suggesting that these microbiota make only a subtle contribution to the birds' adaptive mechanism following P depletion.

2.1 Study 1 – Jejunal transcriptomic profiling of two-layer strains throughout the entire production period

Adewunmi Omolade Omotoso, Henry Reyer, Michael Oster, Siriluck Ponsuksili, Nares Trakooljul, Eduard Muráni, Vera Sommerfeld, Markus Rodehutscord and Klaus Wimmers

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Authors' Contributions: conceptualization, K.W., M.R.; methodology, **A.O.O.,** H.R., M.O., N.T.; formal analysis, H.R., M.O., N.T.; investigation, **A.O.O.,** H.R., M.O., S.P., V.S., M.R., K.W.; resources, S.P., E.M., K.W.; data curation, H.R., M.O., V.S.; writing-original draft preparation, **A.O.O.**; writing-review and editing, **A.O.O.**, H.R., M.O., S.P., N.T., E.M., V.S., M.R., K.W.; visualization, **A.O.O**., H.R., M.O.; supervision, H.R., K.W.; project administration, V.S., M.R., K.W.; funding acquisition, M.R., K.W. All authors have read and agreed to the published version of the manuscript.

The jejunum plays crucial roles for the digestion and absorption of nutrients and minerals and for barrier functions that are essential for a healthy, productive life cycle of farm animals, including laying hens. Accordingly, knowledge of the molecular pathways that emerge in the intestine during development, and particularly at the beginning of laying activity, will help to derive strategies for improving nutrient efficiency in laying hens. In this study, jejunal samples were obtained from two high-yielding layer strains at five developmental stages (weeks 10, 16, 24, 30 and 60 of life) for RNA-sequencing, alongside the profiling of blood plasma parameters to approximate the dynamics of mineral homeostasis. The results reflected a marked distinction between the pre-laying and laying phase as inferred from levels of parathyroid hormone, triiodothyronine, estradiol, vitamin D, and Ca. Moreover, the expression patterns of the intestinal mucosa responded directly to the changing metabolic and nutritional profiles at the beginning of the laying phase in maturing high-yielding strains of laying hens. These comprise signaling events namely RANK/RANKL signaling and cellular senescence. Taken together, the timing of sexual maturity of laying hens demands closer examination to unravel metabolic requirements and associated endogenous mechanisms.

2.2 Study 2 – Transcriptional responses in jejunum of two-layer chicken strains following variations in dietary calcium and phosphorus levels

Henry Reyer, Michael Oster, Siriluck Ponsuksili, Nares Trakooljul, Adewunmi Omolade Omotoso, Muhammad Arslan Iqbal, Eduard Muráni, Vera Sommerfeld, Markus Rodehutscord and Klaus Wimmers

BMC Genomics 22, 485 (2021). https://doi.org/10.1186/s12864-021-07814-9

Authors‘ Contribution: KW, MR and SP designed the research; HR, MO, SP and KW supervised the experiment; HR, MO, NT, SP and VS performed the experiments; HR, KW, MO and SP curated and analyzed the data; HR, **AOO,** MAI and MO performed statistical data analysis; **AOO,** EM, HR, KW, MO, MR and SP participated in the interpretation of the data; HR drafted the manuscript; HR and MO prepared figures; all authors contributed to the preparation and editing of the manuscript.

Calcium (Ca) and phosphorus (P) are essential nutrients that are linked to a large array of biological processes. Disturbances in Ca and P homeostasis in chickens are associated with a decline in growth and egg laying performance and environmental burden due to excessive P excretion rates. Improved utilization of minerals in particular of P sources contributes to healthy growth while preserving the finite resource of mineral P and mitigating environmental pollution. In the current study, high performance Lohmann Selected Leghorn (LSL) and Lohmann Brown (LB) hens at peak laying performance were examined to approximate the consequences of variable dietary Ca and P supply. The experimental design comprised four dietary groups with standard or reduced levels of either Ca or P or both (n = 10 birds per treatment group and strain) in order to stimulate intrinsic mechanisms to maintain homeostasis. Jejunal transcriptome profiles and the systemic endocrine regulation of mineral homeostasis were assessed (n = 80). Results: Endogenous mechanisms to maintain mineral homeostasis in response to variations in the supply of Ca and P were effective in both laying hen strains. However, the LSL and LB appeared to adopt different molecular pathways, as shown by circulating vitamin D levels and strain-specific transcriptome patterns. Responses in LSL indicated altered proliferation rates of intestinal cells as well as adaptive responses at the level of paracellular transport and immunocompetence. Endogenous mechanisms in LB appeared to involve a restructuring of the epithelium, which may allow adaptation of absorption capacity via improved micro-anatomical characteristics. Conclusions: The results suggest that LSL and LB hens may exhibit different Ca, P, and vitamin D requirements, which have been neglected in the supply recommendations. There is a demand for trial data showing endogenous factors

of Ca and P homeostatic mechanisms, such as vitamin D, at local and systemic levels in laying hens.

2.3 Study 3 – Broiler physiological response to low phosphorus diets at different stages of production

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Authors‘ Contribution – conceptualization: H.R., M.O., K.W., methodology: **A.O.O.**, H.R., M.O., formal analysis: **A.O.O.,** H.R., M.O., investigation: **A.O.O.,** H.R., M.O., resources: S.M., S.P., K.W., data curation: **A.O.O.,** H.R., M.O., writing - original draft preparation: **A.O.O.,** writing - review and editing: **A.O.O.,** H.R., M.O., S.M., S.P., K.W., visualization: **A.O.O.,** supervision: H.R., M.O., K.W., project administration: K.W., funding acquisition: K.W.

Phosphorus (P) inclusion in broiler diets needs to meet the physiological demands at a specific developmental stage to ensure the performance, health, and welfare of the birds and minimize nutrient losses. Toward a more efficient utilization of P in broiler husbandry, a timed nutritional conditioning strategy might enhance the endogenous mechanisms of mineral homeostasis and thus reduce dietary P supply of mineral sources. In this study, following a variable P supply in the starter phase, the effects of a dietary P depletion of broiler chickens were investigated at different developmental stages. Physiological adaptation mechanisms were elucidated based on zootechnical performance, endocrine parameters, regulation of intestinal P transport, bone characteristics, and health aspects. The results revealed a marked response to P depletion at the earliest developmental phase, after which indications of effective compensatory mechanisms were detectable with advancing ages. Potential mechanisms that enable broilers to maintain mineral homeostasis primarily include endocrine control mediated by calcitriol actions, as well as intestinal P uptake and mineral mobilization from the bone. Conclusively, the precise timing, duration, and extent of a P depletion strategy in the broiler chicken might be considered for optimized nutrient utilization.

2.4 Study 4 – Jejunal Microbiota of Broilers fed varying Levels of Mineral Phosphorus

Adewunmi Omolade Omotoso, Henry Reyer, Michael Oster, Siriluck Ponsuksili, Klaus Wimmers

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Authors‘ Contribution – conceptualization: H.R., M.O., K.W., methodology: **A.O.O.**, H.R., M.O., formal analysis: **A.O.O.,** H.R., M.O., investigation: **A.O.O.,** H.R., M.O., resources: S.P., K.W., data curation: **A.O.O.,** H.R., M.O., writing - original draft preparation: **A.O.O.,** writing - review and editing: **A.O.O.,** H.R., M.O., S.P., K.W., visualization: **A.O.O.,** supervision: H.R., M.O., K.W., project administration: K.W., funding acquisition: K.W.

Efforts to achieve sustainable phosphorus (P) inputs in broiler farming which meet the physiological demand of animals include nutritional intervention strategies that have the potential to modulate and utilize endogenous and microbiota-associated capacities. A temporal P conditioning strategy in broiler nutrition is promising as it induces endocrinal and transcriptional responses to maintain mineral homeostasis. In this context, the current study aims to evaluate the composition of the jejunal microbiota as a functional entity located at the main absorption site involved in nutrient metabolism. Starting from a medium or high P supply in the first weeks of life of broilers, a depletion strategy was applied at growth intervals from d 17-24 and d 25-37 to investigate the consequences on the composition of the jejunal microbiota. The results on fecal mineral P, calcium (Ca), and phytate contents showed that the diets applied to the depleted and non-depleted cohorts were effective.

Microbial diversity in jejunum was represented by alpha diversity indices which appeared unaffected between dietary groups. However, chickens assigned to the dietary P depletion groups showed significantly higher abundances of *Facklamia*, *Lachnospiraceae*, and *Ruminococcaceae* compared to non-depleted control groups. Based on current knowledge of microbial function, these microorganisms make only a minor contribution to the birds' adaptive mechanism in the jejunum following P depletion.

Microbial taxa such as *Brevibacterium*, *Brachybacterium*, and genera of the *Staphylococcaceae* family proliferated in a P-enriched environment and might be considered biomarkers for excessive P supply in commercial broiler chickens.

2.5 Personal contribution to the experimental studies 1 - 4

I declare that my contribution to each of the experimental studies reported in this project is elaborately documented as follows.

I conducted the wet laboratory analysis for samples collected in studies 1, 3 and 4 with activities ranging from:

- Nucleic acids isolation (RNA) from chicken tissue samples including jejunum mucosa scrapings in study 1, jejunum and kidney samples in study 3 and DNA extraction in the jejunal digesta in study 4.
- Nucleic acids purification and amplification using the polymerase chain reaction (PCR) (studies 1, 3 and 4)
- Amplicon quality control measures using spectrophotometry and agarose gel electrophoresis (studies 1, 3 and 4)
- cDNA synthesis and purification (studies 1 and 3)
- Molecular primers design for candidate genes of interest, e.g., transcellular mineral P and Ca transporters (study 4)
- Candidate gene expression analysis of transcellular mineral P and Ca transporters using the real-time quantitative polymerase chain reaction (RT-qPCR) (study 4)
- Flexural bone bending test for strength analysis (study 4)
- Plasma/serum hormones and metabolite analysis using Enzyme-linked immunoassay techniques (ELISA) and Fuji Dri chemistry, respectively (studies 1 and 3)

I conducted the dry laboratory activities for all studies 1, 2, 3 and 4, which entail;

- Experimental data analysis (transcriptomic and statistical), results interpretation and manuscript drafting (studies 1, 2, 3, and 4)

CHAPTER 3

GENERAL DISCUSSION

3 General Discussion

The experiments elaborately documented in studies 1-4 provide a robust, in-depth overview of the laying hens and broiler chickens' transcriptional, endocrinal, and microbial responses to varied levels of dietary minerals. Specifically, the studies focused on the responses elicited by two-layer hen strains over the five productive time points (weeks 10, 16, 24, 30 and 60) in study 1 and those fed diets with varying levels of dietary P and Ca (i.e., Con, LCaP, LCa, LP) in study 2. In contrast, study 3 investigated the response of the broiler chickens to depleted levels of P in an array of tissues involving the jejunum and kidney (transporters) and bone (morphology). The 4th study reported the jejunal microbiota response to varied dietary P as well as the resultant fecal output of the minerals and phytate in broiler chickens.

All experiments analyzed endocrinal determinants via either blood plasma/serum, hormones, and metabolites to approximate the dynamics of mineral homeostasis within both biosystems as they aimed to achieve mineral homeostasis, efficiency, and resource allocation.

Primarily, transcriptional and microbiomic investigations in studies 1, 2, 3 and 4 focused on jejunum as the preferred intestinal segment of interest, precisely due to the longer length of the villi projections in the tissue, which influences significant absorptive function [De Verdal, 2010].

3.1 Endocrinal determinants crucial for the approximation of mineral homeostasis in laying hens and broiler chickens

The following plasma hormones such as calcidiol, calcitriol, parathyroid hormone (PTH), triiodothyronine (T_3), oestradiol (E2) and metabolites, e.g., calcium (Ca), phosphorus (P), magnesium (Mg), albumin, alkaline phosphatase (ALP), were analyzed to approximately the dynamics of mineral P and Ca homeostasis in the LB and LSL hens in studies 1 and 3. In contrast, the serum levels of the listed hormones and metabolites were accessed in broiler chickens in study 4, excluding E2, ALP, and Mg.

3.2 Age and diet effect on endocrinal profiles of laying hens and broilers chickens

The blood plasma parameters of LB and LSL hens in studies 1 and 2 were investigated to approximate the dynamics of mineral homeostasis.

This was accessed via the plasma hormone and metabolite actions exhibited by the hens over five developmental time points (pullet, grower, onset of lay, peak of lay and senescence) in study 1. The findings revealed a production-specific response in both the LB and LSL laying hens with significantly increased levels of Ca, Mg and albumin, while corresponding levels of P reduced initially with subsequent re-adjustment at the onset of laying (week 24) in both LB and LSL strains.

Physiologically, this signals the preparedness of the layers to meet the metabolic demand of Ca for eggshell calcification processes at the start of egg laying, with the increase in plasma Ca levels for eggshell calcification necessitating the reduction in plasma P for attain balance [Pelicia, 2009]. More so, the increased plasma Ca levels at week 24 compared with those of weeks 10 and 16 and the corresponding lowered plasma P levels reflect intrinsic processes adopted by the bird to maintain homeostasis between both macro-minerals as mediated by actions of hormones such as PTH and calcitriol [Sinclair-Black, 2023]. Similarly, endocrinal profiles in study 3, involving the broiler cohorts fed depleted levels of dietary P across the starter (days 1-10), grower (days 11-24) and finisher (days 25-37) developmental stages, showed significantly reduced serum levels of P in chickens fed low P diets with a correspondingly (numerically) higher serum Ca levels in the same birds compared to those fed the M and H diets at the day 17. In fact, within biological systems, P and Ca exhibit an antagonistic stoichiometric relationship usually to maintain homeostasis, i.e., higher levels of P hamper the surge of Ca and vice versa [Sinclair-Black, 2023]. However, this chemical phenomenon of P is not restricted to Ca alone but is also observed with other cationic high-valency minerals such as iron (Fe^{2+}), zinc (Zn^{2+}), magnesium (Mg^{2+}), potassium (K^{+}), and manganese (Mn^{2+}) [Humer, 2014]. As a result of the high affinity of P to form chemical bond complexes with other cationic high-valency minerals, absorption of these minerals is inhibited [Humer, 2014]. Regarding findings in study 1, a surge in plasma Ca levels was observed at week 24, driven by the need for Ca to meet production demands. However, increased dietary Ca levels might alternately encumber phytate degradation and mineral digestibility in the gut, lowering P uptake at the onset of lay [Sommerfeld, 2020a].

In study 3, broiler chickens fed the depleted P diets at the early growth phase showed lowered serum P concentration with correspondingly surged serum Ca and calcitriol (1,25 $(OH)_2$ vitamin D_3) levels compared to those fed the M and H diets. The continuously elevated calcitriol levels throughout the development phases suggest the bird's efforts to meet metabolic P demand and maintain mineral equilibrium via intestinal P absorption. Plasma calcitriol (1,25 $(OH)_2$ vitamin D_3) concentrations were also pronounced in the LB and LSL hens in study 1, with significantly higher levels at the onset of lay (week 24) compared to periods preceding the onset of lay (weeks 10 and 16). Contrastingly, plasma concentrations of calcitriol did not differ significantly between dietary treatments ($p > 0.05$) in layer cohorts in study 2 but showed high individual variability, wherein LSL hens have higher plasma calcitriol levels compared to LB hens, indicating the possibility of better Ca absorption and mineralization than LB hens. More so, it

has also been observed that LSL hens tend to have higher egg weights than LB hens [Sommerfeld, 2020b].

Calcitriol (bioactive form of vitamin D) modulates optimal intestinal absorption of minerals; hence, its surge is evident in both the egg-type fowl at the onset of lay (week 24) when Ca is needed to meet egg production demands in study 1. A similar observation was found in the meat-type broiler fed the depleted P diet at day 17 in the study 3, where sufficient levels of P to meet growth and performance potentials was needed [Berndt and Kumar, 2009]. In addition, the calciostatic action of PTH was observed in the LB and LSL hens in study 1, revealing significantly higher plasma levels of the hormones at the onset of lay (week 24) compared to periods preceding the onset of lay. However, plasma PTH levels were unresponsive to the varied dietary macro-minerals in LB hens, but significantly higher levels in LSL hens fed the LCaP diet compared with those fed the Con diet ($p = 0.04$) were observed in layers reported in study 2. More so, serum concentrations of PTH remained unaffected by diet at all experimental stages in the broilers reported in study 3.

PTH is often regarded as a hormonal “calciostat” that prevents hypocalcemia, modulating Ca homeostasis by indirectly mediating osteoclastic bone resorption to mobilize Ca and P, as well as the renal reabsorption of Ca and excretion of P [Blaine, 2015; Moe, 2008; Urist, 1967], usually under insufficient diet supplementation. Moreover, the crosstalk between calcitriol and PTH enables the regulation of systemic Ca and P levels via sophisticated feedback loops in the organismal biosystem. The increase in PTH in layers in study 1 suggests the efforts by the hens to maintain appropriate levels of circulating Ca throughout production [Gloux, 2019]. However, the unperturbed levels of PTH in the broiler cohorts in study 3 might be attributable to the fowl-specific utility and metabolism of Ca, which is less intense compared to the layer to define performance [Li, 2017; Pierce, 2009].

Furthermore, temporal profiling of the plasma calcidiol ($25(OH)_2$ Vit D_3), the storage form of vitamin D was significantly lowered from the onset of laying at week 24 in both LB and LSL hens in study 1, revealing a significantly higher level in the LB hens compared to LSL hens at senescence (week 60). Notably, calcidiol levels at week 30 were numerically increased in LB compared to LSL strains ($p = 0.051$). Hence, it is conceivable that calcidiol produced in the liver is deposited in the egg yolk as an embryonic reservoir as the hens peak in production [Qin, 1995]. Similarly, in study 2, a marked strain effect was observed in the plasma calcidiol levels, which differed significantly, with higher levels in LB compared to LSL across all dietary groups. More so, the significant difference in calcidiol concentrations in response to the diets was observed in LB layers fed the low P and Ca diet (LCaP) (39.26 ± 3.35 ng/ml) compared to

those fed LP (39.26 ± 3.35 ng/ml) in LB ($p = 0.03$) [Reyer, 2021a]. Contrastingly, serum concentrations of calcidiol were unaffected by diet at all experimental stages in the broiler in study 3. The observations regarding plasma calcidiol levels in layer experiments in studies 1 and 2 agree with previous experiments that reported LB hens exhibit a higher bone mass and breaking strength of humeral and tibia bones compared to LSL, whereas bone density remained unaffected [Habig, 2013; Silversides, 2012]. Albeit the LB and LSL hens shared similarity in their productive capacity, which directly involves the metabolism, mineralization and turn-over of Ca, it is evident that the laying hens indeed recruit differing endocrinal, transcriptional and metabolic routes in response to environmental stimuli to define their distinct phenotypes [Omotoso, 2021: Reyer, 2021a; Sommerfeld, 2020a; Sommerfeld, 2020b].

Plasma concentrations of oestradiol (E2) were significantly elevated from the onset of laying at week 24 in both LB and LSL hens in study 1, suggesting the attainment of sexual maturity logically connotes the start of egg production. Oestradiol, the most potent form of estrogen, is secreted primarily by the laying hen's ovaries and is responsible for the overall maturation and development of the female reproductive system. Regarding laying hen, it regulates process of yolk protein formation (vitellogenesis) in the oocytes and the activation of yolk precursors in the liver during sexual maturation [Denslow, 1999]. In addition, it contributes to the formation of the medullary bone in the laying hen [Dacke, 1993]. Based on this information, it is inferable, that a strain-specific strategy for maintaining a long-term response to metabolic demands in the LB hens was observed due to the higher plasma levels of oestradiol, which persisted till senescence (week 60).

At the onset of lay in study 1, plasma T3 concentrations decreased with a corresponding increase in the plasma oestradiol levels. Based on the observed increase in the latter, it is inferable that the laying hens traded off between somatic body development and reproductive capacity development. This suggests that the hens prioritized reproductive development over somatic body development at the onset of lay [McNabb, 2017; Sechman, 2009]. However, in the diet experiment in study 2, plasma T_3 concentrations showed no significant difference between dietary groups. The strain-specific responses observed in the LB < LSL at week 16, also suggests an adaptive response for body growth relative to production in the LSL, this is buttressed explicitly by the fact that the LSL hens have lower body weight but a higher egg weight than LB hens [Sommerfeld, 2020b]. In the broiler experiment reported in study 3, serum concentration of T_3 was significantly reduced in chickens fed the L P diet at the early growth phase. Indeed, P deprivation, especially in P-sensitive broilers, induces hypothyroidism and systemic growth reduction at the early stages of development [Parmer, 1987; Jianhua, 2000].

Conclusively, based on the interaction of the endocrinal profiling in the studies discussed, it is conceivable that as soon as the external situations, e.g., supply of appropriate/adequate dietary mineral requirement or the physiological status of the animal (sexual maturation) changes, intrinsic adaptive endocrinal determinants respond to maintain mineral homeostasis in domestic fowl. These mechanisms include an immediate short-term regulatory action for absorption or excretion of minerals mediated by an interplay of different hormones to targeted tissues/organs through feedback loop mechanisms.

3.3 Age and diet effect on transcriptomic profiles of two laying hen strains

Jejunal transcriptomic profiles between the LB and LSL hens accessed overall developmental time points (weeks 10, 16, 24, 30 and 60) in study 1 revealed a total of 82 differentially expressed genes (DEGs) involved in different biological processes, including the formation of extracellular matrix (*COL9A1*, *CRTAC1*, *MMRN2*), adaptive immunity (*CD8A*, *GBP6*, *HCK*) and micro- and macronutrient utilization (*HFE, SLC27A5*) after pathway enrichment analysis. Similarly, the diet-specific comparisons between LB and LSL laying hens fed either of four experimental diets (Con, LCaP, LCa and LP) in study 2 revealed 1020 DEGs intersecting all four diet group comparisons. These DEGs represent the strain-specific differences in jejunal nutrient utilization, metabolism and immunity. They are involved in biological processes including ‘metabolism of xenobiotics by cytochrome P450’, ‘glutathione metabolism’, ‘arginine and proline metabolism’, ‘drug metabolism’, and ‘histidine metabolism‘ after enrichment analysis [Reyer, 2021a].

Indeed, given the observed strain-specific transcript abundance reported in studies 1 and 2, considerable strain/genetic variability exists between the LB and LSL hens. This observation have been elaborately documented in previously and recently reported studies. The disparities in traits exhibited by the LB and LSL hens in response to age effects and environmental factors such as dietary mineral includes phytate degradation and transcellular mineral P and Ca absorption [Sommerfeld, 2020b], egg quality [Wistedt, 2019], bone parameters [Khanal, 2019], temporal distribution and shifts in immune cells towards innate and humoral responses for immunocompetence [Schmucker, 2021; Hofmann, 2021], and identification of allelic specific expression and imbalance [Iqbal, 2023].

Recent studies that investigated the immune system (systemic and lymphatic distribution of leukocyte subsets) of the LB and LSL during adolescence and the egg-laying period reported an increase in counts of all splenic lymphocyte types considered and γδ T cells in the blood from weeks 9/10 to 15/16, suggesting a response to novel pathogens encounter at the adolescence [Schmucker, 2021]. At the transitional phase from the pre-laying production stage

(weeks 15/16) to the onset of lay (weeks 23/24), a marked decrease in the number of γδ T cells and cytotoxic T cells (CTL; CD45+/CD4−/TCRγδ−/CD8α+) was observed, remaining low through the course of the laying period [Schmucker, 2021]. The authors concluded that egg-laying activity altered the immune system toward a more pronounced humoral and innate immune response. Overall, it was evident that although there are variations in the immune traits exhibited by the LB and LSL hen strains, age-related immunological patterns were similar [Schmucker, 2021].

Comparisons of DEGs within and between each of the two-layer strains across the selected production stages were highest at the transitional phase between the pre-lay stage (week 16) and the onset of lay (week 24) in study 1, suggestive of a transcriptional shift and responses in preparedness for egg production. In study 2, the highest DEGs (4540) between the LB and LSL hens were observed in hens fed the low P diet, indicative of transcriptional response to reduced P levels within the layers for adaptation.

The temporal profiling of LB and LSL hen jejunal transcript abundance overall productive developmental time points in study 1 identified 18 significant profiles, including 10 for LB and 8 LSL hens. Three profiles each (profiles #9, #41 and #18) were further selected to represent the downstream analysis for identifying enriched molecular pathways. Pathways were considered significantly activated or inactivated at an IPA-predicted absolute z-score > 2.

Profile #9 consisted of inhibited genes which consistently decreased with advancing productive period and were reflective of pathways generally associated with mitochondrial energy transduction and cellular growth processes, namely, “sirtuin pathway”, “mitochondria dysfunction pathway”, “oxidative phosphorylation pathway”, “JAK/SAT signaling pathway” expressed in both the LB and LSL hens and “CD27 signaling in lymphocytes pathway” in the LB hens. Recently, reports on genes with an allelic-specific expression were associated with these molecular pathways in an RNA-seq based study on discovering genetic variants and allele-specific expression between the two-layer strains [Iqbal, 2023]. Furthermore, enriched genes, e.g., *SIRT2*, *SIRT6* and *SIRT7* in the sirtuin pathway contribute to cellular energy metabolism processes, which enable adaptive responses to metabolic and oxidative stress through metabolic energy dispensation and homeostasis in synergy with the mitochondria [Nogueiras, 2012; Bosch-Presegué, 2013]. Additionally, the enrichment of mitochondrial dysfunction and oxidative phosphorylation pathways in profile #9 buttress the mitochondrial theory of ageing in which the sophisticated roles of cellular energy dispensation and respiration of the mitochondria diminish with advancing age due to the accumulation of reactive oxygen species (ROS), thus becoming dysfunctional [Chistiakov, 2014; Harman, 1972]. Hence, this

suggests the decline in cellular energy-dependent processes (e.g. intestinal cell renewal and proliferation) in the layers strains as they mature, approaching senescence [Rossiello, 2022; Lidzbarsky, 2018]. Also identified were inhibited DEGs in the JAK/STAT signaling pathway in both LB and LSL strains [Omotoso, 2021].

The JAK/STAT pathway is crucial in intestinal epithelial repair and regeneration processes via the activation of growth factors and cytokines [Truong, 2017]; hence, the inhibition of the JAK/STAT signaling pathway alongside the oxidative phosphorylation over the developmental stages (week 10-60) in both hen strains suggests a gradual shift in resource allocation from the initial modulation of cellular growth processes that maintenance the intestinal epithelium [Omotoso, 2021]. Previous studies on gut transcriptomic profiling in mouse models identified aging-associated changes in mRNAs attributable to cell cycle, oxidative stress and apoptosis, specifically within the intestinal epithelial stem cells (IESCs) [Moorefield, 2017].

The abundance of mRNA transcripts clustered in Profile #41 showed an increasing pattern over all stages of production, mainly observed in LSL hens. Molecular pathways enriched Profile #41 included immune-related processes as well as epithelial repair control and regeneration, e.g., “Leukocyte extravasation signaling pathway," "Regulation of the epithelial-mesenchymal transition pathway," "NF-B signaling pathway," and "STAT3 pathway." The observed Leukocytes and NF-B, which are primary regulators of the innate and adaptive immune response system, suggest an age-related activation of the immune system in the LSL hens [Reyer, 2021a; Hofmann, 2021]. DEGs clustered in Profile #18 overlapped between the LB and LSL hens in study 1. It comprised activated mRNA abundances with a sustained increase from the onset of laying (week 24) to the senescent phase of production (week 60). Molecular pathways enriched profile #18 includes “RANK signaling in osteoclast pathway”, “senescence pathway”, and “NGF and HGF signaling pathways”. Hens exhibited strain-specific pathways with the cardiac hypertrophy signaling pathway in the LB hens and the UVA-induced MAPK signaling pathway in the LSL hens. Due to the expression pattern of genes in this profile, direct effects of dietary change or secondary effects of sexual maturity and the nutrient demand with the onset of lay are conceivable [Drozdowski, 2006].

Interestingly, the RANK signaling has been implicated in the adaptation of the laying hens to the onset of egg laying, coupled with the endogenous release of Ca to meet production demand which occurs under the synergistic actions of pro-resorption endocrinal factors such as calcitriol, PTH and oestradiol, in conjunction with transcriptional modulation of the RANKL/RANK signaling pathway [Mizoguchi, 2014; Takahashi, 2014; Boyle, 2003; Carabotti, 2015]. In addition, the RANK signaling is also associated with pro-immune activities

within the epithelium, specifically via the modulation of differentiated sentinel micro fold cells (M cells) present in the follicle-associated epithelium (FAE), which covers the gut-associated lymphoid tissues (GALT) [Knoop, 2011; Knoop, 2009].

Considering the expression pattern of profile #18 for the RANK signaling pathway, it is inferable that both LB and LSL hen strains exhibited a consistently low expression of pro-bone resorption DEGs during the pre-lay stages, specifically weeks 10 and 16 [Omotoso, 2021]. However, there was a surge in expression at the onset and peak of egg laying, weeks 24 and 30, respectively. This surge may have been attributed to the increased metabolic demands during egg laying. Ultimately, this surge reached a plateau during the post-peak production stage. It was expected that the HGF and NGF signaling pathways would be activated in both hen breeds, which suggests a greater degree of gut-brain communication in achieving enteric homeostasis during the egg-production periods [Carabotti, 2015].

Based on the transcriptional responses observed in study 2, it appears that LB and LSL hens have distinct mineral requirements. This inference is drawn from the differences in jejunal gene expression patterns exhibited by each hen. Notably, the LSL hens' response to the LP diet activated pathways related to "ribosomal protein synthesis" and "regulation of cellular signaling cascades". Conversely, the LB hens' response to LP diets was contingent on Ca supply [Reyer, 2021a]. More so, the adaptive Ca metabolism actions exhibited by the LSL hens via a plethora of regulatory genes involved in ‘Ca-ion binding and transport’, ‘Ca release-activated Ca channels’ might allow better coping mechanisms to Ca depletions than the LB strain [Reyer, 2021a]. The LSL hens have also been reported to have higher eggshell weight compared to the LB hens, a trait possibly attributable to their corresponding efficient utilization and metabolism Ca [Sommerfeld, 2020b]. Thus, taken together, it is conceivable to opine that the LB and LSL hens may have unique dietary needs that should be considered.

Immune-related pathways, such as the 'intestinal immune network for IgA production' (KEGG) and 'IL-4 signaling' (IPA), were identified in the LSL post DEGs-derived comparisons between the birds fed the Con and LP diets [Reyer, 2021a]. Similarly, reduced dietary P was reported to enhance immune parameters such as B cells in the blood and IgA concentrations in bile in both laying hen strains, with a comparatively higher level in the LSL hens compared LB [Hofmann, 2021]. Furthermore, transcriptomic analyses of dietary treatments in the LB hen strain in study 2 revealed increased expression of genes involved in ‘Focal adhesion’ and ‘GP6 signaling’ in the LCaP diet compared to LP-fed laying hens [Reyer, 2021a]. The GP6 proteins are central signaling receptors in collagen formation and function, alongside fibronectins, integrins, laminins, and tenascins, constituting components of the extracellular matrix (ECM) [Simon-

Assmann, 1995; Beaulieu, 1997]. In conclusion, the transcriptomic response of the LB and LSL hens to the experimental diets investigated in study 2 suggests that the birds are capable of adapting to dietary changes via the recruitment of endogenous mechanisms.

3.4 Intestinal and renal mineral absorption in laying hens and broiler chickens

Jejunal and renal transcellular transport of P was examined in broiler chickens in response to a depleted dietary P regimen in study 3. Elsewhere, transcriptional investigation into the ileal transcellular transport of minerals (P and Ca) in laying hens in response to the varied supply of dietary levels of Ca and P was reported [Sommerfeld, 2020b]. Comparatively, the ileum was the preferred tissue choice to access insights into the transcellular mineral uptake in [Sommerfeld, 2020b] due to its extensive retention time of ingested feed compared to those of the duodenum and jejunum, coupled with the goals of harnessing insights into strain and dietary factors influencing mechanisms of phytate degradation and its disappearance within the small intestine of both hen strains.

