Hatching and Nutritional Strategies to Improve Broiler Performance and Flock Homogeneity through Modulation of Gut Health and Microbiota

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Preface

Dear reader,

I am pleased to present to you my PhD thesis, the result of a journey that began years ago when I was a young veterinarian with a passion for animal health, production and welfare. That journey took me through a master's degree in animal nutrition, and ultimately to the KU Leuven laboratory of Prof. Nadia Everaert, where I embarked on research to explore different access-to-feed and nutritional strategies to improve broiler performance and flock homogeneity.

First and foremost, I would like to thank my promotor, Nadia Everaert. Nadia, you believed in me from the very beginning and gave me the freedom to explore, think, and grow both as a scientist and as a person. Your trust allowed me to take ownership of my work, while your guidance shaped my professional development in ways I could not have foreseen. You seemed to anticipate challenges and opportunities before I did, steering me in the right direction at crucial moments. I will always admire your foresight, leadership, and unwavering support throughout this journey. I also extend my sincere thanks to my co-promotor, Prof. Martine Schroyen (University of Liège). Your insightful feedback and balanced perspective helped me refine my work, and your encouragement gave me the confidence to keep pushing forward. I am deeply grateful for your time, expertise, and constructive criticism, which have strengthened the scientific rigor of this thesis.

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To my family, my parents, who gave me the foundation for all my achievements; my siblings, whose encouragement has been unwavering; my wife, whose love, patience, and understanding carried me through the most intense phases of this journey; and my daughter, whose smiles and laughter reminded me of the joy and purpose beyond academia, you are the reason I could keep going. This achievement is as much yours as it is mine. Finally, I want to thank all the friends and communities that have been part of my life outside the laboratory. Having spaces to relax, recharge, and enjoy life made it possible to face the challenges of a PhD with renewed energy.

As I conclude this stage of my academic journey, I look ahead with gratitude for what I have learned, the people I have met, and the opportunities that lie ahead. I hope you enjoy reading this thesis as much as I enjoyed the process of creating it.

Abstract

Body weight (BW) variation within broiler flocks is a persistent challenge in poultry production, with implications for economic efficiency, animal welfare, and sustainability. Despite genetic uniformity and standardized management practices, significant growth differences often emerge, and the biological factors driving these disparities remain insufficiently understood. This thesis aimed to identify the gut-related biological factors underlying BW divergence and to evaluate hatching and nutritional strategies to improve the performance of underperforming birds and reduce BW variability. We hypothesized that BW differences are driven by distinct gut microbiota and host physiological profiles, and that early-life interventions could enhance gut health and narrow performance gaps.

In the first part of this thesis, the role of gut microbiota in BW divergence was investigated by comparing low BW (LBW) and high BW (HBW) male Ross 308 chicks, classified on day 7 and followed until day 38. Cecal microbiota composition and predicted function, along with volatile fatty acid (VFA) profiles, were assessed on days 7, 14, and 38 using 16S rRNA sequencing, PICRUSt2 functional prediction, and gas chromatography. Microbial diversity and composition were strongly influenced by BW category. HBW broilers were enriched with VFA-producing taxa, including unclassified *Lachnospiraceae*, *Alistipes*, and *Faecalibacterium*, while LBW birds showed greater abundances of *Lactobacillus*, *Akkermansia*, and *Escherichia-Shigella*. HBW birds had higher acetate concentrations at day 14, whereas LBW birds showed higher isocaproate and isobutyrate levels at earlier and later stages. Predicted functional potential was greater in HBW microbiota, suggesting a more metabolically active microbial

community. These results indicate that BW divergence is closely associated with differences in microbiota composition, metabolic potential, and VFA production patterns.

The second study in Part 1 built upon the first by shifting the focus from microbial factors to host-related mechanisms underlying BW divergence. Male Ross 308 chicks (n = 908) were ranked at day 7 into LBW and HBW groups and monitored for growth, visceral organ development, intestinal permeability, histomorphology, and ileal gene expression profiles at days 7, 14, and 38. A panel of 79 genes related to gut barrier integrity, immune function, nutrient transport, hormones, metabolism, and oxidation was quantified using high-throughput qPCR. HBW broilers remained heavier throughout the production cycle, primarily due to higher feed intake. They had shorter relative small intestine length but greater villus height and villus-to-crypt ratios, indicating superior absorptive capacity. LBW birds displayed increased intestinal permeability on day 38 and upregulation of immune-related genes such as *TNF-α* on day 7 and *CYP450* on day 38, reflecting a potentially more inflammatory gut environment. In contrast, HBW birds upregulated genes associated with barrier function, nutrient transport, and oxidative metabolism, suggesting a more efficient intestinal physiology. Multivariate modelling (PLSR) identified sets of key genes at each age that accurately discriminated BW phenotypes, providing potential molecular biomarkers for early prediction of growth potential.

In the second part of the thesis, three targeted interventions were evaluated to improve the performance of broilers (underperforming) and reduce BW variability. The first intervention assessed the impact of on-farm hatching (HOF) on growth performance, intestinal development, barrier function, immunity, and gene expression. Male Ross 308 chicks hatched either in a hatchery or on-farm were monitored until day 38. HOF chicks had higher day 1 BW, but this advantage disappeared within the first week. Nonetheless, HOF birds exhibited enhanced intestinal morphology; wider duodenal villi, deeper ileal crypts, and greater submucosal thickness and higher relative bursal weight, suggesting improved immune organ development. Gene expression analysis revealed that HOF chicks upregulated immune-related genes (e.g., *IL-8*, *IL-6*, *IFN-γ*, *AVBD9*) and oxidative stress response genes (e.g., *HIF1A*), whereas HH chicks upregulated certain barrier and nutrient transporter genes. Although

performance benefits were transient, HOF improved mucosal morphology and immune modulation, indicating potential long-term health advantages.

The second intervention tested whether in ovo injection of sodium butyrate (SB) could improve growth and gut health, particularly in chicks with low hatch weight (LHW). Ross 308 eggs were injected on incubation day 12 with saline or SB at 0.1%, 0.3%, or 0.5%. Post-hatch, chicks were classified as high or low hatch weight, creating a 4 × 2 factorial design. SB supplementation did not affect hatchability but significantly modulated growth, intestinal morphology, gene expression, and cecal microbiota, with effects varying by SB dose and hatch weight category. The 0.3% SB dose produced the most consistent benefits in LHW birds, leading to the highest final BW, upregulation of gut barrier genes (*CLDN1*, *TJP1*), anti-inflammatory cytokines (*IL-10*), and mucin (*MUC6*), along with improved microbiota diversity and enrichment of beneficial taxa. High HW birds generally performed better than low HW birds on control dose, but SB narrowed the performance gap between low and high HW broilers, particularly at the optimal dose.

The third intervention examined whether dietary structural components could improve the performance of LBW broilers and reduce BW disparity with HBW birds. At day 7, 1400 Ross 308 males were classified into LBW or HBW groups, with LBW birds receiving one of four diets: control (fine corn), coarse corn, oat hulls, or a combination of both. HBW birds received the control diet. By day 38, oat hull supplementation (3%) led to the greatest improvement in BW among LBW birds, significantly reducing the gap with HBW controls. Structural components improved gizzard development, intestinal morphology, and gene expression related to barrier integrity, nutrient transport, and immunity, while reducing cecal concentrations of certain branched-chain VFAs associated with protein fermentation. Microbiota shifts in LBW birds fed structural diets included increased beneficial taxa and reduced potential pathogens.

Collectively, this thesis shows that BW divergence in broilers is associated with distinct microbiota and host physiological profiles established early in life. Interventions such as optimal-dose in ovo sodium butyrate application for low hatch weight chicks and dietary oat hull inclusion for LBW broilers

can improve gut health, narrow performance gaps, and enhance flock uniformity. These insights contribute to the development of precision nutrition and management strategies aimed at improving both flock performance and economic efficiency in commercial broiler production.

Samenvatting

Variatie in lichaamsgewicht (BW) binnen vleeskuiken-koppels blijft een aanhoudende uitdaging in de pluimveeproductie, met directe gevolgen voor product-efficiëntie, voederconversie en uniformiteit bij slacht. Ondanks genetische uniformiteit en gestandaardiseerde managementpraktijken treden er toch aanzienlijke groeiverschillen op, en de biologische factoren die deze ongelijkheden veroorzaken, zijn nog niet volledig begrepen. Dit proefschrift onderzoekt eerst microbiota- en gastheergerelateerde factoren die samenhangen met BW-verschillen bij vleeskuikens, en evalueert vervolgens voedings- en managementstrategieën om de prestaties van onderpresterende dieren te verbeteren en de BW-variatie te verminderen.

In het eerste deel van dit proefschrift werd de rol van de darmmicrobiota bij BW-verschillen onderzocht door mannelijke Ross 308-kuikens met een laag (LBW) en hoog (HBW) lichaamsgewicht op dag 7 te selecteren en te volgen tot dag 38. De samenstelling en voorspelde functies van de microbiota in de ceca, evenals vluchtige vetzuurprofielen (VFA), werden geanalyseerd op dag 7, 14 en 38 met behulp van 16S rRNA-sequencing, PICRUSt2-functionele voorspelling en gaschromatografie. De microbiële diversiteit en samenstelling werden sterk beïnvloed door de BW-categorie. HBW-kuikens waren verrijkt met VFA-producerende taxa, waaronder ongeclassificeerde *Lachnospiraceae*, *Alistipes* en *Faecalibacterium*, terwijl LBW-kuikens hogere aantallen *Lactobacillus*, *Akkermansia* en *Escherichia-Shigella* vertoonden. HBW-kuikens hadden hogere acetaatconcentraties op dag 14, terwijl LBW-kuikens hogere gehalten isocapronaat en

isobutyraat hadden in vroege en latere fasen. De voorspelde functionele capaciteit was groter bij HBW-microbiota, wat wijst op een meer metabool actieve microbiële gemeenschap. Deze resultaten tonen aan dat BW-verschillen sterk samenhangen met verschillen in microbiotasamenstelling, metabolisch potentieel en VFA-productiepatronen.

De tweede studie in deel 1 borduurde voort op de eerste door de focus te verschuiven van microbiële factoren naar gastheer-gerelateerde mechanismen die aan de basis liggen van BW-verschillen. Mannelijke Ross 308-kuikens (n = 908) werden op dag 7 ingedeeld in LBW- en HBW-groepen en gevolgd voor groei, ontwikkeling van inwendige organen, intestinale permeabiliteit, histomorfologie en ileale genexpressie op dag 7, 14 en 38. Een panel van 79 genen, gerelateerd aan darmbarrière, immuunfunctie, nutriëntentransport, hormonen, metabolisme en oxidatie, werd geanalyseerd met high-throughput qPCR. HBW-kuikens bleven gedurende de hele productieronde zwaarder, voornamelijk door een hogere voeropname. Zij hadden een relatief kortere dunne darm, maar grotere villushoogte en hogere villus-cryptverhoudingen, wat wijst op een hogere absorptiecapaciteit. LBW-kuikens vertoonden een verhoogde intestinale permeabiliteit op dag 38 en een verhoogde expressie van immuungerelateerde genen zoals *TNF-α* (dag 7) en *CYP450* (dag 38), wat kan duiden op een meer ontstekingsgevoelige darmomgeving. Daarentegen vertoonden HBW-kuikens een hogere expressie van genen die verband houden met barrièrefunctie, nutriëntentransport en oxidatief metabolisme, wat wijst op een efficiëntere darmfysiologie. Multivariate modellen (PLSR) identificeerden genensets die op elke leeftijd de BW-fenotypes accuraat onderscheidden en mogelijk bruikbaar zijn als moleculaire biomarkers voor vroege voorspelling van groeipotentieel.

In het tweede deel van dit proefschrift werden drie gerichte interventies geëvalueerd om de prestaties van vleeskuikens te verbeteren en de BW-variatie te verkleinen. De eerste interventie beoordeelde de impact van on-farm hatching (HOF) op groeiprestaties, darmontwikkeling, barrièrefunctie, immuunrespons en genexpressie. Mannelijke Ross 308-kuikens, uitgebroed in een broederij of op het bedrijf, werden gevolgd tot dag 38. HOF-kuikens hadden op dag 1 een hoger BW, maar dit voordeel

verdween na de eerste week. Niettemin vertoonden HOF-kuikens verbeterde darmmorfologie, waaronder bredere duodenumvilli, diepere ileale crypten en een dikkere submucosa, evenals een hoger relatief bursa-gewicht. Genexpressieanalyse toonde aan dat HOF-kuikens immuungerelateerde genen (o.a. *IL-8*, *IL-6*, *IFN-γ*, *AVBD9*) en oxidatieve stressgenen (*HIF1A*) opreguleerden, terwijl HH-kuikens bepaalde barrière- en nutriëntentransport-genen opreguleerden. Hoewel de prestatievoordelen tijdelijk waren, verbeterde HOF de mucosale darmstructuur en immuunmodulatie.

De tweede interventie testte of in ovo-injectie van natriumbutyraat (SB) de groei en darmgezondheid kon verbeteren, vooral bij kuikens met een laag uitkomstgewicht (LHW). Ross 308-eieren werden op incubatiedag 12 geïnjecteerd met fysiologisch zout of SB in 0,1%, 0,3% of 0,5%. Na uitkomst werden de kuikens ingedeeld in hoog of laag uitkomstgewicht (4 × 2-factorieel design). SB had geen invloed op de uitkomstpercentages, maar beïnvloedde wel significant de groei, darmmorfologie, genexpressie en cecale microbiota, met dosis- en gewichtsspecifieke effecten. De 0,3%-dosis gaf de meest consistente voordelen voor LHW-kuikens, met de hoogste eind-BW, opregulatie van darmbarrièregenen (*CLDN1*, *TJP1*), anti-inflammatoire cytokinen (*IL-10*) en mucine (*MUC6*), evenals verbeterde microbiotadiversiteit en meer gunstige taxa.

De derde interventie onderzocht of structurele componenten in het dieet de prestaties van LBW-kuikens konden verbeteren en het verschil met HBW-kuikens konden verkleinen. Op dag 7 werden 1400 Ross 308-kuikens ingedeeld in LBW- en HBW-groepen. LBW-kuikens kregen één van vier diëten: controle (fijn maïs), grof maïs, haverdoppen of een combinatie van beide. HBW-kuikens kregen het controledieet. Op dag 38 gaf haverdoppen (3%) de grootste BW-verbetering bij LBW-kuikens en verkleinde significant het verschil met HBW-kuikens. Structurele componenten verbeterden de spiermaagontwikkeling, darmmorfologie en expressie van genen gerelateerd aan barrièrefunctie, nutriëntentransport en immuunrespons, terwijl bepaalde VFA's in de blindedarm werden verlaagd en de microbiotasamenstelling gunstig werd veranderd.

Samenvattend laat dit proefschrift zien dat BW-verschillen bij vleeskuikens samenhangen met specifieke microbiële en fysiologische profielen die

vroeg in het leven worden vastgesteld. Gerichte interventies, zoals optimale in ovo-toediening van natriumbutyraat bij kuikens met een laag uitkomstgewicht en dieet-aanvulling met haverdoppen bij LBW-kuikens, kunnen de darmgezondheid verbeteren, de groeiprestaties verhogen en de uniformiteit van de toom versterken. Deze inzichten dragen bij aan de ontwikkeling van precisievoeding en managementstrategieën voor het optimaliseren van prestaties en economische efficiëntie in de commerciële vleeskuikenproductie.

Abbreviations

ADFI	Average daily feed intake
ADF	Acid detergent fiber
ADG	Average daily gain
ADL	Acid detergent lignin
ANOVA	Analysis of variance
ASV	Amplicon sequence variant
BCFA	Branched-chain fatty acid
BW	Body weight
CCK	Cholecystokinin
CC	Coarse corn
CD	Crypt depth
CV	Coefficient of variation
ED	Embryonic day
EU	European Union
FCR	Feed conversion ratio
FDR	False discovery rate

FITC-d	Fluorescein isothiocyanate dextran
GALT	Gut-associated lymphoid tissue
GC	Gas chromatography
GF	Germ-free
GIT	Gastrointestinal tract
GHRL	Ghrelin
GLP	Glucagon-like-peptide
GMD	Geometric mean diameter
GSD	Geometric standard deviation
HBW	High body weight
HCl	hydrochloric acid
HH	Hatchery hatched
HOF	Hatching on-farm
HS	Hatching system
HSP	Heat shock protein
HW	Hatch weight
HHW	High hatch weight
IgY	Immunoglobulin Y
LBW	Low body weight
LDA	Linear discriminant analysis
LEfSe	Linear discriminant analysis effect size
LHW	Low hatch weight
LPS	Lipopolysaccharides
MALT	Mucosa-associated lymphoid tissue

MT	Microbiota transplantation
NDF	Neutral detergent fiber
NK	Nature killer cells
NLRs	NOD-like receptors
OH	Oat hulls
OTU	Operational taxonomic unit
PAMPs	Pathogen-associated molecular patterns
PBS	Phosphate buffer saline
PCA	Principal component analysis
PCoA	Principal coordinate analysis
PERMANOVA	Permutational multivariate analysis of variance
Picrust2	Phylogenetic investigation of communities by reconstruction of unobserved states
RMSECV	Root mean squared error of cross-validation
ROX	Reactive oxygen species
SB	Sodium butyrate
SCFA	Short-chain fatty acid
SD	Standard deviation
SLC	Solute carrier
TLRs	Toll-like receptors
TJ	Tight junction
VFA	Volatile fatty acid
VH	Villus height

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Chapter 1

General introduction

In this chapter, the reader is introduced to the broiler industry problem of performance variability within commercial broiler flocks, a persistent challenge that affects growth uniformity, feed efficiency, and economic returns. The gut microbiota and host-related factors that may underlie these differences between high and low performing birds are outlined, including variation in microbial composition, fermentation profiles, intestinal morphology, and functional gene expression. Finally, three targeted strategies, early access to feed via on farm hatching, in ovo sodium butyrate injection, and dietary feed structure modification are presented as potential approaches to improve the growth and gut health of underperforming birds.

1.1 Introduction

Modern broiler chickens and laying hens both descend from the red junglefowl (*Gallus gallus*), with genetic contributions from the grey junglefowl (*Gallus sonneratii*), a species still found in the tropical forests of South and Southeast Asia[1]. These wild ancestors represent the closest living relatives of today's domestic chicken, which has become the most numerous livestock species on the planet, surpassing 80 billion birds globally and outnumbering humans nearly ten to one[2].

According to the European Union (EU) stats, broiler meat consumption has been increasing more than any other type of meat, with a four-fold increase to 23.4 kg per capita and year since the 1980s[3]. Since the initial domestication of the red junglefowl around 8,000 years ago, the domestic chicken has undergone remarkable biological transformation[4]. The wild junglefowl is a relatively small bird, with adult males typically weighing 800–1200 grams and females 500–700 grams[5]. By comparison, modern broilers have been intensively selected for rapid growth and feed efficiency. For example, while a broiler in 1957 reached about 900 grams at 56 days of age, the same age today yields a bird exceeding 4.2 kilograms. These changes reflect over a 400% increase in growth rate and a 50% improvement in feed conversion ratio since the 1950s[6].

This extraordinary advancement has been largely driven by genetic selection focused on growth performance and body composition, supported by innovations in tailored nutrition. As a result, a current commercial broiler can reach a market weight of 2.3 kilograms within just 35 days, consuming only 3.2 kilograms of feed[7]. This dramatic evolution in growth performance and metabolic efficiency suggests that the environmental, nutritional, and management requirements for maintaining optimal health, welfare, and productivity in today's newly hatched chicks may differ substantially from those of previous generations.

1.2 Chicken meat

The future of broiler chicken production is closely tied to rising global demand for affordable animal protein. As the world population approaches an estimated 10 billion by 2050, food production systems are under pressure to expand by 50–90% to meet nutritional demands. Among

all livestock sectors, poultry meat is projected to experience the highest growth rate, given its relatively low cost, short production cycle, fewer religious or cultural restrictions, and perceived health benefits compared to red meats[8,9]. According to the *OECD-FAO Agricultural Outlook 2024–2033*, worldwide consumption of poultry meat is expected to increase by 15% between 2023 and 2032, with Europe projected to see a 4% rise over the same period[10] .

The EU remains a significant contributor to global poultry production, accounting for 8.6% of the world's total output equivalent to approximately 10.6 million tonnes. From 2012 to 2022, broiler production in the EU grew by nearly 20%, now amounting to around 6.1 billion birds annually. With an average per capita consumption of 23.4 kg in 2022, chicken ranks as the second most consumed meat in the EU, following pork. Over the next decade, EU poultry meat production is projected to rise marginally by 0.2%, while consumption is expected to increase by 3%, contrasting with the anticipated decline in pork and beef consumption. Notably, the number of broilers slaughtered in the EU is about 25 times greater than that of pigs, highlighting the dominant role of poultry in Europe's meat supply[11].

Today's broiler industry operates within a tightly integrated structure, typically involving hatcheries, grow-out farms, and centralized slaughterhouses. Chickens reach market weight in just over five weeks, creating a highly responsive production cycle. Looking forward, continued growth in global poultry production is anticipated, particularly in developing countries where demand is rising most rapidly. The majority of this growth will be driven by intensive production systems, while alternative systems such as organic or free-range account for less than 5% of EU output[11]. As competition and production intensify, profitability is increasingly influenced by input costs particularly feed, chicks, and technology.

1.3 Body weight variability within broiler flocks

The broiler chicken industry relies on a vertically integrated breeding structure. At the top of the genetic pyramid are the purebred lines, owned and managed by a few multinational breeding companies. These lines are intensely selected for economically valuable traits and give rise to great-

grandparent and grandparent stocks. Crosses of grandparent lines produce hybrid parent stock, which are distributed to integrated producers for the commercial production of meat-type broilers. This system, established since the 1950s, has underpinned significant advances in growth rate, carcass yield, and overall production efficiency[6].

While early selection focused primarily on rapid growth, recent decades have prioritized traits such as breast muscle yield and feed efficiency[12]. Research indicates that approximately 85–90% of this progress is attributable to genetic selection, with the remainder due to improvements in nutrition and husbandry[13]. However, alongside these gains, modern broilers are more susceptible to physiological and metabolic disorders such as excessive fat deposition[14], skeletal abnormalities[15], cardiopulmonary conditions[16], and altered immune responses[17]. Although modern broilers have relatively lean breast meat, they are prone to excessive abdominal and subcutaneous fat due to intensive selection for rapid growth and high feed efficiency. This imbalance between muscle growth and metabolic regulation can lead to fat deposition in non-muscle tissues, particularly under nutrient oversupply or environmental stress, reducing carcass yield and processing efficiency.

Despite genetic homogenization, significant variation in body weight (BW) persists within broiler flocks. Intra-flock variability in broilers is assessed through several complementary statistical and biological metrics, each describing different aspects of dispersion and flock uniformity. The coefficient of variation (CV), calculated as the standard deviation divided by the mean body weight × 100, is the most widely used parameter as it standardizes variability relative to the flock's mean body weight, enabling comparisons across ages, hybrids, and flock sizes[18]. However, CV alone may not capture the full distributional pattern of variability. Additional measures such as the standard deviation (SD) provide absolute dispersion values useful for within-flock tracking, while the range (difference between the heaviest and lightest birds) reflects extreme divergence but is sensitive to outliers. Percentile-based measures (e.g., 10th and 90th percentiles) describe the tails of the distribution and help identify suboptimal subpopulations, whereas skewness and kurtosis reveal asymmetry or clustering within the flock[19]. Advanced approaches such as Gini coefficients, Lorenz curves, and hierarchical clustering have also been

employed to visualize inequality and detect subgroup structures within a population[20–23].

In well-managed commercial flocks, the CV for body weight generally ranges from 8% to 10%[18,19,24,25], while values exceeding 12–15% indicate poor uniformity[26]. Vasdal et al.[18] observed that uniformity varied between 11% and 18% even among flocks of the same hybrid, age, and management standard. Similarly, Griffin et al.[27] reported higher variability in 42-day-old males (CV = 14.2%) than in females (CV = 12.8%). Lundberg et al.[28] noted that CVs of 11–18% are commonly observed in mixed-sex flocks, whereas 8–10% are typical for male-only groups. Industry benchmarks suggest that CVs of ~8–10 % correspond to acceptable uniformity, whereas CVs above ~12 % mark poor uniformity[29].

Although flock uniformity is often evaluated at slaughter age, it should ideally be monitored longitudinally to capture its development over time. Key assessment points include day 0 to estimate hatch weight variation arising from egg size and incubation conditions; day 7–14 to evaluate the early post-hatch adaptation phase when divergence accelerates; day 21–28 to assess mid-grow-out effects related to feed and environment; and day 35–42 to quantify final market uniformity[19]. Tracking CV dynamics across these stages provides insight into whether management interventions mitigate or exacerbate heterogeneity. In commercial practice, breeding companies such as Ross and Cobb recommend assessing body weight in approximately 1% of the flock at each weighing event to obtain a representative estimate of flock uniformity, ensuring reliable monitoring of performance variation throughout the production cycle[29].

From an economic perspective, there is a notable scarcity of studies quantifying the direct impact of flock heterogeneity on farm-level profitability. Most existing research addresses indirect economic consequences through associated production inefficiencies such as increased mortality, poorer feed conversion ratio (FCR), or suboptimal management performance[18,28]. Higher CV in BW have been consistently linked to these parameters, reflecting biological and managerial inefficiencies that ultimately erode profitability. Only a limited number of studies have demonstrated that flock uniformity functions as a key

performance indicator and economic driver in commercial practice. Madsen and Pedersen[30] reported that in the United States, wholesale purchasers require carcasses within narrow weight ranges, and deviation from these specifications incurs substantial economic penalties for processors. Similarly, in Australia, uniformity-related downgrades were estimated to cause annual losses exceeding AUD 127 million, assuming 5% of the 1.16 million tonnes of broiler meat produced were downgraded by 40% due to weight non-compliance[31]. Despite these indications, systematic evaluations of the direct financial impact of within-flock body weight variation on farm economics remain limited, highlighting the need for integrated bioeconomic models to quantify its magnitude and inform precision management strategies.

Flock uniformity is a standardized and objective measure, routinely recorded at slaughter through automated systems, and is increasingly considered an indicator of both production efficiency and animal welfare. Indeed, poor uniformity may reflect unequal access to resources, social stress, or subclinical health issues, which could compromise the well-being of smaller or slower-growing individuals[32].

1.4 Environment basis of body weight variability

BW variation in broilers is largely shaped by a combination of extrinsic factors encountered from the embryonic stage through to the end of the production cycle, with initial hatch weight (HW) being one of the earliest and most influential contributors.

HW is a primary determinant of early growth and subsequent performance, showing a strong positive correlation with BW during the first weeks of life[33]. Chick uniformity at placement is also crucial, as initial BW significantly impacts final BW, every 1 g increase at hatch may result in a 7–13 g increase at slaughter[34]. While some argue that the early advantage diminishes over time, multiple studies confirm a strong positive correlation between early BW (day 7 or 21) and slaughter weight[35,36]. Heavier chicks generally possess greater energy reserves, more advanced organ development, and enhanced thermoregulatory and immune competence, enabling faster early growth and superior feed conversion. Lighter chicks often lag behind in growth, contributing to increased BW variability and reduced flock uniformity, which can persist until market age

despite optimal management. HW reflects cumulative influences of breeder age, egg size, storage conditions, and incubation management, making it a key practical indicator for identifying chicks with good growth potential[37].

Egg size and composition is a critical determinant of HW and early chick quality, as larger eggs provide more nutrients and energy for embryonic development[38]. On average, each gram increase in egg weight corresponds to approximately 0.7–0.8 g higher HW[39]. Heavier chicks from optimally sized eggs are generally more physiologically mature, with better-developed intestinal and hepatic tissues, and superior post-hatch growth[38]. However, extremely small or excessively large eggs may compromise embryonic development, hatchability, and chick quality due to limited nutrient reserves or internal incubation constraints[40,41]. Many studies have confirmed that the morphology (weight and shape of the egg), physical traits (internal quality of eggs), and biochemical traits (e.g., pH of the yolk or the protein, cholesterol level, content of macro- and microelements, and level of lysozyme) of hatching eggs are important factors that influence the proper development of the embryo[42].

Egg size is influenced primarily by breeder-related factors such as age, genotype, nutrition, and health status[40]. Older hens tend to produce larger eggs with more yolk and albumen, whereas genetic and nutritional factors modulate yolk deposition and eggshell quality, establishing the baseline for embryonic growth potential[43]. Eggs from younger breeders tend to be smaller, resulting in lighter chicks with delayed growth, whereas eggs from older breeders may yield larger chicks, however, excessively large eggs often produced by older breeders may experience reduced hatchability due to longer oxygen diffusion distances and steeper internal thermal gradients, which can cause late embryonic mortality or weak chicks[40].

Egg storage conditions further modulate embryonic development, hatch weight, and chick quality. Prolonged storage (>7 days) or elevated temperatures (>21°C) can increase embryonic mortality, disrupt yolk and albumen integrity, and reduce HW[44,45]. Insufficient turning or improper positioning compromises gas exchange and blastoderm orientation, while prewarming and short-term preincubation can partially restore embryonic metabolism, synchronize development, and improve hatchability[46]. The

effects of storage are modulated by breeder age, egg size, and genetic line, as larger eggs from older hens may experience greater weight loss and altered internal composition during storage. Storage-induced variability in embryonic development contributes to differences in HW and early post-hatch growth[47].

After hatching, the timing of chick emergence commonly expressed as the hatch window further influences early growth[48]. The hatch window is defined as the interval between the first and last chicks to hatch, which typically ranges from 24 to 48 hours in commercial broiler flocks. Early-hatched chicks may remain in the hatcher for extended periods before access to feed and water, resulting in progressive depletion of yolk reserves, delayed gastrointestinal development, and reduced early BW gain[49]. In contrast, late-hatched chicks generally have shorter exposure in the hatcher and immediate access to feed and water post-placement, but may compete with larger, early-hatched chicks for resources. These temporal differences in hatching contribute to within-flock variation in BW, intestinal development, and nutrient absorption efficiency. Studies have shown that prolonged post-hatch fasting in early-hatched chicks can reduce yolk utilization, impair gut maturation, and compromise immune competence, whereas prompt access to feed can partially mitigate these effects[50]. Consequently, both the duration and distribution of the hatch window, combined with initial HW and egg-related factors, play a critical role in shaping early growth trajectories, BW variability, and overall flock uniformity.

Early chick management practices, including transport duration, brooding temperature, and uniform access to feed and water during the first 48 hours post-hatch, are critical for synchronized development. Suboptimal brooding can lead to dehydration, delayed gut maturation, and suppressed immune development, disproportionately affecting some chicks and increasing BW variability[51]. Health status, including subclinical infections and gut health, can further impair feed conversion and exacerbate growth differences within the flock. Subclinical infections, uneven vaccine responses, or compromised gut health may limit feed efficiency in affected birds, increasing flock variability. Birds experiencing mild illness may survive but show retarded growth compared to healthy

counterparts. In flocks with poor biosecurity or inconsistent vaccination practices, this can be a major contributor to BW variation.

Stocking density affects birds' access to feed and water, where both excessively low and high densities can increase competition or restrict movement, respectively, thus increasing BW differences. Birds housed at inappropriate densities experience unequal access to feeders and drinkers, increased competition, and stress. While extremely low stocking density may promote dominance by fast-growing birds, excessively high density can restrict movement and access to resources, both leading to increased variation[52].

Feed quality and nutrient balance further shape BW outcomes, with low protein or energy-dense diets leading to excessive variation due to uneven growth rates. Diets deficient in protein or imbalanced in energy-to-protein ratios can lead to uneven growth and increased BW variation. Suboptimal nutrient density may cause compensatory growth in some birds while others remain stunted, resulting in flock performance heterogeneity[52]. Finally, social dynamics within the flock, such as hierarchical pecking order or competition at feeders, particularly in mixed-sex groups, can influence nutrient intake. Dominant birds may restrict access for subordinates, creating nutritional imbalances that exacerbate BW variation[32].

To mitigate environmental influences on body weight variability, coordinated strategies must be implemented across the broiler breeder, hatchery, and farm levels. At the breeder flock level, management practices play a decisive role in determining chick variability. Maintaining flock uniformity is fundamental, as inconsistent breeder weights result in heterogeneous egg sizes and subsequent chick variability[53]. Optimized nutrition ensures uniform body condition and consistent egg composition, while rotational replacement of breeder groups minimizes the variability associated with flock aging[54]. Strict egg quality control excluding cracked, misshapen, or weak-shelled eggs prevents the production of suboptimal chicks, as such eggs often compromise embryonic development and hatch weight. Proper storage conditions (16–18°C, 70–80% humidity) with regular egg turning prevent excessive moisture loss and developmental arrest, whereas prewarming before incubation synchronizes embryonic growth and hatching time[55].

At the hatchery level, egg grading by weight is routinely practiced to exclude extreme sizes from incubation batches, thereby promoting more synchronized hatching and reducing chick heterogeneity. Controlling the hatch window (typically 24–48 h) is vital, as prolonged post-hatch fasting of early-emerging chicks can impair intestinal development and immune function. Hatchery feeding systems and early post-hatch placement strategies are increasingly implemented to narrow developmental gaps among chicks. Large-scale hatcheries often face additional challenges in filling high-capacity broiler farms (multiple houses with up to 50,000 birds per house) with chicks of uniform origin, as eggs are sourced from multiple breeder farms of varying age, health, and management. This logistical complexity can introduce variability before the chicks even reach the farm. To mitigate this, hatcheries aim to consolidate batches from similar breeder ages and origins whenever possible.

At the broiler farm level, when residual variation persists after hatching, on-farm chick sorting based on weight or sex may be applied to reduce within-pen competition and allow targeted nutritional management. Although Neto et al.[19] found that grouping day-old chicks by weight alone did not improve final uniformity, subsequent research suggests that success largely depends on nutritional precision, especially amino acid balancing. Studies by Gous[56] demonstrated that dietary supplementation with limiting amino acids such as methionine and lysine markedly improves growth uniformity reducing CV in live weight by up to 40% and optimizing breast meat yield. These findings emphasize that sorting practices are only beneficial when combined with tailored nutritional strategies that meet the specific growth potential of different subgroups.

On-farm, maintaining precise environmental control (temperature, ventilation, and lighting uniformity) and applying targeted nutritional supplementation remain crucial to preventing further divergence in growth. When poor uniformity is already established, feed optimization particularly through highly digestible and consistent ingredients can help recover performance.

Finally, continuous monitoring and data-driven management are essential for sustained improvement. Breeding companies recommend weighing subpopulation of the flock to accurately estimate uniformity and detect

early deviations. Modern automated poultry scales provide real-time insights into growth distribution, enabling corrective interventions in nutrition or environment.

1.5 Biological basis of body weight variability

1.5.1 Genetic variation

Broilers are among the most genetically uniform livestock species, with production relying on multi-generational purebred lines and their crosses. These lines exhibit low heterozygosity and are highly related genetically, and decades of intensive selection have further reduced genetic variation, resulting in low overall diversity[57]. Consequently, when reared under uniform conditions and provided with the same diet, broilers are expected to show relatively consistent growth and reduced variation in final BW.

Despite extensive genetic selection, residual heterozygosity and polymorphisms persist within broiler populations, contributing to phenotypic variation[58,59]. These subtle genetic differences can affect not only growth performance but also gut development, metabolic efficiency, and host–microbiota interactions, thereby generating variation in body weight (BW) among birds reared under identical conditions. Within a broiler population, individuals differ in multiple traits, notably their potential rates of protein accretion and capacity for lipid deposition under nutrient-limited conditions. These traits typically exhibit low coefficients of variation (CV: 0.02–0.10), reflecting the tight regulation imposed by intensive selection. Interactions between residual genetic and epigenetic variation and environmental stressors can affect immune maturation, feed conversion efficiency, and nutrient absorption, producing divergent growth trajectories among genetically similar birds.

In commercial production, however, these genetic potentials are often constrained by environmental and nutritional limitations. Factors such as feed quality, temperature, or stocking density can shift the BW distribution, sometimes skewing it. Smaller birds may fail to thrive and be culled, while larger birds may not reach their growth potential under limiting conditions[60]. This illustrates how environmental pressures can modify the genetic growth curve, making BW variation often more reflective of extrinsic constraints than intrinsic capacity.

1.5.2 Gut health and function

Part of the performance variation can be attributed to factors such as health, breed, sex, diet, genetics, and environmental conditions, but in an experimental setting where these variables are held constant, performance variance still persists[32]. Another explanation for this residual variation lies in intrinsic biological factors, including differences in gut physiology, immune development, metabolic programming, and host–microbiota interactions (Fig. 1.1). Individual variation in the maturation of the gastrointestinal tract (GIT) can influence nutrient absorption efficiency, enzyme activity, and mucosal immunity, thereby impacting growth performance even under identical rearing conditions. Variation in the development of intestinal epithelial structures, including tight junction proteins and mucosal layers, affects intestinal barrier function, a critical defense against pathogens and toxins. Impaired barrier function, often subclinical, can lead to increased intestinal permeability, low-grade inflammation, and compromised nutrient utilization, ultimately suppressing growth and amplifying intra-flock variation[62]. Moreover, early-life microbial colonization differs between individuals due to subtle genetic or epigenetic cues and stochastic colonization events, which can lead to long-term effects on gut health, immune responses, and metabolic function[32].

Additionally, neuroendocrine regulation particularly mechanisms controlling appetite, satiety, and energy homeostasis, can differ between individuals, impacting feed intake even when feed is uniformly available. Additionally, mitochondrial function, oxidative stress resilience, and the capacity for cellular repair can vary among individuals, further influencing growth rates independent of extrinsic inputs. Epigenetic modifications, potentially triggered during embryogenesis or early post-hatch life, may also regulate gene expression patterns associated with nutrient metabolism and immune function, contributing to lasting inter-individual performance differences. Altogether, these intrinsic factors interact dynamically with environmental cues, and even in meticulously standardized settings, the biological individuality of each bird continues to manifest in divergent growth trajectories.

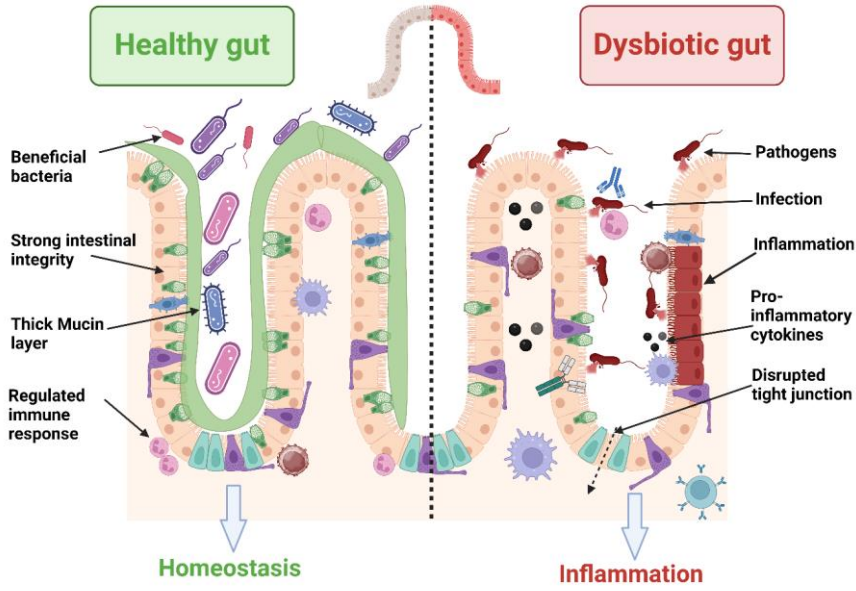

Fig 1.1 A comparison of healthy and dysbiotic gut health. A healthy gut features well-structured villi covered by an intact mucus layer, a balanced immune response, a diverse and beneficial microbiota, and optimal intestinal barrier function. In contrast, a dysbiotic gut is characterized by shortened or damaged villi, a thinner or disrupted mucus layer, imbalanced microbiota composition with an overabundance of pathogenic bacteria, impaired tight junctions, and an inflammatory immune response. This image was developed based on the literature from Aruwa et al.[63] and created using BioRender.com.

1.5.3 Gastrointestinal tract development

The immediate post-hatch period represents a critical developmental window in broilers, during which the GIT undergoes rapid morphological and functional changes to support the transition from yolk-derived lipids to a complex, carbohydrate-rich external diet. This transition must occur swiftly to ensure efficient nutrient digestion and utilization, particularly in modern broiler strains where BW can increase by up to 300% within the first week of life. This rapid early growth is made possible by the allometric development of the digestive system, including the proventriculus, gizzard, and small intestine, which reach peak growth between days 4 and 8. Schematic representation of the different sections of the gastrointestinal tract of a chicken is given in Fig. 1.2. During this time, the GIT expands in length and mass, villi proliferate and elongate, crypt depth increases, and the secretion of digestive enzymes is upregulated. These

changes collectively increase the gut surface area and improve nutrient absorption capacity[64].

A well-developed digestive system is essential from embryogenesis to market age, influencing key performance indicators such as feed conversion ratio (FCR), growth rate, and metabolic efficiency[65]. However, as the commercial broiler's rearing period continues to shorten, many physiological systems including the GIT may not reach full maturity by slaughter age[64]. Consequently, any disruption or delay in early GIT development can compromise lifetime productivity and limit the opportunity for compensatory growth later in life.

Feed ingestion during the first week triggers extensive morphological remodeling of the gut, characterized by significant increases in villus height, number, and crypt depth, which enhance the absorptive efficiency of the intestine[66]. Simultaneously, the immune component of the GIT, particularly the gut-associated lymphoid tissue (GALT), begins to mature rapidly. GALT, a component of mucosa-associated lymphoid tissue (MALT), comprises several lymphoid structures including caecal tonsils, Peyer's patches, the bursa of Fabricius, Meckel's diverticulum, and scattered lymphoid aggregates, each hosting specialized immune cell populations critical for early immune competence[67].

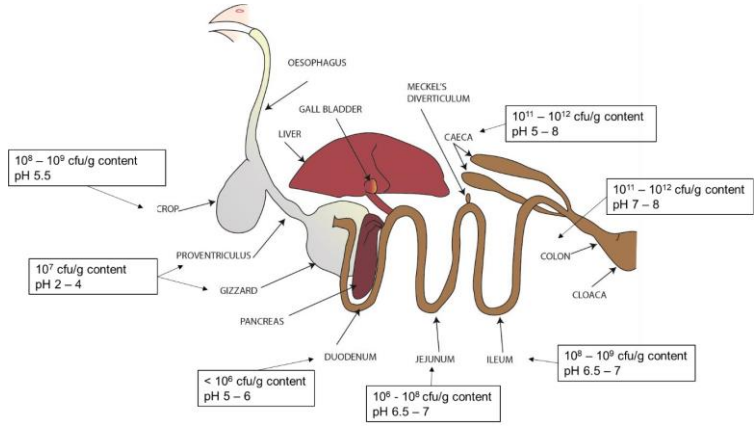

Fig 1.2 Schematic representation of the different sections of the gastrointestinal tract of a chicken [adapted from Anonymous (2020)]. The figure indicates the average pH values for each section[68] as well as the microbial density per gram of content[69]. Abbreviations: CFU, colony-forming unit.

1.5.4 Gizzard

The gizzard, or ventriculus, is a muscular organ composed of densely myelinated smooth muscle fibers located just posterior to the glandular stomach, or proventriculus. It plays a central role in mechanical digestion, particularly in grinding coarse and fibrous feed particles to a critical particle size suitable for further enzymatic digestion in the intestine. Feed materials that exceed this threshold are retained in the gizzard until adequate mechanical breakdown is achieved[70].

While the proventriculus is responsible for secreting digestive enzymes such as pepsinogen and hydrochloric acid (HCl), its small size and limited retention time constrain its digestive contribution. As a result, the majority of mechanical and initial enzymatic digestion occurs within the gizzard. Notably, reflux of digesta from the gizzard back into the proventriculus allows for repeated exposure to gastric secretions, thereby enhancing proteolytic efficiency[70,71].

The effectiveness of protein digestion by pepsin and HCl is closely tied to the intensity of gizzard contractions and the retention time of feed material. Stronger gizzard motility not only prolongs feed exposure to digestive enzymes but also enhances acidification, contributing to improved microbial control and overall gut health[72]. Beyond digestion, the gizzard plays a regulatory role in feed intake. A well-developed and active gizzard may delay gastric emptying, thereby promoting satiety and reducing excessive feed consumption[73]. Interestingly, birds with heavier gizzards, often indicative of higher functional activity have been associated with improved feed efficiency[73].

1.5.5 Pancreas

The pancreas is a vital digestive organ that contributes to nutrient breakdown through the secretion of key enzymes, including proteases, lipases, and amylases. These enzymes play essential roles in the digestion of proteins, fats, and carbohydrates, respectively, supporting efficient nutrient utilization and growth[64]. Given the high starch content of commercial broiler diets, differences in pancreatic amylase secretion and consequently in the capacity to digest starch into glucose may partly explain the variability in growth rates and feed efficiency observed within flocks[74]. Enhanced pancreatic function may enable more effective

carbohydrate digestion and energy utilization, supporting faster growth and improved feed conversion. Azadinia et al.[74] observed that although pancreatic size relative to BW remained constant, heavier pancreases explained part of the variation in body weight at 42 days and feed intake between days 21 and 42.

1.5.6 Liver

The liver is a central metabolic organ that plays a crucial role in nutrient metabolism, energy storage, and detoxification, all of which are essential for supporting growth and productivity in broilers[75]. One of its primary digestive functions is the secretion of bile into the duodenum, facilitating the emulsification and absorption of dietary lipids[76]. Beyond lipid digestion, the liver processes nutrients absorbed from the intestine, converting them into metabolically useful forms and storing glycogen, fat-soluble vitamins, and minerals. It also regulates blood composition by metabolizing hormones, drugs, and metabolic waste, thus maintaining internal homeostasis during rapid growth phases[77]. Studies have shown that liver weight, as a percentage of BW, correlates positively with BW and feed intake, and negatively with FCR, highlighting its role in supporting growth efficiency in broilers[74].

1.5.7 Small intestine size

Intestinal size, measured through weight or length, serves as a key indicator of gut development[78]. Rapid post-hatch growth of the intestine is essential to support increased nutrient intake and subsequent muscle accretion in modern broilers[64]. Although the relative weight of the intestine tends to decline after the first week of life, this is offset by increased intestinal length and mass as birds age. Modern broiler strains exhibit significantly greater intestinal lengths compared to slow-growing lines, underlining the link between GIT development and enhanced growth potential[79].

1.5.8 Villi and crypts

Rapid maturation of the small intestine post-hatch involves increased villus height, crypt depth, and submucosal thickness, key features that enhance the absorptive surface area of the gut[80]. These morphological features are critical for efficient nutrient uptake and overall gut function (Fig. 1.3). The villus height-to-crypt depth (VH:CD) ratio serves as a reliable indicator of

gut health. Indeed, longer ileal villi and elevated VH:CD ratios have been linked to improved feed intake, greater BW gain, and lower FCR, underscoring their importance in broiler performance, while a lower ratio often reflects intestinal stress or pathogenic challenge[81].

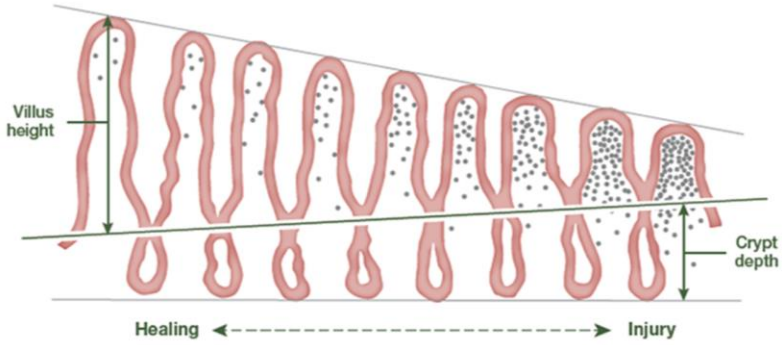

Fig 1.3 Schematic representation of the relationship between villus height and crypt depth across different stages of intestinal injury and healing. Villi become shorter and crypts deeper with increasing severity of injury (left to right), while the reverse pattern indicates mucosal healing (right to left). Figure adapted from Daveson et al.[82].

1.5.9 Nutrient transport

Another key mechanism that may contribute to individual growth variation in broilers involves the efficiency of nutrient absorption, particularly mediated by specialized transporter proteins in the intestinal epithelium[83]. Following digestion, the uptake of amino acids, peptides, and monosaccharides is facilitated by transporter proteins located on the brush border and basolateral membranes of enterocytes. These transporters are largely encoded by the solute carrier (SLC) gene family, which comprises over 395 genes grouped into 52 families[84]. The expression levels of these transporters directly affect the rate at which nutrients cross the intestinal barrier and enter systemic circulation, ultimately influencing the growth potential of individual birds. Increased expression of transporters such as GLUT2, PEPT1, and CPT1 has been consistently observed in high performing broilers, supporting improved glucose and peptide absorption[85]. These findings collectively highlight that variation in the expression of nutrient transporter genes can significantly affect the efficiency of nutrient uptake and utilization, providing a molecular explanation for divergent growth patterns within broiler flocks raised under identical conditions.

1.5.10 Barrier function

The intestinal barrier plays a central role in maintaining gut health, nutrient absorption, and immune defense in broilers, particularly during early life when the GIT is rapidly developing[86]. Its first line of defense is the mucus gel layer, which overlays the epithelial lining and is primarily composed of mucin glycoproteins, secretory immunoglobulin A, and antimicrobial peptides such as β-defensins, cathelicidins, and lysozyme[87]. This layer is produced by goblet cells interspersed along the epithelium, with MUC2 being the predominant mucin responsible for forming the structural mesh of the mucus (Fig. 1.1). MUC2 not only protects against mechanical and enzymatic stress but also regulates microbial colonization by preventing direct contact between bacteria and epithelial cells[88]. Beneath this layer lies the intestinal epithelium, a selectively permeable barrier that regulates the absorption of water, electrolytes, and nutrients while preventing the entry of harmful substances. Tight junction proteins, primarily claudins (e.g., CLDN1 and CLDN5), seal the paracellular space between enterocytes and maintain epithelial polarity and transepithelial resistance. These junctions are genetically regulated and may upregulate in response to damage as a compensatory mechanism to restore barrier function[89]. Intraepithelial lymphocytes (IELs), including natural killer cells, T-cells, B-cells, and heterophils, are distributed throughout the apical and basal regions of the villi, contributing to mucosal immunity and surveillance[90]. However, various factors such as age, diet, microbial imbalances, infections, environmental stress, or chronic inflammation, can impair this barrier by reducing mucin secretion or disrupting tight junction integrity. Increased intestinal permeability allows passive diffusion of antigens and pathogens, leading to reduced feed intake, impaired nutrient absorption, enteric diseases, and compromised growth performance. Birds with poorly functioning intestinal barriers often exhibit lower BW, while high-performing birds typically maintain robust mucosal defenses and epithelial integrity[62].

1.5.11 Immune function

The chicken immune system, like that of other vertebrates, is composed of innate and adaptive components that work in concert to defend against pathogens while maintaining tolerance to commensal microbes and dietary antigens[91]. At hatch, the immune system is immature and largely

dependent on maternally derived antibodies, particularly immunoglobulin Y (IgY), transferred through the egg yolk[92]. This passive immunity provides early systemic protection but is transient and may not fully match the microbial challenges in the post-hatch environment, especially in commercial systems where chicks are reared separately from their mothers. The innate immune system, acting as the first line of defense, includes physical barriers such as the mucus layer, epithelial cells with tight junctions, antimicrobial peptides (e.g., β-defensins, lysozyme), and intraepithelial lymphocytes like natural killer cells and macrophages[93]. Pattern recognition receptors, particularly toll-like receptors (TLRs), detect pathogen-associated molecular patterns (PAMPs) and trigger the release of cytokines such as IL-1β, IL-6, and TNF-α, initiating inflammation and bridging to adaptive responses[94]. As the immune system matures, adaptive immunity becomes functional, typically from around 2 to 3 weeks of age in chickens, through the activation of T and B lymphocytes. T-helper (Th) cells differentiate into subsets: Th1 (targeting intracellular pathogens via IFN-γ), Th2 (stimulating B cells for antibody production), Th17 (responding to extracellular bacteria and fungi), and regulatory T cells (Tregs), which suppress excessive immune activation. B cells are responsible for producing IgM, IgA, and IgY antibodies, with IgA playing a central role in mucosal immunity[95]. However, the intense genetic selection for rapid growth and improved feed efficiency in broilers has inadvertently compromised immune robustness. Fast-growing birds often exhibit reduced antibody production, lower vaccine responsiveness, and weaker mucosal defenses, a phenomenon supported by the resource allocation theory, suggesting that prioritizing growth traits diverts energy away from immune development[96,97]. These immunological trade-offs, coupled with suboptimal microbial colonization and delayed feed access post-hatch, can lead to intestinal inflammation, impaired barrier function, and increased susceptibility to enteric diseases. Consequently, a less efficient immune system not only threatens health and welfare but also contributes to performance variability and economic loss at the flock level.

1.5.12 Digestive neuropeptide hormones

Another critical mechanism underlying growth variation in broilers is the regulation of appetite, which directly influences feed intake and, consequently, BW and production efficiency[98]. In chickens, feed

preferences and consumption patterns are shaped early in life, and even subtle differences in initial feed intake can lead to divergent growth trajectories and impact flock uniformity[99]. Appetite regulation is orchestrated by a complex interplay between the gut and brain, often referred to as the gut–brain axis, which integrates nutrient sensing with hormonal and neural signals. Within the small intestine, nutrient detection triggers the secretion of several key gut-derived hormones that modulate gastrointestinal motility, digestion, and satiety. Among these, ghrelin is known to stimulate appetite by promoting gastric emptying and increasing growth hormone release, thereby encouraging feed intake. However, its role in avian species is contradictory and often considers appetite suppressing hormone[100]. Cholecystokinin (CCK) acts as a satiety signal, regulating gallbladder contraction, pancreatic enzyme secretion, and reducing food consumption[101]. Another anorexigenic hormone, peptide YY, is secreted postprandially and sends inhibitory signals to the hypothalamus, dampening further feeding behavior[102]. Additionally, glucagon-like peptide-1 (GLP-1) plays a role in slowing gastric emptying and promoting satiety[103]. The secretion and activity of these hormones are modulated by nutrient type, gut microbial activity, and physiological state, thereby influencing individual differences in appetite and growth performance[99,104]. In broilers, an imbalance in these regulatory pathways, whether due to genetic predisposition, early-life diet, microbial colonization, or environmental stress, can lead to variable feed intake and divergent growth patterns.

1.5.13 Gut microbiota

The gut microbiota forms a complex, dynamic ecosystem that plays a central role in the development of host immunity, nutrient metabolism, and overall performance[105]. During early life, the chick's GIT undergoes rapid colonization by microbes, previously thought to occur post-hatch but now understood to begin during embryonic development, with at least partial inheritance from the maternal hen. Despite this early exposure, modern commercial hatching practices disrupt natural maternal transfer of microbiota, as breeder flocks and broilers are raised separately. Consequently, chicks are deprived of vertical microbial transmission, which under natural conditions would occur through contact with feathers, nesting material, and feces of the mother hen[106]. Kubasova et

al.[107] demonstrated that even 24 hours of contact with a hen could establish a microbiota resembling that of the adult within one week, an opportunity lost in hatchery environments, which may hinder immune maturation and pathogen defense in broilers.

Following hatch, microbial abundance and diversity increase rapidly. The early-life microbiota is highly variable and sensitive to environmental inputs, maturing gradually with the chick's adaptation to farm conditions. While definitions of microbiota stability vary, evidence suggests stabilization occurs between 14 and 21 days of age, and may shift again in response to dietary transitions such as grower feed introduction[108]. Once mature, the established microbial community becomes more resistant to change.

The microbiota plays dual roles in pathogen defense and nutrient absorption. Beneficial microbes outcompete pathogens for adhesion sites, secrete bacteriocins, and contribute essential metabolites including vitamins and short-chain fatty acids (SCFAs), which in turn influence immune regulation and energy metabolism[109]. Notably, the ceca harbor the highest microbial diversity, functioning as anaerobic fermentation chambers that break down indigestible carbohydrates and contribute significantly to energy harvesting and gut health[110].

The composition of the microbiota is shaped primarily by environmental factors, with host genetics playing a lesser role, especially in genetically uniform commercial broiler lines[111]. Disruptions in microbial composition, termed *dysbiosis*, are associated with poor performance. Dysbiosis typically features reduced microbial diversity, lower populations of beneficial anaerobes (e.g., butyrate producers), and increased abundance of opportunistic pathogens like those in the Enterobacteriaceae family (e.g., *Escherichia coli*). These facultative anaerobes thrive under inflammatory conditions and can dominate in oxygen-enriched environments, disrupting mucosal integrity and nutrient absorption[112]. Butyrate-producing bacteria decline under such conditions, making butyrate a reliable biomarker for gut health[113]. butyrate serves as a primary energy source for enterocytes, supporting intestinal barrier integrity, modulating inflammation, and promoting optimal epithelial renewal.

Conversely, beneficial genera like *Lactobacillus* play crucial roles in maintaining microbial balance and enhancing host defense. These facultative aerobes produce antimicrobial compounds such as lactic acid and bacteriocins, which are proteinaceous toxins that inhibit the growth of closely related or pathogenic bacteria, and compete with pathogens through competitive exclusion. For instance, *Lactobacillus acidophilus* has been shown to inhibit *Salmonella* colonization[114]. Furthermore, *Lactobacillus* abundance in the ceca has been positively associated with BW and feed efficiency[115], supporting its role in productivity.

1.5.14 Relationship of gut microbiota with body weight

The gastrointestinal tract of broiler chickens houses a metabolically active microbial ecosystem that plays a critical role in digestion, immunity, and growth regulation. In modern broiler systems, where genetic selection emphasizes rapid growth and feed efficiency, variations in microbial composition and function have emerged as a potential explanatory factor for divergence in BW among birds rsssaised under similar conditions[32].

Positive and negative correlation of gut bacterial taxa with BW, feed intake and feed efficiency of chicken is given in Table 1.1. BW is an ultimate performance outcome, it primarily depends on two underlying components such as feed intake and feed efficiency. Therefore, bacterial taxa that influence either feed consumption or nutrient utilization may indirectly affect final BW. However, many studies reported microbial associations with body weight as an independent variable, without specifying whether these effects were mediated through intake, efficiency, or other physiological mechanisms. For this reason, correlations with body weight were presented separately to remain consistent with the original sources.

Interestingly, several bacterial taxa showed opposite relationships across performance indicators for example, *Lactobacillus* or *Ruminococcus* being positively correlated with BW but negatively with feed efficiency or intake. These contrasting associations indicate that bacterial effects on performance are context-specific, strain-dependent, and influenced by factors such as gut location, bird age, diet composition, and trial conditions. It is also plausible that some bacteria modulate BW through mechanisms beyond feed intake or efficiency, such as by improving gut

integrity, stimulating immune responses, or producing bioactive metabolites (e.g., SCFAs) that enhance nutrient absorption and energy metabolism. These findings emphasize that microbial contributions to growth are multifactorial, reflecting the complex interplay between host physiology, microbial function, and environmental context.

SCFA production is the results of fiber degradation and fermenetation by bacteria. SCFAs (acetate, propionate, butyrate) are generally associated with beneficial effects such as maintaining gut barrier integrity, regulating immune responses, and providing energy to colonocytes[32]. However, their effects can vary depending on the relative proportions and total concentrations, for instance, excess acetate or propionate may influence lipid metabolism and insulin sensitivity differently[116]. BCFAs, including isobutyrate, isovalerate, and 2-methylbutyrate, arise from the fermentation of branched-chain amino acids. Elevated BCFA levels in broilers often indicate exscessive protein fermentation in the hindgut, which may be associated with reduced nutrient efficiency, poor gut health, or dysbiosis[117]. Nevertheless, small amounts of BCFAs are normal byproducts of microbial metabolism and are not inherently harmful. The effects of SCFAs and BCFAs in broilers depend not only on their type and total concentration but also on their relative proportions in the gut, as well as the overall microbial and host context. Therefore, interpreting gut microbial fermentation requires consideration of both total levels and relative ratios of SCFAs and BCFAs, rather than relying solely on absolute concentrations.

Microbial community structure analyses reveal important insights into BW variability. While Abdel-Kafy et al.[83] found no significant differences in α-diversity between HBW and low body weight (LBW) groups, β-diversity analyses showed distinct microbial community compositions between the groups. In contrast, Lundberg et al.[32] reported higher α-diversity and more uniform microbiota in HBW birds. At the phylum level, Firmicutes and Bacteroidetes dominate the chicken gut, accounting for over 97% of microbial abundance, with HBW birds exhibiting a higher Firmicutes/Bacteroidetes (F/B) ratio [32], often interpreted as indicative of greater energy extraction efficiency[118]. However, microbial heterogeneity may arise due to age, sex, geography, management, and notably, pen-

specific effects through behaviors like coprophagy, emphasizing the need for sufficient pen replication in microbiota studies[32,119].

The metabolic outputs of the gut microbiota further reflect performance divergence. Butyrate, the most studied SCFA, improves gut integrity, enhances epithelial proliferation, and reduces inflammation via histone deacetylase inhibition. It also upregulates tight junction proteins such as claudins, occludin, and ZO-1, strengthening mucosal barrier function[120]. Furthermore, butyrate and other SCFAs activate G-protein-coupled receptors (GPR41, GPR43), modulating systemic energy balance, lipid metabolism, and mucosal immunity[121,122]. Consistently, studies have reported greater abundance of butyrate-producing taxa in HBW birds[32], aligning with performance benefits such as improved feed conversion and growth[120].

Table 1.1 Positive and negative correlation gut bacterial taxa with body weight, feed intake and feed efficiency of chicken.

Parameter	Positive correlation	Negative correlation	Gut section	Age	Reference
Body weight	*Bacteroides Enterococcus Ruminococcaceae UCG-010*	*Ruminococcaceae UCG-013*	Jejunum	37 day	[123]
	Alistipes	*Ruminococcaceae UCG-013*	Caecum	37 day	
	Faecalibacterium, Ruminococcus	*Bifidobacterium, Lactobacillus*	Crop	18 day	[124]
	Methanobrevibacter, Bifidobacterium	*Akkermansia, Lactobacillus, Streptococcus*	Ileum	18 day	[124]
	Lactococcus	*Akkermansia, Anaerovibrio, Prevotella*	Caecum	18 day	[124]
	Faecalibacterium	*Escherichia-Shigella, Enterococcus, Streptococcus*	Caecum	37 day	[32]
Feed intake	Turicibacteraceae, Enterobacteriaceae, Ruminococcaceae, Lachnospriaceae, Peptostreptococcaceae Clostridiaceae and Enterococcaceae,	Lactobacillaceae, Clostridiales, Dehalobacteriaceae, Christensenellaceae	Caecum	42 day	[125]
	Blautia, Clostridium, Unclassified Ruminococcaceae, unclassified Lachnospriaceae	Butyricicoccus	Caecum	35 day	[126]
FCR	Campylobacteraceae, Lachnospiraceae, Moraxellaceae	Enterobacteriaceae, Ruminococcaceae, Synergistaceae	Fecal	35 day	[127]
Feed effciency	*Lactobacillus*, *Akkermansia*	Faecalibacterium	Caecum	60 week	[128]
	Faecalicocus	Unclassified Lachnospiraceae, Oscillibacter,	Caecum	35 day	[129]
	Clostridiales, Faecalibacterium	Bacteroides, Oscillospira	Caecum	64 day	[130]

1.5.15 Role of microbiota in intestinal Immune function

The long-standing trade-off between immune competence and growth performance is a defining challenge in modern broiler production[96]. Intensive genetic selection for rapid weight gain and low FCRs has

inadvertently deprioritized immune system robustness, particularly in high-performing birds. As resources are diverted toward muscle accretion and metabolic output, low-performing birds often exhibit compensatory immune activation, systemic inflammation, and impaired gut function, conditions that redirect energy away from growth and exacerbate performance disparities within flocks.

One of the most critical, yet underappreciated, regulators of this trade-off is the intestinal microbiota. Beyond its role in nutrient metabolism, the gut microbiota serves as a pivotal architect of the avian immune system, influencing both its development and function[131]. While innate immunity can emerge in germ-free (GF) birds, adaptive immune maturation is heavily reliant on microbial cues. In GF models, deficiencies in gut-resident B and T lymphocytes, impaired immunoglobulin class switching (from IgM to IgA/IgY), and reduced mucosal antibody production highlight the indispensable role of the microbiota in shaping immune flexibility and competence[132]. Thus, the immune system is not only a regulator of microbial composition but also a system fundamentally programmed by it. Conceptual illustration of gut microbial balance (eubiosis) versus imbalance (dysbiosis) in poultry is given in Fig. 1.4.

This mutualism is particularly important during early life, when microbial colonization acts as an instructive signal for the development of GALT, including Peyer's patches and cecal tonsils. PRRs such as TLRs and NOD-like receptors (NLRs) detect microbial-associated molecular patterns, triggering cytokine cascades, immunoglobulin secretion, and antimicrobial peptide production[94]. A balanced microbiota facilitates the expansion of regulatory T cells (Tregs) and anti-inflammatory cytokines like IL-10, establishing immune tolerance and mucosal homeostasis. In contrast, dysbiosis frequently seen in underperforming birds, leads to chronic activation of NF-κB signaling and elevated pro-inflammatory mediators (e.g., IL-1β, IL-6, TNF-α), with associated growth penalties due to immune energy reallocation[62].

Crucially, these immune-microbiota dynamics are programmed early in life. The first 72 hours post-hatch constitute a critical window for microbial imprinting and intestinal programming. Hatchery-hatched birds, due to delayed access to feed and microbial seeding, often show delayed

colonization and dominance of facultative aerobes like *Escherichia-Shigella*[133]. These opportunists fail to promote beneficial immune priming and are linked with increased gut permeability and inflammatory tone. In contrast, birds exposed early to complex microbiota, such as through on-farm hatching or maternal microbial transfer develop more stable, diverse microbial communities enriched with *Lactobacillus*, *Bifidobacterium*, and *Faecalibacterium*[106]. These taxa not only reinforce mucosal development and enhance early SCFA production but also prime the adaptive immune system for functional resilience.

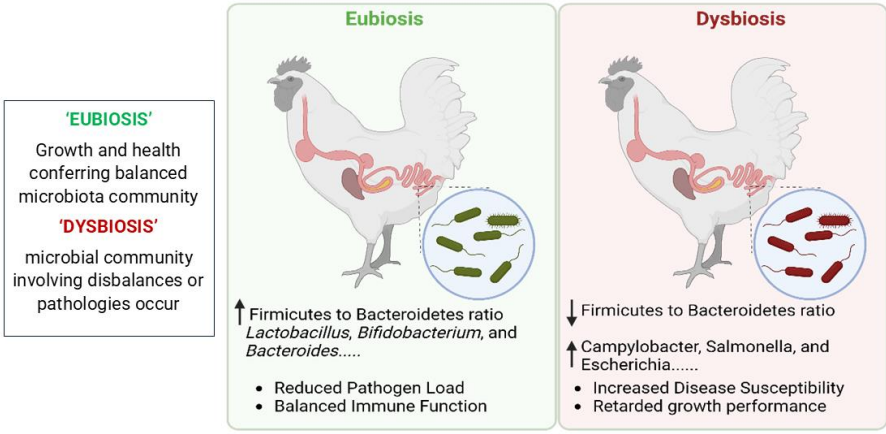

Fig 1.4 Conceptual illustration of gut microbial balance (eubiosis) versus imbalance (dysbiosis) in poultry. Eubiosis is characterized by a higher Firmicutes to Bacteroidetes ratio and the presence of beneficial genera such as Lactobacillus, Bifidobacterium, and Bacteroides, leading to reduced pathogen load and balanced immune function. In contrast, dysbiosis involves a disrupted microbial ratio with increased prevalence of pathogenic genera such as Campylobacter, Salmonella, and Escherichia, resulting in increased disease susceptibility and impaired growth. Figure based on Ducatelle et al. (2023) and created using BioRender.com.

1.6 Strategies to overcome the physiological limitations

Given the inherent physiological constraints faced by newly hatched chicks especially those at risk of underperformance, it becomes evident that the early post-hatch period offers a critical window for intervention. The immature digestive system of hatchlings is often insufficient to support the rapid skeletal muscle accretion characteristic of modern broilers. This mismatch between nutrient demand and digestive capacity contributes significantly to early growth variation and may have lasting effects on

performance trajectories. Even marginal improvements in gut development, achieved within the first 48 to 72 hours post-hatch, can translate into significant downstream benefits. Interventions that promote villus growth, enzyme secretion, microbial colonization, and barrier function during this period may effectively reduce the biological gap between low- and high-performing birds. Several targeted strategies, either standalone or in synergistic combinations, may show potential to overcome early-life physiological limitations.

1.6.1 Breeder hen nutrition

One of the earliest opportunities to influence gut development and immune function in broilers lies in maternal nutrition. Nutrients from the breeder hen's diet are readily transferred to the developing embryo via the egg, offering a practical route to prenatal programming[134,135]. The success of n-3 fatty acid-enriched eggs has paved the way for enriching hatching eggs with functional nutrients like conjugated linoleic acid, vitamins D and E, selenium, folic acid, and carotenoids[136]. Early exposure to essential fatty acids influences cell membrane composition, immune development, and the production of inflammatory mediators, with potential benefits for gut maturation and early growth. Such programming may also enhance cytokine responses and improve early resilience to pathogens. Although some studies report modest improvements in early post-hatch growth, consistent effects on performance beyond the first week are less clear[137]. Variability in results may reflect differences in breeder age, egg handling, or baseline nutrition[135,137]. Nonetheless, breeder diet manipulation remains a feasible and underutilized strategy, especially when combined with post-hatch interventions, to support underperforming chicks from the earliest developmental stages.

1.6.2 In ovo stimulation and nutrition via hatching eggs

Early-life interventions, particularly during the embryonic period, offer a critical window to enhance the physiological development of broilers. Among these, in ovo feeding of nutrients directly into the egg has emerged as a promising tool to overcome early-life physiological limitations. Originally developed for vaccine delivery, in ovo injection is widely practiced in commercial hatcheries due to its efficiency, precision, and minimal stress on the embryo. This same platform can be repurposed for nutritional interventions, targeting the late stages of embryogenesis

(typically day 17–18 of incubation)[138]. During this period, the embryo begins ingesting amniotic fluid, a protein-rich medium into which nutrients can be delivered to stimulate GIT development[139].

A variety of compounds have been evaluated for in ovo use, including simple carbohydrates (e.g., dextrin, maltose, sucrose), amino acids, vitamins, minerals, creatine, glycerol, and L-carnitine. These nutrients aim to accelerate intestinal maturation, enhance enzyme activity, and improve nutrient transporter expression. Notably, studies have reported increased villus surface area, carbohydrase activity, carbohydrate absorption, and brush border transporter activity within 3 days post-hatch[140–142]. Uni and Ferket[138] observed that in ovo-fed chicks hatch with intestinal development equivalent to 2-day-old control chicks, effectively advancing gut maturity.

Developmentally, the intestinal epithelium begins differentiation around embryonic day (ED) 14, while the immune system initiates T and B cell development from ED 10–12[143,144]. This timeline aligns with the window for microbial and nutritional programming, highlighting the potential of in ovo bioactive stimulation (e.g., prebiotics or immune modulators) on day 12 to modulate microbiota composition, mucosal immunity, and long-term performance[145].

While early growth benefits of in ovo stimulation and feeding are consistently observed particularly in the first week post-hatch, their persistence into the grow-out phase remains variable. This may be partially explained by compensatory growth mechanisms, which allow initially underdeveloped birds to catch up, thereby narrowing performance gaps over time. Despite strong experimental support, commercial adoption of in ovo feeding remains limited. Challenges include the need for specialized injection equipment, additional capital investment, and logistical integration into hatchery operations.

1.6.3 On-farm hatching and early access to feed

The critical window immediately following hatch represents a defining period in a broiler chicken's life trajectory. Traditional hatchery operations present inherent challenges, as chicks hatch across a 36-48 hour window, with collection typically occurring once approximately 95% have hatched.

This standard practice, combined with subsequent processing and transportation requirements, can result in chicks experiencing feed and water deprivation for up to 72 hours[146]. While newly hatched chicks possess residual yolk sac reserves, these resources serve specific biological functions beyond mere sustenance. The yolk primarily provides essential phospholipids for intestinal cell membrane formation and delivers maternal antibodies crucial for early passive immunity. Research has demonstrated that early feeding actually enhances yolk utilization, as intestinal movements facilitate yolk material transfer through Meckel's diverticulum into the digestive tract[147].

A delay of 36 to 48 hours in access to feed has been associated with increased mortality, impaired growth, and an unfavorable feed-to-gain ratio[148]. It also negatively affects gastrointestinal development, delaying the structural and functional maturation of the intestine, reducing nutrient absorption, and impairing gut health by disrupting gut barrier function and immune system development[149,150]. Moreover, the timing of feed intake impacts the integrity of tight junctions in the intestinal epithelium, which is essential for maintaining gut barrier function and preventing pathogen invasion[151]. Delayed feeding in chickens has been shown to impair intestinal structure, reduce nutrient absorption and compromise gut integrity[151,152].

Immediate post-hatch nutrition yields multiple developmental advantages. Early access to feed promotes nutritional maturity, accelerates gastrointestinal development, enhances digestive capabilities, and establishes favorable long-term metabolic patterns[153]. The evolution of hatching practices has led to innovative solutions addressing the challenges of delayed feeding and transportation stress. A groundbreaking development in this field is the concept of hatch on-farm (HOF), which fundamentally reimagines the traditional hatching practices. This approach involves transferring eggs at day 18 of incubation directly to broiler farms, where they complete their hatching process with immediate access to nutrition and water. HOF systems offer several distinct advantages over conventional hatchery practices. By eliminating transportation stress and providing immediate nutrition access, these systems stimulate gastrointestinal development and enhance early growth trajectories. Immediate post-hatch nutrition accelerates immune organ

development, enhances vaccination responses, and strengthens disease resistance capabilities[154]. The timing of initial feed intake also plays a crucial role in establishing gut microbiota populations, which in turn affects immune system development and overall gastrointestinal health. Feed presence stimulates secretory activity in the stomach, liver, pancreas, and small intestine, promoting intestinal mucosa development[155]. The gastrointestinal system undergoes rapid structural and functional changes, with early feeding stimulating enhanced villus development and increased absorptive capacity[152]. These mechanical and chemical stimuli influence gut hormone production, affecting appetite regulation, metabolic efficiency, and growth patterns[156]. Additionally, early feeding helps maintain intestinal barrier integrity through proper tight junction formation, reducing susceptibility to pathogen invasion[151]. HOF chicks tended to be heavier than traditionally hatched chickens until 21 d of age, but the advantage was lost thereafter. A tendency for improved feed efficiency for HOF birds was observed at 1.5 and 2.0 kg BW[157]. Importantly, the results showed that the on-farm hatching might be beneficial for broiler welfare, as it reduced wet litter, foot dermatitis, and total mortality. Importantly, chickens from young breeder flocks appear to benefit more from the HOF system due to their smaller size and higher sensitivity to suboptimal conditions[158].

Commercially available HOF systems now offer various implementation options, each with distinct operational characteristics and investment requirements. These systems represent different approaches to achieving the same goal: optimizing early-life conditions for enhanced broiler performance and welfare. However, the implementation of HOF systems requires careful consideration of practical constraints and economic factors. While these systems eliminate certain traditional challenges, they introduce new logistical considerations and require specialized equipment and staff training. The decision to adopt HOF technology must balance potential performance benefits against implementation costs and operational complexities. This technology is now commercially available through different Dutch suppliers offering specific systems (Nestborn (Exergen), One2Born (one2Born B.V.), Patio system (Vencomatic), and X-treck (Vencomatic), varying in labor requirements, ease of use, and investment.

1.6.4 Dietary physical structure modifications

The physical structure of poultry feed refers to its form and particle size, which can range from fine mash particles to larger coarse particles, and from small crumbles to full pellets. Particle size determines the surface area available for digestive enzymes, while feed form (e.g., mash, crumble, or pellet) influences how particles are presented and consumed by the bird. Traditionally, broilers have been considered to benefit from small feed particles because of their increased surface area and greater accessibility to enzymatic digestion, enhancing nutrient digestibility and supporting efficient growth[159]. However, there is growing evidence that coarser particles can also be physiologically beneficial, as they stimulate gizzard activity, slow digesta passage, and promote more complete mechanical and chemical digestion[160].

In addition to physical structure, the nutrient composition of the feed particularly its fiber fraction also plays a crucial role in gastrointestinal development. Inclusion of coarse particles or insoluble fiber sources, can significantly improve gizzard development by prolonging digesta retention time in the upper GIT, from the crop to the gizzard[161,162]. A well-developed gizzard enhances reverse peristalsis via cholecystokinin (CCK) signaling[163], increases secretion of hydrochloric acid and digestive enzymes, and improves nutrient exposure to enzymatic hydrolysis, thereby enhancing digestibility, gut motility, and energy utilization[70]. These effects are particularly evident with coarse oat hulls, which increase gizzard weight, acidification, and pepsin activation, improving protein breakdown[164].

Although fiber contributes little directly to energy supply in chickens due to limited fermentability[165], insoluble fiber has substantial physical and physiological roles. It stimulates mechanical digestion, reduces gizzard pH, creating an unfavorable environment for pathogens and can beneficially modulate gut microbiota[166,167]. In contrast, excessive soluble fiber increases digesta viscosity and slows passage rate, which can impair nutrient absorption and reduce performance[168]. Strategic inclusion of insoluble fiber, such as 2–3% insoluble fiber, has been shown to improve starch digestibility, enhance gizzard function, and reduce enteric disorders[72,169].

However, early-age broilers possess limited gizzard functionality, which may reduce their capacity to handle coarse diets efficiently, leading to initial reductions in feed intake or weight gain. As the birds mature, their gizzards adapt and benefit more markedly from structural components, improving feed conversion and weight gain[170]. Thus, the benefits of dietary structure are often age-dependent and may need to be phased appropriately.

1.7 Outline and objectives

The aim of this thesis was to investigate the factors that distinguish high-performing from low-performing broilers and to explore strategies for improving the growth and gut health of underperforming birds. First, the research focused on characterizing key biological factors, including GIT development, intestinal health, and microbiota colonization, contributing towards divergence in BW of broilers within a flock. Second, the influence of early access to feed on these parameters and its potential role in determining growth performance was assessed. Finally, based on these insights, two targeted intervention strategies were evaluated: in ovo injection of sodium butyrate (SB) during incubation and post hatch dietary modifications through feed structure adjustments. The organizational outline of the thesis is presented in Fig. 1.5.

Chapter 1, the general introduction, provides an overview of modern broiler production systems, emphasizing the persistent challenge of performance variability among birds within a single flock. It discusses key biological and hatching factors including hatching systems (HS), gut development, and microbial colonization that influence early growth. Special focus is placed on the critical post-hatch period, when nutritional and environmental factors can shape long-term performance outcomes. The chapter introduces the concept of targeting underperforming individuals as a practical approach to improve overall flock productivity and outlines early-life and nutritional strategies investigated in subsequent chapters.

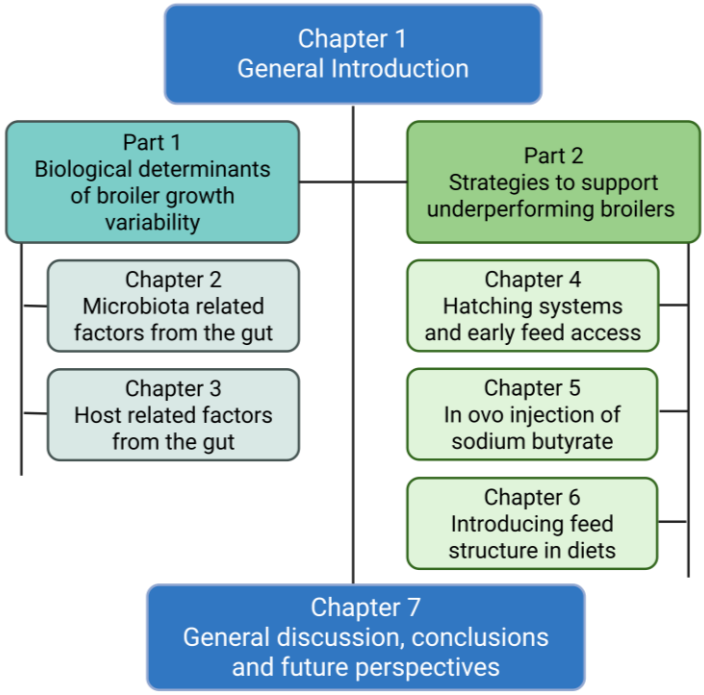

Fig 1.5 Schematic overview of the thesis' structure.

The experimental part consists of two parts.

Part 1: Biological determinants of broiler BW variability

- Chapter 2 (Published in *Animal Microbiome*) investigates whether early differences in growth performance are associated with variation in gut microbiota composition and functionality. The objective was to identify microbial taxa and metabolic profiles linked to high or low BW, with the hypothesis that HBW chicks harbor more metabolically active microbiota supporting nutrient utilization and growth. Cecal microbiome profiling and measurement of fermentation products were combined with diversity metrics and functional predictions to explore how microbial communities may influence or reflect individual bird development.
- Chapter 3 (Published in *Poultry Science*) focuses on host-related gut factors that may contribute to BW variability. The aim was to determine whether differences in gut structure, intestinal size,

histomorphology, and gene expression underpin growth disparities, with the hypothesis that HBW chicks exhibit superior gut development and functionality compared with LBW chicks. Tissue sampling and molecular analyses assessed nutrient absorption, immune function, barrier integrity, metabolism, oxidation, and hormonal regulation, helping to identify potential physiological bottlenecks in underperforming birds.

Part 2: Strategies to support underperforming broilers

- Chapter 4 (Published in *Poultry Science*) compares conventional hatchery hatching with on-farm hatching (HOF). The objective was to test whether immediate access to feed and water post-hatch improves early growth trajectories and intestinal development, hypothesizing that HOF chicks would show enhanced gut maturation and early performance.
- Chapter 5 (Published in *Journal of Animal Science and Biotechnology*) evaluates in ovo injection of sodium butyrate (SB) at day 12 of incubation in chicks with different hatch weights. The aim was to determine whether SB can promote gut development, modulate immunity, and reduce the performance gap between low and high HW chicks, with the hypothesis that optimal SB dosing supports favorable early-life programming.
- Chapter 6 (Published in *Animal Nutrition*) investigates the effects of dietary structural components, including coarse corn and oat hulls, on LBW broilers. The objective was to assess whether modifying feed structure enhances gut development, nutrient utilization, and growth, with the hypothesis that structural diets reduce BW disparities between LBW and HBW birds.

Chapter 7 provides a general discussion, integrating findings across all experimental chapters. It highlights the interplay between early-life interventions, gut health, and growth outcomes, reflects on practical implications for improving flock-level efficiency, and identifies limitations, knowledge gaps, and future directions for precision nutrition and management in broiler production.

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Chapter 2

Assessing the impact of hatching system and body weight on the growth performance, caecal short-chain fatty acids, and microbiota composition and functionality in broilers

The work presented in this chapter is adapted from:

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Abstract

Variations in body weight (BW) remain a significant challenge within broiler flocks, despite uniform management practices. Chicken growth traits are influenced by gut microbiota, which are in turn shaped by early-life events like different hatching environments and timing of first feeding. Chicks hatched in hatcheries (HH) experience prolonged feed deprivation, which could adversely impact early microbiota colonization. Conversely, hatching on-farm (HOF) allows early feeding, potentially fostering a more favorable gut environment for beneficial microbial establishment. This study investigates whether BW differences among broilers are linked to the disparities in gut microbiota characteristics and whether hatching systems (HS) impact the initial microbial colonization of broilers differing in BW, which in turn affects their growth patterns. Male Ross-308 chicks, either hatched in a hatchery or on-farm, were categorized into low (LBW) and high (HBW) BW groups on day 7, making a two-factorial design (HS × BW). Production parameters were recorded periodically. On days 7, 14, and 38, cecal volatile fatty acid (VFA) and microbiota composition and function (using 16S rRNA gene sequencing and PICRUSt2) were examined. HOF chicks had higher day 1 BW, but HH chicks caught up within first week, with no further HS-related performance differences. The HBW chicks remained heavier attributed to higher feed intake rather than improved feed efficiency. HBW group had higher acetate concentration on day 14, while LBW group exhibited higher isocaproate on day 7 and isobutyrate on days 14 and 38. Microbiota analyses revealed diversity and composition were primarily influenced by BW than by HS, with HS having minimal impact on BW-related microbiota. The HBW group on various growth stages was enriched in VFA-producing bacteria like unclassified *Lachnospiraceae*, *Alistipes* and *Faecalibacterium*, while the LBW group had higher abundances of *Lactobacillus*, *Akkermansia* and *Escherichia-Shigella*. HBW microbiota presented higher predicted functional potential compared to the LBW group, with early colonizers exhibiting greater metabolic activity than late colonizers. Despite differences in hatching conditions, the effects of HS on broiler performance were transient, and barely impacting BW-related microbiota. BW variations among broilers are likely linked to differences in feed intake, VFA profiles, and distinct microbiota compositions and functions.

2.1 Introduction

Based on the findings of the literature review in Chapter 1, it was determined that broiler flock heterogeneity is multifactorial in nature, and that gut microbiota may be a potentially influential factor. Chicken growth traits are influenced by gut microbiota, which are in turn shaped by early-life events like different hatching environments and timing of first feeding. Therefore, this chapter focuses on exploring the interplay between early-life microbial colonization, hatching systems, and broiler growth trajectories. Despite advances in genetic selection and optimized management practices, achieving uniformity in body weight (BW) at market age remains a significant challenge in broiler production[1]. Variation in final BW, typically expressed as the coefficient of variation (CV), is of particular concern, as a high CV is associated with reduced feed efficiency, increased mortality, and greater rates of market rejection[2–4].

The gut microbiota has emerged as a significant factor influencing the physiological characteristics and performance of chickens[5]. The resident gut microbiota possess the capacity to extract energy from otherwise indigestible feed components via fermentation, producing high-energy by-products such as short-chain fatty acids (SCFAs)[6]. These microbial-derived metabolites can modulate various host physiological functions, including metabolism, immunity, and intestinal barrier integrity[7].

The composition and functional capabilities of the intestinal microbiota have been extensively investigated for their potential links to broiler growth performance, however, the results have been varied and contradictory. Han et al.[8] reported a negative correlation between microbial diversity in the caecum and BW, while Abdel-Kafy et al.[9] found no differences in microbial diversity between chickens varying in growth rate. Certain bacterial genera considered beneficial, such as *Bacteroides* and *Lactobacillus*, have been associated with high weight gain and improved growth[10], but *Lactobacillus* has also been negatively correlated with BW in both the ileum and caecum[11]. Additionally, the Proteobacteria genus *Escherichia–Shigella* has been negatively correlated, while the Firmicutes genus *Clostridium coccoides* has been positively correlated with weight gain[12]. These discrepancies may be attributed to differences in

chicken genotypes, sex, geographical regions, rearing conditions, sampling time points, and intestinal sites analyzed.

A few studies have comprehensively examined distinct gut microbial signatures and functional profiles in broilers exhibiting extreme differences in BW. A recent investigation by Lundberg et al.[13] identified taxa such as *Lachnospiraceae*, *Faecalibacterium*, and *Butyricicoccus* to be enriched in high body weight (HBW) broilers, while *Akkermansia* and *Escherichia-Shigella* were more abundant in low body weight (LBW) counterparts on day 37. Furthermore, Lee et al.[14] found that higher abundances of *Shuttleworthia* and *Faecalibacterium* in HBW male chickens on day 35 post-hatch. However, the majority of studies have focused on a single time point, typically near or at market age, limiting the understanding of dynamic gut microbial changes during early life.

First gut microbiota colonization has been reported to influence microbiome succession and host growth in later stages[15]. While the influence of early life experiences on broiler development has been acknowledged, limited research has explored the specific effects of hatching conditions on broiler microbiota and subsequent growth patterns. Traditionally, broiler chicks hatch in artificial incubators under a relatively sterile environment (egg and incubator sterilization) without maternal-offspring interaction[12]. Additionally, hatchery-hatched (HH) chicks face delayed access to feed and water due to long hatching windows and hatchery logistic procedures[16]. This implies the lack of proper early exposure to microorganisms particularly to those with beneficial effects, increasing the likelihood of exposure to environmental pathogens[17].

Alternatively, hatching on-farm (HOF) involves the transportation of embryonated eggs to the broiler house on day 18 of incubation, allowing immediate access to feed and water for chicks at hatching[16]. This approach has the potential to foster a more favorable environment for early gut development and beneficial microbiota colonization. Since chickens on farms encounter a wide variety of microorganisms present in litter, feed, water, and air, thus the conditions during the hatching process can play a crucial role in shaping the initial colonization of the gut microbiota. For example, it was highlighted that chicks originating from hatcheries often exhibit delayed and highly variable development of their gut microbiota[18].

This variability is anticipated to be reduced in chicks with early access to feed, as demonstrated by the observed similarities between the microbiota of their diet and that of their intestines[19].

To delve deeper into the aforementioned aspects, we designed a study to investigate the intricate relationships between BW and caecal microbiota dynamics and the impact of different hatching systems (HS) on microbial signatures in birds with different BWs. The aim in this study was to explore whether broilers with varying BWs have differences in performance indices, caecal volatile fatty acids (VFAs), microbiota community structures, and predicted functionality on days 7, 14, and 38 under shared management conditions. Thereby, we extend beyond existing studies that majorly focus on single time points, particularly at slaughter age. Consequently, we characterized crucial changes in intestinal microbiota also during early life stages that influence the succession of gut microbiota and subsequent growth trajectories. We also investigated how HS may differentially impact initial microbiota colonization in broilers with different weights and shape their post-hatch microbiota development and growth patterns.

2.2 Materials and methods

This animal study was approved by the Katholieke Universiteit Leuven Ethical Committee for Animal Experimentation (Ethical protocol P045/2022, Belgium) and was performed at TRANSfarm, the research facility for animal experimentation of KU Leuven (Lovenjoel, Belgium).

2.2.1 Animals, Housing and Management

This study involved Ross 308 male chicks, sourced from eggs intended for both HS and originating from the same 40-week-old parent flock. These eggs were obtained from Hatchery Belgabroed N.V. (Merksplas, Belgium). The HOF system involved obtaining fertilized eggs and transporting them to the barn after candling on embryonic day 18. The eggs were placed on the wood shavings at optimal housing conditions with regulated eggshell temperatures (36.1 – 37.2 °C) to support optimal embryonic development. Chicks started hatching on embryonic day 19. Once 75% of the chicks had hatched, the primary focus shifted from regulating the eggshell temperature to maintaining the chicks' body temperature between 39.5 and 40.5 °C. Chicks were provided 24 hours of light to facilitate their

immediate access to feed and water upon hatching. The HH chicks hatched in a hatchery (Belgabroed N.V., Belgium) under standard procedures. The hatch window typically lasts 24-36 hours, after which chicks were removed from the hatcher[20]. Following grading, sexing, and other processes, chicks were transported 108 km to the farm, which took approximately 2 hours. Consequently, for some chicks, it was more than 40 hours before placing into the pens and accessing feed and water, considering the hatch window, hatchery protocols, and transportation time.

Following standard commercial practices, the day of arrival of HH chicks at the broiler house was designated "day 1" for both HS. On this day, HOF chicks underwent manual grading and sexing, including culling of chicks with deformities. By the end of day 1, the temperature of the barn was set at 33 °C, gradually decreasing by approximately 0.5 °C daily until it reached 21.5 °C on day 21, remaining constant for the remainder of the experiment. Birds were reared on a concrete floor with wood shavings as bedding material, provided with one hour of darkness on day 1, increasing to six hours from day 7 onwards. They had unrestricted access to water and received three-phase commercial diets (starter, grower, and finisher) without exposure to antibiotics (Table S2.1).

2.2.2 Study design

The study included 908 day-old male Ross 308 broiler chicks, 454 of which were from each of the two HS (Fig. 2.1). For each HS, chicks were co-reared until day 7, and then grouped into BW categories as follows: low (LBW, n = 147), birds falling below the mean BW by half the standard deviation (½×SD); middle (n = 167), birds within the mean BW and ± ½×SD; and high (HBW, n = 140), birds surpassing the mean BW by half the SD (½×SD). The middle BW birds were excluded from the study. The study design was a 2 × 2 factorial arrangement, investigating two main factors: HS (HH vs. HOF) and BW (LBW vs. HBW), and their interaction (HS × BW). The chicks were reared in the same management conditions following the commercial stocking density limits, housed in 28 pens ((1.3m2/pen, 7 replicate pens per experimental group) of LBW (n=21/pen) and HBW (n=20/pen). The LBW pens each had one extra bird so as to reach a similar stocking density to that of the HBW pens.

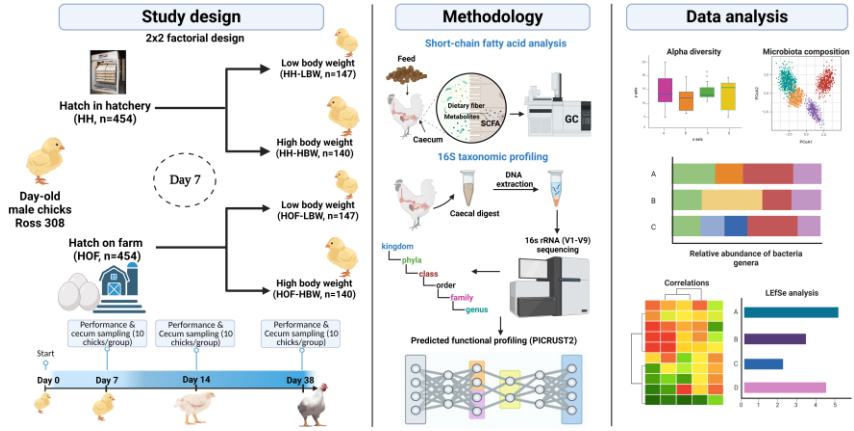

Fig. 2.1 Flow chart of the study design, timeline, and parameters investigated. This image was created with Biorender.com.

2.2.3 Growth performance

Birds were weighed individually on days 0, 7, 14, 28, and 38 post-hatch and feed intake was recorded per pen on days 7, 14, 28, and 38. Mortalities and postmortem weight were recorded for the calculation of average daily gain (ADG), average daily feed intake (ADFI), and mortality-corrected feed conversion ration (FCR). The CV (%) for weight uniformity in each group was calculated on days 7, 14, 28, and 38 by taking the ratio of the standard deviation (SD) to the mean BW and multiplying by 100.

2.2.4 Chick sampling

On days 7, 14, and 38, ten birds from each experimental group were killed by electronarcosis followed by decapitation for sampling purposes. Digesta samples were carefully collected from both caeca, placed in 2 mL vials, snap-frozen, and stored at -80 °C until further analysis of microbiota and VFAs.

2.2.5 Volatile fatty acid analysis

The level of short- (SCFAs; acetate, propionate, butyrate, valerate, and caproate) and branched- (BCFAs; isobutyrate, isovalerate, and isocaproate) chain fatty acids were determined using a method previously detailed by Van Craeyveld et al.[21] with minor modifications. Briefly, 450-500 mg caecal content was mixed with 100 µl of a 2-methylhexanoic acid, followed by the addition of 200 µL of 25% NaCl solution and 9.2 M sulfuric acid each. Subsequently, 800 µL diethyl ether was added to extract organic

acids, followed by centrifugation at 3800 × g for 5 minutes at 4 °C. The resulting supernatants were transferred to a reactive vial containing 0.2 – 0.3 g of activated anhydrous sodium sulfate and centrifuged at 3800 × g for 6 minutes at 4 °C before analysis. VFAs were quantified by gas chromatography (GC) on an HP 6890 Series GC System. This system had an Automatic Liquid Sampler (7683 Series Injector, Agilent Technologies) for cool on-column injection, a flame ionization detector, and a DB-FFAP capillary column (Agilent J&W GC Columns, 30 m length, 0.32 mm internal diameter, 0.25 µm film thickness). Nitrogen served as the carrier gas flowing at a 25 mL/min rate. The column temperature was maintained at 130°C, while the injector and detector temperatures were set to 195°C.

2.2.6 DNA extraction and 16S rRNA gene amplicon sequencing

DNA was extracted from approximately 250 mg of caecal digesta for 16S rRNA gene markers using the QIAamp PowerFecal Pro DNA Kit (Qiagen Benelux B.V., Venlo, the Netherlands) in accordance with the manufacturer's standard protocol. The concentration of obtained DNA was determined using a Nanodrop 2000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA), while quality was assessed by 1% agarose gel electrophoresis. The full-length (V1-V9) 16S rRNA gene was amplified via PCR using the universal primers 27F: AGRGTTYGATYMTGGCTCAG and 1492R: RGYTACCTTGTTACGACTT, with sample-specific PacBio barcode sequences added. A ZymoBIOMICS™ Microbial Community DNA Standard (P/N: D6306, Lot ZRC190811) containing genomic DNA from six phylogenetically diverse bacteria was used as a positive control, and DNA from ultrapure water was used as a negative control. DNA libraries were generated from the amplified DNA, and sequencing was performed using the PacBio platform by the VIB Nucleomics Core (Leuven, Belgium).

2.2.7 Sequence processing workflow

After sequencing, further data analysis was performed in R (v4.2.3, R Foundation, Vienna, Austria). The raw sequence data obtained from PacBio long-read amplicon sequencing underwent additional processing steps, including quality filtering, denoising, and removal of chimeric sequences, following the established long-read workflow by Callahan et al.[22]. After filtering and denoising, ASVs were inferred using the DADA2 R package. The ASVs were taxonomically classified by comparing them against the SILVA database (release 138) at a 99% shared identity using the

Naive Bayes Classifier method. Downstream analysis focused on bacterial domain sequences, and positive control was excluded from the analysis, as it was included to verify the accuracy of the taxonomic assignment. Reads were decontaminated based on the negative control, which identified *Bradyrhizobium elkanii*, unclassified *0319-6G20*, and unclassified *Acidibacter* spp. as contaminants, and these were removed from the ASV table accordingly, resulted in 2776 ASVs on day 7, 2839 ASVs on day 14, and 4118 ASVs on day 38. The α-diversity and β-diversity were calculated in R using the phyloseq package (v1.40.0). For α-diversity, the rarefaction of the ASV table was performed to the minimum sample depth. Three α-diversity indices were calculated: Chao1, Shannon, and Simpson, which indicate microbial richness, overall diversity, and evenness, respectively. Two-way analysis of variance (ANOVA) was used for each α-diversity measure to compare the effects of HS, BW, and their interaction. β-diversity was determined using the Bray-Curtis dissimilarity matrix, which was obtained from the distance function in phyloseq and visualized via principal coordinate analysis (PCoA). Multivariate effects of HS and BW on β-diversity were evaluated by non-parametric permutational multivariate analysis of variance (PERMANOVA) using the adonis2 function with 999 permutations from the vegan package (v2.6.4). The differential abundance of cav ecal microbiota was calculated using LEfSe in R using the microbiome package (v1.18.0). The default parameter of LDA > 2 was used with a significance threshold of $P < 0.05$. The obtained *P*-values were further adjusted FDR through the Benjamini-Hochberg method, with a stringent criterion of FDR < 0.05. The results were then visually represented based on the Log10 (LDA score). PICRUSt2 was used to predict the functional capabilities of the microbial communities in the different BW groups. This functional profiling was derived from the 16S rRNA gene sequences and utilized the MetaCyc Metabolic Pathway Database as a reference[23]. The data obtained from PICRUST2 was analyzed through two-way ANOVA with FDR < 0.05. Principal component analysis (PCA), an unsupervised pattern recognition method, was used in R using the factoextra (v 1.0.7) package, to provide an overview of the predicted function data patterns between HS-BW groups.

2.2.8 Statistical analysis

Shapiro-Wilk's test in R was performed to evaluate the normality of data. Following the confirmation of normality, the BW data on day 1 for HH and HOF chicks was analyzed by Student's t-test. The data on growth performance and VFA from day 7 onward were used to conduct the two-way ANOVA and Tukey's post hoc test. The HS and BW were used as the fixed effects and the pens were considered as a random effect to account for potential confounding variation due to pen location and differing numbers of birds per pen. For all statistical tests, a P-value threshold of 0.05 was used to determine statistical significance, while a P-value between 0.05 and 0.10 indicated a trend toward significance. Spearman correlation analysis was performed in R using the psych package (v2.3.12) to determine the correlation between LEfSe-identified abundant bacterial genera and the BW and caecal VFA of broilers.

2.3 Results

2.3.1 Growth performance

HS significantly influenced BW at placement ($P < 0.05$), with HOF chicks exhibiting higher BW on day 1 (45.1 ± 3.14 g, n = 454) compared to HH chicks (42.2 ± 2.92 g, n = 454). This difference in BW between HS disappeared by day 7, and the chicks hatched in either system no longer differed in any performance indices thereafter (Table 2.1, $P > 0.05$). The chicks from both HS were categorized into LBW and HBW groups on day 7, revealing a significant difference in BW ($P < 0.05$). From day 7 onwards, there was no point at which chicks in the LBW group were able to catch up and they consistently demonstrated lower BW ($P < 0.05$) on days 14, 28, and 38 compared to chicks in the HBW group. Similarly, ADG was lower ($P < 0.001$) in chicks of the LBW group than in those in the HBW group except during 29 – 38 days. Lower initial BW was accompanied by a lower feed intake, and chicks in the LBW group demonstrated lower ADFI ($P < 0.05$) than chicks in the HBW group during 7 – 14 days, 15 – 28 days, 29 – 38 days, and 7 – 38 days, respectively. The FCR was lower ($P = 0.021$) in the LBW group than in the HBW group during the overall period (7 – 38 days). The CV for BW [CV(%) = flock heterogeneity] was lower in the HBW group on days 14, 28, and 38 than in the LBW group ($P < 0.05$). There was no interaction ($P > 0.05$) between HS and BW for any growth performance measurements. Finally, no differences in mortality ($P > 0.05$) were

observed between LBW and HBW birds of either HS over the 38-day period (data not shown).

Table 2.1 Growth performance of low (LBW) and high (HBW) body weight broilers hatched in the hatchery (HH) or on-farm (HOF).

[1]Items	[2]Groups (n = 7 pen/group)				SD	*P*-values		
	HH-LBW	HH-HBW	HOF-LBW	HOF-HBW		HS	BW	HS × BW
BW, g								
Day 7	166[b]	206[a]	159[b]	211[a]	23.7	0.698	<0.001	0.268
Day 14	451[b]	572[a]	446[b]	563[a]	62.9	0.364	<0.001	0.724
Day 28	1716[b]	2025[a]	1701[b]	2012[a]	160.6	0.876	<0.001	0.426
Day 38	2962[b]	3259[a]	2946[b]	3248[a]	177.1	0.623	<0.001	0.770
ADG, g BW/day								
7-14 days	41.1[b]	52.3[a]	40.7[b]	51.5[a]	5.82	0.362	<0.001	0.808
15-28 days	90.2[b]	103.3[a]	89.7[b]	102.9[a]	7.65	0.746	<0.001	0.822
29-38 days	125.3	124.2	124.6	123.8	9.40	0.881	0.796	0.974
7-38 days	94.3[b]	103.7[a]	93.6[b]	103.4[a]	5.81	0.701	<0.001	0.891
ADFI, g feed/day								
7-14 days	47.0[b]	57.5[a]	46.5[b]	58.9[a]	6.67	0.740	<0.001	0.499
15-28 days	117.2[b]	137.0[a]	117.1[b]	136.2[a]	11.21	0.827	<0.001	0.903
29-38 days	184.7[b]	198.9[a]	189.9[b]	200.2[a]	13.03	0.470	0.012	0.682
7-38 days	123.1[b]	139.0[a]	124.7[b]	139.4[a]	9.31	0.630	<0.001	0.779
FCR, g feed/g BW								
7-14 days	1.16	1.16	1.19	1.20	0.198	0.336	0.858	0.933
15-28 days	1.34	1.36	1.36	1.39	0.206	0.479	0.464	0.958
29-38 days	1.53	1.61	1.54	1.66	0.261	0.732	0.056	0.522
7-38 days	1.40[b]	1.45[a]	1.42[b]	1.45[a]	0.143	0.621	0.021	0.366
CV in BW (%)								
Day 7	4.9	4.8	5.0	4.9	0.28	0.679	0.102	0.956
Day 14	6.9[b]	5.2[a]	7.4[b]	5.7[a]	1.72	0.321	0.007	0.892
Day 28	8.7[b]	6.8[a]	9.2[b]	7.1[a]	1.37	0.868	≤0.001	0.168
Day 38	12.9[b]	7.9[a]	13.8[b]	8.2[a]	3.08	0.472	≤0.001	0.700

[1]BW: body weight; ADG: average daily gain; ADFI: average daily feed intake; FCR: feed conversion ratio; CV: coefficient of variation (inversely related to flock uniformity). [2]HH-LBW: hatchery-hatched low BW group, HH-HBW: hatchery-hatched high BW group, HOF-LBW: hatched on-farm low BW group, HOF-HBW: hatched on-farm high BW group. Except for BW data, the pen was considered as an experiment unit. Data are presented as mean and pooled standard deviation (SD). Values in a row with different superscript letters (a,b) indicate significant difference at $P < 0.05$.

2.1.1 Caecal microbiota

A total of 3,763,252 reads obtained from 120 samples were used in the microbiota analysis, resulting in an average of 31,360 reads per sample with a SD of 11,410 reads (Range: minimum = 11,711 and maximum = 60,170). To ensure uniformity in the α-diversity analysis, the sample with the minimum number of reads (11,711) was established as the cut-off threshold for rarefying all samples.

2.1.1.1 α-diversity

α-diversity metrics were not affected by HS at any time point (Fig. 2.2, P > 0.05). However, BW significantly influenced α-diversity, with higher Chao1 index values on day 7 (P < 0.001) and day 38 (P = 0.033) and increased Shannon and Simpson index values on day 38 (P < 0.001) in chicks of the LBW group than those in the HBW group. No interaction between HS and BW for α-diversity was deemed significant at any time point (P > 0.05).

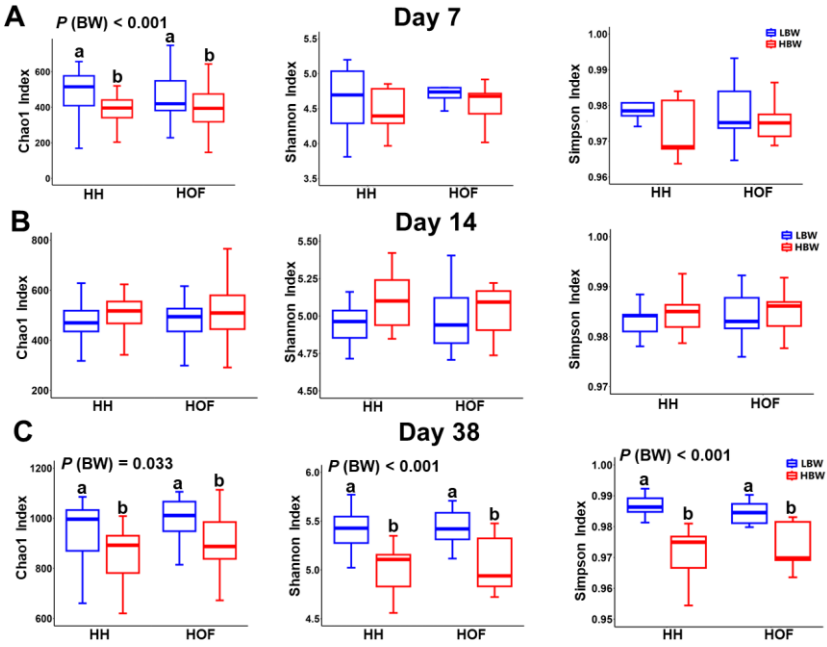

Fig. 2.2 α-diversity measures (Chao1, Shannon and Simpson index) of the caecal microbiota of low (LBW) and high (HBW) body weight chickens hatched in the hatchery (HH) and on-farm (HOF) systems on day 7 (A), day 14 (B), and day 38 (C). Individually sampled chickens were considered as experimental unit (n = 10 per group). α-diversity measures were evaluated by two-way ANOVA and significant differences were only found for BW, indicated with different letters with P (BW) < 0.05.

2.3.2.2 β-diversity

β-diversity analysis using Bray-Curtis distances did not show any differences for the HS at any time point (Fig. 2.3). However, β-diversity was significantly different between LBW and HBW groups on days 7 and 38, with two distinct clusters based on the BW groups were observed (P = 0.002 and R2 = 0.042 for day 7, and P = 0.001 and R2 = 0.027 for day 38, Fig. 2A and C). The interaction between HS and BW for β-diversity was found to be non-significant throughout the study (P > 0.05).

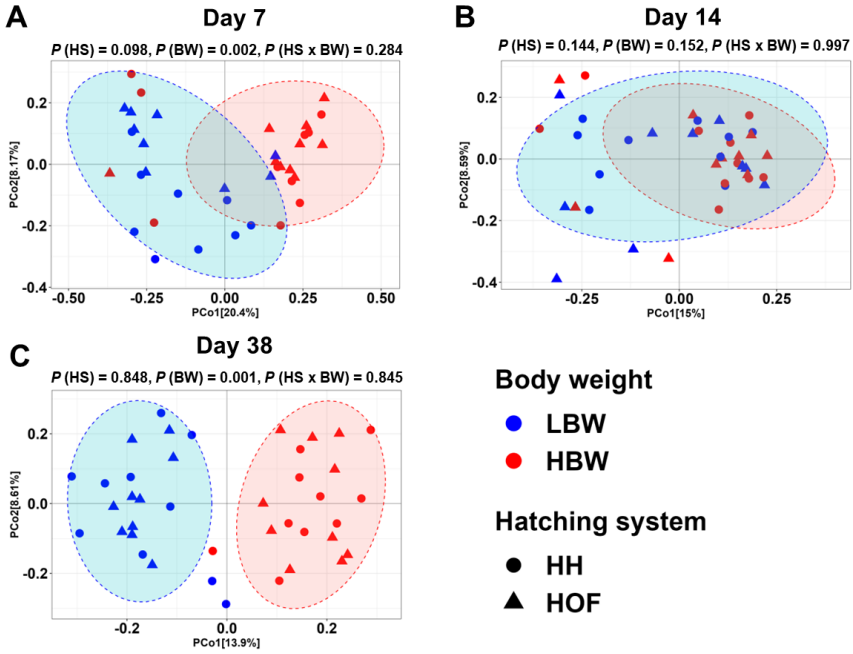

Fig. 2.3 Principal coordinate analysis (PCoA) for log-transformed Bray–Curtis dissimilarity matrices of caecal microbiota of low (LBW) and high (HBW) body weight (BW) chickens hatched in the hatchery (HH) or on-farm (HOF) on day 7 (A), day 14 (B), and day 38 (C). The color of the dots represents BW groups and their shapes represent the hatching system (HS). Individually sampled chickens were considered as experimental unit (n = 10 per group). Multivariate effects of HS and BW on β-diversity were evaluated by non-parametric permutational multivariate analysis of variance (PERMANOVA) and significant differences were only found for BW. The P-values for HS, BW, and their interaction are indicated with different letters with P (BW), P (HS), and P (HS × BW), respectively.

2.1.1.1 Core microbiota composition

Compositional analysis consistently identified Firmicutes as the predominant phylum in chickens from both HS throughout the study (Table S2.2). This phylum represented ~99% of the total relative abundance on day 7, ~97% on day 14, and ~93% on day 38. Following Firmicutes, Bacteroidota, and Proteobacteria emerged as the next dominant phyla across all three time points, with Cyanobacteria joining in notable relative abundance by day 38. At the genus level, HH and HOF chickens exhibited a distinctive dominance of unclassified *Lachnospiraceae*, and *Lactobacillus* on day 7 (16–30% and 10–22% respectively), followed by the *[Ruminococcus] torques group* (10–13%) and *Lachnoclostridium* (3–5%, Fig. 2.4). By day 14, the dominant genera included unclassified *Lachnospiraceae* (14–20%) and *Faecalibacterium* (15–17%), along with *Lactobacillus* and the *[Ruminococcus] torques group* at 6–10% and 7–8%, respectively. By day 38, the most predominant genera were the unclassified *Clostridia vadinBB60 group* (9–16%) and unclassified *Lachnospiraceae* (11–12%), followed by *Faecalibacterium* (7–9%), *Lactobacillus* (6–9%), and *Blautia* (6–9%).

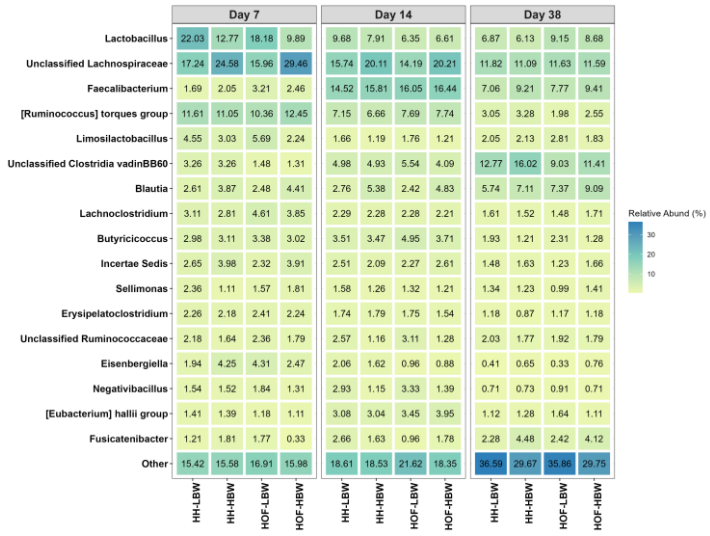

Fig. 2.4 Relative abundance of caecal bacterial genera in low (LBW) and high (HBW) body weight chickens hatched in the hatchery (HH) or on-farm (HOF) on days 7, 14, and 38. Values indicate the mean relative abundance (%) of the top 17 genera (Y-axis). Individually sampled chickens were considered as experimental unit (n = 10 per group).

2.1.1.1 Differential abundance of bacteria

Linear discriminant analysis (LDA) effect size (LEfSe) analysis was used to determine the differential phylum abundance between groups using a false discover rate (FDR) cut-off value of 0.05 with a minimum LDA score of 2. At the phylum level, no differences were observed for HS (HH vs. HOF) or the HS × BW interaction. BW-dependent differences were observed at the phylum level on days 7 and 38 (Fig. 2.5A and B). Bacteroidota phylum was differentially enriched in HBW chickens on days 7 and 38, while Proteobacteria was more abundant in LBW chickens on days 7 and 38 along with Cyanobacteria on day 38 (FDR < 0.05). No differential abundance was found at the phylum level on day 14.

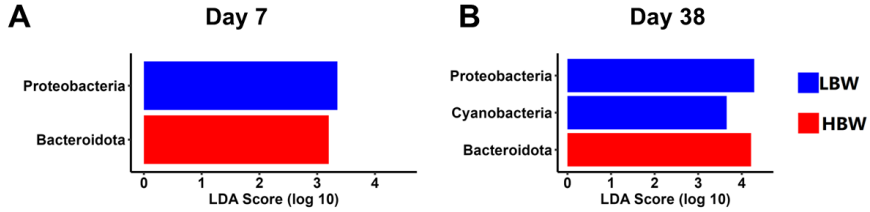

Fig. 2.5 Differential abundance of phyla (FDR < 0.05 and |LDA| > 2) in samples from low (LBW) and high (HBW) body weight chickens on day 7 (A), and day 38 (B). No differences were observed on day 14.