3.4.1 Ileal transcellular mineral (P, Ca) transport in laying hens

The transcriptional investigation of the ileal transcellular transport of minerals (P and Ca) in two laying hens strains fed either of the four experimental diets (P+Ca+, P− Ca+, P+Ca−, and P− Ca−) selected three mineral cotransporters each for P (*SLC20A1, SLC20A2*, *SLC34A2*) and Ca (*ATP2B1*, *CALB1*, *NCX1*), alongside the *Gallus gallus GAPDH* and *ACTB* genes for housekeeping [Sommerfeld, 2020b]. Findings on genes encoding transcellular P transport (*SLC20A1* and *SLC34A2*) in the ileum of laying hens were higher in the LB hens compared to LSL (LB > LSL; FC= 1.60 and 1.34, respectively; $p \leq 0.007$) [Sommerfeld, 2020b]. Genes encoding transcellular Ca transport *ATP2B1* ($p = 0.077$) and *NCX1* ($p = 0.083$) had 24% higher and 25% lower ileal expressions in the LB hens compared to the LSL [Sommerfeld, 2020b]. However, *CALB1* expression was higher in LSL fed the P− diet compared to LB hens receiving the same. More so, the higher dietary P level led to a higher P intake and a higher P excretion. It is conceivable that the utilization of P might not be influenced by the dietary P level, considering the complex stoichiometric balance between P and Ca in the laying hen [Sinclair-Black, 2023]. It also stands to reason that higher levels of Ca in the diet might have led to the decreased intake and utilization of P, as evidenced by the numerically lower mRNA copy numbers of *SLC34A2* in hens fed diets with higher Ca levels compared to those fed lower levels. Elsewhere, dietary Ca levels have been reported to impact the ileal phytate P degradation. Tamin *et al.* (2004) reported that using two dietary Ca levels (0 or 0.5% of inclusion), 69% and 25.4% degradation of ileal phytate, respectively, was observed without the addition of phytase. However, on the supplementation of 500 FTU/kg of 3-phytase *Aspergillus ficuum* or 6-phytase

Peniophora lycii phytase, the degradation of ileal phytate increased to 79.5% and 76.2% in diets with no Ca and to 58.9% and 44.9% in diets with 0.5% Ca. In this context, it is inferable that the lower P intake associated with the general lower feed intake might be due to the higher dietary Ca concentration. However, it is noteworthy that the P excretion was not affected by Ca, which implies that, although less P reached the small intestine, the same amount was excreted, possibly due to excess Ca (Ca+ diet treatment) that might have complexed with P hampering the absorption, hence, leading to excretion. Conclusively, the gene expression profiles of transcellular mineral transporters revealed a vivid strain-specific expression in response to the varying dietary minerals [Sommerfeld, 2020b]

3.4.2 Jejunal and renal transcellular mineral P transport in broiler chickens

Similarly, in order to access the roles of the jejunum and kidney, four transcellular P co-transporters namely; *SLC20A1, SLC20A2, SLC34A1* and *SLC34A2*, were investigated in the jejunum and kidneys of broiler chickens at different developmental stages under the different P diet regimen. During the early growth phase, broilers were fed one of three experimental diets containing different levels of dietary P (L, M and H). Subsequently, a low dietary P level was supplied at the grower and finisher stages [Omotoso, 2023]. The application of a P depletion strategy due to environmental concerns was intended to realize an improved mineral utilization of the broiler chicken via the optimization of intestinal P absorption, bone retention, and reabsorption by the kidney. In order to access the roles of the jejunum and kidney in this context, four transcellular P co-transporters namely; *SLC20A1*, *SLC20A2*, *SLC34A1* and *SLC34A2,* were investigated in the jejunum and kidneys of broiler chickens at different developmental stages under the different P diet regimen.

Findings on the jejunal and renal mRNA transcript abundance of P co-transporters revealed a significant difference on day 17, with an increased jejunal expression of *SLC34A2* (L>H; FC= 2.27) observed in L-fed broilers compared to those fed the H diet. Similarly, renal *SLC20A2* transcripts differed between broilers fed the H diet compared to those fed the L diet (L<H; FC = 1.96) and those fed the M diet (M<H; FC = 1.99) [Omotoso, 2023]. Renal *SLC34A1* mRNA abundances differed significantly between broilers fed the L and M diet (L>M; FC = 2.04). Furthermore, renal *SLC34A2* mRNA abundance increased (L<H; FC = 1.96) in the animals fed H compared to those fed the L diet. On days 24 and 37, the expression of jejunal and renal sodium-phosphate co-transporter remained unaffected between the dietary groups [Omotoso, 2023].

In fact, the gene expression profiling of P transport in broilers fed varied P diets revealed a vivid tissue-specific (jejunum, *SLC34A2* and kidney; *SLC34A1*) expression. Transcriptionally,

SLC34A2 plays an essential role in the transcellular P transport mechanism within the intestine of rodents [Sabbagh, 2009], humans [Xu, 1999] and broiler chickens [Omotoso, 2023]. However, lower mRNA expression of *SLC34A2* has been reported in pigs [Wubuli, 2019]. The jejunal *SLC34A2* response to the depleted P strategy suggests that intestinal P availability in the diets elicited the abundance of sodium/phosphate co-transporters in jejunal cells to maintain homeostasis and adaptation at the earlier growth phase [Hu, 2018].

Comparatively, the appreciable abundance of *SLC34A2* mRNA transcripts in the ileum of laying hens and the jejunum of broiler chickens [Sommerfeld, 2020b; Omotoso, 2023], validate its relevance in facilitating transcellular P transport in the intestine of the domestic fowl. Several studies have highlighted an increased expression of genes encoding transcellular P transport in the small intestine of both broiler chickens [Proszkowiec-Weglarz, 2019; Hu, 2018; Rousseau, 2016; Li, 2012] and laying hens [Wang, 2022; Sommerfeld, 2020b; Li, 2018] on exposure to deficient P diets, implicating a transcriptional response to luminal P concentrations in conjunction with endocrinal factors, such as calcitriol.

In addition, the renal-specific expression of the *SLC34A1* gene in the L-fed broilers at day 17 suggests a transcriptional regulatory role in the reabsorption of P at the proximal tubule to promote P homeostasis. Moreover, a higher abundance of renal type III sodium/phosphate co-transporters was observed in broilers of the H group compared to L and M (*SLC20A2*) and M group (*SLC20A1*), respectively. Previous studies on murine models identified responses of the type III sodium/phosphate co-transporters to changes in dietary P contents [Candeal, 2017; Marks, 2019]. However, their precise contribution to the poultry's P regulation and homeostasis remains unclear [Marks, 2019].

Indeed, the transcellular pathway seemed to be the preferential intestinal P absorption route under dietary P restrictions [Marks, 2019]. This is inferable, given the comprehensive expression and response of the birds through jejunal and renal routes under dietary P deficit conditions, which severely impacted the birds at the early (day 17) development stages. Hence, reducing P supply in early life had limited success in conditioning for a mineral prudent phenotype with high P efficiency in later life [Omotoso, 2023]. As a result, a practical implementation of a P depletion must meticulously consider the timing, duration, and extent of the P depletion strategy in broiler chickens. Thus, it is recommended that early-life conditioning strategies for mineral efficiency (e.g., P depletion) in the broiler should be initiated no earlier than the early grower phase of development when the birds are more tolerant [Omotoso, 2023].

3.5 Osteo-physiological response of the domestic fowl to varied dietary minerals

In order to assess the role of the bone in the compensatory homeostatic mechanism explored by the broiler chickens under the dietary P depletion strategy, osteo-physiological parameters, including the bone-breaking strength, length, diameter, weight and ash, were analysed. Findings on the osteo-physiological response of the birds highlighted significant differences in the bone force (breaking strength), weight, length, diameter, and ash between broilers fed the low P diet compared to those fed the M and H diet at day 17. Specifically, bone force differed significantly between broilers fed the depleted P and those fed the recommended at day 24 and between the recommended and high P groups at day 37. The femora bone diameter differed significantly between broilers fed the depleted P (MLL) and the MMM and HHH. Values for bone weight, length, and ash remained unaffected. In this context, it is conceivable that osteo-physiological responses exhibited by the broiler chickens fed the L diet compared to those fed M and H, was aggravated by age and the reduced pool of P within the organismal biosystem, hence the severity in response, which was highest at the earlier developmental phase.

Physiologically, the lowered bone mineralization observed at the earlier developmental phase might be attributable to either a reduced P availability needed to drive bone ossification processes [Shao, 2019; Taylor, 2013] or increased bone mobilization processes from the bone to meet other physiological processes [Li, 2020]. This is inferred from the fact that the bone accounts for about 80% of total body P [De Groote, 1997], and is stored in the Ca-complex form of hydroxyapatite $Ca_{10}(PO_4)_6(OH)_2$, crucial for musculoskeletal development [Penido, 2012].

Broadly, the optimal development of skeletal structure in the domestic fowl is crucial for their overall productivity and welfare. However, dietary mineral fluctuations such as over- or under-supply of dietary P and Ca demand of the bird have a significant impact on bone traits [Driver, 2006], leading to fractures and other bone-related abnormalities such as hypophosphatemic rickets, osteomalacia, osteoporosis. Bone fractures, including keel, femur or tibia, are a significant welfare problem in laying hens and broiler chicken farming, respectively. In the laying hens, the incidence of keel fracture is predominant and often associated with the extensive exogenous resorption of the hens bone for eggshell calcification processes [Toscano, 2020], which, when unreplenished exogenously through diets, increases the brittleness of the bone resulting in a fracture as the bird interacts with its housing environments, e.g., cages [Toscano, 2020]. Therefore, research-related efforts towards deciphering precise dietary mineral balance, stock improvements, housing and environmental enrichment [Rufener, 2020;

Riber, 2018] and early-life conditioning strategies [Valable, 2018] need to be adopted as potential preventive strategies for bone fractures in the domestic fowl.

3.6 Response of the broiler's jejunal microbiota to varied dietary P

The experiment reported in study 4 consolidated the previous research presented in study 3, forming a comprehensive continuum of investigation. The study aimed at identifying the functional contributory role of the jejunal microbial composition to the compensatory mechanism for mineral homeostasis and efficiency adopted by broilers subjected to a P depletion strategy at the early developmental phase. The study commenced with birds fed a medium or high P supply in the first weeks of life, followed by a depletion strategy applied at growth intervals from d 17-24 and d 25-37 to investigate the effect on the jejunal microbiota community. The mineral P and Ca intake and their corresponding fecal levels were also analysed.

Notably, it was crucial to integrate this research interface because the intestinal microbiota contains specific phosphatase-secreting microbes such as the *Bifidobacteria* [Haros, 2005] and isolates of *Lactobacillus* [Kim, 2007], which are capable of hydrolyzing phytate and release inorganic P to the host for absorption. Moreover, the dietary supply of macro-minerals such as P and Ca to the monogastric species has been reported to modulate the gut microbiota [Ptak, 2015], thus indicating the microbiota as a potent, functional entity involved in nutrient metabolism [Grice, 2012].

3.6.1 Effect of mineral P and Ca intake on the resultant fecal mineral levels

Broadly, the resultant fecal P levels mirrored the dietary P intake in the depleted, recommended, and high P feeding groups, suggesting that the feed formulations have been effective. Previous studies in the broilers [Rama Rao, 2006] have also reported similar complementary relationships between high dietary minerals intake and their resultant fecal excrement and vice versa.

Interestingly, at day 24, fecal Ca levels of broilers in the depleted P groups differed significantly from those in the non-depleted groups (HL>HH). At the same time, total fecal Ca levels were unaffected by the diets at d 17 and 37. In addition, results from broiler chickens fed reduced inorganic P showed increased serum calcitriol levels in study 3, alongside increased intestinal Ca-binding protein levels leading to improved Ca absorption reported elsewhere [Wasserman, 1992; Friedlander, 1977].

Taken together, the analyses revealed evidence of possible increased bone resorption in the depleted broiler chickens compared with the non-depleted groups. This could indicate increased mineral mobilization of P from the bone to meet physiological demands in the depleted P group

and, as a result, impede further absorption and retaining of Ca pool within the organism in efforts to maintain stoichiometric mineral P and Ca balance, hence the increased excretion of Ca. Furthermore, a comprehensive review of the current recommendations for total Ca content in broiler feed formulations concluded that dietary Ca might be overestimated [David, 2023], affecting P absorption rates [Selle, 2009]. Notably, higher P excretion rates due to mineral P supplements above recommendations have also been demonstrated in other monogastric species, such as pigs [Reyer, 2021b], with no added benefit observed for bone mineralization [Gerlinger, 2021]. Results in this study indicate that broilers fed the high P diet received mineral fractions that exceeded their metabolic demands for growth or maintenance with no additional benefit for the measured traits but resulted in unnecessary fecal losses, as reported elsewhere [Li and Bryden, 2017].

Therefore, a well-tailored depleted/reduced P strategy remains a powerful tool for limiting the P content of manure under consideration of Ca:nPP ratios [Knowlton, 2004] while strengthening the bird's P resource allocation and efficiency.

3.6.2 Effect of mineral P and Ca intake on the resultant fecal phytate levels

At d 17, the analysis of fecal phytate revealed a significantly higher level in H animals compared to M animals. At d 24 and d 37, the P depletion groups ML, HL, MLL, and HLL showed lower fecal phytate levels compared to the non-depleted groups HH and HHH but did not differ significantly from groups MM and MMM. The significantly lowered phytate concentrations in the feces of birds fed medium P than in birds fed high P levels at d 17 and d 24 suggests the hydrolysis of phosphoric ester forms, mediated by a phytase secreted by the broiler’s enteral microbiota. The P-depleted groups exhibited a nearly maximal phytate degradation at d 24 and d 37, further validating the domestic fowl’s intestinal phytate degradation capacity to meet metabolic P demand [Ingelmann, 2019].

3.6.3 Relative Abundance of broiler chickens jejunal microbiota fed varied P diets

In the current study, the overall microbial diversity represented by alpha diversity indices revealed no alterations based on dietary P depletion. Based on the microbial dissimilarity analysis, an age-dependent separation of profiles was observed. Accordingly, previous studies have reported the apparent effects of age on the microbial community that colonizes the broiler's GIT [Zhou, 2021; De Cesare, 2019]. Moreover, the dominance of the *Lactobacillus,* as highlighted by the taxonomic plot, was consistent with several previously reported studies [Künzel, 2021; Kers, 2018; Borda-Molina, 2016], where *Lactobacillus* presence in the gut accounted for the majority of the microbiota fraction. As a result, *Lactobacillus* can be referred to as the “core microbiota” in the present study. The threshold for defining a microbial taxa as

the core microbiota hinges on the predominant abundance compared other taxa within the microbial community. For example; Ngunjiri *et al.* (2019) defined core microbiota taxa in their study at maximum coverage 75%, Clavijo *et al*. (2022) at 50% coverage while Roth *et al.* (2022) set the detection limit to classify a taxa as a core member at 97% of the total sample number.

Functionally, the abundance of *Lactobacillus,* a known probiotic in the gut, has been positively correlated with beneficial functions, influencing the gut in one or several ways, including the improvement of gut physiology, structure, integrity and function [Khan, 2020], as well as increased body weight gain in the chicken [Zhang, 2022; Lokapirnasari, 2019]. More so, it is inferable that the prevalence of *Lactobacillus* might indicate a low complexity of jejunal microbiota in broiler chickens. Given the response of the broilers' jejunal gut microbiota response to depleted P diets at day 24, it is inferable that the shift in microbiota community made only a subtle contribution to the birds' adaptive mechanism towards maintaining P homeostasis but responded more based on the availability or scarcity of the macro-mineral. Furthermore, a recent study that reported on active core microbiota of two high-yielding layer strains fed dietary Ca and P 20% lower than recommended indicated that reduced dietary Ca and P supplementation had a minor effect on the microbiota compared to the strong influence of the bird's genetic background [Roth, 2022].

Conversely, at d 37, an incremental shift in the gut of broilers fed the HLL diet compared to those that received HHH was observed for unclassified genera belonging to families *Lachnospiraceae* and *Ruminococcaceae*. Both *Lachnospiraceae* and *Ruminococcaceae* microbiota families have been reported as beneficial in the human GIT, implicated in the fermentation of carbohydrates [Duncan, 2007], coupled with the degradation of resistant polysaccharides, e.g., starch and cellulose, facilitating digestion of plant-based diets [Collier, 2008]. The identified taxa may be of interest in further studies to reshape the microbial composition for improved nutrient utilization from dietary P sources.

In contrast to the microbes whose abundance increased following the P depletion diet at day 24, *Brachybacterium*, *Brevibacterium*, and genera of the *Staphylococcaceae* family were observed to significantly increase in abundances in the jejunum of non-depleted P groups compared to the depleted, which suggests that these microbial genera rely on a P enriched intestinal environment to proliferate and possibly their disappearance under P scare enteral milieu. For example, several species of *Brevibacterium* were described as phosphate-accumulating probiotics, which might be more prevalent in high P supply [Anand, 2019]. The results suggest that the increased proliferation of these mentioned microbial taxa under the high dietary P supply could be a biomarker for excessive P intake in commercial broiler chickens.

Furthermore, the current microbial profiles in this study may support the hypothesis that an increase in mammalian intestinal P levels stimulates microbial short-chain fatty acid (SCFA) production [Heyer, 2015]. This also agrees with a study in broilers in which low P and low Ca (P-Ca-) diets resulted in a decrease in SCFA (DL-lactate and acetic acid) in the ileum, and subsequently, an increase in these parameters was observed after phytase supplementation [Ptak, 2015]. In addition, a recent study in chickens reported that dietary P deficiency decreased SCFA production due to reduced cellulose fermentation, which suggests intestinal P content modulates the abundance of fibrolytic bacteria [Li, 2022].

A firm body of evidence exists that diet is a potent factor driving the gut microbiota's structure, abundance and diversity. Recently, nutritionists have made an effort to manage and nurture the benefits of the gut microbiota, including promoting a healthy gut environment via the applicability of probiotics, prebiotics and synbiotics in monogastric livestock nutrition [Pourakbari, 2021]. Probiotics are living microorganisms that modulate the composition and diversity of host gut microbes [Pourakbari, 2021; Martin, 2019], increasing the abundance of beneficial bacteria such as *Lactobacillus, Bifidobacterium.* Prebiotics are non-digestible feed ingredients that selectively stimulate the growth and activity of beneficial bacteria in the gut [Hill, 2014]. Taken together, pre and probiotics form synbiotics, which aid the collective beneficial goals of both practices, including improving gut development and immune function fortification, enabling optimal overall health and performance of the bird. Deeper insights into the interactions between probiotics, prebiotics, synbiotics and the existing gut microbiota for phytate degradation and P and Ca resource allocation and efficiency would be of value in poultry livestock farming.

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4 General Conclusion

The laying hens and broiler chicken both exhibited sophisticated interactive network of intrinsic homeostatic mechanisms to maintain P balance. These mechanisms involve transcriptional (e.g., differential transcript abundance and molecular pathways enrichment), endocrinal (hormone and metabolites dynamics) and gut microbial (shift of microbioat composition) responses accessed in a plethora of tissues/organs such as the jejunum, ileum, kidney and the bone for mineral P and Ca balance.

The endocrinal and transcriptional responses elicited in the laying hens confirm an age-dependent, strain-specific response with respect to mineral homeostasis and efficiency. The longitutinal assessment of endocrinal determinants for mineral homeostasis dynamics in the laying hens revealed the most conspicuous shift at the transitional phase from the pre-laying (week 16) to the onset of lay (week 24), chiefly mediated by calcitriol (bioactive form of vitamin D), and PTH, via a feedback loop mechanism. Notably, the contributory effect of oestradiol (E2) and triiodothyronine (T_3) played a crucial role in the attainment of sexual maturity by the layers, which is associated with the shift in dietary Ca demand at the onset of egg production, proving to be the most significant developmental phase in the entire production cycle in the laying hens.

The transitional phase from the pre-lay (week 16) to the onset of lay (week 24) was the most important developmental phase of the laying hens, evidenced by the highest number of DEGs, which modulated different molecular pathways to define their respective strain-specific phenotypes. Strain-specific metabolic attributes were identified between the LB and LSL hen strains regarding mineral utilization and phytate degradation. LSL hens exhibited a higher capacity for phytate degradation, whereas LB hens utilize an active transcellular mineral transport mechanism. Furthermore, there was noticeable difference in calcium-dependent performance, with LSL hens producing higher quality eggs in terms of weight, while LB hens exhibit greater bone mineralization and turnover. These results suggest a distinctive mineral and vitamin D metabolism, which was further reflected at the transcriptional level. These findings present promising avenues for further research into mineral efficiency and resource allocation-related studies for optimal performance and welfare optimisation in the laying hen.

Broiler chickens exhibited a robust response to depleted dietary P levels at various stages of development via the synergistic interplay of determinants, comprising hormonal as well as osteophysiological responses evident in the variable calcitriol levels and bone mineralisation and integrity parameters. Overall, broiler chickens elicited a tissue-specific transcellular

transporters expression, i.e. jejunal (*SLC34A2*) and renal (*SLC34A1*) essential to facilitate active intestinal absorption and renal re-absorption of P at a most critical growth phase (early) in response to P depletion. These endogenous responses (endocrinal, transcriptional and bone) aided the birds in compensating for the dietary P deficit in the early productive life, optimizing mineral efficiency to facilitate adaptation in the later productive life.

The input of the gut microbiota in the compensatory adaptive mechanism in the broiler chickens in this study was subtle in groups fed dietary P depletion compared to their non-depleted cohorts. There are no additional benefits for excess P supplementation in commercial broiler production, as the dietary P supply, P intake, and fecal P content were parallel. However, the observation of increased proliferation of microbial taxa in the jejunum of broilers fed the surplus P diets might present potential microbial biomarkers for excessive P supply in commercial broiler production.

The use of low mineral P supplementation in poultry nutrition has multiple benefits for the animal, humans, and the environment. Further research should be conducted with emphasis on the timing of initiating the depletion, the length of depletion, and age/strain-specific requirements of the bird to achieve maximal homeostasis for efficiency and reduced environmental burden of the mineral. To optimize the metabolic and immunological benefits of the gut microbiota, concise application of pro, pre, and synbiotic techniques to gut health and functionality is necessary.

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6 Appendix

6.1 Jejunal transcriptomic profiling of two-layer strains throughout the entire production period

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Jejunal transcriptomic profiling of two layer strains throughout the entire production period

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The jejunum plays crucial roles for the digestion and absorption of nutrients and minerals and for barrier functions that are essential for a healthy, productive life cycle of farm animals, including laying hens. Accordingly, knowledge of the molecular pathways that emerge in the intestine during development, and particularly at the beginning of laying activity, will help to derive strategies for improving nutrient eficiency in laying hens. In this study, jejunal samples were obtained from two high-yielding layer strains at five developmental stages (weeks 10, 16, 24, 30 and 60 of life) for RNA-sequencing, alongside the profiling of blood plasma parameters to approximate the dynamics of mineral homeostasis. The results reflected a marked distinction between the pre-laying and laying phase as inferred from levels of parathyroid hormone, triiodothyronine, estradiol, vitamin D, and calcium. Moreover, the expression patterns of the intestinal mucosa responded directly to the changing metabolic and nutritional profiles at the beginning of the laying phase in maturing high-yielding strains of laying hens. These comprise signaling events namely RANK/RANKL signaling and cellular senescence. Taken together, the timing of sexual maturity of laying hens demands closer examination to unravel metabolic requirements and associated endogenous mechanisms.

Laying hens provide an affordable, safe and high-quality animal protein source in the form of eggs required to meet the nutritional demands of the growing human population which currently stands at 7.7 billion[1]. The global annual egg production is estimated at 76 million tonnes[2,3], showing a trend of the continuous increase in the number of layers, achieved mainly by populous countries such as China and India in an attempt to mitigate food insecurity associated challenges[4]. The European Union accounts for the second-largest share of world egg production after China, with an estimated number of over 400 million laying hens. Animals of the Lohmann Brown (LB) and Lohmann Selected Leghorn (LSL) strains are widely used across husbandry systems[5,6]. Both LB and LSL strains have been improved for egg production performance and have been extensively monitored at the levels of bone quality, egg quality and behaviour[7]. Their egg production performance is approximately identical, however, LB and LSL layer strains significantly differ in gene expression profiles of cerebrum, egg qual-ity parameters (egg and eggshell weights), mineral metabolism (bone-breaking strength, phytate degradation, trans- and paracellular transport), and immune responsiveness[8–12]. Importantly, the attainment of sexual maturity in pullets (~18 weeks) through to the onset of laying (~24 weeks) represents a significant physiological shift within the layers' metabolic demand. More so, this developmental phase encompasses the cumulative inputs and interconnectivity of different biological factors spanning nutrients and mineral metabolism, neuro-endocrinal complexes, hepatic, skeletal and the immune systems[13–15]. The small intestine, specifically the jejunum is tasked with the vital role of nutrient and mineral absorption (e.g., glucose, calcium, phosphorus), amongst other regulatory and crucial functions such as, barrier integrity, immune defense, lipid metabolism and endocrinal functions all of which ultimately contribute to the overall health and stability in production performance of the hens[16,17].

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Furthermore, the developmental transition of the layers from pullets to growers (pre-layers) to layers (onset of lay), its peak in egg production and the senescence are strongly mediated intrinsically by the temporal expression of gene transcripts[18,19], supported by the dynamics of the endocrine status and their interaction with the environment to depict these physiological outcomes. Thus, the dietary regimen for layers is adjusted to meet requirements for the respective production stages, e.g. higher dietary calcium at the onset of laying (3.5–4.5% in dry matter) compared to grower phase (0.9–1.2% in dry matter) and pre-layer phase (2.0–2.5% in dry matter)[20], albeit this recommendation might be outdated and in need of a scientific re-evaluation[9,21].

Transcriptomics, a current genomic appraisal method widely employed in the study of several species populations, laying hens inclusive, provides relative ease in the detection of differential gene expression. Thus, its use as a genomic appraisal tool is quite significant. Transcriptomic studies have been conducted with the Lohmann layers to uncover temporal differential gene expression patterns in oviduct development and defense in pre-laying and laying hens[22]. Conversely, transcriptomic insight into the developmental process in laying hens through the enteral routes (jejunum) continues to be limited. Clearly, intrinsic mechanisms throughout the entire production period including the utilization of nutrients should be exploited. The multifaceted function and synergistic inclusion of the small intestine in various biological complexes are associated with development and maturity in the laying hens, coupled with the similarities in production and the different adaptive strategies adopted for mineral homeostasis, immune and bone traits. We hypothesize that knowledge of differentially expressed genes (DEGs) and molecular pathways related to development, growth and the onset of laying will contribute to further improvements in nutrient eficiency and productivity.

The present study investigated differentially abundant mRNA transcripts and enriched pathways in the jejunum of two-layer strains (LB and LSL). Jejunal samples were collected throughout the entire production period at weeks 10, 16, 24, 30 and 60 of life for high-throughput RNA sequencing, incorporating blood parameters to approximate the dynamics of mineral homeostasis.

Results

Blood plasma profiling. Plasma levels of calcium, magnesium and albumin showed production period-specific responses, which significantly increased along the developmental phases in both LB and LSL laying hens (Fig. 1, Table S1). The plasma levels of triiodothyronine (T3) and calcidiol (25OH-vitamin D3) were significantly lowered while levels of calcitriol (1,25 $(OH)_2$-vitamin D3) and estradiol (E2) were significantly elevated from the onset of laying at week 24. A significant reduction with subsequent re-adjustment of the inorganic P levels was observed at the onset of laying at week 24 in both LB and LSL strains. The PTH levels were significantly increased at week 24 in both LB and LSL strains compared to other time periods. Alkaline phosphatase activity (ALP) significantly differed between growing and senescent LSL hens, while no significant differences between consecutive time points was observed in LB hens. Regarding strain differences, levels of triiodothyronine were found to be significantly higher in LSL strain compared to LB hens at week 16. For ALP, the activity was significantly higher at week 10 in the LSL as compared to LB hens. At week 60, estradiol and calcidiol levels were significantly higher in LB hens compared to LSL hens, while calcitriol was significantly higher in LSL hens compared to the LB hen strain. Notably, calcidiol levels at week 30 were numerically increased in LB compared to LSL strains ($p = 0.051$).

Identification of differentially expressed genes (DEGs). The DEGs were obtained by comparing the expression of jejunal mRNA from LB and LSL layer strains independently for each of the five production stages. The integration of the resultant DEGs revealed unique sets of production-stage specific genes found to be differentially abundant between both strains (Fig. 2A). In particular, 220 DEGs were identified between LB and LSL hens at week 10, while 262, 877, 259 and 284 DEGs were identified between the LB and LSL hens at weeks 16, 24, 30 and 60 respectively.

The strain comparison at pre-layer stages (week 10 and 16) revealed a total of 43 and 50 DEGs (Table S2). At the onset of laying in week 24, a total of 601 unique DEGs were identified, whereas weeks 30 and 60 showed 33 and 52 stage-specific DEGs, respectively, between LB and LSL strains. Interestingly, as the laying hens developed through the production periods, a total of 82 genes were consistently differentially expressed between both strains (Table S3). These specific differentially expressed genes over all the developmental stages were molecularly implicated in the immune modulation (*HCK*, *MTURN*, *CD8A*, *GBP6*), nucleotide-binding and chromosomal maintenance (*WRAP53*, *CELF5*, *MMRN2*), barrier integrity/extracellular matrix (*TMIGD1*, *COL9A1*, *LRFN5*, *CRTAC1*), and complex lipid synthesis (*SLC27A5*). Temporal DEGs exhibited within LB and LSL laying hen strains across the five production stages (Fig. 2B) were also deciphered by the comparison of the jejunal mRNA expression with the highest number of DEGs between week 16 and 24 (Table S4). The overlap of DEGs analyzed during this period is 69.5% (3399 genes) between the two strains.

STEM, functional annotation and pathway enrichment analysis. Considering the 5 production stages as time series, a total of 13,676 and 13,921 genes were used to analyze the transcriptional patterns in LB and LSL hens, respectively. The STEM analysis highlighted 10 significant profiles in the LB layers strain and 8 significant profiles in LSL (Fig. 3). Profiles #9 and #41 were selected for detailed analyses due to their linear time-course expression patterns in relation to the overall experiment. To approximate transcriptional shifts related to altered metabolic demands at onset of laying, profile #18 was considered for detailed analyses. Genes included in profile #18 represent a considerable overlap with DEGs identified in the contrast between week 16 and 24 (Supplementary Fig. S1). Moreover, additional DEGs from the week 16 to 24 comparisons are assigned to profiles #9 and #41.

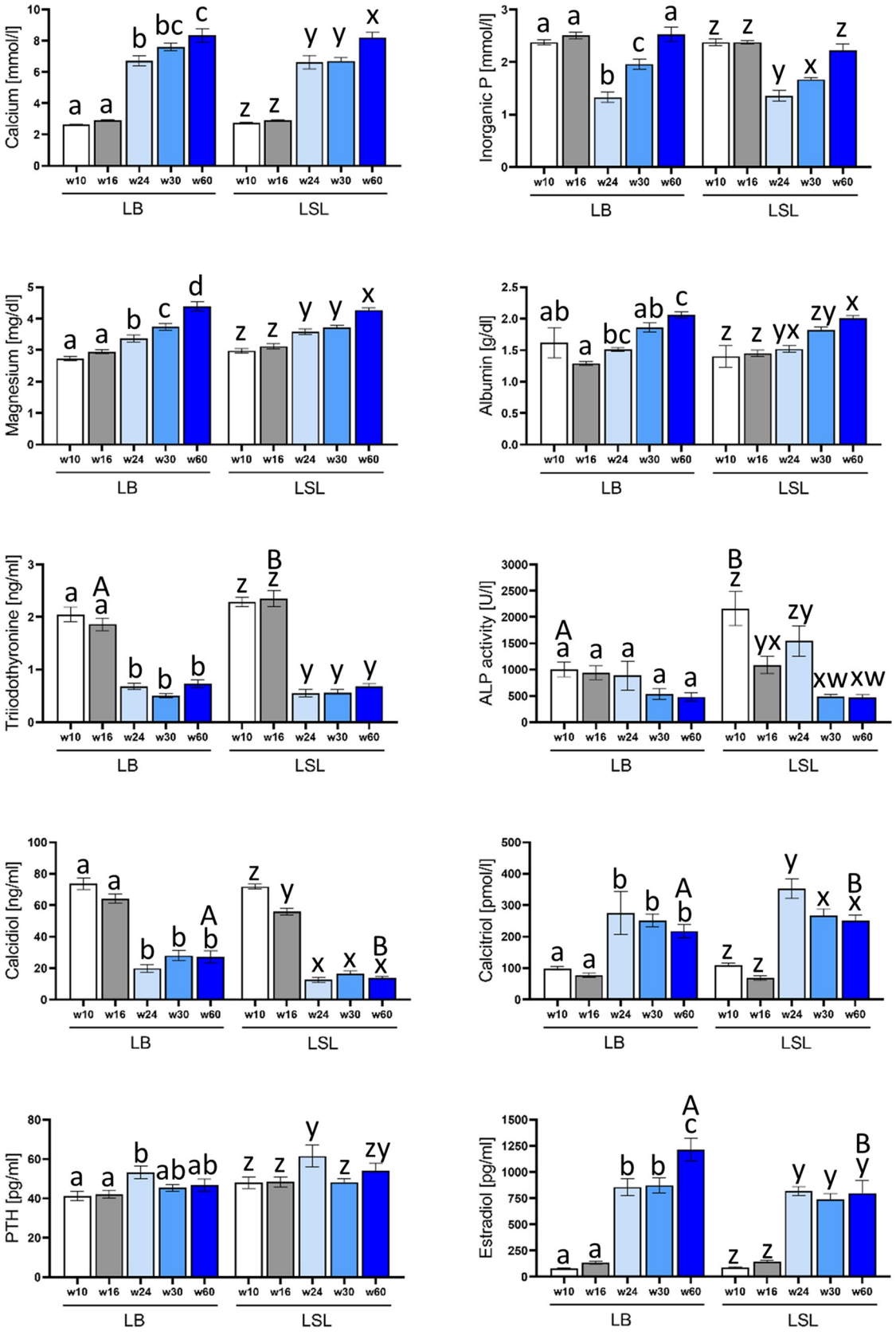

Figure 1. Plasma parameters referring to endogenous mechanisms to maintain mineral homeostasis with respect to the selected production stages (weeks 10, 16, 24, 30 and 60) of the LB and LSL laying hens. Values are displayed as means ± SE. Data for inorganic P and calcium were adopted from[9]. Superscripts indicate statistical significance ($P < 0.05$) between laying hen strains (capital letters) and production stages (small letters). PTH—parathyroid hormone; ALP—Alkaline phosphatase.