The differential abundance of bacterial genera between HH and HOF chickens was determined on days 7, 14, and 38 using LEfSe analysis using an FDR cut-off value of 0.05 with a minimum LDA score of 2. (Fig. 2.6). On day 7, HH chicks exhibited enriched *Escherichia-Shigella*, *Lactobacillus*, and unclassified *Clostridia vadinBB60 group* (FDR < 0.05), while HOF chicks showed greater relative abundance of unclassified *Lachnospiraceae*, *Lachnoclostridium*, *Faecalibacterium*, and *Oscillibacter* (Fig. 2.6A). By day 14, HH chicks were enriched in *Lactobacillus*, *Lachnospiraceae NK4A136 group*, and *Ruminococcus*, while HOF chicks had a higher abundance of *Incertae Sedis*, *Bilophila*, and unclassified *Desulfovibrionaceae* (Fig. 2.6B). By day 38, microbiota differences between HS substantially reduced, with only four genera showing differential abundance. The HH chicks had an higher abundance of unclassified *Clostridia vadinBB60 group*, while HOF chicks had a higher abundance of *Shuttleworthia*, *Lactobacillus*, and *Blautia* (Fig. 2.6C).

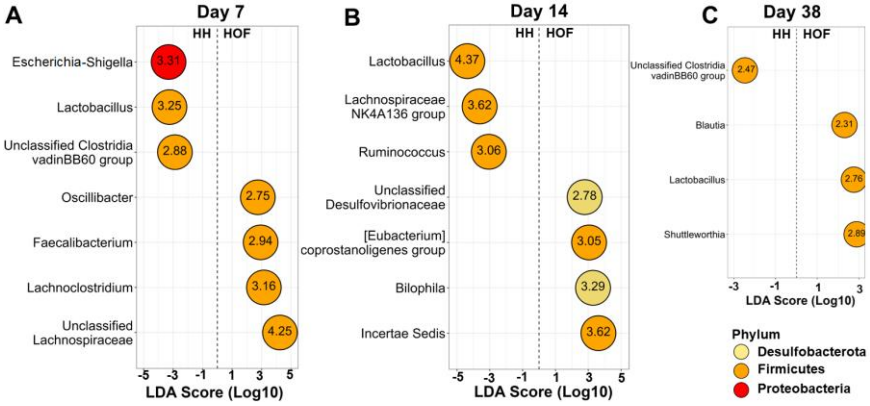

Fig. 2.6 LEfSe results of differentially abundant genera for chicks hatched in the hatchery (HH) vs. on-farm (HOF) on day 7 (A), day 14 (B), and day 38 (C). Only genera with an FDR ≤ 0.05 and an absolute value of LDA > 2 are presented. The left-hand side of each plot indicates bacterial genera enriched in HH chickens, and on the right-hand side, bacterial genera enriched in HOF chickens.

Differential abundance of bacterial genera between BW groups was also determined on days 7, 14, and 38 using LEfSe analysis (Fig. 2.7). On day 7, the LBW group showed enrichment in seven genera, including *Escherichia-Shigella*, *Streptococcus*, *Limosilactobacillus* and *Lactobacillus*, while the HBW group had higher abundance in five genera, including unclassified *Lachnospiraceae*, *Christensenellaceae R-7 group*, and *Alistipes* (Fig. 2.7A). By day 14, LBW group were significantly enriched with four genera, including *Lachnospiraceae NK4A136 group*, unclassified *Ruminococcaceae* and *Negativibacillus*, while the HBW group exhibited increased abundance in five genera, such as unclassified *Lachnospiraceae*, *Subdoligranulum*, *Romboutsia*, and *Blautia* (Fig. 2.7B). The differences in microbiota composition between BW groups increased over time, with the LBW group on day 38 showing differential enrichment of 21 genera, including *Escherichia-Shigella*, *Enterococcus*, *Bilophila*, *Streptococcus*, and *Akkermansia*, compared to the HBW group, which exhibited increased abundances of six genera, including *Faecalibacterium*, unclassified *Clostridia vadinBB60 group*, and *Alistipes* (Fig. 2.7C). A few interactions between HS and BW were observed for microbiota differential abundance analysis (Fig. S2.1). Specifically, *Lactobacillus* was enriched in HH-LBW chicks on day 7, while unclassified *Lachnospiraceae* was enriched in HOF-HBW chicks (Fig. S2.1 A). Two genera were differentially abundant on day

38, with HOF-LBW birds having a higher abundance of unclassified *Desulfovibrioceace*, while HH-HBW chickens had an overabundance of unclassified *Clostridia vadinBB60 group* (Fig. S2.1 B). No significant differences for HS × BW interaction were observed on day 14.

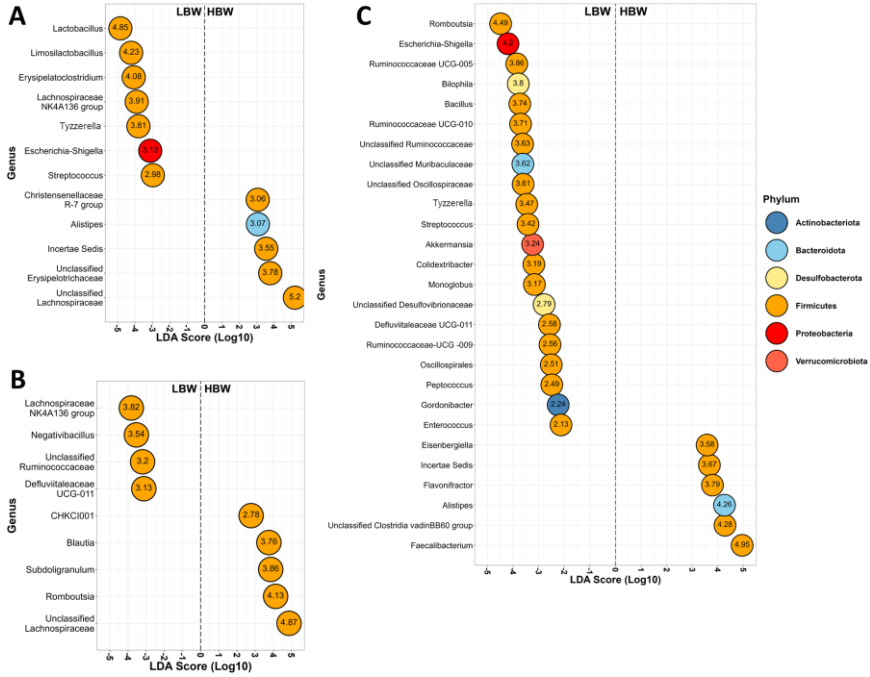

Fig. 2.7 LEfSe results of the differential abundance of genera in the caecal microbiota of chickens with low (LBW) and high (HBW) body weight on day 7 (A), day 14 (B), and day 38 (C). Only genera with an FDR ≤ 0.05 and with an absolute value of LDA > 2 are presented. The lefthand side of plots indicates bacterial genera enriched in LBW chickens, and on the right hand side, bacterial genera enriched in HBW chickens.

2.3.3 Concentration of volatile fatty acids in caecum

HS did not significantly influence VFAs, but BW and the interaction between HS and BW affected their concentrations on various days (Table 2.2). On day 7, Isocapraote production was higher in the LBW group. The HBW group demonstrated higher acetate and total SCFA concentrations on day 14, while the LBW exhibited higher levels of isobutyrate on days 14 and 38, as well as increased isovalerate and total branched-chain fatty acids (BCFAs) on day 14. Furthermore, propionate and total BCFA concentrations on day 38 tended to be higher in the LBW group. An

interaction between HS and BW was observed on day 7, with the HH-LBW group exhibiting higher valerate concentrations. A tendency towards an interaction between HS and BW was noted, with acetate and total SCFA levels tending to be higher in the HOF-HBW group on day 7, and valerate levels tending to be higher on day 14.

Fig. 2.8 shows Spearman correlations between VFA concentrations and differentially enriched bacterial genera across 3 time points, with the highest number of significant (positive) correlations found on day 38. Correlations with an FDR < 0.05 and |R| > 0.33 were considered significant and were indicated with an asterisk. *Blautia* correlated positively with acetate concentration on day 14 but negatively correlated with butyrate on day 38. *Lactobacillus* was positively correlated with propionate, valerate, isovalerate, isocaproate and total BCFAs on day 7, and isobutyrate on day 38. *Limosilactobacillus* showed a negative correlation with acetate, isovalerate, and total SCFAs on day 7. *Negativibacillus* positively correlated with isobutyrate on day 14. *Romboutsia* showed a positive correlation with propionate on day 14, and a negative correlation with butyrate on day 38. *Christensenellaceae R-7 group* positively correlated with propionate and total SCFAs on day 14 *Escherichia-Shigella* correlated positively with isovalerate on day 7, negatively with propionate and butyrate on day 14, and positively with isobutyrate on day 38. *Flavonifractor* positively correlated with acetate, butyrate, caproate, and total SCFA production on day 38.

Table 2.2 Caecal volatile fatty acid (VFA) concentrations (mM/g wet digesta) of low (LBW) and high (HBW) body weight broiler chickens hatched in the hatchery or on-farm.

[1]Items	[2]Groups (n=10/group)				SD	[3]*P*-values		
	HH-LBW	HH-HBW	HOF-LBW	HOF-HBW		HS	BW	HS × BW
Day 7								
Acetate	200.5	155.8	182.9	249.6	73.61	0.212	0.713	0.074
Propionate	6.5	4.8	5.1	6.0	3.61	0.948	0.788	0.429
Butyrate	25.9	21.1	24.5	28.9	8.31	0.393	0.950	0.208
Valerate	1.39^{a}	0.74^{bc}	0.56^{c}	0.94^{b}	0.596	0.212	0.566	**0.047**
Caproate	0.04	0.02	0.01	0.03	0.022	0.134	0.540	0.109
Total SCFAs	234.6	182.6	213.2	285.7	79.02	0.210	0.750	0.068
Isobutyrate	0.96	0.77	0.87	0.98	0.358	0.349	0.806	0.734
Isovalerate	0.57	0.35	0.53	0.61	0.214	0.215	0.411	0.107
Isocaproate	0.26^{a}	0.19^{b}	0.22^{a}	0.21^{b}	0.041	0.332	**0.019**	0.095
Total BCFAs	1.79	1.31	1.62	1.79	0.552	0.528	0.533	0.185
Day 14								
Acetate	185.2^{b}	217.3^{a}	177.5^{b}	207.8^{a}	45.31	0.545	**0.032**	0.955
Propionate	10.9	11.4	10.8	13.4	5.79	0.648	0.423	0.571
Butyrate	40.3	40.6	37.6	39.8	14.78	0.551	0.641	0.683
Valerate	2.21	2.14	1.76	2.30	0.641	0.432	0.123	0.077
Caproate	0.07	0.06	0.06	0.07	0.031	0.728	0.851	0.356
Total SCFAs	238.8^{b}	277.4^{a}	225.4^{b}	263.4^{a}	53.79	0.524	**0.041**	0.875
Isobutyrate	1.42^{a}	0.97^{b}	1.16^{a}	0.87^{b}	0.520	0.247	**0.022**	0.588
Isovalerate	1.18^{a}	0.80^{b}	1.09^{a}	0.91^{b}	0.440	0.932	**0.049**	0.489
Isocaproate	0.18	0.15	0.17	018	0.093	0.688	0.697	0.603
Total BCFAs	1.25^{a}	0.87^{b}	1.09^{a}	0.88^{b}	0.427	0.586	**0.028**	0.501
Day 38								
Acetate	212.2	227.1	209.1	218.3	88.56	0.840	0.661	0.922
Propionate	14.1	12.4	14.8	11.5	4.86	0.860	0.079	0.860
Butyrate	41.8	43.5	42.8	42.9	15.7	0.963	0.850	0.878
Valerate	1.91	2.05	2.17	1.91	0.586	0.741	0.743	0.302
Caproate	0.07	0.06	0.08	0.06	0.042	0.634	0.242	0.725
Total SCFAs	270.5	285.2	269.1	274.8	102.0	0.862	0.762	0.895
Isobutyrate	1.63^{a}	1.41^{b}	1.67^{a}	1.34^{b}	0.438	0.927	**0.048**	0.710
Isovalerate	1.46	1.42	1.43	1.14	0.435	0.237	0.236	0.392
Isocaproate	0.23	0.25	0.26	0.24	0.029	0.335	0.832	0.167
Total BCFAs	3.33	3.07	3.35	2.72	0.810	0.511	0.086	0.475

[1]SCFAs: short-chain fatty acids (Acetate, propionate, butyrate, valerate, and caproate); BCFAs: branched-chain fatty acids (Isobutyrate, Isovalerate, and Isocaproate). [2]HH-LBW: hatchery-hatched low BW group, HH-HBW: hatchery-hatched high BW group, HOF-LBW: hatched on-farm low BW group, HOF-HBW: hatched on-farm high BW group. Individually sampled chickens were considered as experimental unit. [3]HS: main effect of hatching system; BW: main effect of body weight; HS × BW: interaction between HS and BW. Data are presented as mean and pooled standard deviation (SD). Values in a row with different superscript letters ([a,b]) indicate significant difference at $P < 0.05$.

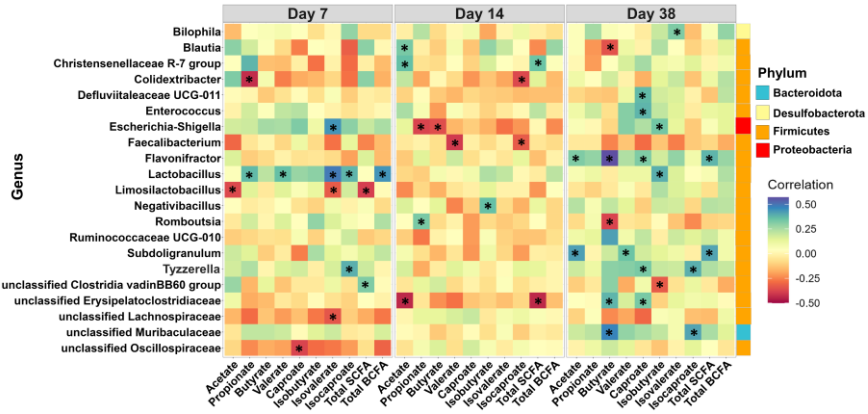

Fig. 2.8 Heatmap of Spearman correlation on days 7, 14 and 38 between caecal VFA concentrations and relative abundance of bacterial genera that were differentially enriched among BW groups based on the LEfSe analysis. Bacterial genera are color-labeled with their corresponding phyla (right side of the figure). Correlations with an FDR < 0.05 and |R| > 0.33 were considered significant and were indicated with an asterisk.

2.3.2 Microbiota functional profiling

The principal component analysis (PCA) of the metabolic pathways associated with caecal microbiota showed a clear separation between the LBW and HBW groups on day 7 (Fig. 2.9A), However, over time, the functional profiles of the microbiota converged across BW groups, as evidenced by the lack of distinct separation on days 14 and 38 (Fig. 2.9B and C). The PICRUST2 (Phylogenetic Investigation of Communities by Reconstruction of Unobserved States) output was analyzed by two-way ANOVA with FDR cut-off value of 0.05 and results revealed 25 significantly different microbial pathways on day 7 between the LBW and HBW groups (Fig. 2.10A). The LBW group showed enrichment in microbial pathways involved in the biosynthesis of cell wall components (UDP-N-acetyl-D-glucosamine, and teichoic acid), nucleotides (pyrimidine), and fermentation (lactate and butanoic acid). Microbial pathways enriched in the biosynthesis of amino acids (Thiamine, phenylalanine, tyrosine, and

glutamate), cofactors (tetrapyrrole, NAD), and vitamins (folate) were higher in the HBW group compared to the LBW group. It is worth mentioning that the LBW group also demonstrated a higher relative abundance in one pathway of amino acid biosynthesis (aspartate). Moreover, bacteria of the HBW group exhibited enrichment in degradation pathways of simple and complex carbohydrates (fucose, starch, glycerol, sucrose, fructuronate, glucuronate, and other sugars) compared to the LBW group. On day 14, five microbial pathways were enriched in both LBW and HBW groups, primarily associated with cofactor synthesis (biotin, menaquinol, and 1,4-dihydroxy-6-naphthoate) and amino acid degradation (histidine, Fig. 2.10B). On day 38, only one function related to polyamine synthesis was enriched in LBW compared to HBW birds (Fig. 2.10C). PICRUST2 functional prediction analysis revealed 11 differentially abundant microbial pathways between HH and HOF groups on day 7, and 5 differential pathways on day 14, with no significant differences observed on day 38 (Fig. S2.2 and S2.3). Interaction between HS and BW was observed solely on day 7, with the HOF-HBW group demonstrating a higher abundance of starch degradation and Calvin Benson Bassham cycle pathways compared to the other groups.

2.3.2 Correlation of bacterial genera with body weight

To further identify the bacterial genera associated with BW, Spearman correlation analysis was performed using the genera differentially enriched based on the LEfSe results (Fig. 2.11). On day 7, 6 out of 12 genera showed significant correlations with BW, including unclassified *Lachnospiraceae*, unclassified *Erysipelotrichaceae* and *Incertae Sedis* positively correlated, and *Lactobacillus*, *Lachnospiraceae NK4136* and *Limosilactobacillus* negatively correlated (Fig. 2.11A). On day 14, 2 out of 9 genera exhibited correlations, with unclassified *Lachnospiraceae* favorably correlated and unclassified *Ruminococcaceae* negatively correlated with BW (Fig. 2.11B). On day 38, 3 out of 27 genera showed significant correlations with BW, including *Eisenbergiella* positively correlated, while *Akkermansia*, *Bilophila*, and unclassified *Desulfovibrionaceae* were negatively correlated with BW (Fig. 2.11C).

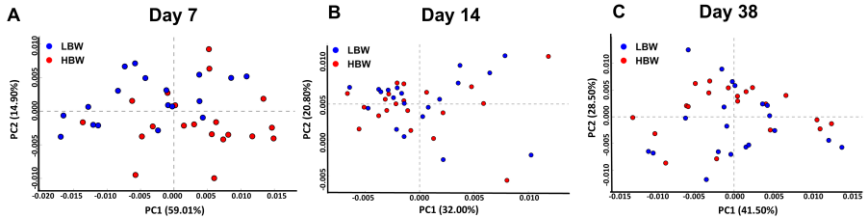

Fig. 2.9 Principal component analysis of predicted pathways of the differential microbiota in low (LBW) and high (HBW) body weight groups on day 7 (A), day 14 (B), and day 38 (C).

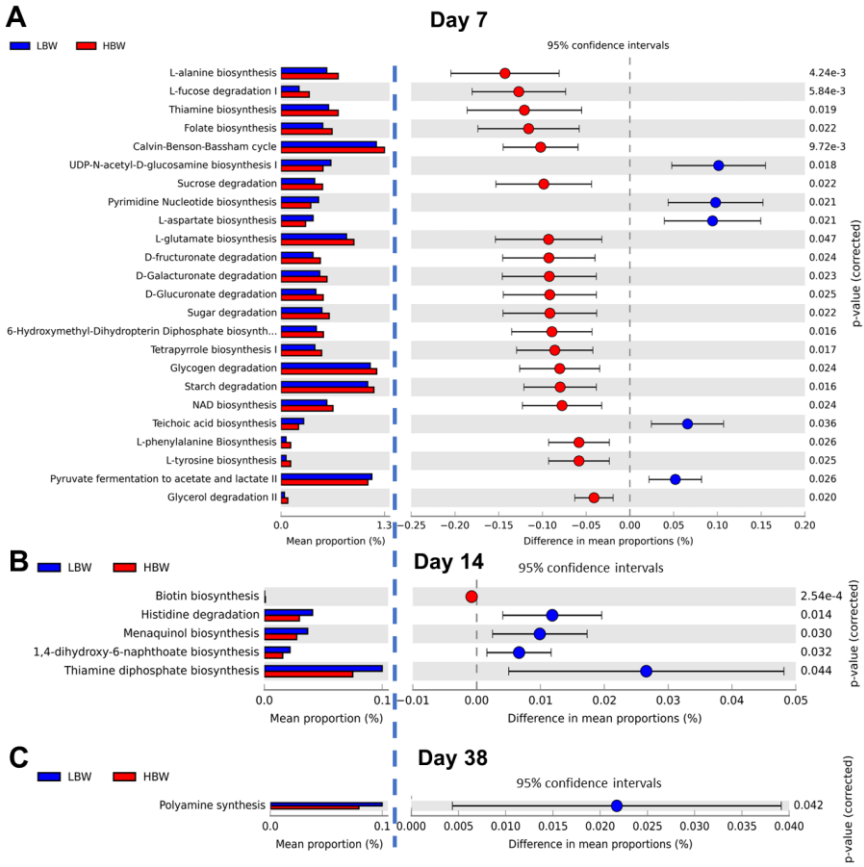

Fig. 2.10 Predicted functions of the cecal microbiota of low (LBW) and high. (HBW) body weight broilers of both hatching systems (HS) on day 7 (A), day 14 (B), and day 38 (C). Only differentially regulated metabolic pathways are shown (FDR < 0.05).

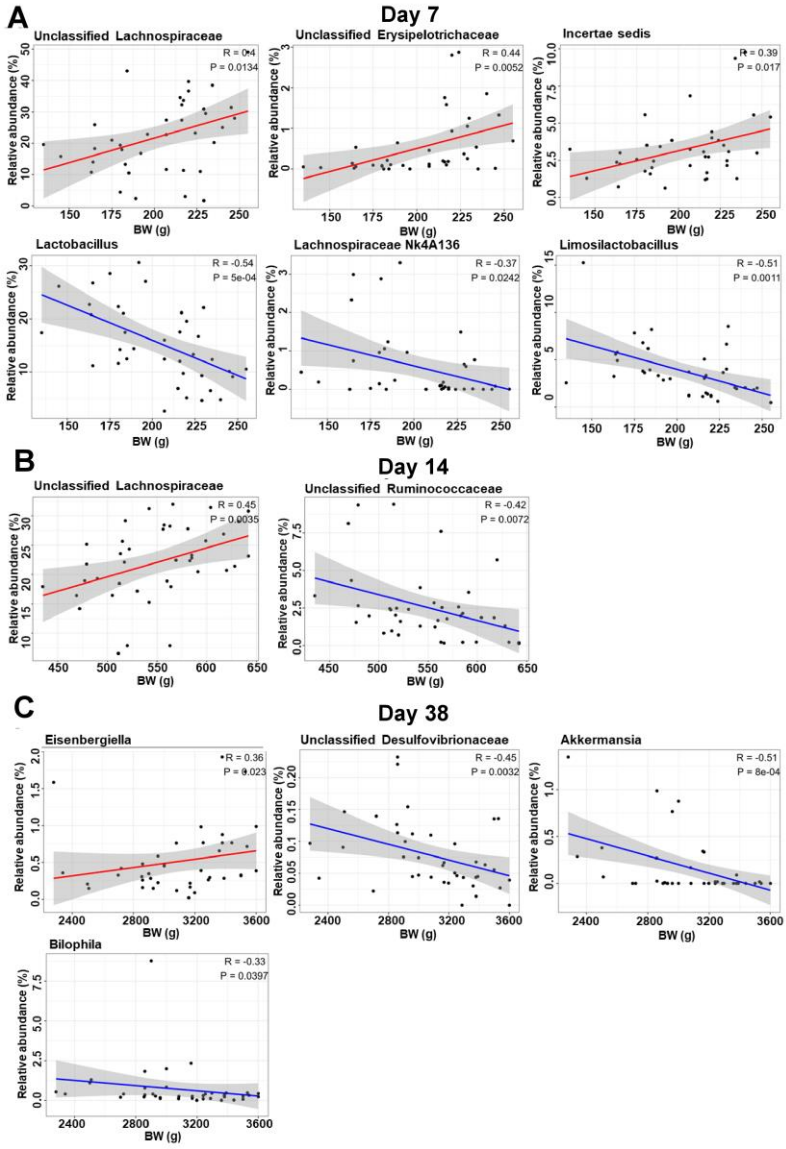

Fig. 2.11 Spearman correlation between body weight (BW) and differentially abundant bacterial genera identified via LEfSe analysis in broiler chickens of both hatching systems (HS) on day 7 (A), day 14 (B), and day 38 (C). Only those features with a P-value less than 0.05 and an absolute correlation coefficient (|R|) greater than 0.30 are shown. The line of best fit is represented by a solid line (red = positive correlation, blue = negative correlation), while the gray shaded area around the line depicts the 95% confidence interval.

2.4 Discussion

Our findings revealed variations in growth indices, VFA concentrations, and gut microbiome characteristics among broilers with different BW. HS resulted in transient effects on growth performance and exerted limited changes in caecal microbiota composition. Considering our initial hypothesis that HS might influence the investigated physiological mechanisms in broilers with varying BW and consequently affect their post-hatch growth patterns, we observed barely any interaction effect between HS and BW. Given the observed independent actions of HS and BW, we will present a separate discussion of these factors.

2.4.1 Effect of hatching system

2.4.1.1 Growth performance

HOF had only short-term effects on broiler performance, as the initial body weight differences between HH and HOF chicks disappeared within the first week after placement. This rapid recovery suggests that HH chicks displayed a compensatory response following early-life feed and water deprivation during the prolonged hatch window and transport[16,24,25]. Such a response could involve increased feed intake, improved feed efficiency, or both, but the exact mechanism cannot be confirmed without direct measurements of feed intake and metabolism. Juul-Madsen et al.[26] observed a catch-up growth in chicks deprived of feed for 24 hours, reaching the weight of early-fed chicks by day 8, but chicks deprived for 48 hours failed to reach similar weights even at 6 weeks of age. Similar to de Jong et al[16], the relatively short duration of feed deprivation in the present study may not have been prolonged enough to induce significant and persistent effects on performance.

2.4.1.2 Microbiota composition

HS failed to demonstrate any effect on α and β diversity of gut microbiota in the present study, aligning with the findings of de Jong et al.[27], who observed no differences in diversity or composition of microbiota between HH and HOF chicks across all ages. However, our investigation did identify HS-dependent differences in caecal microbial communities across all time points, with a noteworthy increase in the *Escherichia-Shigella* abundance in HH chicks on day 7. The enrichment of these potentially pathogenic bacteria in HH chicks during early-life emphasize the importance of

hatching environment, as they can potentially cause subclinical or clinical disease and impact the performance of broiler. Despite the HS-related microbial variations during the early stage, the microbiota community composition gradually converged over time, with few bacterial genera being different between HH and HOF chickens by slaughter age. Jong et al.[27] showed that broiler chicks subjected to different hatching conditions did not exhibit differences in their gut microbiota composition from the outset of the study. Similarly, Simon[28] reported ileal bacterial composition differences in broilers and laying hens fed immediately post-hatch versus those with feed deprivation for 72 hours, but no significant differences persisted from day 21 onwards. This suggests that as the birds mature and undergo similar rearing conditions, the influence of the hatching environment and initial feeding time on the gut microbiome becomes less pronounced. Other factors, such as diet, housing conditions, BW, and age, likely take over and a exert stronger impact on shaping the gut microbial community composition.

2.4.2 Effect of body weight

2.4.2.1 Growth performance

The LBW chicks were unable to overcome setbacks in weight throughout the study, even when reared under identical management conditions to their heavier counterparts. The chickens in the HBW group exhibited higher ADG during the starter and grower phases. Although weight gains were similar between BW groups in the finisher phase, HBW birds maintained weight advantage due to their initial higher weight and faster early growth. These findings emphasize the significance of first-week weight on subsequent growth and slaughter weight of broilers as supported by literature showing a high positive correlation between chick weight at 7 days and harvest weight[29]. Consistent with a previous study on broilers[30], HBW chicken showed increased feed intake, possibly requiring more feed to sustain rapid growth. It further suggests that variations in feed intake since the initial days led to divergent weight gains, consequently impacting growth homogeneity directly.

2.4.2.2 Microbiota composition

Reduced microbial diversity was observed in the HBW group on days 7 and 38, consistent with certain studies[8,31], even though some others suggest

that HBW chickens might harbor more diverse bacteria than their LBW counterparts[13]. The LBW group demonstrated an age-dependent shift in microbiota development, initially harboring higher levels of immature and variable taxa, mainly comprising aerotolerant bacteria, such as most Enterobacteriaceae and Lactobacillaceae, which gradually transitioned to other microbial communities after day 14. This early microbiota profile suggests a less mature microbial composition in LBW chickens that evolves over time, leading to unstable microbiota communities and contributing to high species richness[32]. In contrast, the HBW group established microbial patterns typical of adult chickens from the outset, dominated by obligate anaerobic taxa from Firmicutes and Bacteroidetes including unclassified *Lachnospiraceae* and *Alistipes*. This age-dependent microbiota succession has been corroborated by previous studies, which found that newly-hatched chicks are initially dominated by rapidly colonizing bacterial groups like *Escherichia-Shigella* and *Streptococcus*, followed by a subsequent increase in the abundance of *Lactobacillus* from day 3 to day 14 of age[33]. Later in life, representatives from the phylum Bacteroidetes colonize and dominate the gut[34]. The early establishment of mature microbiota in HBW chickens may confer intestinal microbial stability and improved resilience to disturbances. Bilal et al.[35] reported that the presence of mature microbiota in day-old chicks can accelerate gut development, positively impacting overall health and productivity.

The unclassified *Lachnospiraceae* emerged as a biomarker in the HBW group, consistently enriched and strongly correlated with BW on days 7 and 14. *Lachnospiraceae* members are known for their ability to break down plant fibers and produce SCFAs, particularly butyrate, which promotes intestinal health, and host growth, and has immunomodulatory benefits[6,36]. *Christensenellaceae R-7* was significantly higher in HBW chickens on day 7, previously found positively correlated with BW and muscle fiber diameter[37]. *Alistipes* was also recognized as biomarkers in the HBW group on days 7 and 38. Alistipes is an efficient colonizer of the caeca, promotes the growth of broiler chickens by producing SCFAs[38], and has been shown to be more abundant in HBW chicken[39]. The genera *Blautia* and *Subdoligranulum* were found as biomarkers in the HBW group on day 14. *Blautia* was previously identified in HBW broilers[13], and generates acetate by converting acetyl-CoA from pyruvate through the Wood-

Ljungdahl pathway by fermenting both glucose and indigestible dietary fibers[40]. *Subdoligranulum* represents a sign of improved gut health as it produces SCFAs (i.e. butyrate) and influence gut physiology[41]. *Faecalibacterium*, a saccharolytic butyrate-producing bacterium, has been used as a probiotic in livestock[14], and emerged as a potential biomarker for enhanced performance in the later stages of life. *Eisenbergiella*, and the unclassified *Clostridia vadin BB60* group, capable of degrading complex plant polysaccharides like cellulose and hemicellulose[42], were prominent members of the gut microbiota in HBW chickens on day 38 compared to the LBW counterparts. *Flavonifractor* was also increased in HBW chickens[13], consistent with the previous study, and has been involved in SCFA production[43]. *Romboutsia* produces SCFAs, especially butyrate, which was enriched in the HBW group on day 14 and became abundant in the LBW group on day 38, previously positively associated with BW and ADG in broilers[44].

The enrichment in the phylum Proteobacteria and the genus *Escherichia-Shigella* on days 7 and 38 of the LBW group suggestes these genera to be potential biomarkers for lower BW in both early and later growth stages. The identified *Escherichia-Shigella* species in our study (*E. coli, S. boydii, S. dysenteriae, S. flexneri,* and *S. sonnei*) can be associated with colibacillosis and Shigellosis, leading to economic losses, reduced productivity, and compromised food safety[13]. Certain *Enterococcus* strains are intestinal commensals in farm animals, play crucial role in gut health, and are used as probiotics in poultry. However, some strains invade the intestinal mucosa and cause systemic infections[45]. Although *Enterococcus* species (E. faecalis and E. faecium) are often used as probiotics, their higher abundance in LBW chickens suggests that their presence does not always correlate with improved performance and may be associated with reduced productivity. Dolka et al.[46] reported that E. faecalis and E. faecium are sometimes considered opportunistic pathogens that can adversely affect growth in chickens under specific circumstances. *Streptococcus*, an opportunistic pathogen often causing secondary infections[47], was abundant in the LBW group. The LBW group also presented a higher abundance of the genus *Akkermansia*, previously linked to lower BW in broilers[8,13]. Involved in mucin degradation, this genus is considered a biomarker for lipid metabolism and has been demonstrated to be

beneficial in addressing obesity[48]. The genus *Bilophila* demonstrated a negative association with BW and has been reported in high abundance in intestinal diseases and inflammation in chickens[49]. This genus unclassified *Ruminococcaceae* on day 14 was found to be more enriched in the LBW group, which is in agreement with the observations of Farkas et al.[50]. Similarly, the pectin-degrading genus *Monoglobus* and the gut health-promoting genus *Lachnospiraceae NK4A136* group were also more abundant in LBW chickens. Although the precise mechanisms by which these bacteria influence LBW chickens are not fully understood, other factors such as feed intake or FCR may have a relevant impact on caecal microbiota besides BW, warranting further exploration. We found a negative correlation between *Lactobacillus* and BW, consistent with other studies linking this genus to decreased chicken productivity[11,51]. *Lactobacilli* are highly dependent on the amino acids available in the small intestine[52]. The possibly reduced protein digestion and lower absorption capacity in the small intestine of LBW chicks may have increased protein bypass to the lower intestine, providing easily available substrates to *Lactobacilli*, and consequent activation of this microbial group in the caecum[53]. Some studies highlighted the implication of higher *Lactobacillus* levels with broiler growth reduction due to impaired fat absorption linked to the deconjugation of bile acids[54]. In our study, chicks categorized as LBW on day 7 were 21% lighter than the Aviagen target for male Ross 308 broilers and 22% lighter than HBW chicks in the study, reflecting the underperforming category typically can be observed in commercial settings. By day 38, both LBW and HBW groups exceeded expected Aviagen thresholds, with a 300 g (10%) difference between categories, which is a smaller gap than typically observed commercially at slaughter age[13]. Thus, our study primarily explained performance-related microbial biomarkers that more effectively account for the exceptional growth performance of HBW chicks, rather than emphasizing the factors contributing to poor performance in LBW birds.

Volatile fatty acid differences between BW groups

Most SCFAs showed significantly or numerically higher concentrations in the HBW group, while BCFAs were increased in the LBW group. SCFAs have been related to BW changes, with elevated acetate levels observed in overweight human individuals[55]. The BCFAs are generated through protein

fermentation in the cecum and are often associated with unfavorable shifts in the microbial community and increased ammonia production[56]. Specific bacterial genera enriched in LBW chickens, including *Negativibacillus* and *Escherichia-Shigella*, positively correlated with BCFA isobutyrate on days 14 and 38, respectively, aligning with prior study[57] linking *Escherichia-Shigella* abundance to cecal isobutyrate concentration. Conversely, in the HBW group, *Blautia* abundance positively correlated with acetate on day 14, while *Flavonifractor* enrichment on day 38 showed positive correlations with acetate, butyrate, caproate, and total SCFA concentrations. These observations suggest that specific bacteria in each BW category can produce certain types of VFAs, which could influence intestinal health in a BW-dependent manner.

Predicted function of caecal microbiota

During earlier days, lower taxonomic but higher functional differences existed between LBW and HBW microbiota. By day 38, taxonomic differences of microbiota increased while functional differences decreased suggesting microbiota in both groups were likely fulfilling similar functional roles in later stages. Early colonizers demonstrated greater versatility and metabolic activity compared to the late colonizers, corroborating a previous study on infants where microbiota at 1 month of age were more functionally active and independent compared to 6 months[58]. The early life differences between BW groups resulted in higher positive microbial functionalities in HBW chickens, allowing them to have an initial performance boost, resulting in faster growth, finally reaching an equilibrium on day 38. The HBW group exhibited enrichment of microbial genes involved in biosynthesis pathways (amino acids, cofactors, and vitamins). It is speculated that this might have contributed to their better performance as previous studies have reported an association between microbial functions related to nutrient biosynthesis and increased weight gain[9]. The higher feed intake observed in HBW chickens suggests that a greater quantity of feed components, which would otherwise be indigestible by the host, likely reached the ceca for microbial utilization and fermentation. The HBW group possessed a gut microbiota better adapted to utilizing both complex and simple carbohydrates, potentially producing essential nutrients including SCFAs, thereby facilitating rapid weight gain. The LBW group exhibited enrichment in the microbial

pathway for pyruvate fermentation to acetate and lactate. The higher abundance of *Lactobacillus*, which ferments pyruvate into lactate, may negatively impact mucosal barrier function and host health[59], yet metabolic cross-feeding enables lactate-utilizing bacteria to convert it into other metabolites[60]. LBW group also exhibited enrichment in the microbial pathway responsible for UDP-N-acetyl-D-glucosamine biosynthesis, a precursor for cell wall peptidoglycan, lipopolysaccharide, and the enterobacterial common antigen, as observed in *Escherichia coli*[61].

Predicting functional activities based solely on taxonomic composition or genomic data may not fully reflect the dynamic and context-dependent nature of microbial metabolism. To address these limitations, future studies are suggested to complement 16S rRNA gene amplicon sequencing with metatranscriptomics or metabolomics approaches, which can provide more direct and comprehensive insights into the functional potential and metabolic activities of the gut microbiome.

Interaction effect of HS and BW on microbiota

Previous studies have suggested that variations between low and high-weight birds might originate before their placement in the barn[13], influenced by factors like hatching environment, chick transportation, and access to first feed. The interaction between HS and BW showed no impact on α and β diversities. Nevertheless, some initial interactions between HS and BW were noted concerning early-life microbiota composition, but these interactions markedly decreased over time. Our findings indicate that factors associated with the hatching conditions do not have long-term impact on BW-related microbiota characteristics of birds. Instead, selection by the host (i.e. BW of birds) emerged as a more potent driver for shaping the intestinal microbiota, overshadowing the effects of hatching conditions.

2.5 Conclusions

We observed that HS had only short-lasting effects on chicken performance and microbiota composition, and barely showed an impact on BW-related differences in the variables investigated. The disparities in growth among broilers were primarily driven by the bird's initial BW, rather than the hatching conditions. A higher BW in the first week allows

chicks to maintain an advantage over the chicks with a lower BW, shaping differences in feed intake and microbiota characteristics, and subsequently influencing overall performance. SCFAs (which are beneficial) were higher in the HBW group and BCFAs (which are unfavorable) were higher in the LBW group. Genera like unclassified *Lachnospiraceae* early on, and *Faecalibacterium* and *Clostridia vadin BB60 group* in later growth stages could serve as biomarkers for enhanced performance in broilers. Conversely, *Escherichia-Shigella* and *Streptococcus* appear to be a biomarker for suboptimal performance during early and later growth stages. The HBW group demonstrated enrichment of gut-health-promoting taxa, which may have contributed to enhanced performance through various mechanisms such as better utilization of feed, enhanced metabolic activity, biosynthesis of essential nutrients, production of energy-rich metabolites, and modulation of the immune system. Our study further strengthens the understanding regarding the microbiota characteristics that impact broiler performance across growth stages under uniform rearing conditions. These findings provide potential insights for developing strategies to modulate and establish a more uniform and beneficial microbiota in underperforming broilers, thereby ensuring greater uniformity.

Apart from microbiota factors, to further explore the host related factors of growth heterogeneity and flock uniformity, the following chapter will investigate how differences in intestinal structure, function, and gene expression between LBW and HBW broilers contribute to performance outcomes. This multi-layered analysis integrates gut morphology, permeability, and transcriptomic data to uncover molecular mechanisms underlying growth variability in broilers.

2.6 References

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Chapter 3

Linking gastrointestinal tract structure, function, and gene expression signatures to growth variability in broilers: a novel interpretation for flock uniformity

The work presented in this chapter is adapted from:

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Abstract

Variation in body weight (BW) within broiler flocks is a significant challenge in poultry production. Investigating differences in gut-related parameters between low (LBW) and high BW (HBW) chicks may provide insights into the underlying causes of BW heterogeneity. 908 day-old male broiler chicks were reared until day 7 and then ranked into LBW and HBW groups. Thereafter, performance parameters were compared between BW groups periodically. On days 7, 14, and 38, visceral organ characteristics, intestinal permeability, and duodenal and ileal histomorphology were examined. Expression profiles were analyzed for 79 ileal genes related to gut barrier function, immune function, nutrient transport, gut hormones, nutrient receptors, metabolism, and oxidation using high-throughput qPCR. Student's t-tests were performed to compare measurements. Multivariate statistics, including partial least square regression (PLSR) analysis, were applied to identify combinations of key genes discriminating BW groups, offering predictive capability for phenotypic variations. The HBW group remained heavier at each timepoint, which could be explained by higher feed intake. The HBW group had shorter relative small intestine length but higher villus height and villi height/crypt depth ratios. The LBW group demonstrated increased intestinal permeability on day 38. The LBW group showed upregulation of immune response genes including *TNF-α* on day 7 and *CYP450* on day 38, while the HBW group showed higher *AHSA1* and *HSPA4* expressions on day 7. The LBW group had upregulation of the metabolism genes *mTOR* and *EIF4EBP1* on day 7 and the satiety-induced hormone cholecystokinin on day 14, while the HBW group tended to increase expression of the hunger hormone ghrelin on day 38. Genes related to gut barrier function, nutrient transport, and oxidation categories were consistently upregulated in the HBW group. PLSR models revealed 4, 12, and 11 sets of key genes highly predictive of BW phenotypes on days 7, 14, and 38, respectively. These findings suggest that growth rates are linked to the intestinal size, structure, and function of broiler chickens, offering insights into the underlying mechanisms regulating BW.

3.1 Introduction

Based on the findings from Chapter 2, it was determined that while differences in gut microbiota composition are associated with body weight (BW) variation in broilers, they do not fully explain the performance differences observed between low (LBW) and high BW (HBW) individuals under uniform rearing conditions. These results suggest this heterogeneity appears to extend beyond conventional breeding targets and is likely shaped by complex interactions among gut structure, function, and host regulation. The gastrointestinal tract (GIT) in birds is a central organ for growth, which typically undergoes rapid development in the early stage of life to meet nutritional and immunological needs[1]. Disparities in gut structure and function between LBW and HBW broilers could significantly impact production efficiency, even under standardized rearing conditions.

One of the mechanisms that potentially influences growth variations in LBW and HBW chickens could be the efficiency of nutrient uptake within the small intestine. The small intestine's significance for nutrient digestion and absorption in chickens is widely recognized[2]. An optimally developed GIT with efficient histomorphological characteristics is pivotal for shaping long-term growth, metabolism, and overall health[3]. Nutrient uptake is mediated by transporter proteins located at the brush border or basolateral membranes of the intestinal epithelia, governing the flux of nutrients from the intestinal lumen to the bloodstream[4]. Among these transporters, amino acid, peptide, and monosaccharide transporters belong to the solute carrier (SLC) gene family, comprising 395 transporter genes across 52 families[5]. The regulation and expression of these specific nutrient transporters profoundly influence animal growth and development[6], with increased mRNA expression of glucose transporters previously associated with higher BW in chickens[7].

Intestinal permeability is another gut-related mechanism believed to be capable of potentially influencing growth variation, given its critical role in integrity of the intestinal barrier regulated by tight junction (TJ) proteins[8]. In addition to TJ proteins, mucin also contributes significantly to this complex defense mechanism by serving as a physical barrier against harmful pathogens and toxins[9]. Increased permeability can result in bacterial translocation and the entrance of toxin compounds into the body, affecting nutrient absorption and growth efficiency[10]. Furthermore,

the gut microbiome directly affects the development and function of the mucosal immune system[11]. Differences in microbiota between chickens of extreme BWs have been reported[12], with LBW chickens often showing a higher abundance of potential pathogens[13], which can trigger differential immune responses in birds differing in weight. Host-pathogen interaction in chickens can lead to shifts in energy distribution, potentially prioritizing immunity over rapid growth, impacting growth rates and potentially contributing to differences in broiler BW as observed in practice.

The next conceivable mechanism for growth variation concerns the regulation of appetite, which plays a crucial role in animal growth[14], given the direct relationship between feed intake and economic traits in broilers[15]. Gut hormones significantly modulate the feed intake in chickens, thereby influencing weight gain[16]. Feed preferences are established early in the life of birds[15], and differences in feed intake from the outset can elicit diverse responses in chickens and directly influence growth homogeneity. Understanding the role of mediators affecting feed intake in broilers is essential due to its involvement in major physiological processes like growth, immunity, and production.

GIT size, structure, and function along with intestinal gene expression profiles have been identified as potential mechanisms in controlling the growth rate of chickens with different genotypes[9,17–19]. However, our understanding of GIT development and intestinal gene expression patterns contributing to intra-flock variance among broilers under uniform management practices remains limited. Moreover, existing studies often focus on single time points, typically at slaughter age, overlooking crucial changes in intestinal physiological functions during earlier life stages that influence later growth trajectories. In this study, we investigated differences in visceral organ size, gut permeability, small intestine morphology and gene expression profiles related to gut barrier function, immune responses, nutrient receptors and transporters, neuropeptide gut hormones, metabolism, and oxidation between LBW and HBW broilers across different growth stages. Our objective was to elucidate mechanisms driving growth variations between LBW and HBW broilers reared under uniform management conditions on days 7, 14, and 38.

3.2 Materials and methods

This animal study was approved by the Katholieke Universiteit Leuven Ethical Committee for Animal Experimentation (Ethical protocol P045/2022, Belgium) and was performed at TRANSfarm, the research facility for animal experimentation of KU Leuven (Lovenjoel, Belgium).

3.2.1 Animals, housing and management

A total of 908 day-old male ROSS 308 broiler chicks were obtained from Belgabroed NV (Merksplats, Belgium) and housed in separate floor pens (1.3 m² each) at the research facility. The floor of the pens was cobvered with a 3 cm layer of wood shavings, serving as bedding material. The initial barn temperature was set at 33 °C and was systematically lowered by approximately 0.5 °C each day until reaching 21.5 °C on the 21st day, after which it was held constant at that level. A 1-hour dark period was initially provided until day 7, after which it was extended to 6 h for the remainder of the study period. The 38-day experiment ensured uniform rearing conditions and *ad libitum* access to the same feed and water for all chicks. The chicks were vaccinated against Newcastle disease and Gumboro on day 16 and had no exposure to antibiotics. Chicks were fed crumbled, sieved pellets as starter feed (1-14 days), transitioning to pelleted grower feed (15-28 days), and then to finisher feed (29-38 days, Table S2.1).

3.2.2 Study design

The chicks were weighed on day 7, and they were categorized into three groups based on their BW: LBW, middle BW, and HBW. LBW chicks (n = 294, 32%) were those with weights below the mean BW by half the standard deviation (½ × SD), while HBW chicks (n = 280, 31%) exceeded the mean BW by ½ × SD. Middle BW chicks (n = 334, 37%), falling within the mean BW ± ½ × SD, were excluded from the study. The experimental setup ensured uniform conditions for all birds, with 28 pens utilized in total (14 replicate pens per group) allocated for LBW (21 chicks/pen) and HBW (20 chicks/pen) groups. The LBW pens accommodated one additional bird to maintain a similar stocking density (kg/m²) to that of the HBW pens.

3.2.3 Growth performance

Individual bird weights and feed consumption per pen were recorded on days 7, 14, 28, and 38. Mortality was noted as it occurred. Subsequently,

average daily gain (ADG), mortality-corrected average daily feed intake (ADFI), and feed conversion ratio (FCR) were calculated.

3.2.4 Sampling and visceral organ measurements

On days 7, 14, and 38 post-hatch, 20 birds per experimental group were sacrificed for sampling by electronarcosis followed by decapitation. Following the killing, organ dissection included the stomach (proventriculus and gizzard), small intestine, liver, pancreas, spleen, bursa of Fabricius, and heart. The small intestine weight was weighed without evacuating the digesta and its length recorded. Relative organ weights were calculated as grams per 100 grams of BW, and small intestine length as centimeters per 100 grams of BW. Duodenal and ileal segments (approximately 5 cm long from midpoint) were collected for histomorphological analysis. Ileal tissue samples were rapidly snap-frozen and stored at -80 °C for subsequent target gene expression analysis. Fourteen chickens from each group were randomly chosen to evaluate intestinal permeability using fluorescein isothiocyanate dextran (FITC-dextran, Molecular weight 4 kda; Sigma-Aldrich, USA).

3.2.5 Intestinal histomorphology

Duodenum and ileum sections were rinsed with 1x phosphate-buffered saline followed by immersion in a 4% formaldehyde solution for 48 h for fixation, before preservation in 70% ethanol. Subsequently, the sections were embedded in paraffin and sectioned using a microtome. The resulting sections were then mounted on glass slides, stained with Alcian Blue-Periodic Acid Schiff, and examined under a microscope at 20x magnification. The selected sections were analyzed using NDP.view2 software (Hamamatsu Photonics K.K., Hamamatsu, Japan). For each sample, twenty well-oriented villus-crypt units were evaluated. The recorded parameters included villus height (VH), crypt depth (CD), VH:CD ratio, villus width, and the thickness of the submucosa and tunica muscularis layers.

3.2.6 Intestinal permeability

A solution containing FITC-dextran (2.2 mg/mL/bird) was administered via oral gavage, and blood samples (1 mL) were collected from the jugular vein 2.5 h post-administration. The obtained blood samples were centrifuged at 3000 × g for 15 min at 4 °C to isolate the plasma. Diluted plasma samples

and standard solution (1:5 PBS) were pipetted in duplicates into 96-well microplates, and fluorescence intensity measurements were then performed using spectrophotometry (Victor3, PerkinElmer Inc. USA) with an excitation wavelength of 485 nm and an emission wavelength of 530 nm. The absolute FITC-dextran concentration was calculated based on the standard curve as ng/mL of blood. The relative concentration of FITC-dextran was calculated as ng/mL/100 g BW. Normalization of plasma FITC-dextran values to BW accounts for variations in BW and blood volume between BW groups. This allows for a more accurate comparison of intestinal permeability across chickens of different weights.

3.2.7 RNA extraction

RNA was extracted using the ReliaPrep™ RNA Miniprep Systems (Promega Corporation, USA) Kit as per the manufacturer's guidelines. RNA quantity and quality were determined via NanoDrop 2000 (Thermo Fisher Scientific, USA), while integrity was confirmed using 1% agarose gel electrophoresis.

3.2.8 Primer design and validation

The study analyzed the expression of 92 genes in the ileum, selected for their roles in various physiological functions based on published literature. Exon-exon-spanning primers were either obtained from previous studies or designed using the NCBI Primer-Blast tool (Table S3.1). These primers, under 30 nucleotides, produced amplicons not exceeding 150 base pairs. Efficiency and specificity of all primers were assessed on a QuantStudio 6 Real-Time PCR Systems (Thermo Fisher Scientific, USA) using three-fold serial dilutions of a pool of cDNA from all samples. The validation of PCR products was carried out using agarose gel electrophoresis, which confirmed the presence of a single product, as well as through the analysis of melting curves during real-time PCR.

3.2.9 Reverse transcription and preamplification

Reverse transcription of 50 ng of RNA was carried out using a Reverse Transcription Master Mix (Standard BioTools, USA) as per the manufacturer's guidelines. A primer mix was made by pooling 1 µL each of forward and reverse primers with Tris EDTA buffer (Thermo Fisher Scientific) to a final volume of 400 µL. This primer mix was then combined with Fluidigm PreAmp Mastermix (Standard BioTools, USA) to form a preamplification mix. In a 96-well PCR plate, 3.75 µL of preamplification

mix was mixed with 1.25 µL of cDNA samples, and subjected to thermal cycling conditions: 2 min at 95 °C, followed by 14 cycles of 95 °C for 15 s and 60 °C for 4 min. Exonuclease I (New England Biolabs, USA) was subsequently used to remove unincorporated primers. After treatment, samples were diluted ten-fold with Tris EDTA buffer and stored at –20 °C.

3.2.10 High-throughput qPCR

Quantitative PCR (qPCR) was performed using the BioMark™ HD instrument with a 96.96 Dynamic Array™ integrated fluidic circuits (IFCs) specifically designed for gene expression (Standard BioTools, USA). A total of three IFCs were used, each dedicated to samples from different timepoints (days 7, 14, and 38), tested separately. A pool was created by combining 10 µL from each individual sample. Pre-amplified cDNA of the pooled samples was diluted threefold for primer efficiency and standard curve setup for each IFC. Non-template controls were included to monitor for contamination and nonspecific amplification. The sample mix comprised 0.25 µL 20X DNA Binding Dye (Standard BioTools, USA) and 2.5 µL 2X SSoFast™ EvaGreen® Supermix with low ROX (Biorad, Hercules, USA). The assay mix contained 2.5 µL 2X Assay Loading Reagent (Standard BioTools, USA) and 2.25 µL of 1x DNA Suspension Buffer (TEKnova, USA). Thermal cycling in BioMark™ HD machine involved denaturation at 95 °C for 60 s, followed by thirty cycles of denaturation at 96 °C for 5 s, and annealing/elongation at 60 °C for 20 s. The raw data of gene expression were extracted using SBI Real-Time PCR software (v1.0.2, Standard BioTools, USA). Relative mRNA concentrations were determined using the standard curve of the pooled samples on each respective IFC. Housekeeping genes' expression stability according to the experimental groups and sampling time points was calculated using NormFinder[20], and four housekeeping genes (TBP, B2M, NDUFA, and B-ACTIN) proved most stable over the groups and time points. The relative gene expression level for each target and housekeeping gene was calculated using the Pfaffl method[21], and the geometrical mean of the relative expression of the four housekeeping genes (TBP, B2M, NDUFA, and B-ACTIN) was used to normalize all samples.

3.2.11 Statistical analysis

The data regarding growth performance, visceral organ characteristics, intestinal histomorphology, in vivo gut permeability, and ileum gene

expression were analyzed via a linear mixed model in R (v4.2.3, R Foundation, Vienna, Austria). The BW effect was used as a fixed effect and the pen effect was considered a random effect to account for the possible confounding variation due to pen location and number of animals in each pen. Prior to analysis, normal distribution of the data was confirmed via Shapiro-Wilk's test. Subsequently, Student's t-test was applied to assess statistical significance, with *P* values < 0.05 indicating significance and values within 0.05 < P < 0.10 considered as trends. For gene expression data, P-values were adjusted for false discovery rate (FDR) using the Benjamini–Hochberg method[22], with a significance threshold set at < 0.05. Results are presented as means alongside a pooled SD, which combines the variability observed in all samples.

Principal Component Analysis (PCA), an unsupervised pattern recognition technique, was used in R using the factoextra package (v 1.0.7) to visualize overall patterns of gene expression data across BW groups. Genes were used as variables, with samples as individual data points, while BW was included as a categorical variable. Permutational multivariate analysis of variance (PERMANOVA) analysis was performed on PCA scores to assess whether there are significant differences between groups in terms of the overall multivariate structure captured by the principal components (PCs). Heatmaps were generated in R using the pheatmap package (v 1.0.12) to visualize sample variability, with gene expression values scaled by row. Heatmaps were based on Pearson's correlation distance and ward clustering method for two-way hierarchical clustering analysis.

Using gene expression datasets, partial least square regression (PLSR) models were built as an alternative to identify a combination of key genes predicting the growth rate of LBW and HBW broilers. Per day, nine cross-validation splits were created with two or three samples per BW group. The number of latent variables was determined for the highest R-squared of the cross-validation set (R^2_{CV}). The outlier analysis was performed by examining the Q residuals, the Hotelling T^2, and manual inspection of aberrant gene expression levels in the data. Then, the PLSR model was further optimized by applying a variable importance in projection (VIP). Thereby, each variable was considered significant if its score was 1 or higher. The PLSR analysis was performed using the PLS toolbox (v8.7 2019,

Eigenvector Research, Wenatchee, WA) within Matlab (v2018b, Mathworks, Natick, MA).

Pearson's correlation analysis was used to establish and quantify the relationship between BW and other performance parameters, intestinal size, structure, and function in R using the package corrplot (v0.92). Correlations with a $P < 0.05$ and $|R| > 0.30$ were reported.

3.3 Results

3.3.1 Growth performance

The average BW of birds was 43.2 ± 2.88 g upon placement in the barn. On day 7, these chicks were divided into two distinct weight categories designated as LBW and HBW groups, showing a statistically significant difference ($P < 0.05$, Fig. 3.1). Thereafter, the LBW group consistently maintained a lower BW ($P < 0.05$) on days 14, 28, and 38, when compared to their HBW counterparts. The ADG in LBW chicks demonstrated a significant reduction ($P < 0.001$) compared to HBW chicks during the periods of 7-14 days, 15-28 days, and the overall study duration of 7-38 days. However, the birds within the LBW group exhibited similar ADG during the 29-38-day period. Furthermore, LBW chicks exhibited lower ADFI ($P < 0.05$) than the HBW chicks throughout the study, while FCR was lower ($P = 0.021$) in the LBW group than in the HBW group during the overall period of 7-38 days.

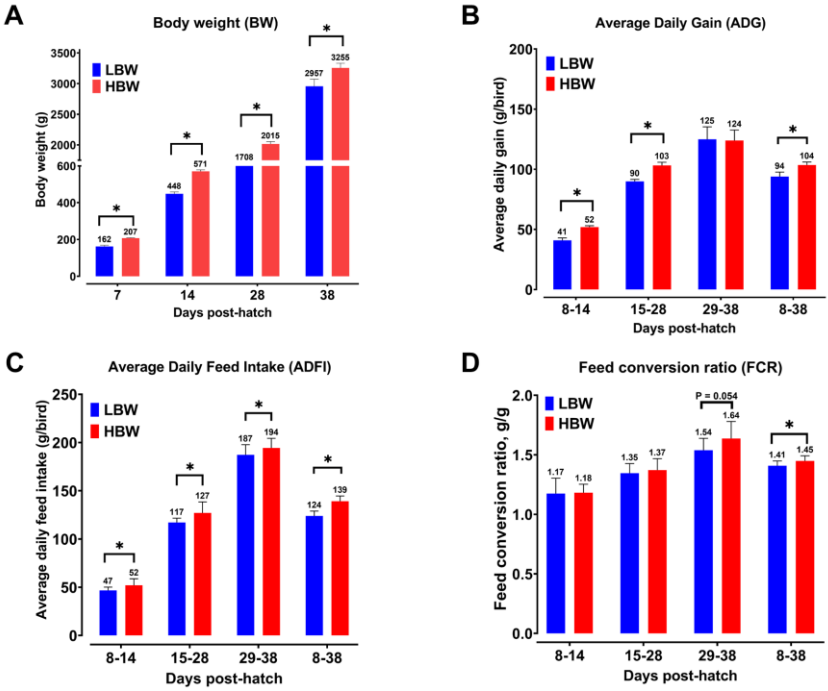

Fig. 3.1 Body weight **(A)**, average daily gain **(B)**, average daily feed intake **(C)** and feed conversion ratio **(D)** of low (LBW, n = 14 pens) and high (HBW, n = 14 pens) body weight (BW) groups. Except for BW data, a pen was considered an experimental unit. BW was recorded from individual birds. Data are presented as mean and standard deviation (SD). Values with (*) indicate significant differences at $P < 0.05$ (Student's t-test).