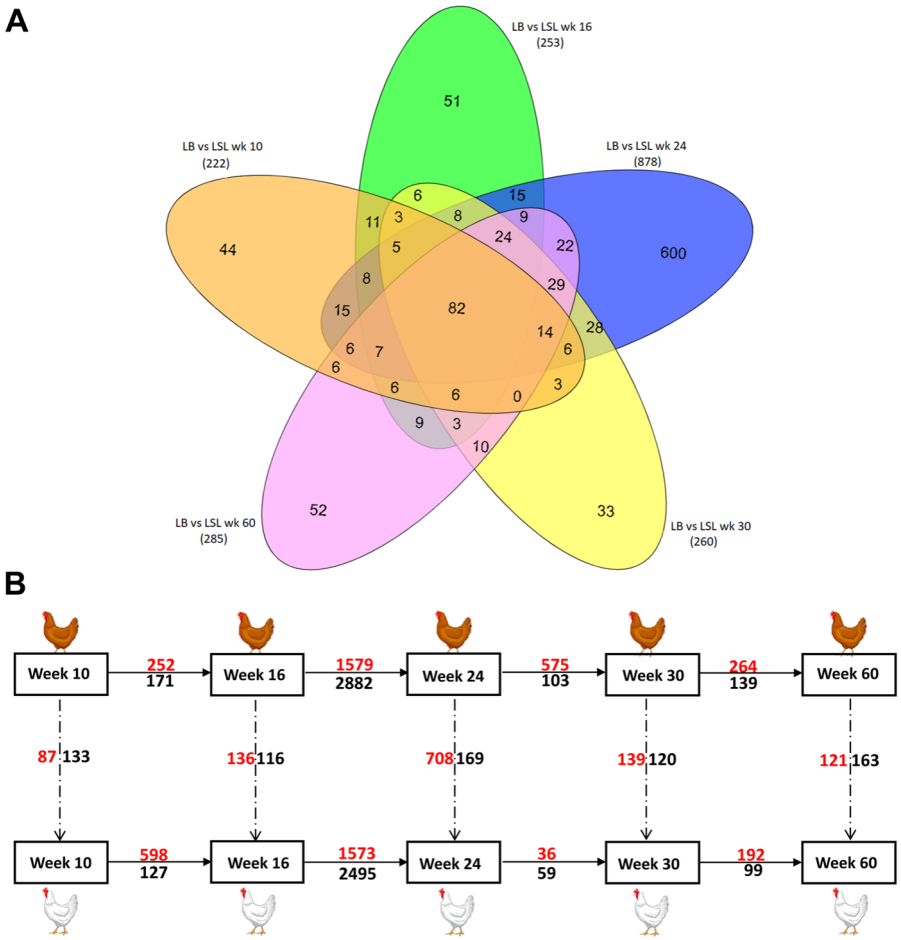

Figure 2. Number of DEGs derived from jejunal mucosa related to selected production stages in LB and LSL laying hens. (**A**) Venn diagram depicting the differentially expressed genes between LB and LSL for each production stage (week 10, 16, 24, 30 and 60) as well as their overlaps among different production stages. The total number of stage-specific DEGs is given in brackets. (**B**) Comparisons of DEGs within (horizontal) and between (vertical) each of the two laying hen strains across the selected production stages. Values in red and black represent numbers of upregulated and downregulated DEGs. LB—Lohmann Brown; LSL—Lohmann Selected Leghorn.

Selected expression data from the STEM profiles were submitted to IPA for functional annotation analysis (Fig. 4, Tables S5, S6). Interestingly, genes clustered in profile #9 in the LB (1631 DEGs) and LSL (1994 DEGs) strains were involved in the mitochondrial energy transduction processes over the production stages, whereas profile #41 comprised genes enriched in the cell-cycle and mitosis/DNA damage regulation checkpoint prior to division and differentiation. Profile #18 exhibited enrichment in RANK/RANKL signaling and cellular senescence in LB and LSL layers.

Discussion

The onset of egg production in the laying hen is preceded by a myriad of interconnected biological processes, which spans the endocrine secretion of hormones and feedback mechanisms among the target organ (ovary) and other organ systems (jejunum, bone, kidney, liver, parathyroid) to modulate nutrient and mineral utilization according to changing needs. The physiological shift into the onset of laying is characterized by an intensification of the calcium metabolism crucial for eggshell calcification[23], which is driven exogenously by the adequate supply of dietary nutrients and endogenously by the osteoclastic resorption of the medullary bone, which serves as a calcium reservoir[14,24,25]. Furthermore, alongside calcium, mineral P is equally important owing to its numerous physiologic functions in skeletal development, blood buffering, mineral metabolism, and energy signaling[25,26] which are pivotal for optimal production in the laying hen.

Dynamics of mineral homeostasis throughout the production period. There was a considerable increase in plasma calcium levels and a reduction in plasma P levels with the onset of laying in both strains. This reflects the increase in dietary calcium at week 24 compared to weeks 10 and 16 which hampers intestinal phytate degradation and mineral digestibility resulting in lower P uptake[9]. However, PTH was increased at week

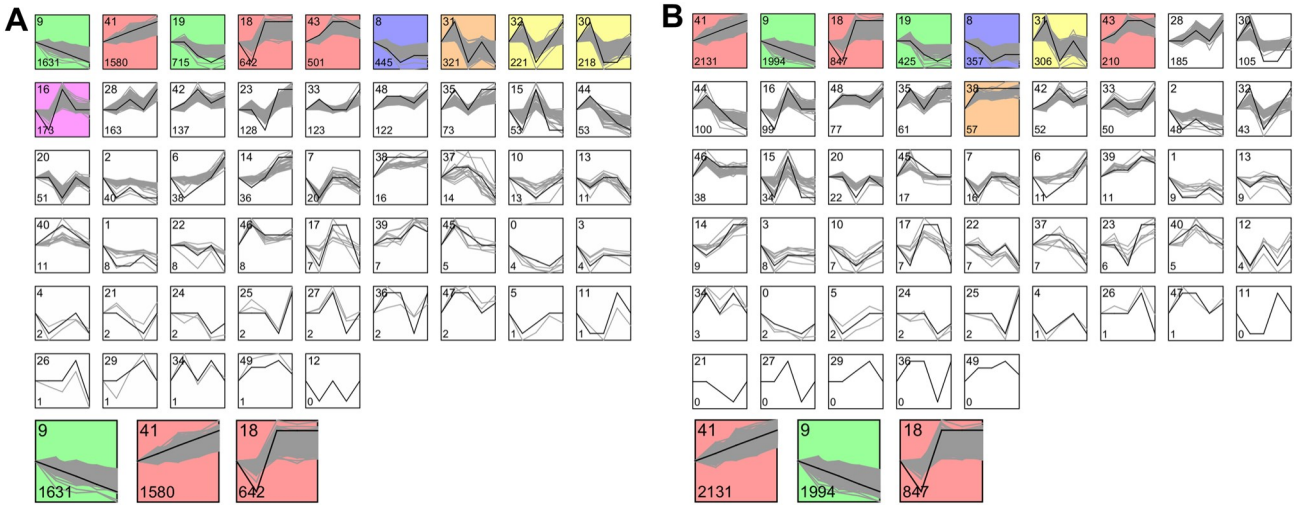

Figure 3. Time-series analysis of production stage-specific jejunal transcripts expressed in LB (**A**) and LSL (**B**). Expression patterns (grey lines) over the five selected production stages at week 10, 16, 24, 30 and 60 were clustered into profiles. Colors are assigned to only significant profiles ($p < 0.05$) and ordering is based on number of genes displayed in the lower left corner. The profile number is shown in the upper left corner. Highlighted profiles #9, #41, and #18 were selected for detailed analyses via IPA.

24 in both LB and LSL strains compared to pre-laying period. Indeed, PTH favors the endocrinal regulation of calcium homeostasis via activation of osteoclastic bone resorption and renal reabsorption of calcium[25,27]. Since the calcium content of the feed may not meet the immediate needs at the beginning of the laying period, an increase in PTH production triggers the mobilization of bone calcium reserves to match metabolic calcium requirements. However, intestinal capacity for mineral uptake is known to increase in laying hen at onset of laying[28].

Consequently, at the laying peak (around week 30) a re-increase in plasma P was observed in both LB and LSL layers, which suggests adapted intestinal fluxes. Interestingly, calcitriol levels were increased from week 24 compared to the pre-laying period. In physiological conditions, increased calcitriol levels prompt decreased PTH levels[29,30]. However, in this study, PTH levels from week 30 onwards were still relatively high. This can be explained by the need to prevent a calcium deficiency due to a competing calcium demand for eggshell production and the associated fluctuation in calcium[23,31–33]. These regulations account for dramatic change in bone metabolism at sexual maturity driven by endocrine secretion. Consequently, triiodothyronine levels were decreasing while estradiol levels were found to be increased in both laying hen strains to induce egg laying capacity while terminating somatic growth and development[34–36].

Estradiol, a most potent form of estrogen, is secreted principally by the ovaries of the hen and mediates the overall maturation and development of the female reproductive system. It has a regulatory role in the induction of vitellogenesis, the activation of yolk precursors in the liver[37] and contributes to the formation of the medullary bone[15]. In this context, the vitamin D system undergoes dramatic changes, which implicates the regulation of mineral homeostasis via bone remodeling and resorption. At week 24, calcidiol levels dropped whereas calcitriol levels increased compared to the pre-laying period in both strains. It is conceivable that the synthesized calcidiol from liver is deposited in the egg yolk as embryonic reservoir[38]. However, the calcitriol level in conjunction with increased estradiol level account for osteoblastic formation of medullary bone during the entire productive period[39] and thus provides a stock of mobilizable calcium. Notably, the consecutive increase on levels of plasma magnesium might counteract the very high calcium plasma levels and affect on blood viscosity[40]. The increasing albumin levels might account for egg production in both laying hen strains.

Regarding the observed strain effects, the levels of triiodothyronine were increased in LSL compared to LB laying hens at week 16. This might reflect compensatory response to the body growth since LSL hens have lower body weight compared to LB hens[9]. Furthermore, at week 60 the hen strains differed in plasma levels of estradiol (LB > LSL), calcidiol (LB > LSL), and calcitriol (LB < LSL). This reflects different strategies to ensure long-term metabolic demands. Beside the dietary shifts at the onset of laying, the endocrinal profiles clearly show a physiologic shift that leads to a pre-laying and an egg-laying period engaging a large number of organs including kidney, liver, bone, ovary, and jejunum.

Longitudinal evaluation of jejunal gene expression throughout the production period. Transcriptomically, the 82 genes expressed differentially between the LB and LSL hens, consistently over all five developmental stages (Fig. 2A), are connected to biological processes along immunity (*CD8A*, *GBP6*, *HCK*), extra cellular matrix formation (*COL9A1*, *CRTAC1*, *MMRN2*), and micro- and macronutrient utilization (*HFE*, *SLC27A5*). Interestingly, the transcript abundance of avidin encoding gene (*AVD*) is consistently higher in LB compared to LSL irrespective of production stage, which suggests strain-specific alterations of biotin levels[41]. It is conceivable that these strain-specific transcript abundances are due to the observed genetic differences between

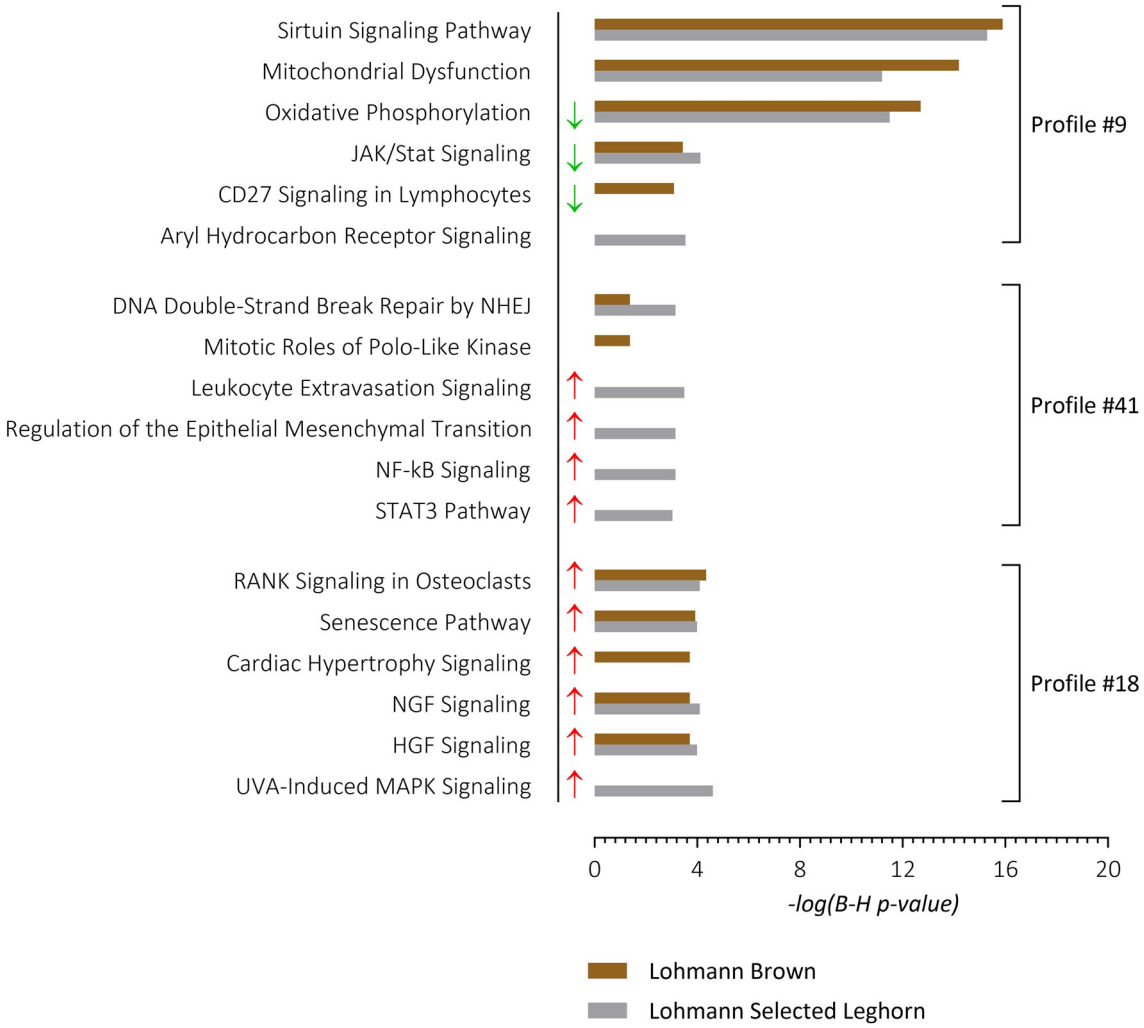

Figure 4. Top 5 canonical pathways predicted from selected STEM profiles in LB and LSL laying hens. Analyses comprise profiles #9 (consistently lower mRNA abundances with advancing production periods), #41 (consistently higher mRNA abundances with advancing production periods), and #18 (sustained increase in mRNA abundances from onset of laying). Arrows indicate significantly activated (red) and inhibited (green) pathways over the time course from week 10 to week 60 referring to the z-score. NHEJ—Non-homologous end joining.

LB and LSL laying hens that affect immune competence, e.g. resistance to endoparasite infection[42,43]. Additionally, the analyses of production stage-specific jejunal transcripts identified a number of genes within each strain that followed specific expression patterns (Fig. 3). The pathway analysis for the profiles #9, #41, and #18 for each strain over the developmental stages revealed enrichment in pathways, which spans mitochondrial energy transduction, cell-cycle regulation, DNA damage repair mechanisms and RANK/RANKL-induced immune modulation related to physiological growth and maturation (Fig. 4).

Genes assigned to profile #9 were gradually decreasing in expression throughout the production period. This profile highlighted overlapping pathways that encompass mitochondrial energy transduction and cellular growth processes in the LB and LSL layer strains, such as the sirtuin pathway, oxidative phosphorylation, mitochondria dysfunction, and JAK/STAT signaling. Members of the sirtuin family are nicotinamide dinucleotide (NAD+) dependent deacylases, of which *SIRT2*, *SIRT6* and *SIRT7* were enriched in profile #9. They are implicated in several molecular regulatory process e.g. cellular metabolism, energy metabolism, and cell survival. The sirtuin pathway enables effective adaptive response to metabolic, oxidative and genotoxic stress through metabolic homeostasis mechanism by acting as cellular sensors for energy abundance and modulating metabolic processes in conjunction with the mitochondria[44,45].

Mitochondrial dysfunction and oxidative phosphorylation enriched in profile #9 corroborates the mitochondrial theory of ageing. Enriched genes represented all five complexes of the mitochondrial electron transport chain. This is indicative of an overall decline of energy-dependent processes (e.g. intestinal cell renewal and proliferation processes) which were considered optimal at an earlier stage of production, but possibly experienced a reactive oxidative species (ROS) associated decline over time[46,47]. ROS play essential roles in proper oxygen sensing, maintenance of cellular redox state, cell signaling and the regulation of cell proliferation and differentiation at

lower concentrations[48–50]. However, the long-term accumulation of ROS with advancing age may result in the loss of the mitochondria integrity, functionality and ultimately dysfunction. Indeed, mitochondria are speculated to play a key role in delaying or accelerating the aging process especially in tissues with a high demand in energy[51].

The JAK/STAT signaling pathway has been reported to modulate the adaptive and innate immune component of layers' intestinal mucosal as well as epithelial repair and regeneration, via the activation of growth factors and cytokines[52]. In this regard, the transcription factor encoding genes *STAT2*, *STAT3*, *STAT5A* and *STAT6* were enriched in profile #9, indicating the transmission of effects at the level of gene expression represented by this pathway. The onset of laying in particular has been shown to have effects on the immune system as analyzed in the blood, spleen, and cecal tonsils of LB and LSL laying hens[12]. Moreover, corresponding analyses in the same individuals highlighted regulatory roles of miRNA within the JAK/STAT signaling[53]. The significant inhibition of the JAK/STAT signaling pathway and of oxidative phosphorylation over the developmental stages from week 10 to week 60 in both laying hen strains suggests a gradual shift in resource allocation from the initial modulation of cellular growth processes to the maintenance of the intestinal epithelium.

Genes allotted to profile #41 showed an increasing trend over the developmental stages whereby in both laying hen strains these mainly involve different molecular pathways, i.e. pathways related to cell cycle regulation and cell division in LB and pathways related to immunity and regulation of epithelial repair and regeneration in LSL. Specifically, pathways related to the innate immune system involving leukocytes and NF-κB, as the main regulator of innate immune responses, were shown to be activated in LSL with increasing age[54]. This buttresses the adaptive responses of the LSL strain via eficiency in paracellular transport and immune competence[10]. An overlap in predicted pathways of both hen strains was observed for DNA Double-Strand Break Repair by Non-Homologous End Joining, as evidenced by the clustered expression patterns of *ATM*, *DCLRE1C*, *LIG4*, *MRE11*, *PARP1* and *XRCC5*. This might reflect the accumulating number of senescent gut cells in both layer strains over the production stages and aging.

The expression profiles of genes assigned to profile #18 showed a considerable and sustained increase in expression with the beginning of the laying period in week 24. Most of the highlighted pathways based on this profile, including RANK signaling, senescence pathways, HGF and NGF signaling pathways, overlapped between the two laying hen strains. Due to the pattern, direct effects of dietary change or secondary effects of sexual maturity and the nutrient demand with the onset of lay are conceivable[55]. The direct dietary effects would be applicable to the enrichment of the cellular senescence pathways, which might occur due to the fourfold increase of calcium content in the diet and corresponding changes in gastrointestinal pH and microbiota.

Furthermore, RANK signaling has been associated with the gastrointestinal tract through its pro-immune activities within the epithelium, specifically, via the mediation of the development and differentiation of sentinel M cells present in the follicle-associated epithelium (FAE) which covers the gut-associated lymphoid tissues (GALT)[56–58]. In adaptation to the onset of egg laying, the endogenous release of calcium to meet production demand occurs under the collaborative actions of endocrinal pro-resorption factors such as calcitriol, PTH and estradiol, in conjunction with transcriptional modulation of the RANKL/RANK signaling pathway[59–62].

The RANK signaling pathway was predicted to be activated in both laying hen strains with an increase in the expression over the time course from week 10 to week 60, i.e., a steady low expression of pro-bone resorp-tion DEGs at the pre-lay stages, followed by a surge at the onset and peak of production, possibly due to the increased metabolic demands during production and, a plateau in the post-peak production stage, which is reflective of senescence. Additionally, the HGF and NGF signaling pathways were predicted to be activated in both hen strains, suggesting an increased gut-brain crosstalk for the attainment of enteric homeostasis over the production periods[62].

Materials and methods

Ethical statement. The animal experimentation was performed at the Agricultural Experiment Station of the University of Hohenheim, Germany, in accordance with relevant guidelines and regulations and approved by the Animal Welfare Committee of the University of Hohenheim. The experimental protocol is in strict compliance with the German Animal Welfare Legislation and approved by the Regierungspräsidium Tübingen, Germany (Project No.: HOH50/17TE) and in accordance with the ARRIVE guidelines.

Experimental chicken population and sample collection. Two strains of laying hens were used for this trial (Fig. 5). As described previously, LB (n = 50) and LSL (n = 50) laying hens were fed a corn-soybean based diet with recommended calcium levels[9]. The feed formulations covered starter, grower, pre-laying (PL), and laying diets (layer 1, layer 2, layer 3)[9]. In all formulations, plant-based phytases were minimized and exogenous phytases of microbial origin were not included[9]. Birds were sampled at weeks 10, 16, 24, 30 and 60 of life to cover relevant periods of the production cycle, i.e. pullets, pre-layer, onset of laying, peak of laying, and senescence. The sampling comprised ten birds per strain with the progeny of the same ten fathers per strain at each of the sampling stages. Following stunning, hens were sacrificed by exsanguination at 0900–1200 h. Plasma samples were prepared from trunk blood in heparin-containing tubes by centrifugation (10 min at 2500×g) and stored at − 80 °C until analysis. After removal of the gastrointestinal tract, a 2 cm jejunum samples were collected approximately 3 cm distal to the duodenal loop. The samples were cut open, the mucosa was thoroughly rinsed with a 0.9% NaCl solution and scraped for each bird over the respective production stages (LB, n = 50; LSL, n = 49). Samples were frozen on dry ice and stored at − 80 °C until RNA extraction.

Measurement of blood parameters. The levels of albumin, magnesium and alkaline phosphatase activ-ity were analysed in plasma samples using the Fuji DriChem 4000i commercial assays (FujiFilm, Minato, Japan). The calcium and phosphorus values of the same samples were determined photometrically as part of the previ-

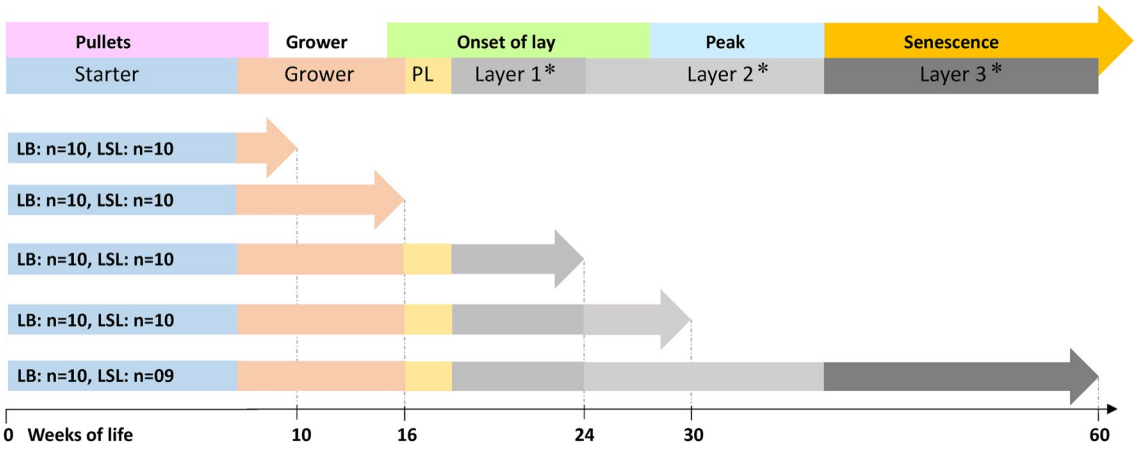

Figure 5. Dietary regimen for LB and LSL laying hen strains throughout the entire production period. Jejunal mucosa scrapings and plasma samples were retrieved at week 10, 16, 24, 30, and 60 to cover relevant production stages. Asterisks indicate a four-fold increase in the dietary calcium level to address the physiological demands from the onset of egg production. LB—Lohmann Brown; LSL—Lohmann Selected Leghorn; PL—pre-laying.

ous work[9] Hormones were measured in duplicate using commercially available enzyme-linked immunosorbent assays (ELISA). ELISA kits were used with strict adherence to the manufacturer's instructions for estradiol (EIA-2693, DRG, Marburg, Germany), 1,25(OH) vitamin D (AC-62F1, Immunodiagnostic Systems GmbH, Frankfurt am Main, Germany), triiodothyronine (EIA-4569, DRG, Marburg, Germany), parathyroid hormone (CSB-E118880Ch, CusaBio, Houston, USA) and 25(OH) vitamin D (EIA-5396, DRG, Marburg, Germany). For data analysis, a linear model was applied including the production stages, laying hen strains, hen father and slaughter order with the 'lm' function of the 'stats' package[63]. Pairwise comparison of means between experimental groups was achieved with the Tukey posthoc statistics embedded in 'stats' R package. Differences between hen strains and production stage were considered significant at $P<0.05$.

RNA extraction and sequencing. Total RNA was isolated with TRIzol Reagent (Invitrogen, Karlsruhe, Germany) from all 99 jejunal samples RNA was purified with the RNeasy Mini Spin kit including an additional DNase digestion (Qiagen, Hilden, Germany). The quantity and quality of final RNA were determined through spectrophotometry using the NanoDrop ND-2000 (Peqlab, Erlangen, Germany) and Bioanalyzer 2100 devices (Agilent Technologies, Waldbronn, Germany). RNA integrity numbers (RIN) were between 7.0 and 9.6. Sequencing libraries with a unique index for each sample were generated via stranded mRNA library preparation kit (Illumina, San Diego, CA, USA). Prior to sequencing, individual libraries were pooled. Paired-end sequencing was performed on a Illumina HiSeq 2500 device with 2×101 bp reads. Retrieved raw data were provided to the EMBL-EBI (www.ebi.ac.uk/arrayexpress) database (E-MTAB-9137).

Processing and analysis of gene expression analysis. Quality control and preprocessing of raw sequencing reads was performed using FastQC (version 0.11.7) and Trim Galore (version 0.5.0; https://www.bioinformatics.babraham.ac.uk/projects/). Low-quality reads (mean Q-score<20) and short length reads (<30 bp) were removed. The resulting reads were mapped to the chicken genome assembly (GRCg6a, Ensembl release 95) using Hisat2 (version 2.1.0; http://daehwankimlab.github.io/hisat2/). Read counts for each gene were summarized with HTseq (version 0.11.2)[64]. The average number of pair-end reads per jejunal sample was 20.0 ± 2.9 million. The entire dataset was checked for sample outliers using the arrayQualityMetrics package in R[65]. Subsequently, differentially expressed genes (DEGs) were retrieved via DESeq2 applying the in-build normalization method[66]. The count data were initially filtered to remove very low abundant transcripts and retain observations with 5 or more counts in at least 8 animals of the entire data set. For comparison of the two laying hen strains within each production stage, a base model to identify DEGs was performed using the DESeq2[66]. In order to identify DEGs in the contrasts of the production stage within each of the two strains an additional a statistical model was applied including hen father as a fixed effect. DEGs met the criteria of p-value<0.01 and |Log2FC|>1.5. Q-values were estimated to calculate the false positive rate<0.01[67]. Differentially expressed genes revealed in the contrast of the production stages between both layer strains LB and LSL were visualized using the InteractiVenn[68].

Gene clustering using short time-series expression miner (STEM). STEM, a java application suitable for the analysis of longitudinal gene expression data[69], was employed to gain insight into the temporal expression of genes via the comparison, clustering and visualization of expression patterns and their associated genes over the 5 production stages in the LB and LSL layer strains. Therefore, count-based data was transformed to regularized log values over all production stages for the two-layer strains. The median of individual values was generated per production stage and strain and submitted for the STEM analysis. The STEM clustering method was adopted with filtering threshold at a false discovery rate (FDR)<0.05[70].

Functional annotation and pathway enrichment analysis of DEGs. Initially, the online tool g:profiler was used to convert the chicken Ensembl IDs to human orthologue gene symbols (https://biit.cs.ut.ee/gprofiler/orth)[71]. Ingenuity Pathway Analysis (IPA, Qiagen Redwood City, www.qiagen.com/ingenuity) was used to further derive biological interpretation of the resultant profiles from STEM. Temporal differentially expressed genes clustered per profile, along with their corresponding base-mean values, gene symbols and fold changes for the entire production period (week 10 vs. week 60) were submitted to IPA for the identification of canonical pathways based on the Ingenuity® Knowledge Base. Human orthologous gene symbols for 12,047 (LB) and 12,214 (LSL) chicken transcripts were considered in IPA analysis. Canonical pathway significance was tested at an adjusted P-value (Benjamini-Hochberg) < 0.05. Pathways were considered significantly activated or inactivated at an IPA-predicted absolute z-score > 2. Cancer-related pathways were excluded from the results derived from IPA.

Conclusions

The onset of egg production, its peak, and senescence involve a cascade of several biological complexes, which are characterized by the interrelatedness of diet and physiological transition mediated by endocrinal regulation and transcript expression at each production stage. The attainment of sexual maturity in laying hens and its associated shift in dietary calcium intake at onset of egg production proves to be the most crucial developmental stage in the entire production cycle as proven by the conspicuous shifts in blood plasma metabolites levels. In particular, the high calcium requirement from the start of the laying required subtle coordination between PTH and the vitamin D system from week 24, which seems crucial to ameliorate production. Thus, the transcriptomic investigation of the jejunum from LB and LSL laying hens revealed several signaling pathways substantiating the complexity and importance of the jejunum in its contribution to the overall health and maintenance for optimum production across the entire developmental period in the layers. The study shows that both strains cope with changes in metabolic demands to reach comparable egg production performance by partially recruiting different pathways. The strains differ in pathways related to immunity, barrier and age-related tissue and cell integrity during all production periods, which could be due to genetic differences between the strains and deserve further investigation. However, insights into the host-microbiota interaction, specifically its influence on the gut-brain complex will further strengthen the knowledge and facilitate the management to improve mineral utilization and egg production.

Data availability

The raw data were deposited in the EMBL-EBI (www.ebi.ac.uk/arrayexpress) database under accession number E-MTAB-9137.

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Author contributions

Conceptualization, K.W., M.R.; methodology, A.O.O., H.R., M.O., N.T.; formal analysis, H.R., M.O., N.T.; investigation, A.O.O., H.R., M.O., S.P., V.S., M.R., K.W.; resources, S.P., E.M., K.W.; data curation, H.R., M.O., V.S.; writing-original draft preparation, A.O.O.; writing-review and editing, A.O.O., H.R., M.O., S.P., N.T., E.M., V.S., M.R., K.W.; visualization, A.O.O., H.R., M.O.; supervision, H.R., K.W.; project administration, V.S., M.R., K.W.; funding acquisition, M.R., K.W. All authors have read and agreed to the published version of the manuscript.

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Competing interests

The authors declare no competing interests.

Additional information

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6.2 Transcriptional responses in jejunum of two layer chicken strains following variations in dietary calcium and phosphorus levels

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Transcriptional responses in jejunum of two layer chicken strains following variations in dietary calcium and phosphorus levels

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Abstract

Background: Calcium (Ca) and phosphorus (P) are essential nutrients that are linked to a large array of biological processes. Disturbances in Ca and P homeostasis in chickens are associated with a decline in growth and egg laying performance and environmental burden due to excessive P excretion rates. Improved utilization of minerals in particular of P sources contributes to healthy growth while preserving the finite resource of mineral P and mitigating environmental pollution. In the current study, high performance Lohmann Selected Leghorn (LSL) and Lohmann Brown (LB) hens at peak laying performance were examined to approximate the consequences of variable dietary Ca and P supply. The experimental design comprised four dietary groups with standard or reduced levels of either Ca or P or both (n = 10 birds per treatment group and strain) in order to stimulate intrinsic mechanisms to maintain homeostasis. Jejunal transcriptome profiles and the systemic endocrine regulation of mineral homeostasis were assessed (n = 80).

Results: Endogenous mechanisms to maintain mineral homeostasis in response to variations in the supply of Ca and P were effective in both laying hen strains. However, the LSL and LB appeared to adopt different molecular pathways, as shown by circulating vitamin D levels and strain-specific transcriptome patterns. Responses in LSL indicated altered proliferation rates of intestinal cells as well as adaptive responses at the level of paracellular transport and immunocompetence. Endogenous mechanisms in LB appeared to involve a restructuring of the epithelium, which may allow adaptation of absorption capacity via improved micro-anatomical characteristics.

Conclusions: The results suggest that LSL and LB hens may exhibit different Ca, P, and vitamin D requirements, which have so far been neglected in the supply recommendations. There is a demand for trial data showing the mechanisms of endogenous factors of Ca and P homeostasis, such as vitamin D, at local and systemic levels in laying hens.

Keywords: laying hen line, mineral requirements, mineral homeostasis

Background

Sufficient dietary supply of calcium (Ca) and phosphorus (P) is essential for all vertebrates to ensure various biological processes including bone formation, blood clotting, cell proliferation and energy metabolism. In avian species, the egg laying phase in general and high laying rates in particular generate extra demands on mineral

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homeostasis and nutrient flows. The continous process of eggshell formation and yolk production during the laying period requires high amounts of dietary Ca [1]. In fact, Ca accounts for 40 % of the eggshell weight in the form of $CaCO_3$. The sources of Ca and P for laying hens are derived from mineral supplements and plant-derived compounds. However, depending on the feedstuff components, up to 80 % of P occurs in the form of inositol phosphates with a considerable variation in their abundance [2, 3], which are available for intestinal absorption only following enzymatic cleavage [4]. Therefore, phytases of microbial origin are added to the feed to increase the intestinal availability [5]. In addition, diets of highly productive laying hen strains are supplemented with high-quality inorganic phosphates to meet required levels of dietary available P or nonphytate P. The inefficient use of P makes monogastric animal species significant P excretors and thus a major source of P input into the environment [6]. To reduce the environmental impact of animal production and to preserve the valuable natural resources of P, measures on digestibility and nutrient utilization are needed to increase the use of plant P taking into account management strategies and animal-based approaches.

In vertebrates, mechanisms of P homeostasis are largely conserved and closely linked to Ca metabolism. In particular the dietary Ca/P ratio has to meet physiological ranges and has a strong impact on health and performance data [7]. Due to the stoichiometric equilibrium of Ca and P and the tight regulation of the Ca/P ratio in serum and body fluids, measures to maintain mineral homeostasis during the laying period will affect both minerals. This includes absorption, storage and excretion processes at the level of gastrointestinal tract, bone, and kidney, which are strictly controlled by a number of known and as yet unknown regulators, transporters and endocrine and paracrine signals. Key regulators are the parathyroid hormone (PTH), the active form of vitamin D3 (calcitriol), calcitonin and fibroblast growth factor 23 (FGF23). PTH is synthesized by the parathyroid glands and its secretion depends largely on the Ca concentration in serum, which is sensed by the Ca-sensing receptor (CASR) [8]. Downstream functions of PTH comprise the short-term and sustained activation of molecular pathways that are involved in maintaining serum Ca levels mainly via improved bone resorption and renal Ca reabsorption, while enhancing renal P excretion [9]. Moreover, PTH receptors have been detected in the duodenum of chickens where they mediate a direct effect on intestinal Ca transport and influence P absorption processes [10]. In general, intestinal Ca and P absorption is achieved via para- and transcellular transport processes, which are responsive to dietary mineral supply [11]. In particular, the jejunum and duodenum are considered to be the primary sites of P absorption in the gastrointestinal tract [12]. Vitamin D3 controls Ca and P homeostasis through direct actions on the intestine, kidney, and bones and through feedback inhibition of PTH production in the parathyroid. These actions are mainly mediated by binding of the activated vitamin D receptor to vitamin D response elements (VDRE) in the promoter regions of various target genes [13]. Regarding laying hens, it has been shown that Ca and P utilization are strongly dependent on vitamin D [14]. FGF23, which is derived from osteoblasts and osteocytes, affects serum concentrations of P and PTH, as well as renal P transporter expression and the formation of active vitamin D in the kidney [15].

The laying performance of commercial laying hens is exceptionally high and requires high dietary standards, especially with regard to mineral supply. Two important representative layer strains are Lohmann Selected Leghorn (LSL) and Lohmann Brown (LB) with a similar laying performance over the production period [16]. Nevertheless, there are distinct differences between the two strains in terms of body weight and immunity as well as in bone metabolism and phytate degradation [17–19]. The current study is based on a previous experimental trial using high performing laying hens of these distinct genetic origins (LSL and LB) to investigate the dietary impact of variable Ca and P supply [19]. This study extends the previous investigations by assessing an endocrine and metabolic pattern in plasma as well as molecular transcriptional responses at the level of the small intestine obtained by RNA sequencing. Specifically, the proximal part of the jejunum, as the main site of mineral absorption, is the focus of the study. This approach aims to elucidate the jejunal contribution to the complex regulation of mineral homeostasis in individuals at peak performance. It is hypothesized that diets low in P and/or Ca trigger phenotypic and molecular adaptations in laying hens to orchestrate e.g. mineral absorption and storage in order to maintain mineral homeostasis. Strain-specific transcriptional responses can identify genotype-environment interactions to be incorporated into strategies for targeted resource management.

Results

Average body weight differed significantly between strains but not between diet groups within strain. The dietary groups comprised the control group (Con) and groups with diets low in Ca and P (LCaP), low in Ca (LCa), and low in P (LP). The average body weights (LSmeans) at slaughter (week 31) for LSL were 1648 g (Con), 1683 g (LCaP), 1641 g (LCa), and 1599 g (LP) [19]. The corresponding body weights (LSmeans) of LB animals were 1784 g (Con), 1809 g (LCaP), 1929 g

(LCa), and 1838 g (LP). Statistical analysis of zootechnical data of these birds was performed by Sommerfeld et al. 2020 [19] and revealed that body weight was significantly higher in LCa hens of the LB strain compared to Con animals. Plasma levels of albumin, magnesium, T3 and activity of alkaline phosphatase were not significantly different between treatments ($p > 0.05$, Fig. 1). For vitamin D, levels of the storage form calcidiol were significantly higher in LB compared to LSL across all dietary groups ($p \leq 0.03$). Moreover, significant differences in calcidiol concentrations were observed for LCaP (39.26 ± 3.35 ng/ml) compared to LP (39.26 ± 3.35 ng/ml) in LB ($p = 0.03$). Plasma concentrations of the active form, 1,25(OH) vitamin D (calcitriol), showed a high individual variability and did not differ significantly between treatments ($p > 0.05$). While PTH levels were not affected by diet in LB hens, it was significantly higher under the LCaP diet than the Con diet in LSL hens ($p = 0.04$).

The comparison of expression profiles of LSL and LB in the four experimental diets by the base model showed considerable differences between laying hen strains (Fig. 2, Additional file 1). For the Con diet, the comparison between strains revealed 2426 differentially expressed genes (DEGs; $p < 0.01$, $p_{adj} < 0.029$). For the LCaP diet, 1911 DEGs ($p < 0.01$, $p_{adj} < 0.041$) were identified between LSL and LB. The diets reduced in either Ca or P resulted in a number of 2680 ($p < 0.01$, $p_{adj} < 0.028$) and 4540 DEGs ($p < 0.01$, $p_{adj} < 0.012$) between strains, respectively. The intersection of the strain comparisons for all four dietary groups revealed 1020 genes, which are considered to represent the strain-specific differences in jejunal nutrient utilization and metabolism. Interestingly, all 1020 DEGs showed a consistent expression pattern in terms of their mRNA abundances over all four diets, with 527 upregulated (LSL > LB) and 493 downregulated (LSL < LB) genes. This information was

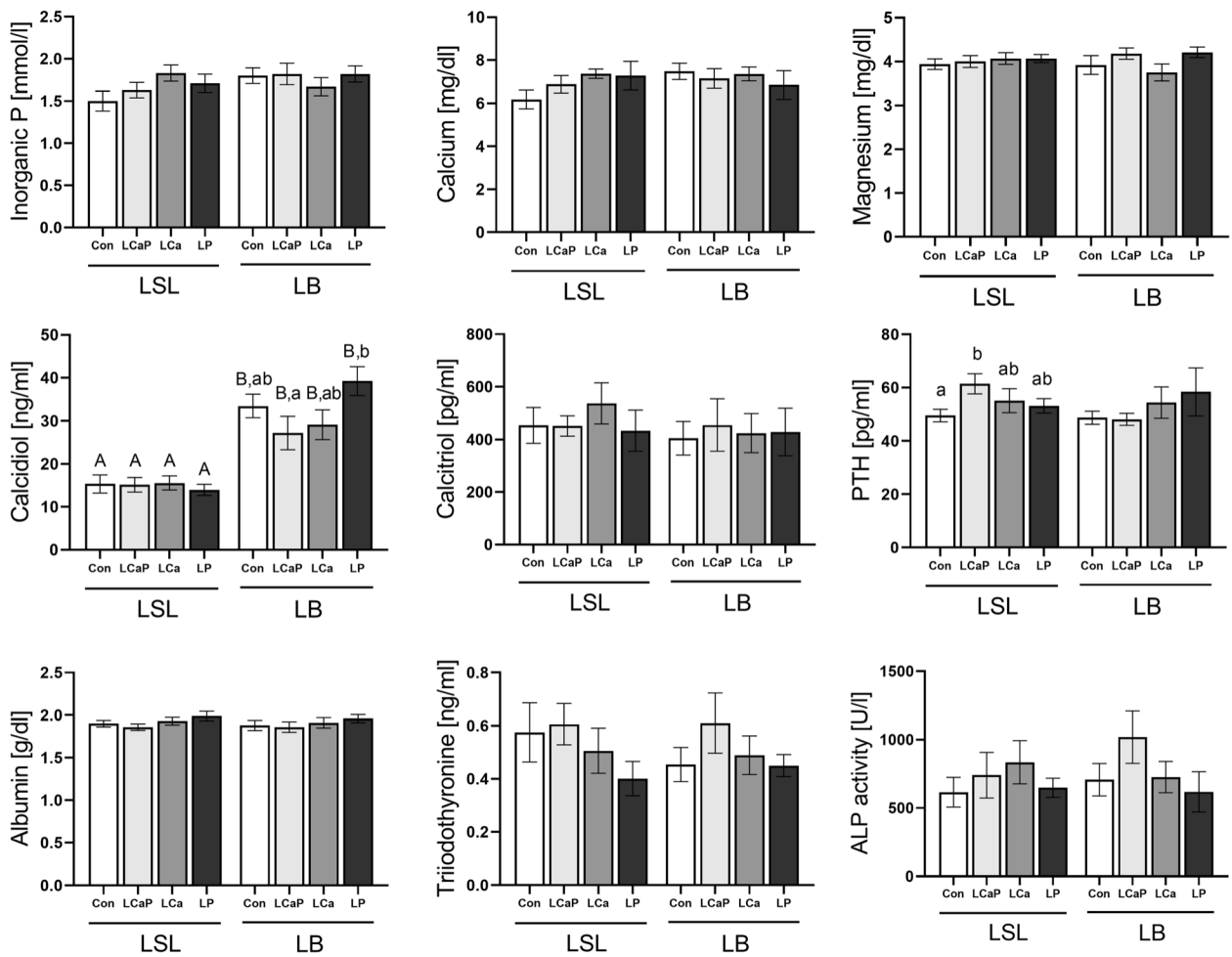

Fig. 1 Plasma parameters referring to mineral homeostasis and growth of LSL and LB laying hens fed a standard control diet (Con), reduced Ca and P levels (LCaP), reduced Ca levels (LCa) or reduced P levels (LP). Values are displayed as mean ± SE. Data for inorganic P and Ca were taken from [17]. Superscripts indicate statistical significance ($p < 0.05$) between hen strains as capital letters or within strains between dietary groups as small letters

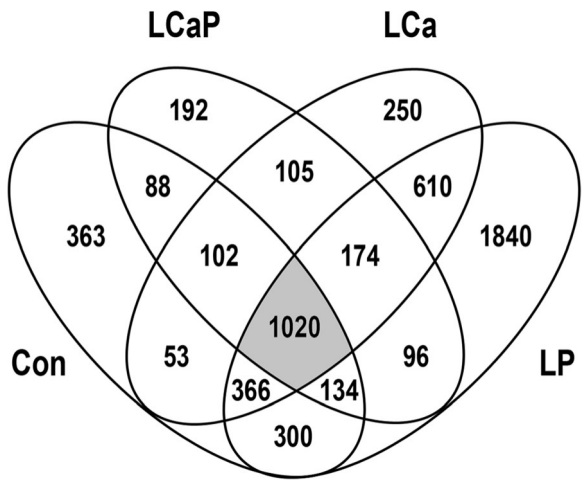

Fig. 2 Venn diagram of differentially expressed genes identified in diet-specific comparisons between LSL and LB laying hens fed a standard control diet (Con), reduced Ca and P content (LCaP), reduced Ca content (LCa) or reduced P content (LP). The 1020 DEGs that are present in all dietary comparisons represent the laying hen strain-specific transcriptional patterns in the jejunum

subjected to pathway enrichment analysis using the KEGG database, highlighting those genes out of the 1020 that accumulate in certain pathways (Fig. 3). Enriched pathways considering the significance threshold ($p_{adj} < 0.05$) include 'metabolism of xenobiotics by cytochrome P450', 'glutathione metabolism', 'arginine and proline metabolism', 'drug metabolism', and 'histidine metabolism' (Fig. 3).

After filtering, 13,123 and 12,703 genes were included in the analysis for contrasting diets within LSL and LB, respectively. The variable selection approach based on gene expression data revealed no clear separation of all four groups within a hen strain (Fig. 4). However, in LSL the LP group is partly separated from the Con group in the first component, which explained 17 % of the variance. Samples of the LCa and LCaP groups largely overlapped. For LB, the LP group was found to be separated to some extent from the LCaP group when the first component was considered. Moreover, these two dietary groups were partly distinct from Con and LCa, which largely overlap on the two components. Correspondingly, the differential gene expression analysis revealed a considerable number of DEGs exclusively for the Con –

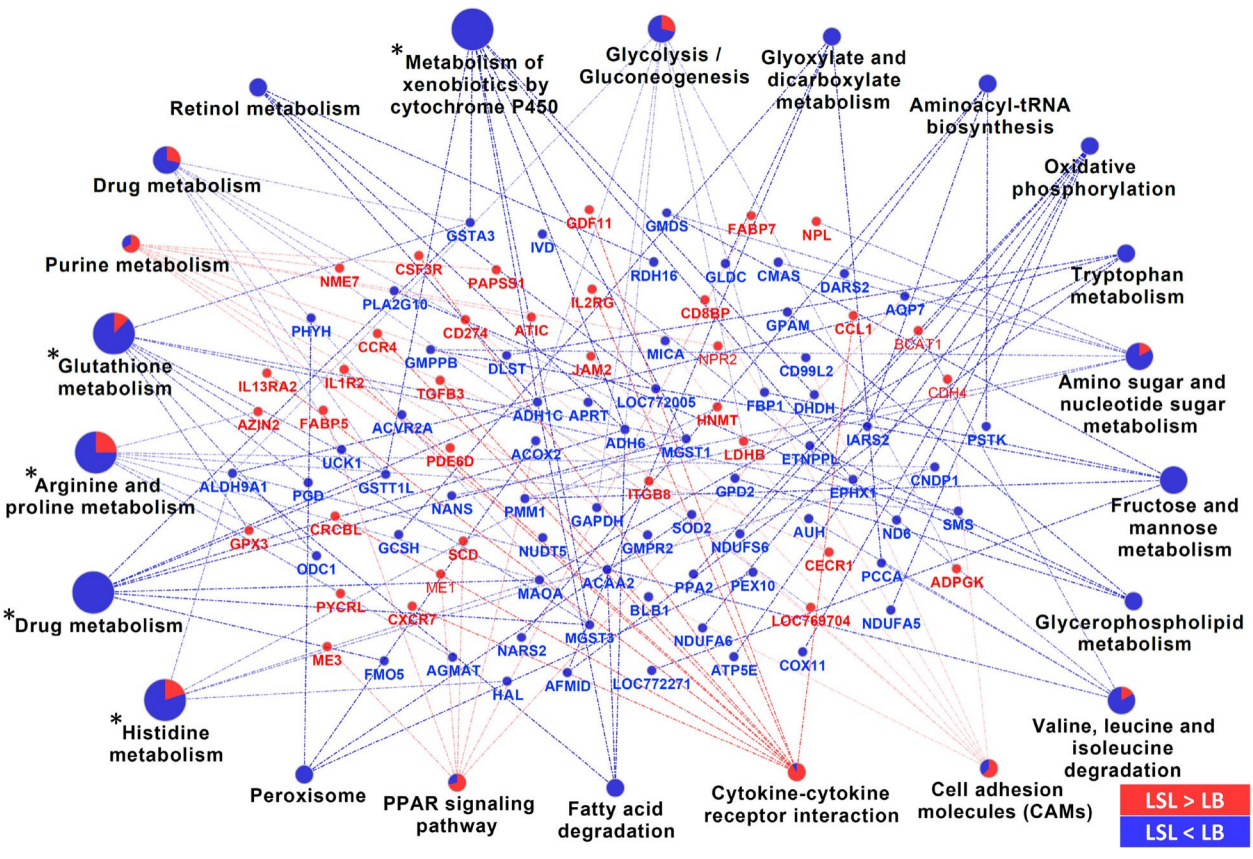

Fig. 3 KEGG pathway analysis of 1020 genes found to be consistently differentially expressed between LSL and LB strains covering all four dietary comparisons. The size of the pathway term represents the term p-value. Terms indicated by an asterisk were considered significant (Benjamini-Hochberg adjusted p-value < 0.05)

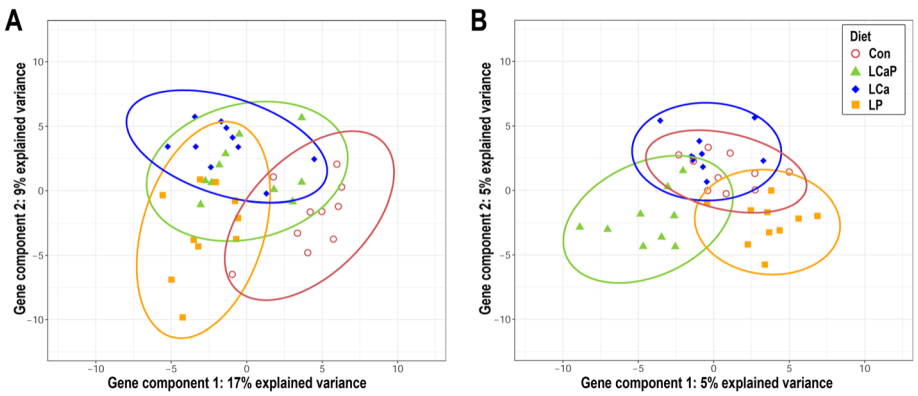

Fig. 4 Principal component analysis of the LSL (A) and LB (B) laying hens fed a standard control diet (Con), reduced Ca and P levels (LCaP), reduced Ca levels (LCa) or reduced P levels (LP). The plots represent the first two components, which are derived from a variable selection approach (sPLS-DA) based on jejunal gene expression profiles

LP contrast in LSL and the LCaP – LP contrast in LB (Additional file 1). For Con – LP, 503 DEGs were detected in LSL. For LB, the contrast between LCaP and LP revealed the highest number of DEG with in total 568 genes. The comparison of expression profiles of other groups within each strain revealed in only minor alterations at the transcriptional level (Additional file 1).

Based on the DEGs identified in reasonable numbers for the contrasts Con-LP in the LSL strain and LCaP-LP in the LB strain, biological pathway analyses were performed using the KEGG database (Figs. 5 and 6). For the contrast between Con and LP in LSL, the 'ribosome' pathway was found to be enriched ($p_{adj} < 0.05$; Fig. 5). Three pathways including 'intestinal immune network

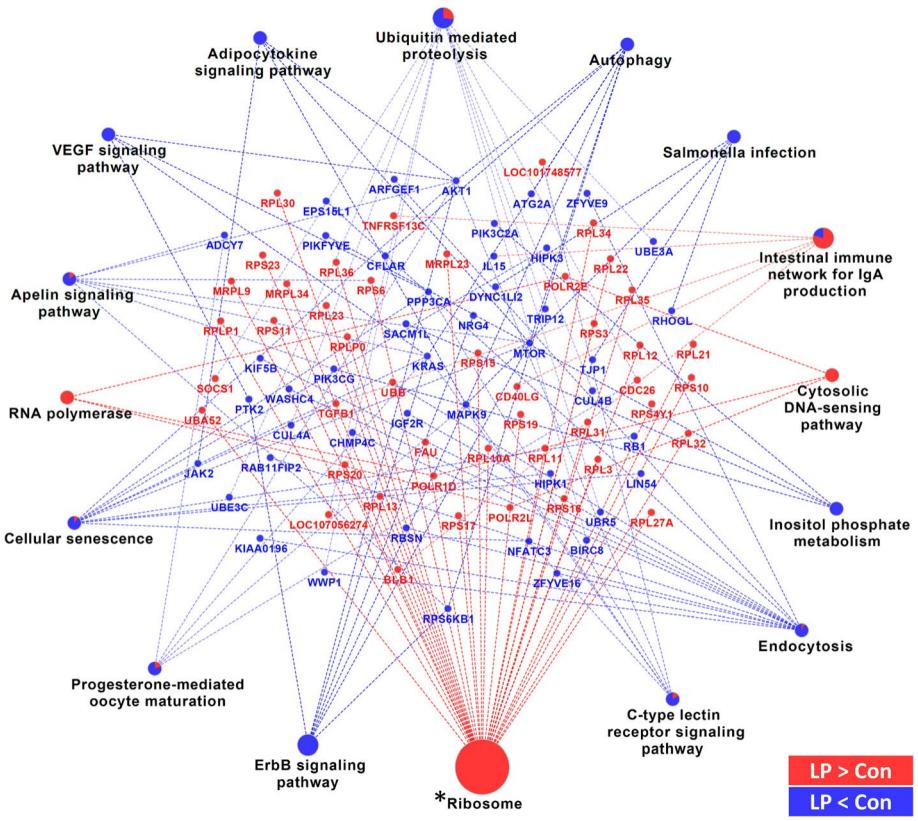

Fig. 5 KEGG pathway analysis of genes found to be differentially expressed between Con and LP hens of the LSL strain. The size of the pathway designation represents the p-value. Terms indicated by an asterisk were considered significant (Benjamini-Hochberg adjusted p-value < 0.05)

for IgA production' (p_{adj} = 0.05), 'ubiquitin mediated proteolysis' (p_{adj} = 0.06) and 'ErbB signaling pathway' (p_{adj} = 0.10) tended to be significantly enriched (Fig. 5). DEGs contributing to the 'ribosome' pathways were entirely upregulated in the LP group compared to control animals (LP > Con), whereas for 'ubiquitin mediated proteolysis' the majority of genes were lower abundant in LP chicken compared to Con animals. Thematically overlapping pathways were identified using IPA (Table 1). Predicted activation state (z-score) of these pathways revealed a significant activation of 'EIF2 signaling' (z-score = -3.0) in the LP chickens compared to Con, whereas for the 'regulation of eIF4 and p70S6K signaling' and 'mTOR signaling' a trend for inhibition of this pathways was observed in the LP group (z-scores = 1.89). In addition, an enrichment of genes in 'IL-4 signaling' and 'glucocorticoid receptor signaling' was identified.

For the comparison of LCaP and LP groups in LB chickens, 'focal adhesion' was found to be the most enriched pathway with 22 involved genes (Fig. 6). The majority of DEGs in this pathway were found to be more abundant in the LCaP than in LP group. In general, most of the DEGs were more abundant in the LCaP chickens compared to LP animals. Other enriched pathways and corresponding DEGs including 'cell adhesion molecules', 'extracellular matrix interactions', and 'regulation of actin cytoskeleton' are summarized in Fig. 6. For IPA, the DEGs were found to be significantly enriched in nine canonical pathways (Table 1). Among these, the 'GP6 signaling pathway' and the 'Apelin Liver Signaling Pathway' were predicted to be activated in the LCaP group compared to the LP group. Overall, the results point to an involvement of DEGs in pathways of inflammation, cell adhesion, and extracellular matrix formation.

Discussion

For many decades, the Ca and P requirements of laying hens have been an important research subject to ensure laying performance [20, 21], it receives additional attention due to the intention to preserve mineral resources in animal-based food production [22]. The complex dynamics of Ca and P metabolism impede the precise assessment of the dietary Ca and P supply in respect to genetics and age [23, 24]. Current dietary recommendations for laying hens range between 32 and 44 g/kg for Ca and between 1.5 and 4.5 g/kg for non-phytate P, depending on age [25]. Interestingly, the requirements for Ca and P of LSL and LB strains are currently assumed identical, although there are significant differences between the strains regarding traits related to mineral utilization. The LB hens have been reported to exhibit a higher bone mass and a higher breaking strength of

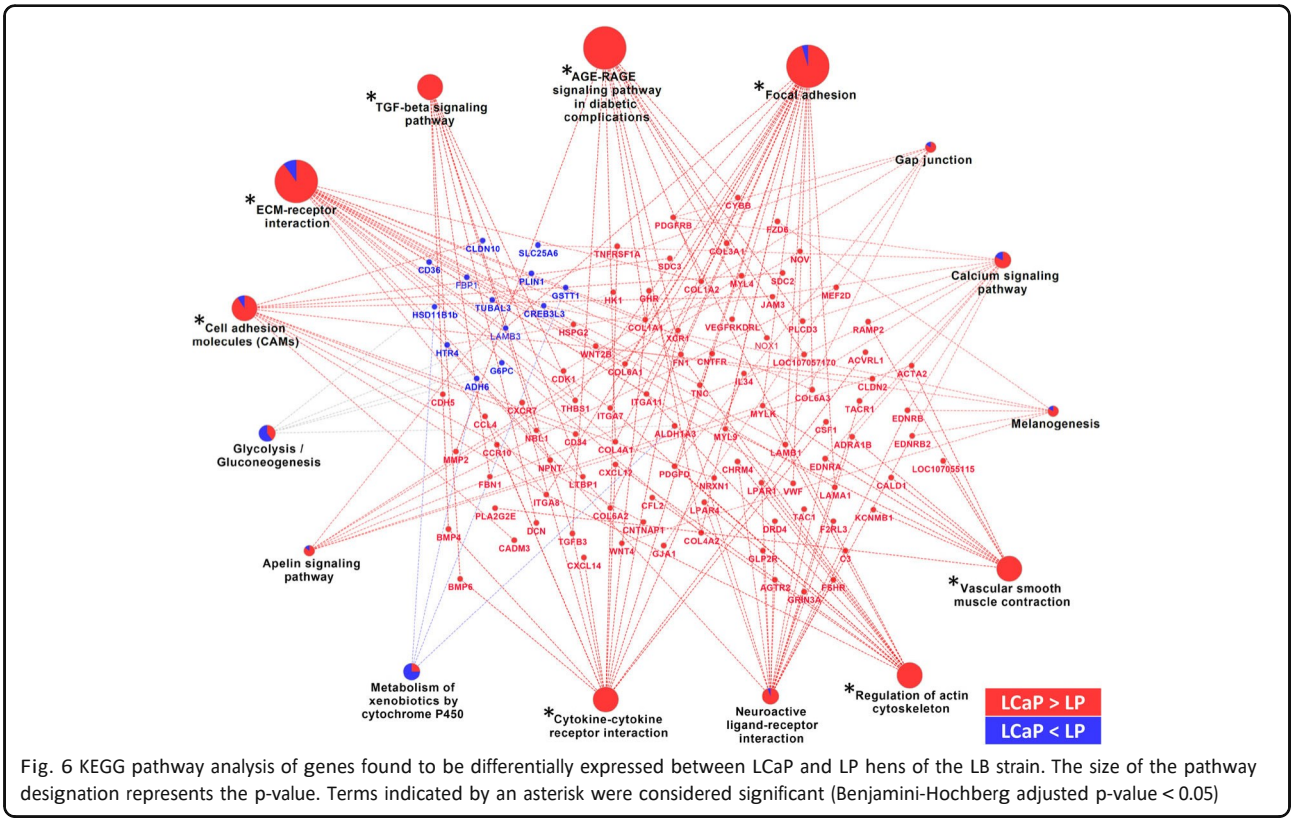

Fig. 6 KEGG pathway analysis of genes found to be differentially expressed between LCaP and LP hens of the LB strain. The size of the pathway designation represents the p-value. Terms indicated by an asterisk were considered significant (Benjamini-Hochberg adjusted p-value < 0.05)

Table 1 Canonical pathways enriched by DEGs comparing Con and LP groups of LSL laying hens and LCaP and LP of LB laying hens

Canonical pathways	p_{adj}-value	z-score*	Molecules
Lohmann Selected Leghorn (LSL): Con vs. LP			
EIF2 Signaling	< 0.001	3.00	AKT1,ATF3,EIF3K,FAU,KRAS,PAIP1,PIK3C2A,PIK3CG, RPL10A,RPL11,RPL12,RPL13,RPL21,RPL22,RPL23,RPL27A, RPL3,RPL30,RPL31,RPL32,RPL34,RPL35,RPL36,RPLP0, RPLP1,RPS10,RPS11,RPS15,RPS16,RPS17,RPS19,RPS20, RPS23,RPS3,RPS4X,RPS6,UBA52,XIAP
Regulation of eIF4 and p70S6K Signaling	< 0.001	-1.89	AKT1,EIF3K,EIF4EBP2,FAU,KRAS,MTOR,PAIP1,PIK3C2A, PIK3CG,RPS10,RPS11,RPS15,RPS16,RPS17,RPS19, RPS20,RPS23,RPS3,RPS4X,RPS6,RPS6KB1
mTOR Signaling	0.005	-1.89	AKT1,EIF3K,FAU,KRAS,MTOR,PIK3C2A,PIK3CG,PRR5L, RPS10,RPS11,RPS15,RPS16,RPS17,RPS19,RPS20,RPS23, RPS3,RPS4X,RPS6,RPS6KB1
IL-4 Signaling	0.013		AKT1,JAK2,KRAS,MTOR,NFAT5,NFATC3,NR3C2, PIK3C2A,PIK3CG,RPS6KB1,SOCS1
Glucocorticoid Receptor Signaling	0.034		AKT1,BAG1,GTF2A1,GTF2H1,JAK2,KAT2B,KRAS,MAPK9, MED14,NCOA2,NFAT5,NFATC3,NR3C2,PIK3C2A,PIK3CG, POLR2E,POLR2L,PPP3CA,TAF4,TGFB1,YWHAH
Lohmann Brown (LB): LCaP vs. LP			
Agranulocyte Adhesion and Diapedesis	< 0.001		ACTA2,CCL23,CD34,CDH5,CLDN10,CLDN2,CXCL12, CXCL14,FN1,JAM3,MMP10,MMP11,MMP17,MMP2, MMP9,MYH11,MYL4,MYL9,PODXL,PPBP,TNFRSF1A
Granulocyte Adhesion and Diapedesis	< 0.001		CCL23,CDH5,CLDN10,CLDN2,CXCL12,CXCL14,JAM3, MMP10,MMP11,MMP17,MMP2,MMP9,PPBP,SDC2, SDC3,THY1,TNFRSF1A
GP6 Signaling Pathway	< 0.001	3.77	COL13A1,COL16A1,COL18A1,COL1A1,COL1A2,COL3A1, COL4A1,COL4A2,COL4A6,COL5A1,COL5A2,COL6A1, COL6A2,COL6A3,LAMA1,LAMB1,LAMB3,NOX1
Axonal Guidance Signaling	< 0.001		ADAM19,ADAMTS1,ADAMTS13,ADAMTS9,BMP4,BMP6, CFL2,CXCL12,ECEL1,EFNB1,EPHB2,FZD6,GNB1L,HHIP, LINGO1,MMP10,MMP11,MMP17,MMP2,MMP9,MYL4, MYL9,NRP2,NTN3,NTN4,PDGFD,PLCD3,PLXND1,PTCH2, RASD2,SDC2,SEMA3F,TUBA4A,UNC5B,UNC5C,WNT2B, WNT4
Inhibition of Matrix Metalloproteases	< 0.001	-1.13	HSPG2,MMP10,MMP11,MMP17,MMP2,MMP9,SDC2, TFPI2,TIMP2
Apelin Liver Signaling Pathway	0.002	2.45	COL18A1,COL1A1,COL1A2,COL3A1,IRS1,PDGFRB
Intrinsic Prothrombin Activation Pathway	0.014	1.34	COL18A1,COL1A1,COL1A2,COL3A1,PROS1
Inhibition of Angiogenesis by TSP1	0.029	0.00	CD36,HSPG2,MMP9,NOS3,SDC2,THBS1
Leukocyte Extravasation Signaling	0.044	1.90	ACTA2,CDH5,CLDN10,CLDN2,CXCL12,CYBB,JAM3,MMP10, MMP11,MMP17,MMP2,MMP9,NOX1,THY1,TIMP2

* z-score: pathways with an absolute z-score ≥ 2 were considered significant. Positive and negative values indicate activation (in LSL: LP > Con; in LB: LCaP > LP) and inhibition (in LSL: LP < Con; in LB: LCaP < P)

humeral and tibia bones compared to LSL, whereas bone density was unaffected [17, 26]. However, Khanal et al. observed that with high Ca content in the pre-lay diet, LSL had higher femur mineral density, ash content and breaking strength at the onset of the laying period compared to LB [27]. They concluded that LSL hens have a higher capacity than LB hens to accumulate excess feed Ca in the bones. Obviously, the utilization of micronutrients in LSL and LB hens is based on strain-specific sophisticated metabolic routes, a fact which has been demonstrated for e.g. lysine [28]. Results of the current layer hen trial suggest similar conclusions for Ca and P. Although the analyses demonstrated unaffected plasma Ca and P levels between the strains [19], a clear and marked strain-specific effect was observed for plasma calcidiol (LB > LSL). The difference in the vitamin D system is found exclusively at the level of the respective storage form and is not reflected at the level of the active calcitriol. Due to the required hydroxylation process for the synthesis of calcidiol, this indicates the liver as an important target tissue to initiate local and systemic responses to maintain mineral homeostasis.