3.3.2 Visceral organ development

The chicks in the LBW group demonstrated higher relative heart weights on days 7 and 14 (P = 0.006 and P < 0.001, respectively, Table 3.1), as well as higher stomach and bursa relative weights on day 38 (P < 0.010). A tendency for increasing relative pancreas weight on day 14 was observed in LBW birds (P = 0.083). The LBW group demonstrated longer small intestine length on days 7, 14, and 38 (P < 0,001), despite lower relative weights of the small intestine on days 7 (P < 0.001) and 14 (P = 0.020).

Table 3.1 Visceral organ weights (g/100 g body weight) and small intestine length (cm/100 g body weight) of chickens from the low (LBW) and high (HBW) weight groups.

Items	Days	[1]Groups		SD	P value
		LBW	HBW		
Heart (g)	Day 7	0.79[a]	0.70[b]	0.120	0.006
	Day 14	0.85[a]	0.76[b]	0.082	<0.001
	Day 38	0.49	0.49	0.066	0.679
Liver (g)	Day 7	4.30	4.34	0.423	0.426
	Day 14	3.42	3.26	0.400	0.192
	Day 38	1.96	2.06	0.280	0.301
Spleen (g)	Day 7	0.07	0.07	0.027	0.207
	Day 14	0.08	0.09	0.018	0.633
	Day 38	0.12	0.11	0.032	0.548
Pancreas (g)	Day 7	0.44	0.42	0.087	0.591
	Day 14	0.40	0.36	0.077	0.083
	Day 38	0.17	0.15	0.040	0.168
Bursa (g)	Day 7	0.19	0.16	0.056	0.882
	Day 14	0.25	0.22	0.069	0.149
	Day 38	0.17[a]	0.13[b]	0.044	<0.001
Stomach (g)	Day 7	6.59	6.30	0.651	0.155
	Day 14	4.75	4.38	0.535	0.033
	Day 38	1.94	1.68	0.384	0.032
Small intestine (g)	Day 7	16.80[a]	19.95[b]	1.393	<0.001
	Day 14	15.20[a]	15.85[b]	1.596	0.020
	Day 38	8.01	7.37	1.265	0.113
Small intestine (cm)	Day 7	61.68[a]	50.11[b]	7.899	<0.001
	Day 14	29.29[a]	26.57[b]	2.669	<0.001
	Day 38	7.28[a]	6.21[b]	0.924	<0.001

[1]LBW: low body weight group (n = 20), HBW: high body weight group (n = 20). The experimental unit was considered as individually sampled chickens. Data are presented as mean and pooled standard deviation (SD). [a-b]Values with different superscripts in a row differ at $P < 0.05$ (Student's t-test).

3.3.3 Duodenum and ileum histomorphology

The LBW group exhibited shorter duodenal VH on days 7 and 38 ($P \leq 0.001$ and $P \leq 0.049$, respectively), a lower VH:CD ratio, and a thinner tunica muscularis layer on day 7 compared to the HBW group ($P < 0.05$, Table 3.2). Birds in the HBW group demonstrated increased ileal VH ($P \leq 0.001$

and P = 0.002, respectively) and greater VH:CD ratios on days 7 and 14 (P ≤ 0.001 and P = 0.013, respectively, Table 3.3).

Table 3.2 Duodenal histological characteristics of the chickens from low (LBW) and high (HBW) weight groups.

[1]Items	Days	[2]Groups		SD	P value
		LBW	HBW		
VH (µm)	Day 7	1353[b]	1496[a]	120.7	≤0.001
	Day 14	1885	1964	160.3	0.155
	Day 38	2003[b]	2141[a]	218.6	0.049
CD (µm)	Day 7	137	134	27.4	0.562
	Day 14	199	212	40.7	0.381
	Day 38	201	191	65.4	0.649
VH:CD	Day 7	9.9[b]	11.9[a]	2.51	0.015
	Day 14	9.71	9.70	2.10	0.962
	Day 38	11.3	12.0	3.93	0.582
Villus Width (µm)	Day 7	154	154	18.5	0.890
	Day 14	178	186	26.1	0.246
	Day 38	187	199	27.8	0.207
Sub mucosa (µm)	Day 7	22.4	23.1	3.91	0.548
	Day 14	21.8	22.9	3.62	0.330
	Day 38	28.4	28.9	4.71	0.680
Tunica muscularis (µm)	Day 7	127[b]	138[a]	16.7	0.042
	Day 14	149	150	23.1	0.981
	Day 38	199	186	33.5	0.348

[1]VH: villus height, CD: crypt depth, VH:CD: ratio of VH to CD. [2]LBW: low body weight group (n = 20), HBW: High body weight group (n = 20); The experimental unit was considered as individually sampled chickens. Data are presented as mean and pooled standard deviation (SD). [a-b] values with different superscripts in a row differ at P < 0.05 (Student's t-test).

Table 3.3: Ileal histological characteristics of the chickens from low (LBW) and high (HBW) weight groups.

[1]Items	Days	[2]Groups		SD	P value
		LBW	HBW		
VH (µm)	Day 7	504[b]	579[a]	67.1	≤0.001
	Day 14	606[b]	688[a]	86.8	0.002
	Day 38	1007	1087	147.7	0.089
CD (µm)	Day 7	126	123	17.9	0.599
	Day 14	188	174	28.8	0.133
	Day 38	160	160	27.2	0.995
VH:CD	Day 7	4.1[b]	4.8[a]	0.73	≤0.001
	Day 14	3.3[b]	3.8[a]	0.70	0.013
	Day 38	6.4	6.9	1.14	0.196
Villus Width (µm)	Day 7	151	137	18.2	0.224
	Day 14	179	182	15.6	0.539
	Day 38	156	163	34.8	0.674
Sub mucosa (µm)	Day 7	20.8	20.3	2.14	0.429
	Day 14	26.9	28.2	3.86	0.315
	Day 38	34.0	38.2	9.15	0.346
Tunica muscularis (µm)	Day 7	111	116	21.7	0.615
	Day 14	149	154	21.1	0.420
	Day 38	191	223	53.9	0.197

[1]VH: villus height, CD: crypt depth, VH:CD: ratio of VH to CD. [2]LBW: low body weight group (n = 20), HBW: High body weight group (n = 20); The experimental unit was considered as individually sampled chickens. Data are presented as mean and pooled standard deviation (SD). [a-b] values with different superscripts in a row differ at $P < 0.05$ (Student's t-test).

3.3.4 Intestinal permeability

Absolute plasma FITC-dextran levels on d 7, 14, and 38 did not differ between BW groups (Fig 3.2). When considering the plasma concentration of FITC-dextran relative to the BW of birds, the LBW group demonstrated a trend towards increased plasma FITC-dextran levels on day 7, and had significantly higher levels on day 38 compared to the HBW group ($P \leq 0.001$).

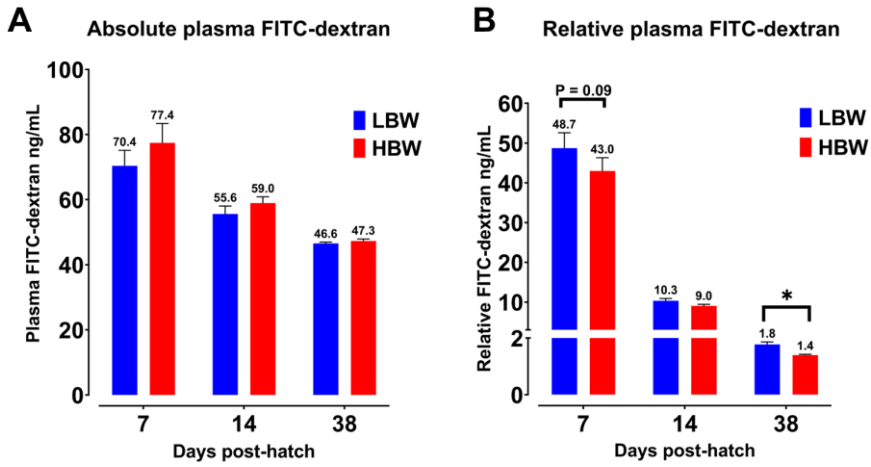

Fig. 3.2 Plasma absolute (ng/mL; **A**) and relative (ng/mL/100 g body weight; **B**) fluorescein isothiocyanate dextran (FITC-dextran, 2.2 mg/mL/bird) levels of chickens from low (LBW, n = 14) and high (HBW, n = 14) body weight groups. Data are presented as mean and standard deviation (SD). Values with (*) significantly differ at $P < 0.05$ (Student's t-test).

3.3.5 Ileum gene expression

One sample from the LBW group on day 7 and one sample from HBW on day 38 completely failed during high-throughput qPCR and were subsequently excluded from the study. Due to technical problems, *CLDN4*, *JAM 3*, *T1R1*, *TLR4*, *SLC5A9*, *FABP*, and *FABP1* on day 7, *IL-4*, *IL-10*, and *TLR4* on day 14, and *OCLN*, *IL-4*, *FABP*, and *FABP1* on day 38 were withdrawn from the study because of their low mRNA levels in all samples.

3.3.5.1 Principal component analysis and heatmap clustering

The PCA on day 7 showed a distinct clustering of samples based on their BW groups, with LBW and HBW samples separated along the PC1 axis (Fig. 3.3A). This observation was further validated by the PERMANOVA analysis, which confirmed the substantial differences in the gene expression data represented by PCs, was significantly associated with the BW groups ($P = 0.002$). In contrast, on days 14 and 38, we still observed variation in the gene expression in PCA while the separation for BW groups was less evident, indicating that over time, the expression of genes converged across groups. Furthermore, the PERMANOVA analysis did not identify any distinct separation in gene expression profiles between BW groups at

these later growth stages (P = 0.325 and P = 0.169, respectively, Figure 3.3B and C).

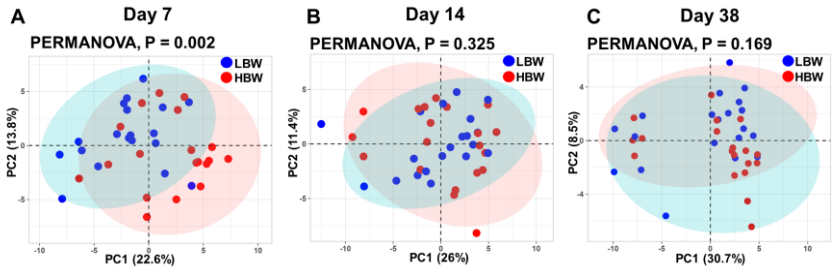

Fig. 3.3 Principal component analysis (PC1 and PC2) based on the gene expression in the ileum of low (LBW) and high (HBW) body weight groups on days 7 (A), 14 (B), and 38 (C).

Two-way hierarchical cluster analysis (Fig. 3.4, Fig. S3.1 and S3.2) revealed the overall variance in gene expression profiles among the samples from both BW groups on days 7, 14 and 38, respectively. On day 7, we identified three distinct clusters based on gene expression patterns, while five clusters were identified based on BW. The majority of the samples from the HBW group tended to cluster together and showed higher expression of genes within the first row cluster, which contains genes related to gut barrier function (*CLDN2*, *CLDN3*, *ZO-1*, *ZO-2*, *MUC2*, *MUC13* and *MUC5ac*), immune response (*AHSA1* and *HSPA4*), nutrient transport (*SLC1A1*, *SLC5A1*, *SLC7A1*, *SLC7A6*, *SLC7A9* and *SLC30A1*), gut hormone (*PYY*), metabolism (*COX-16*) and oxidation (*GPX7*). The HBW samples exhibited lower expression of genes in the other two row clusters. The two-way hierarchical clustering of samples and genes on days 14 and 38 was not as distinct, aligning with PCA and PERMANOVA findings. Neither the samples nor the genes showed clear clustering for BW groups or their biological functions, respectively.

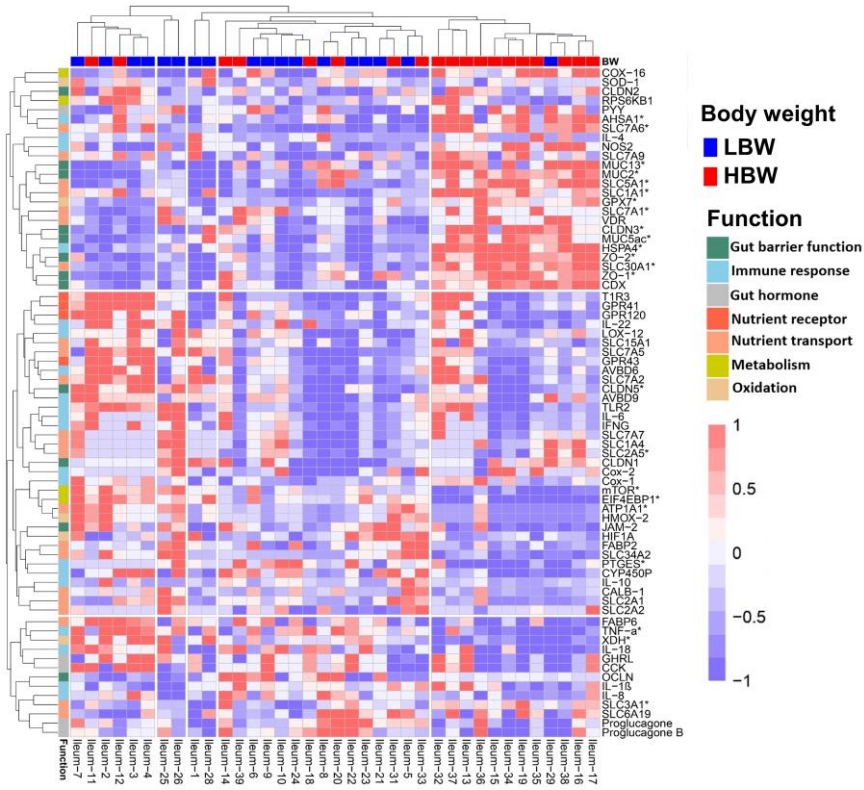

Fig 3.4 Two-way hierarchical cluster analysis showing the expression level of the genes analyzed in the ileum between low (LBW, n = 19) and high (HBW, n = 20) body weight groups on day 7. Samples are represented on the x-axis and genes on the y-axis. The red color indicates high expression while blue indicates low expression. Gene functions (y-axis) were labeled with different colors. The dendrogram on the left of the heatmap clusters genes with similar expression patterns, while the dendrogram on the top groups samples with similar gene expression profiles. Genes with (*) indicate significant differences between BW groups based on the univariate analysis (Student's t-test).

3.3.5.2 Differential gene expression analysis

To further investigate the number of genes differentially expressed between BW groups, a univariate analysis approach using Student's t-test was performed. Genes with an FDR corrected P-value less than 0.05 were considered significantly different between groups and presented in Fig. 3.5. On day 7, the HBW group showed higher expression of genes associated with gut barrier function, including *CLDN3* (P = 0.021), *ZO-1* (P = 0.009), *ZO-2* (P = 0.004), *MUC2* (P = 0.006), *MUC13* (P = 0.016), and

MUC5ac (P = 0.026) (Fig. 3.5A). In contrast, LBW group showed a tendency towards increased *CLDN5* expression (P = 0.076). The LBW group exhibited higher expression of genes related to the immune response, such as *TNF-α* (P = 0.025) and a tendency for increased *PTGES* expression (P = 0.079). However, the HBW group showed increased expression of *AHSA1* and *HSPA4* genes (P = 0.015 and P = 0.001). Regarding nutrient transport, the HBW group demonstrated upregulation of various SLC genes, including *SLC1A1* (P = 0.004), *SLC3A1* (P = 0.025), *SLC7A6* (P = 0.031), *SLC5A1* (P = 0.005), and *SLC30A1* (P ≤ 0.001), while showing a decrease in *SLC2A5* (P = 0.041) and a tendency for decreased *ATP1A1* expresions (P = 0.072). The LBW group exhibited higher expression of the metabolism-related genes *EIF4EBP1* (P = 0.002) and *mTOR* (P = 0.002), as well as altered expression of oxidation-related genes, with increased *XDH* (P ≤ 0.001) and decreased *GPX7* (P = 0.025).

On day 14, the LBW group showed down-regulation of the gut barrier-related genes *CLDN1* (P = 0.010) and *MUC5ac* (P = 0.012), while showing higher expression of *CCK* (P ≤ 0.001), a gut hormone (Fig. 3.5B). The HBW group exhibited upregulation of the nutrient transporter genes *SLC1A1* (P = 0.033) and *SLC2A1* (P = 0.048). On day 38, the HBW group tended to have increased expression of the barrier-related gene *JAM 3* (P = 0.074), the gut hormone *GHRL* (P = 0.071) and the nutrient receptor gene *GPR120* (P = 0.035) (Fig. 3.5C). Additionally, the HBW chicks exhibited higher expression of nutrient transport genes, including *SLC1A1* (P = 0.041), *SLC3A1* (P = 0.031), *SLC7A9* (P = 0.017), *FABP2* (P = 0.017), and *VDR* (P = 0.009). In the oxidation and metabolism categories, *XDH* was significantly higher (P = 0.007), while *RPS6KB1* (P = 0.068) tended to be higher in HBW chickens. The LBW group demonstrated a higher expression of the immune-related gene *CYP450* (P = 0.021).

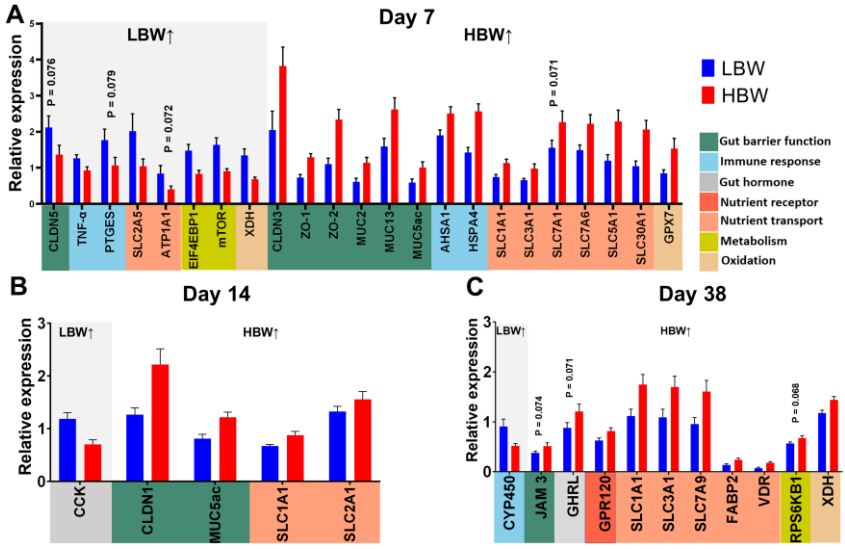

Fig. 3.5 Significantly different genes between low (LBW) and high (HBW) groups on days 7 (A), 14 (B), and 38 (C). Genes that were significantly upregulated in the LBW group are shown on the left side of the bar plot under the gray color background, while genes that were significantly upregulated in the HBW group are shown on the left side (white background). Gene functions are labeled with various colors (x-axis). Statistical analysis was conducted using the Student's t-test with an FDR-adjusted P value < 0.05 to control for false discovery rate.

3.3.6 Partial least square regression models

Following PCA, we acquired valuable insights into the overall gene expression patterns delineating between the BW groups. Furthermore, differential gene expression was determined for each day through a Student's t-test. This section employs targeted PLSR analysis to identify the most effective combination of key genes and their role in explaining the variance in BW between the two BW groups. The PLSR models identified the genes with VIP ≥ 1 as the most important discriminatory features between the groups on days 7, 14, and 38 (Fig. 3.6). As a result, all three PLSR models were comprised of two factors based on the minimal root mean squared error of cross-validation (RMSECV). On day 7, the PLSR model identified a combination of four genes related to gut barrier function (*ZO-1*), immune response (*HSPA4*), nutrient transport (*SLC1A4*), and oxidation (*XDH*) as highly predictive of the BW phenotype (Fig. 3.6A). The model yielded an R^2CV value of 0.4048, indicating that the expression of these 4 genes could explain 40.48% of the variance in BW (Fig. 3.6D).

On day 14, the PLSR model identified 12 genes as important predictors of BW, with an R^2CV of 0.4582, explaining 45.82% of the variability in BW (Fig. 3.6B and E). The majority of the genes were upregulated in the HBW group and were related to gut barrier function (*CLDN1*, *ZO-2*, and *MUC5ac*), immune response (*HSPA4* and *IL-18*), nutrient transport (*SLC1A1*), metabolism (*RPS6KB1*), and oxidation (*HMOX-2*). However, gut hormone (CCK and *Proglucagon B*), nutrient receptor (*GPR120*), and metabolism (*EIFEBP1*) genes were identified as higher in the LBW group. By day 38, the PLSR model identified a combination of 11 genes as a discriminatory factor between BW groups, with an R^2CV of 0.2439 (Fig. 3.6C). A higher number of genes were identified in the HBW group compared to the LBW group, and the majority of these genes were related the nutrient transport (*SLC1A1*, *SLC7A9*, *SLC30A1*, *FABP2* and *VDR*) as well as some genes from other categories such as gut barrier function (*CLDN5*), nutrient receptor (*GPR120*), metabolism (*RPS6KB1*) and oxidation (*XDH*). However, the predictive power of the gene expression data was lower at this late time point compared to the earlier days, it still held the capacity to explain 24.39% of the variance in BW.

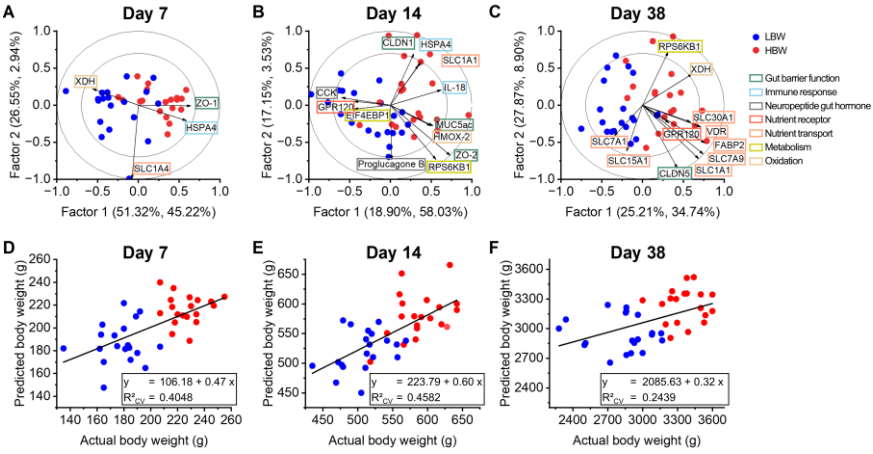

Fig. 3.6 Partial least squares regression (PLSR) models illustrating the relationship between ileal gene expression and body weight in low (LBW) and high (HBW) body weight broilers on days 7 (A), 14 (B), and 38 (C). The colored frames around the genes denote gene function, while the outer and inner circles depict the 100 and 50% explained variance, respectively. The percentage of x and y variance per factor is presented in parentheses. Additionally, linear fit equations and R-squared values on the cross-validation sets (R^2_{CV}) for the PLSR models of days 7 (D), 14 (E), and 38 (F) are presented.

3.3.7 Pearson's correlation between body weight and growth parameters, visceral organ size, and intestinal structure and function

Fig. 3.7 shows Pearson correlations between BW and growth parameters, visceral organ size, and intestinal structure and function across 3 time points, with the highest number of significant correlations found on day 7. Positive correlations were observed between BW, ADG, and ADFI on days 7 and 14, while FCR exhibited a significant positive correlation with BW only on day 38. BW showed a positive correlation with ileal VH but was negatively correlated with relative small intestine length throughout the study period. Relative heart weights were negatively correlated with BW during the first two weeks, and the relative weights of the bursa of Fabricius demonstrated a negative correlation with BW on days 14 and 38. On day 14, the liver's relative weight was negatively correlated with BW, while the stomach's relative weight showed negative correlations on days 7 and 14. Additionally, the pancreas' relative weight demonstrated a negative correlation with BW on day 38. Similarly, the correlation between BW and relative plasma FITC-dextran was negative on day 38. BW on day 7 correlated positively with the expression of genes related to gut barrier function (*CLDN3*, *ZO-1*, *ZO-2*, *MUC2*, *MUC13*, and *MUC5ac*), and nutrient transporters (*SLC1A1*, *SLC3A1*, *SLC7A9*, *SLC7A6*, and *SLC5A1*). In contrast, negative correlations were observed with genes associated with the immune response (*TNF-α*), metabolism (*EIF4EBP1* and *mTOR*), and oxidation (*XDH*). Certain genes associated with nutrient transporters, such as *SLC34A2* and *ATP1A1*, showed negative correlations with BW. On day 14, BW correlated positively with genes related to barrier function (*ZO-2* and *MUC5ac*), immune response (*IL-18*), oxidation (*HMOX-2*), and negatively with the expression of the digestive hormone-related gene *CCK*. On day 38, BW correlated positively with gene expression related to nutrient receptors (*GPR120*), metabolism (*RPS6KB1*), and oxidation (*XDH*).

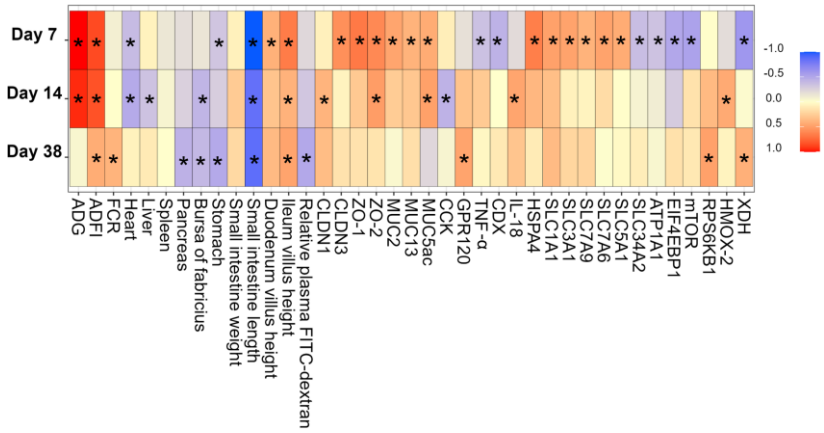

Fig. 3.7 Pearson's correlation between body weight (BW) and growth parameters, visceral organ weights, and ileum gene expression levels on days 7, 14, and 38. Only statistically significant correlations ($P < 0.05$) with an absolute correlation coefficient ($|R|$) greater than 0.30 are shown and marked with an asterisk (*).

3.4 Discussion

The categorization of broilers based on their day 7 BW relative to the flock mean revealed several important biological concepts. Despite being subjected to identical management practices, broilers with lower initial BW failed to exhibit any catch-up growth, suggesting that day 7 BW is a strong indicator of chicks' growth potential and slaughter weight[23]. The HBW group demonstrated higher ADG compared to the LBW group during the starter and grower periods, while their FCR remained comparable. This suggests that the discrepancy in ADG is primarily due to higher voluntary feed intake by the HBW group rather than more efficient utilization of dietary nutrients, suggesting differential feed intake behavior as a discerning factor in elucidating the growth patterns that contributed to divergent weight gain. In agreement, a similar study in pigs linked the growth lag in LBW piglets to reduced feed intake, which decreased the nutrient supply for pre- and postweaning growth[24].

Heavier relative visceral organs require higher nutrients, leading to increased maintenance energy expenditures and lower chicken performance[25]. Consistent with these findings, the LBW group had significantly heavier hearts during the early growth stages and larger bursa of Fabricius in the later stage. Longer small intestines are commonly believed to enhance nutrient absorption and facilitate growth in chickens.

Contrary to this assumption, our findings observed a shorter small intestine length in HBW chickens, suggesting the presence of a metabolic energy-saving mechanism in these chickens, wherein nutrients may be partitioned more efficiently towards growth and weight gain, rather than diverting resources to maintain a longer small intestine[26,27]. Despite the shorter intestinal length, the increased development of villi in HBW chickens compensated for this reduction. The HBW group demonstrated higher VH and VH:CD ratios, which could provide an expanded surface area underlying a higher nutrient requirement, thereby contributing to their accelerated growth rate[12]. Increased weights of the stomach, liver, and pancreas are indicators of improved digestive efficiency[28,29]. Pearson correlation analyses identified these digestive organs as being heavier in the LBW group at various growth stages, which may reflect a developmental adaptation to maximize digestive capacity and promote growth.

The GIT, constantly exposed to a variety of foreign antigens, necessitates rapid mucosal restoration mechanisms in the event of tissue damage[30]. Increased inflammation can destroy the intestinal structure and impair TJ integrity[8,30], facilitating the translocation of antigens and toxins into the systemic circulation. In our study, in vivo gut permeability and gene expression results indicated that there was a disrupted gut barrier function in the LBW chickens, with consistently and significantly decreased relative mRNA expression of TJ genes, including *CLDN1*, *CLDN3*, *CLDN5*, *ZO-1*, and *ZO-2* compared to the HBW chickens. Several studies have linked compromised intestinal TJs in chickens to impaired health and performance[31]. In contrast, the HBW chickens demonstrated higher expression of mucin producing genes, including *MUC2*, *MUC13*, and *MUC5ac*, likely reflective of the strong ability for more efficient clearance of bacteria and a robust protective barrier against pathogen colonization[9,32]. This is corroborated by the fact that the *MUC2* gene expression has been used as a marker for gut health in poultry and other species[32], and has been shown to reduce *Salmonella* adhesion in the jejunum[9].

The HBW chickens exhibited increased relative mRNA expression of *SLC2A1* and *SLC5A1*, which are linked to glucose efflux. Previous studies have linked higher mRNA levels of glucose transporters with an increased

BW in chickens[7,12]. The LBW chickens demonstrated increased expression of the fructose transporter *SLC2A5* on day 7, suggesting an adaptive response to address potential nutritional challenges due to lower feed intake. Additionally, the HBW chickens consistently demonstrated higher mRNA levels of various amino acid transporters across different growth stages, including *SLC1A1*, *SLC3A1*, *SLC7A1*, *SLC7A6*, and *SLC7A9*, indicating their capability to support rapid growth and weight gain through increased amino acid uptake. The *FABP2* gene, known for its role in lipogenesis and fatty acid transport in broilers[33], is also recognized as a marker for gut barrier health and epithelial content in humans and pigs[30]. In the present study, gene expression of *FABP2* was higher in HBW chickens on day 38, suggesting enhanced free fatty acid absorption, increased epithelial cell content, and a strengthened intestinal barrier function. Furthermore, upregulation of genes like the vitamin D receptor (*VDR*) and *SLC30A1* in HBW chickens suggests enhanced absorption of crucial nutrients like calcium and zinc, important for intestinal function and overall growth.

Previous studies have reported higher relative gene expression of pro-inflammatory mediators, including *TNF-α* in LBW chickens compared to their HBW counterparts[34], with overwhelming production of pro-inflammatory cytokines being indicative of an inflammatory state[35]. In line with these findings, our study found significantly higher gene expression of proinflammatory cytokine *TNF-α* and a tendency for increased *PTGES* on day 7 in LBW chicken's ileum. *TNF-α* is a pleiotropic cytokine, and has been shown to potentially affect barrier function by downregulating tight junction proteins[36]. *PTGES* is a fundamental gene required for the synthesis of prostaglandins, which are well-known inflammatory mediators[37]. As such, the elevation of *PTGES* has been linked to intestinal inflammation[38]. In addition to cyclooxygenase pathway, cytochrome P450 (CYP450) enzyme provide an alternative pathway for the metabolism of arachidonic acid, a polyunsaturated fatty acid, into eicoanoides[39]. These eicosanoids, including prostaglandins, thromboxanes, and leukotrienes, modulate immune cell activity and are generally considered pro-inflammatory molecules due to their potent effects on inflammation, oxidative stress, and immune response stimulation[40,41]. On day 38, the LBW chickens exhibited higher expression of *CYP450* gene compared to their heavier counterparts, suggesting potential implications for

eicosanoid production and inflammatory processes. This finding aligns with previous research linking growth restriction of chickens to a predisposition for pro-inflammatory states and an increased risk of inflammatory disorders[34]. The increased immune response in LBW chickens may not solely result from active infection but rather suggests a basal immune system activation, possibly adapting to counteract an imbalanced gut microbiota, rich in opportunistic pathogenic bacteria. Indeed, the gut microbiota plays a pivotal role in shaping the host's immune response[11], with previous studies reported an imbalanced microbiome composition in LBW chickens, characterized by a higher abundance of opportunistic pathogens like *Escherichia-Shigella*[13]. Gram-negative pathogenic bacteria contribute to the release of lipopolysaccharides that induce the expression of inflammatory mediators[42]. This increased immune response can place a significant nutritional burden on the host[43], diverting nutrient resources toward immunity at the cost of rapid growth. On the other hand, *AHSA1* gene, which encodes a protein responsible for activating the ATPase activity of the heat shock protein (HSP) 90 chaperone, was upregulated in the HBW group on day 7. This protein plays a crucial role in the stress response and regulation of Hsp90-dependent cellular pathways in broiler chickens[44]. HBW group also exhibited a higher expression of the *HSPA4* gene, a member of the HSPs, on day 7. HSPs play a critical role in gut health and immune regulation, acting as molecular chaperones for maintaining gut epithelium integrity and effective intestinal barrier function[45]. The univariate analysis revealed a higher expression of *GPR120* mRNA on day 38 in the HBW group, a receptor that binds unsaturated long-chain fatty acids and derivatives[46]. *GPR120* monitors fatty acid concentrations in gastrointestinal and oral tissues[47], and is also highly expressed in adipose tissues and pro-inflammatory macrophages. Its activation mediates anti-inflammatory effects, which reduces the inflammatory signaling responses induced by lipopolysaccharide and *TNF-α* cytokine[48]. Thus, the upregulation of this gene in HBW chickens further confirms their enhanced capacity to regulate lipid metabolism and inflammatory processes and maintain gastrointestinal homeostasis.

Feed intake is known to be strongly correlated with weight gain in broilers[49]. Differential expression of gut hormones, such as the increased

proglucagon B and *CCK* in the LBW group on day 14 and the tendency for higher *GHRL* in the HBW group on day 38, may have contributed to the divergent feed intake behaviors and subsequent growth variation between BW groups. *Proglucagon B*, which was identified as a predictive gene for LBW by PLSR analysis on day 14, is a precursor for glucagon, glucagon-like-peptide-1 (GLP-1), and glucagon-like-peptide-2 (GLP-2), known to have pronounced effects on appetite and food intake regulation[50]. Glucagon reduces BW and adiposity in humans by suppressing appetite and modulating lipid metabolism[51]. CCK hormone serves as a satiation signal and contributes to the feeling of fullness and satisfaction after eating[52], might be leading to a reduction in feed intake in LBW group. Studies have shown that administering CCK reduces feed intake in chickens[53], while inhibiting CCK-A receptors promotes growth and increases BW[54]. The HBW group had higher feed intake than LBW group, which is corroborated by previous findings[55,56] reporting higher GHRL, known as the "hunger hormone," is involved in increasing feed intake and weight gain in chickens by transmitting the hunger signal to the brain before feed intake. *mTOR* and *EIF4EBP1* are central regulators of cellular processes such as protein synthesis, cell growth, and metabolism[44]. Their increased expression in the LBW group on day 7 suggests an attempt to stimulate cellular growth and proliferation as a response to developmental challenges in early life.

Modern fast-growing broilers are highly susceptible to the detrimental effects of excessive reactive oxygen species (ROS) resulting from cellular metabolism, which contributes to intestinal oxidative stress[57]. These ROS adversely affect the antioxidant system in the gut and lead to health problems[58]. The HBW chickens exhibited higher gene expression of antioxidant enzymes, including *GPX7* on day 7, *HMOX-2* on day 14, and *XDH* on day 38, indicating their ability to combat excess free radicals and maintain homeostasis through an activated antioxidation system. Interestingly, *XDH* exhibited dual behavior, with higher expression in the LBW chickens on day 7 but higher in the HBW chickens on day 38, suggesting that some genes may play different roles across growth stages.

The univariate approach with the Student’s t-test provided a list of individual genes exhibiting significant expression changes but did not account for the potential combined effects of multiple genes. The PLSR model's strength lies in its ability to identify combination of co-expressed

genes whose expression highly correlates with the underlying observed phenotypes, regardless of the expression level. On day 7, the PLSR model selected only four genes (*ZO-1*, *HSPA4*, *SLC1A4* and *XDH*) as the most important discriminatory features, while adding extra genes did not improve model performance. This suggests that these four genes represented a concise set of biomarkers effectively capturing the underlying molecular differences between BW groups in the first week. In contrast to day 7, the PLSR models for days 14 and 38 required a larger number of genes, indicating more information from different genes was needed to explain the BW variance at later growth stages. The PLSR analysis revealed an early genetic signature explained by genes involved in gut barrier function (*ZO-1*), immune response (*HSPA4*), and oxidation (*XDH*) that transitioned towards a lasting profile of genes regulating nutrient transport (*SLC1A1*, *SLC7A1*, *SLC15A1*, *SLC7A9*, *SLC30A1*, *FABP2* and *VDR*), nutrient receptor (*GPR120*), gut hormone (*CCK* and *Proglucagone B*) and metabolism (*RPS6KB1* and *EIF4EBP1*) as determinants of BW phenotypes over time. Interestingly, genes associated with gut barrier integrity and oxidation remained as consistent predictors of BW phenotype across all time points. The PLSR models based on gene expression data explained 40% and 45% of the BW variance on days 7 and 14 by identifying 4 and 12 key genes, respectively. However, by day 38, the model's predictive accuracy decreased substantially, possibly due to the convergence of growth rate differences between BW groups during the late growth stage. Nevertheless, the robust predictive accuracy of the PLSR models on days 7 and 14 offers a valuable tool for early identification of the growth potential, informing strategies to improve broiler flock uniformity.

The study aimed to standardize conditions from placement to slaughter for all broiler birds, yet significant variations in growth rates were observed. Egg age and storage conditions were similar for all the chicks in our study as they originated from one flock, of 40-week old parents, and all eggs were placed in 1 incubator, hence minimizing factors related to parent flock and incubation. Hatch weight is a good predictor of subsequent performance, with heavier chicks typically showing improved growth rates [59]. On day 1, chicks had similar BW with low variance, indicating minimal differences in hatch weights. Despite the low genetic

variations within highly inbred broiler lines, residual heterozygosity and genetic polymorphisms yet exists, which may contribute to phenotypic variation among chicks within a shared environment[60]. Additionally, variability in gut microbiota composition among individuals within a flock can influence nutrient digestion, absorption, gut integrity, and immune function, thereby impacting growth trajectories[13]. Moreover, epigenetic changes induced by early-life conditions such as incubation conditions, chick transportation and environmental stress can impact bird performance by altering physiological mechanisms and metabolic pathways. LBW and HBW chicks were housed separately with ad-libitum feed and ample feeder space to reduce competition. However, observed differences in feed intake behaviors among BW categories, influenced by unknown physiological and environmental factors, may have altered nutrient availability and signaling molecules in the gut, subsequently impacting gut health and host responses.

3.5 Conclusions

The findings suggest that variability in feed intake and gut-related traits partly explains why chickens raised under identical management conditions exhibit differences in BW. HBW birds exhibited more efficient digestive physiology characterized by shorter relative intestinal length but higher absorptive capacity (longer VH, greater VH:CD ratio), and enhanced expression of genes involved in maintaining gut barrier integrity and nutrient transport. In contrast, the LBW group demonstrated more energy-intensive visceral organ development, activation of pro-inflammatory response genes, and increased intestinal permeability across various growth stages, potentially leading to higher maintenance energy requirements. The findings further suggest divergent hormonal regulation of appetite and feed intake as a significant driver of the observed variation in growth rates among broilers. PLSR predictive models identified combinations of genes as highly predictive of BW phenotypes, with high model predictive power during early growth stages. These findings suggest that the divergence in BW outcomes is driven, at least in part, by differences in the gene expression of various intestinal functions between birds, offering insights into the molecular mechanisms governing growth. The insights gained in this study shed light on the underlying gut-related regulatory mechanisms involved in broiler growth rates and also

highlighted the importance of tailored management practices to optimize production efficiency and flock uniformity.

Given the significant impact of early-life differences in gut function and gene expression, implementing targeted strategies during this critical period is essential for improving GIT development, gut health, and feed intake behavior, especially in LBW chickens. Such strategies can be implemented through management practices like providing early access to feed and making nutritional and dietary modifications. These approaches may help address gut health deficiencies in LBW chickens and thereby reduce BW heterogeneity. The following chapter will explore the hatching system and early feed access to determine whether they have a significant and lasting impact on broiler performance and intestinal health.

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Chapter 4

Effects of on-farm hatching versus hatchery hatching on growth performance, gut development, and intestinal health and function in broiler chickens

The work presented in this chapter is adapted from:

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Abstract

An alternative hatching system (HS) known as hatch on-farm (HOF) provides early access to feed compared to hatch in hatchery (HH) system. Early feeding may promote favorable gut development, potentially improving intestinal health and broiler performance. Previous studies have assessed the effects of HOF on chick quality, welfare and performance, its impacts on gut health remain inconclusive. A total of 560 Ross 308 male chicks were reared until d 38, hatched either in a hatchery (n = 280) or on-farm (n = 280), with 14 replicates per system and 20 birds per pen. Production parameters were periodically monitored. Digestive and immune organ characteristics, intestinal permeability and histomorphology were assessed on d 7, 14, and 38. High-throughput qPCR analyzed 79 ileal genes regarding barrier integrity, immune function, nutrient transporters, gut hormones, metabolism, and oxidation. HOF chicks had higher d1 body weights than HH chicks ($P < 001$), but this advantage disappeared within first week, with no subsequent performance differences. HOF chickens demonstrated increased duodenal villus width on d 7 and 14, and increased ileal crypt depth and submucosal thickness on d 7 ($P < 0.05$). Relative bursal weight was higher on d 14 ($P = 0.018$) and tended to be higher on d 38 in HOF chickens ($P = 0.094$). Intestinal permeability remained unaffected ($P > 0.05$), while HH chicks showed upregulation of gut barrier genes such as *MUC5ac* on d 7 and *CLDN2* and *MUC2* on d 14 ($P < 0.05$). HH chicks also showed upregulation of nutrient transports including *VDR* on d 7 and *SLC30A1* and *SLC5A9* on d 38, and decreased expression of the appetite-suppressing hormone *CCK* on d 7 ($P < 0.05$). HOF chicks upregulated immune-related genes, including *IL-8* on d 7, *IL-6*, *IFN-γ*, *AVBD9* on d 14, and *NOS2* on d 38 ($P < 0.05$), and the oxidation gene *HIF1A* on d 38 ($P = 0.039$). In conclusion, although the HOF showed only transient growth advantages, it enhanced mucosal morphology and modulated immunity, indicating improved intestinal health.

4.1 Introduction

Based on the findings from Chapter 2 and 3, it was inferred that early-life factors related to gut microbiota composition (Chapter 2) and intestinal development (Chapter 3) both play critical roles in shaping growth performance and BW variability in broilers. These results collectively emphasize that the early post-hatch period is pivotal for establishing microbial colonization and physiological maturation, which together influence long-term growth potential and flock uniformity. Consequently, early-life management strategies that enhance gut health and function, particularly in low-BW chicks, may help reduce BW heterogeneity under uniform rearing conditions.

One such strategy is optimizing the timing of first feed access. In commercial hatchery systems, chicks often experience a feed and water deprivation period of up to 48–72 hours due to hatch window variation and transport logistics[1]. This delay can negatively affect gastrointestinal development, impair intestinal barrier function, alter microbiota colonization, and reduce growth performance[2–4].

Recent advancements in hatching practices aim to mitigate these challenges by addressing delayed feeding and transportation. Two notable approaches currently implemented in commercial poultry production are hatchery feeding and hatch on-farm (HOF) systems. Hatchery feeding involves hatching chicks in the hatchery with immediate access to feed, although it does not eliminate the need for transportation to broiler farms[5]. In contrast, the HOF system allows 18-day incubated eggs to be transported directly to the barn, facilitating hatching on the farm with immediate access to feed and water[1,6]. The HOF system has demonstrated several welfare and performance benefits, including a reduced incidence of footpad dermatitis and better litter quality[7,8]. It has also been associated with transient advantages in body weight and intestinal development until 21 days of age, with compensatory growth observed in hatchery-hatched (HH) chicks, allowing them to attain similar weights at slaughter age. Importantly, chickens from young breeder flocks appear to benefit more from the HOF system due to their smaller size and higher sensitivity to suboptimal conditions[6,8,9]. Nonetheless, the implementation of the HOF system requires careful consideration of potential limitations, including

logistical complexities, costs, and the need for specialized equipment and training.

Despite extensive research on the effects of HOF systems on broiler welfare and performance[5,7–9], there is a significant gap in understanding their comprehensive impact on gut health and related physiological processes. Early feeding is crucial for the development of the immune system and increased disease resistance in comparison to delayed-fed chickens[10]. The timing of the first feed intake significantly impacts gut microbiota colonization[11], which directly influences the enteric immune system[12]. Differences in the intestinal microbiota between HOF and HH chickens have been reported[13], which may cause differential immune responses in birds with early compared to delayed feeding. Immediate post-hatch feeding accelerates the development of immune organs[14] and can prevent reduced bursa weight, poor vaccination responses, and decreased disease resistance associated with delayed feeding[3,15]. Feeding stimulates the digestive system, including the stomach, liver, pancreas, and small intestine to secrete compounds that support the growth of the intestinal mucosa[16]. Early luminal stimulation by feed may positively affect intestinal morphology and nutrient uptake. Luminal nutrients also stimulate structural and functional regulations in the intestine through a process involving different gut hormones[17]. These hormonal responses are crucial for appetite regulation, metabolic efficiency, and overall growth performance. Moreover, the timing of feed intake impacts the integrity of tight junctions in the intestinal epithelium, which is essential for maintaining gut barrier function and preventing pathogen invasion[18]. On the other hand, delayed feeding in chickens has been shown to impair intestinal structure, reduce nutrient absorption and compromise gut integrity[19–21].

The aim of the current study was to investigate the impact of HOF compared to the HH system on growth performance and gut health-related parameters in broiler chickens. This includes measurements of growth performance, digestive and immune organ characteristics, intestinal permeability and morphology, and gene expression patterns associated with various intestinal functions, including gut integrity, immune function, nutrient transport and receptors, gut hormones, metabolism, and oxidative processes across various growth stages. It was

hypothesized that on-farm hatching, which eliminates transportation stress and enables early access to feed, would result in better growth performance and improved intestinal development and health in HOF chickens compared to conventionally hatched chickens, up to slaughter age.

4.2 Materials and methods

This animal study was approved by the Katholieke Universiteit Leuven Ethical Committee for Animal Experimentation (Ethical protocol P045/2022, Belgium) and was performed at TRANSfarm, the research facility for animal experimentation of KU Leuven (Lovenjoel, Belgium).

4.2.1 Animals, Housing and Management

For this study, Ross 308 male broiler chicks were sourced from the same 40-week-old breeder eggs and were hatched either in a commercial hatchery or on-farm. For the HOF system, 18th day incubated eggs were transported, after candling, to the broiler house, carefully placed on litter material, and provided with feed, with optimal hatching conditions being maintained. In brief, the ambient temperature of the broiler house was regulated to maintain the eggshell temperature within the range of 36.1 °C and 37.2 °C with relative humidity of 40-50 %. Chicks began hatching on embryonic d 19, two days earlier than the commercial age. As hatching progressed, the temperature regulation was shifted to maintain the chick's body temperature between 39.5 °C to 40.5 °C. Male chicks hatched slightly later than females, as a larger proportion of female chicks emerged earlier during the hatching process. Continuous light was provided to ensure prompt access to feed and water to chicks after hatch. HOF birds were manually picked up, graded, and sexed. Deformed HOF chicks were promptly culled by decapitation. The shells of the hatched eggs were shredded into the pens. In contrast, HH chicks were incubated at a commercial hatchery (Belgabroed NV, Merksplas, Belgium), with a hatch window of 24-36 hours. After hatching, chicks were subjected to standard hatchery procedures including grading, sexing, and vaccination before they were transported to the broiler farm. The broiler farm was 108 km from the hatchery, and transportation took around 2-3 hours, which led to it taking more than 40 hours before most of the chicks received feed and water.

Following standard commercial practices, the day on which the HH chicks arrived at the broiler farm was designated as day 1 for both hatching system (HS). From that day, standard broiler house settings were implemented, with the ambient temperature gradually reduced from 33.5 °C to 21.5 °C by d 21, and then maintained at 21.5 °C until d 38. On d 1, one hour of darkness was provided, which increased to six hours from d 7 onward. All chicks received vaccinations against Newcastle disease virus on d 1 and 16, and for Gumboro on d 16. All birds received feed and water ad-libitum, with three-phase commercial diets (starter diet: d 1 to 14, grower diet: d 15 to 28, and finisher diet: d 29 to 38). Specific details of the ingredients and chemical composition of the diets fed to the birds are given in Table S2.1.

4.2.2 Study design

A total of 560-day-old Ross 308 male chicks were used, 280 from each of the two HS. Birds were housed in 28 pens (1.3 m2/pen and 14 replicate pens per HS) with 20 chicks each. By d 38, the study concluded with 14 to 15 birds per pen, corresponding to a stocking density of 33 kg/m².

4.2.3 Growth performance measurements and sampling

Individual animals were weighed on d 1, 7, 14, 28, and 38. Performance parameters including average daily gain (ADG), average daily feed intake (ADFI), and mortality-corrected feed conversion ratio (FCR) were calculated per pen for each diet phase. The coefficient of variation (CV, %) in body weight of both HS was also calculated on d 7, 14, 28, and 38. Twenty birds per HS were randomly selected (1-2 broilers per pen) for sample collection on d 7, 14, and 38, and euthanized by a trained person using electronarcosis followed by decapitation. For histomorphological examination, duodenum and ileum sections (small intestine starts after Meckel's diverticulum) from the midpoint were obtained. Additionally, ileal tissues were snap-frozen and stored at -80°C for high-throughput qPCR gene expression analysis.

4.2.4 Relative weights of digestive and immune organs

Digestive viscera (heart, liver, pancreas, stomach – both proventriculus and ventriculus – and small intestine), as well as immune organs (spleen and bursa), were carefully removed (n = 20/group), and their weights were recorded on a scale with a precision of ± 0.01 g. The small intestine weight

was recorded without removing the digesta. Afterward, their relative weights as grams per 100 grams of live body weight were calculated. The small intestine length was measured and its relative length was expressed as centimeters per 100 grams of live body weight.

4.2.5 Intestinal histomorphology

The duodenum and ileum samples (n = 20/group) were fixed in a 4% formaldehyde solution for 48 hours, after which the formaldehyde was replaced with 70% ethanol, following the standard procedure used by the GIGA Immunohistology Platform (ULiège, Belgium). One slide per sample was prepared, and histological sections were stained with Alcian Blue-Periodic Acid Schiff. Microscopy images of the slides were captured at 20x magnification and examined using specialized software (NDP.view2, Hamamatsu, Japan). Measurements were taken from 20-well-oriented villus-crypt units and morphometric parameters analyzed included villus height (VH), crypt depth (CD), the ratio between VH and CD (VH:CD), villus width, and the thickness of both the submucosal layer and the tunica muscularis as described by previous research[22].

4.2.6 Intestinal permeability

To evaluate intestinal permeability, one bird was randomly selected from each pen for each HS group (n = 14). Fluorescein isothiocyanate dextran (FITC-d, 4 kDa; Sigma-Aldrich, St. Louis, MO, USA) was administered orally via gavage at a concentration of 2.2 mg/mL per bird. Following a 2.5-hour administration period, blood samples (1 mL) were obtained from the jugular vein, which were centrifuged at 3000 × g for 15 minutes at 4°C to isolate plasma. Subsequently, 1:5 dilutions of the plasma samples and standard solutions were prepared using phosphate buffer solution. Duplicate aliquots were transferred to 96-well microplates for fluorescence measurements. Spectrophotometric analysis was carried out on Victor3 instrument (PerkinElmer Inc., Waltham, MA, USA) with excitation wavelength at 485 nm and emission wavelength at 530 nm. FITC-d concentrations in plasma samples, expressed in ng/mL, were derived from a standard curve generated during the analysis.

4.2.7 Gene expression through high throughput qPCR

4.2.7.1 Primer design and validation

A list of 92 genes (13 housekeeping genes and 79 target genes) associated with different ileal physiological functions was selected based on a thorough literature review. These genes were analyzed for their expression using high-throughput qPCR, and a brief description of their main functions is provided in Table S3.1. Validated primer sequences were adapted from previous research[23], which established a robust ileum gene expression panel for evaluating intestinal health in broiler chickens under various environmental conditions. Additionally, validated primers from other studies were incorporated into our ileum gene panel to ensure comprehensive coverage of important genes relevant to the intestinal health and function of broilers (Table S3.1).

Primers were cross-checked using the NCBI Primer-Blast tool to span exon-exon junctions, preventing genomic DNA amplification. Target specificity was confirmed using in silico analysis through NCBI databases, ensuring no significant cross-reactivity with non-target sequences. Experimental validation involved melting curve analysis following qPCR amplification. All melting curves showed a single peak, indicating specific amplification without side products or primer dimers. Single-product amplification was further verified through agarose gel electrophoresis, where single sharply defined bands appeared at the expected molecular weight for each amplicon. The primers were optimized for efficiency between 90-110%, with R^2 values >0.99 using three-fold serial dilutions of a pooled cDNA derived from all samples on a QuantStudio 6 Real-Time PCR System (Thermo Fisher Scientific). No-template and no-RT controls confirmed the absence of contamination, and consistent Ct values across biological replicates (CV < 5%) demonstrated reliable performance.

4.2.7.2 RNA isolation, reverse transcription and preamplification

RNA was isolated from ileal tissue samples (n = 20/group) using a commercial kit (ReliaPrep™ RNA Miniprep Systems, Promega) according to the provided protocol. The quantity and quality of the isolated mRNA were assessed using spectrophotometry (Nanodrop 2000, Thermo Fisher Scientific), and RNA integrity was verified through 1% agarose gel electrophoresis. cDNA was prepared from extracted RNA using RT

MasterMix (Standard BioTools). A preamplification step was carried out in a 96-well qPCR plate using a primer mixture with PreAmp Mastermix (Standard BioTools). The thermal cycling conditions for this step were: initial denaturation at 95°C for 2 min, followed by 14 cycles of 95°C for 15 sec and 60°C for 4 min. Next, a clean-up step was performed using Exonuclease I under specific thermal conditions to eliminate unincorporated primers. The Exonuclease I-treated PreAmp reactions were diluted 10-fold and stored at -20°C until the next step.

4.2.7.3 High-throughput qPCR

The BioMark™ HD system (Standard BioTools) was used for high-throughput qPCR, following a protocol described in our previous study [24]. Three 96.96 Integrated Fluid Circuits (IFCs) were run, each corresponding to the samples obtained on d 7, 14, and 38, respectively. Before qPCR, the sample mix was prepared by combining 2.25 µL pre-amplified Exo-I treated cDNA samples with 2.5 µL of 2x SSoFast™ EvaGreen® Supermix (Bio-Rad, Hercules, CA, USA) and 0.25 µL of 20x DNA Binding Dye (Standard BioTools). The assay mix was prepared by combining 0.5 µL of each forward and reverse primer (100µM) with 2.5 µL of 2x Assay Loading Reagent (Standard BioTools) and 2.25 µL of low EDTA DNA suspension buffer. The sample and assay mixtures were then transferred into the IFC. The qPCR was performed using a fast program with an initial denaturation at 95°C for 60 sec, followed by 30 cycles of denaturation: 96°C for 5 sec and 60°C for 20 sec. The standard curve based on pooled pre-amplified cDNA samples was used to calculate relative mRNA concentrations. Four reference genes (TBP, B2M, NDUFA, and B-ACTIN) were identified as the most stable across experimental conditions via the NormFinder algorithm[25]. The Pfaffl method[26] was used to calculate the relative expression of all genes, with normalization of target genes achieved by using the geometric mean of the reference genes' expression.

4.2.8 Statistical analysis

Prior to the statistical analysis, the normality of data distribution was confirmed using the Shapiro–Wilk test in R (v4.2.3). The effects of HS on investigated variables were analyzed using a linear mixed-effects model for each sampling time. The pen was used as a random factor to account for any confounding effect caused by pen location and different number of animals in pens, while HS was used as a fixed effect. A significance

threshold was set at $P < 0.05$, and a trend was considered for values between 0.05 and 0.10. *P* values for ileal gene expression data were adjusted using the Benjamini-Hochberg approach[27] to correct for multiple testing, with $P < 0.05$ set as the significance threshold. Principal component analysis (PCA) was performed to visualize sample clustering between HS based on gene expression data using factoextra package (v1.0.7) in R. Furthermore, Permutational Multivariate Analysis of Variance (PERMANOVA) was performed in R using adonis2 (v2.6.4) to test for multivariate effects of HS on sample clustering in PCA. Heatmaps were generated that show sample variability and gene expression levels using the pheatmap package (v1.0.12) in R. The heatmaps for two-way hierarchical clustering analysis were based on Pearson's correlation distance and Ward's clustering method, with gene expression levels scaled per gene.