The study implies different mineral requirements in LSL and LB, which is substantiated by the fact that

differences in gene expression patterns of jejunum were identified in different contrasts between the two strains. While low P supply in LSL induced considerable transcriptional responses compared to Con, hens of the LB strain showed marked differences under low P supply depending on the Ca supply. LSL were reported to have an increased demand and utilization of Ca resulting in eggs with higher eggshell weights compared to LB [29]. Moreover, the regulation of several genes and their products associated with the 'Ca-ion binding and transport' and 'Ca release-activated Ca channels' establish this pattern in LSL. These adaptive actions of the Ca metabolism might enable LSL to cope better with moderate dietary Ca restrictions than LB hens. However, it needs to be considered that nutritional recommendations for the two strains are identical, while the optimum might be better matched for one than for the other.

For the two strains studied, the dietary reduction of Ca and/or P was reflected to varying degrees in the gene expression patterns in the gut. For the LSL strain, marginal differences were observed for the group comparisons Con vs. LCaP and Con vs. LCa, whereas the reduction of dietary P supply resulted in considerable changes in intestinal gene expression compared to the control. These findings suggest that the animals investigated were generally able to adapt to the dietary changes by endogenous mechanisms. This was also reflected in plasma levels and performance traits, which were found to be mostly inconspicuous compared to Con, although body weight was significantly increased in hens of the LB strain submitted to a low Ca [19]. Merely increased plasma PTH levels in the LCaP group compared to Con point to active adaptation mechanisms to maintain Ca and P homeostasis. Indeed, PTH is an important regulator of osteoclast activity and may mediate intensified bone resorption in the LCaP group of LSL hens in order to mobilize additional Ca and P [30].

For LSL, the dominant dietary treatment in terms of identified intestinal DEGs was the lowered P diet (LP group) in comparison to Con. The integration of functional annotations of corresponding DEGs showed that the main pathways initiated by LP treatment include ribosomal protein synthesis and the regulation of cellular signaling cascades. In accordance, the protein biosynthesis pathway was recently found to be affected in the intestinal epithelium of Japanese quail with divergent P utilization efficiency [31]. In this quail study, high P utilization efficiency was ascribed to an accelerated cell proliferation in the intestine. The current list of DEGs further revealed an impairment of the mTOR signaling pathway, which has important functions in cell proliferation, differentiation, growth, and metabolism [32]. These differences are mainly driven by the differential abundance of ribosomal proteins and ubiquitin proteins. Effects of the diet composition on proliferation of intestinal cells are described for several nutritional components including non-starch polysaccharides, short chain fatty acids, and vitamins [33]. Non-starch polysaccharides affect viscosity of digesta in the intestine and induce renewal of the epithelium due to delayed nutrient absorption. Moreover, there are also effects of dietary mineral supplements including Ca and P described to affect intestinal cell proliferation in rodents [34].

Among the differentially abundant genes, a number of transcripts encoding for transport proteins were obtained. These comprised anion transmembrane transporters (SLC4A4, SLC4A8, and SLC35B3) as well as some molecules involved in mineral homeostasis such as ATP2C1 and CASK. ATP2C1 regulates Ca concentrations in cytosol and plays an important role for protein synthesis in the endoplasmic reticulum [35]. CASK encodes for a Ca/calmodulin dependent serine protein kinase mediating intracellular effects downstream of plasma membrane Ca pumps [36]. No effects on the most common transcellular Ca and P transporters were identified between LP and Con animals of LSL. However, paracellular transport processes might be affected through changes in TJP1 and ADAM10 expression. Both genes affect cell-to-cell adhesion and influence the mobility of molecules in the paracellular area [37, 38]. Consequently, the lower abundance of TJP1 in LP compared to Con might increase the selective permeability for ions but conversely might also increase the risk of transferring intestinal microbes, toxins, and antigens into tissues [39]. In fact, intestinal signaling pathways related to the immune response, in particular 'intestinal immune network for IgA production' (KEGG) and 'IL-4 signaling' (IPA), were considered influenced in the comparison of Con and LP through the functional enrichment analysis. IgA production relies on antibodies that are produced on the basis of bacterial antigens and that aim to counteract toxins and pathogenic microbes at the contact surface between host and intestinal microbiota [40]. In chickens identified to be more resistant to Salmonella infections, it was found that the pathway of IgA production was activated compared to more susceptible individuals [41]. However, any change in the dietary composition, even changes in individual minerals, drives alterations of the intestinal microbial community and requires an adaptation of the host's defence mechanisms [42, 43].

In LB hens, the plasma calcidiol levels were higher compared to LSL hens. Moreover, higher calcidiol levels were found in LB hens fed LP compared to LCaP diets. Plasma levels of calcidiol reflect the vitamin D status, which is affected by long-term feed supply and individual vitamin D metabolism (e.g. hepatic hydroxylation processes, renal clearance), since under current housing

conditions UV-mediated endogenous vitamin D synthesis is not present in laying hens [44]. Thus, LB hens fed the LCaP diet might use a higher proportion of the available vitamin D to counteract mineral shortage and maintain mineral homeostasis. The analyses of dietary treatments in the LB strain revealed increased expression of genes involved in 'GP6 signaling' and 'Focal adhesion' in LCaP compared to LP fed laying hens. A considerable number of DEGs encoding collagens and matrix metalloproteinases (MMP) have been identified. Indeed, the GP6 proteins are major signaling receptors in collagen formation and function, which together with integrins, tenascins, fibronectins and laminins constitute the main components of the extracellular matrix (ECM) [45, 46]. The MMP are a family of zinc-dependent proteinases that are secreted to the extracellular space or localized to the cell surface in a premature state. Once activated, MMP are collectively able to cleave all components of the ECM [47]. Focal adhesions constitute a large macromolecular assembly of proteins namely vinculin, talin, paxillin, zyxin, and α-actinin that associate with integrins in order to facilitate the anchorage of the cell and the ECM but also to support cell migration [48, 49]. The recruited cellular components that form a focal adhesion remain anchored to the ECM to allow sequential cell migration, which is of particular importance for intestinal enterocytes and immune cells [50]. Indeed, the DEGs assigned to both agranulocyte and granulocyte adhesion pathways are responsible for facilitating the migration of immunocompetent cells in the intestinal endothelium. Taken together, the results obtained for the comparison of LCaP with LP diets in LB hens point to a number of DEGs encoding collagens and MMPs that might have an impact on ECM formation. Apparently, the amounts of dietary Ca and P have to be considered to trigger structural changes in the intestinal epithelium. In fact, a previous jejunal transcriptome study in broilers linked the increased expression of genes encoding for collagen and ECM to increased villus length for improved nutrient uptake and energy utilization [51]. Results in the present study suggest therefore a vulnerability of LB hens for inadequate Ca intake. Further analysis of the microanatomy in laying hens are required to map potential phenotypic adaptations related to the maintenance of mineral homeostasis. A limitation of the study is that although the sampling times of the current study were standardised and largely controlled, the calcium requirement of egg-laying hens, which is hormonally regulated, varies considerably depending on oviposition and the circadian cycle [52]. To some extent, this could also influence gene expression patterns in the gastrointestinal tract, as the demands on the entire organism shift due to the higher calcium requirement for eggshell calcification processes [53].

Conclusions

Differences between LSL and LB hens, which are present in genetic and morphological aspects, are also reflected in the transcriptional profile of the jejunum. Accordingly, the response to varying levels of dietary Ca and P concentrations differs in the two laying hen strains. LSL hens might be more prone to effects of varying dietary mineral supply on intestinal cell proliferation rate, while adaptive responses occur at the level of the paracellular transport and immune competence. The endogenous mechanisms in LB hens might involve the formation of extracellular matrix for compensatory improvement of the absorptive capacity. The results of the current study indicate that LSL and LB laying hens have different mineral and vitamin D requirements owing to different transcriptome, which potentially might be exploited to reduce mineral resources. In terms of environmental protection and poultry management, there is both a need and a possibility to further specify the requirements for dietary Ca, P, and vitamin D supply in LSL and LB laying hen strains.

Methods

Birds and diets

The animal trial was conducted at the Agricultural Experiment Station of the University of Hohenheim, Germany, and was approved by the Regierungspräsidium Tübingen, Germany (HOH50/17TE). Procedures were in accordance with the German Animal Welfare Legislation.

The trial is based on two modern strains of laying hens supplied with variable Ca and P levels [19]. In particular, for each of the strains Lohmann Selected Leghorn (LSL, n = 40) and Lohmann Brown (LB, n = 40; Lohmann Tierzucht GmbH, Cuxhaven, Germany), four dietary groups (n = 10 per group) with varying Ca levels and non-phytate P levels (standard vs. reduced) were formulated as described previously [19]. In brief, except for Ca and P levels the composition of the corn-soybean based diets met current recommendations [54]. Differences in Ca and P levels were achieved by varying levels of mineral monocalcium phosphate and limestone. The analyzed Ca and total P levels of the control diet (Con) were 39.5 g/kg dry matter (DM) and 5.3 g/kg DM. The treatment groups were supplied with diets low in Ca and P (LCaP), low in Ca (LCa), and low in P (LP). The retrieved dietary Ca and P contents were 34.4 g/kg DM and 4.7 g/kg DM for LCaP, 35.1 g/kg DM and 5.3 g/kg DM for LCa, and 40.3 g/kg DM and 4.7 g/kg DM for LP diets, respectively. The feed was formulated to minimize plant based phytases and no additional phytases of microbial origin were added. For each of the two strains LSL and LB, the four diet groups consisted of 10 birds each, with the progeny of the same 10 fathers in each of

the groups. Birds were group-housed in pens for the first 26 weeks and then were rehoused individually into metabolism unity cages in a randomized complete block design, which resulted in 10 replicates per strain and diet. The feeding trial lasted 21 days in metabolism units in which each bird was fed individually. Chickens were phenotyped for zootechnical and physiological parameters as described elsewhere [19]. At the age of 31 weeks, the hens were stunned and subsequently slaughtered by exsanguination Prior to slaughter, each bird was subjected to a two-hour feed withdrawal period, followed by a one-hour re-feeding period. Sampling started at 9 am, with twenty birds slaughtered at 15-minute intervals on each of four consecutive days, with the order of slaughter recorded. Trunk blood was collected in heparin-containing tubes and centrifuged to obtain plasma samples (10 min at 2500 × g). Samples were stored at -80 °C until analysis of plasma parameters. Approximately 3 cm distal to the duodenal loop, a jejunum sample of 2 cm in length was collected from each bird.The mucosa was rinsed with 0.9 % NaCl solution and then scraped off with a scalpel. Samples were frozen on dry ice and stored at -80 °C upon RNA extraction.

Measurement of blood parameters

The plasma samples collected at slaughter were analysed to measure the levels of albumin, magnesium and alkaline phosphatase activity using commercial assays via the Fuji DriChem 4000i according to manufacturer's instructions (FujiFilm, Minato, Japan). Hormone measurements were performed in duplicate with commercially available enzyme-linked immunosorbent assays (ELISA). Corresponing kits were processed according to manufacturer's instructions for parathyroid hormone (CSB-E118880Ch, CusaBio, Houston, USA), triiodothyronine (EIA-4569, DRG, Marburg, Germany), 25(OH) vitamin D (EIA-5396, DRG), and 1,25(OH) vitamin D (AC-62F1, Immunodiagnostic Systems GmbH, Frankfurt am Main, Germany). For statistical analysis of the mentioned blood parameters and hormones, a linear model was applied including dietary group, laying hen strain, hen father and slaughter order (R language, version 3.6.2, package stats). Differences were considered significant at $P \leq 0.05$.

RNA extraction and sequencing

Total RNA was extracted from all 80 jejunal samples using TRIzol Reagent (Invitrogen, Karlsruhe, Germany) according to the manufacturer's protocol. Subsequently, mRNA was extracted using the Rneasy Mini Spin kit including an additional Dnase digestion (Qiagen). The quantity and quality of final mRNA was determinded using NanoDrop ND-2000 (Peqlab, Erlangen, Germany) and Bioanalyzer 2100 devices (Agilent Technologies, Waldbronn, Germany). RNA integrity numbers were between 7.1 and 9.4. Sequencing libraries were prepared with the stranded mRNA library preparation kit (Illumina, San Diego, CA, USA). Sample-specific, tagged libraries ($n = 80$) were pooled and sequenced on the Illumina HiSeq 2500 in a paired-end setup with 2 × 101 bp reads. The corresponding raw data were deposited in the EMBL-EBI (https://www.ebi.ac.uk) database under accession number E-MTAB-9109.

Sequencing data processing and gene expression analysis

Initially, raw sequencing reads were checked for quality and preprocessed using FastQC (version 0.11.7) and Trim Galore (version 0.5.0; https://www.bioinformatics.babraham.ac.uk/projects/). Reads with low quality (mean Q-score < 20) and short length (< 30 bp) were filtered out. Remaining reads were mapped to the current chicken genome assembley (GRCg6a, Ensembl release 95) using Hisat2 (version 2.1.0; http://daehwankimlab.github.io/hisat2/). Gene-specific read counts were extracted with HTseq (version 0.11.2; https://htseq.readthedocs.io/en/master/). Differentially expressed genes (DEG) between the experimental groups were obtained using DESeq2 (DOI: https://doi.org/10.18129/B9.bioc.DESeq2). Initially, outlier detection approaches, including the distance between individual data sets, the distribution of signal intensities and the quality and quantity of the individual data were assessed using the arrayQualityMetrics R package (DOI: https://doi.org/10.18129/B9.bioc.arrayQualityMetrics). One sample (from LB LCaP group) was excluded from analysis due to low sequencing depth (Additional file 2). The count data were filtered to retain only genes with observations of 5 or more counts in at least 10 animals. Firstly, a base model to identify DEGs between the two laying hen strains within each dietary group was performed. Secondly, a statistical model was designed to reveal DEGs in the contrasts of diets within each of the two strains. This model included hen father as fixed effect to account for genetic relationship of animals. Genes were considered as significantly differentially expressed meeting the criteria of p-value < 0.01 and Benjamini-Hochberg adjusted p-value < 0.15. Normalized count data was further used to select the most important genes to distinguish between dietary groups using the sparse Partial Least Squares discriminant analysis (sPLS-DA) function of the mixOmics R package [55]. The variable selection approach considered the first two components with 50 variables each. The differentiation of groups was presented in a scatter plot. KEGG pathway enrichment analysis of the identified DEGs was performed using Cytoscape software (version 3.6.1) with the ClueGO plugin (version 2.5.1). The Clue GO plug-in generates functionally clustered KEGG annotation Networks for a list of DEGs.

The p-values were calculated by right-sided hypergeometric tests and Benjamini-Hochberg adjustment was used for multiple testing correction. KEGG pathways with an adjusted p-value < 0.05 and comprising at least five DEGs were considered significant.

Abbreviations
Ca: Calcium; CASR: Ca-sensing receptor; Con: experimental group `Control´; DEG: Differentially expressed genes; DM: dry matter; ELISA: enzyme-linked immunosorbent assays; FDR: false discovery rate; FGF23: fibroblast growth factor 23; GO: gene ontology; KEGG: Kyoto Encyclopedia of Genes and Genomes; LB: Lohmann Brown; LCa: experimental group `low Ca´; LCaP: experimental group `low Ca and P´; LP: experimental group `low P´; LSL: Lohmann Selected Leghorn; P: Phosphorus; PTH: parathyroid hormone; sPLS-DA: sparse Partial Least Squares discriminant analysis; VDRE: vitamin D response elements

Supplementary information

The online version contains supplementary material available at https://doi.org/10.1186/s12864-021-07814-9.

Additional file 1 Differentially expressed genes in the jejunum mucosa in LSL and LB laying hens following the variations of dietary calcium and phosphorus levels. Associated statistics refer to strain or dietary effects (base mean – average of the normalized counts over all samples included in the respective analysis, p-value, Benjamini-Hochberg adjusted p-value, fold change).

Additional file 2 Sequencing statistics, including read count and number of mapped reads for the jejunum samples used for mRNA sequencing.

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Authors' contributions
KW, MR and SP designed the research; HR, MO, SP and KW supervised the experiment; HR, MO, NT, SP and VS performed the experiments; HR, KW, MO and SP curated and analyzed the data; HR, AOO, MAI and MO performed statistical data analysis; AOO, EM, HR, KW, MO, MR and SP participated in the interpretation of the data; HR drafted the manuscript; HR and MO prepared figures; all authors contributed to the preparation and editing of the manuscript. The author(s) read and approved the final manuscript.

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Availability of data and materials
shttps://www.ebi.ac.uk) under accession number E-MTAB-9109. Information on the chicken genome assembly (GRCg6a, Ensembl release 95), which was considered for read mapping and gene annotation (e.g., gene identifiers in Additional file 1), is accessible in the Ensembl database (http://ftp.ensembl.org/pub/release-95/fasta/gallus_gallus/).

Declarations

Ethics approval and consent to participate
The animal trial was approved by the local authority, the Regierungspräsidium Tübingen, Germany (HOH50/17TE) and performed at the Agricultural Experiment Station of the University of Hohenheim, Germany. All procedures were in accordance with the German Animal Welfare Legislation and complied with the European Union Directive 2010/63/EU on the protection of animals used for scientific purposes.

Consent for publication
Not applicable.

Competing interest
The authors declare that they have no competing interests.

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Publisher's Note

6.3 Broiler physiological response to low phosphorus diets at different stages of production

Broiler physiological response to low phosphorus diets at different stages of production

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ABSTRACT Phosphorus (P) inclusion in broiler diets needs to meet the physiological demands at a specific developmental stage to ensure the performance, health, and welfare of the birds and minimize nutrient losses. Toward a more efficient utilization of P in broiler husbandry, a timed nutritional conditioning strategy might enhance the endogenous mechanisms of mineral homeostasis and thus reduce dietary P supply of mineral sources. In this study, following a variable P supply in the starter phase, the effects of a dietary P depletion of broiler chickens were investigated at different developmental stages. Physiological adaptation mechanisms were elucidated based on zootechnical performance, endocrine parameters, regulation of intestinal P transport, bone characteristics, and health aspects. The results revealed a marked response to P depletion at the earliest developmental phase, after which indications of effective compensatory mechanism were detectable with advancing ages. Potential mechanisms that enable broilers to maintain mineral homeostasis primarily include endocrine control mediated by calcitriol actions, as well as intestinal P uptake and mineral mobilization from the bone. Conclusively, the precise timing, duration, and extent of a P depletion strategy in the broiler chicken might be considered for optimized nutrient utilization.

Key words: broiler chicken, dietary mineral depletion, mineral homeostasis, nutritional conditioning, vitamin D metabolism

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INTRODUCTION

The comprehensive realization of the genetic potential of broiler chickens requires the provision of optimal environmental conditions, which prioritizes a well-founded nutritional regimen specifically with regard to mineral supply, including phosphorus (P) and calcium (Ca). The inclusion of P as a dietary macromineral in broiler farming is essential to drive various physiological processes. These comprise bone mineralization and integrity, acid-base balance, phospholipid and nucleotide formation, nerve function and cellular energy metabolism (ATP), which are critical to the sustenance of growth, productivity and overall welfare of the bird (Proszkowiec-Weglarz and Angel, 2013).

In practice, the amount of the bioavailable P in plant-based diets fed to broilers usually is insufficient, mainly due to the limited or lacking production of endogenous mucosal phosphatases required to facilitate intestinal P release. Therefore, inorganic P sources and exogenous phytases are obligatory to compensate for the low rate of digestible P obtained from cereals and other plant-based feed (Singh, 2008; Dersjant-Li et al., 2015). Nutritional strategies regarding broiler dietary P need to avoid, on the one hand, excessive P supplementation resulting in increased P excretion with the risk of environmental pollution and, on the other hand, avoid undersupply, which might negatively impact development, performance and health (Campbell et al., 2017; Tay-Zar et al., 2019). Hence, P supplementation in broiler diets needs to consider the birds' physiological demands at a specific developmental stage. In fact, adequate P supply in early development is critical for bone mineralization and body growth, prompting the need for high dietary mineral intake in the early stages (Rama Rao et al., 2003; Coto et al., 2008). During the maturation of the bird, the supply of P and administered phytase levels make an important contribution to the maintenance of physiological processes, including muscle differentiation, lipid metabolism and immune

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functions (Li et al., 2016; Schmeisser et al., 2017; Nari et al., 2020).

Therefore, life-time responses to nutritional conditioning for P efficiency in the broiler and its age-specific demand have shown that broilers fed depleted P at the starter phase manifested adaptive mechanisms of maintaining P homeostasis to support adequate bone mineralization and the modulation of other physiological attributes. The nutritional studies conducted by Yan et al. (2005), in which broilers were subjected to P depletion from d 19 of life, showed efficient P utilization and unchanged weight development in depleted birds compared to controls throughout their life-time, but compensatory actions at the bone level. Furthermore, in an experimental design on broiler chickens with P depletion and P repletion, there was an effect on zootechnical traits during P depletion, which was compensated during P repletion, suggesting a capacity to compensate for an initial depleted mineral status in broiler chickens (Letourneau-Montminy et al., 2008). As for the presumed mechanism, depleted P levels have been reported to stimulate the expression of genes encoding the transcellular P transport in the intestine to drive rapid recovery and equilibrium for the limited mineral supply (Yan et al., 2007; Proszkowiec-Weglarz and Angel, 2013; Lederer, 2014).

Endocrinal regulators such as parathyroid hormone (PTH) and calcitriol ($1,25(OH)_2$ vitamin D3) act on respective responsive organs, including the intestine, kidney and bone to maintain mineral homeostasis (Bergwitz and Jüppner, 2010; Proszkowiec-Weglarz and Angel, 2013). PTH prevents hypocalcaemia and acts on bone to mobilize Ca and P and reduces high serum P levels by promoting renal P excretion (Blaine et al., 2014). Moreover, PTH acts on calcitriol regulation, which in turn increases mineral absorption in the small intestine and reduces PTH expression in the parathyroid glands (Demay et al., 1992; Brenza et al., 1998). Thus, the interaction of PTH and calcitriol enables the regulation of systemic Ca and P levels via sophisticated feedback loops in the organismal biosystem. Due to variable dietary P supply, the broiler executes compensatory mechanisms by regulating serum calcitriol levels and transcellular P transport in the small intestine via variable abundances of sodium/phosphate co-transporters (Rousseau et al., 2016; Hu et al., 2018). The P retention and body reserves play a critical role in the timing and success of maintaining mineral homeostasis. The broiler's compensatory adaptation to variable P-supply, which incorporates hormonal, transcriptional and bone interactions, must be examined over all developmental stages (starter, grower, and finisher) to achieve economic growth rates with lower P supply in diets.

We hypothesize that the depletion of dietary P supply and its timing contribute to endogenous adaptive responses for P efficiency during the productive life of the broiler and its age-specific requirements. Comprehensive phenotyping which commences immediately post-hatch until market weight with P supply below, equal to or above current recommendations in the starter phase and its subsequent reduction within the groups in grower and finisher phases will identify limits and opportunities for the efficient use of mineral P in broiler chicken farming. The objective of the present study was to evaluate the effects of a variable P supply throughout the entire production phases via measurements for growth performance, endocrine control, transcellular P transport, bone mineralization, and health aspects in an array of tissues such as blood, jejunum, kidney, and bone.

MATERIALS AND METHODS

Ethical Statement

The study was approved by the Scientific Committee of the Research Institute of Farm Animal Biology (FBN), and the experimental setup was generally licensed by the ethics committee of the state Mecklenburg-Western Pomerania, Germany (LALLF MV 7221.3-1-051/16).

Broiler Chickens, Housing, Experimental Diets, and Design

The study was conducted at a poultry research facility and comprised Ross 308 broiler hatchlings of both sexes (n = 165). Hatchlings with an average body weight of 41.2 g were obtained from WIMEX Agrarprodukte GmbH (Regenstauf, Germany) and were raised on wood shavings as litter material in pens of 3.8 m^2 per dietary group. At any phase, the animal density in pens was below 25 kg/m^2, which assured that the current organic standards for broiler space requirements were fulfilled. Each pen was equipped with nipple drinkers and feeders for unrestricted access to water and feed. Lighting and temperature followed recommendations throughout starter (d 1–10; duration: 20 h; intensity: 20 lux; temperature: 30–35°C), grower (d 11–24; duration: 17 h; intensity: 20 lux; temperature: 30–35°C), and finisher phases (d 25–37; duration: 17 h; intensity: 20 lux; temperature: 30–35°C) (Aviagen, 2018). Broiler chickens have been subjected to an oral vaccination against Newcastle Disease at d 9 of life. Broilers were distributed in a completely randomized design where birds received a wheat-corn-soybean meal-based diet without phytase supplementation to minimize exogenous phytase activity. Diets were formulated without the addition of non-starch polysaccharide enzymes. All diets were fed in pelleted form and were formulated according to nutrient recommendations (GfE, 1999) except for P (Tables 1 and 2). This resulted in 3 dietary groups with recommended (M; according to Ross, 2014), lower (L; 50%), or higher (H; +50%) amounts of non-phytate P (nPP) fed during the starter developmental stage. The level of soluble phosphorus was quantified in the eluate. Phytase activity was analyzed spectrophotometrically with an LOD of 180 FTU/kg (EN ISO 30024). Birds were randomly assigned to 1 of 3 dietary groups of 55 animals

Table 1. Composition of the experimental diets for broiler chickens at starter, grower, and finisher phases.

		Starter (d 1–10)			Grower (d 11–24)			Finisher (d 25–37)		
Ingredient	Unit	50% nPP	100% nPP	+50% nPP	50% nPP	100% nPP	+50% nPP	50% nPP	100% nPP	+50% nPP
Wheat	%	30.0	30.0	30.0	32.0	32.0	32.0	33.5	33.5	33.5
Soybean meal (44% CP)	%	26.0	26.0	26.0	27.0	27.0	27.0	24.0	24.0	24.0
Corn, pre-treated[1]	%	19.4	19.4	19.4	21.0	21.0	21.0	24.0	24.0	24.0
Soybean concentrate (64% CP)	%	11.0	11.0	11.0	6.5	6.5	6.5	5.0	5.0	5.0
Soybean oil	%	5.4	5.4	5.4	6.3	6.3	6.3	6.3	6.3	6.3
Calcium carbonate	%	1.69	1.16	0.63	1.37	0.94	0.48	1.28	0.87	0.40
Cellulose powder	%	1.24	0.60	-	1.10	0.50	-	1.10	0.60	-
Corn starch, pre-gelatinized	%	1.1072	1.1172	1.0472	0.6573	0.7173	0.6273	0.8674	0.8374	0.8874
Brewer's dried yeast	%	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
Vitamin & trace element premix[2]	%	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
Monocalcium phosphate, (23% P)	%	0.44	1.60	2.80	0.33	1.30	2.35	0.27	1.21	2.23
Salt, NaCl	%	0.43	0.43	0.43	0.43	0.43	0.43	0.43	0.43	0.43
Choline Cl (50%)	%	0.39	0.39	0.39	0.37	0.37	0.37	0.35	0.35	0.35
DL-Methionine	%	0.35	0.35	0.35	0.34	0.34	0.34	0.29	0.29	0.29
Calcium propionate	%	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3
Lysine HCl	%	0.21	0.21	0.21	0.21	0.21	0.21	0.22	0.22	0.22
Manganese sulphate (33% Mn)	%	0.028	0.028	0.028	0.028	0.028	0.028	0.028	0.028	0.028
Zinc sulphate (36% Zn)	%	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01
Copper sulphate (24% Cu)	%	0.004	0.004	0.004	0.004	0.004	0.004	0.004	0.004	0.004
Vitamin D3 (500.000 IU/g)	%	0.00078	0.00078	0.00078	0.0007	0.0007	0.0007	0.0006	0.0006	0.0006
Threonine	%	-	-	-	0.05	0.05	0.05	0.05	0.05	0.05
ME	kcal/kg	2,988	2,988	2,988	3,059	3,059	3,059	3,107	3,107	3,107
Sum	%	100	100	100	100	100	100	100	100	100
Calcium	%	1.04	1.04	1.04	0.90	0.90	0.90	0.84	0.84	0.84
Phosphorus, total	%	0.51	0.78	1.05	0.47	0.69	0.93	0.44	0.65	0.88
Phosphorus, nPP[3]	%	0.26	0.52	0.78	0.23	0.45	0.68	0.21	0.42	0.64

[1]Corn, pre-treated – hydrothermal treatment.

[2]Vitamin & trace element premix (SNIFF Spezialdiäten GmbH, Soest, Germany) provided per kg of feed: vitamin A (retinyl acetate), 15.000 IE; vita-min D3 (cholecalciferol), 1.100 IE; vitamin E (all-rac-alpha-tocopheryl acetate), 100 mg; vitamin K3 (menadione), 7 mg; Fe (as FeSO4), 100 mg; Zn (as ZnSO4), 50 mg; Mn (as MnSO4), 30 mg; Cu (as CuSO4), 5 mg; Se (as Na2SeO3), 0.1 mg; I (as Ca(IO3)2), 2.0 mg.

[3]nPP, non-phytate phosphorus.

each (Figure 1). For the grower stage at d 11, a subset of sex-balanced broiler chickens were transferred into single cages (45 cm £ 45 cm £ 45 cm) equipped with nipple drinkers and feeders to record individual feed intake and body weight (Figure 1). Animals in the cages had visual contact with their conspecifics. The assignment to the respective dietary group, that is, M or H, was maintained to ensure adaptation to the new housing environment and grower feed. From d 17, broiler chickens kept in pens as well as those in cages were subjected to a dietary P depletion. Thus, broiler chickens were offered lowered dietary P levels in grower (ML, HL) and finisher phases (MLL, HLL) compared to the starter phase (Figure 1). Accordingly, the experimental design also included non-depleted control groups for the respective stages, that is, MM and HH for grower and MMM and HHH for finisher phases. Animals in pens were kept until d 24, whereas those in individual cages were kept until d 37. Due to high losses among the chickens that received the L diet in the starter phase, the trial was continued only with animals of the M and H groups (details are presented in the results section). Individual body weight of broiler chickens was recorded on d 10, d 17, d 24, and d 37 of life to capture the respective developmental phases.