4.3 Results

4.3.1 Growth performance

The percentages of non-hatched eggs and culled deformed chicks were only recorded for the HOF system, which were 1.82 % and 1.19 %, respectively. On d 1, the BW of HOF chicks was significantly higher than that of HH chicks (45.7 ± 3.14 g vs. 42.2 ± 2.89 g) ($P < 0.001$, Table 4.1). The difference in BW between HS disappeared by d 7 and remained statistically similar thereafter. There was no point at which the ADG, ADFI, or FCR differed significantly between the HS ($P > 0.05$). The HOF system showed numerically higher CV in body weight than the chicks in HH system, however, these differences did not reach statistical significance ($P > 0.05$).

Table 4.1 Performance indicators of chickens hatched in the hatchery (HH) or on-farm (HOF).

Indicator	Days of age	HH	HOF	SD	*P* value
Body weight (g)	1	42.2[b]	45.7[a]	3.60	<0.001
	7	187.1	191.1	23.70	0.929
	14	507.0	511.7	62.90	0.892
	28	1863.7	1857.1	159.79	0.906
	38	3115.5	3098.6	179.66	0.897
ADG (g/g)	1-14	32.9	33.1	4.50	0.831
	15-28	96.9	96.2	7.10	0.917
	29-38	125.1	124.2	9.39	0.876
	1-38	85.0	84.5	4.67	0.825
ADFI (g)	1-14	41.9	41.02	3.70	0.513
	15-28	127.1	126.7	11.18	0.921
	29-38	191.8	195.1	13.03	0.521
	1-38	112.8	113.1	7.60	0.905
FCR (g/g)	1-14	1.19	1.18	0.080	0.892
	15-28	1.35	1.37	0.869	0.466
	29-38	1.60	1.58	0.128	0.743
	1-38	1.33	1.34	0.038	0.729
CV (%)	7	4.85	4.95	0.28	0.679
	14	6.05	6.55	1.72	0.868
	28	7.75	8.15	1.37	0.321
	38	10.4	11.01	3.09	0.472

Abbreviations: ADG = average daily gain, ADFI = average daily feed intake, FCR = feed conversion ratio, CV = coefficient of variation. Body weight was recorded from individual birds, while a pen was considered the experimental unit for all other measurements. Data are presented as mean and pooled standard deviation (SD).

4.3.2 Relative weights of digestive and immune organs

HH chicks demonstrated higher relative heart weights on d 7 (P = 0.041), and HOF chicks demonstrated higher relative weight of the bursa of Fabricius on d 14 (P = 0.018, Table 4.2). In addition, HOF chicks tended to have increased relative liver and small intestine weights on d 14 (P = 0.060 and P = 0.059, respectively) and higher relative bursa weights on d 38 (P = 0.094) as compared to HH chicks.

Table 4.2 Relative visceral organ weights (g/100 g body weight) and small intestine length (cm/100 g body weight) of chickens hatched in the hatchery (HH, n = 20) or on-farm (HOF, n = 20).

Organ	Days of age	HH	HOF	SD	*P* value
Heart	7	0.78[a]	0.71[b]	0.199	0.041
	14	0.79	0.82	0.081	0.106
	38	0.47	0.50	0.065	0.164
Liver	7	4.38	4.27	0.420	0.731
	14	3.22	3.46	0.399	0.060
	38	2.01	2.01	0.279	0.977
Spleen	7	0.07	0.08	0.030	0.549
	14	0.09	0.08	0.021	0.477
	38	0.11	0.12	0.032	0.855
Pancreas	7	0.43	0.44	0.080	0.682
	14	0.38	0.39	0.077	0.749
	38	0.16	0.16	0.039	0.931
Bursa of fabricius	7	0.17	0.17	0.056	0.121
	14	0.21[b]	0.26[a]	0.070	0.018
	38	0.14	0.16	0.131	0.094
Stomach	7	6.59	6.31	0.700	0.178
	14	4.51	4.61	0.535	0.522
	38	1.79	1.83	0.398	0.707
Small intestine weight	7	18.40	18.35	1.393	0.889
	14	15.25	15.80	1.601	0.059
	38	7.68	7.70	1.265	0.970
Small intestine length	7	55.94	55.84	7.903	0.954
	14	27.46	28.40	2.713	0.194
	38	6.68	6.80	0.924	0.619

Data are presented as mean and pooled standard deviation (SD).

4.3.3 Intestinal permeability

FITC-d levels were not significantly different between HH and HOF chickens at any of the time points (P > 0.05; Fig. 4.1).

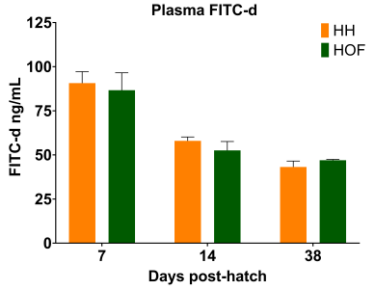

Fig. 4.1 Plasma fluorescein isothiocyanate dextran (FITC-d, ng/mL) levels 2.5 h after oral administration to chickens hatched in the hatchery (HH, n = 14) or on-farm (HOF, n = 14) on 7, 14 and 38 days post-hatch.

4.3.4 Intestinal histomorphology

Compared with HH chicks, HOF chicks demonstrated an increased duodenal villus width on d 7 and 14 (P = 0.031 and 0.030, respectively) and a thicker submucosal layer (P = 0.045) on d 7 (Table 4.3). In addition, HOF chicks had deeper ileal crypts (P = 0.018) and tended to have a lower VH:CD ratio on d 7 (P = 0.099) than HH chicks (Table 4.4).

Table 4.3 Duodenal histomorphological characteristics of chickens hatched in the hatchery (HH, n = 20) or on-farm (HOF, n = 20).

Indicator	Days of age	HH	HOF	SD	*P* value
VH (µm)	7	1418	1431	120.7	0.690
	14	1917	1933	160.2	0.773
	38	2077	2068	218.5	0.899
CD (µm)	7	132	140	27.4	0.366
	14	200	211	40.7	0.421
	38	188	205	65.4	0.447
VH:CD	7	11.1	10.6	2.51	0.536
	14	10.1	9.3	2.13	0.323
	38	12.3	11.5	3.95	0.351
Villus width (µm)	7	148[b]	160[a]	18.5	0.031
	14	175[b]	193[a]	26.1	0.030
	38	191	195	27.8	0.612
Submucosa (µm)	7	21.6[b]	23.8[a]	3.90	0.045
	14	21.7	23.0	3.62	0.309
	38	29.0	28.3	4.75	0.623
Tunica muscularis (µm)	7	133	132	16.7	0.830
	14	151	149	23.1	0.972
	38	190	195	33.5	0.608

Abbreviations: VH = villus height, CD = crypt depth, VH:CD = ratio of VH to CD. Data are presented as mean and pooled standard deviation (SD).

Table 4.4 Ileal histomorphological characteristics of chickens hatched in the hatchery (HH, n = 20) or on-farm (HOF, n = 20).

Indicator	Days of age	HH	HOF	SD	*P* value
VH (µm)	7	536	547	67.1	0.517
	14	646	648	86.8	0.944
	38	1075	1019	147	0.235
CD (µm)	7	118[b]	131[a]	17.9	0.018
	14	180	182	28.8	0.774
	38	162	158	27.2	0.590
VH:CD	7	4.6	4.3	0.72	0.099
	14	3.5	3.6	0.75	0.652
	38	6.8	6.6	1.21	0.551
Villus width (µm)	7	152	144	18.2	0.222
	14	177	184	15.5	0.180
	38	170	149	34.8	0.217
Sub mucosa (µm)	7	20.0	21.0	2.13	0.456
	14	27.2	27.9	3.91	0.545
	38	34.1	38.2	9.24	0.359
Tunica muscularis (µm)	7	111	116	21.7	0.579
	14	153	150	21.1	0.697
	38	189	225	53.8	0.146

Abbreviations: VH = villus height, CD = crypt depth, VH:CD = ratio of VH to CD. Data are presented as mean and pooled standard deviation (SD).

4.3.5 Ileum gene expression

During the high-throughput qPCR, three samples from the HH group on d 7 and one sample from the HOF group on d 38 had to be excluded because of technical problem. No expression readouts were obtained from some genes due to technical issues and these were withdrawn from the statistical analysis (Table S3.1).

4.3.6 Principal component analysis and heatmap clustering

On d 7, the first two principal components (PCs) combined accounted for 37.5 % of the total variability. However, the variability of these components did not effectively separate the samples into distinct clusters based on HS treatment (P = 0.362; Fig. 4.2A). PERMANOVA further showed no statistically significant relationship between HS groups and the variability in gene expression as captured by PCs. On d 14 and 38, the PCA

results showed that PC1 explained 26 % and 30.7 % of the total variance, respectively. However, at these later growth stages, the separation of samples based on the HS was also not distinctly observable. PERMANOVA further confirmed the absence of substantial differences in gene expression profiles between the HH and HOF treatments (P = 0.255 for d 14 and P = 0.427 for d 38; Fig. 4.2B and 4.2C).

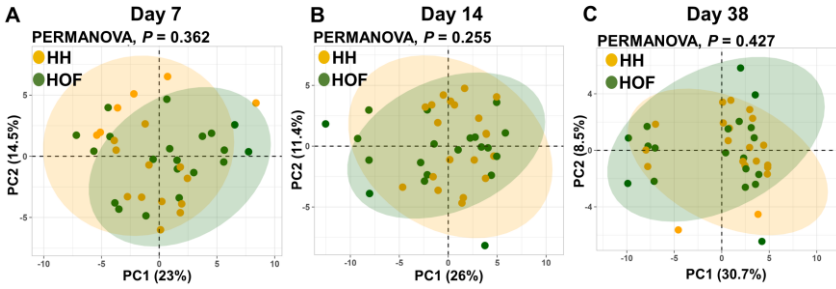

Fig. 4.2 Principal component analysis of gene expression data obtained from the ileum of chickens hatched in the hatchery (HH) or on-farm (HOF) on days 7 (A), 14 (B), and 38 (C).

4.3.7 Heatmap clustering

The heatmaps provide a visual representation of the gene expression variability across samples from both HS groups (Fig. 4.3, S4.1, and S4.2). Two-way hierarchical analysis on d 7, 14, and 38 revealed no clear clustering of samples or genes according to HS conditions or biological functions. However, three distinct gene expression clusters were identified at each age. On d 7, the first-row cluster had genes that tended to be co-expressed and were associated with gut barrier function, immunological response, nutrition transport, gut hormone, metabolism, and oxidation. On d 14, the first row of cells showed co-expression of genes related to gut barrier function. The second-row cluster consisted primarily of nutrient transport genes, while the third-row cluster contained mostly immune response genes. On d 38, the first-row cluster showed co-expression of genes primarily from the immune response category. The second-row cluster contained mostly nutrient transport genes and gut barrier function genes. The third-row cluster contained genes associated with both gut barrier function and immune response.

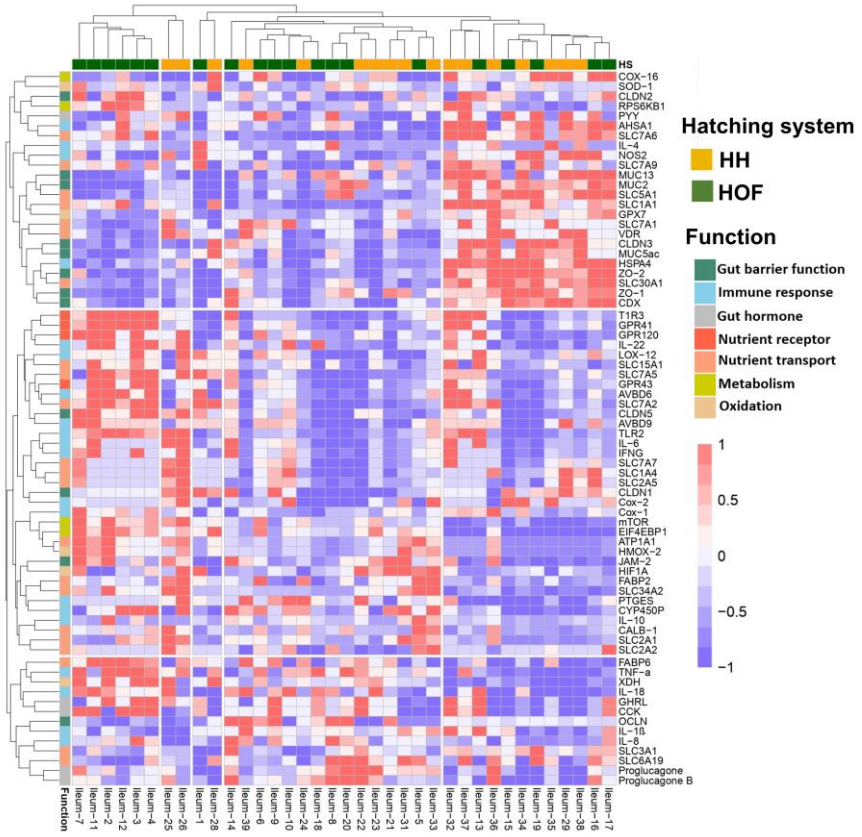

Fig. 4.3 Heatmap of ileal gene expression levels on day 7 of chickens hatched in hatchery (HH, n = 17) or on-farm (HOF, n = 20). The x-axis represents individual samples, while the y-axis shows the genes. Expression levels are color-coded, with red corresponding to high expression and blue indicating low. Gene functions are denoted by different colors on the y-axis. The left dendrogram clusters genes with similar expression patterns, and the top dendrogram groups samples with similar gene expression profiles.

4.3.8 Differential gene expression analysis

On d 7, HH chicks showed higher expression of *MUC5ac* (P = 0.048) and *VDR* (P = 0.015), genes associated with gut barrier function and nutrient transport, respectively (Fig. 4.4A). In contrast, HOF chicks had higher expression of *CCK* (P = 0.041) and *IL-8* (P = 0.009) genes associated with gut hormones and the immune response, respectively. On d 14, HH chicks demonstrated higher expression of *CLDN2* (P = 0.029) and *MUC2* (P = 0.046), with a tendency toward increased *ZO-2* (P = 0.062; Fig. 4.4B), genes

related to gut-barrier function, compared to HOF chicks. HOF chicks had higher expression of the immune-related genes *AVBD9* (P = 0.047), *IFN-γ* (P = 0.048), and *IL-6* (P = 0.040). However, the expression of the other immune-related genes *IL-18* (P = 0.052) and *COX-1* (P = 0.093) tended to decrease in HOF chicks. Additionally, HOF chicks had a tendency toward higher expression of *T1R3* (P = 0.084), a nutrient receptor-related gene. On d 38, HOF chicks showed upregulation of *ZO-1* (P = 0.018), *HIF1A* (P = 0.039), and *NOS2* (P = 0.035), genes related to barrier function, oxidation, and the immune response, respectively (Fig. 4.4C). In contrast, HH chickens showed upregulation of nutrient transport-related genes *SLC5A9* (P = 0.045) and *SLC30A1* (P = 0.002), with a tendency toward higher *s* (P = 0.068) expression.

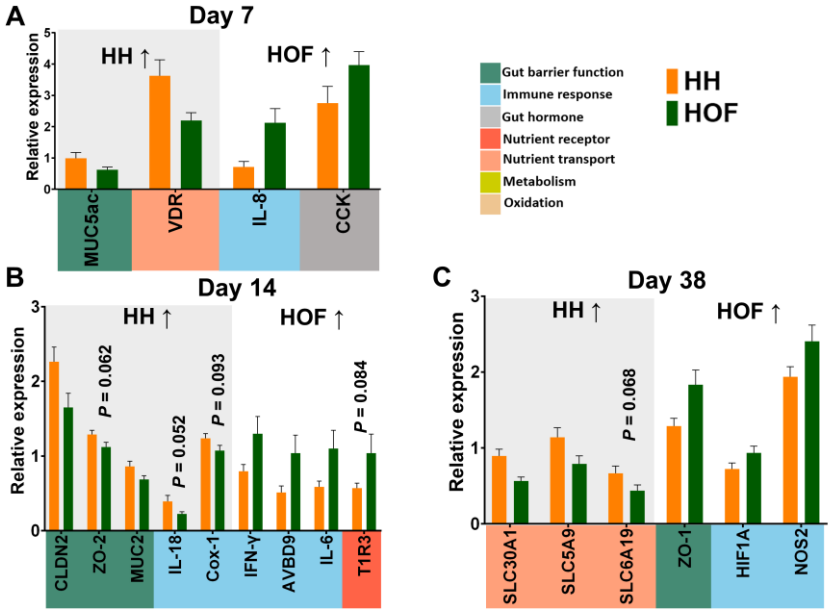

Fig. 4.4 Differential gene expression (Student's t-test with an FDR-adjusted *P* value < 0.05) between chickens hatched in the hatchery (HH) or on-farm (HOF) chickens on days 7 (A), 14 (B), and 38 (C). The gray background section shows genes upregulated in HH chickens, while white background section shows those upregulated in HOF chickens. Gene functions are annotated by color codes on the x-axis.

4.4 Discussion

Although HOF chicks had a temporary body weight advantage on d 1, it did not persist beyond the first week. This initial body weight advantage is likely due to the favorable start in the HOF system, characterized by immediate feeding and the absence of transportation. HOF chicks had immediate feeding, unlike the 48 – 72 hours delay in traditional HH practices due to prolonged hatch windows and hatchery protocols. Early nutritional access is crucial, as previous studies have demonstrated its positive impact on broiler body weight and feed intake by 7 days of age[20]. Even brief early fasting, as short as 24 hours, negatively impacts weight gain during the starter phase[28]. Furthermore, the HOF system likely minimized the adverse effects of transportation. Bergoug et al.[29] reported that transportation negatively impacts BW in chickens up to 21 days of age, with transported chickens showing lower BW compared to those that were not transported. The presence of transportation, combined with initial deprivation of feed and water, might have negatively affected the development of HH chicks and have resulted in their lower body weight at placement[4,30]. Although long-term effects of the HOF system on broiler performance were not observed, implementing this system resulted in immediate improvements in early growth rates as compared to the HH system. HOF chicks exhibited a temporary BW advantage until first week, which can be attributed to the immediate post-hatch access to feed and water, facilitating nutrient intake and hydration during a critical developmental period. In the current study, HH chickens experienced an average post-hatch feed deprivation of approximately 40 hours, which did not appear long enough to cause significant long-term performance differences between the two HS. Boyner[31] similarly reported that HH chicks can effectively compensate for early-life setbacks, including short-term feed deprivation and transportation stress, minimizing their impact on overall performance. Additionally, male chicks were hatched slightly later than females, which aligns with previous studies[32,33]. Since our study exclusively used male chicks, their relatively shorter time to access feed may have contributed to the observed short-term BW advantage in HOF chicks.

The findings showed that duodenal and ileal VH and the VH:CD ratio remained unaffected by the feed deprivation associated with the HH

system. This observation aligns with previous studies[4,8], which reported no deleterious effects of delayed feeding due to the HH system on intestinal morphology. However, it contrasts with studies showing higher duodenal and ileal VH and CD in early-fed chickens[34] or decreased villus surface area and height in fasted broilers[35,36]. Discrepancies across studies may stem from different sampling times. Uni et al.[37] reported that the duodenal villus surface area in feed-deprived chicks, which was initially reduced, recovered after 4 days. Our first sampling occurred on d 7 post-hatch, which may have been too late from the critical window to capture the full spectrum of transitional effects of HS on intestinal morphology. Future investigations should consider earlier sampling points, specifically on days 1-3 post-hatch, to better understand the effects of feed deprivation on intestinal development. HH chickens had lower duodenal villus width on d 7 and 14, and decreased submucosa thickness and ileal crypt depth on d 7. These findings suggest that while some intestinal features catch up with those of HOF chickens, the persistent reduction in some parameters suggests that feed deprivation may have lasting impacts on certain intestinal traits. The reduction in villus width and CD in HH chicks could be interpreted as a physiological response to the absence of early luminal stimulation by feed. In contrast, the enlargement of histomorphological parameters in HOF chicks may be related to early nutrient availability, which facilitated more rapid intestinal development. This is consistent with a previous study in ducklings, where early feeding resulted in increased villi height, villi width and crypt width compared to delayed feeding[38]. In contrast to prior studies[7,39], our study found a significant difference in relative heart weights between HS, with HH chicks showing higher relative heart weights on day 7 compared to HOF chicks. This suggests that hatching conditions may influence cardiovascular development. Stressors associated with conventional hatchery practices, such as continuous darkness, high noise levels, and handling stress, could have contributed to physiological stress responses in HH chicks. Although stress responses were not measured in this study, perinatal stress is known to activate the hypothalamic-pituitary-adrenal axis, potentially triggering adaptive changes in organ development, including the cardiovascular system [40]. The increased relative heart weight in HH chicks may reflect a compensatory mechanism to mitigate stress-induced challenges during early post-hatch development.

The findings indicated that the HS exerted no significant influence on intestinal permeability, as measured by FITC-d concentrations in plasma. This observation suggests that intestinal barrier function was maintained despite differences in early feeding practices between the HH and HOF systems. Although early fasting is often linked to altered gene expression related to gut barrier integrity[18,41], higher mRNA expression of barrier function genes was observed in HH chicks during the early growth stages. Upregulated genes included *MUC5ac* on d 7, and *CLDN2*, *ZO-2,* and *MUC2* on d 14, all of which are essential for tight junction formation and mucin production, critical to intestinal barrier function[42,43]. Their upregulation may indicate an adaptive response to feed deprivation, explaining the intact intestinal integrity in HH chicks.

Early feeding is key to immune maturation, as the timing of the first feed significantly impacts gut microbiota colonization, which directly affects immune development[11,13,44,45]. Feeding triggers rapid bacterial growth in the intestine[46], suggesting that diet shapes immune function by altering the gut microbiota. Consistent with previous studies[3,15], the findings of the current study demonstrated that early feeding, facilitated by the HOF system, led to increased relative bursa weights on d 14 and 38 compared to the HH birds. This immune stimulation was further evidenced by increased expression of immune-related genes in the ileum of the HOF chickens. An enhanced immune response in early-fed chickens has been linked to higher levels of T and B cells in the bursa[3]. Hollemans et al.[47] reported that early feeding improves the humoral immune response against infections at young ages and reduces the risk of disease and mortality.

HOF chicks showed higher *IL-8* expression on d 7, a pro-inflammatory cytokine crucial for heterophil recruitment and bacterial clearance[48,49]. Furthermore, chicks in the HOF system had higher expression of *IL-6*, *AVBD9*, and *IFN-γ* on d 14 as compared to those in the HH system. *IL-6*, a pleiotropic cytokine, aids in infection response and tissue repair through *IL-8* activation[50,51]. AVBD9, an antimicrobial peptide, directly kills microbes and stimulates cytokine production and dendritic cell differentiation[52]. It effectively targets both Gram-positive and Gram-negative bacteria and has strong fungicidal activity[53,54]. *IFN-γ*, produced by nature killer (NK) cells and T lymphocytes, activates macrophages, enhancing viral inhibition, antigen

presentation, and pathogen elimination[55–57]. On d 38, HOF birds demonstrated elevated *NOS2* expression, boosting pathogen-targeting nitric oxide[58], and increased *HIF1A,* linked to anti-inflammatory responses and cellular adaptation to hypoxia[59]. Despite broilers' typical trade-off between rapid growth and immune support[60], the upregulation of immune genes in HOF birds did not lead to tissue damage or reduced production. This implicates that the HOF system can promote early immune maturation, particularly in intensive production systems, and support operations transitioning to antibiotic-free production by enhancing disease resilience while maintaining growth performance.

On d 7, a higher *VDR* expression was observed in HH birds compared to HOF chicks. *VDR* regulates genes involved in calcium and phosphorus transport[21], suggesting a compensatory response to optimize nutrient absorption after feed availability in the intestinal lumen. HH birds also showed unexpected upregulation of *SLC5A9* and *SLC30A1* genes related to glucose and zinc transport on d 38, potentially due to delayed feeding effects. The timing of initial feeding after hatch could have significant implications for appetite regulation and possibly feed intake patterns in broiler chickens. Lower *CCK* expression was observed in HH chicks on d 7. CCK is a well-known gut hormone that plays a crucial role in appetite suppression[61]. Reduced *CCK* expression in HH birds during early life may result from increased appetite due to delayed feeding, potentially altering feeding behaviors that led to compensatory growth. This finding is corroborated by a previous study[62], which reported that plasma CCK levels in rats decreased rapidly in response to feed deprivation for up to five days and returned to control levels after just one day of refeeding. In addition, the hormone CCK plays a role in stimulating gut reflexes and promoting the release of bile acids and pancreatic enzymes, which collectively enhance digestive efficiency[63].

4.5 Conclusions

This study explored a new HS, where chicks hatch on-farm, which was compared to the standard hatchery system. The findings demonstrated short-term advantage of the HOF system on chicken growth performance as compared to the HH system. Age-related compensatory growth occurred in HH chicks within the first week, after which both HS groups showed similar growth trajectories. The delayed feeding associated with

HH did not fundamentally alter intestinal permeability. Instead, HH birds showed an adaptive upregulation of genes associated with intestinal barrier function, suggesting a mechanism to maintain gut integrity despite early feed deprivation. HOF birds had improved intestinal architecture, higher bursa weight, and higher expression of immune-related genes, suggesting that early feeding facilitated by the HOF system aids in intestinal development and supports the immune system. It is important to note that the present study was performed in healthy and non-challenged conditions; hence, the potential impacts of the HOF system on the immune system may become more apparent in stressful or challenged settings. Overall, the findings of this study demonstrate the potential of the HOF system as an effective management strategy for supporting the immune system and intestinal health in broiler chickens, which may significantly improve overall health and productivity; however, it does not improve the homogeneity of BW in broilers.

Collectively, these results emphasize the significance of early-life nutritional and management interventions in promoting gut development and resilience in broilers. However, individual variability at hatch such as differences in hatch weight (HW) continues to present a challenge for uniform flock performance. In particular, low HW (LHW) chicks are often at a disadvantage, displaying suboptimal growth and intestinal development. As early support strategies remain critical for improving the performance of these birds, the following chapter explores whether targeted in ovo stimulation interventions during incubation period, such as sodium butyrate (SB) injection, can enhance gut health and performance outcomes especially in LHW chicks.

4.6 References

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Chapter 5

In ovo sodium butyrate administration differentially impacts growth performance, intestinal barrier function, immune response, and gut microbiota characteristics in low and high hatch-weight broilers

The work presented in this chapter is adapted from:

Akram, M. Z., Everaert, N., & Dunisławska, A. (2024). In ovo sodium butyrate administration differentially impacts growth performance, intestinal barrier function, immune response, and gut microbiota characteristics in low and high hatch-weight broilers. Journal of Animal Science and Biotechnology, 15(1), 165.

Abstract

Hatch weight (HW) affects broiler growth, and low HW (LHW) often leads to suboptimal performance. Sodium butyrate (SB) has been shown to promote growth through enhanced intestinal health. This study investigated how broilers with different HWs responded to in ovo SB injection and whether SB could enhance gut health and performance in LHW chicks. Ross 308 broiler eggs were injected on incubation d 12 with physiological saline (control) or SB at 0.1% (SB1), 0.3% (SB3), or 0.5% (SB5). Post-hatch, male chicks from each treatment were categorized as high HW (HHW) or LHW and assigned to 8 groups in a 4 × 2 factorial design. Production parameters were recorded periodically. Intestinal weight, length, and gene expression related to gut barrier function and immune response were examined on d 14 and 42. Cecal microbiota dynamics and predicted functionality were analyzed using 16S rRNA gene sequencing. SB treatments did not affect hatchability. HHW-control group exhibited consistently better weight gain and FCR than LHW-control group. SB dose-dependently influenced performance and gut health in both HW categories, with greater effects in LHW broilers at 0.3%. LHW-SB3 group attained highest body weight on d 42, exceeding controls but not significantly differing from HHW-SB3 group. LHW-SB3 group showed upregulation of gut-barrier genes *CLDN1* in ileum, *TJP1* in jejunum and anti-inflammatory cytokine *IL-10* in both jejunum and ileum on d 14. Additionally, LHW-SB3 group upregulated mucin-producing *MUC6* gene in ileum, while HHW-SB5 group increased pro-inflammatory *IL-12p40* cytokine in caecum on d 42. LHW-SB3 group demonstrated shorter relative intestinal lengths, while HHW-SB5 had longer lengths. HHW-control group had higher bacterial diversity and growth-promoting bacteria while LHW-control group harbored the potential pathogen *Helicobacter*. SB reshaped gut microbiota biodiversity, composition, and predicted metabolic pathways in both HW categories. The LHW-SB3 group exhibited highest alpha diversity on d 14 and most beneficial bacteria at all timepoints. HHW-SB5 group presented increased pathogenic *Escherichia-Shigella* and *Campylobacter* on d 42. In conclusion, HW significantly affects subsequent performance and SB has differential effects based on HW. LHW chicks benefited more from 0.3% SB, showing improvements in growth, intestinal development, health, and gut microbiota characteristics.

5.1 Introduction

As demonstrated in Chapter 5, early access to feed through on-farm hatching (HOF) provided short-term advantages in growth performance, intestinal morphology, and immune gene expression compared to the conventional hatchery-hatched (HH) system. However, these benefits were largely transient, with HH chicks exhibiting compensatory growth and convergence in performance parameters after the first week. While early post-hatch feeding clearly supports early development, its limited long-term impact under non-challenging conditions suggests the need for additional or complementary strategies that act even earlier during embryonic development.

Stimulation of the developing embryo through in ovo injection has emerged as a promising strategy to promote the maturation of the gastrointestinal tract (GIT) and immune system before hatch. Administering bioactive compounds at critical embryonic stages may trigger epigenetic and trophic effects that influence intestinal development, immune function, and overall growth long after hatch. This early-life programming has the potential to improve post-hatch performance and resilience in broiler chickens, particularly in birds predisposed to suboptimal development.

One of the most influential early-life factors in broiler production is hatch weight (HW), which strongly predicts post-hatch performance[1]. Chicks with low HW (LHW) are often biologically disadvantaged, displaying reduced growth, poor feed efficiency, compromised intestinal development, and increased expression of inflammatory markers[2,3,4]. These issues are frequently accompanied by an imbalance in gut microbiota and impaired immune responses[5,4], making LHW birds particularly vulnerable to environmental and nutritional stressors.

Butyric acid, a short-chain fatty acid (SCFA), has gained attention as a feed additive in poultry production due to its potential benefits on gut health, growth performance, and immune modulation [6]. Butyrate accelerates gut epithelial cell proliferation, improves mucosal morphology, and enhances weight gain and carcass characteristics in chickens[7,8]. It also exerts immunomodulatory effects by inducing host defense peptides, modulating cytokine expression, and increasing IgG and IgA levels[9–11].

Furthermore, it has been shown to reduce the incidence of intestinal inflammation, thereby contributing positively to overall gut health[12]. Butyrate supports beneficial microbiota growth by lowering the intestinal pH, creating an unfavorable environment for pathogenic bacteria[13]. As a result, the digestion and absorption of nutrients are enhanced, effectively improving the growth performance of animals[14].

Early-life interventions in broiler chickens, particularly during the 21 d incubation period, can significantly impact their long-term health and performance. The small intestine initiates differentiation and morphological changes around embryonic d 14 (ED14), while immune system development begins around ED10, with T cells and B cells developing around ED12[15,16]. The microbiota in the egg, especially within the yolk sac and amniotic fluid, shifts throughout embryonic development, indicating that the native bacteria present in the egg may play a role in development[17]. Given this developmental timeline, in ovo butyrate stimulation on incubation d 12 can shape GIT related parameters, which may have lasting effects on overall broiler performance throughout the production cycle. Previous studies have shown that in ovo administration of bioactive substances on d 12 of incubation can effectively modulate the gut microbiota and immune response[18].

Previous studies have demonstrated the effects of butyrate on broiler chickens' health status and intestinal response with normal HWs. However, it remains unknown whether LHW chicks respond similarly to butyrate as their normal HW counterparts or whether butyrate can mitigate delayed growth effects in LHW chicks. LHW chickens typically exhibit slower growth rates and suboptimal feed efficiency compared to their HHW counterparts[2]. These differences could be linked to variations in intestinal development, gut microbiota composition, and immune function[3,5]. Given these differences, it is plausible that LHW chickens may respond differently to in ovo butyrate administration. This study is the first to investigate the effects of HW on growth performance, intestinal development and function, and microbiota composition in broilers and how these effects are influenced by in ovo sodium butyrate (SB) injection. We hypothesized that in ovo SB administration would improve performance, support intestinal barrier function, regulate the immune

response, and modulate the gut microbiota composition and function more effectively in LHW chickens than in their HHW counterparts.

5.2 Materials and methods

The bird rearing and slaughter procedures followed the Ethics Committee guidelines and complied with the Polish Act on the Protection of Animals Used for Scientific or Educational Purposes (15 January 2015), implementing EU Directive 2010/63/EU.

5.2.1 Eggs and in ovo injection

Ross 308 breeder eggs with an average weight of 66.5 ± 1.93 g originating from a 40-week-old breeding flock were obtained from a commercial hatchery (Drobex-Agro, Solec Kujawski, Poland). All the eggs were incubated under standard conditions (37.8°C and 60% relative humidity). On the 12th d of incubation after candling, the eggs were randomly divided into four treatment groups (*n* = 300 eggs/group). Eggs were then injected into the air chamber with 0.2 mL of physiological saline (0.9% sodium chloride; Fresenius Kabi, Warsaw, Poland) or one of three doses of SB (molecular weight: 110.09 g; Merck Life Science, Warsaw, Poland). The treatment groups were as follows: (1) control (0.9% NaCl), (2) 0.1% SB (SB1), (3) 0.3% SB (SB3), and (4) 0.5% SB (SB5). The in ovo injection procedure was performed following the method described by Dunisławska et al.[19], and the eggs were incubated for 21 d.

5.2.2 Post-hatch chick selection and management

At hatch, the hatchability of each in ovo SB treatment was recorded. The weights of the male chicks were recorded, and the chicks were categorized based on their HWs. In each in ovo treatment group, chicks were divided into low and high HW groups, with 72 chicks per group, resulting in 576 chicks who continued in the experiment. LHW chicks had a BW of 45.6 ± 2.30 g, while HHW chicks weighed 55.1 ± 2.83 g. This created a 4 (SB) × 2 (HW) factorial arrangement, with six replicate pens per group and 12 chicks per pen (Fig. 5.1). The pens contained wheat straw litter as bedding material and had a single feeder and drinker. Uniform rearing conditions with appropriate ventilation, litter management, lighting programs, and stocking densities were provided as recommended by the Aviagen Ross 308 guidelines. The temperature of the barn was initially set at 33°C, which

decreased by approximately 0.5°C daily until it reached 21.5°C on d 21, after which it remained constant. Broilers had ad-libitum access to feed and water and had diets formulated for starter (1–14 d), grower (15–35 d), and finisher (35–42 d) phases; these diets contained 23.0%, 21.5%, and 19.5% crude protein and 3000, 3100, and 3200 kcal/kg metabolizable energy, respectively.

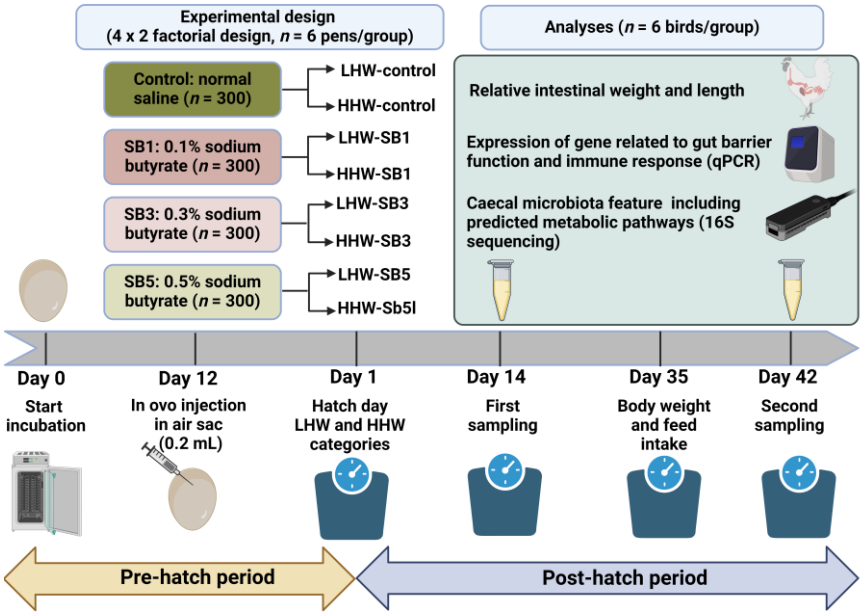

Fig. 5.1 Flow chart illustrating the study design, timeline, and parameters investigated. This diagram was created using Biorender.com.

5.2.3 Growth performance parameters and sample collection

Individual BW and feed intake per pen were recorded at the end of each diet phase to calculate the average daily gain (ADG), average daily feed intake (ADFI), and feed conversion ratio (FCR). ADFI and FCR were adjusted for leftover feed and bird mortality. On d 14 and 42, six birds per group were stunned by percussive blows to the head and then decapitation in accordance with European Commission Council Regulation No 1099/2009 of 24 September 2009 on the protection of animals at the time of killing[20]. After sacrifice, gut development parameters, including the weight and length of the small intestine segments (duodenum, jejunum, ileum) and the cecum, were measured. The relative organ weights and intestine lengths are expressed as g/100 g BW and cm/100 g BW, respectively.

Mucosa scrapings from the jejunum, ileum, and cecum were collected in RNA stabilizing buffer (fix RNA, EURx, Gdańsk, Poland) and stored at -80°C until RNA extraction. The cecal digesta was collected, placed on dry ice, and stored at -80°C until DNA extraction for microbiota analysis.

5.2.4 Gene expression in the intestinal mucosa

5.2.4.1 RNA extraction

RNA was extracted from 100 mg of intestinal mucosal scrapings using an RNA extraction solution (EURx, Gdańsk, Poland) and a TissueRuptor homogenizer (Qiagen, Germany). The homogenate was centrifuged with 0.2 mL of chloroform (Chempur, Poland), and RNA was purified using a universal RNA purification kit (EURx, Poland). RNA quantity and quality were assessed using a NanoDrop 2000 spectrophotometer (Thermo Scientific, USA), and RNA integrity was examined on a 2% agarose gel.

5.2.4.2 Quantitative real-time PCR (qPCR)

Gene expression of gut barrier components (*CLDN1*, *TJP1,* and *MUC6*) and immune-related cytokines (*IL-1β*, *IL-12p40,* and *IL-10*) was quantified via qPCR, with *ACTB* and *G6PDH* serving as reference genes (Table S5.1). The RNA was reverse transcribed into cDNA using a smART First Strand cDNA Synthesis Kit (EURx, Poland). Each sample was analyzed in duplicate with a LightCycler 480 System (Roche Diagnostics, Basel, Switzerland). The qPCR reactions were conducted in a 12.5 µL total volume and included 6.25 µL of SYBR Green I dye (EURx, Gdańsk, Poland), 1 µmol/L each of the forward and reverse primers, and 140 ng of cDNA. The qPCR protocol involved an initial denaturation step at 95°C for 15 min, followed by 40 cycles of amplification (95°C for 15 s, 58°C for 20 s, and 72°C for 20 s), and a melting curve analysis. Relative gene expression was calculated using the $^{\Delta\Delta}$Ct method and quantified with the $2^{-\Delta\Delta Ct}$ formula as described by Livak and Schmittgen[21].

5.2.5 Microbiota analysis

5.2.5.1 DNA extraction

DNA was isolated from approximately 150 mg of cecal digesta using a Stool DNA Purification Kit (EURx, Poland) following the manufacturer's instructions. DNA quantity and quality were assessed as described in the RNA extraction section. The DNA samples were stored at -80°C until further analysis.

5.2.5.2 ONT MinION (16 S, V1-V9) library preparation and sequencing

DNA was prepared for prokaryotic metagenome sequencing using a 16S barcoding kit (SQK-16S024, Oxford Nanopore Technologies, Oxford, UK), with PCR amplification of the full hypervariable region (V1–V9) using universal 16S forward (27F): 5'-AGAGTTTGATCMTGGCTCAG-3' and reverse (1492R): 5'-CGGTTACCTTGTTACGACTT-3' primers. The obtained amplicons were purified with 30 µL of AMPure XP beads (Beckman Coulter, USA) and eluted in 10 µL of 10 mM Tris-HCl buffer to a final library concentration of 100 fmol. The generated sequencing libraries were sequenced on a MinION Flow Cell (FLO-MIN-106, Oxford Nanopore Technologies, Oxford, UK) for 48 h, and the obtained data were processed into FASTQ files using the Ont-guppy-cpu basecaller (v 6.4.6, Oxford Nanopore Technologies, Oxford, UK) in super accurate mode.

5.2.5.3 Bioinformatics workflow

Raw reads underwent initial processing, which included demultiplexing, trimming, and quality-based filtering, using an Ont-guppy-cpu barcoder (v 6.5.7) and Nanofilt (v 2.8.0) software. Filtered FASTQ files were subsequently imported into QIIME 2 (v 2023.9) for downstream analysis. Dereplication of sequences was performed using vsearch[22], followed by de novo clustering of operational taxonomic units (OTUs) with an identity threshold of 85%. Taxonomic classification of clustered OTUs was performed against the SILVA database (release 138) using QIIME 2-vsearch with an 85% identity threshold. Alpha diversity metrics, calculated using the Shannon and Simpson indexes, were calculated after rarefying the OTU table to the minimum sample depth in R (v4.2.3). Differences in alpha diversity metrics between groups were assessed using two-way ANOVA. The Bray–Curtis distance was used for the comparison of beta diversity data among groups via R and was visualized through principal coordinate analysis (PCoA). The significance of multivariate effects on beta diversity was tested using nonparametric permutational multivariate analysis of variance (PERMANOVA). Significant differences in the microbial communities were detected with linear discriminant analysis (LDA) effect size (LEfSe) in R with a minimum LDA threshold of 3.0. The obtained *P* values were further subjected to a false discovery rate (FDR) analysis using the Benjamin–Hochberg method. Phylogenetic investigation of the

communities by reconstruction of unobserved states (PICRUSt2) was used to predict the functional capabilities of the microbial communities in the different groups using two-way ANOVA ($P < 0.05$), using the MetaCyc metabolic pathway database as a reference[23].

5.2.6 Statistical analysis

The normality of the data was assessed through the Shapiro–Wilk test in R. One-way ANOVA was applied to the hatching data. Two-way ANOVA was used to determine the significant effects of HW, SB, or their interaction on growth performance, intestinal weight and length, or gene expression. The means were separated by post hoc tests with Tukey's multiple comparison test, and the significance level was considered at $P < 0.05$. Heatmaps were generated in R using the pheatmap package (v 1.0.12) to visualize sample variability, with predicted metabolic pathway values scaled by row. Heatmaps were based on Pearson's correlation distance and ward clustering method for two-way hierarchical clustering analysis. Correlations between the most abundant bacterial genera and between bacterial genera and metabolic pathways were assessed by Pearson's correlation analysis.

5.3 Results

5.3.1 Hatchability and growth performance

Hatchability was not affected by in ovo SB treatments ($P > 0.05$; Fig. S5.1). At hatching, BW differed significantly between the LHW and HHW categories ($P < 0.001$; Table 5.1), with HHW chicks having higher BW. A significant interaction effect between HW and SB on BW was observed on d 35 and 42 ($P = 0.029$ and $P = 0.045$, respectively). On d 35, the LHW-SB3 group had greater BW than both the LHW-control and HHW-SB5 groups. By d 42, the LHW-SB3 chicks had greater BW than LHW and HHW control groups but did not differ significantly from the HHW-SB3 and LHW-SB1 groups. ADG showed an interaction effect between HW and SB ($P < 0.001$), with LHW-SB1 having greater ADG during 1-14 d, while LHW-SB3 demonstrated greater ADG in all subsequent growth stages. The HHW-control group had greater ADG than the LHW-control group throughout the study. The ADFI was affected by the main effect of SB only during 15-35 d, with SB5-treated chicks showing higher feed intake and SB1-treated chicks showing lower intake ($P = 0.029$; Table 5.2). The FCR exhibited a

significant interaction between HW and SB on 15-35 d and 36-42 d (P = 0.034 and P < 0.001, respectively). The LHW-SB3 group had the lowest FCR values for both periods, while the HHW-SB5 during 15–35 d and LHW-SB5 during 36–42 d showed the highest FCR values. Regardless of HW, the main effect of SB revealed that SB3-treated chicks were most feed efficient during 15-35 d, 36-42 d, and the overall 1-42 d period, while SB5-treated chicks were least efficient (P < 0.05).

Table 5.1 Effect of in ovo sodium butyrate administration on the body weight and average daily gain of broiler chickens with different hatch weights.

[1]Item		Body weight (g)				Average daily gain (g/d)			
		1 d	14 d	35 d	42 d	0 – 14 d	15 – 35 d	36 – 42 d	0 – 42 d
HW	**SB**								
HHW	Control	55.18	399	1917^{ab}	2418^{b}	24.85^{bc}	71.96^{bc}	72.73^{c}	56.87^{bc}
	SB1	55.08	411	1891^{ab}	2365^{bc}	25.78^{bc}	70.45^{c}	67.45^{d}	54.93^{cd}
	SB3	55.06	397	2028^{ab}	2571^{ab}	24.74^{c}	76.15^{a}	75.92^{bc}	59.42^{b}
	SB5	55.20	335	1668^{c}	2282^{bc}	20.34^{d}	63.50^{d}	84.60^{a}	54.91^{cd}
LHW	Control	45.12	377	1723^{bc}	2201^{c}	24.01^{c}	64.80^{d}	67.87^{d}	51.80^{d}
	SB1	45.48	441	1944^{ab}	2499^{ab}	28.55^{a}	71.26^{bc}	79.05^{b}	59.23^{b}
	SB3	46.05	416	2050^{a}	2651^{a}	26.77^{ab}	77.48^{a}	87.01^{a}	63.36^{a}
	SB5	45.78	379	1912^{ab}	2342^{bc}	24.10^{c}	72.67^{b}	61.93^{e}	53.68^{cd}
SD		2.301	54.5	221.3	238.7	3.112	4.831	8.434	3.773
Main effects									
HW									
HHW		55.13^{a}	386	1876	2409	23.93^{b}	70.52^{b}	75.18^{a}	56.53
LHW		45.61^{b}	403	1907	2423	25.86^{a}	71.55^{a}	73.97^{b}	57.02
SB									
Control		50.15	388	1820^{b}	2310^{c}	24.43^{c}	68.38^{c}	70.30^{c}	54.34^{c}
SB1		50.28	426	1918^{ab}	2432^{b}	27.17^{a}	70.86^{b}	73.25^{b}	57.08^{b}
SB3		50.56	408	2039^{a}	2611^{a}	25.76^{b}	76.82^{a}	81.47^{a}	61.39^{a}
SB5		50.49	357	1790^{b}	2312^{c}	22.22^{d}	68.09^{c}	73.27^{b}	54.30^{c}
***P* value**									
HW		<0.001	0.087	0.576	0.668	0.079	0.029	0.027	0.256
SB		0.4191	0.108	<0.001	0.025	0.023	<0.001	<0.001	<0.001
HW × SB		0.419	0.061	0.029	0.045	<0.001	<0.001	<0.001	<0.001

[1]HW: hatch weight; SB: sodium butyrate inclusion level. Control: HHW or LHW chicks from eggs injected with 0.2 mL of 0.9% NaCl. SB1, SB3, SB5: HHW or LHW chicks from eggs injected with 0.2 mL of 0.1%, 0.3%, or 0.5% SB, respectively. The data are presented as the mean and pooled standard deviation (SD) (n = 6 pens/group). [a–d]Values with different superscripts in a column indicate statistical significance at P < 0.05 (two-way ANOVA followed by Tukey's HSD test).

Table 5.2 Effect of in ovo sodium butyrate administration on the feed intake and feed conversion ratio of broiler chickens with different hatch weights.

[1]Item		Average daily feed intake (g/bird/d)				Feed conversion ratio			
		0 – 14 d	15 – 35 d	36 – 42 d	0 – 42 d	0 – 14 d	15 – 35 d	36 – 42 d	0 – 42 d
HW	**SB**								
HHW	Control	35.3	134.9	178.3	113.7	1.46	1.89^{ab}	2.51^{bc}	1.96
	SB1	34.9	132.9	180.9	113.6	1.39	1.92^{ab}	2.70^{ab}	2.00
	SB3	33.9	134.1	177.6	112.8	1.41	1.74^{b}	2.35^{cd}	1.83
	SB5	33.4	142.1	184.2	117.7	1.68	2.27^{a}	2.13^{d}	2.11
LHW	Control	34.2	133.1	181.4	113.5	1.47	2.10^{a}	2.69^{ab}	2.12
	SB1	35.2	132.9	181.6	114.0	1.27	1.89^{ab}	2.32^{cd}	1.86
	SB3	33.4	133.5	182.6	113.8	1.28	1.74^{b}	2.12^{d}	1.75
	SB5	33.8	136.2	177.8	113.5	1.44	1.89^{ab}	2.92^{a}	2.04
SD		3.27	4.17	3.72	5.07	0.177	0.210	0.301	0.174
Main effects									
HW									
HHW		34.4	135.9	180.2	114.4	1.49	1.96	2.42	1.98
LHW		34.2	133.9	180.9	113.7	1.37	1.91	2.51	1.94
SB									
Control		34.8	133.9^{b}	179.9	113.6	1.46	1.99^{a}	2.60^{a}	2.04^{b}
SB1		35.1	132.9^{c}	181.3	113.7	1.33	1.91^{ab}	2.51^{a}	1.93^{bc}
SB3		33.6	133.8^{b}	180.1	113.2	1.35	1.74^{b}	2.24^{b}	1.79^{c}
SB5		33.6	139.1^{a}	181.0	115.6	1.56	2.08^{a}	2.53^{a}	2.08^{a}
***P* value**									
HW		0.882	0.165	0.668	0.770	0.069	0.448	0.181	0.537
SB		0.840	0.029	0.865	0.901	0.059	0.010	0.005	0.013
HW × SB		0.982	0.491	0.064	0.887	0.573	0.034	<0.001	0.324

[1]HW: hatch weight; SB: sodium butyrate inclusion level. Control: HHW or LHW chicks from eggs injected with 0.2 mL of 0.9% NaCl. SB1, SB3, SB5: HHW or LHW chicks from eggs injected with 0.2 mL of 0.1%, 0.3%, or 0.5% SB, respectively. The data are presented as the mean and pooled standard deviation (SD) (n = 6 pens/group). [a–d]Values with different superscripts in a column indicate statistical significance at P < 0.05 (two-way ANOVA followed by Tukey's HSD test).

5.3.2 Relative weights and lengths of the intestine

On d 14, HW, SB, or their interaction had no significant effect on the relative weights of the intestine ($P > 0.05$; Table 5.3). However, there was a significant interaction between HW and SB for relative intestinal lengths ($P < 0.05$). The duodenum was shortest in the LHW-SB1 group and longest in the HHW-SB5 group ($P = 0.001$). The LHW-SB3 group had the shortest jejunum and ileum lengths, while the HHW-SB5 group had the longest length ($P = 0.011$ and $P = 0.015$, respectively). The relative cecal lengths were shorter in the LHW-SB1 and LHW-SB3 groups than in the HHW-SB5 group ($P = 0.030$). On d 42, HW, SB, and their interaction significantly affected various intestinal parameters ($P < 0.05$; Table 5.4). The jejunum relative weight was higher in the LHW category compared to the HHW category ($P < 0.001$), with the LHW-SB3 group showing the highest weight. Similar trends were observed for ileum and cecum weights, with higher values in the LHW category than in the HHW category. The ileum relative weight in the LHW category was higher in the LHW-SB1 group compared to the LHW-SB5 group ($P = 0.013$), while the cecum relative weight did not significantly differ among the LHW groups ($P < 0.001$). For the relative length of the jejunum, the LHW-SB3 group had the shortest length, and the HHW-SB5 group had the longest length ($P < 0.001$).

Table 5.3 Effect of in ovo sodium butyrate administration on the relative weights (g/100 g of body weight) and lengths (cm/100 g per body weight) of intestines in broiler chickens with different hatch weights on d 14.

Items		Relative weights				Relative lengths			
		Duodenum	Jejunum	Ileum	Cecum	Duodenum	Jejunum	Ileum	Cecum
HW	**SB**								
HHW	Control	1.71	1.92	1.25	0.84	5.64^{cde}	11.99^{b}	9.93^{b}	5.38^{abc}
	SB1	1.87	2.11	1.50	1.14	5.52^{de}	12.05^{b}	11.23^{ab}	4.87^{bc}
	SB3	1.72	2.18	1.43	1.05	6.38^{ab}	12.64^{b}	11.48^{ab}	5.31^{abc}
	SB5	1.84	2.67	1.74	0.85	6.94^{a}	14.68^{a}	13.31^{a}	5.95^{a}
LHW	Control	1.99	2.16	1.72	1.11	6.12^{bcd}	12.89^{ab}	11.74^{ab}	5.66^{ab}
	SB1	1.86	2.52	1.59	0.91	5.33^{e}	11.82^{b}	10.14^{b}	4.59^{c}
	SB3	1.68	2.20	1.52	1.08	5.73^{bcde}	10.93^{b}	9.85^{b}	4.67^{c}
	SB5	1.59	2.51	1.59	0.62	6.35^{abc}	12.77^{ab}	12.81^{a}	5.01^{abc}
SD		0.278	0.473	0.295	0.391	0.626	1.424	1.748	0.659
Main effects									
HW									
HHW		1.79	2.22	1.48	0.97	6.12	12.84^{a}	11.49	5.38^{a}
LHW		1.78	2.35	1.61	0.93	5.89	12.10^{b}	11.14	4.98^{b}
SB									
Control		1.85	2.04	1.49	0.98	5.88^{b}	12.44^{b}	10.84^{b}	5.52^{ab}
SB1		1.87	2.32	1.55	1.03	5.43^{c}	11.94^{b}	10.67^{b}	4.73^{c}
SB3		1.70	2.19	1.48	1.07	6.06^{b}	11.79^{b}	10.66^{b}	4.99^{bc}
SB5		1.72	2.59	1.67	0.74	6.65^{a}	13.73^{a}	13.06^{a}	5.48^{a}
***P* value**									
HW		0.202	0.325	0.122	0.731	0.218	0.041	0.397	0.011
SB		0.165	0.323	0.328	0.160	<0.001	<0.001	<0.001	0.001
HW × SB		0.202	0.187	0.062	0.332	0.001	0.011	0.015	0.030

[1]HW: hatch weight; SB: sodium butyrate inclusion level. Control: HHW or LHW chicks from eggs injected with 0.2 mL of 0.9% NaCl. SB1, SB3, SB5: HHW or LHW chicks from eggs injected with 0.2 mL of 0.1%, 0.3%, or 0.5% SB, respectively. The data are presented as the mean and pooled standard deviation (SD) (n = 6 birds/group). $^{a–d}$Values with different superscripts in a column indicate statistical significance at $P < 0.05$ (two-way ANOVA followed by Tukey's HSD test).

Table 5.4 Effect of in ovo sodium butyrate administration on the relative weights (g/100 g of body weight) and lengths (cm/100 g per body weight) of intestines in broiler chickens with different hatch weights on d 42.

Items		Relative weights				Relative lengths			
		Duodenum	Jejunum	Ileum	Cecum	Duodenum	Jejunum	Ileum	Cecum
HW	**SB**								
HHW	Control	0.74	1.26^{d}	0.92^{d}	0.54^{b}	1.45	3.19^{bc}	3.20	1.69
	SB1	0.72	1.23^{d}	0.90^{d}	0.51^{b}	1.44	3.27^{abc}	3.42	1.62
	SB3	0.89	1.40^{cd}	1.01^{cd}	0.48^{b}	1.43	3.23^{bc}	3.52	1.68
	SB5	0.73	1.31^{cd}	1.13^{cd}	0.51^{b}	1.41	3.62^{ab}	3.46	1.67
LHW	Control	1.8	3.19^{a}	2.13^{ab}	1.17^{a}	1.51	3.86^{a}	3.58	1.84
	SB1	1.65	2.84^{ab}	2.27^{a}	1.08^{a}	1.43	3.30^{abc}	3.32	1.54
	SB3	1.44	3.26^{a}	1.88^{ab}	0.98^{a}	1.26	2.93^{c}	3.29	1.41
	SB5	1.47	2.04^{bc}	1.61^{bc}	0.83^{a}	1.35	3.15^{bc}	3.35	1.66
SD		0.487	0.896	0.619	0.301	0.132	0.419	0.485	0.246
Main effects									
HW									
HHW		0.77^{b}	1.30^{b}	0.99^{b}	0.51^{b}	1.43	333	3.40	1.67
LHW		1.59^{a}	2.83^{a}	1.97^{a}	1.02^{a}	1.39	3.31	3.39	1.61
SB									
Control		1.27	2.23^{ab}	1.53	0.85^{a}	1.48^{a}	3.53^{a}	3.39	1.77
SB1		1.18	2.04^{b}	1.59	0.78^{a}	1.43^{b}	3.29^{b}	3.37	1.58
SB3		1.16	2.33^{a}	1.45	0.73^{a}	1.35^{c}	3.08^{c}	3.41	1.55
SB5		1.10	1.68^{c}	1.37	0.57^{b}	1.38^{b}	3.38^{b}	3.42	1.67
***P* value**									
HW		<0.001	<0.001	<0.001	<0.001	0.168	0.867	0.814	0.433
SB		0.399	0.028	0.456	<0.001	0.046	0.021	0.247	0.154
HW × SB		0.076	<0.001	0.013	<0.001	0.162	<0.001	0.389	0.137

[1]HW: hatch weight; SB: sodium butyrate inclusion level. Control: HHW or LHW chicks from eggs injected with 0.2 mL of 0.9% NaCl. SB1, SB3, SB5: HHW or LHW chicks from eggs injected with 0.2 mL of 0.1%, 0.3%, or 0.5% SB, respectively. The data are presented as the mean and pooled standard deviation (SD) (n = 6 birds/group). $^{a–d}$Values with different superscripts in a column indicate statistical significance at P < 0.05 (two-way ANOVA followed by Tukey's HSD test).

5.3.3 Gene expression in intestinal mucosa

5.3.3.1 Jejunum

On d 14, the expression of *TJP1* and *IL-10* in the jejunum was significantly influenced by the interaction between HW and SB (P = 0.037 and P = 0.007, respectively; Fig. 5.2A), with the LHW-SB3 group exhibiting the highest expression and the LHW-SB5 group exhibiting the lowest expression. *MUC6* expression was affected by SB (P < 0.001), with SB3-treated groups showing higher levels regardless of HW. On d 42, significant interactions between HW and SB were observed for *CLDN1* and *MUC6* expressions (P = 0.047 and P = 0.039, respectively; Fig. 5.2B). The HHW-SB1 group had higher *CLDN1* expression compared to all the LHW groups receiving in ovo SB injection but did not differ from the LHW and HHW control groups. *MUC6* expression was lower in birds receiving in ovo SB injections (both LHW and HHW) than in control birds, with the LHW-control group showing the highest *MUC6* expression. *IL-12p40* expression revealed a significant main effect of SB (P = 0.014), with the SB5-treated groups exhibiting greater expression than the other groups.

5.3.3.2 Ileum

On d 14, the expression of *CLDN1*, *TJP1*, and *IL-10* in the ileum was significantly influenced by the interaction between HW and SB treatment (P = 0.016, P = 0.028, and P = 0.048, respectively; Fig. 5.3A). The LHW-SB3 group showed the highest *CLDN1* expression compared to the LHW and HHW control groups, and the LHW-control group had lower *CLDN1* levels than the HHW-control group. *TJP1* expression was higher in the LHW-SB3 group than in the HHW-control and LHW-SB5 groups, though not significantly different from other groups. All in ovo SB groups had higher *IL-10* expression compared to the HHW-control group, with the LHW-SB3 group showing the highest levels. The LHW-control group also had higher *IL-10* expression than the HHW-control group. For *MUC6* expression, LHW chicks that received in ovo SB had higher levels, which increased with increasing SB dose (P < 0.05). On d 42, significant interactions between HW and SB were seen for *CLDN1* and *MUC6* (P = 0.002 and P = 0.024, respectively; Fig. 5.3B). The LHW-SB1 group had higher *CLDN1* levels than all other in ovo SB groups, with no significant difference from the LHW and HHW controls. The LHW-SB3 group had the highest *MUC6* expression among all the SB groups.

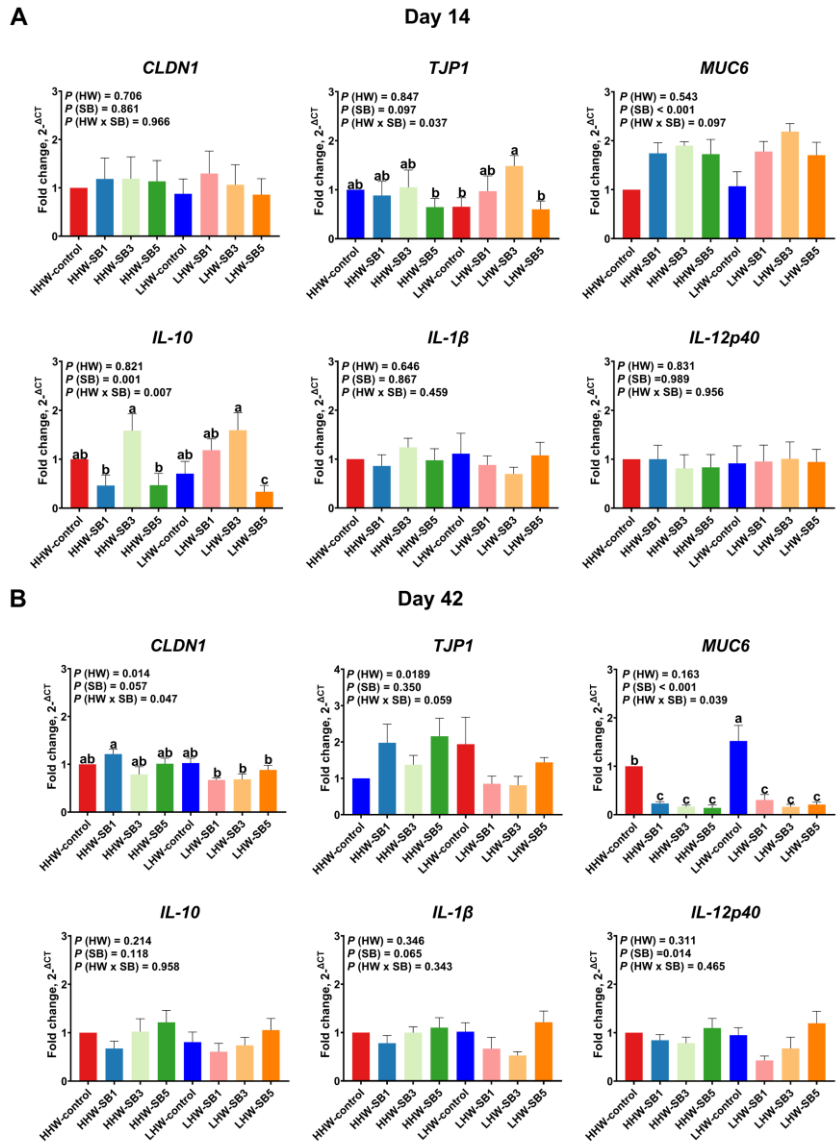

Fig. 5.2 The relative expression of gut barrier and immune-related genes in the jejunal mucosa of high (HHW) and low (LHW) hatch weight chickens on d 14 (A) and d 42 (B) that had received three levels of sodium butyrate in ovo (SB1: 0.1%, SB3: 0.3%, SB5: 0.5%) or 0.9% NaCl (control) in ovo. Two-way ANOVA was applied to determine the fold change of the relative expression of genes (n = 6 birds/group) and P values are indicated by different letters corresponding to P(HW), P(SB), and P(HW×SB).

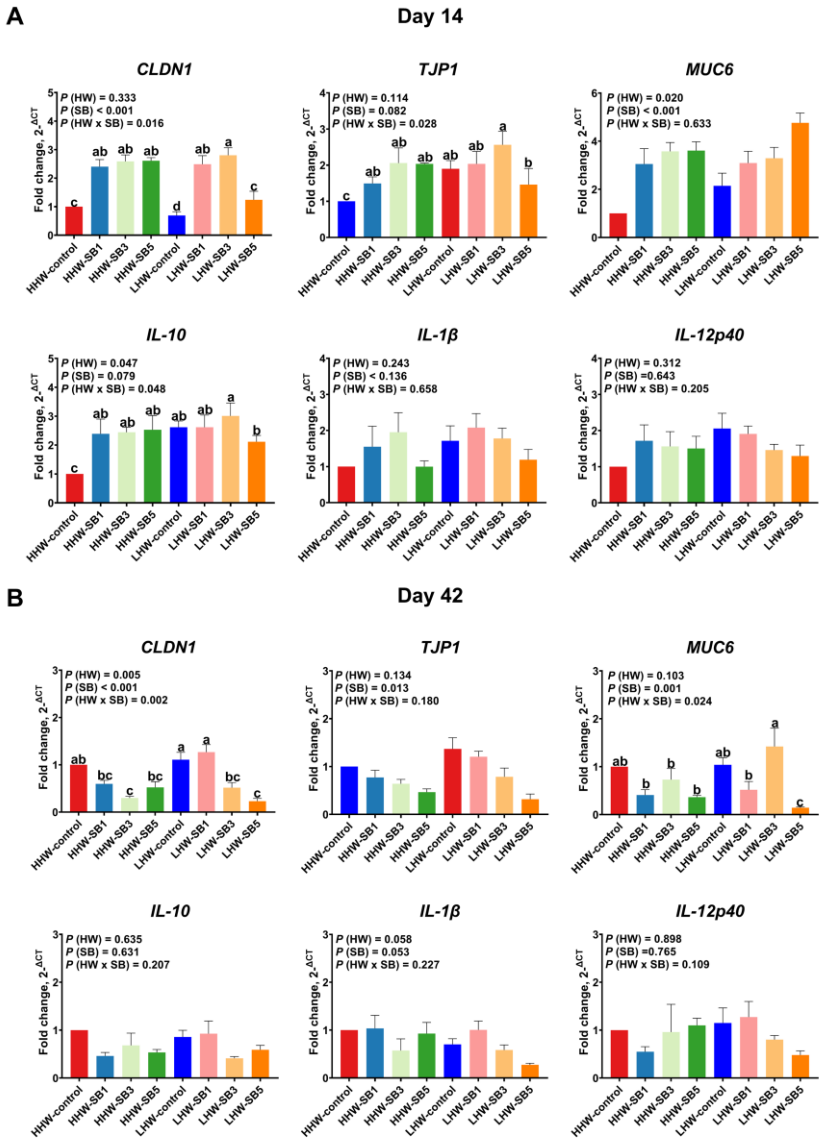

Fig. 5.3 The relative expression of gut barrier and immune-related genes in the ileal mucosa of high (HHW) and low (LHW) hatch weight chickens on d 14 (A) and d 42 (B) that had received three levels of sodium butyrate in ovo (SB1: 0.1%, SB3: 0.3%, SB5: 0.5%) or 0.9% NaCl (control) in ovo. Two-way ANOVA was applied to determine the fold change of relative expression (n = 6 birds/group) and P values are indicated by different letters corresponding to P(HW), P(SB), and P(HW×SB).

5.3.3.3 Caecum

On d 14, *CLDN1* and *TJP1* expressions were affected by HW and SB interactions (P = 0.014 and P = 0.012, respectively; Fig. 5.4A), with HHW-SB3 showing higher *CLDN1* expression than all the other groups. *TJP1* expression was significantly upregulated in the HHW-SB5 group compared to the HHW-SB1 and LHW-SB3 groups. SB treatment also had a significant main effect on *MUC6* and *IL-1β* expressions (P = 0.004 and P < 0.001). *MUC6* expression increased with increasing SB dose, while *IL-1β* decreased with increasing dose. On d 42, *CLDN1* and *IL-12p40* expressions were significantly influenced by the interaction between HW and SB (P < 0.001 and P = 0.032, respectively; Fig. 5.4B). The LHW-SB1 group exhibited the highest *CLDN1* expression, which did not differ significantly from that of the HHW-SB1 and HHW-control groups. Additionally, the LHW-control group presented lower *CLDN1* expression than the HHW-control group. The HHW-SB1 group showed higher expression of *IL-12p40* than all the other groups, except for the HHW-SB5 group. SB treatment also had a significant main effect on *MUC6* expression (P < 0.001), with SB3-treated groups showing higher levels regardless of HW.

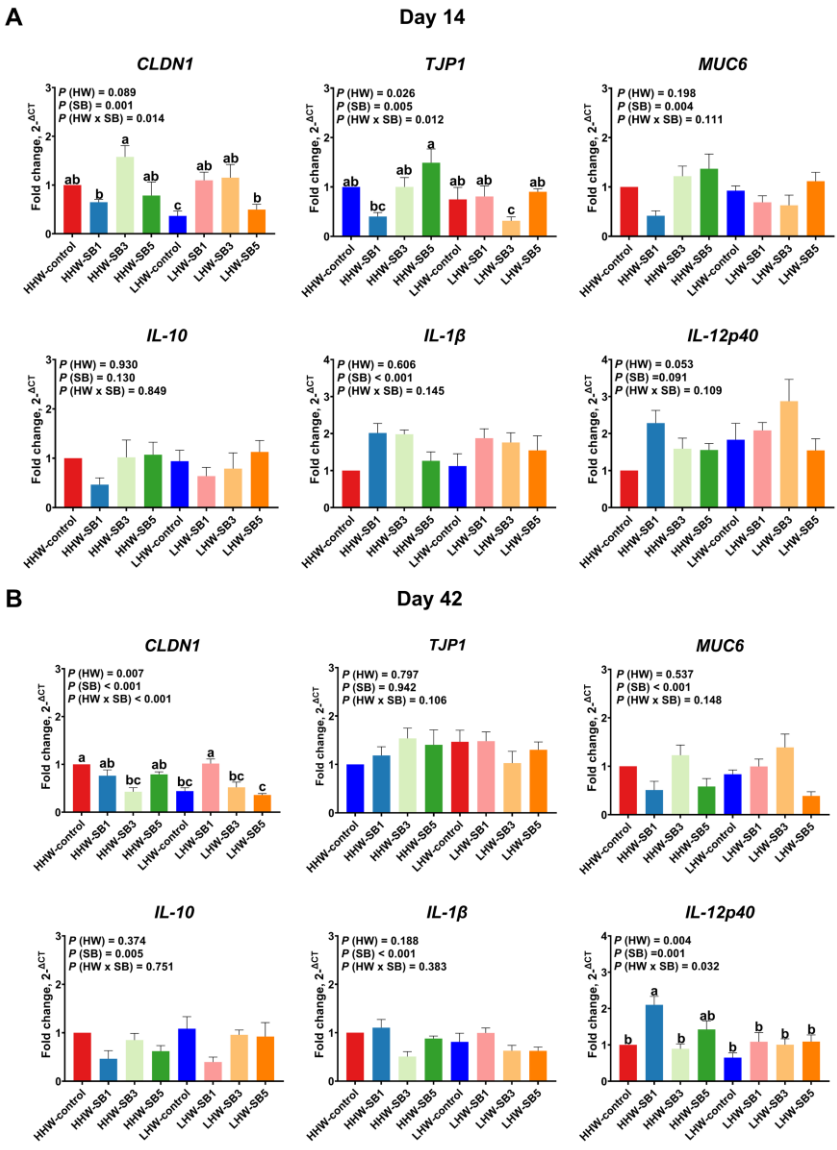

Fig. 5.4 The relative expression of gut barrier and immune-related genes in the cecal mucosa of high (HHW) and low (LHW) hatch weight chickens on d 14 (A) and d 42 (B) that had received three levels of sodium butyrate in ovo (SB1: 0.1%, SB3: 0.3%, SB5: 0.5%) or 0.9% NaCl (control) in ovo. Two-way ANOVA was applied to determine the fold change of relative expression (n = 6 birds/group) and P values are indicated by different letters corresponding to P(HW), P(SB), and P(HW×SB).

5.3.4 Microbiota analysis

5.3.4.1 Temporal changes and core microbiota composition

Cecal metagenome sequencing generated 3,971,213 reads, with 41,366 ± 28,810 (mean ± SD) reads per sample. After quality filtering, 2,573,048 reads remained, with an average of 26,802 reads per sample. Compositional analysis revealed considerable inter-individual variability and a significant shift in gut microbiota from d 14 to d 42 post-hatch (Fig. 5.5). On d 14, the microbiota was dominated by Firmicutes phylum (79%–99%), with minor contributions from Epsilonbacteraeota (0%–20%), Proteobacteria (0.2%–1.5%), and Bacteroidota (~0%–1.8%, Fig. 5A). As the chickens matured to d 42, Firmicutes remained the most abundant phylum but its dominance decreased substantially (38%–64%), leading to a significant increase in Bacteroidetes (4%–34%) and Epsilonbacteraeota (15%–32%, Fig. 5.5C). A few low-abundance previously undetected phyla also emerged at this stage, including Cyanobacteria (5%–13%), Lentisphaerae (0.5%–3.5%), Tenericutes (0.1%–0.2%) and Verrucomicrobia (0.004%–0.76%), indicating diversification of the microbial ecosystem. At the genus level, on d 14, prominent early colonizers such as *Lactobacillus* (5%–25%), unclassified *[Ruminococcus] torque group* (4.8%–19%), unclassified *Lachnospiraceae* (3%–17%) and *Faecalibacterium* (7%–15%) were observed (Fig. 5.5B). By d 42, the *Lactobacillus*-dominated community had transitioned to one where *Helicobacter* was the most prevalent genus (12%–28%), and this change was accompanied by an increase in the *Rikenellaceae RC9 gut group* (0%–22%), *Campylobacter* (0.7%–13%), and *Clostridiales vadinBB60 group* (5.4%–9.5%, Fig. 5.5D). Despite the consistency of core genera across individuals, many low-abundance genera collectively made up more than 20% of the community on both days, representing a highly variable component of the gut ecosystem.

5.3.4.2 Alpha and beta diversity

On d 14, the Shannon index of alpha diversity showed a significant interaction between HW and SB ($P = 0.044$; Fig. 5.6A). The Shannon index was highest in the LHW-SB3 group, while it was lowest in the LHW-control group. The HHW-control group also had a greater Shannon index than the LHW-control group. On d 42, SB had a significant effect on both the Shannon and Simpson indexes ($P < 0.05$; Fig. 5.6C and 5.6D), with SB3-

treated groups showing higher values regardless of HW. However, the HW and HW × SB interactions did not significantly affect alpha diversity on d 42. Beta diversity analysis via PERMANOVA of the Bray–Curtis distance showed significant HW × SB interaction effects on the microbiota composition on both d 14 ($P = 0.028$) and d 42 ($P < 0.001$; Fig. 5.7A and 5.7B). The control groups (LHW and HHW) formed distinct clusters, while the SB-treated groups exhibited similar clusters, indicating that SB had a homogenizing effect on the microbiota composition. The Bray–Curtis dissimilarity boxplot also showed that the SB-treated groups had microbiota profiles closer to each other than the LHW and HHW control groups (Fig. S5.2).

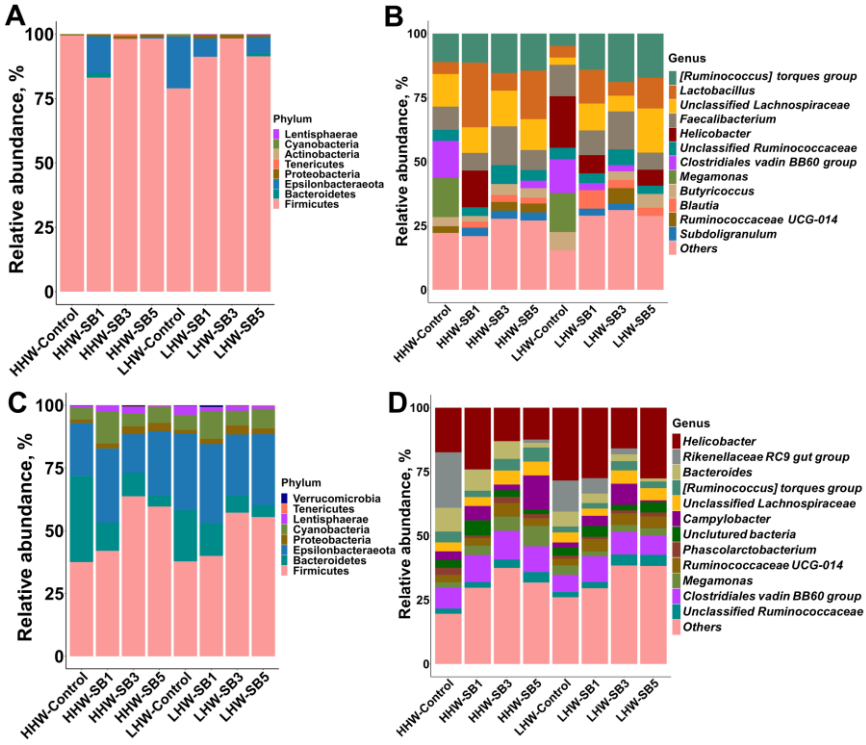

Fig. 5.5 Relative abundance of cecal bacterial phyla and genera in high (HHW) and low (LHW) hatch weight chickens on d 14 (A and B) and 42 (C and D) that had received three levels of sodium butyrate (SB1: 0.1%, SB3: 0.3%, SB5: 0.5%) or 0.9% NaCl (control) in ovo. The data are from individually sampled chickens (n = 6 birds/group).

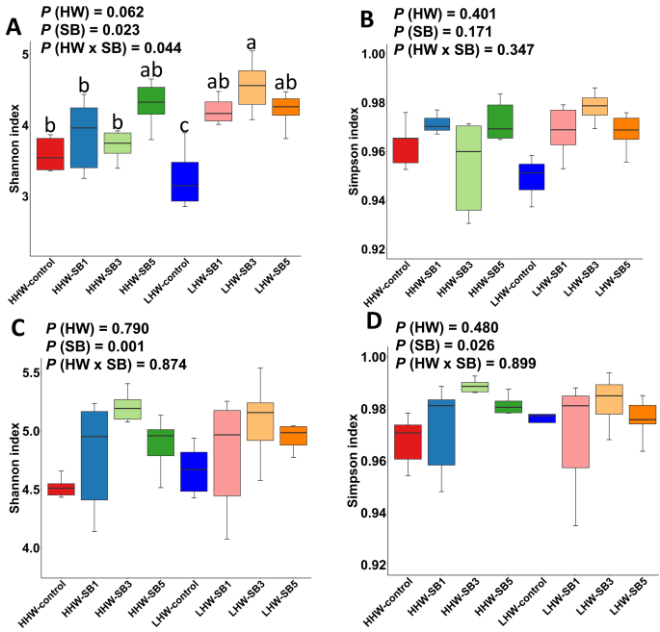

Fig. 5.6 Alpha diversity of the cecal microbiota, measured using the Shannon and Simpson indexes, in high (HHW) and low (LHW) hatch weight chickens on d 14 (A) and d 42 (B) that had received three levels of sodium butyrate in ovo (SB1: 0.1%, SB3: 0.3%, SB5: 0.5%) or 0.9% NaCl (control) in ovo. Two-way ANOVA was applied to the alpha diversity metrics (n = 6 birds/group) and P values are indicated by different letters corresponding to P(HW), P(SB), and P(HW×SB).

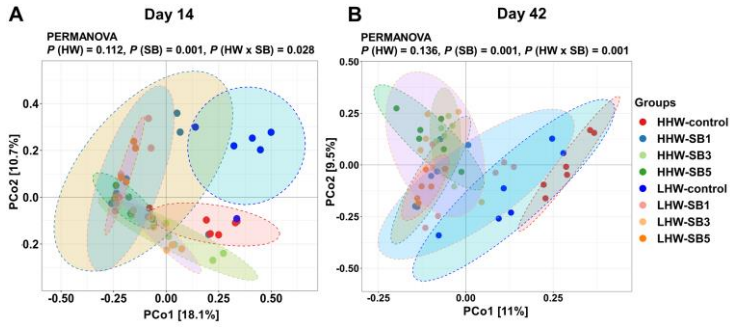

Fig. 5.7 Principal coordinate analysis (PCoA) generated based on Bray–Curtis distance comparing the gut microbiota composition of high (HHW) and low (LHW) hatch weight chickens on d 14 (A) and d 42 (B) that had received three levels of sodium butyrate (SB1: 0.1%, SB3: 0.3%, SB5: 0.5%) or 0.9% NaCl (control) in ovo. A nonparametric permutational multivariate analysis of variance (PERMANOVA) was applied to the Bray–Curtis distance and P values are indicated by different letters corresponding to P(HW), P(SB), and P(HW×SB).

5.3.4.3 Differential abundance of bacterial genera

On d 14, LEfSe analysis identified 24 differentially abundant genera across all groups (Fig. 5.8A, LDA cut-off value ≥ 3.0, FDR < 0.05). In the HHW category, the control group exhibited enrichment of the *Clostridial vadinBB60 group*, *Megamonas*, and *Family XIII UCG-001*. The HHW-SB1 group showed higher *Lactobacillus* abundance. The HHW-SB3 group was enriched in the *Ruminococcaceae UCG-013* and *[Ruminococcus] gauvreauii group*. The HHW-SB5 group presented high enrichment of *Fusicatenibacter*, *Romboutsia*, *Tyzzerella 3*, and *Sellimonas*. In the LHW category, the control group exhibited differential abundances of *Helicobacter*, *Lachnospiraceae UCG-010*, and *Gastranaerophilales*. The abundance of *Lachnospiraceae NK4A136 group*, *Eisenbergiella*, and *Tyzzerella* was increased in the LHW-SB1 group. The LHW-SB3 group was enriched in *Faecalibacterium*, *[Ruminococcus] torques group*, *Ruminiclostridium 9*, and *Anaerotruncus*. The LHW-SB5 group had higher abundance of *unclassified Lachnospiraceae*, *Lachnospiraceae FE2018*, and *Defluviitaleaceae UCG-011*.

On d 42, the analysis revealed 26 bacterial genera exhibiting differential abundance among all groups (Fig. 5.8B, LDA cut-off value ≥ 3.0, FDR < 0.05). In the HHW category, the control group demonstrated enrichment of the *Rikenellaceae RC9 gut group*, *Bacteroides*, and *Parabacteroides*. The HHW-SB1 group showed higher *unclassified Barnesiellaceae*, while HHW-SB3 had increased VadinBE97, *Ruminiclostridium 9*, and *Parasutterella* abundances. The HHW-SB5 group had a greater abundance of *Escherichia-Shigella*, *Gallibacterium*, and *Campylobacter*. In the LHW category, the control group showed enrichment of *Peptococcus*. The LHW-SB1 group exhibited higher abundance of unclassified *Flavobacteriaceae*, *Cerasicoccus*, and *Prevotella 7*. The LHW-SB3 group had a higher abundance of *GCA-900066575*, *Oscillibacter*, *unclassified Ruminococcaceae*, *Sutterella*, *Flavonifractor*, and *Intestinimonas*. The LHW-SB5 group exhibited increased abundances of *Streptococcus*, *Eisenbergiella*, *Ruminiclostridium*, and *Ruminococcus 1*.

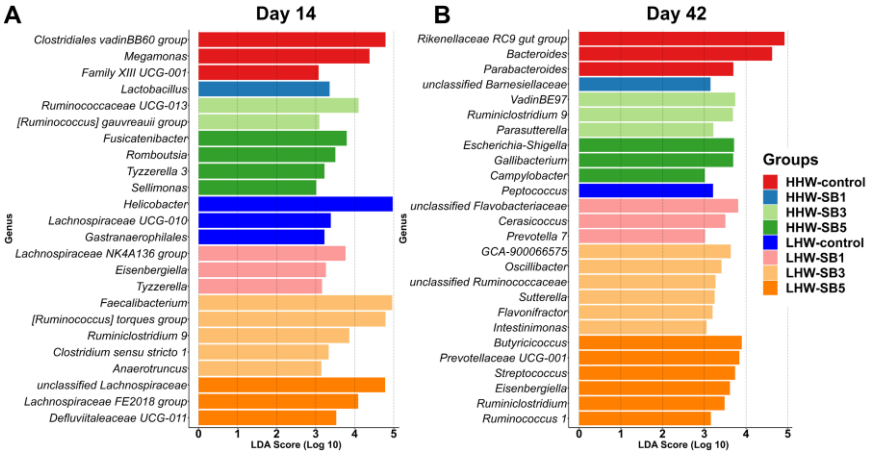

Fig. 5.8 Differentially enriched cecal bacterial genera in high (HHW) and low (LHW) hatch weight chickens on d 14 (A) and d 42 (B) that had received three levels of sodium butyrate (SB1: 0.1%, SB3: 0.3%, SB5: 0.5%) or 0.9% NaCl (control) in ovo. LEfSe analysis was performed (n = 6 birds/group) using an FDR < 0.05 and a linear discriminate analysis (LDA) score of ≥ 3.0 as the thresholds.

5.3.4.4 Predicted functionality of the cecal microbiota

Metabolic pathway analysis using the MetaCyc database identified 321 pathways on d 14 and 315 pathways on d 42 across all groups. Two-way hierarchical clustering of the top 50 pathways, including those related to fermentation, sugar metabolism, amino acid biosynthesis, genetic processing, and cell wall components, revealed distinct groupings (Fig. S5.3). On d 14, clustering revealed three groups: LHW-SB3 and HHW-SB3 clustered together with higher levels of amino acid biosynthesis and galactose and starch degradation pathways; HHW-control and LHW-control formed another cluster; and the remaining groups were separated (Fig. S5.3A). By d 42, HHW-SB3 exhibited a distinct pattern of decreased amino acid biosynthesis, while LHW-SB1 and HHW-SB1 grouped together with higher activity in genetic processing and cell wall pathways (Fig. S5.3B).

Two-way ANOVA revealed significant differences in only two metabolic pathways on d 14 and four pathways on d 42 (Fig. 5.9). On d 14, the LHW-SB3 group exhibited a greater abundance of the gondoate biosynthesis pathway, though not significantly different from HHW-SB3 group (Fig. 5.9A). The HHW-SB3 group had the highest levels of microbial genes involved in serine and glycine biosynthesis, while the HHW-SB1 group had

the lowest. On d 42, the HHW-control group had a greater abundance of the pyrimidine deoxyribonucleoside salvage pathway, and HHW-SB3 showed greater enrichment of the bifidum fermentation pathway (Fig. 5.9B).

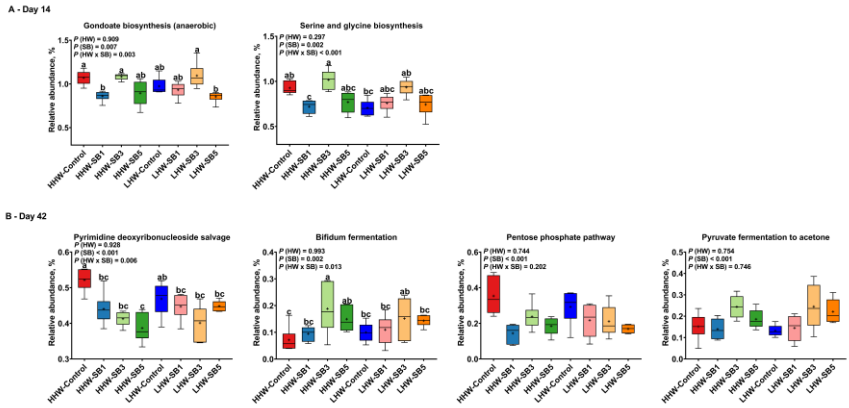

Fig. 5.9 Predicted microbial metabolic pathways of high (HHW) and low (LHW) hatch weight chickens on d 14 (A) and d 42 (B) that had received three levels of sodium butyrate (SB1: 0.1%, SB3: 0.3%, SB5: 0.5%) or 0.9% NaCl (control) in ovo. Only significantly different metabolic pathways are shown (P value < 0.05).

5.3.5 Correlations between bacterial genera and metabolic pathways

Pearson's correlation analysis revealed relationships among the top 12 most abundant bacterial genera and 25 metabolic pathways (Fig. 5.10). On d 14, *unclassified Ruminococcaceae* were positively correlated with several genera, including *Subdoligranulum* and *Ruminococcaceae UCG-014* (Fig. 5.10A). *Ruminococcaceae UCG-014* was positively correlated with *Faecalibacterium*, while Blautia was positively correlated with the *[Ruminococcus] torques group*. *Helicobacter* and *Lactobacillus* were negatively correlated with most genera. On d 42, the *[Ruminococcus] torques group* was positively correlated with *Faecalibacterium*, and *unclassified Lachnospiraceae* was positively correlated with *Ruminococcaceae UCG-014* (Fig. 5.10B). Most genera maintained negative correlations with *Helicobacter*. For metabolic pathways, on d 14, *Lactobacillus*, *Megamonas*, and *Helicobacter* formed a distinct cluster with negative correlations with most pathways (Fig. 5.10C). *Faecalibacterium* positively correlated with gondoate biosynthesis and serine-glycine

biosynthesis, while *unclassified Lachnospiraceae* and the *[Ruminococcus] torques group* positively correlated with pyruvate fermentation to isobutanol and glycogen degradation I. On d 42, *Megamonas was* negatively correlated with most pathways. *Helicobacter* was positively correlated with L-isoleucine and L-tryptophan biosynthesis but negatively correlated with pyrimidine nucleobase salvage (Fig. 5.10D). *Lactobacillus* abundance was negatively correlated with 4-aminobutanoate degradation V but was positively correlated with bifidum fermentation. The *Rikenellaceae RC9 gut group* was positively correlated with several pathways, including those related to the pentose phosphate pathway and pyrimidine deoxyribonucleoside salvage and was negatively correlated with L-arginine biosynthesis I.

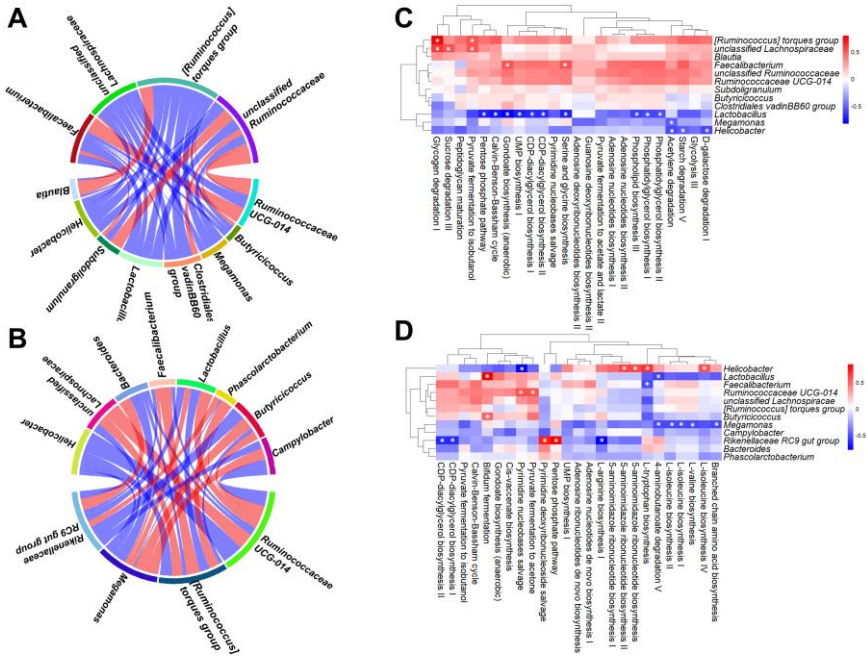

Fig. 5.10 Chord diagram showing Pearson's correlations between the most abundant bacterial genera on d 14 (A) and d 42 (B). Chord width reflects the strength of the correlations, with red indicating positive correlations and blue indicating negative correlations. Heatmap of Pearson's correlations between specific bacteria and the 25 most abundant predicted metabolic pathways on d 14 (C) and d 42 (D). Significant correlations ($P < 0.05$) are marked with an asterisk, with red representing positive correlations and blue representing negative correlations.

5.4 Discussion

The current study revealed the significant impact of HW on broiler performance, showing substantial advantages for HHW chicks over their LHW counterparts when both were administered physiological saline as a control. HHW-control group exhibited higher BW, ADG, and improved FCR compared to LHW-control group, consistent with previous studies reporting that HHW chicks generally outperform LHW chicks on control diets[1,24].

The hatchability did not differ across treatments, which suggested that none of the treatments adversely affected embryonic viability. In the present study, in ovo SB administration demonstrated growth-promoting effects by improving ADG and the FCR, which is consistent with the findings of previous reports on the positive impacts of dietary supplementation or in ovo butyrate administration on broiler performance[8,25]. However, the effects were dose dependent. The 0.3% SB dose produced the most favorable results, which was consistent with the findings of Saleha et al.[26], who reported that weight gain and performance improved with this dose. In contrast, a higher dose of 0.5% proved too high for in ovo stimulation, negatively affecting ADG and FCR, suggesting an optimal dose range for SB beyond which negative impacts may occur. Similarly, Pineda-Quiroga et al.[27] reported similar observations, finding that high inclusion rates of SB decreased broiler weight and feed intake while worsening the FCR at every growth stage.

In ovo SB administration influenced growth in both HW categories. However, the beneficial effects were more pronounced in LHW broilers at the 0.3% inclusion level, suggesting that the optimal SB dosage might help bridge the performance gap between LHW and HHW chicks. SB may improve weight gain in chickens by upregulating nutrient transporter activity, stimulating intestinal cell proliferation, modulating tight junction protein expression, and improving nutrient digestibility[28–30]. It also creates an acidic environment in the gut, which minimizes the load of pathogens[7]. During the early post-hatching period, butyrate production in the intestines is generally insufficient due to inadequate microbiota colonization[31]. This deficiency is likely more severe in low-weight chicks, which are known to have compromised gut health and an unbalanced microbiota composition compared to their heavier counterparts[3–5]. In ovo

SB injection likely addressed this deficiency by supplying an optimal amount of butyrate at a critical developmental stage, thereby improving gut function and overall performance in LHW chicks. In contrast, SB has shown limited effects on production and gut health parameters in healthy and unchallenged chickens[32–34]. Therefore, HHW chicks would have been less responsive to SB marginal benefits, as high-performing broilers typically have better initial gut development and face fewer gut-related challenges[4].

The study also revealed divergent effects of SB on intestinal development between HW categories. The HHW-SB5 group, despite suboptimal performance, had longer intestines, contradicting the typical correlation between longer intestines and improved nutrient absorption[7]. In contrast, the LHW-SB3 group, despite having shorter intestines, exhibited better performance, suggesting a potential metabolic nutrient-saving mechanism induced by SB, where energy is redirected from intestinal maintenance to growth and muscle development. Furthermore, the LHW-SB3 group had a greater relative jejunum weight on d 42, likely due to the trophic effect of butyrate on epithelial cells, which enhances cell proliferation, differentiation, and maturation[35], resulting in an increase in absorptive surface area.

In ovo, SB administration led to increased expression of *CLDN1*, *TJP1*, and *MUC6* across various intestinal segments, suggesting that SB may protect the mucosal epithelium from injury and alleviate enteropathic stress by enhancing gut barrier function and mucus secretion. Song et al.[28] similarly found that in feed butyrate administration has a protective effect in necrotic enteritis-challenged broilers by alleviating gut barrier injuries through the upregulation of the jejunal *CLDN1*, *CLDN4* and *occludin* genes. Butyrate enhances intestinal barrier function by accelerating the assembly of tight junctions through AMP-activated protein kinase activation[36], which suggests that SB induces epithelial cell differentiation toward tight junction cells, which could improve intestinal health and integrity. Although gut barrier-related gene expression also increased in other groups, the LHW-SB3 group exhibited the most pronounced upregulation, indicating a particularly beneficial effect on gut barrier function in these chickens. The divergent responses of the HHW and LHW categories to SB injection could be related to differences in intestinal health and

development. Butyrate tends to exert more significant effects under stressful conditions[28,37,38] but has minimal impact on the gut epithelium of healthy chickens[32]. Since low-weight chickens often face gut health challenges such as delayed GIT development and compromised barrier function[4], SB injection likely benefits them more than their heavier counterparts. Our observations also revealed varying immune responses among different HW categories following in ovo injection of SB. IL-10 is a potent anti-inflammatory cytokine produced by activated macrophages that plays a crucial role in enhancing intestinal barrier function and attenuating intestinal inflammation[39]. LHW chicks receiving 0.3% SB showed increased *IL-10* expression, suggesting that localized anti-inflammatory effects likely contributed to enhanced gut health. Conversely, 0.5% SB in HHW chicks resulted in increased *IL-12p40* expression, indicating potential inflammation. IL-12p40, a subunit of IL-12, is involved in regulating cell-mediated immune responses and inducing inflammatory mediators[40]. It is well established that overwhelming production of pro-inflammatory cytokines is energetically expensive due to the metabolic demands on immune cells and the negative effects of prolonged inflammation such as anorexia and tissue degradation[34].

Cecal microbiota analysis revealed a shift from a Firmicutes-dominated community to a more diverse ecosystem with increased Bacteroidetes abundance over time, consistent with the findings of previous studies[41]. Our study revealed greater alpha diversity in the HHW-control group on d 14 than in the LHW-control group, suggesting an advantage in gut microbial development for heavier chicks[42]. Consistent with the findings of previous studies[43], SB injection significantly impacted the biodiversity of the microbiota in both HW categories, with the LHW-SB3 group showing the highest Shannon index of alpha diversity on d 14. This increased diversity, particularly in LHW chicks, may be crucial for improving gut health and performance, as higher bacterial diversity is linked to better gut health and infection resistance[44]. PCoA further showed that in ovo SB administration resulted in a significant separation of microflora, implying that SB altered the composition of the flora compared to the controls.

LEfSe analysis revealed that the HHW-control group exhibited a greater proportion of beneficial bacteria, including the genus *Megamonas*, which

plays a crucial role in fermenting glucose into acetate and propionate, as well as cellulose-degrading bacteria such as the *Clostridiales vadin BB60 group*, *Bacteroides*, *Parabacteroides*, and the *Rikenellaceae RC9 gut group*[45–47]. In addition, *Bacteroides* has immune-modifying functions and inhibits inflammatory cytokines[48]. The LHW-control group had an increased abundance of the pathogenic *Helicobacter* genus, implying that *Helicobacter* species, particularly *Helicobacter pylori* and *Helicobacter pullorum*, are known to negatively impact GIT structure, health, and growth performance in broilers[3,49]. These pathogens may cause gastroenteritis in chickens and pose potential risks to human health through meat contamination[50]. The LHW-SB3 group had a greater abundance of the genus *Faecalibacterium*, a genus associated with high performance in male broilers[5], and a reduced abundance of this genus is often linked to inflammatory diseases[51]. *Faecalibacterium prausnitzii*, the only known species in this genus, is a potent butyrate producer and probiotic in livestock[52]. A correlation analysis showed that *Faecalibacterium* was positively correlated with several predicted metabolic pathways, including gondoate biosynthesis, a known antimicrobial agent against Gram-negative pathogenic bacteria[53], which is beneficial to host health. Other taxa in this group that contribute to SCFA production and weight gain included *Flavonifracto, [Ruminococcus] torques group*, *Ruminococcaceae UCG-10*, *Anaerotruncus*, *Intestinimonas*, *Sutterella*, and *Oscillibacter*[54–56]. An increase in the proportion of these beneficial bacteria in the LHW-SB3 group was expected to positively impact intestinal health and overall performance. However, the 0.5% SB treatment in the HHW group resulted in higher abundances of pathogenic genera such as *Escherichia-Shigella*, *Galibacterium*, and *Campylobacter*, which might be correlated with their limited growth response and increased expression of pro-inflammatory cytokine *IL-12p40*. *Gallibacterium anatis*, a Gram-negative bacterium from the *Pasteurellaceae* family, typically resides in the respiratory and reproductive tracts, and significantly impacts animal welfare and productivity by causing peritonitis and mortality[57]. Similarly, *Escherichia-Shigella* and *Campylobacter* are known to be associated with intestinal inflammation and dysbiosis, and their proliferation often results in adverse effects on growth and overall health in chickens[5,58].

The predicted metabolic pathway analysis revealed that the LHW-SB3 and HHW-SB3 samples clustered together on d 14, indicating similar metabolic responses to SB despite initial weight differences. The LHW-SB3 group exhibited the highest abundance of microbial pathways involved in the production of gondoic acid, a known antimicrobial agent effective against Gram-negative bacteria[53]. The enrichment of this metabolic pathway, combined with the increased expression of *IL-10* in the LHW-SB3 group indicates the potential for reduced inflammatory responses and the exclusion of Gram-negative bacteria, which are commonly linked to enteric diseases. The HHW-SB3 group exhibited relative enrichment in the bifidum fermentation pathway, which improves gut health through acetate and lactate production[59]. Additionally, the glycine-serine microbial pathway was more abundant in the HHW-SB3 group, indicating increased amino acid synthesis. Glycine is crucial for modern broiler chickens due to its limited endogenous synthesis[60]. Glycine also has anti-inflammatory effects, suppressing transcription factors, free radicals, and cytokine production in macrophages[61], which is beneficial to host health.

In ovo SB may exert different effects than in-feed administration due to the timing and duration of exposure. In ovo SB injection delivers a single, critical dose early in development, likely inducing epigenetic and microbiota changes. These alterations may trigger cascading physiological effects that persist until d 42 post-hatch. Future research should focus on larger-scale trials to validate these findings and explore the underlying epigenetic and microbiota-mediated mechanisms more comprehensively.

5.5 Conclusions

HW had a positive effect on subsequent broiler growth performance and the HHW-control group demonstrated better growth performance and a more favorable gut microbiota characteristics. Butyrate seemed to exert a more significant effects on LHW chicks at 0.3% inclusion level, likely due to their compromised gut health. This led to significant improvements in intestinal development, strengthened gut barrier function, increased anti-inflammatory cytokine production, and beneficial cecal microbiota characteristics, collectively contributing to enhanced growth performance. The effects of SB were dose dependent, with adverse outcomes observed at higher concentrations (0.5%), impacting performance, the gut microbiota, and the expression of intestinal genes.

These results provide insights into optimizing SB use for broilers with varying HWs. The potential for targeted intervention is particularly promising for LHW chicks, presenting an opportunity to reduce BW variance among broilers to improve overall flock uniformity.

In ovo injection showed promising results in supporting gut health and performance in LHW chicks; however, it requires substantial standardization and scalability before widespread industry adoption. As an alternative, we also investigated dietary strategies involving feed structure modifications, which can be more easily implemented at the feed mill level. The following chapter explores the effects of coarse corn and oat hulls inclusion in the diet to improve intestinal health and reduce performance disparities in underperforming broilers.

5.6 References

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Chapter 6

Effects of coarse corn or oat hulls on growth performance, intestinal health, and microbiota modulation in underperforming broilers

The work presented in this chapter is adapted from:

Akram, M. Z., Sureda, E. A., Corion, M., Comer, L., Zhao, H., Schroyen, M., & Everaert, N. (2025). Effects of coarse corn or oat hulls on growth performance, intestinal health, and microbiota modulation in underperforming broilers. Animal Nutrition (In press).

Abstract

Intra-flock body weight (BW) variability in broilers increases production costs, as underperforming chicks often show suboptimal gut development and performance. Increasing grain particle size and dietary fiber content has been shown to improve digestive efficiency and intestinal health. This study investigated whether dietary inclusion of coarse corn (CC) and oat hulls (OH) could improve gut health and reduce the performance gap between low- and high-BW (LBW and HBW) broilers. On d 7, 1,400 Ross 308 male broilers were categorized as LBW or HBW, with 504 LBW chicks assigned to four isocaloric and isonitrogenous diets with 10% fine corn (LBWC), 7% CC with 3% fine corn (LBW+CC), 3% OH with 10% fine corn (LBW+OH), or 7% CC and 3% OH (LBW+CO). HBW chicks received a 10% fine corn diet (HBWC). Each group had 6 replicates with 21 chicks per pen. The HBWC group showed the highest BW at each timepoint ($P < 0.05$). By day 38, LBW+OH chicks had significantly reduced the weight difference with HBWC chicks and significantly outperformed LBWC chicks ($P < 0.001$), whereas other groups showed intermediate values. CC and OH, individually or combined, reduced intestinal permeability on d 14 ($P = 0.014$) and increased gizzard weights on d 21 and 38 ($P < 0.05$). The LBW+OH group showed increased pancreas relative weight on d 21 ($P = 0.005$) and villus height ($P = 0.042$) on d 38. Additionally, LBW+OH group reduced isobutyrate and isovalerate levels in caecum ($P < 0.05$) on d 21 and upregulated ileal genes related to gut barrier function (*CLDN1*, *CLDN4*, *CLDN5*), amino acid and glucose transporters (*SLC15A1*, *SLC1A1*, *SLC2A1*), and immune function (*NOS2*, *TLR4*) on d 14, and sodium-phosphate transporter *SLC34A2* on d 38. LBW+CC birds had increased valerate concentrations on d 21 ($P = 0.002$) and upregulated *SLC15A1* on d 38. *Lactobacillus* was enriched in the caecum of HBWC birds, while *Escherichia-Shigella* was abundant in LBWC birds on d 14, with CC and OH promoting beneficial bacterial shifts in LBW groups. Overall, incorporating structural components into diets, particularly 3% OH, enhanced gastrointestinal development, intestinal integrity, and growth performance in LBW broilers. These improvements reduced disparities in BW between LBW and HBW birds, thereby contributing to more uniform flock performance at slaughter age.

6.1 Introduction

As shown in chapter 5, in ovo administration of sodium butyrate improved gut health and performance in low hatch weight (LHW) broilers, highlighting the potential of early-life interventions to reduce body weight (BW) variability. However, despite its biological efficacy, in ovo application poses practical challenges in terms of standardization and scalability in commercial hatchery settings. Therefore, in the current chapter, we explored a more easily adoptable post-hatch strategy modifying feed structure through inclusion of coarse corn (CC) and oat hulls (OH) to support gut development and improve performance in underperforming broilers.

Optimal broiler growth and production fundamentally depend on efficient nutrient digestion and absorption, which are intrinsically linked to a well-functioning digestive system[1]. A range of dietary strategies to support GIT development have gained attention, including dietary modification with insoluble fiber sources and coarse feed particles[2,3]. Adding insoluble fiber sources such as oat hulls (OH) has been found to improve nutrient retention, digestion, and growth in broilers[4]. Similarly, the addition of coarse corn (CC) to pelleted diets enhances protein digestibility, energy utilization, live performance, and litter quality[3]. These benefits are attributed to the physical properties of these feed ingredients, which stimulate gizzard development[5], and increase pancreatic enzyme secretion, such as amylase and chymotrypsin, driven by gizzard activity. Furthermore, a well-developed gizzard promotes the release of cholecystokinin (CCK), which stimulates reverse peristalsis, prolonging digesta transit time and enabling more thorough digestion[6,7]. Consequently, slower digesta transit increases nutrient digestion and absorption by maximizing the contact time with absorptive cells[8]. Besides the chemical composition and particle size, the physical structure of feed including pellet size and hardness may influence digestive development. Harder pellets can further stimulate gizzard activity by resisting breakdown, thereby enhancing the mechanical stimulation of the digestive tract[9]. While the breakdown of fiber in poultry is minimal in terms of energy provision, it may still influence the nutritional value of feed through interactions with other nutrients. Unlike soluble fiber, which can hinder nutrient digestion and absorption due to increased digesta

viscosity[10], insoluble fiber supports chicken growth by improving the digestibility of other feed ingredients[4]. However, the effects of insoluble fiber can be context-dependent. At inappropriate inclusion levels or depending on the fiber source and bird physiology, insoluble fiber may impair nutrient utilization or lead to undesirable outcomes such as wet litter or sticky droppings, often considered antinutritional effects in broiler production systems[11]. The physical attributes of OH and CC may also exert microbiota-modulating effects. The lignin-rich matrix of fiber materials just as in OH can act as a fermentable substrate for beneficial microbes[12], producing short-chain fatty acids (SCFAs) with anti-inflammatory and trophic effects on the gut epithelium. Similarly, the structural complexity of CC may support the proliferation of specific bacterial taxa that favor gut health and metabolic efficiency[13]. While individual studies have explored the effects of CC and OH on nutrient digestibility and growth performance, their impact on gut microbiota and intestinal health is relatively underexplored.

The positive effects of coarse grain particles and insoluble fiber on broiler performance are well documented[5,14]. However, their potential benefits in LBW broilers, which are characterized by impaired growth performance and physiological development, remain elusive. Given the growth impairments in LBW broilers, we hypothesize that structural diets incorporating CC and OH can stimulate gizzard activity, restore intestinal health, and reduce the performance gap between LBW and high body weight (HBW) birds. Thus, the objective of this study was to investigate the individual and synergistic effects of CC and OH on the growth performance, GIT development, intestinal health, and microbiota characteristics of LBW broilers.

6.2 Materials and methods

The study was performed at TRANSfarm, KU Leuven, Bierbeek, Belgium, following approval from the KU Leuven Ethical Committee for Animal Experimentation under project number 112/2023.

6.2.1 Experimental diets

Isocaloric and isonitrogenous wheat-based broiler diets were formulated to meet nutritional requirements across different growth phases (Table 6.1). All broilers received a crumbled-form pre-starter diet in the first week. Thereafter, four pelleted experimental diets were formulated in a commercial feed mill (Vanden Avenne, Ooigem Belgium) using conditioning with expander: a commercial broiler diet with 10% finely ground corn (Control), a diet formulated with 7% CC and 3% finely ground corn (CC), a diet containing 10% finely ground corn and 3% OH (OH), and a combination diet with 7% CC and 3% OH (CO). The grower diets were fed until day 16, followed by the finisher diets provided until the end of the trial.

Fine corn, wheat, and soybean meal were ground using a hammer mill fitted with a 4 mm sieve, while coarse corn was processed using a roller mill with sequential gap settings of 1.8, 1.6, and 1.5 mm. Oat hulls, initially pelleted, were reground using a roller mill with fixed gaps of 3.6 mm. All experimental diets were pelleted using a die with 3.2 mm holes and a roll-die distance of 0.2 mm. Pelleting involved an expander time of approximately 5 seconds at ~20 bar and 80°C, followed by a conditioning phase at 65°C for ~10 seconds with 2.1% steam addition and 12% initial feed moisture.

Table 6.1 Composition and nutrient content of the wheat-based diet.

Ingredients (g/100 g)	Pre-starter d 1–7	Grower d 8 to16				Finisher d 17 to 38			
	All	Control	CC	OH	CO	Control	CC	OH	CO
Wheat	43.48	52.93	52.92	49.35	49.35	55.89	55.89	53.47	53.47
Soyabean meal	28.23	26.14	26.14	28.55	28.55	21.82	21.82	24.74	24.74
Coarse corn	0.00	0.00	7.00	0.00	7.00	0.00	7.00	0.00	7.00
Oat hull	0.00	0.00	0.00	3.00	3.00	0.00	0.00	3.00	3.00
Fine corn	15.00	10.00	3.00	10.00	3.00	10.00	3.00	10.00	3.00
Soya oil	4.77	4.83	4.83	5.49	5.49	4.83	4.83	5.08	5.08
Sunflower meal	3.57	2.40	2.40	0.00	0.00	3.90	3.90	0.00	0.00
Monocalcium Phosphate	1.29	0.89	0.89	0.92	0.92	0.93	0.93	0.97	0.97
Limestone	1.26	0.93	0.93	0.92	0.92	0.84	0.84	0.82	0.82
Salt	0.20	0.22	0.22	0.22	0.22	0.21	0.21	0.22	0.22
Na-bicarbonate	0.20	0.17	0.17	0.16	0.16	0.18	0.18	0.17	0.17
Choline 75%	0.09	0.09	0.09	0.09	0.09	0.09	0.09	0.09	0.09
Lysine	0.66	0.48	0.48	0.42	0.42	0.50	0.50	0.44	0.44
Methionine	0.36	0.29	0.29	0.30	0.30	0.26	0.26	0.28	0.28
L-Valine	0.12	0.06	0.06	0.06	0.06	0.05	0.05	0.06	0.06
L-Isoleucine	0.07	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02
L-Arginine	0.06	0.00	0.00	0.00	0.00	0.00	0.00	0.02	0.03
L-Threonine	0.21	0.15	0.15	0.14	0.14	0.14	0.14	0.30	0.30
Vitamine E	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01
Vitamine premix[1]	0.39	0.31	0.31	0.31	0.31	0.30	0.30	0.30	0.30
Decoxx[2]	0.05	0.05	0.05	0.05	0.05	0.00	0.00	0.00	0.00
Phytase[3]	0.008	0.008	0.008	0.008	0.008	0.008	0.008	0.008	0.008
Xylanase[4]	0.00	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01
Nutrient levels[5]									
Metabolizable Energy, kcal/kg	2975	2925	2925	2925	2925	2950	2950	2950	2950
Crude Protein, %	21.30	20.20	20.18	20.17	20.21	19.00	19.09	19.02	19.04
Crude Fat, %	5.92	5.23	5.46	5.82	5.93	5.66	5.68	6.08	5.84
Crude fiber, %	3.18	3.07	3.06	3.58	3.57	3.22	3.21	3.56	3.55
NDF, %	8.37	9.32	11.11	17.7	15.46	9.01	8.45	12.83	11.38
ADF, %	4.61	4.58	4.83	5.01	5.41	4.37	4.21	4.95	4.49
ADL, %	0.63	0.62	0.62	0.70	0.68	0.69	0.67	0.71	0.73
Digestible lysine, %	1.22	1.08	1.05	1.10	1.08	1.00	1.01	1.04	1.01
Calcium, %	0.7	0.69	0.69	0.72	0.73	0.69	0.73	0.69	0.68
Phosphorus, %	0.70	0.57	0.51	0.59	0.56	0.59	0.60	0.57	0.57
Sodium, %	0.14	0.13	0.13	0.14	0.14	0.15	0.14	0.14	0.14
Chloride, %	0.20	0.21	0.19	0.22	0.20	0.19	0.21	0.23	0.20
Potassium, %	0.94	0.90	0.88	0.94	0.93	0.85	0.85	0.87	0.88
Magnesium, %	0.21	0.20	0.21	0.18	0.19	0.18	0.17	0.19	0.18

[1]Biotine premix together provided per kg feed: Vit A 10.000 IU, Vit D3 2750 IU, 25-hydroxycho2ecalciferol 0.056 mg, Vit E 90 mg, copper 15 mg, iron 15 mg, manganese 85 mg, zinc 50 mg, iodine 2 mg, and selenium 0.4 mg. [2]Provided per kg feed: 30.3 mg of decoquinate. [3]Provided per kg of feed: 500 FTU. [4]Provided per kg feed: 10 IU. [5]Metabolizable energy was calculated, while all other nutrient levels were analyzed. Control = a commercial broiler diet with 10% finely ground corn; CC = a diet formulated with 7% CC and 3% finely ground corn; OH = a diet containing 10% finely ground corn and 3% OH; CO = a diet containing 7% CC and 3% OH; NDF = neutral detergent fiber; ADF = acid detergent fiber; ADL = acid detergent lignin.

The particle size distribution of the OH was assessed through dry sieving in duplicate. Results indicated that 62% of particles were >4 mm, 10% between 3.15–4 mm, 13% between 2–3.15 mm, 11% between 1–2 mm, and 4% <1 mm. The particle size distribution (%), geometric mean diameter (GMD) and geometric standard deviation (GSD) of the experimental feeds were determined through wet sieving in duplicate (Table 6.2). A 20 g feed sample was soaked in 400 mL of distilled water for 1 h at room temperature. The sample was then sieved using a vibratory sieve shaker (Retch, Aartselaar, Belgium) equipped with sieves with mesh sizes of 2000, 1000, 500, 200, 90, 50, and 38 µm. The feed and water suspension was deposited onto the top sieve, and sieving was performed for 10 min with a water flow rate of 2.0–2.3 L/min, followed by 1 min without water flow to drain excess moisture. The fractions retained on each sieve were collected separately in Falcon tubes, freeze-dried, and stored in a desiccator until weighing. The average particle size (d_{av}) was calculated according to the equation: $d_{av} = \sum di \times mi$

where di represents the mesh size of sieve i, and mi represents the mass percentage of the fraction retained on sieve i.