Table 2. Wet-chemical analysis of the broiler chicken diets (g/kg as fed basis).

		Starter (d 1–10)			Grower (d 11–24)			Finisher (d 25–37)		
Ingredient	Unit	50% nPP[1]	100% nPP	+50% nPP	50% nPP	100% nPP	+50% nPP	50% nPP	100% nPP	+50% nPP
Dry matter	g/kg	908	907	909	903	904	904	905	903	902
Crude protein	g/kg	243	248	243	225	217	221	201	203	198
Crude ash	g/kg	59	63	65	55	59	62	50	53	57
Crude fat	g/kg	55	63	61	48	48	58	68	68	68
Sucrose	g/kg	50.7	51.5	52.2	53.8	52.5	52.2	53	52.4	53.3
Total starch	g/kg	347	347	354	365	363	362	397	393	399
Calcium	g/kg	10	9.8	9.9	8.7	9.1	9.2	7.9	8.1	8.4
Phosphorus, total	g/kg	4.7	7.2	9.5	4.3	6.4	8.6	4	6.1	8.1
Phosphorus, soluble	g/kg	2.5	3.7	4.9	2.5	3.5	4.6	2.2	3.4	4.4
Magnesium	g/kg	2.1	2	2	1.8	1.9	1.9	1.8	1.7	1.7
Potassium	g/kg	9.7	9.8	9.9	9.0	9.2	9.3	8.5	8.5	8.5
Sodium	g/kg	1.8	1.7	1.8	1.8	2.1	2	1.7	1.8	1.8
ME	kcal/kg	2,892	2,988	2,988	2,844	2,820	2,892	3,059	3,035	3,035
Phytase activity[2]	FTU/kg	211	<180	314	267	218	<180	267	194	199

[1]nPP, non-phytate phosphorus.

[2]Phytase activity was analyzed spectrophotometrically with an LOD of 180 FTU/kg (EN ISO 30024).

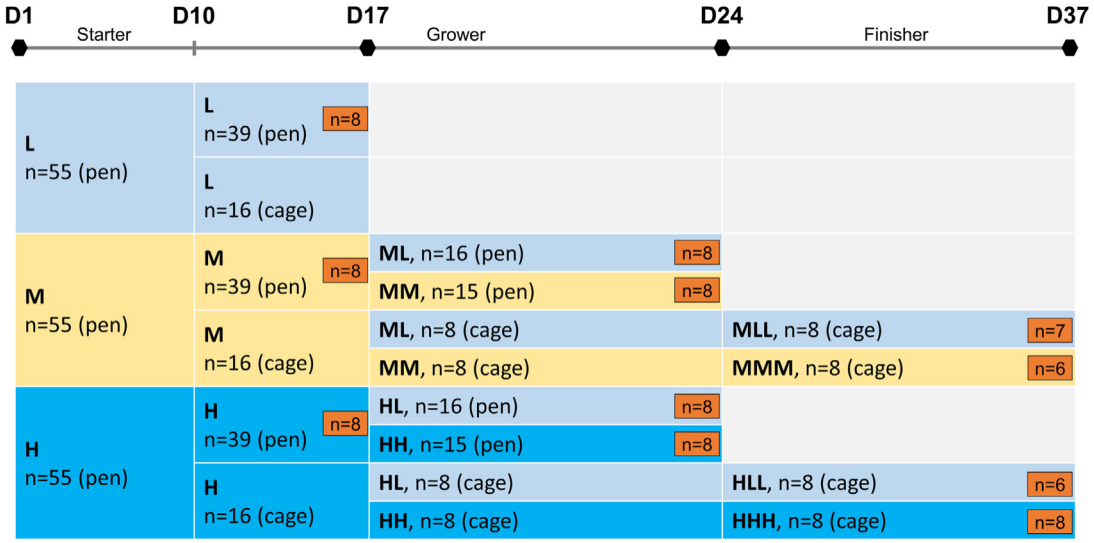

Figure 1. Experimental design. Broiler chickens received 1 of 3 experimental diets containing lower (L), medium (M), or higher (H) levels of die-tary P / non-phytate P (nPP) between d 1 and 17. Following an initial dietary assessment during the starter phase, a depletion strategy using a low dietary P level was applied during the grower (ML vs. MM; HL vs. HH) and finisher stages (MLL vs. MMM; HLL vs. HHH). The feeding trial included (i) animals housed in pens until d 24 and (ii) animals housed in individual cages until d 37. A subset of broiler chickens (n = 6–8) were sam-pled on d 17, 24, and 37 to obtain blood, jejunum, kidney, and bone samples as indicated by orange rectangles.

Serum and Tissue Sample Collection

A total of 83 broiler chickens were sampled at the 3 sampling stages, that is, d 17 (n = 24 from pens), d 24 (n = 32 from pens), d 37 (n = 27 from cages) as outlined in Figure 1. Sampling comprised 4 birds per sex per dietary group at the grower sampling stages (d 17, d 24) and at least 3 birds per sex per dietary group at the finisher sampling stage (d 37). At the respective growth stages, birds were randomly selected, anaesthetized by electrical stunning, and slaughtered between 09h00 and 12h00. Trunk blood was collected in anticoagulant-free tubes and allowed to clot for 20 min. Serum was prepared by centrifugation at 3500 £ g for 15 min. Serum samples were stored at 80°C until further analysis. Furthermore, sections of jejunal tissue (»3 cm in length) were collected proximal to the Meckel's Diverticulum. Jejunal samples were rinsed with a 0.9% NaCl solution, snap-frozen in liquid nitrogen and stored at 80°C until RNA extraction. Moreover, the right kidneys were sampled, snap-frozen in liquid nitrogen and stored at 80°C until RNA extraction. Finally, the right femurs of the birds were collected and stored at 0°C until further analysis.

Serum Minerals and Hormones Measurement

Serum samples were analyzed to determine calcium, inorganic P, and albumin levels using commercial assays for the Fuji DriChem 4000i device according to the manufacturer's instructions (FujiFilm, Minato, Japan). In addition, hormone measurements were prepared in duplicate and measured using commercially available enzyme-linked immunosorbent assay (ELISA) kits. Corresponding kits for ELISA were processed according to the manufacturer's instructions for PTH (CSB-E11880Ch, CUSABIO, Houston, TX), triiodothyronine (EIA-4569, DRG, Marburg, Germany), calcidiol (EIA-5396, DRG), and calcitriol (IDS-AC-62F1, Immunodiagnostic Systems, Frankfurt am Main, Germany). The raw data was processed according to 4-parameter logistic curve analysis.

Bone Breaking Strength and Ash

Femur samples were thawed overnight at room temperature. Individual bones were weighed, and the length, width, and maximum diameter (epiphysis) of the femur samples were measured to ascertain linear bone growth. The bone-breaking strength (force) was estimated at the calculated midpoint (50% of length) using a 3-point bending/flexural test device (WINOPAL Forschungsbedarf, Elze, Germany). The proximal and distal epiphyses of individual femur was placed horizontally on the 2 supporting anvils of the bending test device. Relative to the length of the femurs an adjusted diaphyseal free span (fulcrum point) ranging between 2.0 and 3.0 cm was set. The vertical loading anvil with a capacity of 50 kg was then applied to the mid-diaphysis of each bone at a speed of 2 mm/s until the bone failed. A computerized monitor recorded the load-displacement curve illustrating the estimated fracture load. Moreover, the ash percentage of femora was analyzed. Separated diaphyseal regions of the femur from the prior bending test were homogenized with a high-speed grinder. Approximately 1.5 g of homogenized samples were transferred in triplicates to pre-heated and weighed porcelain crucibles for incineration at 600°C for 7 h in the muffle furnace. Afterwards, samples were left overnight at 105°C in the drying cabinet. Samples were charred on the Bunsen burner and incinerated for 7 h in the muffle furnace at 600°C. The resultant material was cooled at room temperature. After adding a few drops of 30% hydrogen peroxide

Table 3. List of genes encoding annotated sodium/phosphate co-transporters in broiler chickens.

Gene symbol	Aliases	Ensembl ID	Gene name
SLC20A1	GLVR1, PiT-1	ENSGALG00000013740	Solute carrier family 20 member 1
SLC20A2	GLVR-2, Ram-1, PiT-2	ENSGALG00000038336	Solute carrier family 20 member 2
SLC34A1	NaPi-2a, NPT2a, NaPi-IIa	ENSGALG00000003075	Solute carrier family 34 member 1
SLC34A2	NaPi-2b, NPT2b, NAPi-IIb	ENSGALG00000014372	Solute carrier family 34 member 2

(H_2O_2), samples were transferred to the drying cabinet (105°C for 1 h). Samples were returned to the muffle furnace for incineration at 600°C for 10 min. Afterward, bone ash was allowed to cool in the desiccator for approximately 30 min followed by final weight measurement on a precision scale. The bone ash percentage was calculated (ash% = final weight / initial weight £ 100).

RNA Isolation, Purification, and cDNA Synthesis

Prior to RNA extraction, snap-frozen jejunal, and renal samples were transferred into a sterile ceramic mortal placed in a liquid nitrogen bath and pulverized with a pestle to homogenize. Subsequently, total RNA was isolated from the pulverized samples using TRI Reagent with adherence to the manufacturer's guidelines (Sigma-Aldrich, Taufkirchen, Germany). DNase I was used for DNA digestion followed by purification with column-based NucleoSpin RNA II-Kit (Macherey-Nagel, Düren, Germany). RNA concentration was determined using the NanoDrop ND-2000 spectrophotometer (Thermo Fisher Scientific, Dreieich, Germany). Further integrity test was done by visualization via agarose gel electrophoresis. The presence of genomic DNA contamination was checked by polymerase chain reaction (PCR) amplification of the chicken GAPDH gene using an intron-spanning primer set (forward primer: 5′-AGTCGGAGTCAACGGATTTG -3′; reverse primer: 5′ -CTGCCCATTTGATGTTGCTG- 3′). Subsequently, cDNA was synthesized using 1,500 ng of RNA, together with random primers (Promega, Mannheim, Germany), oligo d(T) nucleotides, and RNAsin plus (Promega), in the presence of SuperScript III Reverse Transcriptase (Invitrogen, Karlsruhe, Germany) according to the manufacturer's instructions. The cDNA samples were diluted with Aqua dest. to a final volume of 200 mL and stored at 20°C. Further verification for the absence of genomic DNA in the synthesized cDNA was conducted with another PCR for chicken GAPDH employing SupraTherm Taq DNA polymerase (GeneCraft, Münster, Germany).

Quantitative Real-Time PCR

To assess the contribution of transcellular P transporters in the kidney and intestine, gene expression of solute carrier family 20 and solute carrier family 34 members were analyzed. Presently, there are 4 known candidate genes encoding chicken transcellular P transporters (Table 3), namely; solute carrier family 20 member 1 (SLC20A1), solute carrier family 20 member 2 (SLC20A2), solute carrier family 34 member 1 (SLC34A1), and solute carrier family 34 member 2 (SLC34A2). Gene expression analysis was conducted using these 4 genes alongside b-actin (ACTB) as a housekeeping gene, to quantify the transcript abundance via real-time PCR assay. The gene-specific primers, corresponding annealing temperatures, and resulting fragment lengths are stated in Table 4. Individual cDNA samples were analyzed in duplicate on the LightCycler 480 System (Roche, Mannheim, Germany). Reactions were performed in a final volume of 12 mL comprising Light Cycler 480 SYBR Green I Master mix (Roche) and gene-specific primers. The temperature profiles included an initial denaturation step at 95°C for 5 min, followed by 45 cycles comprising denaturation at 95°C

Table 4. Gene-specific primers used for mRNA expression analysis via RT-qPCR.[1]

Gene symbol	Primer sequence (5′-3′)	Melting temperature (°C)	Amplicon length (bp)
SLC20A1	FOR: CTCTCGTCGTCTGGTTCTTTG	60	95
	REV: CTTCTCCATCAGCGGACTTTC	60	
SLC20A2	FOR: TGCTGCTACCATTGCTATTAACG	60	161
	REV: TTCTCTTCATCCAGGGGCATAC	60	
SLC34A1	FOR: CTTTTGCTGGTGCTACAGTGC	61	167
	REV: CGTGATGATTTTCAGCAGGTC	61	
SLC34A2	FOR: CTGATCTTGCCATCGGTCTC	60	170
	REV: TCCAGCCAGCCAAGTAAAAG	60	
ACTB*	FOR: CCTCTTCCAGCCATCTTTCTT	60	254
	REV: TAGAGCCTCCAATCCAGACA	60	

Abbreviations: ACTB, beta-actin; FOR, Forward; REV, Reverse; SLC20A1, solute carrier family 20 member 1; SLC20A2, solute carrier family 20 member 2; SLC34A1, solute carrier family 34 member 1; SLC34A2, solute carrier family 34 member 2.

[1]Primers used for the expression analysis were designed using the Primer-BLAST software on the NCBI platform (https://www.ncbi.nlm.nih.gov/tools/primer-blast/).

*Housekeeping gene.

for 10 s, annealing at the specific temperature for 15 s, and extension/fluorescence acquisition at 72°C for 25 s. The quality and specificity of amplified products were assessed by the melting curve analysis. For all assays, threshold cycles were converted to copy numbers of respective transcripts using standard curves generated by amplifying serial dilutions of the corresponding PCR standard (10^7 to 10^1 copies). Transcript copy numbers were factorial normalized based on ACTB expression values and log2 transformed prior to further analysis.

Statistical Data Analysis

The experimental design comprised 3 dietary P groups (L, M and H), with a subsequent dietary P depletion (ML, HL, MLL, HLL) and their controls (MM, HH, MMM, HHH) across the 3 distinct developmental stages (starter, grower, finisher) between 2 sexes of Ross 308 broilers raised under 2 housing conditions (individual cages, pens). The body weight and feed intake for the broiler chickens at the respective growth stages were measured and used to determine the body weight gain and feed conversion ratio (FCR). Traits were analyzed within each phase using the linear model: $g_{ij} = m + m_i + n_j + b_W + e_{ij}$, where g_{ij} are the measurements of the response variable (i.e., zootechnical traits, bone traits, serum traits and gene expression), m represents the overall mean, m_i represents effect of the dietary P group, n_j represents sex effect, b_W is the linear effect of the covariates individual body weight or femur weights as stated in the result section and e_{ij} represents the residual error. Mortality rates per diet group per phase were analyzed for association via Fisher's Exact Test. Analyses were performed using the R package stats and lmerTest (R core team, 2019; package; Kuznetsova et al., 2017). The pairwise comparison of means between dietary groups was achieved with the embedded Tukey post-hoc test. Differences were considered as statistically significant at $P \leq 0.05$.

RESULTS

In this study, the effects of dietary P depletion were investigated at 3 developmental stages throughout the productive life span, starting with a variable P supply immediately after hatching. The feeding regimen included P supply below, at, or above current recommendations early in life and subsequent P reduction within these groups. Responses of broilers were ascertained via performance, serum metabolites, bone parameters, and mRNA expression of transcellular sodium/phosphate co-transporters.

Zootechnical Parameters

Based on an average body weight of 41.2 g at d 1, the dietary treatments within the starter phase resulted in a body weight of 204 § 3 g (L; n = 62; mean § SE), 311 § 4 g (M; n = 60), and 312 § 4 g (H; n = 59) at d 10, which is statistically significant in the comparison between L and both M and H groups. Data for chickens kept in individual cages from day 10 onwards is presented in Table 5. At the early grower phase (d 10–17), L fed broiler chickens kept in cages showed significantly reduced body weight, feed intake, weight gain, and FCR compared to other dietary groups (Table 5). For body weights at d 17, similar results were obtained from birds kept in pens regarding group L (n = 29; 354 § 14 g), M (n = 35; 748 § 11 g), and H (n = 35; 769 § 13 g). The L group showed a significantly increased mortality in the phase from d 1 to 17 (31%) compared to M (5%) and H groups (7%). With advancing development, that is, the grower phase (d 17–24), feed intake, and mortality revealed no significant differences between the dietary groups for individual cages (Table 5) and pens for groups ML (n = 13; 1,308 § 21 g), HL (n = 13; 1,333 § 41 g), MM (n = 15; 1,266 § 30 g), and HH (n = 14; 1,352 § 46 g). Body weight and body weight gain significantly differed between the depleted P diet and control groups (Table 5). In addition, chickens of the ML group

Table 5. Body weight, feed intake, body weight gain and feed conversion ratio (FCR) of broiler chickens housed in individual cages and fed divergent amounts of dietary P throughout experimental phases. All values are displayed as mean § SE.

Phase	Diet (n)	Body weight (g)	Feed intake (g)	Body weight gain (g)	FCR (g/g)
d 10	L (n = 16)	226 § 4^{b}			
	M (n = 16)	335 § 6^{a}			
	H(n= 16)	332 § 4^{a}			
d 10–17	L (n = 10)	377 § 15^{b}	229 § 14^{b}	147 § 17^{b}	1.73 § 0.19^{a}
	M (n = 16)	613 § 14^{a}	406 § 18^{a}	278 § 13^{a}	1.49 § 0.08^{ab}
	H(n= 16)	629 § 18^{a}	396 § 16^{a}	298 § 17^{a}	1.35 § 0.03^{b}
d 17–24	ML (n = 8)	1093 § 41^{b}	646 § 39	480 § 23^{c}	1.35 § 0.07^{a}
	HL (n = 8)	1136 § 52^{ab}	627 § 27	528 § 25^{bc}	1.19 § 0.02^{b}
	MM (n = 7)	1201 § 52^{ab}	670 § 30	586 § 33^{ab}	1.15 § 0.02^{b}
	HH (n = 8)	1252 § 55^{a}	687 § 36	601 § 38^{a}	1.15 § 0.02^{b}
d 24–37	MLL (n = 7)	2171 § 145	1858 § 158	1086 § 71^{b}	1.80 § 0.12
	HLL (n = 6)	2159 § 199	1852 § 271	1177 § 115^{ab}	1.94 § 0.13
	MMM (n = 6)	2450 § 188	2021 § 210	1254 § 68^{ab}	1.69 § 0.08
	HHH (n = 8)	2632 § 94	2244 § 79	1436 § 39^{a}	1.63 § 0.03

Abbreviations: H, high P diet; HH, high-high P diet; HL, high-low P diet; HLL, high-low-low P diet; HHH, high-high-high P diet; L, low P diet; M, medium P diet; ML, medium-low P diet; MM, medium-medium P diet; MLL, medium-low-low P diet; MMM, medium-medium-medium P diet.

$^{a-c}$Column-wise disparity of superscripts indicates statistical significance ($P < 0.05$) between dietary P groups within phase.

revealed significantly higher FCR than the other groups. Finally, at the finisher phase (d 24–37), zootechnical data such as body weight, feed intake, FCR, and mortality were unaltered between dietary groups (Table 5).

Serum Mineral and Hormone Measurements

On d 17, L fed broiler chickens showed significantly reduced serum levels of P and triiodothyronine (T3) and significantly increased levels of albumin and calcitriol compared to those fed M and H diets (Figure 2). On d 24, significant differences were observed in serum levels of P, calcium, albumin, and calcitriol (Figure 3). The P depleted groups (ML, HL) showed reduced serum P levels but increased levels of calcium, albumin, and calcitriol compared with the control groups (MM, HH). On d 37, serum calcitriol levels differed significantly between P depleted groups (MLL, HLL) and animals fed MMM, but not compared to broilers fed HHH (Figure 4). Concentrations of calcidiol and PTH were unaffected by diet at all experimental stages.

Bone Trait Measurement

On d 17, L fed broiler chicken significantly differed for bone traits (force, weight, diameter, and ash) compared to those fed M and H diets (Table 6). On d 24, significant differences were observed in the bone fracture load of broilers fed the P depleted groups (ML, HL) and those fed MM. Analyses of other bone traits such as bone weight, length, diameter, and ash analyses showed no diet-dependent differences. On d 37, broiler chickens fed depleted P (MLL, HLL) significantly differed from those fed the MMM and HHH diet for breaking force. Femora bone diameter differed significantly between broilers fed the depleted P (MLL), and broilers fed the MMM and HHH. Values for bone weight, length, and ash remained unaffected.

Temporal Gene Expression of Jejunal and Renal Transcellular Phosphorus Transporters

On d 17, divergent P diets elicited significant differences in jejunal and renal mRNA expressions of sodium/phosphate co-transporters in the birds (Table 7). Specifically, increased jejunal expression of SLC34A2 (L>H; FC= 2.27) was observed in L fed broilers compared to those that received an H diet. In addition, renal mRNA expression of SLC20A1 significantly increased (M<H; FC = 3.61) in broilers fed the H diet compared to the M diet. Likewise, renal SLC20A2 transcripts differed between broilers fed the H diet compared to those fed the L diet (L<H; FC = 1.96) and those fed M diets (M<H; FC = 1.99). Renal SLC34A1 mRNA abundances differed significantly between broilers fed the L and M diet (L>M; FC = 2.04). Furthermore, renal SLC34A2 mRNA abundance increased (L<H; FC = 1.96) in the animals fed H compared to those fed the L diet. A tissue-specific expression was observed for both sodium/phosphate co-transporter II genes, with SLC34A2 predominantly expressed in the jejunum and SLC34A1 in the kidney. On d 24 and 37, jejunal and renal sodium/phosphate co-transporters expression were unaffected between the dietary groups.

DISCUSSION

Efficient nutrient utilization in broiler chickens needs to account for endogenous responses to variable P supply to benefit from enhanced P absorption in the intestine, P retention in the bone, and P reabsorption by the kidney. The age-appropriate provision of dietary P is particularly relevant in the early growth phase, when a sufficient amount of dietary P is necessary to meet physiological needs for health and tissue integrity (Baradaran et al., 2021). At the same time, excess dietary P must be prevented due to environmental burden, which has led to the development of feeding strategies tailored to meet age-specific requirements (Abbasi et al., 2019). In the current study, broilers fed the L diet during the early growth phase (d 1–17) had lower body weight and feed intake, while weight gain and FCR increased, accompanied by an increased mortality compared to broilers fed the M or H diets. This suggests that it is only possible to a limited extent to condition a thrifty phenotype with high P efficiency in later life by reducing P supply in early life. This observation contrasts with previous studies that reported broilers raised on dietary nPP levels as low as 0.25% and 1.0% calcium on d 1 to 21 could thrive without microbial phytase supplementation, although weight reductions of up to 15% were observed (Waldroup et al., 2000). It was also reported that dietary P depletion at early and late developmental stages can be applied to reduce P excretion, whereby dietary Ca intake has been shown to be a significant influencing factor (Rousseau et al., 2016). However, the dietary mineral composition applied to birds of the L group in our study (0.26% nPP, 1.04% Ca; Table 1) proved to be insufficient in the early growth phase. The inconsistent observations regarding the potential for P reduction between the studies might be due to the differences in dietary formulations and the interplay of feed components, including varying Ca:P ratios and the level and degradation of plant-based phytase (Sommerfeld et al., 2018). In addition, strain-specific metabolic requirements are also conceivable. In fact, a genetic contribution to P utilization has been shown in chickens and other species, including pigs and quails (Reyer et al., 2019; Vollmar et al., 2020). In addition, the decreased feed intake in broiler chickens raised on the L starter diet may indicate a humoral control of feeding behaviour, although in vertebrates the mechanisms for sensing P by integrating signals from different tissues are still largely unclear (Michigami et al., 2018).

A low P diet impacted bone traits of broilers chickens during each phase, including bone fracture load, weight, length, diameter, and ash, compared to the chickens in

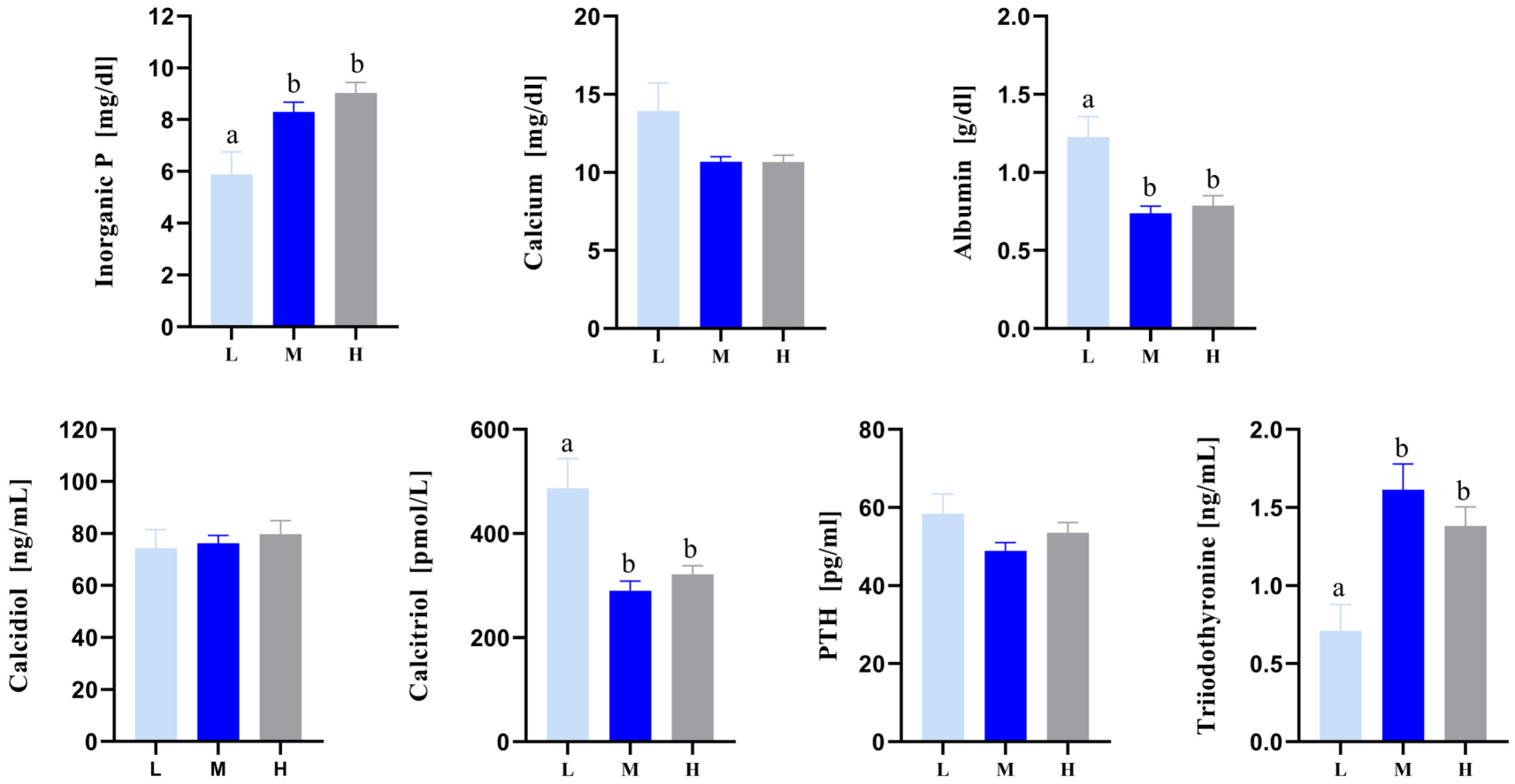

Figure 2. Serum parameters of broiler chickens fed divergent amounts of dietary P until 17 d of life. Values are displayed as means § SE. Superscripts indicate statistical significance ($P < 0.05$) between the dietary groups. PTH, parathyroid hormone; L, low P diet (n = 8); M - medium P diet (n = 8); H, high P diet (n = 8).

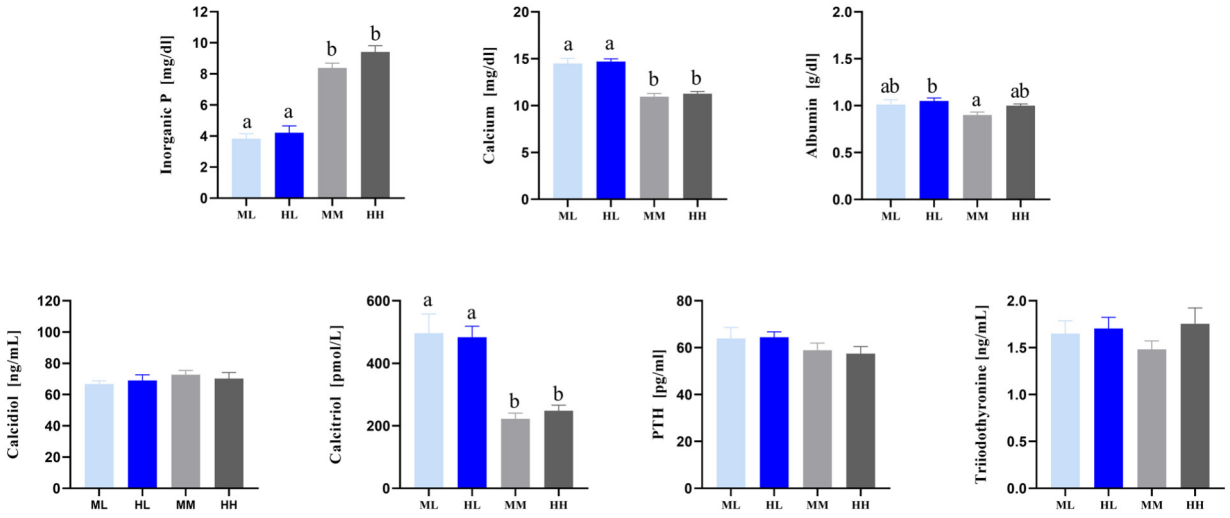

Figure 3. Serum parameters of broiler chickens fed divergent amounts of dietary P until 24 d of life. Values are displayed as means § SE. Superscripts indicate statistical significance ($P < 0.05$) between the dietary groups. PTH, parathyroid hormone; ML, medium-low P diet (n = 8); MM, medium-medium P diet (n = 8); HL, high-low P diet (n = 8); HH, high-high P diet (n = 8).

the M and H diet groups. The lowered bone mineralization indicates a reduced P availability to drive ossification (Taylor et al., 2013; Shao et al., 2019) or an increased mobilization of bone retained minerals to meet growth and other physiological processes (Li et al., 2020). Although the bone parameters obtained are in a comparable range to recent reports (Sözer et al., 2019; Eusemann et al., 2022), no conclusive statement on optimal mineralization in broilers can be concluded from these values. In the current study, broilers showed femoral sensitivity to P depletion, as P serves as a significant component of the bone due to its deposition in the complex form of hydroxyapatite ($Ca_5(PO_4)_3(OH)$) and is essential for the musculoskeletal development. Reduced feed intake of the L diets compared to M and H diets at d 17 certainly limited the available P pool within the birds resulting in age-inappropriate skeletal development. However, a previous study reported that broiler chickens fed depleted P diets at the grower phase showed similar consequences for bone traits, but also exhibited an increased propensity for bone mineralization processes when mineral deficiencies were replenished later in life (Valable et al., 2018).

Serum P concentrations of broilers fed the L diets until d 17 were lowest compared to broilers that received the M and H diets. This was further triggered due to the reduction in the broilers' feed intake. Contrastingly, serum calcitriol (active vitamin D) levels were highest in broilers fed the L diet compared to broilers fed the M and H diet. Calcitriol plays a pivotal role in the broiler's homeostatic regulation of P by mediating increased intestinal absorption, usually under the conditions of nutritional mineral deficit to attain equilibrium (Berndt and Kumar, 2009). Hence, vitamin D metabolism is essential for adaptation throughout the feeding phases studied to ensure P homeostasis. However, the physiological effect of the endogenous responses to the L diet in the early phase is clearly limited, as broiler chickens

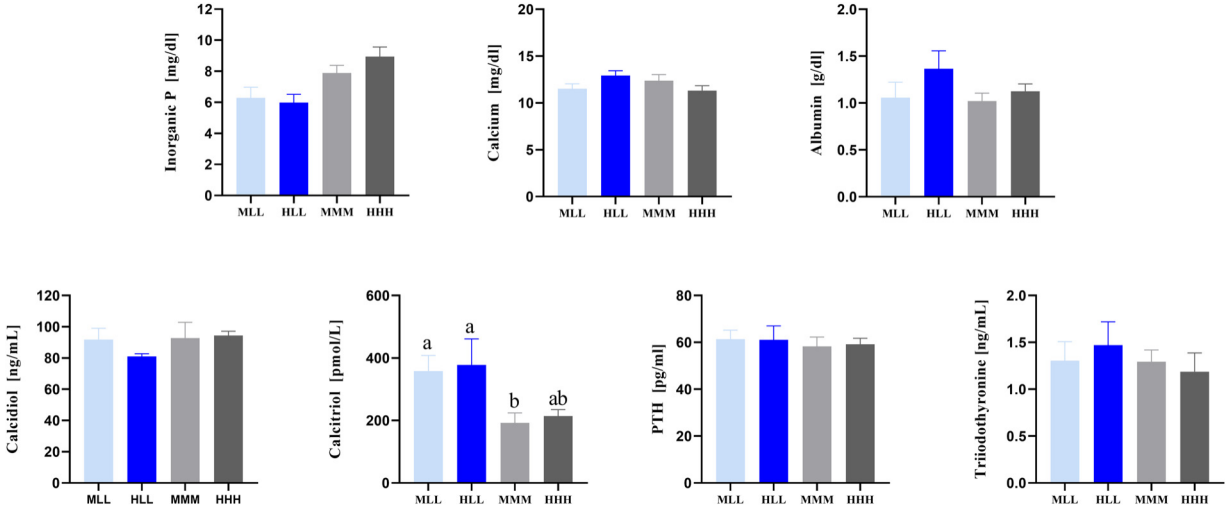

Figure 4. Serum parameters of broiler chickens fed divergent amounts of dietary P until 37 d of life. Values are displayed as means § SE. Superscripts indicate statistical significance ($P < 0.05$) between the dietary groups. PTH, parathyroid hormone; MLL - medium-low-low P diet (n = 7); MMM, medium-medium-medium P diet (n = 6); HLL, high-low-low P diet (n = 6); HHH, high-high-high P diet (n = 8).