Except stated otherwise, all chemical analyses were done using AOAC methods (AOAC, 2016). Crude protein was determined by the Kjeldahl method (Method 990.03), and crude fat was measured via Soxhlet extraction (method 920.39). Crude fiber was analyzed using the fiber bag technique (method 978.10). Neutral detergent fiber (NDF) and acid detergent fiber (ADF) contents were determined following the procedures described by Van Soest et al.[15], while acid detergent lignin (ADL) was measured following method 973.18. Digestible lysine levels were calculated based on the analyzed amino acid profile, determined using high-performance liquid chromatography following method 982.30. Calcium, phosphorus, sodium, potassium, and magnesium were quantified using inductively coupled plasma optical emission spectrometry following method 985.01. Chloride content was determined using potentiometric titration following method 943.01.

Table 6.2 Wet sieving particle size distribution (%) and geometric mean diameter (GMD) of experimental diets used in pre-starter, grower and finisher phases for low- and high-weight broilers.

Sieve size (mm)	Pre-starter	Grower (d 8 to16)				Finisher (d 17 to 38)			
		Control	CC	OH	CO	Control	CC	OH	CO
2.0	1.3	1.0	10.1	5.4	7.4	4.7	13.7	5.4	15.5
1.0	6.1	7.6	16.8	8.4	14.1	9.4	18.9	11.4	25.1
0.5	10.2	9.1	12.1	13.1	12.7	13.0	12.2	11.7	10.0
0.2	12.7	11.0	8.4	9.8	10.6	9.7	7.6	8.7	6.3
0.09	10.2	9.4	5.1	8.4	5.3	8.4	4.6	7.5	3.8
0.05	10.2	7.2	6.1	6.4	6.4	6.4	5.5	5.7	4.5
0.038	6.3	9.4	3.0	8.4	3.2	8.4	2.7	7.5	2.3
<0.038	43.1	45.3	38.4	40.2	40.3	40.1	34.7	42.0	32.6
>1.0	7.4	8.6	26.9	14.0	21.5	14.1	32.6	16.8	40.6
GMD ± GSD	360 ± 54.1	372 ± 48.7	582 ± 57.6	416 ± 51.5	532 ± 82.2	413 ± 45.9	621 ± 108.2	458 ± 53.3	698 ± 101.2

Control = a commercial broiler diet with 10% finely ground corn; CC = a diet formulated with 7% CC and 3% finely ground corn; OH = a diet containing 10% finely ground corn and 3% OH; CO = a diet containing 7% CC and 3% OH. GSD = geometric standard deviation.

6.2.2 Animals, husbandry, and data collection

A total of 1400-day-old male Ross 308 broiler chicks (initial BW: 44.5 ± 3.21 g) were obtained from a commercial hatchery (Belgabroed NV, Belgium). All chicks were fed a pre-starter diet until d 7, after which individual BW was recorded, and the birds were categorized into low-, medium-, and high-weight groups on the basis of BW distribution. Day 7 BW is a strong predictor of final BW, and selection at this age enables biologically relevant classification of LBW and HBW phenotypes before compensatory growth mechanisms begin to manifest, which typically occur during later stages of development[16]. The chicks on the lower end of the BW range were designated as LBW (n = 504),) and further divided into four treatment groups: LBWC, LBW+CC, LBW+OH, and LBW+CO, each comprising 126 chicks receiving either the control diet or one of the experimental diets (CC, OH, or CO). Additionally, the birds on the higher end of the BW (HBWC, n = 126) received the control diet with fine corn, whereas the remaining medium BW chicks (n = 770) were excluded from the experiment. The experiment consisted of five groups with six replicate

pens per group (1.3 m² per pen), with each pen containing 21 birds. Pen floors were covered with 3 cm of wood shavings. Feed and water were provided ad libitum throughout the study. The light schedule started with 1 h of darkness on d 1, increasing by 1 h per day to 6 h of darkness, which was maintained thereafter. The initial temperature was set at 33°C and decreased by 0.5°C daily until it reached 21.5°C on day 21, after which it remained constant.

6.2.3 Sampling and measurements

BW was individually recorded, and feed intake was recorded per pen after each dietary phase. The average daily gain (ADG), mortality-corrected average daily feed intake (ADFI) and feed conversion ratio (FCR) were calculated for the grower and finisher phases. The birds weighing nearest to the pen's average weight (n=12), were sacrificed through electrical stunning followed by decapitation on d 14, 21, and 38. Dissection was performed, and the weights of the gizzard, liver, pancreas, small intestine, and cecum were determined. The small intestine and cecum weights were measured without emptying the digesta, and their length was also recorded. The relative organ weights were expressed as g/100 g BW, and the relative lengths of the small intestine and cecum were calculated as cm/100 g BW. Digesta samples from both caeca were collected, placed in 2 mL vials, snap-frozen in liquid nitrogen, and stored at -80°C for microbiota and volatile fatty acid (VFA) analysis. Ileum sections from the midpoint were taken for histomorphological examination on d 14, 21 and 38, while ileal tissue samples were snap-frozen and stored at -80°C for high-throughput qPCR gene expression analysis on d 14 and 38. Two chickens per pen in each group were randomly selected on d 14, 21 and 38 for intestinal permeability tests via fluorescein isothiocyanate dextran (FITC-dextran, 4 kDa; Sigma–Aldrich, St. Louis, MO).

6.2.4 Ileal histomorphology

Ileum samples were fixed in 4% formaldehyde for 48 h and afterwards stored in 70% ethanol. Histology sections were embedded in paraffin, sectioned, and stained with alcian blue-periodic acid–Schiff according to the standard procedure of the GIGA immunohistochemistry platform (ULiège, Belgium). The microscopy images were analyzed via NDP.view2 software (Hamamatsu Photonics, Japan). Villus height (VH) and crypt

depth (CD) were measured for 20-well-oriented villus-crypt units per bird, and the VH/CD ratio was calculated.

6.2.5 Intestinal permeability

FITC-dextran solution (2.2 mg/mL/bird) was administered orally, and blood samples (1 mL) were collected from the jugular vein 2.5 h post gavage. Blood samples were centrifuged at 4°C at 3000 × g. A standard series was created, and plasma samples diluted in phosphate-buffered saline (1:5) were analyzed in duplicate via a 96-well microplate reader (CLARIOstar Plus, BMG LABTECH, Germany) with an excitation wavelength of 485 nm and an emission wavelength of 530 nm. Plasma FITC-dextran concentrations (ng/mL) were calculated using a standard curve. The relative concentration of FITC-dextran was calculated as ng/mL/100 g BW.

6.2.6 Cecal volatile fatty acid analysis

Short-chain fatty acids (SCFAs: acetate, propionate, butyrate, valerate, and caproate) and branched-chain fatty acids (BCFAs: isobutyrate, isovalerate, and isocaproate) were measured according to a modified method from previous study[17]. Briefly, approximately 250 mg of cecal content was weighed into 2 mL Eppendorf tubes, placed on ice, and mixed with 50 µL of MHA-2 internal standard solution and 80 µL of 6 M HCl. The samples were vortexed and were left on ice for 20 min and then mixed with 25% NaCl and tertiary-butyl methyl ether. After centrifugation at 4°C (10,000 × *g* for 5 min), 600 µL of the supernatant was transferred to a 1.5 mL Eppendorf tube containing anhydrous sodium sulfate, vortexed, and centrifuged again. A 200 µL aliquot was pipetted into screw-neck vials with conical glass inserts and stored at -20°C until analysis. VFAs were quantified via an HP 6890 Series GC System equipped with an automatic liquid sampler, flame ionization detector, and DB-FFAP capillary column (30 m length, 0.32 mm internal diameter, 0.25 µm film thickness; Agilent Technologies, Santa Clara, CA). The carrier gas was nitrogen at a flow rate of 25 mL/min, with the column at 130°C and the injector and detector at 195°C. SCFA and BCFA concentrations were calculated in mM/g wet digesta on the basis of calibration curves.

6.2.7 Gene expression through high-throughput qPCR

6.2.7.1 Selection of genes, primer design and validation

A total of 92 genes (13 housekeeping genes and 79 target genes) involved in intestinal barrier function, nutrient transport, immune response, metabolism, and oxidative homeostasis were selected based on published literature[18,19]. The expression of these genes was analyzed, and details on the genes and their primary functions are provided in Table S3.1.

Primers were designed using the NCBI Primer-Blast tool to span exon-exon junctions to minimize genomic DNA amplification. Specificity was confirmed by melting curve analysis following qPCR amplification, which revealed single peaks for all primers, indicating that there was no non-specific amplification or primer-dimer formation. Verification using agarose gel electrophoresis revealed single and distinct bands at the expected molecular weights for each amplicon. The primer efficiency was optimized between 90% and 110%, with R^2 values exceeding 0.99, using 3-fold serial dilutions of pooled cDNA derived from all samples on a QuantStudio 6 Real-Time PCR System (Thermo Fisher Scientific, Waltham, MA).

6.2.7.2 RNA extraction

Total RNA was extracted from ileal tissue samples using the ReliaPrep™ RNA Miniprep System (Promega Corporation, Madison, WI) according to the manufacturer's protocol. The RNA concentration and purity were determined by spectrophotometry (Nanodrop 2000, Thermo Fisher Scientific, Waltham, MA), whereas the integrity of the RNA was verified on 1% agarose gel electrophoresis.

6.2.7.3 Reverse transcription, preamplification, and high-throughput qPCR

The BioMark™ HD system (Standard BioTools, South San Francisco, CA) was used for high-throughput qPCR, following a protocol described in our previous study[18,19]. cDNA synthesis was performed using RT MasterMix (Standard BioTools, South San Francisco, CA). Preamplification was conducted in a 96-well qPCR plate with a primer mixture and PreAmp Mastermix (Standard BioTools, South San Francisco, CA) under the following thermal conditions: 95°C for 2 min, then 14 cycles of 95°C for 15

s and 60°C for 4 min. Exonuclease I treatment was then applied to remove unincorporated primers, and pre-amplified cDNA samples were diluted 8-fold. Prior to high-throughput qPCR, we prepared a sample mixture by combining 2.25 µL of exonuclease-treated, pre-amplified cDNA with 2.5 µL of 2x SSoFast™ EvaGreen® Supermix (Biorad, Hercules, CA) and 0.25 µL of 20x DNA-binding dye (Standard BioTools). The assay mixture contained 0.5 µL of each primer (100µM), 2.5 µL of 2x Assay Loading Reagent (Standard BioTools), and 2.25 µL of low-EDTA DNA suspension buffer (TEKnova, Hollister, CA). Both sample and assay mixtures were loaded into 96.96 Integrated Fluid Circuits (IFCs), and qPCR was conducted with initial denaturation at 95°C for 60 s, followed by 30 cycles of 96°C for 5 s and 60°C for 20 s. The standard curve, generated from dilutions of the pooled pre-amplified cDNA, was used to calculate relative mRNA levels. Four reference genes (*TBP*, *B2M*, *NDUFA*, and *B-Actin*) were identified as the most stable under the experimental conditions using the NormFinder algorithm[20]. Relative gene expression was calculated using the Pfaffl method[21] through normalization of target genes to the geometric mean of the reference genes' expression levels. Genes showing poor amplification efficiency, high Cq variability between technical replicates, or mean Cq values above 30 were excluded from downstream statistical analyses to ensure reliability of the expression data.

6.2.8 DNA extraction, 16S rRNA gene amplicon sequencing and bioinformatics

Cecal digesta DNA was extracted using the QIAamp PowerFecal Pro DNA Kit (Qiagen Benelux B.V., Venlo, Netherlands) following the manufacturer's protocol. DNA concentration and quality were assessed following the same protocol as described in the RNA extraction section. For sequencing library preparation, the V3–V4 region of the 16S rRNA gene was amplified using primers 341F (5′-CCTAYGGGRBGCASCAG-3′) and 806R (5′-GGACTACNNGGGTATCTAAT-3′), each with sample-specific barcodes. Libraries were sequenced on an Illumina NovaSeq 6000 platform, which produced 250 bp paired-end reads. Ultrapure water was included as a negative control to monitor sequencing quality.

The raw sequences were subjected to quality filtering, trimming, and demultiplexing in QIIME2 (v2024.2) with the default settings. Low-quality

reads were removed, and amplicon sequence variants (ASVs) were generated using DADA2. Taxonomic assignment of ASVs was conducted with the Naïve Bayes classifier against the SILVA database (release 138) at a 99% similarity threshold. For statistical analysis, the QIIME2 artifacts were imported into R (v4.2.3, R Foundation, Vienna, Austria). Microbial diversity metrics (Shannon and Simpson indices) were calculated as alpha diversity measures in R using the phyloseq package (v1.40.0) after rarefaction to the minimum sample depth, and groups were compared via the Kruskal–Wallis test. Beta diversity was evaluated with Bray–Curtis dissimilarity and visualized through principal coordinate analysis (PCoA). Group differences were assessed using non-parametric permutational multivariate analysis of variance (PERMANOVA) with 9999 permutations (vegan package, v2.6.4). Differential microbial abundance at the genus level was determined using linear discriminant analysis effect size (LEfSe) using the microbiome package (v1.18.0), with an LDA score threshold of ≥ 2.0 and significance set at $P < 0.05$. False discovery rate (FDR) adjustment was applied using the Benjamini-Hochberg method[22], and the results were visualized as log10 (LDA score) values.

6.2.9 Statistical analysis

Data normality was assessed using the Shapiro–Wilk test in R before conducting statistical analyses. Outliers were identified and removed based on values exceeding quartile 3 + 1.5 × interquartile range or falling below quartile 1 − 1.5 × interquartile range. Growth performance, digestive organ characteristics, ileal histomorphology, intestinal permeability, and ileum gene expression were analyzed using a one-way ANOVA model as follows:

$$Yij = \mu + Ti + \epsilon ij$$

where Yij is the dependent variable; μ is the overall mean; Ti is the fixed treatment effect; ϵij is the residual error term.

Pairwise comparisons were conducted using Tukey's HSD test. $P < 0.05$ was set significant difference and $0.05 < P \leq 0.10$ as a tendency. For gene expression data, P values were adjusted for FDR using the Benjamini–Hochberg method. All the results are reported as the means with a pooled standard deviation (SD), which combines the variability observed in all

samples. Principal component analysis (PCA) was performed to visualize sample clustering on the basis of gene expression data using the factoextra package (v1.0.7) in R. The multivariate effects of the treatments on sample clustering were tested using PERMANOVA with the adonis2 function (v2.6.4) to test for multivariate effects of dietary treatments on sample clustering in PCA. Heatmaps were plotted to show the sample variability and gene expression levels using the pheatmap package (v1.0.12) in R. Two-way hierarchical clustering of the heatmap data was performed using Pearson's correlation distance and Ward's clustering methods, with gene expression levels scaled per gene.

6.3 Results

6.3.1 Growth Performance

On d 7 and 16, the BW of the HBWC group was higher than that of all LBW groups ($P < 0.001$; Table 6.3). Among LBW groups on d 16, the LBW+OH group had higher BW than the LBWC group, LBW+CC chicks and LBW+CO chicks had intermediate weights. By d 38, the HBWC group maintained the highest BW, exceeding the LBWC group ($P < 0.001$). LBW+OH birds showed a significantly reduced difference in BW compared to HBWC birds and had significantly higher BW than LBWC birds, while LBW+CO and LBW+CC birds showed intermediate values.

From d 8 to16, the HBWC group had higher ADG than all other groups ($P < 0.001$). Among the LBW groups, the LBW+OH group exhibited greater ADG than the LBWC group. Over the full study period (d 8 to 38), HBWC birds maintained the highest ADG, significantly surpassing LBWC, LBW+CC, and LBW+CO birds ($P = 0.001$). The LBW+OH group attained the highest ADG among all LBW groups, significantly exceeding the LBWC group.

From d 8 to 16, the HBWC group had higher ADFI than all LBW groups ($P = 0.023$). Over the entire study period, HBWC birds had the highest ADFI, significantly surpassing LBWC and LBW+CO birds ($P = 0.005$). LBW+CC and LBW+OH birds showed intermediate ADFI values that did not differ significantly from HBWC or LBWC birds.

Table 6.3 Effects of coarse corn and oat hulls on the growth performance of low-weight broilers.

[1]Parameters	[2]Groups					SD	*P*-value
	HBWC	LBWC	LBW+CC	LBW+OH	LBW+CO		
BW, g							
7 d	203.3[a]	164.5[b]	166.6[b]	165.9[b]	164.5[b]	16.45	<0.001
16 d	675.8[a]	583.9[c]	598.5[bc]	611.7[b]	602.3[bc]	61.12	<0.001
38 d	3087[a]	2796[b]	2928[ab]	2983[a]	2941[ab]	105.43	<0.001
ADG, g/d							
8 to 16 d	52.5[a]	46.6[c]	47.6[bc]	49.5[b]	48.6[b]	2.45	<0.001
17 to 38 d	109.6	100.6	105.9	107.9	106.5	5.36	0.086
8 to 38 d	80.1[a]	72.4[c]	76.1[b]	77.3[ab]	76.3[b]	4.31	0.001
ADFI, g/d							
8 to 16 d	71.7[a]	56.2[b]	57.6[b]	58.1[b]	56.8[b]	9.98	0.023
17 to 38 d	146.1	134.7	142.7	140.0	137.4	7.40	0.062
8 to 38 d	124.5[a]	111.9[b]	118.0[ab]	116.2[ab]	114.0[b]	6.60	0.005
FCR, g/g							
8 to 16 d	1.22	1.20	1.20	1.19	1.17	0.161	0.735
17 to 38 d	1.33	1.33	1.33	1.31	1.29	0.069	0.850
8 to 38 d	1.34	1.31	1.31	1.29	1.27	0.070	0.608

[1]BW: body weight; ADG: average daily gain; ADFI: average daily feed intake; FCR: feed conversion ratio. SD: standard deviation. [2]HBWC = high BW chickens fed a commercial broiler diet with 10% finely ground corn; LBWC = low body weight chickens fed a commercial broiler diet with 10% finely ground corn; LBW+CC = low body weight chickens fed a commercial broiler diet with 7% coarse corn and 3% finely ground corn; LBW+OH = low body weight chickens fed a commercial broiler diet with 10% ground corn and 3% oat hulls; LBW+CO = low body weight chickens fed a commercial broiler diet with 7% coarse corn and 3% oat hulls. The BW was measured individually using the animal as the experimental unit. The ADG ADFI and FCR were calculated from 6 replicates of each group using the pen as an experimental unit. [a-c]Values with different superscripts in a row differ at $P < 0.05$.

6.3.2 Relative lengths and weights of digestive organs

On d 21, the LBW+OH group had the highest relative pancreas weight, exceeding that of the HBWC group (P = 0.005, Table 6.4). By d 38, the LBW+CO group showed a lower relative liver weight compared to the HBWC group (P = 0.027), whereas the other groups had liver weights comparable to HBWC birds. The gizzard relative weight was higher in LBW+CC, LBW+OH, and LBW+CO birds than in both HBWC and LBWC birds on d 21 and 38 (P = 0.016 and P = 0.044, respectively).

Table 6.4 Effects of coarse corn and oat hulls on the relative weights (g/100 g of live body weight) and lengths (cm/100 g of live body weight) of the digestive organs of low-weight broilers.

Parameters	Days of age	[1]Groups					SD	*P*-value
		HBWC	LBWC	LBW+CC	LBW+OH	LBW+CO		
Pancreas, g	14	0.38	0.35	0.39	0.39	0.35	0.097	0.733
	21	0.29^{b}	0.34^{ab}	0.33^{ab}	0.37^{a}	0.33^{ab}	0.045	0.005
	38	0.16	0.17	0.18	0.16	0.17	0.04	0.745
Liver, g	14	2.89	3.06	3.05	2.91	2.86	0.393	0.703
	21	2.72	2.77	2.87	2.78	2.79	0.265	0.758
	38	2.51^{a}	2.35^{ab}	2.34^{ab}	2.38^{ab}	2.21^{b}	0.023	0.027
Gizzard, g	14	2.05	1.89	2.01	2.09	1.97	0.223	0.220
	21	1.60^{b}	1.55^{b}	1.78^{a}	1.83^{a}	1.82^{a}	0.259	0.016
	38	0.90^{b}	0.93^{b}	1.01^{a}	1.05^{a}	0.99^{a}	0.191	0.044
Small intestine, g	14	7.97	7.90	7.80	7.45	7.15	0.907	0.137
	21	11.49	7.62	7.22	6.98	6.98	6.475	0.374
	38	5.18	4.92	5.36	4.69	5.21	0.748	0.215
Cecum, g	14	0.72	0.83	0.78	0.70	0.78	0.207	0.560
	21	0.84	0.89	0.79	0.92	0.91	0.228	0.627
	38	0.69	0.66	0.75	0.71	0.76	0.191	0.688
Small intestine, cm	14	23.69	25.93	25.71	24.70	23.69	3.173	0.089
	21	15.33	15.81	16.36	15.99	15.94	1.387	0.339
	38	6.82	7.17	7.35	7.01	7.55	0.871	0.273
Cecum, cm	14	3.85	4.37	5.01	4.04	4.02	0.506	0.160
	21	2.64	2.89	2.85	2.91	2.86	0.342	0.291
	38	1.27	1.46	1.38	1.42	1.50	0.230	0.143

[1]HBWC = high BW chickens fed a commercial broiler diet with 10% finely ground corn; LBWC = low body weight chickens fed a commercial broiler diet with 10% finely ground corn; LBW+CC = low body weight chickens fed a commercial broiler diet with 7% coarse corn and 3% finely ground corn; LBW+OH = low body weight chickens fed a commercial broiler diet with 10% ground corn and 3% oat hulls; LBW+CO = low body weight chickens fed a commercial broiler diet with 7% coarse corn and 3% oat hulls. SD = standard deviation. $^{a\text{-}b}$Values with different superscripts in a row indicate a significant difference (n = 12, P < 0.05).

6.3.3 Ileal histomorphology

On d 21, HBWC birds had greater VH compared to LBWC and LBW+CC birds (P = 0.005, Table 6.5). VH in LBW+OH and LBW+CO birds was intermediate, significantly exceeding LBW+CC but comparable to HBWC birds. By d 38, LBW+OH birds showed the highest VH, significantly surpassing LBWC birds (P = 0.042). On d 14, CD was lowest in LBW+CC compared to LBWC, LBW+OH, and LBW+CO birds.

Table 6.5 Effects of coarse corn and oat hulls on the villus height, crypt depth and villus height to crypt depth of low-weight broilers.

Parameters	Days of age	[1]Groups					SD	*P*-value
		HBWC	LBWC	LBW+CC	LBW+OH	LBW+CO		
Villus height, µm	14	548.8	541.3	525.3	542.5	544.9	53.90	0.863
	21	704.9^{a}	633.9^{bc}	605.8^{c}	663.1^{ab}	653.3^{abc}	68.35	0.005
	38	955.5^{ab}	881.8^{b}	929.1^{ab}	998.9^{a}	951.8^{ab}	95.77	0.042
Crypt depth, µm	14	148.0^{ab}	161.2^{a}	142.0^{b}	163.6^{a}	163.9^{a}	20.73	0.017
	21	168.9	164.4	157.2	161.4	158.6	19.42	0.608
	38	139.6	131.4	140.1	132.9	139.6	21.26	0.769
Villus height/crypt depth	14	3.72	3.40	3.76	3.36	3.36	0.517	0.123
	21	4.26	3.88	3.87	4.14	4.18	0.557	0.284
	38	6.90	6.82	6.79	7.64	6.90	1.008	0.201

[1]HBWC = high BW chickens fed a commercial broiler diet with 10% finely ground corn; LBWC = low body weight chickens fed a commercial broiler diet with 10% finely ground corn; LBW+CC = low body weight chickens fed a commercial broiler diet with 7% coarse corn and 3% finely ground corn; LBW+OH = low body weight chickens fed a commercial broiler diet with 10% ground corn and 3% oat hulls; LBW+CO = low body weight chickens fed a commercial broiler diet with 7% coarse corn and 3% oat hulls. SD = standard deviation. $^{a-c}$Values with different superscripts in a row indicate a significant difference (n = 12, P < 0.05).

6.3.4 Intestinal permeability

On d 14, 21, and 38, absolute plasma FITC-dextran concentrations did not differ significantly among groups (P > 0.05, Table 6.6). On d 14, relative plasma FITC-dextran levels were lower in the LBW+CC, LBW+OH, and LBW+CO groups compared to the LBWC group (P = 0.014) and were comparable to the HBWC group. On d 38, LBWC birds had a significantly higher relative plasma FITC-dextran concentration than HBWC birds (P = 0.022), while LBW groups fed CC, OH or their combination had intermediate values that were not significantly different from those of the HBWC group.

Table 6.6 Effects of coarse corn and oat hulls on plasma absolute and relative fluorescein isothiocyanate dextran levels in low-weight broilers.

Days of age	[1]Groups					SD	*P*-value
	HBWC	LBWC	LBW+CC	LBW+OH	LBW+CO		
	Absolute plasma FITC-dextran (ng/mL)						
14	45.7	55.8	46.1	48.2	48.3	10.49	0.119
21	52.2	53.0	51.1	51.4	51.9	4.95	0.931
38	59.7	64.8	64.0	62.1	63.1	5.45	0.429
	Relative plasma FITC-dextran (ng/mL/100 g BW)						
14	7.27^{b}	10.05^{a}	8.17^{b}	8.29^{b}	8.03^{b}	2.027	0.014
21	4.23	4.98	4.72	4.65	4.75	0.593	0.063
38	1.88^{b}	2.31^{a}	2.12^{ab}	2.03^{ab}	2.08^{ab}	0.283	0.022

[1]HBWC = high BW chickens fed commercial broiler feed with fine corn; LBWC = low body weight chickens fed a commercial broiler diet with 10% finely ground corn; LBW+CC = low body weight chickens fed a commercial broiler diet with 7% coarse corn and 3% finely ground corn; LBW+OH = low body weight chickens fed a commercial broiler diet with 10% ground corn and 3% oat hulls; LBW+CO = low body weight chickens fed a commercial broiler diet with 7% coarse corn and 3% oat hulls. SD = standard deviation. $^{a-b}$Values with different superscripts in a row indicate a significant difference (n = 12, $P < 0.05$).

6.3.5 Cecal VFA composition

Isobutyrate tended to be lower in the LBW+CO group on d 14 (P = 0.091). On d 21, valerate concentration was highest in the LBW+CC group, significantly exceeding that of the LBW+OH group (P = 0.002, Table 6.7). Isobutyrate, isovalerate and total BCFAs on the same day were significantly lower in the LBW+OH group compared to the LBWC group (P = 0.005, P = 0.003, and P = 0.029, respectively).

6.3.6 Ileal gene expression

6.3.6.1 Principal component analysis and heatmap clustering

The PCA on d 14 and 38 showed no distinct clustering of experimental groups (Fig. S6.1). This was supported by PERMANOVA, which revealed no significant associations between principal component variability and gene expression differences on d 38, though a tendency for differentiation was observed on d 14 (P = 0.073). Heatmaps visualized gene expression variability across samples from all groups (Fig. S6.2 and S6.3). Consistent with PCA, two-way hierarchical clustering on d 14 and 38 demonstrated that neither samples nor genes grouped consistently by experimental group or functional category.

Table 6.7 Effects of coarse corn and oat hulls on cecal volatile fatty acids (mM/g wet digesta) in low-weight broilers.

Parameters	Days of age	[1]Groups					SD	*P*-value
		HBWC	LBWC	LBW+CC	LBW+OH	LBW+CO		
Acetate	14	250.0	226.8	225.7	265.6	210.4	81.34	0.368
	21	246.8	259.1	245.3	255.8	243.1	65.38	0.978
	38	244.0	320.0	252.3	277.9	278.3	106.87	0.477
Propionate	14	24.8	20.5	21.4	22.6	20.8	3.75	0.125
	21	23.5	25.3	23.6	22.2	21.2	6.23	0.468
	38	27.6	31.8	29.8	31.3	27.6	8.98	0.704
Butyrate	14	59.8	48.1	43.1	61.2	44.1	20.33	0.111
	21	59.5	56.6	45.5	57.5	53.2	22.59	0.644
	38	64.9	81.0	69.2	71.4	75.1	39.03	0.695
Valerate	14	3.3	3.2	3.3	3.6	3.2	0.55	0.564
	21	3.41^{a}	3.34^{ab}	3.54^{a}	3.04^{b}	3.26^{ab}	0.423	0.002
	38	4.24	4.26	4.69	4.39	4.02	1.103	0.535
Total SCFAs	14	320.9	290.5	291.1	352.9	271.9	107.34	0.314
	21	333.2	344.3	317.9	338.5	320.6	85.18	0.912
	38	340.7	437.1	356.0	385.1	385.0	146.49	0.436
Isobutyrate	14	2.7	3.6	2.9	2.6	2.5	0.94	0.091
	21	2.57^{ab}	2.93^{a}	2.78^{a}	2.31^{b}	2.51^{ab}	0.351	0.005
	38	3.71	3.26	3.83	3.68	3.91	0.986	0.564
Isovalerate	14	2.3	2.8	2.7	2.7	2.4	0.91	0.392
	21	2.31^{ab}	2.63^{a}	2.41^{ab}	2.03^{b}	2.24^{ab}	0.325	0.003
	38	3.41	2.91	3.57	3.29	3.42	0.913	0.479
Total BCFAs	14	4.9	6.1	5.1	5.1	4.9	1.71	0.491
	21	4.89^{ab}	5.56^{a}	4.94^{ab}	4.34^{b}	4.76^{ab}	0.751	0.029
	38	7.12	6.17	7.40	6.97	7.33	1.867	0.496

[1]HBWC = high BW chickens fed commercial broiler feed with fine corn; LBWC = low body weight chickens fed a commercial broiler diet with 10% finely ground corn; LBW+CC = low body weight chickens fed a commercial broiler diet with 7% coarse corn and 3% finely ground corn; LBW+OH = low body weight chickens fed a commercial broiler diet with 10% ground corn and 3% oat hulls; LBW+CO = low body weight chickens fed a commercial broiler diet with 7% coarse corn and 3% oat hulls. SD = standard deviation. $^{a-b}$Values with different superscripts in a row indicate a significant difference (n = 12, P < 0.05).

6.3.6.2 Differential gene expression

To identify differentially expressed genes, one-way ANOVA with Tukey's HSD post hoc test was performed, considering genes with an FDR-adjusted $P < 0.05$ as significant (Table 6.8).

On d 14, the LBW+OH group showed the highest expression of the tight junction genes *CLDN1*, *CLDN4*, and *CLDN5; CLDN1* was even significantly higher than that of the LBWC and HBWC groups (FDR = 0.004), while *CLDN4* expression was significantly higher compared to the HBWC group (FDR = 0.003) and *CLDN5* was significantly higher compared to the LBWC

group (FDR = 0.001). The gut hormone *CCK* gene was upregulated in LBWC birds compared to LBW+CC and LBW+OH birds (FDR = 0.021), with intermediate levels in HBWC and LBW+CO birds. Expression of the nutrient receptor *T1R1* was significantly higher in LBW+OH birds than in all other groups (FDR = 0.011). Among the immune function genes, *TLR4* and *NOS2* (pattern recognition receptor and anti-inflammatory marker, respectively) showed the highest expression in LBW+OH birds (FDR = 0.021 and FDR = 0.044, respectively), although *TLR4* was not significantly different from LBWC birds, and *NOS2* was not significantly different from LBW+CO birds. The inflammatory marker *IL-6* was significantly upregulated in LBW+CC birds relative to HBWC, LBWC, and LBW+OH birds (FDR = 0.017). Genes associated with nutrient transport, including *SLC15A1* (peptide transporter) and *SLC1A4* (amino acid transporter), were upregulated in LBW+OH birds compared with LBWC and HBWC birds (FDR < 0.001 and FDR = 0.008, respectively). Additionally, the expression of genes such as *SLC2A1* (glucose transporter) and *VDR* (vitamin D receptor) tended to increase in LBW+OH birds (FDR = 0.064 and FDR = 0.085, respectively), whereas the expression of *CALB1* (calcium-binding protein) tended to increase in HBWC birds (FDR = 0.081). The expression of *HMOX2*, a gene associated with intestinal oxidation, was increased in the LBW+OH group (FDR = 0.045).

On d 38, *CLDN1* expression was significantly upregulated in HBWC birds compared to LBW+CC birds (FDR = 0.027). *CLDN2* expression tended to be higher in LBW+CC birds (FDR = 0.079). The expression of the proinflammatory cytokine *IL-18*, an immune-related gene, was highest in HBWC birds, significantly exceeding LBW+CC, LBW+OH and LBW+CO groups (FDR = 0.003). Among nutrient transporters, *SLC15A1* (peptide transporter) was significantly upregulated in LBW+CC birds (FDR < 0.001), whereas *SLC2A2* (glucose transporter) was highest in HBWC birds (FDR = 0.008). *SLC34A2* expression (sodium-phosphate cotransporter) was significantly upregulated in LBW+OH compared to HBWC (FDR = 0.049), while *SLC30A1* (zinc transporter) showed a tendency toward higher expression in the LBW+CO group (FDR = 0.084).

Table 6.8 Effects of coarse corn and oat hulls on the relative expression of genes involved in various intestinal functions in the ileum on d 14 and 38. Only significantly different or tended to be different genes are shown.

Genes	Function	[1]Groups					SD	FDR-value
		HBWC	LBWC	LBW+CC	LBW+OH	LBW+CO		
d 14								
CLDN1	BF	1.34^{b}	1.37^{b}	1.10^{b}	2.26^{a}	1.42^{b}	0.827	0.004
CLDN4	BF	0.54^{b}	0.98^{ab}	0.51^{b}	1.62^{a}	1.57^{a}	1.084	0.003
CLDN5	BF	1.58^{ab}	0.86^{bc}	0.45^{c}	1.89^{a}	0.74^{bc}	1.019	0.001
CDX	BF	0.98	1.05	0.99	1.33	1.39	0.440	0.083
CCK	GH	1.72^{ab}	2.33^{a}	1.13^{b}	0.97^{b}	1.73^{ab}	1.052	0.021
T1R1	NR	0.89^{b}	1.09^{b}	0.92^{b}	1.70^{a}	0.95^{b}	0.618	0.011
NOS2	IF	0.87^{b}	0.83^{b}	1.03^{b}	1.50^{a}	1.11^{ab}	0.588	0.044
TLR4	IF	0.56^{b}	0.96^{ab}	0.52^{b}	1.14^{a}	0.67^{b}	0.758	0.021
IL-6	IF	0.24^{b}	0.22^{b}	0.90^{a}	0.32^{b}	0.56^{ab}	0.535	0.017
SLC15A1	NT	0.64^{c}	0.62^{c}	1.63^{ab}	2.07^{a}	1.23^{bc}	0.881	<0.001
SLC1A4	NT	1.00^{b}	1.02^{b}	0.88^{b}	1.94^{a}	1.26^{ab}	0.752	0.008
SLC2A1	NT	0.98	0.85	0.98	1.70	1.45	0.816	0.064
VDR	NT	1.39	1.20	1.01	1.51	0.59	0.894	0.085
CALB1	NT	1.60	1.09	1.20	1.14	1.09	0.500	0.081
HMOX2	Ox	0.71^{b}	0.78^{b}	0.94^{ab}	1.17^{a}	0.94^{ab}	0.397	0.045
d 38								
CLDN1	BF	0.94^{a}	0.51^{ab}	0.33^{b}	0.69^{ab}	0.79^{ab}	0.613	0.027
CLDN2	BF	1.06	1.23	2.02	1.57	1.39	0.859	0.079
IL-18	IF	1.40^{a}	1.09^{ab}	0.39^{c}	0.50^{bc}	0.58^{bc}	0.695	0.003
TLR4	IF	0.42	0.96	0.27	0.46	0.36	0.641	0.094
SLC15A1	NT	0.78^{b}	1.05^{b}	2.97^{a}	1.34^{b}	1.06^{b}	0.903	<0.001
SLC2A2	NT	1.63^{a}	0.91^{b}	0.76^{b}	0.42^{b}	0.51^{b}	0.515	0.008
SLC34A2	NT	0.45^{b}	0.89^{ab}	1.03^{ab}	1.31^{a}	0.95^{ab}	0.814	0.049
SLC30A1	NT	1.59	1.52	2.17	1.94	2.22	0.764	0.084

[1]HBWC = high BW chickens fed a commercial broiler diet with 10% finely ground corn; LBWC = low body weight chickens fed a commercial broiler diet with 10% finely ground corn; LBW+CC = low body weight chickens fed a commercial broiler diet with 7% coarse corn and 3% finely ground corn; LBW+OH = low body weight chickens fed a commercial broiler diet with 10% ground corn and 3% oat hulls; LBW+CO = low body weight chickens fed a commercial broiler diet with 7% coarse corn and 3% oat hulls. BF = Barrier function, GH = Gut hormone, NR = Nutrient receptor, IF = Immune function, NT = Nutrient transport, OX = Oxidation. SD = standard deviation. FDR = false discovery rate. a-cValues with different superscripts in a row indicate a significant difference (n = 12, FDR < 0.05).

6.3.7 Cecal microbiota

Cecal amplicon sequencing generated 15,130,747 reads, with 86,461 ± 8181 (mean ± SD) reads per sample. After quality filtering, 13,617,672 reads remained, with an average of 77,815 ± 7363 reads per sample.

6.3.7.1 Core microbiota composition

Compositional analysis revealed considerable inter-individual variability and a significant shift in the gut microbiota from d 14 to d 38 post-hatching. On d 14, the Firmicutes phylum dominated (95%–97%), with minor contributions from Bacteroidota (1%–2%), Proteobacteria (1%–3%), and Cyanobacteria (0.2%–1.8%) (Table S6.1). By d 21, Firmicutes remained predominant (93%–96%), but there were slight increases in Actinobacteriota (0.2%–3.6%). On d 38, the dominance of Firmicutes persisted (92%–95%), while Actinobacteriota (1.8%–2.8%) and Cyanobacteria (0.6%–1.2%) remained stable. Additionally, Bacteroidota exhibited minor fluctuations (1%–2%) over time, and low-abundance phyla such as Desulfobacterota (< 0.3%) remained consistently present.

At the genus level, core genera including *Faecalibacterium*, *Lactobacillus*, *unclassified Lachnospiraceae,* and *Ruminococcus* torques *group* accounted for approximately 56% of the total relative abundance in the chickens, as shown in Fig. 6.1. With age, *Lactobacillus* and *unclassified Lachnospiraceae* numerically increased, whereas *Faecalibacterium* and *Ruminococcus torques group* decreased. Despite the consistency of these core genera across individuals, many low-abundance genera collectively made up more than 10% of the community, representing a highly variable component of the gut ecosystem.

6.3.7.2 Alpha and beta diversity

On d 14, the Shannon index of alpha diversity was significantly higher in the LBW+CC group compared to LBWC and HBWC groups ($P < 0.05$, Fig. 6.2), with the LBW+OH and LBW+CO groups showing intermediate values. No significant differences in alpha diversity metrics were observed among groups on d 21 and 38. Beta diversity analysis using Bray-Curtis dissimilarity did not reveal a clear separation in the overall microbiota composition between groups at any timepoint (Fig. S6.4). This lack of separation was confirmed by PERMANOVA, which found no significant

associations between principal component variability and microbiota composition differences (P > 0.05).

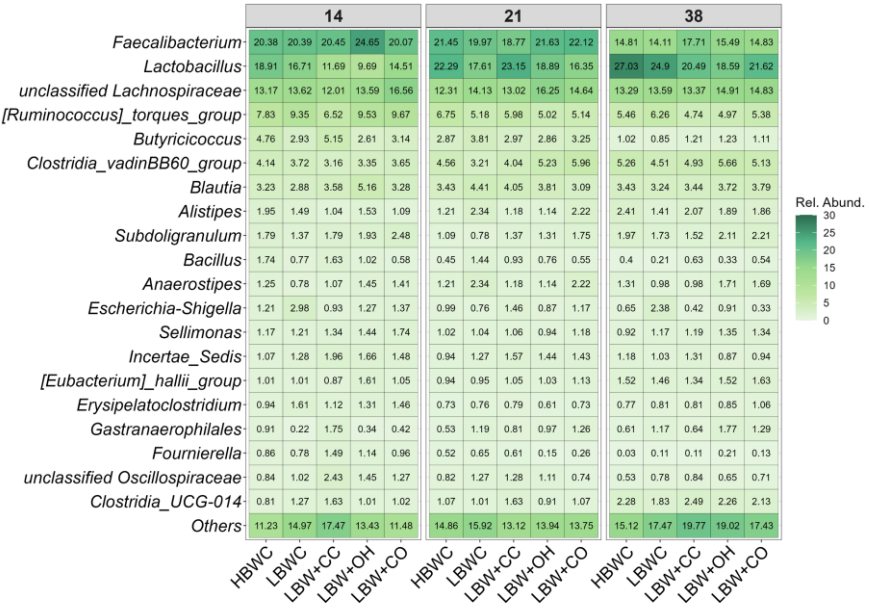

Fig. 6.1 Relative abundance (%) of cecal bacterial genera in broilers from different dietary groups (n = 12). Groups include high body weight broilers fed a commercial broiler diet with 10% finely ground corn (HBWC), low body weight chickens fed a commercial broiler diet with 10% finely ground corn (LBWC), low body weight chickens fed a commercial broiler diet with 7% coarse corn and 3% finely ground corn (LBW+CC), low body weight chickens fed a commercial broiler diet with 10% finely ground corn and 3% oat hulls (LBW+OH), and low body weight chickens fed a commercial broiler diet with 7% coarse corn and 3% oat hulls (LBW+CO) on d 14, 21, and 38. The values indicate the mean relative abundance of bacterial genera.

6.3.7.3 Differential abundance of bacterial genera

LEfSe analysis identified differentially abundant bacterial genera across groups on d 14, 21, and 38 (LDA cut-off ≥ 2.0, FDR < 0.05).

On d 14, a total of 12 genera were identified across groups (Fig. 6.3A). The HBWC group was enriched with *Lactobacillus*, whereas LBWC birds had increased *Escherichia-Shigella*. The LBW+CC group was enriched with *unclassified Oscillospiraceae*, *Ruminococcaceae UCG-005*, *Gastranaerophilales*, the *Christensenellaceae R-7 group*, *Anaerofilum*, *unclassified Ruminococcaceae,* and *Candidatus_Soleaferrea*. The LBW+OH

group showed a higher abundance of *Faecalibacterium* and *Blautia*, whereas the LBW+CO group had higher abundance of *unclassified Lachnospiraceae*.

On d 21, a total of 9 genera were identified (Fig. 6.3B). HBWC birds were enriched with *Anaerostipes*, and LBWC birds with *UCG-008* (family Butyricicoccaceae) and *Colidextribacter*. LBW+CC birds showed higher abundance of *Ruminococcaceae UCG-005*, *Roseburia*, *Romboutsia,* and *Akkermansia*, while LBW+OH birds were enriched with *unclassified Lachnospiraceae*. LBW+CO birds showed increased *Bifidobacterium* abundance.

On d 38, a total of 13 genera were identified (Fig. 6.3C). HBWC birds showed a higher abundance of *Lactobacillus* and *Caproiciproducens*, while LBWC birds were enriched with *Lachnospiraceae_FE2018_group*. LBW+CC birds showed enrichment of *Christensenellaceae R-7 group* and *unclassified Ruminococcaceae*, *Mordavella*, and *Eggerthellaceae*. LBW+OH birds were enriched with *Fournierella* and *Lachnospiraceae_FCS020_group*, whereas LBW+CO birds showed increased abundance of *Romboutsia*, *Enterococcus*, *[Clostridium]_sprioforme* and *Sellimonas*.

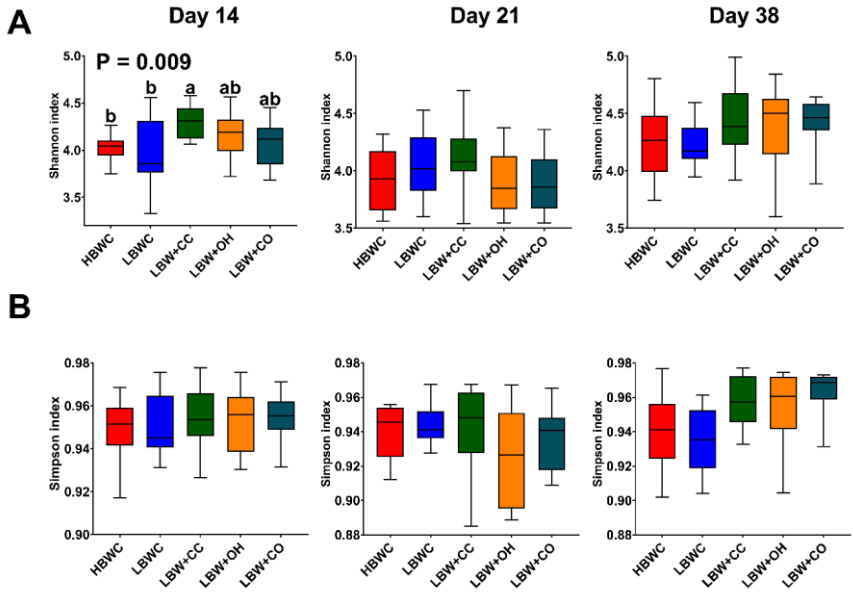

Fig. 6.2 Shannon (A) and Simpson (B) indices of alpha diversity for the cecal microbiota in broilers from different dietary groups (n = 12). Groups include high body weight broilers fed a commercial broiler diet with 10% finely ground corn (HBWC), low body weight chickens fed a commercial broiler diet with 10% finely ground corn (LBWC), low body weight chickens fed a commercial broiler diet with 7% coarse corn and 3% finely ground corn (LBW+CC), low body weight chickens fed a commercial broiler diet with 10% finely ground corn and 3% oat hulls (LBW+OH), and low body weight chickens fed a commercial broiler diet with 7% coarse corn and 3% oat hulls (LBW+CO) on d 14, 21, and 38. Alpha diversity measures were evaluated using Kruskal–Wallis test, with significance threshold set at $P < 0.05$. a-bValues in a row with no common letters differ significantly ($P < 0.05$).

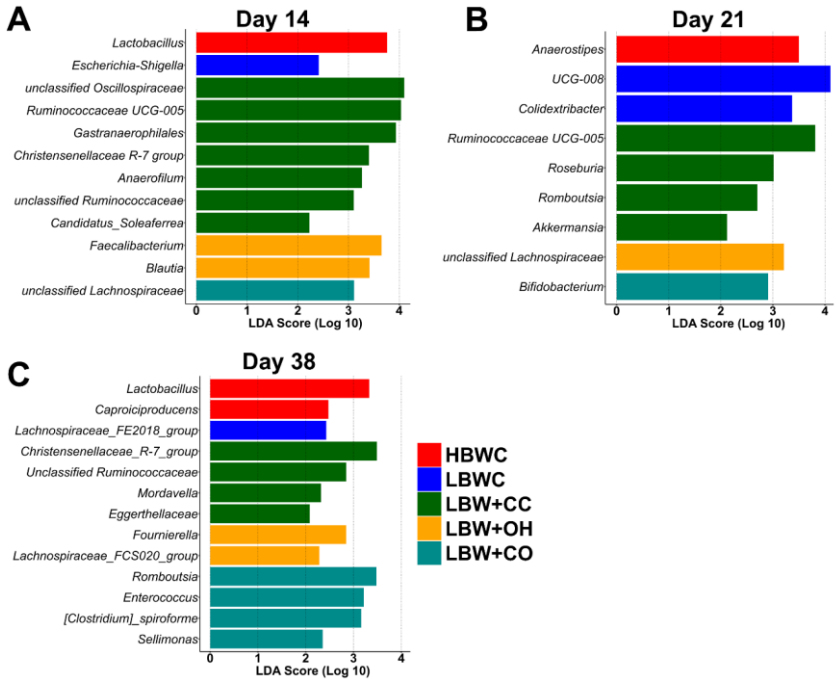

Fig. 6.3 LEfSe results of differentially abundant cecal bacterial genera in broilers from different dietary groups (n = 12). Groups include high body weight broilers fed a commercial broiler diet with 10% finely ground corn (HBWC), low body weight chickens fed a commercial broiler diet with 10% finely ground corn (LBWC), low body weight chickens fed a commercial broiler diet with 7% coarse corn and 3% finely ground corn (LBW+CC), low body weight chickens fed a commercial broiler diet with 10% finely ground corn and 3% oat hulls (LBW+OH), and low body weight chickens fed a commercial broiler diet with 7% coarse corn and 3% oat hulls (LBW+CO) on d 14 (A), d 21 (B), and d 38 (C). Only genera with an FDR ≤ 0.05 and an absolute value of LDA ≥ 2.0 are presented.

6.4 Discussion

The relationship between first-week chick weight and subsequent broiler performance represents a critical determinant of production efficiency, as demonstrated by current findings and supported by previous research[16]. Despite uniform rearing conditions, HBWC chicks consistently outperformed LBWC chicks, with no compensatory growth observed in LBWC broilers throughout the experimental period. The better performance of HBWC birds corresponded with a higher ADFI, better intestinal function and favorable microbiota composition, corroborating earlier findings that heavier chicks possess intrinsic growth potential advantages over lighter chicks under standard conditions[16,18]. In LBWC

chicks, higher expression of *CCK*, a gut hormone involved in satiety signaling and feed intake regulation[23], may explain their lower feed intake. Furthermore, the dominance of *Lactobacillus*, a genus linked to probiotic effects and improved broiler growth[24], in HBWC birds contrasts with the higher abundance of potential pathogen *Escherichia-Shigella* in LBWC chicks on day 14. The presence of this pathogenic genus, known to contribute to intestinal dysbiosis and inflammation[16], likely contributes to the suboptimal performance of LBWC birds.

Dietary interventions with CC and OH, either individually or in combination, improved growth parameters in LBW broilers compared to the LBWC birds. By d 38, notably, the LBW+OH group not only outperformed the LBWC group but also showed a markedly reduced difference in BW compared to the HBWC group, primarily through increased feed intake rather than improved FCR. These findings suggest that OH inclusion modulates the feeding pattern, contributing to compensatory weight gain in LBW broilers. These results align with González-Alvarado et al.[25], who reported increased weight gain and feed intake with 3% soybean or oat hulls, and Kheravii et al.[14], who reported synergistic effects of CC and 2% sugar bagasse on broiler performance. The improved growth response of LBW broilers fed dietary interventions are attributed to enhanced GIT development and function. Dietary CC, OH or their combination rapidly increased gizzard size in LBW broilers, a response driven by increased mechanical demands for particle size reduction. The enlarged gizzard improves gut motility and digestive efficiency, partially mediated by CCK, which increases digesta retention time and pancreatic enzyme secretion, enhancing the interaction between digesta and digestive enzymes for better nutrient digestion[7,26]. Increased transit time in the gizzard also lowers pH, stimulating pepsin activity[27] and accelerating protein denaturation, thereby improving protein digestion. Interestingly, ileal CCK expression was not significantly altered by dietary modifications, supporting findings that CCK may play a less central role in regulating pancreatic enzyme secretion in birds compared to mammals[28]. The LBW+OH group exhibited increased relative pancreas weight, reflecting adjustments for increased enzyme production necessary for fibrous diet digestion. Previous research has demonstrated that insoluble fibers increase the production of pancreatic enzymes, including

chymotrypsin[29,30]. Although enzyme activity and nutrient digestibility were not directly measured in this study, the results align with previous findings showing that structural diets promote gizzard development, thereby enhancing pancreatic enzyme secretion, nutrient digestibility and growth performance in broilers[13,31]. Dietary OH inclusion improved VH in LBW birds, counteracting the reduction in absorptive surface area observed in LBWC broilers. This aligns with findings by Moradi et al.[32], who reported that OH stimulates mucosal development, which leads to improved intestinal morphology and nutrient absorption. At the molecular level, OH in LBW birds induced nutrient-sensing receptor *T1R1* upregulation, enhancing their capacity to detect and respond to essential nutrients including amino acids and fatty acids[33]. This was accompanied by the upregulation of nutrient transporters (*SLC15A1*, *SLC1A4*, and *SLC2A1*) on d 14, facilitating di- and tri-peptide, neutral amino acids, and glucose absorption. Additionally, *VDR*, which mediates vitamin D uptake, showed a tendency towards increased expression in the LBW+OH group, suggesting improved mineral utilization. These adaptations might have enabled LBW+OH birds to compensate for their initial growth disadvantage. The LBW+CC group also showed upregulation of the di- and tri-peptide transporter *SLC1A4* on d 38, indicating that CC can increase nutrient absorption during later growth stages. Nevertheless, its contribution to compensatory growth was less pronounced than that observed with OH. The structural properties of insoluble fibers, forming a bulky and spongy matrix, increase the interaction of digestive enzymes with digesta[34]. This enhances nutrient availability for enzymatic activity, improving nutrient absorption and retention, and supporting better growth performance. The higher nutrient availability in the intestinal lumen of LBW+OH birds likely activated nutrient transporters, consistent with Kheravii et al.[14], who found dietary insoluble fiber (2% sugar bagasse) upregulated amino acid transporters in the small intestine. Additionally, OH stimulates pancreatic amylase secretion[35], leading to increased carbohydrate digestion and glucose absorption.

The LBWC group exhibited increased intestinal permeability on both d 14 and 38 than HBWC group, as indicated by increased plasma FITC-dextran concentrations. This finding aligns with Akram et al.[18], who associated low BW in broilers with impaired gut barrier function and increased

inflammation, facilitating pathogen translocation and elevating metabolic demands. Dietary OH reduced intestinal permeability, likely via upregulated expression of tight junction proteins *CLDN1*, *CLDN4*, and *CLDN5*, enhancing gut barrier integrity in LBW+OH broilers. This aligns with previous studies suggesting that insoluble fibers improve intestinal barrier function and host immunity by promoting SCFA production[36]. Unlike a previous study[37], this study found a significant effect of OH on certain immune function genes. The observed upregulation of *NOS2* and *TLR4* in the LBW+OH group on day 14 suggests enhanced activation of innate immune pathways. *NOS2*, which is critical for nitric oxide production, contributes to pathogen clearance by promoting antimicrobial activity[38], whereas *TLR4* is integral for recognizing microbial components and activating downstream inflammatory signaling pathways, such as *NF-κB*, which regulates cytokine production[39]. The LBW+OH group demonstrated increased expression of *HMOX2*, a gene involved in antioxidant processes in the ileum, suggesting their increased capacity to mitigate free radicals in the intestine to maintain intestinal homeostasis[18]. These molecular alterations point to the effects of OH as a fermentable substrate for the gut microbiota, leading to the production of SCFAs that may influence *NOS2*- and *TLR4*-mediated pathways.

Dietary interventions showed minimal effects on overall SCFA concentrations but significantly influenced valerate levels, which were highest in LBW+CC broilers on day 21. Valerate has been associated with improved broiler performance and reduced necrotic enteritis[40]. BCFAs, primarily derived from protein fermentation in the cecum, are associated with undesirable microbial shifts and elevated nitrogenous metabolites[41]. The reduced BCFAs in the LBW+OH group on d 21 suggest that OH improved protein digestion in the small intestine, limiting cecal protein fermentation and production of microbial metabolites associated with gut dysbiosis, as corroborated by a previous study in weaner pigs[42].

Microbiota diversity, as indicated by the Shannon index, was greater in the LBW+CC group than in other groups on day 14, suggesting that CC feeding had a positive influence on microbial richness during early growth. No significant differences in alpha diversity were observed among CC- or OH-fed groups at later time points, which is consistent with the findings of previous studies[13,43]. In the LBW+CC group, the enrichment of the

Christensenellaceae R-7 group and *Romboutsia* suggested their roles in enhancing nutrient metabolism. The *Christensenellaceae R-7 group* is a biomarker for broiler growth and muscle development[16], while *Romboutsia*, a butyrate producer, supports intestinal health and energy availability, contributing to better feed efficiency[44]. Meanwhile, OH promoted *Faecalibacterium* and *Blautia* abundance suggested their involvement in modulating gut architecture, SCFA production, and energy metabolism. *Faecalibacterium*, particularly *F. prausnitzii*, is a key butyrate producer known to reduce inflammation, strengthen intestinal barrier integrity and improvements in morphological traits[45,46], as reflected by reduced intestinal permeability, upregulated tight junction gene expression and increased VH in the LBW+OH group. *Blautia*, known for fermenting insoluble fibers such as those in OH, produces acetate, a key SCFA that promotes gut health and increases energy metabolism[47]. This may indicate that the use of OH as a substrate significantly influences the number of favorable bacteria. In the LBW+CO group, *unclassified Lachnospiraceae* were enriched on d 14 and their abundance in LBW+OH broilers on d 21 indicated their central role in fiber degradation and SCFA production, particularly butyrate. Butyrate is known to promote intestinal health and modulate immune function[48].

The particle size of coarsely ground corn in this study did not seem to be the same as that of particles generated after milling when they were subjected to secondary grinding. Secondary grinding, which involved expander conditioning and pelleting, further reduced the particle size in CC and CO diets, substantially minimizing differences with control diet. Despite this reduction, the CC and CO diets positively influenced gizzard development and broiler performance. These results align with the findings of Ebbing et al.[49], where secondary grinding using an expander and pelleting significantly reduced the initial particle size obtained after milling but enhanced nutrient digestibility. This outcome suggests that, beyond particle size alone, other physical characteristics such as pellet hardness and durability may also play a role in stimulating gizzard activity. Harder pellets that resist disintegration during early digestion can increase the mechanical function of the gizzard and prolong digesta retention, potentially improving feed intake and nutrient utilization[9]. However, pellet hardness and durability were not directly assessed in this study, which

limits our ability to determine their specific contribution to the observed effects. Future studies should consider incorporating direct measurements of pellet physical quality to better understand how structural features interact with particle size in influencing digestive function. Maintaining coarse particles in broiler pellets is challenging, especially in high-throughput feed mills using conditioning with expander. The intense mechanical and thermal processes often break down larger particles into finer fractions. Balancing particle preservation with pellet durability and process efficiency is critical, as coarse particles are nutritionally beneficial but prone to degradation during production.