Table 6. Femoral bone traits of broiler chickens fed divergent amounts of dietary P throughout experimental phases. All values are displayed as mean § SE.

Day of sampling	Diet (n)	[y]Fracture load (N)	Weight (g)	[z]Length (cm)	[z]Diameter (cm)	[z]Ash (%)
d 17	L (n = 8)	30.9 § 4.23[a]	1.9 § 0.09[a]	4.0 § 0.08[a]	0.5 § 0.02[a]	7.1 § 0.24[a]
	M (n = 6)	231.1 § 13.19[b]	3.9 § 0.27[b]	5.2 § 0.11[b]	0.6 § 0.02[b]	15.2 § 0.60[b]
	H(n= 7)	211.3 § 9.91[b]	3.8 § 0.21[b]	5.2 § 0.07[b]	0.7 § 0.02[b]	16.1 § 0.64[b]
d 24	ML (n=8)	199.7 § 7.73[ac]	6.8 § 0.32	6.4 § 0.05	0.8 § 0.01	18.4 § 2.05
	HL (n = 8)	214.7 § 13.17[c]	6.8 § 0.32	6.3 § 0.08	0.8 § 0.03	18.0 § 1.64
	MM (n = 6)	293.4 § 42.60[b]	6.7 § 0.45	6.3 § 0.08	0.8 § 0.03	17.2 § 1.38
	HH (n = 7)	253.3 § 22.34[abc]	6.5 § 0.51	6.2 § 0.14	0.8 § 0.04	20.8 § 1.09
d 37	MLL (n = 7)	192.8 § 16.79[ab]	10.5 § 0.98[ab]	7.5 § 0.20	0.9 § 0.04[a]	16.1 § 0.79
	HLL (n = 6)	176.7 § 15.28[b]	9.8 § 0.60[a]	7.3 § 0.16	0.9 § 0.02[ab]	16.1 § 0.85
	MMM (n = 5)	259.2 § 43.06[c]	11.3 § 1.46[ab]	7.3 § 0.19	1.1 § 0.09[b]	18.5 § 1.59
	HHH (n = 7)	261.3 § 11.39[c]	11.8 § 0.74[b]	7.6 § 0.13	1.1 § 0.04[b]	17.6 § 0.81

Abbreviations: H, high P diet; HH, high-high P diet; HL, high-low P diet; HLL, high-low-low P diet; HHH, high-high-high P diet; L, low P diet; M, medium P diet; ML, medium-low P diet; MM, medium-medium P diet; MLL, medium-low-low P diet; MMM, medium-medium-medium P diet.

[y]Body weight factored as covariate.

[z]Femoral weight factored as covariate.

[a–c]Column-wise disparity of superscripts indicates statistical significance ($p<0.05$) between dietary P groups within phase.

showed higher serum albumin and lower serum triiodothyronine (T_3) concentrations compared to broilers fed the M and H diets. Albumin plays a physiological role in maintaining colloidal osmotic pressure and acts as a marker for renal integrity. Serum levels indicate a severe malnourishment of broilers fed the L diet, suggesting dehydration as well as catabolic metabolism due to the reduction in feed intake (Wadden et al., 1990). In fact, P deprivation has been reported to induce hypothyroidism, and therefore, systemic growth reduction, especially at the early stages of development in the broiler chicken (Parmer et al., 1987; Jianhua et al., 2000). The skeleton represents a target tissue for T_3, which contributes to the regulation of bone turnover (Williams, 2013). Decreased T_3 levels are associated with fracture risk and may be related to impaired bone resorption and formation via reduced osteoblast differentiation and function (Vestergaard et al., 2005; Tuchendler and Bolanowski, 2014). Studies in murine bone cells revealed that a higher T_3 concentration increased bone resorption and made the osteoblasts more sensitive to the actions of PTH (Schmid et al., 1986). Hence, higher serum calcium levels coupled with high levels of T_3 are probably due to the interactive effects of T_3 on bone resorption and recruitment of osteoblasts in response to PTH.

Significant differences were observed in serum concentrations of P and Ca between the depleted P groups (ML, HL) and controls (MM, HH) at d 24, indicative of the birds' attempt to achieve mineral homeostasis for both micronutrients with advancing age. Broadly, broilers from the grower phase onwards (d 18–37) exhibited the capacity to tolerate the effects of dietary P for zootechnical properties (Waldroup et al., 2000; Bar et al., 2003). In this context, endocrine control

Table 7. Gene expression levels of sodium/phosphate co-transporters expressed in jejunum and kidney of broiler chickens fed divergent amounts of dietary P throughout experimental phases. Transcript copy numbers were presented as log2 values (mean § SE).

Day of sampling	Tissue	Diet (n)	SLC20A1	SLC20A2	SLC34A1	SLC34A2
d 17	Jejunum	L (n = 8)	13.8 § 0.44	14.4 § 0.56	8.4 § 0.91	17.7 § 0.30[a]
		M (n = 8)	14.2 § 0.45	13.9 § 0.61	7.0 § 0.81	17.0 § 0.36[ab]
		H (n = 8)	14.0 § 0.39	13.8 § 0.59	5.8 § 0.56	16.5 § 0.25[b]
	Kidney	L (n = 8)	13.4 § 0.48[ab]	14.4 § 0.23[a]	21.2 § 0.26[a]	8.2 § 0.20[a]
		M (n = 8)	12.0 § 0.41[a]	14.4 § 0.25[a]	20.2 § 0.29[b]	8.5 § 0.29[ab]
		H (n = 8)	13.9 § 0.21[b]	15.4 § 0.20[b]	20.4 § 0.19[ab]	9.1 § 0.23[b]
d 24	Jejunum	ML (n = 8)	12.2 § 0.97	12.4 § 0.22	5.8 § 0.59	16.6 § 0.37
		HL (n = 8)	9.9 § 1.93	11.8 § 0.40	7.3 § 0.28	15.9 § 0.49
		MM (n = 8)	10.7 § 1.45	11.8 § 0.20	5.6 § 0.62	16.0 § 0.31
		HH (n = 8)	11.3 § 1.02	12.1 § 0.22	3.9 § 1.25	16.1 § 0.24
	Kidney	ML (n = 8)	13.5 § 0.43	13.4 § 0.45	20.1 § 0.69	7.6 § 0.65
		HL (n = 8)	15.3 § 1.34	15.9 § 1.46	23.9 § 1.31	10.0 § 1.46
		MM (n = 8)	13.5 § 0.90	13.7 § 1.16	19.3 § 2.33	9.3 § 1.21
		HH (n = 8)	12.6 § 0.94	13.7 § 0.17	20.5 § 0.44	7.5 § 0.28
d 37	Jejunum	MLL (n = 7)	13.3 § 1.09	13.1 § 0.54	8.5 § 0.77	16.1 § 0.82
		HLL (n = 6)	13.0 § 0.74	11.7 § 0.99	11.2 § 0.84	15.5 § 0.41
		MMM (n = 6)	13.4 § 0.91	12.2 § 0.98	9.8 § 0.78	14.3 § 0.92
		HHH (n = 8)	13.2 § 0.65	12.6 § 0.47	8.3 § 0.87	16.0 § 0.82
	Kidney	MLL (n = 7)	14.0 § 0.58	14.7 § 0.90	22.0 § 1.04	9.8 § 1.30
		HLL (n = 6)	14.5 § 1.38	14.9 § 1.18	21.5 § 1.05	8.9 § 1.07
		MMM (n = 6)	14.8 § 0.26	14.3 § 0.27	19.4 § 1.05	8.2 § 0.46
		HHH (n = 8)	15.8 § 0.86	15.0 § 0.95	20.8 § 0.32	9.0 § 0.96

Abbreviations: H, high P diet; HH, high-high P diet; HL, high-low P diet; HLL, high-low-low P diet; HHH, high-high-high P diet; L, low P diet; M, medium P diet; ML, medium-low P diet; MM, medium-medium P diet; MLL, medium-low-low P diet; MMM, medium-medium-medium P diet.

[a–b]Column-wise disparity of superscripts indicates statistical significance ($P < 0.05$) between dietary P groups within tissue within phase.

mechanisms represent the adaptive response to address reductions of P supply. Notably, the effect of the serum calcitriol remained elevated in L diet broilers throughout the entire developmental phase, indicating subtle effects such as the continued endocrine mediation of intestinal absorption and renal reabsorption of P (Li et al., 2021).

Dietary effects on other serum metabolites (P, Ca, albumin, calcidiol, PTH, and T_3) were not present in the depleted P groups and controls at d 37. However, broilers fed L diets at the grower and finisher phases showed effects on bone traits compared to the broilers fed the control and H diets. Although bone fracture load was reduced at d 24, that is after only 1 week on an L diet, the results indicate intact tissue development and absence of pathophysiologic abnormalities. The broiler chickens fed high P diets across developmental phases showed no merits for zootechnical, endocrine, or bone traits compared with birds fed recommended and depleted P levels. It must be noted that numerical differences in the respective traits exist between the experimental groups, which require the validation of the results with a larger sample size. In view of the current results, providing safety margins for dietary P supply do not reveal beneficial outcomes for the bird, but rather exacerbates environmental impacts further associated with the scarcity of P resources (Campbell et al., 2017). This observation is in line with previous findings focusing on high P bioavailability (Gautier et al., 2018).

The jejunum has been reported as the primary site of intestinal P absorption in the broiler (Hurwitz and Bar, 1970). Notably, intestinal P absorption in the broiler occurs via paracellular (passive) and transcellular (active) transport mechanisms. Whereas the former involves the selective molecule diffusion or inhibition through alternative sealing or pore-forming characteristics via the tight-junction protein permeability gradients, for example, claudins (Marks, 2019). The latter entails the recruitment of sodium/phosphate dependent co-transporters located at the brush border membrane (Eto et al., 2006). The transcellular pathway is thought to be the preferential intestinal P absorption route under dietary P restrictions (Marks, 2019). The expression of jejunal SLC34A2 in response to the diet in the current study suggests that intestinal P availability in the diets affected the abundance of sodium/phosphate co-transporters in jejunal cells to maintain homeostasis and adaptation at the earlier growth phase (Hu et al., 2018). In fact, several studies highlighted an increased expression of genes encoding transcellular P transport in the small intestine of both broiler chickens and laying hens on exposure to deficient P diets, implicating a cellular response to luminal P concentrations via endocrinal factors, for example, calcitriol (Rousseau et al., 2016; Hu et al., 2018; Knopfel et al., 2019; Proszkowiec-Weglarz et al., 2019; Sommerfeld et al., 2020). In addition, the expression of the SLC34A1 gene in the kidney of L fed broilers indicated its contributory regulatory role, including reabsorption of P at the proximal tubule to achieve P homeostasis. The renal type III Na^+-P co-transporters were more abundant in broilers of the H group compared to L and M (SLC20A2) and M group (SLC20A1), respectively. Previous studies on murine models identified responses of the type III Na^+-P co-transporters to changes in dietary P contents (Candeal et al., 2017; Marks, 2019). Albeit the type III Na^+-P co-transporters were expressed in response to P levels within the organism's biosystem, suggesting possible transmembrane transport and intracellular utilization, their precise functional potential for P regulation remains unclear (Marks, 2019).

Genes encoding sodium/phosphate co-transporters in the jejunum and kidney remained unaffected throughout the grower and finisher phase (d 18–37), in contrast to observable responses at the endocrine and bone level. This observation suggests that broilers use other compensatory mechanisms to achieve P efficiency in addition to subtle changes in active transcellular P transport in the gut and kidney. In other studies, a low-P diet has not consistently been shown to increase mRNA abundance of transcellular P transporters (Just et al., 2018; Reyer et al., 2021). This could indicate complementary paracellular P transport, post-transcriptional modifications, or miRNA-mediated regulation mechanisms. However, it also broadly informs the broilers' capacity to cope with the P nutrient challenge, for example, depletion, through synergistic interactions of endocrinal and genetic factors towards zootechnical performance and bone traits. Adequate P supply in the early growth phase is thus the basis for long-term physiological P efficiency in the finisher phase (Valable et al., 2020; Baradaran et al., 2021).

In summary, broiler chickens showed physiological responses to different dietary P levels at different developmental stages, as shown by the interplay between serum P and Ca levels, endocrine responses in terms of calcidiol, calcitriol, PTH, and triiodothyronine levels, bone strength and mineralization, and jejunal and renal P transporter gene expressions to maintain P homeostasis for production and welfare. Based on these results, the threshold for P deprivation for environmental concerns should be set no earlier than the late start/early growth phase, as physiological adaptation mechanisms to P deficiency seem more effective than in the early growth phase. In this study, a one-third reduction in P intake in the early growth phase up to d 17 resulted in severe developmental abnormalities that could not be tolerated and compensated. Consequently, nutritional strategies such as the efficient application of phytases and targeted P reduction in the late rearing or early finishing phase are conceivable. The feeding management might be in accordance to the MLL group, as broilers at this age show higher tolerance and faster compensation capacity without compromising production as well as health traits by the adaptation mechanisms investigated in this study.

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DISCLOSURES

The authors declare no conflicts of interest.

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6.4 Jejunal Microbiota of Broilers fed varying Levels of Mineral Phosphorus

Poultry Science

Jejunal microbiota of broilers fed varying levels of mineral phosphorus

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ABSTRACT Efforts to achieve sustainable phosphorus (P) inputs in broiler farming which meet the physiological demand of animals include nutritional intervention strategies that have the potential to modulate and utilize endogenous and microbiota-associated capacities. A temporal P conditioning strategy in broiler nutrition is promising as it induces endocrinal and transcriptional responses to maintain mineral homeostasis. In this context, the current study aims to evaluate the composition of the jejunal microbiota as a functional entity located at the main absorption site involved in nutrient metabolism. Starting from a medium or high P supply in the first weeks of life of broilers, a depletion strategy was applied at growth intervals from d 17 to 24 and d 25 to 37 to investigate the consequences on the composition of the jejunal microbiota. The results on fecal mineral P, calcium (Ca), and phytate contents showed that the diets applied to the depleted and non-depleted cohorts were effective. Microbial diversity in jejunum was represented by alpha diversity indices which appeared unaffected between dietary groups. However, chickens assigned to the dietary P depletion groups showed significantly higher abundances of Facklamia, Lachnospiraceae, and Ruminococcaceae compared to non-depleted control groups. Based on current knowledge of microbial function, these microorganisms make only a minor contribution to the birds' adaptive mechanism in the jejunum following P depletion. Microbial taxa such as Brevibacterium, Brachybacterium, and genera of the Staphylococcaceae family proliferated in a P-enriched environment and might be considered biomarkers for excessive P supply in commercial broiler chickens.

Key words: phosphorus excretion, intestinal microbiota, mineral supply, mineral homeostasis, nutritional conditioning

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INTRODUCTION

Dietary phosphorous (P) is essential to all life forms owing to its multifaceted functions within the organismal biosystem. In the broiler, the significance of P as a mineral constituent of the diet has been established due to its pivotal role in physiological processes relating to the bird's growth and productivity when efficiently utilized. In plant-based diets, P is stored as the salt form of phytic acid [myo-inositol 1,2,3,4,5,6-hexakisphosphate; $InsP_6$], also referred to as phytate. However, broilers have limited capacity to utilize phytate-P due to inadequate production of enteral phytase/phosphatases needed to hydrolyze phytate (Rama Rao et al., 1999), accounting for environmental P losses (Panagos et al., 2022). On conventional farms, both mineral P and phytases of microbial origin are supplemented to diets. The latter is used to enzymatically degrade phytate and release inorganic P, a practice prohibited in organic livestock production (Council of the European Union, 2007). However, apart from such exogenous routes regarding the enteric bioavailability of various P-sources, the broiler chicken has demonstrated the capacity to efficiently allocate P resources through endogenous mechanisms involving mineral deposition in the bone, mediated by endocrinal and transcriptional control and adaptation in the intestine and kidney (Shao et al., 2018; Omotoso et al., 2023).

With regard to the efficient utilization of plant-bound P, the diversity and functional contribution of the intestinal microbiota is also an interesting target. The microbiota represents a dynamic constituent of the gastrointestinal tract (GIT) that colonizes after hatching and is defined by several factors that can be broadly divided into i) host characteristics, for example, bird age, strain, sex, GIT section, and ii) the environmental factors, for example, husbandry system, feed, geographic location, and biosecurity (Kers et al., 2018; Ngunjiri

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et al., 2019). Interestingly, colonization cascades have been described in which intestinal pioneer colonizers could shape the subsequent microbiota composition of individual birds (Rubio, 2019). In fact, the microbiota is acknowledged to contribute to the dynamics of complex structural, metabolic and immunological processes that define the host's health, welfare and age-appropriate development (Rubio et al., 2014).

The intestinal microbiota contains specific phosphatase-secreting microbes such as the Bifidobacteria (Haros et al., 2005) and isolates of Lactobacillus (Kim et al., 2007), which are capable of hydrolyzing phytate and release inorganic P to the host for absorption. Moreover, the dietary supply of macrominerals such as P and calcium to the monogastric species has been reported to modulate the gut microbiota (Ptak et al., 2015; Reyer et al., 2021), thus, indicating the microbiota as a potent, functional entity involved in nutrient metabolism (Grice and Segre, 2012).

Furthermore, accumulating scientific studies on the chicken microbiota focused on the distal ileocecal region of the GIT, for example, the caeca or colon, due to the high diversity of the microbial community in this GIT section crucial to mediate the hydrolysis of phytate which determines the corresponding levels of P and inositol phosphates excreted to the environment (Witzig et al., 2015; Yan et al., 2017). However, the homeostasis and metabolism of P are initiated in the proximal small intestine, specifically, the jejunum, where active and passive transport processes enable P uptake and thus facilitate increased absorption and utilization after enzymatic phytate degradation (Hurwitz and Bar, 1970). Hence, investigating the broiler's jejunal microbiota composition under different dietary P supply might be informative.

Additionally, due to P loss concerns, recent studies which investigated the broiler's physiological adaptation to moderate dietary P reductions initiated at the early growth phase highlighted the bird's capacity to incorporate different intrinsic compensatory mechanisms to define nutrient efficiency with maturity (Valable et al., 2018; Baradaran et al., 2021). Endogenous mechanisms observed include the synergy between endocrinal regulators (e.g., calcitriol), gene expression (e.g., SLC34A2), and the contribution of intrinsic organismal P reservoirs such as the bone.

Physiologically, calcitriol ($1,25(OH)_2$ vitamin D) mediates transcellular P uptake in the intestine via its receptor (VDR) which acts on the promoter of the sodium-dependent P transporter to stimulate its expression. The bone facilitated P homeostasis due to its remodeling attributes (e.g., dynamics of mineral P storage and resorption), by enabling effective P resource allocation and adaptation in response to the reduced dietary P intake (Hu et al., 2018; Li et al., 2020). Moreover, previous studies in pigs fed variable dietary P levels showed significantly differential abundances of the intestinal microbial genera, suggesting the possibility of targeted manipulation of the microbial community in the intestine by feeding interventions for an improved intestinal phytate utilization (Reyer et al., 2021).

Therefore, we hypothesized that the jejunal microbiota of broilers fed varying dietary P levels from the early grower until finisher phase synergizes with the endogenous mechanisms adopted by the bird to maintain P homeostasis. The objective of the present study was to evaluate the jejunal microbiota composition of broilers subjected to P depletion throughout the grower and finisher stages. Additionally, corresponding measurements of total fecal P, calcium, and phytate were conducted to approximate the unutilized fractions.

MATERIALS AND METHODS

Ethical Statement

The animal experimental setup was approved by the Scientific Committee of the Research Institute for Farm Animal Biology (FBN) and licensed by the animal welfare and ethics committee of the state Mecklenburg-Western Pomerania, Germany (LALLF 7221.3-1-051/16).

Experimental Birds, Management, and Diets

The feeding trial refers to a larger experiment involving previously reported performance data (Omotoso et al., 2023). The experiment was conducted at the poultry research facility of the FBN. In this study, a total of n = 110 Ross 308 broiler hatchlings of both sexes obtained from WIMEX Agrarprodukte GmbH was used (Regenstauf, Germany). In brief, birds were raised on wood shavings as litter material in pens of 3.8 m^2 with a stocking density below 25 kg/m^2, which meets current organic standards for broiler spacing requirements. Each pen was equipped with feeders and nipple drinkers for unrestricted access to feed and water. Birds were randomly allotted to 2 dietary groups comprising 55 animals each housed in 1 pen per treatment. From the grower stage at d 11, a total of 39 birds per group remained in the respective pens, while a subset of sex-balanced broiler chickens (n = 16 per dietary group) were transferred into individual metabolic units (45 cm £ 45 cm £ 45 cm) equipped with feeders and nipple drinkers to enable access to feed and water. The metabolic units were designed to ensure the birds' visual contact with their conspecifics and guarantee to record individual bird's zootechnical parameters including feed intake. The dietary regimen comprised starter (d 1–10), grower (d 11–24), and finisher diets (d 25–37). The wheat-corn-soybean meal-based diet was formulated without the addition of nonstarch polysaccharide enzymes or phytase. The fed diets for starter, grower, and finishers were pelleted and formulated according to the nutrient recommendations of the Gesellschaft für Ernährungsphysiologie (GFE, 1999) except for P (Supplemental Table 1). The experimental diets contained either recommended (M; 100% according to Ross, 2014) or higher (H; +50%) amounts of nonphytate P (nPP)

in the respective grower and finisher feeds. From d 17, half of the broilers in cages were subjected to dietary P depletion (50%), wherein chickens were offered lowered dietary P levels in the grower stage (i.e., ML, HL) and finisher phases (MLL, HLL). Accordingly, the experimental design also included the non-depleted (recommended and high) groups for the respective stages, that is, MM and HH for grower and MMM and HHH for the finisher phases, respectively. As previously published (Omotoso et al., 2023), the analyzed values for crude protein (CP) in grower diets (L: 225 g/kg; M: 217 g/kg; H: 221 g/kg) and finisher diets (L: 201 g/kg; M: 203 g/kg; H: 198 g/kg) as well as metabolizable energy (ME) in grower diets (L: 2,844 kcal/kg; M: 2,820 kcal/kg; H: 2,892 kcal/kg) and finisher diets (L: 3,059 kcal/kg; M: 3,035 kcal/kg; H: 3,035 kcal/kg) were comparable.

Jejunal Digesta and Fecal Sample Collection

A total of n = 75 broilers were considered in this study with an emphasis on d 17, d 24, and d 37. For jejunal digesta collection at d 17 (n = 16 from pens), d 24 (n = 32 from pens), and d 37 (n = 27 from single cages), birds were anesthetized by electrical stunning and slaughtered by exsanguination. Whole jejunum tissue (approx. 5 cm) was collected proximal to the Merkel's diverticulum and cut lengthwise to sample the intestinal digesta gently with a spatula. Digesta were snap-frozen in liquid nitrogen and stored at 80°C until DNA isolation. Additionally, deposited fecal samples from a period of 4 h were collected from birds housed in single cages at d 17, d 24, and d 37. Fecal samples were stored at 20°C until further analysis.

DNA Isolation, 16S rRNA Gene Amplicon Sequencing and Bioinformatics Analysis

Microbial DNA was isolated from the broiler digesta samples using the DNeasy PowerLyzer PowerSoil Kit (QIAGEN, Hilden, Germany) according to the manufacturer's guidelines. The samples were incubated at 70°C and 95°C for 10 min each before bead-beating with Precellys 24 homogenizer (PEQLab Biotechnology GmbH, Darmstadt, Germany). DNA concentration was determined using the NanoDrop ND-2000 spectrophotometer (Thermo Fisher Scientific, Dreieich, Germany). Amplicons of the 16S rRNA gene were synthesized in duplicates using primers specific to the V4 (515F: GTGCCAGCMGCCGCGGTAA and 806R: GGACTACHVGGGTWTCTAAT) hypervariable region alongside adapters and barcodes (Hugerth et al., 2014). The polymerase chain reaction was performed with the GoTaq G2 Hot Start Master Mix (Promega, Walldorf, Germany), with temperature, timing, and cycle regimen set as follows: initial denaturation step at 95°C for 2 min, 35 cycles, denaturation at 95°C for 30 s, annealing at 50°C for 60 s and 72°C for 90 s, and a final extension at 72°C for 10 min. Amplicons were prepared in duplicates, combined, purified, and normalized using a SequalPrep normalization plate (Thermo Fisher Scientific, Darmstadt, Germany). Afterward, libraries were sequenced on a HiSeq 2500 instrument (Illumina, San Diego, CA). After demultiplexing, raw data were analyzed with the mothur software (version 1.44.1) (Schloss et al., 2009). The Silva reference database (release 138) was employed for the global alignment of the 22,942,877 sequence reads, after which annotated operational taxonomic units (OTUs) were retrieved at 97% sequence identity.

Fecal Mineral Content and Phytate Measurement

The freeze-dried fecal samples were weighed, milled, and digested via microwave treatment to solve the analytes and obtain an effective yield. Total P and calcium (Ca) content was ascertained via inductively coupled plasma-optical emission spectroscopy (ICP-OES) (UEA Consulting Ltd., Norwich, UK).

For phytate quantification, a total of 100 mg of fecal samples were added to 500 mL deionized water and homogenized on ice. After a centrifugation step for 20 min at 10,000 rpm at 4°C, the supernatant was stored at 20°C and used for further analyses. The fecal phytate content was analyzed in a microplate format via a colorimetric assay (orb707384, Biorbyt, Cambridge, UK).

Statistical Data Analysis

The broiler chicken's daily P and Ca intake was calculated based on feed intake and the corresponding dietary composition for the respective periods as presented previously (Omotoso et al., 2023). These parameters and fecal P, Ca, and phytate were analyzed at each phase using a linear model: $g_{ij} = m + d_i + s_j + e_{ij}$, where g_{ij} are the measurements of the response variable (i.e., zootechnical traits, fecal minerals), m represents the overall mean, d_i represents effect of the dietary P group, s_j represents sex effect, and e_{ij} represents the residual error. Analyses were performed using the R package stats and lmerTest (Kuznetsova et al., 2017; R Core Team, 2023). The pairwise comparison of means between dietary groups was achieved with the embedded Tukey post hoc test. Microbial alpha diversity parameters including the Shannon diversity index, inverse Simpson index, and species richness using the abundance-based coverage estimator (ACE) were analyzed to ascertain the jejunal microbiota richness, evenness, and diversity in response to the dietary P using vegan package v2.5-7 and phyloseq v1.42.0 embedded in R (R Core Team, 2023). Furthermore, the non-metric multidimensional scaling (NMDS) ordination was visualized based on the Bray-Curtis dissimilarities and the similarities were checked with the analysis of similarities (ANOSIM) approach in vegan package v2.5-7 embedded in R (R Core Team, 2023). The relative abundance of the microbiota was visualized using taxa plot at the genus level employing the R software (R Core Team, 2023). Differences were

considered as statistically significant at $P \leq 0.05$. After subsampling the 16S data for each sample to 96,873 reads, dietary effects on microbial abundance were assessed at the genus level for samples collected at d 24 and d 37 using Wald test statistics embedded within the DESeq2 in R platform (DOI:10.18129/B9.bioc.DESeq2). At least 30 counts in more than 6 individuals were used as filtering criteria at the genera level. Genera with a Benjamini-Hochberg-adjusted P value <0.05 were considered as statistically significant.

RESULTS

The current study investigated the role of the broiler's jejunal microbiota in adapting and contributing to maintain the host's nutrient efficiency following a P depletion strategy.

Mineral Intake of Total Phosphorus and Calcium

The early grower developmental phase (d 10–17) revealed significant differences between the dietary intake of P between the M and H groups, indicating that the feed formulations were effective (Table 1). Following the dietary P depletion (d 17–24), significantly lower levels were observed for P intake in the depleted groups (ML, HL) compared to non-depleted groups (i.e., recommended, MM and high, HH). Moreover, total P intake was higher in HH animals compared to MM animals. The total Ca intake was significantly reduced in HL compared to HH animals. At d 25 to 37, total P intake was significantly reduced in the depleted groups (MLL, HLL) compared to the respective non-depleted animals (MHH, HHH). Moreover, total P and calcium intake were higher in HHH animals compared to MMM animals at this growth phase (d 25–37).

Fecal Content of Total Inorganic Phosphorus and Calcium

At d 17, the analysis of total mineral P in fecal samples revealed a significantly higher level in H (n = 16; 25.87 § 0.75 g) animals compared to M (n = 16; 15.63 § 0.52 g) animals (Figure 1A). At d 24 and d 37, the P depletion groups ML (n = 8; 7.55 § 0.35 g), HL (n = 8; 8.67 § 0.41 g), MLL (n = 7; 8.13 § 1.24 g), and HLL (n = 6; 8.76 § 0.78 g) showed reduced fecal P levels compared to the respective non-depleted groups MM (n = 7; 16.86 § 1.02 g), HH (n = 8; 23.75 § 0.55 g), MMM (n = 6; 18.40 § 0.96 g), and HHH (n = 8; 28.78 § 1.23 g). Additionally, the comparison of the non-depleted groups revealed significantly higher levels of fecal P in HH and HHH compared to MM and MMM (Figure 1A). The Ca levels in the broilers' feces differed significantly at d 24 with higher levels in the depleted HL group (n = 8; 26.85 § 1.03 g) compared to the corresponding non-depleted HH group (n = 8; 21.44 § 0.85 g) (Figure 1B). Total fecal Ca levels were unaffected by the diets at d 17 and 37 (Figure 1B). At d 17, the analysis of fecal phytate revealed a significantly higher level in H (n = 14; 10.36 § 0.96 mmol/g) animals compared to M (n = 13; 3.16 § 0.48 mmol/g) animals (Figure 1C). At d 24 and d 37, the P depletion groups ML (n = 7; 1.09 § 0.27 mmol/g), HL (n = 6; 0.64 § 0.29 mmol/g), MLL (n = 7; 1.96 § 0.76 mmol/g), and HLL (n = 6; 2.07 § 0.67 mmol/g) showed lower fecal phytate levels compared to the non-depleted groups HH (n = 8; 12.61 § 1.68 mmol/g) and HHH (n = 8; 9.19 § 1.36 mmol/g), but did not differ significantly from groups MM (n = 6; 4.24 § 1.72 mmol/g) and MMM (n = 6; 4.92 § 1.96 mmol/g). Additionally, the comparison of the non-depleted groups revealed significantly higher levels of fecal phytate in HH compared to MM at d 24 (Figure 1C).

Table 1. Total phosphorus (P) and calcium (Ca) intake of broilers raised in individual metabolic units and fed divergent amounts of dietary P throughout the developmental phases. Values are displayed as mean § SEM.

Phase	Diet (n)	Total P intake (mg/d)	Ca intake (mg/d)
D 10–17	M (n = 16)	418 § 18[b]	568 § 25
	H (n = 16)	537 § 21[a]	560 § 22
D 17–24	ML (n = 8)	397 § 24[c]	803 § 49[a,b]
	HL (n = 8)	385 § 17[c]	779 § 34[b]
	MM (n = 7)	612 § 28[b]	870 § 39[a,b]
	HH (n = 8)	844 § 45[a]	903 § 48[a]
D 24–37	MLL (n = 7)	572 § 49[c]	1129 § 96[b]
	HLL (n = 6)	570 § 83[c]	1125 § 164[b]
	MMM (n = 6)	948 § 99[b]	1295 § 131[b]
	HHH (n = 8)	1398 § 49[a]	1450 § 51[a]

[a,b,c]Indicate significant differences between groups ($P < 0.05$); L, low P diet; M, medium P diet; H, high P diet. Consecutive letters indicate the dietary treatment in experimental periods.

Alpha Diversity Indices of Jejunal Microbiota

To ascertain the response of the jejunal microbiota to the varied P levels fed to the broiler chickens at the grower and finisher developmental phases, alpha diversity indices which account for the distribution or abundance of OTUs within the population were calculated. Shannon, inverse Simpson, and ACE indices revealed no significant differences between the P dietary groups at d 24 and d 37 (Figure 2A and B).

Composition of Jejunal Microbiota

The non-metric dimensional scaling ordination was used to access the compositional and structural variation of broiler chicken jejunal microbiota. The NMDS analysis revealed an age-based clustering of the jejunal microbial communities (ANOSIM, $r^2 = 0.238$, $P = 0.001$) with no corresponding influence of the varied P diets within age ($P > 0.05$, Figure 3).

Relative Abundance of Jejunal Microbiota

Regarding the relative abundance of microbiota in the broilers' jejunum fed varied P diets, visualization using a taxaplot at the genera level identified the Lactobacillus

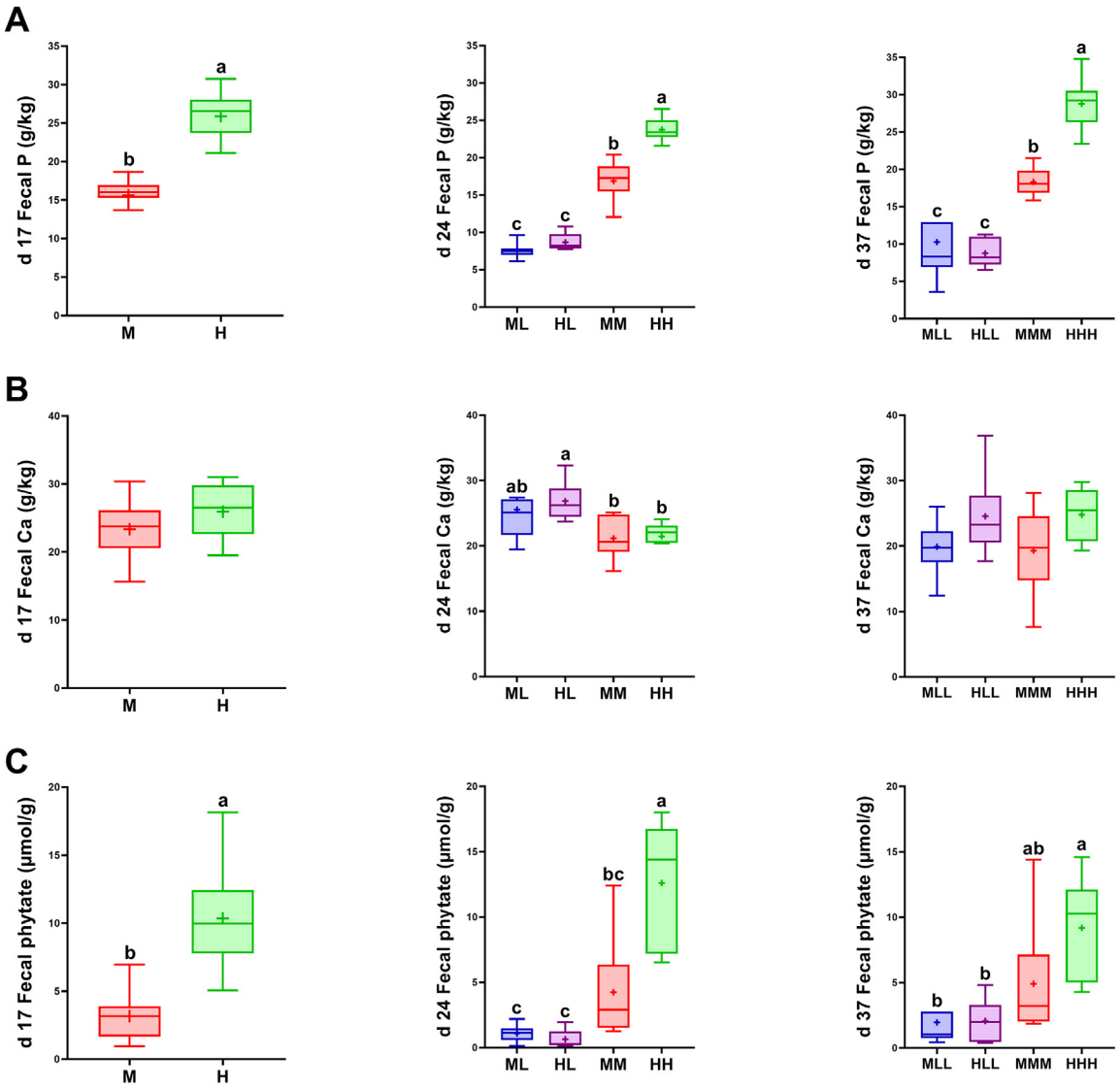

Figure 1. Fecal content of total inorganic phosphorus (A), calcium (B), and phytate (C) of broilers fed varied levels of dietary P at d 17, d 24, and d 37 of life; L, low P diet; M, medium P diet; H, high P diet: Consecutive letters indicate the dietary treatment in each experimental periods. [a,b,c]Indicate significant differences between groups ($P < 0.05$).

of the phylum Firmicutes as the most predominant genus (Figure 4). Further comparisons of relative abundance at genus level between dietary groups revealed 16 and 4 significantly differentially abundant taxa at d 24 and at d 37, respectively (Table 2). At d 24, significantly increased relative abundance of the genera Facklamia (HL>HH) was observed in jejunum of broilers fed depleted P diets compared to non-depleted birds. The relative abundances of genera that decreased in the low-P diets compared to the non-depleted diets included Anaerocolumna (HL<HH), Blautia (HL<HH), Brachybacterium (ML<MM; HL<HH), Brevibacterium (HL<HH; ML<MM), Candidatus arthromitus (HL<HH), Fusicatenibacter (HL<HH), Jeotgalicoccus (ML<MM), Lachnoclostridium (HL<HH), Monoglobus (HL<HH), unclassified genera of Staphylococcaceae (HL<HH), and Staphylococcus (HL<HH; ML<MM). At d 37, a significant differential relative abundance between the depleted and non-depleted P groups was observed in the genera Romboutsia (MLL<MMM), while unclassified genera of Lachnospiraceae (HLL>HHH) and Ruminococcaceae (HLL>HHH) increased in the broilers fed depleted compared to those fed the non-depleted P diets (Table 2). For comparisons of MM and HH diets at d 24, jejunal microbes were observed to be increased in broilers fed the high P diet, including Blautia (HH>MM), Candidatus arthromitus (HH>MM), Eisenbergiella (HH>MM), unclassified genera of Enterobacteriaceae (HH>MM), Monoglobus (HH>MM), and unclassified genera of Selenomonadaceae (HH>MM). In contrast, decreased abundances were found for unclassified genera of Staphylococcaceae (MM>HH) and Aerococcus (MM>HH). At d 37, a decrease in the differential abundance of microbes was observed between the MMM and HHH diets in Escherichia-Shigella (MMM>HHH) and Romboutsia (MMM>HHH). The abundance of Escherichia-Shigella suggests the possibility of a recent infection episode in individuals of the MMM group.

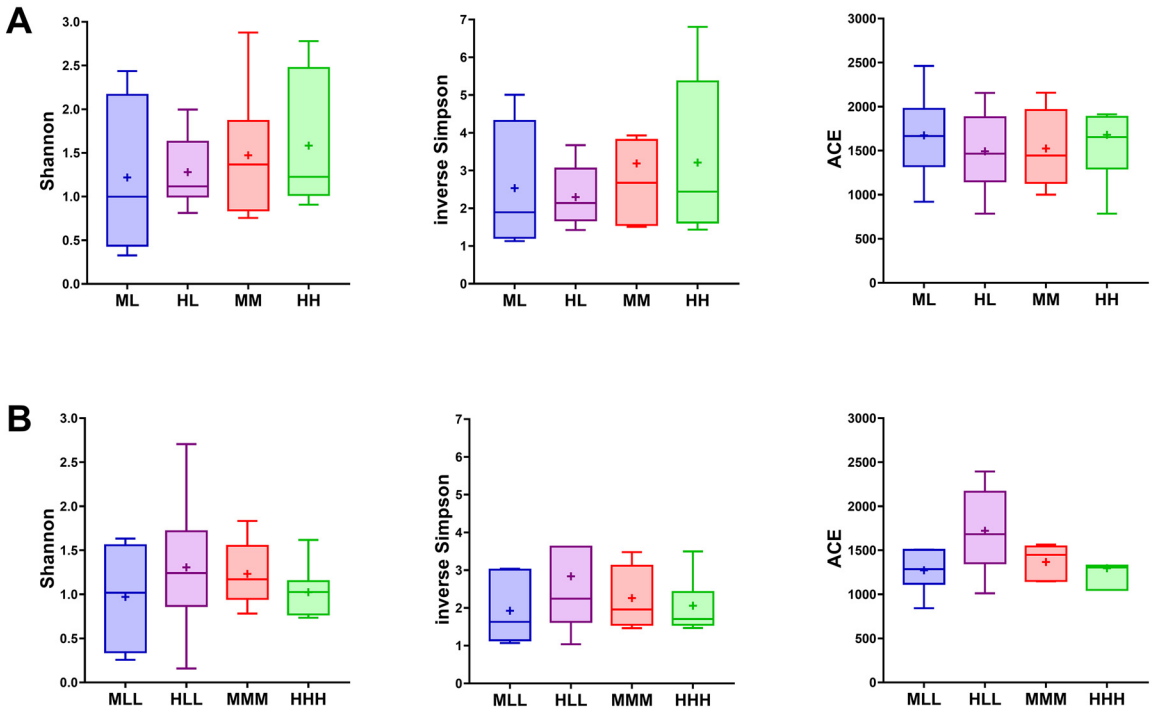

Figure 2. Boxplots showing the alpha diversity of the jejunal microbiota of broilers chickens fed varying amounts of phosphorus (P) at d 24 (A) and d 37 (B). Data based on 16S sequencing were used to calculate the Shannon index, inverse Simpson index and species richness using ACE. ACE, abundance-based coverage estimator; L, low P diet; M, medium P diet; H, high P diet. Consecutive letters indicate the dietary treatment in each experimental period.

DISCUSSION

To mitigate the immediate concerns associated with the environmental loading of P from broiler husbandry (Maguire et al., 2005), studies have investigated effects of moderate dietary P depletion initiated at an earlier growth phase and subsequently repleted with advancing ages for mineral efficiency (Valable et al., 2018; Baradaran et al., 2021). Indeed, our recent analyses on the physiological response of modern high-performance broiler lines challenged with depleted P diets revealed intrinsic mechanisms, spanning hormones, bone traits, as well as intestinal and renal P transporters to maintain P turnover (Omotoso et al., 2023). Subsequently, the resultant fecal P levels mirrored the dietary P intake in the depleted, recommended, and high P feeding groups. A previous study found that broilers fed a balanced Ca: nPP ratio of 2:1 (i.e., 6 g/kg and 3 g/kg DM) had the lowest fecal P excretion, while higher nPP levels at a ratio of 1.33:1 (i.e., 6.0 g/kg and 4.5 g/kg DM) resulted in increased fecal P content (Rama Rao et al., 2006). It was observed in the current study that the birds fed the HL diet had higher levels of fecal Ca at d 24 in compari-son to the non-depleted cohorts. Nevertheless, previous findings on broiler chickens fed reduced inorganic P showed increased calcitriol in the blood and intestinal Ca-binding protein levels leading to improved Ca absorption (Friedlander et al., 1977; Wasserman et al., 1992). In fact, serum Ca levels were elevated in both ML and HL compared with the non-depleted groups (Omotoso et al., 2023). In parallel, the analyses revealed evidence of increased bone resorption in the depleted broiler chickens compared with the non-depleted groups. This could indicate increased mineral mobilization to balance P requirements as a consequence of the applied depletion strategy, with excess Ca being excreted accordingly. Comprehensive studies concluded that

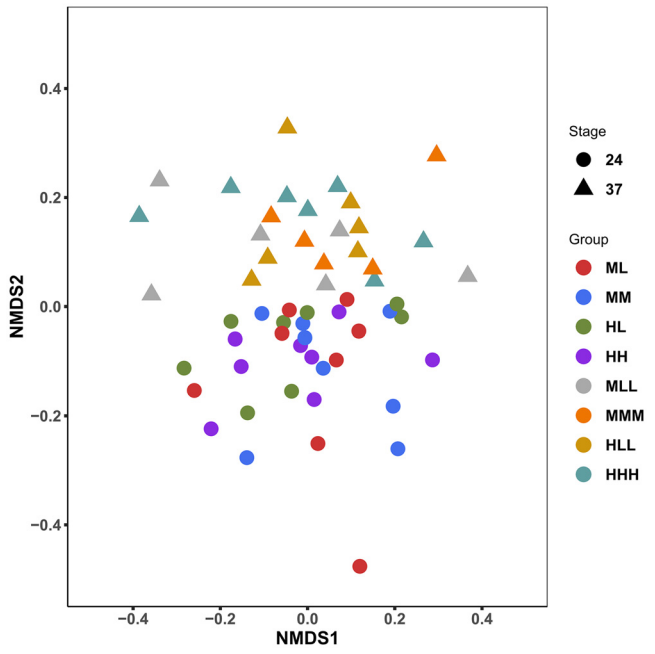

Figure 3. Nonmetric dimensional scaling (NMDS) ordination showing the compositional variation of jejunal microbiota in broilers fed divergent phosphorus (P) diets. Animals were sampled at d 24 (ML, HL, MM, HH) and at d 37 (MLL, HLL, MMM, HHH). L, low P diet; M, medium P diet; H, high P diet. Consecutive letters indicate the dietary treatment in each experimental period.

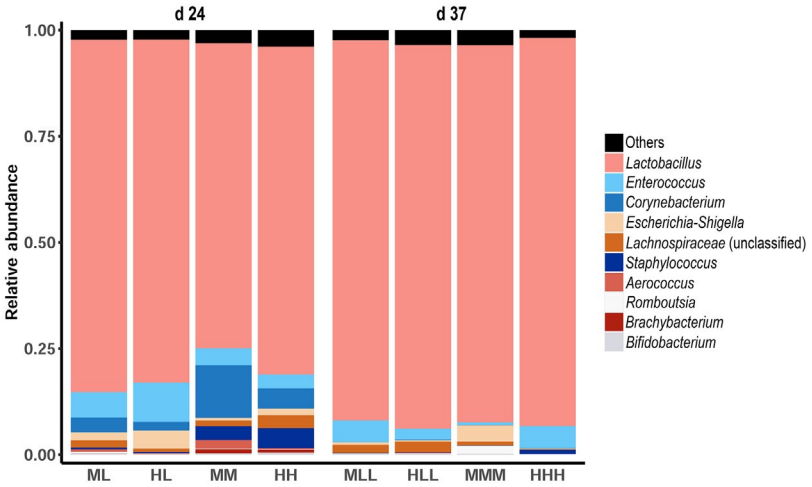

Figure 4. Taxonomic plot showing the 10 predominant relative abundance of the microbial genera in jejunal digesta of broilers fed divergent phosphorus (P) diets. Animals were sampled at d 24 (ML, HL, MM, HH) and at d 37 (MLL, HLL, MMM, HHH). L, low P diet; M, medium P diet; H, high P diet. Consecutive letters indicate the dietary treatment in each of the experimental periods.

current recommendations for total Ca content in broiler feed formulations might be overestimated (David et al., 2023), affecting P absorption rates (Selle et al., 2009). In the current study, the resultant fecal P levels were significantly higher in the broilers fed the high P diets than those fed recommended P levels throughout the grower and finisher periods. Higher P excretion rates due to mineral P supplements above recommendations have also been demonstrated in other monogastric species such as pigs (Reyer et al., 2021), with no added benefit observed for bone mineralization (Gerlinger et al., 2021). Thus, the results indicate that broilers fed the high P diet received mineral fractions that exceeded their metabolic demands for growth or maintenance

Table 2. Relative abundances of genera in the jejunal digesta collected from broilers fed varied levels of dietary phosphorus (P) at the grower and finisher developmental stages. Listed genera differed significantly (Benjamini-Hochberg-adjusted P < 0.05) between dietary groups, with the log2 fold-change expression in a given comparison. Data are presented as mean § SEM.

D 24	Relative abundance of jejunal microbiota within the dietary P groups (%)				Contrast	
Genera	ML	HL	MM	HH	Fold change (log2)	Adjusted P value
Aerococcus	0.6 § 0.360	0.12 § 0.027	2.04 § 1.068	0.15 § 0.082	MM>HH (3.77)	<0.001
Anaerocolumna	0.02 § 0.014	0.003 § 0.002	0.03 § 0.016	0.18 § 0.073	HH>HL (5.55)	<0.001
Blautia	0.09 § 0.047	0.03 § 0.008	0.11 § 0.087	0.22 § 0.120	HH>HL (3.06)	0.010
					HH>MM (3.29)	0.008
Brachybacterium	0.02 § 0.008	0.01 § 0.005	0.95 § 0.522	0.7 § 0.302	HH>HL (5.62)	<0.001
					MM>ML (5.55)	<0.001
Brevibacterium	0.003§ 0.001	0.002 § 0.001	0.04 § 0.026	0.12 § 0.070	HH>HL (4.90)	<0.001
					MM>ML (3.90)	0.028
Candidatus arthromitus	0.02 § 0.015	0.01 § 0.003	0.01 § 0.007	0.14 § 0.047	HH>HL (5.49)	0.001
					HH>MM (3.46)	0.009
Eisenbergiella	0.02 § 0.010	0.02 § 0.017	0.01 § 0.003	0.04 § 0.022	HH>MM (3.20)	0.044
Enterobacteriaceae (uncl.)	0.03 § 0.019	0.07 § 0.028	0.02 § 0.007	0.34 § 0.273	HH>MM (4.07)	0.001
Facklamia	0.04 § 0.021	0.05 § 0.016	0.19 § 0.082	0.004 § 0.002	HL>HH (3.37)	0.017
					MM>HH (5.28)	0.001
Fusicatenibacter	0.05 § 0.031	0.01 § 0.005	0.02 § 0.014	0.11 § 0.056	HH>HL (3.31)	0.017
Jeotgalicoccus	0.05 § 0.023	0.03 § 0.014	0.5 § 0.204	0.03 § 0.017	MM>HH (3.97)	0.008
					MM>ML (3.32)	0.044
Lachnoclostridium	0.02 § 0.008	0.01 § 0.006	0.03 § 0.008	0.09 § 0.029	HH>HL (2.83)	0.007
Monoglobus	0.04 § 0.018	0.01 § 0.004	0.02 § 0.012	0.17 § 0.074	HH>HL (3.51)	0.008
					HH>MM (3.10)	0.027
Selenomonadaceae (uncl.)	0.01 § 0.007	0.03 § 0.015	0.001 § 0.001	0.03 § 0.030	HH>MM (4.01)	0.008
Staphylococcaceae (uncl.)	0.002 § 0.001	0.002 § 0.001	0.26 § 0.150	0.02 § 0.010	HH>HL (3.87)	0.009
					MM>HH (3.83)	0.008
					MM>ML (6.78)	<0.001
Staphylococcus	0.44 § 0.266	0.22 § 0.064	3.3 § 1.872	4.83 § 2.538	HH>HL (4.46)	<0.001
					MM>ML (2.93)	0.039
D 37	Relative abundance of jejunal microbiota within the dietary P groups (%)				Contrast	
Genera	MLL	HLL	MMM	HHH	Fold change (log2)	Adjusted P value
Escherichia-Shigella	0.61 § 0.185	0.28 § 0.092	3.81 § 3.658	0.12 § 0.035	MMM>HHH (4.94)	0.001
Lachnospiraceae (uncl.)	1.87 § 1.673	2.55 § 1.890	0.91 § 0.522	0.18 § 0.051	HLL>HHH (3.86)	0.010
Romboutsia	0.04 § 0.017	0.11 § 0.102	1.86 § 1.789	0.04 § 0.015	MMM>HHH (5.70)	0.002
					MMM>MLL (5.45)	0.010
Ruminococcaceae (uncl.)	0.34 § 0.303	0.53 § 0.446	0.10 § 0.067	0.03 § 0.014	HLL>HHH (4.44)	0.010

L, low P diet; M, medium P diet; H, high P diet. Consecutive letters indicate the dietary treatment in each experimental period.

with no additional benefit for the measured traits but resulted in unnecessary fecal losses as reported elsewhere (Rodehutscord et al., 2012; Li et al., 2017).

The gut microbiota has been associated with crucial homeostatic and metabolic processes, which entail, for example, the hydrolysis of phytate in the broiler's GIT, culminating in the host's productivity and welfare (Rinttila and Apajalahti, 2013; Li et al., 2016). In the current study, the overall microbial diversity represented by alpha diversity indices revealed no alterations based on dietary P depletion. Based on the microbial dissimilarity analysis, an age-dependent separation of profiles was observed. In accordance, previous studies have reported clear effects of age on the microbial community that colonizes the broiler's GIT (De Cesare et al., 2019; Zhou et al., 2021). Furthermore, the dominance of the Lactobacillus in the current study was consistent with several previously reported studies (Borda-Molina et al., 2016; Kers et al., 2018; Künzel et al., 2021), where Lactobacillus presence in the gut accounted for up to 99% of the microbiota fraction. Functionally, the abundance of Lactobacillus in the gut has been positively correlated with beneficial functions, including improved gut physiology and increased body weight gain in the chicken (Lokapirnasari et al., 2019; Zhang et al., 2022). More so, it is inferable that the prevalence of Lactobacillus might indicate a low complexity of jejunal microbiota in broiler chickens.

The analysis of microbial abundances, particularly the fact that hardly any genera increase in abundance following a P depletion, suggests that shifts in the jejunal microbiota make only a minor contribution to maintaining P homeostasis in broilers. Nevertheless, it remains conceivable that transcriptional changes of the abundant microbiota are affecting mineral metabolism, which would need to be tested by metatranscriptomic approaches.

The phytate profile reported in the current work serves as proxy for intestinal microbiota activity. Results show significantly lowered concentrations in birds fed medium P than in birds fed high P levels at d 17 and d 24, indicating hydrolysis of phosphoric ester forms mediated by a phytase that, based on current knowledge, is likely secreted by the intestinal microbiota in broiler chickens. Indeed, phytase activity of microbiota has been mainly observed in the lower part of the intestinal tract such as the caecum (Dersjant-Li et al., 2015). The observed shifts in fecal phytate content matches previous studies (Shastak et al., 2014) and suggests efficiency mechanisms already at early age. Results further suggest that birds fed currently recommended P levels (M diets) mobilize P from plant sources. The P-depleted groups exhibited a nearly maximal degradation of phytate at d 24 and d 37, providing evidence for intestinal dephosphorylation of phytate to meet metabolic demands. However, to what extent the P mobilized from intestinal phytate is available to the host or the microbiota remains further clarification. According to previous work, P mobilization from intestinal inositol phosphates can be assumed to be incomplete as lower phosphoric ester forms, that is, the degradation products of phytate, are present in feces at varying amounts (Gautier et al., 2018). A study in piglets identified microbial taxa that were positively or negatively correlated to intestinal P levels in terms of proliferation (Reyer et al., 2021). In the current study, exclusively the genus Facklamia exhibited an increased abundance in the broiler cohort fed the HL compared to HH diets at d 24. The abundance of Facklamia was previously reported to be related with housing and litter management, that is, with higher abundances in fresh litter compared to reused litter material (Wang et al., 2016; Song et al., 2022). The information on its role in relation to nutrient metabolism in the broiler is absent. For certain species of Facklamia, enzymatic profiles revealed activity for alkaline phosphatases, while acid phosphatase activities were not observed (Lawson et al., 1999). At d 37, an incremental shift in the gut of broilers fed the HLL diet compared to those that received HHH was observed for unclassified genera belonging to families Lachnospiraceae and Ruminococcaceae. Broadly, both the Lachnospiraceae and Ruminococcaceae microbiota families are categorized as beneficial in the human GIT and have been implicated in the fermentation of carbohydrates (Duncan et al., 2007), coupled with the degradation of resistant polysaccharides, for example, starch and cellulose, facilitating digestion of plant-based diets (Collier et al., 2008). The identified taxa may be of interest in further studies to reshape the microbial composition for improved nutrient utilization from dietary P sources.

In contrast to the microbes whose abundance increased after the P depletion diet, Brachybacterium, Brevibacterium, and genera of the Staphylococcaceae family showed significantly lower abundances in the jejunum, which was consistently observed in both P depletion groups compared to the respective non-depleted controls on d 24. Apparently, these microbial families rely on an intestinal milieu with higher available P content, implying overgrowth of these taxa when P resources are scarce. In this context, an increased intestinal abundance of Brachybacterium has been reported from patients with disturbed mineral balance based on chronic kidney disease (Vaziri et al., 2013). Several species of Brevibacterium were described as phosphate-accumulating probiotics, which might be more prevalent in high P supply (Anand et al., 2019). Indeed, some taxa show a dominant growth pattern whereby the microbial community structures are subject to dynamics in their composition with corresponding effects on the ability to respond to abiotic and biotic factors (Niccum et al., 2020). The results suggest that the increased proliferation of these mentioned microbial taxa due to high P supply could be considered a biomarker of excessive P intake in commercial broiler chickens.

At the same time, several other taxa were shown here to be generally responsive to the divergent P supply in the different diets. The microbial profiles may support the current hypothesis that an increase in intestinal P levels in mammals stimulates microbial short-chain fatty

acid (SCFA) production (Heyer et al., 2015). This is consistent with a study in broilers in which a decrease in SCFA, DL-lactate, and acetic acid in the ileum was observed following low P and low Ca diets and subsequently, an increase in these parameters was observed after phytase supplementation (Ptak et al., 2015). This agrees with in vitro studies on the fermentation activity of rumen bacteria, which identified an association between depleted P levels and a reduction in SCFA and bacterial ATP production (Komisarczuk et al., 1987). Moreover, a recent study in chickens reported that dietary P deficiency resulted in decreased SCFA production due to reduced cellulose fermentation, suggesting that intestinal P content modulates the abundance of fibrolytic bacteria (Li et al., 2022).

A body of literature supports some of the observed genera in the context of intestinal SCFA production and P utilization in poultry. Among such taxa are the Blautia, Anaerocolumna, Candidatus arthromitus, and unclassified genera of Selenomonadaceae, observed to increase significantly in broilers fed the high P diet. The Blautia, an anaerobic bacteria specie which clusters into the Clostridium XIVa group, became of particular interest in human microbiomics since the knowledge of its ability to synthesize SCFA (Blaak et al., 2020; Nishiwaki et al., 2022). Similarly, Anaerocolumna, a major taxonomic microbial group in the gut, is reported to mediate the fermentation of complex polysaccharides and degrading highly lignified diets in polygastrics (Shabana et al., 2020). Furthermore, a previous study in piglets reported that the intestinal abundance of Selenomonadaceae genera negatively correlated with P intake, serum P levels and degradation of phytate as well as inositol-5 phosphate in distal parts of the gastrointestinal tract (Reyer et al., 2021). A study on quail (Cortunix japonica) reported a positive correlation between the abundance of genera Candidatus arthromitus and performance and P utilization traits (Vollmar et al., 2020). However, following a contrasting observation in the current broiler study, wherein Candidatus arthromitus was more abundant in the high P group, it is not yet clear whether the abundance of this taxon depends on dietary P levels or the abundance of Candidatus arthromitus improves intestinal P mobilization. It should be noted that due to taxonomic reclassifications, sequences assigned to Candidatus Arthromitus in vertebrates should be considered as Candidatus Savagella (Thompson et al., 2012).

CONCLUSIONS

In summary, the study showed that P intake, fecal P, and fecal phytate contents are parallel, that is, the P-depleted cohorts excreted less P compared to the non-depleted feeding groups and vice versa. The improved phytate degradation in P-depleted broilers efficiently mobilizes phosphorus from plant feed components, which is also largely observed in the groups fed the currently recommended P supply, while higher P-fed groups excrete substantial amounts of unutilized phytate. However, in addition to the distinct mechanisms for improved P utilization, the analysis of the jejunal microbiota shows only a minor shift in microbial taxa between the P-depleted and non-depleted groups. Furthermore, some microbial taxa proliferated in a P-enriched environment and might be considered as biomarkers of excessive P supply in commercial broiler chicken as well as significant SCFA producers. The microbial composition in jejunum made only a minor contribution to the birds' compensatory mechanism for adaptation following P depletion.

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DISCLOSURES

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in the present study.

SUPPLEMENTARY MATERIALS

Supplementary material associated with this article can be found in the online version at doi:10.1016/j.psj.2023.103096.

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6.5 List of scientific publications (peer-review) and presentations

I declare that I am the first author of the publications and presentations listed below. A detailed description of my contribution to the publications and experimental trials was described above.

List of Publications

Omotoso, A.O., Reyer, H., Oster, M., Ponsuksili, S., Trakooljul, N., Muráni, E., Sommerfeld, V., Rodehutscord, M. and Wimmers, K. 2021. Jejunal transcriptomic profiling of two-layer strains throughout the entire production period. *Scientific Reports* 11: 20086 https://doi.org/10.1038/s41598-021-99566-5

Omotoso, A.O., Reyer, H., Oster, M., Maak, S., Ponsuksili, S., Wimmers, K. 2023. Broiler physiological response to low phosphorus diets at different stages of production, *Poultry Science*, 102(2)102351. https://doi.org/10.1016/j.psj.2022.102351

Omotoso, A.O., Reyer, H., Oster. M., Ponsuksili, S. and Wimmers, K. 2023. Jejunal microbiota of broilers fed varying levels of mineral phosphorus *Poultry Science* (2023), doi: https://doi.org/10.1016/j.psj.2023.103096

List of Presentations

Omotoso A.O. Jejunal transcriptomic profiling of two-layer strains throughout the entire production period (PFOWL) Cluster I: Phosphorus in the environment and IV: Phosphorus in Biology, International P-Campus symposium Nov. 16 -17, 2020

Omotoso A.O. Jejunal transcriptomic profiling of two-layer strains throughout the entire production period (PFOWL) Cluster I. FBN doctoral day seminar beginner (November, 2020)

Omotoso A.O. Jejunal transcriptomic profiling of two-layer strains throughout the entire production period (PFOWL) Cluster I Institute for Genome Biology workshop FBN (November, 2021)

Omotoso, A.O. Broiler physiological response to low phosphorus diets at different stages of production. FBN doctoral day seminar advanced (August, 2022)

6.6 Acknowledgement

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6.7 Curriculum Vitae

Adewunmi Omotoso

Date of Birth	17.03.1988
Nationality	Nigerian
Language	English (Native)

Education

02/2020 - 2023	**Doctoral student** Research Institute for Farm Animal Biology (FBN), Institute of Genome Biology Dummerstorf, Germany Thesis: Characterizing endocrinal and transcriptional determinants of phosphorus utilization mediated by the environment-host interaction in laying hens and broiler chickens DFG-PFOWL research phase I
11/2017	**Master of Agriculture (Animal Biotechnology)** Dissertation: Genetic variation and phylogenetic relationship of four rabbit populations in Nigeria based on microsatellite markers
03/2014 – 11/2017	**Master of Agriculture (Animal Biotechnology)** Federal University of Agriculture, Abeokuta (FUNAAB) Nigeria.
07/2011	**Bachelor of Agriculture (Animal Breeding and Genetics)** Dissertation: Genetic distance and the time of divergence of turkey populations in Nigeria based on the use of microsatellite markers
12/2006 - 07/2011	**Bachelor of Agriculture (Animal Breeding and Genetics)** Federal University of Agriculture, Abeokuta (FUNAAB) Nigeria.
06/2004	**West African Examination Council (WAEC)** Senior Secondary School Leaving Certificate Lagos State Model College Kankon Badagry,(LASMOCK) Nigeria

Research Experience

12/2017 – 12/2018	**Postgraduate research assistant** Department of Animal Breeding and Genetics Federal University of Agriculture, Abeokuta (FUNAAB) Nigeria.

Community service

12/2011 – 03/2012	**Red Cross Representative** National Youth Service Corp (NYSC) Gombe Northern Nigeria
04/2012 – 10/2012	**Agricultural Science Instructor & Village Extension Agent** (NYSC) Ekiti-Nigeria **Red Cross Representative** (NYSC) Ekiti Western Nigeria

6.8 Declaration

I hereby declare with my appended signature that I have written this dissertation independently without the aid of any sources beyond those specifically mentioned. Any ideas or concepts derived from external sources directly or indirectly have been cited accordingly. This dissertation has not been presented to any other examining authority in any form.

Dummerstorf, 23/02/2024 _____

Place, Date

_____

Doctoral Candidate Signature