While all structural dietary interventions enhanced gizzard development to a comparable extent, OH addition conferred superior growth benefits in LBW broilers relative to CC. This differential response may reflect the distinct functional properties of OH, particularly its high lignocellulosic content, which provides not only mechanical stimulation but also fermentable substrates that shape the gut microbial landscape. Insoluble fiber can act as a physical matrix, supporting bacterial colonization and enhancing fermentation, leading to higher SCFA production. This improved SCFA profile, combined with the mechanical action of insoluble fiber in enhancing digestive efficiency, contributes to better nutrient utilization and intestinal health, potentially giving OH an advantage over CC. Interestingly, the combination of CC and OH did not consistently outperform OH alone. The simultaneous inclusion of CC and OH may dilute the specific benefits of each component, as CC primarily enhances gizzard activity through its coarse particle size, while OH provides advantages by supporting gut microbial activity and SCFA production. These combined effects may alter the gut environment in ways that reduce the distinct benefits of OH's fiber-driven modulation of gut health, suggesting complex relationships between corn particle size and dietary fiber that warrant further investigation.

6.5 Conclusions

LBWC birds exhibited persistent growth impairments, characterized by compromised intestinal morphology, elevated gut permeability, and dysbiotic microbial signatures compared to HBWC birds, despite uniform post-hatch management. Dietary inclusion of CC and OH, either individually or in combination, enhanced GIT development by increasing

the relative gizzard weight and reducing gut permeability. Among these dietary strategies, OH inclusion had the most significant impact, effectively ameliorating growth depression in LBW broilers, outperforming the LBWC group, and reducing the difference in BW compared to HBWC birds. This was achieved through improved ileal morphology, upregulation of genes associated with gut barrier function, nutrient transport, and immune function. Additionally, OH contributed to better gut health by promoting favorable caecal microbiota and reducing BCFAs. Overall, incorporating structural components, particularly OH at moderate inclusion levels (3%), effectively addressed growth limitations in LBWC birds, aligning their performance with HBWC birds by slaughter age and presenting a promising approach for optimizing production efficiency.

6.6 Reference

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Chapter 7

General discussion, conclusions and future perspectives

This chapter presents the general discussion and conclusions of the dissertation. Additionally, suggestions for future research are provided which can further improve broiler growth and intestinal health, which might contribute to more efficient and sustainable poultry production systems.

7.1 General discussion

In our study, chicks categorized as low body weight (LBW) on day 7 were 21% lighter than the Aviagen target for male Ross 308 broilers and 22% lighter than high body weight (HBW) chicks in the study, reflecting the underperforming category typically can be observed in commercial settings. By day 38, both LBW and HBW groups exceeded expected Aviagen thresholds, with a 300 g (10%) difference between categories, which is a smaller gap than typically observed commercially at slaughter age[1]. Moreover, both low- and high-weight categories exceeded expected Aviagen performance thresholds by day 38. This superior performance across weight categories may be attributed to optimal hygiene, ventilation, and reduced feed competition in our research facility compared to commercial settings. Thus, our studies primarily explained performance-related biomarkers that more effectively account for the exceptional growth performance of HBW chicks, rather than emphasizing the factors contributing to poor performance in LBW birds.

7.1.1 Intra-flock body weight variability represents a persistent challenge in broiler production

Modern broiler chickens represent one of the most genetically uniform livestock species in the world[2]. Decades of intensive genetic selection for production traits have dramatically improved their growth performance, feed conversion ratio, and meat yield, transforming poultry production into a highly efficient protein source[3]. Yet, despite remarkable advancements in genetics, nutrition, and management practices, intra-flock body weight (BW) variability remains a persistent and economically significant challenge[4,5]. In contemporary broiler production, achieving uniformity within a flock, where all birds reach market weight and physiological maturity at similar rates, represents a fundamental goal with profound economic, welfare, and sustainability implications.

While some degree of natural variation is inevitable in any biological system, excessive heterogeneity in commercial flocks undermines production efficiency and creates cascading problems throughout the production chain. From an economic perspective, poor flock uniformity forces producers into suboptimal management decisions, choosing between processing underweight birds that fail to meet market specifications or managing heavier birds that may suffer from metabolic and skeletal disorders. In some production systems, such as in Belgium,

partial depopulation around day 35 is used to mitigate these issues by removing a portion of the flock and creating more space for the remaining birds to reach higher slaughter weights. Although heavier birds often demonstrate superior profitability, aligning with the industry's primary objective of producing high-performing animals, their presence alongside significantly smaller counterparts creates operational challenges. Smaller birds typically exhibit suboptimal feed conversion ratios, produce uneven carcass quality, and incur economic penalties at processing facilities, which often require costly sorting procedures or reject non-conforming carcasses entirely[6]. In modern automated processing systems, substantial size disparities can severely interfere with mechanical operations, including defeathering, evisceration, and portioning, reducing line efficiency and product quality[7].

Beyond economic considerations, intra-flock variability carries significant animal welfare implications that extend throughout the production cycle. Smaller or slower-growing birds often become less competitive for essential resources including feed, water, and optimal positioning within high-density environments, predisposing them to chronic stress, increased disease susceptibility, and physical injury. These birds frequently suffer from compromised gut health, immunosuppression, and stress responses that may not be externally visible, particularly under intensive fast-growth production systems[6]. Consequently, individuals at the lower end of the growth distribution face elevated risks of being culled, trampled, or systematically excluded from feeders and drinkers, raising substantial ethical concerns and complicating welfare assessments and auditing procedures.

At the systems level, BW heterogeneity fundamentally limits the full realization of genetic potential within commercial flocks, introducing biological inefficiencies where identical input resources yield highly variable outcomes. This inefficiency becomes increasingly critical under mounting sustainability pressures, where minimizing environmental impact per unit of meat produced has become paramount for industry viability and social acceptance. Understanding and mitigating the underlying causes of flock variability therefore represents not merely an optimization challenge, but a necessity for sustainable intensification of poultry production.

The present research has revealed that broiler flock uniformity is governed by a complex, multifactorial network involving access to feed, gut

microbiota composition, and host-related physiological factors. Critically, these investigations demonstrate that BW categories established as early as day 7 post-hatch serve as powerful predictors of subsequent performance throughout the entire production cycle. These findings indicate that the factors governing performance heterogeneity operate both independently and synergistically, creating cascading physiological events that ultimately determine individual bird performance within genetically uniform populations. By elucidating these interconnected mechanisms, this work provides a foundation for developing targeted interventions to improve the productivity of (underperforming) broilers, thereby improving production efficiency.

7.1.2 Initial body weight as determinant of body weight divergence

The significance of initial BW on subsequent growth patterns and slaughter weight in broilers was investigated in chapter 2 and 3. Chicks were categorized based on their body weight relative to the flock mean at day 7 post-hatching, highlighting associations with foundational biological concepts and limitations regarding growth potential. Despite exposure to uniform management practices, chicks with LBW did not exhibit catch-up growth, suggesting an association with intrinsic biological factors, possibly involving physiological and behavioral mechanisms, which may have limited compensatory growth typically observable in later developmental stages. During the starter and grower phases, HBW chicks exhibited significantly higher ADG and ADFI compared to LBW chicks, while FCR between the two groups remained comparable. This pattern indicates a strong association between higher growth rates and increased voluntary feed intake rather than improved nutrient utilization efficiency. Thus, early-established feed intake appeared to be associated with subsequent growth trajectories.

The observed divergence in feed intake between BW groups can partially be attributed to differential expression of appetite-regulating gut hormone genes, particularly proglucagon B and cholecystokinin (CCK). LBW birds expressed higher levels of CCK and Proglucagon B, both of which suppress appetite and slow gastric emptying, potentially reducing nutrient intake during critical growth windows (Chapter 3) . Proglucagon B encodes glucagon, glucagon-like-peptide-1 (GLP-1), and glucagon-like-peptide-2

(GLP-2), hormones involved in appetite suppression and lipid metabolism modulation[8,9]. Similarly, CCK serves as a satiation signal, contributing significantly to reduced feed intake through enhanced feelings of fullness[10]. In this study, genes related to appetite-regulating hormones were investigated in the ileum; however, it is worth noting that the duodenum and jejunum may represent more relevant tissues for examining the expression of these hormones, as they are primary sites of nutrient sensing and gut hormone secretion.

Taken together, the disparities in growth among broilers were strongly associated with the bird's initial BW. A higher BW in the first week was associated with maintaining an advantage over chicks with lower BW, coinciding with differences in feed intake and intestinal physiology, and subsequently linked to overall performance outcomes.

7.1.3 Microbiota as diverging factor

Chapter 2 revealed that the caecal gut microbial community differed in association with initial BW categories, with distinct microbial signatures emerging by day 7. HBW birds tended to establish a more mature, functionally stable microbiota early in life, dominated by obligate anaerobes such as unclassified Lachnospiraceae, *Faecalibacterium*, and *Alistipes*, taxa associated with higher production of beneficial SCFAs like butyrate and acetate. In contrast, LBW birds showed a microbial pattern enriched in *Escherichia–Shigella*, *Lactobacillus*, and *Akkermansia*, genera often linked to inflammation and less efficient fermentation.

Microbiota composition was closely associated with growth variability. HBW birds not only had more uniform BW distribution but also lower inter-individual variation in microbiota (lower beta-diversity), indicating a relationship between microbial uniformity and phenotypic uniformity. This aligns with findings from Lundberg et al.[1] showing heavier birds possess more consistent microbiota profiles. High microbiome similarity has been correlated with reduced dysbiosis risk, whereas greater variability is often linked to disease susceptibility and impaired growth[11].

The metabolic potential of the microbiota further explained differences between BW categories. Certain bacteria can metabolize dietary components inaccessible to the host, expanding the nutritional capacity of

the host–microbiome "superorganism" and enhancing energy capture. Studies in mice[12,13] demonstrated that specific gut microbes can confer an energy-efficient phenotype by increasing the abundance of genes involved in fat, protein, and carbohydrate metabolism, leading to greater SCFA production and energy extraction. In our study, HBW microbiota were enriched for pathways related to vitamin and amino acid biosynthesis, fiber degradation, and SCFA production, all supporting nutrient utilization and gut development. Conversely, LBW birds displayed microbial signatures associated with protein fermentation byproducts and mucosal stress. Although microbial functions appeared to converge by slaughter age, the early-life microbial landscape was associated with long-term growth trajectories.

Across three independent trials in this thesis (Chapters 2, 5, and 6), high-performing birds consistently shared a similar microbial signature, with this pattern emerging as early as day 7 and, in some cases, persisting beyond the early growth period: an abundance of obligate anaerobes with established roles in intestinal health, efficient carbohydrate fermentation, SCFA production, and biosynthesis of essential metabolites. Underperforming birds consistently carried dysbiotic profiles enriched in facultative anaerobes such as *Escherichia-Shigella* and *Enterococcus*, taxa associated with gut barrier dysfunction, endotoxin production, and reduced nutrient absorption. This repeated pattern across trials suggests that specific microbial traits, rather than trial-specific factors, are robustly linked to performance.

Importantly, these results suggest the presence of a set of core taxa that remain stable across environments in high-performing birds and are associated with beneficial metabolic outputs and host–microbe interactions. Targeting this core microbiota through diet, management, or microbial interventions rather than attempting to reshape the entire community may offer a more predictable path to improve growth and feed efficiency. The role of early-life microbiota establishment is particularly important, as germ-free animal studies[14,15] show that microbial colonization is essential for proper immune and metabolic programming, and the absence of stable colonizers leads to long-term physiological deficits.

Taken together, the microbiota appears closely linked with flock uniformity, although the present results cannot establish causality. Future studies employing mechanistic approaches, such as fecal microbiota transplantation or longitudinal mediation analyses, will be required to determine whether the microbiota actively contributes to or merely reflects growth divergence. Establishing a mature, robust, and metabolically beneficial microbiota early in life is likely associated with improved performance and flock uniformity, whereas delayed or dysbiotic colonization correlates with growth heterogeneity and long-term production disadvantages.

7.1.4 Visceral organ development

In Chapter 3, the development of visceral organs was explored in relation to BW variability among broilers, highlighting associations with growth performance. Heavier relative weights of visceral organs, such as the GIT and heart, are associated with higher nutrient demands and increased maintenance energy requirements, which may reduce the energy available for growth[16].

Our findings support an association consistent with a resource allocation model wherein LBW birds face greater metabolic costs due to disproportionately large visceral organs. This increased energetic burden may be linked to reduced growth potential. Additionally, the enlargement of immune-related organs like the bursa of Fabricius in LBW birds is associated with sustained or elevated immune investment, suggesting that growth limitations could be influenced not only by digestive inefficiency but also by immune system demands.

In contrast, HBW birds exhibited shorter small intestines but better-developed mucosal structures, specifically higher villus height and villus height-to-crypt depth ratios. This pattern suggests that intestinal length alone does not determine growth efficiency, as absorptive capacity is more dependent on villus structure. Instead, HBW birds appear to achieve superior nutrient absorption through more metabolically efficient gut structures, potentially reducing the energy cost of maintaining unnecessary tissue and thereby supporting improved growth rates[17].

While increased weights of the stomach, liver, and pancreas are typically indicators of enhanced digestive function[18,19], Pearson correlation analyses in our study revealed these organs were actually heavier in LBW birds particularly during later growth satge. This may reflect a adaptive response, where LBW birds expand their digestive organ mass in an attempt to boost nutrient absorption and support catch-up growth. Such compensatory development could explain why LBW chickens achieved ADG comparable to HBW birds during the finisher phase, despite early-life disadvantages. Similar findings were observed in chapter 6, where structural components of diets (Coarse corn and oat hulls) enhanced the gizzard size, which led to improved growth performance of low weight broilers. This suggests that enhancing gut organ development (i.e., Gizzard size) through targeted nutrition and feed structure may accelerate the birds' natural compensatory growth processes, enabling them to overcome early-life deficiencies sooner rather than later and allowing low-performing birds to close the performance gap with their high-performing counterparts well before slaughter age, thereby improving flock uniformity and overall production efficiency.

7.1.5 Intestinal permeability, morphology and nutrient absorption

Chapter 3 revealed that the disparity in growth performance between HBW and LBW chickens can be interpreted in association with differences in intestinal morphology, permeability, and gene expression patterns. HBW birds demonstrated a more functionally efficient gut structure, characterized by taller villi and shallower crypts, which are associated with increased absorptive surface area while potentially reducing the metabolic cost of epithelial renewal. This favorable morphology was accompanied by elevated expression of tight junction genes (*CLDN1*, *CLDN5*, *ZO-1*, *ZO-2*) and mucin genes (*MUC2*), which together reinforce epithelial cohesion and barrier integrity. A tightly regulated epithelial barrier minimizes paracellular permeability and prevents the leakage of luminal antigens and toxins that would otherwise trigger systemic immune responses. In this stable intestinal environment, HBW birds also showed enhanced expression of nutrient transporter genes, such as those encoding amino acid transporters (*SLC1A5*, *SLC7A1*) and glucose transporters (*SGLT1*, *GLUT2*), which is associated with more efficient nutrient uptake and may support superior growth performance.

In contrast, LBW birds exhibited shorter villi and deeper crypts, reflecting a tissue in a heightened state of turnover, possibly associated with inflammation or barrier disruption. These structural features were paralleled by downregulation of tight junction and mucin genes, potentially leading to increased intestinal permeability and a "leaky gut" phenotype linked to inefficient nutrient absorption and chronic immune activation. Compounding this, LBW birds showed lower expression of key nutrient transporter genes, suggesting that even when feed is consumed, the gut is less capable of extracting and utilizing critical macronutrients. Together, these morphological and molecular features in LBW birds create a metabolically costly intestinal environment that diverts resources away from growth toward maintenance and repair, reinforcing a cycle of stunted development. This integrated pattern indicates that growth potential in chickens is not solely dictated by nutrient intake but by the gut's capacity to function as a highly regulated interface between digestion, immunity, and systemic metabolism.

Across all our studies, a consistent pattern emerged that LBW broilers suffer from both structural and functional impairments in the gut that limit their growth potential. Structural dietary components like oat hulls (OH) stimulated mucosal development, increasing villus height and, consequently, absorptive surface area. This morphological improvement was accompanied by the activation of nutrient-sensing and transporter pathways, indicating not only a restoration of structural capacity but also an enhanced functional readiness of the gut to detect, absorb, and utilize key nutrients (Chapter 6). Importantly, dietary structure also supported barrier integrity through the upregulation of tight junction proteins, contributing to reduced intestinal permeability and a more stable internal environment for nutrient assimilation. Similarly, in ovo sodium butyrate (SB) stimulation improved gut barrier function, as evidenced by increased expression of tight junction and mucin genes and underperforming broilers appeared to benefit most from SB administration (Chapter 5). This suggest that OH inclusion and in ovo SB injection can effectively accelerate the birds' natural growth processes by restoring both the form and function of the gut earlier in the production cycle, that may allow LBW birds to overcome early-life disadvantages before slaughter age.

7.1.6 Immune burden diverts growth resources

One of the consistent physiological differences between HBW and LBW broilers observed across trials lies in immune system activation. Birds mounting a sustained immune response often due to subclinical infections or an imbalanced gut microbiota divert essential metabolic resources away from growth processes. In commercial settings, where birds are continuously exposed to a variety of pathogens, both viral and bacterial, the immune system must therefore strike a balance between mounting sufficient protection and avoiding excessive energy expenditure that would otherwise support growth. In Chapter 3, LBW birds showed markedly elevated expression of immune and oxidative stress-related genes, including *TNF-α*, *IL-8*, *CYP450*, and *NOS2*, indicating underlying immune activation even in the absence of overt clinical disease. Mounting such responses is energetically costly, requiring amino acids, energy, and micronutrients for cytokine synthesis, immune cell proliferation, and detoxification processes. This low-grade immune stress likely represents a major contributor to the poor feed efficiency and stunted growth seen in underperforming birds, suggesting that host-driven intestinal variability, even within genetically similar birds, is a powerful source of performance divergence.

The link between gut health, microbiota composition, and immune activation is well established. Gut homeostasis depends on tightly regulated innate immune responses that can distinguish between 'self' and 'non-self,' eliminate pathogens, and avoid inappropriate inflammatory reactions[20]. Disruption of this balance can result in chronic inflammation and impaired growth performance[21]. Germ-free animal studies further demonstrate that the absence of microbial colonization impairs immune development, resulting in immature gut-associated lymphoid tissues, reduced cytokine responsiveness, and weaker pathogen defense, whereas colonization with beneficial consortia promotes balanced immune maturation[14]. In our microbiota-focused trials, LBW birds were enriched in pro-inflammatory taxa such as *Escherichia-Shigella*, known producers of lipopolysaccharides (LPS) that trigger inflammatory signaling cascades. This microbial stimulation, when coupled with impaired barrier integrity, creates a self-reinforcing cycle of inflammation, barrier damage, and nutrient loss. In contrast, HBW birds harbored more stable communities

dominated by obligate anaerobes with fermentative capacities that support gut homeostasis. These birds displayed a more regulated immune profile, with upregulation of anti-inflammatory mediators such as *GPR120* and antioxidant enzymes (*GPX7*, *HMOX2*, *XDH*), suggesting better control of oxidative stress and a reduced need for resource-diverting immune responses.

This reinforces the idea that strategic microbiota modulation either directly via microbial interventions or indirectly through dietary and early-life strategies could recalibrate immune responses in underperforming birds toward a more growth-compatible state. Our in ovo SB injection trial offers direct evidence that targeted early-life interventions can modulate immune tone in underperforming broilers. Low hatch-weight (LHW) chicks receiving 0.3% SB showed elevated *IL-10* expression, an anti-inflammatory cytokine that enhances barrier function and suppresses excessive immune activation, potentially conserving metabolic resources for tissue accretion. These findings align with the broader context showing that butyrate exerts its greatest benefits under conditions of physiological stress or compromised gut function, which are more common in underperforming birds. Comparative gut health and physiological differences between LBW and HBW broiler chickens are give in Fig. 7.1.

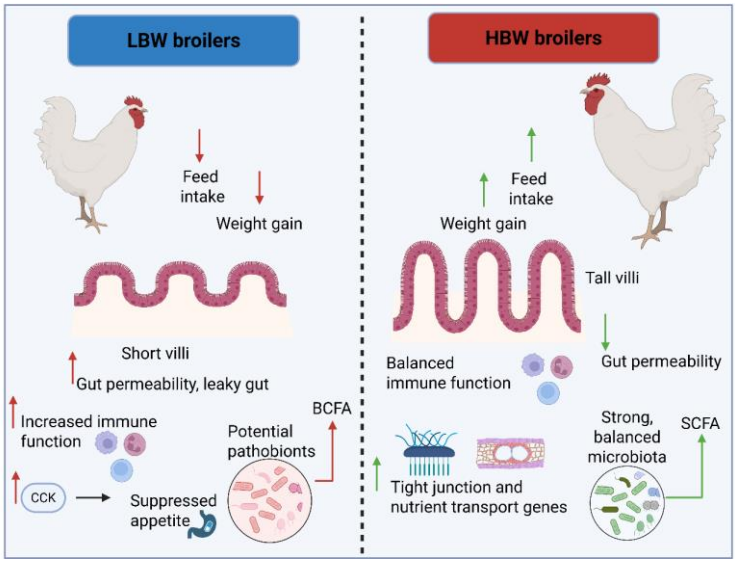

Fig. 7.1 Comparative gut health and physiological differences between low body weight (LBW) and high body weight (HBW) broiler chickens, adapted from Akram et al.[4,22]. LBW broilers exhibit lower feed intake and weight gain, shorter villi, higher gut permeability (leaky gut), elevated immune activation, and higher levels of potential pathobionts and branched-chain fatty acids (BCFA). These changes are associated with suppressed appetite via elevated cholecystokinin (CCK). In contrast, HBW broilers show enhanced feed intake and growth, taller villi, improved gut barrier integrity, balanced immune function, and a strong, diverse microbiota producing short-chain fatty acids (SCFA), supported by upregulation of tight junction and nutrient transport genes.

7.1.7 Strategies to support underperforming broilers

Building on the findings in Part 1, the second part of this thesis tested three practical interventions aimed at improving intestinal health and performance in underperforming broilers.

7.1.7.1 Hatching system and early access to feed

Across the trials in this thesis, early-life conditions emerged as a consistent determinant of developmental trajectories, with the hatching system (HS) providing a management based intervention in how immediate post-hatch environments shape gut, immune, and microbial outcomes. Hatching on-farm (HOF) system has been promoted within the poultry industry for its animal welfare and chick quality benefits primarily because it provides newly hatched chicks with direct access to feed and water, removes the need for transport during a sensitive developmental window[23]. However,

when evaluated in the context of this thesis' broader findings, the performance impact of HOF on final slaughter weight of low and high weight broilers was negligible, with no persistent differences in performance-related parameters compared to conventional HH birds. The transient nature of the growth advantage seen in HOF chicks limited to the initial few days and dissipating within the first week, aligns with the compensatory growth capacity observed in hatchery-hatched (HH) birds[24,25]. In modern broiler strains, moderate feed deprivation (around 40 hours in this study) fortunately results only in a transient growth effect, consistent with literature showing that persistent performance deficits typically arise only after deprivation exceeds 48–72 hours[26,27].

Although growth performance converged, the hatching system did have sustained effects on physiological traits. HOF chicks exhibited improved intestinal histomorphology, greater relative bursal weight, and higher expression of immune-related genes (*IL-6*, *IFN-γ*, *AVBD9*), indicating enhanced early gut and immune development. These adaptations might theoretically improve resilience in antibiotic-free systems or under higher pathogen pressure, conditions in which immune readiness has a clearer link to performance outcomes. Microbiota findings from Chapter 2 further integrated HOF into the broader thesis theme of microbial influence on growth. On day 7, HOF birds had higher alpha diversity and reduced relative abundance of potentially pathogenic taxa such as *Escherichia–Shigella*, paralleling the "favourable" microbial profiles observed in HBW birds. However, these differences in community composition were temporary, with no significant differences in beta diversity or microbiota profiles observed at slaughter age, suggesting that post-placement environmental and dietary factors exert a dominant shaping influence on the mature microbiome.

Recent comparative studies of conventional and on-farm hatching have highlighted another critical dimension: the microbiological characteristics of the hatching environment[28]. Factors such as farm-level sanitation, the microbiological status of eggshells, and the quality of litter material can influence early pathogen exposure, thereby affecting both hatchability and patterns of microbial colonization during the initial stages of life. These dynamics carry important biosecurity implications, as minimizing early

pathogen load may help preserve gut integrity and support immune homeostasis during the critical early growth window. Another factor warranting attention is the interaction between breeder flock age and hatching system. Offspring from young breeder flocks (<35 weeks of age) hatched in alternative systems have more frequently shown improved performance up to slaughter age compared with those from prime or older breeders[29,30]. This effect may be linked to the smaller body size and distinct body composition of chicks from younger breeders at hatch[31], potentially combined with differences in sensitivity to early-life stressors. Future research should investigate whether breeder age and HS can be strategically aligned to optimize early chick development, with particular focus on flocks derived from young breeders.

Taken together, the HOF findings in this thesis suggest that while the HS alone is unlikely to close the performance gap between high- and low-performing birds under typical commercial conditions, it can produce early-life physiological states that resemble those of inherently higher-performing birds better gut micro-architecture, more mature immune tissues, and a transiently more favorable microbiota. These traits may not translate into measurable performance gains in low-challenge environments but could become decisive under conditions of higher disease pressure, antibiotic-free production, or when integrated with other targeted early-life strategies identified in this work.

7.1.7.2 In ovo sodium butyrate injection

Across trials in this thesis, LHW and LBW broilers consistently exhibited a set of interlinked deficits: underdeveloped gut morphology, compromised barrier integrity, dysbiotic microbiota enriched with facultative anaerobes, and elevated expression of pro-inflammatory markers. These factors formed a physiological environment in which nutrient absorption efficiency was reduced, immune burden was elevated, and growth potential was constrained. High hatch-weight (HHW) and HBW birds, in contrast, shared a profile of well-developed villi, intact tight junction architecture, stable microbial communities dominated by SCFA-producing taxa, and a balanced immune–oxidative state. This recurring pattern across different trials suggested that any successful intervention for underperforming birds would need to address multiple interacting physiological systems rather than a single bottleneck.

The in ovo SB trial demonstrated that a single embryonic application of 0.3% SB at day 12 of incubation could partially reprogramme these early-life deficits in LHW birds. When contextualised alongside findings from other chapters, SB appears to exert its benefits through three converging pathways. First, it promoted barrier maturation, reflected in the upregulation of *CLDN1* and *TJP1* and the mucin gene *MUC6*, aligning with the barrier gene profiles observed in HBW birds in earlier studies (Chapter 3). Second, it modulated immune tone through elevated anti-inflammatory *IL-10* expression and reduced pro-inflammatory *IL-12p40* expression in LHW birds, counteracting the low-grade inflammation identified as a performance-limiting factor in the immune activation analyses (Chapter 3). Third, SB accelerated the establishment of a microbiota profile enriched in obligate anaerobes such as *Faecalibacterium* and *Anaerotruncus*, taxa repeatedly associated with higher fermentation capacity and carbohydrate degradation potential in the microbiota divergence studies between low and high BW broilers (Chapter 2).

The effects of in ovo SB were more pronounced in LHW chicks. Unlike HHW birds, which begin life with more mature intestinal architecture and a relatively balanced microbiota, LHW chicks have underdeveloped guts and limited endogenous butyrate production due to delayed colonization by SCFA-producing microbes. As a result, their gut development depends more heavily on external interventions. In ovo SB administration helps compensate for this deficit by delivering butyrate during a critical window of gut receptivity, effectively elevating the growth trajectory of LHW chicks to match that of HHW birds. Although SB provided some benefits to HHW chicks, the impact was limited, likely due to their inherently robust gut development. These findings align with previous research showing that butyrate has reduced efficacy in unchallenged or healthy birds.

These convergences suggest that SB's primary role in LHW broilers may be as a modulator synchronising gut epithelial maturation, immune regulation, and microbial succession in a way that more closely resembles the developmental trajectory of high-performing birds. One novel hypothesis emerging from this integration is that SB acts via dual routes: (1) direct modulation of epithelial gene expression through its histone

deacetylase inhibitor activity, leading to persistent changes in barrier and immune regulation; and (2) indirect shaping of microbiota by altering the biochemical and mucosal environment available for colonisation. This second route aligns with the possibility that SB modifies the endogenous egg-associated microbiome during incubation or primes the gut epithelium to secrete mucins and antimicrobial peptides that shape early colonization, although this remains to be experimentally verified.

From an industry perspective, these findings support the concept of precision prenatal nutrition, where interventions are targeted specifically to at-risk populations such as chicks from younger breeder flocks, those with predicted low hatch weights, or those hatched under suboptimal incubation conditions. However, before such strategies can be applied, it will be important to evaluate whether the effects of SB differ according to breeder flock age. By initiating compensatory growth processes earlier in the production cycle, SB application could help narrow performance variability within flocks, reducing the need for later-life dietary or management corrections. Importantly, the narrow therapeutic window observed in this study underscores the need for careful dose optimisation, as excessive SB (0.5%) provoked inflammatory responses and microbiota disruption, particularly in already robust HHW birds. This research also opens further research avenues. Combining in ovo SB with on-farm hatching or post-hatch dietary interventions may yield additive or synergistic effects on gut function and microbiota stability. Expanding this work into breeder flocks could also reveal whether prenatal microbiota modulation and transcriptomic changes have intergenerational effects, potentially shifting the baseline gut health of commercial broiler populations.

7.1.7.3 Post-hatch coarse particles and oat hulls

Inclusion of dietary coarse corn (CC) or OH improved the performance and gut health of LBW birds, mitigating the growth disadvantages observed in LBW broilers fed the control diet. Notably, supplementation with 3% OH had the most pronounced effect, narrowing the body weight gap between LBW fed OH and HBW control birds by day 38, primarily through increased feed intake rather than enhanced feed efficiency. OH inclusion led to several physiological improvements with beneficial impact on productivity: increased gizzard and pancreas size, enhanced ileal villus

height, reduced intestinal permeability (as shown by lower FITC-dextran levels), and upregulated expression of tight-junction proteins (*CLDN1*, *CLDN4*, *CLDN5*), nutrient transporters (*SLC15A1*, *SLC1A4*, *SLC2A1*, *VDR*), and immune markers (*NOS2*, *TLR4*). Cecal microbiota shifted toward beneficial taxa (e.g., *Faecalibacterium*, *Blautia*), with reduced levels of BCFAs, indicating improved gut health.

CC alone improved early microbial richness and peptide transport but was less effective than OH. Mechanistically, CC primarily enhanced gizzard stimulation, while OH offered both mechanical effects and fermentable lignocellulose that promoted SCFA-related benefits. However, the lack of SCFA increase in the OH group, suggests limited fermentation in the cecum. This could result from low fermentability of OH or inadequate particle size allowing fiber to reach the caecum. Future studies should examine OH fermentability using in vitro fermentation models and assess in vivo particle size distribution along the gut to determine fiber availability in the caecum. The structural characteristics of insoluble fibers create a bulky matrix that increases digesta-enzyme interaction, improving nutrient digestibility and absorption[32]. This likely explains the activation of nutrient transporter genes in the OH group, aligning with findings by Kheravii et al.[33]. OH may also stimulate pancreatic amylase secretion[34], supporting improved carbohydrate digestion and glucose uptake. However, digestive efficiency could be better characterized by measuring apparent ileal nutrient digestibility and the activity of pancreatic and brush-border enzymes, which would confirm whether observed improvements in performance are truly driven by enhanced nutrient utilization. Gut transit time, inferred to be slowed by structural diets, was also not directly measured. Assessing it using markers like titanium dioxide or chromic oxide could confirm whether prolonged digesta retention enhances nutrient absorption and utilization.

Gizzard development emerges as a central mediator of these benefits. Increased muscular activity in OH-fed birds is likely to reduce gizzard pH, increasing gut retention time, improving protein digestion and limiting pathogenic bacterial survival. The balance between insoluble and soluble fiber is relevant, as excessive viscosity from soluble fractions could offset the positive effects of enhanced gizzard function on nutrient utilization.

Gut transit time was also not directly measured. Assessing it using markers like titanium dioxide or chromic oxide could confirm whether prolonged digesta retention enhances nutrient absorption and utilization. Another overlooked factor is gizzard pH. Future work should evaluate gizzard pH to clarify its role in protein digestion and microbial control.

Finally, pellet physical quality, specifically hardness and durability post-pelleting was not assessed. Maintaining coarse particles in broiler pellets is challenging, especially in high-throughput feed mills using conditioning with expander. The intense mechanical and thermal processes often break down larger particles into finer fractions. Balancing particle preservation with pellet durability and process efficiency is critical, as coarse particles are nutritionally beneficial but prone to degradation during production.

7.1.7.4 Are high-performing broilers equally responsive to interventions as low-weight broilers?

In Chapter 5, an in ovo SB injection was applied to both low and high hatch-weight broilers, whereas feed structure modifications in Chapter 6 were implemented exclusively in low-weight birds due to barn space limitations. This raises a key question: Would high hatch-weight broilers respond equally to such interventions, and if so, would the performance gap and BW variability between groups remain unchanged, thus limiting improvements in overall flock uniformity?

Evidence from Chapter 5 and prior studies indicates that low hatch-weight broilers are more physiologically responsive to early-life interventions targeting the gastrointestinal tract. This increased responsiveness likely reflects their developmental limitations at hatch delayed intestinal maturation, compromised intestinal architecture, reduced barrier function, and an underdeveloped or dysbiotic microbiota. These deficits provide greater scope for interventions to produce measurable gains in growth, gut health, and nutrient utilization. High-weight broilers, in contrast, start with more advanced gut development and a favorable microbial profile, leaving less room for further enhancement. As a result, their responses to interventions are typically smaller and less impactful on performance.

Unlike in monogastric mammals such as humans or pigs, individualized or precision interventions are not practically feasible in poultry systems, where birds are reared in large groups in uniform environmental and management conditions. As a result, any strategy must be implemented at the population level. Although such a uniform approach does not allow for individualized optimization, it has the potential to elevate the performance of the flock as a whole, particularly by amplifying improvements in the lower-performing (i.e., low-weight) subpopulation. This targeted benefit could help reduce inter-individual BW variability and promote greater uniformity within the flock, a desirable outcome in commercial broiler production for both processing efficiency and economic return.

7.1.7.5 Practical implications of investigated strategies for underperforming broilers

In the current study, we explored three early-life intervention strategies i.e. on-farm hatching, in ovo SB injection, and feed structure modification, with the aim of enhancing growth performance, intestinal health, and overall productivity in broilers. These approaches were tested independently to evaluate their potential application within modern commercial production systems. From this investigation, a central question emerged: which of these strategies offers the most practical and sustainable benefits for large-scale broiler production?

On-farm hatching has gained popularity due to its welfare-oriented benefits. By allowing chicks to hatch directly on the farm, this method avoids the stressors associated with post-hatch handling and transportation. While it provides a smoother transition into the production environment and supports early feed and water access, its performance-related effects particularly on growth and intestinal development tend to be transient. The initial advantages often diminish over time, suggesting that while on-farm hatching supports chick welfare, it may not be a reliable strategy for long-term performance optimization, particularly since it also extends the overall production cycle by approximately three days due to on-farm incubation.

In contrast, in ovo SB stimulation offers more direct biological stimulation during embryogenesis. By targeting embryonic development, these interventions whether nutritional, immunological, or microbial can

influence early gut developmental pathways through epigenetic and trophic mechanisms. However, the practical integration of in ovo technologies into standard hatchery operations remains a significant challenge. In ovo stimulation, typically applied during the second week of incubation, remains difficult to synchronize with standard hatchery protocols. While in ovo feeding, applied around day 18 during the transfer from setter to hatcher, can coincide with hatchery operations and in ovo vaccination protocols, it remains essential to investigate whether such interventions interfere with vaccine efficacy. Despite promising results, adoption of in ovo technologies in commercial settings is limited, and further development is needed for commercial standardization and scalability.

Of the three, feed structure modification emerges as the most practical and industry-ready intervention. Physical structure in the diet particularly from coarse particles stimulates gizzard function, enhances intestinal development, and supports overall digestive efficiency. Achieving and maintaining optimal particle size, however, is technically challenging in feed mills, as mechanical and thermal processing during pelleting can reduce particle size and compromise the benefits. Beyond particle size, specific ingredients such as agricultural by-products offer both structural and nutritional benefits as well as align with sustainable feed sourcing strategies. As the industry shifts toward utilizing non-human-edible feed resources, by-products often rich in insoluble fibers have gained attention. The perception of fiber in poultry nutrition has shifted from being viewed as a diluent or even an anti-nutritional factor, to a functional component with clear gut health benefits. While broilers can tolerate 3–5% inclusion of insoluble fiber without adverse effects, results from higher inclusion rates have been inconsistent. In our research, oat hulls improved gizzard development, gut integrity, and nutrient transporter expression in LBW birds. Their combined physical and chemical properties suggest they may influence gut physiology beyond mechanical effects. This approach is easily implemented in feed manufacturing and aligns with the industry's move toward circular, resource-efficient production systems. Future work should explore other by-product feed sources and determine optimal inclusion levels for maximizing both gut health and sustainability outcomes.

7.2 Future perspectives

7.2.1 Gut-brain axis in feed intake regulation

The observed differences in feed intake between high and low BW broilers suggest a potentially critical link between feed intake regulation and growth dynamics. High weight chickens consistently consumed more feed, thereby supporting greater growth, while low weight chickens consumed less, resulting in diminished growth performance. These observations imply that feed intake is a key driver of growth differences and is subject to multifactorial regulation.

Feed intake is not solely governed by peripheral signals from the gastrointestinal tract but is also centrally regulated through the hypothalamus and other brain regions via neuropeptides such as neuropeptide Y (NPY), agouti-related peptide (AgRP), proopiomelanocortin (POMC), and others[35]. Importantly, emerging evidence suggests that gut-derived factors, particularly the intestinal microbiota, can modulate central appetite-regulating pathways through the microbiota-gut-brain axis[36] (Fig. 7.2).

Understanding how the gut microbiota influences neuroendocrine signaling involved in feed intake may provide novel insights into the mechanisms underpinning growth disparities in broilers. Microbial metabolites, such as SCFAs, secondary bile acids, and tryptophan-derived compounds, are known to interact with host receptors and potentially alter the expression of central appetite-regulating genes[37]. Therefore, characterizing the microbiota-gut-brain axis in both high and low weight broiler phenotypes could shed light on how microbial signals affect feed intake behavior and overall growth.

Future studies focusing on this axis may help in identifying microbial biomarkers or targets for nutritional or microbial interventions to optimize feed efficiency and growth performance. This line of research could be especially valuable for precision poultry farming, where tailored strategies could be developed to enhance productivity while maintaining animal health and welfare.

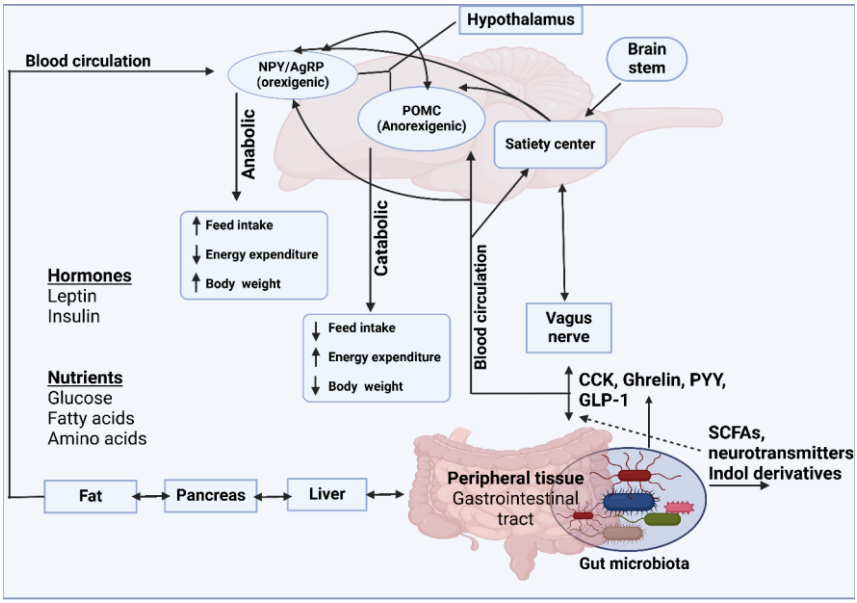

Fig. 7.2 Gut–brain axis in the feed intake regulation in avian species. The figure depicts bidirectional communication between the gastrointestinal tract, peripheral tissues, and the central nervous system. Nutrients and hormones from adipose tissue, pancreas, and liver signal to hypothalamic orexigenic (NPY/AgRP) and anorexigenic (POMC) neurons, modulating feed intake, energy expenditure, and body weight. Gut-derived hormones (CCK, ghrelin, PYY, GLP-1) and microbiota metabolites (SCFAs, neurotransmitters, indole derivatives) act via the vagus nerve and circulation to influence satiety centers and brain stem pathways, integrating metabolic and appetite regulation.

7.2.2 Microbiota engineering for growth optimization

Unlike mammals, early-life microbiota colonization in commercial poultry is primarily shaped by the external environment. This is largely due to the absence of maternal-offspring interaction, as chicks hatch in sanitized incubators under relatively sterile conditions. As a result, the initial gut microbiota of newly hatched chicks remains unsaturated and highly susceptible to environmental influences[38]. This presents a critical window of opportunity to modulate microbial colonization and influence long-term physiological outcomes, including growth, immunity, and behavior.

One promising approach is microbiota transplantation (MT), where gut microbiota from high-performing donor birds are transferred to recipient birds, typically via oral gavage. This technique has shown success in various animal models, including poultry, by promoting more favorable microbial

colonization patterns that correlate with improved growth and feed efficiency[38]. In broilers, MT from high weight phenotypes to low weight birds may serve as a tool to modulate the gut environment in a way that supports better nutrient absorption, enhanced feed intake, and ultimately, improved growth outcomes. Alternatively, less invasive and scalable methods such as spraying microbiota onto eggs, chick transport boxes, feed or litter material have gained attention (Fig. 7.3). Adding microbial supplements, especially probiotics, directly to feed can be challenging, as the pelleting process involves high heat and pressure, which may reduce microbial viability and efficacy. However, these methods allow for early-life microbial exposure, facilitating microbiota colonization during the critical window of immune and metabolic development. Such early interventions may help establish a stable and beneficial microbiota that persists throughout the production cycle.

These microbiota engineering strategies represent practical and potentially cost-effective solutions for improving performance traits in broilers. Future work should focus on identifying optimal donor profiles, standardizing MT protocols, and characterizing the longitudinal effects of microbial interventions on growth, feed conversion ratio, gut integrity, and host-microbe interactions. Additionally, integrating microbiome-based approaches with precision nutrition and genomics could enable the development of targeted microbial consortia tailored for specific broiler genotypes or production goals.

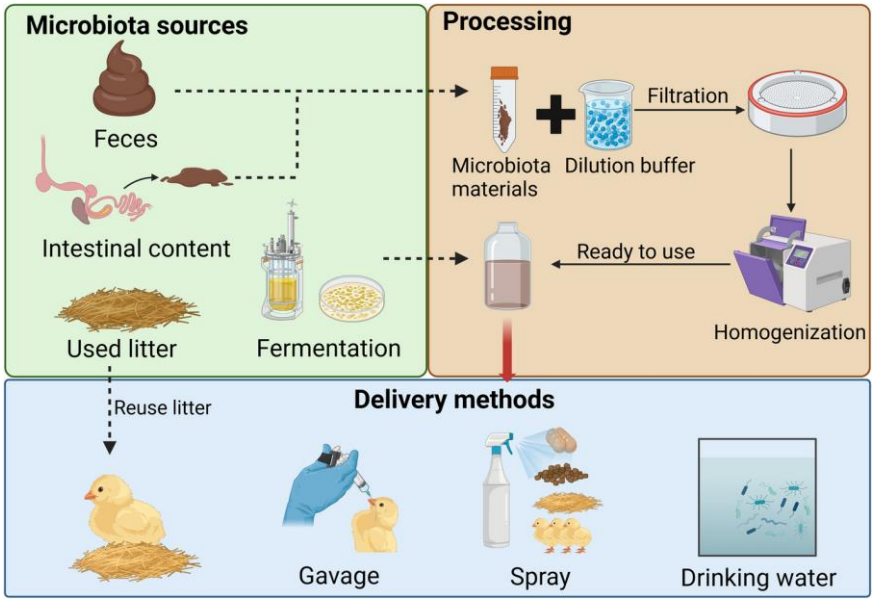

Figure 7.3 Overview of microbiota sources, processing steps, and delivery methods of microbiota transplantation (MT). Microbiota sources for MT include feces, intestinal content, fermentation products, and used litter. Feces and intestinal content are typically diluted with a buffer (e.g., saline or phosphate-buffered saline), homogenized, and filtered to remove large particles, after which they are ready for use. Fermentation products are generated by culturing healthy donor microbiota in nutrient-rich bioreactors for several days, resulting in a ready-to-use product. Delivery methods vary, with gavage being the most common approach. The prepared inoculum can also be sprayed onto eggs, feed, wood shavings, or directly onto chicks. Additionally, microbiota can be delivered through water. Reusing litter directly is also suggested, as it contains mature and abundant microbiota (Adapted from Zhao et al.[38]).

7.2.3 Integrative multi-omics and predictive modeling to understand gut physiology and flock performance

To comprehensively understand gut physiology and its role in lifetime performance, multi-omics approaches offer a powerful solution by integrating various layers of biological information (Fig. 7.4). Applying longitudinal and stage-specific multi-omics analyses combining transcriptomics, metabolomics, and microbiota profiling enables the construction of causal networks that link early-life gut development to long-term growth, health, and productivity outcomes in broilers. These integrative datasets support the development of network-based analytical frameworks, which leverage computational modeling and network analysis to identify key regulatory nodes and interactions between host

genes, microbial taxa, and metabolic pathways. In addition, incorporating machine learning algorithms and predictive models into these frameworks allows for the identification of early-life microbial or molecular biomarkers that are strongly associated with growth performance and flock uniformity. Such predictive tools can enable real-time monitoring of flock status, early diagnosis of underperforming subpopulations, and the implementation of targeted interventions. Ultimately, this approach facilitates precision livestock management by enabling proactive decision-making based on biological insights and data-driven predictions.

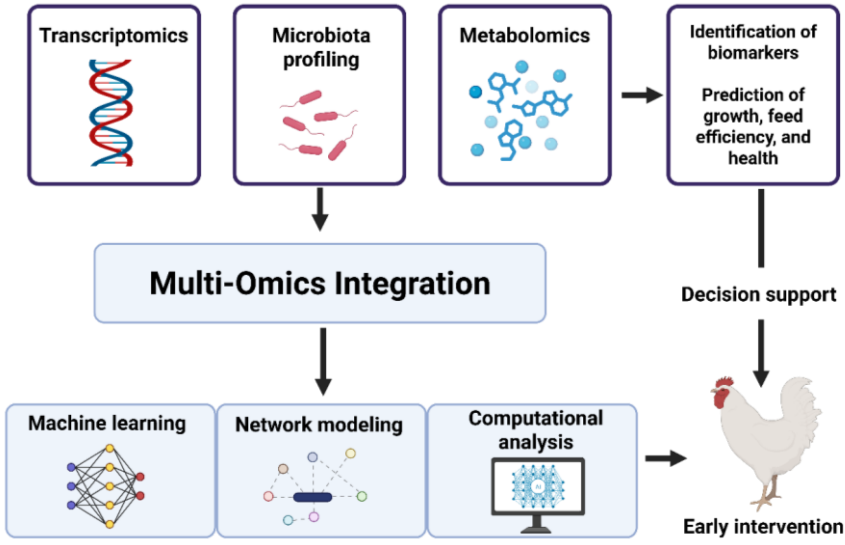

Figure 7.4 Integrative multi-omics framework for biomarker discovery and performance prediction in broilers. Transcriptomics, microbiota profiling, and metabolomics data are integrated to identify key biomarkers associated with growth, feed efficiency, and health. This multi-layered dataset is analyzed using machine learning, network modeling, and computational analysis to predict performance outcomes and guide targeted interventions in poultry production. Figure is created in Biorender.

7.2.4 Agroecology and its integration into poultry production

Industrial poultry production systems have primarily focused on maximizing output efficiency through genetic uniformity, high-energy diets, and controlled environments. These systems have succeeded in achieving remarkable productivity gains but often at the cost of increased environmental burden, dependence on imported feed, animal welfare concerns, and vulnerability to climate fluctuations[39,40]. Furthermore, the

global consolidation of breeding programs has narrowed genetic diversity and reduced the adaptive capacity of modern broiler genotypes to variable or low-input conditions[41].

Agroecology in poultry production refers to the application of ecological principles to design and manage sustainable and resilient farming systems that rely on natural processes rather than synthetic inputs[42]. It emphasizes optimizing the interactions between animals, environment, and management to improve nutrient cycling, animal health, and welfare while minimizing environmental impacts. Core aspects of agroecology include reducing external chemical inputs, enhancing biodiversity, recycling farm resources, and promoting adaptation to local ecological and socio-economic contexts[43].

Integrating agroecological principles into poultry production can counter these limitations by fostering systems that are more self-sufficient, resource-efficient, and locally adapted. Such integration promotes resilience to climate variability, reduces reliance on antimicrobials and external feed inputs, and aligns with growing societal expectations for ethical and sustainable food production. This transition requires reorienting breeding goals from maximizing productivity under controlled environments toward enhancing robustness, adaptability, and welfare in diverse production settings. Breeding for robustness enables poultry to maintain performance under fluctuating nutritional, climatic, and sanitary conditions, ensuring long-term system sustainability[44,45]. Therefore, future poultry breeding strategies should prioritize health, reproduction, and behavioral adaptability, accounting for genotype × environment interactions to identify strains best suited for agroecological systems[46,47].

7.2.5 Considerations on sample size and study power

When designing animal experiments, the number of animals should be determined through a priori power calculations based on the primary outcome, expected effect size, and variability. In our case, body weight was considered the primary outcome, while other parameters such as intestinal morphology, gene expression, and microbiota composition were exploratory. A group size of 10–12 birds can be sufficient when the expected effect size is large and the experimental unit is properly defined; however, it may limit the power to detect smaller differences, especially

in highly variable parameters such as microbiota composition. Since microbial communities are inherently plastic and influenced by multiple environmental and host factors, future studies should incorporate larger sample sizes or repeated measures to better capture biological variation.

7.3 General conclusions

This PhD research elucidates the complex gastrointestinal mechanisms underlying BW variability in broiler flocks, revealing why certain birds fail to achieve their genetic potential despite uniform management conditions. Growth variation is not solely the result of environmental factors but stems from fundamental biological differences that emerge early in development. Small differences in BW at the start of life lead to physiological changes that reinforce and amplify those differences as birds grow. A higher BW in the first week allows chicks to maintain an advantage over lighter counterparts, shaping differences in feed intake, gut physiology, immune balance, microbial colonization, and metabolic regulation. Across trials, HBW birds demonstrated superior nutrient absorption through optimized intestinal architecture, shorter relative gut length, and favorable microbiota that support metabolic and immune homeostasis. This configuration enables preferential nutrient partitioning toward muscle accretion rather than maintenance functions. In contrast, LBW birds become trapped in a metabolically costly compensatory state, where energy is diverted toward visceral organ hypertrophy, inflammatory responses, and gut barrier repair in response to increased intestinal permeability. LBW broilers consistently exhibited delayed gut development, impaired barrier integrity, downregulated nutrient transporter expression, and a dysbiotic, pro-inflammatory microbiota. These deficits translated into lower feed intake, reduced growth, and greater within-flock BW variability. While modern broilers possess substantial compensatory growth capacity, LBW birds often failed to fully recover before slaughter age, suggesting the need for targeted early-life interventions.

This research demonstrates that intra-flock BW variability is a modifiable trait and underperformance can be managed using strategies such as on-farm hatching, in ovo SB injection, and inclusion of structural components in broiler diets. On-farm hatching showed transient impact on the growth

performance of broilers; with minimal impact on intestinal health, indicates that on-farm hatching might primarily be used to advocate to provide stress free start and welfare-wise in poultry. In contrast, in ovo injection of SB on ED12 showed dose-dependent effects without compromising hatchability. Among all doses, impressive results were observed at 0.3% inclusion level, which modulated LHW broilers performance and intestinal health and microbiota characteristics. This suggests that LHW broilers owing to their initial intestinal challenges and setbacks, were more responsive to SB, compared to the HHW broilers which often has less gut related challenges. Although in ovo SB stimulation was applied uniformly to all eggs regardless of HW, LHW broilers exhibited greater responsiveness, indicating that such strategies can be implemented at the population level without the need for individual-specific interventions. However, standardization and scalability of in ovo injection technique are one the constraints for its implications in the broiler industry. Feed structure modification using CC and OH in LBW broilers appeared the most practical and impactful intervention, readily adaptable by feed mills. CC and OH both stimulated gizzard development, gut physiology and broiler growth; however, OH stand out might be due to its physical and chemical structures. OH, a source of insoluble fiber, might have provided not only physical structure, but also a substrate for bacterial fermentation to produce SCFAs and other beneficial metabolites, helping maintain gut health in a more positive way and reducing BW difference between low and high weight broilers.

This thesis provides an integrated understanding of the gut-related determinants of performance variability in broilers and offers practical, biologically grounded strategies to improve the growth and health of underperforming birds. By bridging biological findings with applied interventions, the work contributes to the development of precision poultry nutrition and management practices that enhance both productivity and animal welfare in modern broiler production systems.

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Appendices

Supporting information for Chapter 2

Table S2.1 Composition (%) of the feed offered to broilers during starter, grower, and finisher phases.

Ingredients %	Starter 1 – 14 days	Grower 15 – 28 days	Finisher 29 – 38 days
Maize	15.0	10.0	5.0
Wheat (fine)	33.73	39.20	48.88
Wheat (coarse)	5.0	10.0	10.0
Maize gluten	2.88	3.32	0
Soy oil	4.61	5.02	5.32
Soybean meal	29.86	24.79	25.42
Sunflower meal	2.5	2.5	0
Oat hull (coarse)	1.0	0	0
Sodium bicarbonate	0.23	0.23	0.25
Salt	0.16	0.16	0.16
Choline 75%	0.09	0.09	0.09
[1]Premix	0.30	0.30	0.30
Limestone	1.44	1.31	1.19
Monocalcium Phosphate	1.13	0.92	0.70
Lysine	0.55	0.52	0.30
Methionine	0.26	0.22	0.22
L-Threonine	0.14	0.12	0.09
L-Valine	0.02	0	0
[2]Avi-Deccox	0.05	0.05	0
L-Arginine	0.03	0.02	0.02
Palm oil spray	1.0	1.22	2.03
[3]Phytase	0.01	0.01	0.01
Chemical Composition			
Metabolizable energy (kcal/kg)	3000	3100	3200
Digestible lysine (%)	1.28	1.15	1.02
Crude protein (%)	23.0	21.5	19.5
Calcium (%)	0.96	0.87	0.78
Available phosphorus (%)	0.48	0.44	0.39
Sodium (%)	0.14	0.14	0.14
Chloride (%)	0.18	0.18	0.16
Potassium (%)	1.0	0.90	0.90

[1]Provided per kg feed: Vit A 10.0 IU, Vit D3 2750 IU, 25-hydroxycholecalciferol 0.06 mg, Vit E 90 mg, Copper 15 mg, Iron 15 mg, Manganese 85 mg, Zinc 50 mg, Iodine 2 mg, Selenium 0.4 mg. [2]Provided per kg feed: 30.3 mg decoquinate. [3]Provided per kg feed: 500 FTU.

Table S2.2 Relative abundance (%) of phyla in caecal samples of low (LBW) and high (HBW) body weight (BW) chickens hatched in the hatchery (HH) or on farm (HOF).

Phylum	[1]HS		BW		[2]Interaction				SD
	HH	HOF	LBW	HBW	HH-LBW	HH-HBW	HOF-LBW	HOF-HBW	
Day 7									
Firmicutes	99.7	99.7	99.6	99.8	99.6	99.8	99.7	99.7	0.47
Proteobacteria	0.23	0.19	0.32	0.11	0.39	0.08	0.25	0.13	0.289
Bacteroidota	0.03	0.04	0.00	0.07	0.00	0.05	0.00	0.08	0.333
Others	0.04	0.05	0.05	0.04	0.04	0.04	0.05	0.04	0.022
Day 14									
Firmicutes	97.4	97.6	97.3	97.7	97.2	97.5	97.3	97.8	1.27
Bacteroidota	2.18	1.93	2.24	1.87	2.38	1.98	2.11	1.76	1.060
Proteobacteria	0.26	0.23	0.26	0.22	0.27	0.25	0.25	0.20	0.435
Others	0.21	0.26	0.24	0.23	0.16	0.26	0.32	0.2	0.130
Day 38									
Firmicutes	93.8	94.8	93.7	94.9	93.7	93.9	93.8	95.9	3.66
Cyanobacteria	2.70	2.30	2.99	2.02	2.71	2.69	3.27	1.34	3.010
Bacteroidota	1.62	1.36	1.33	1.65	1.40	1.83	1.25	1.47	0.902
Proteobacteria	1.37	0.94	1.43	0.89	1.69	1.05	1.16	0.73	1.206
Others	0.48	0.6	0.58	0.5	0.48	0.47	0.65	0.53	0.201

[1]HS: hatching system. [2]HH-LBW: hatchery hatched low BW group (n = 10), HH-HBW: hatchery hatched high BW group (n = 10), HOF-LBW: hatched on-farm low BW group (n = 10), HOF-HBW: hatched on-farm high BW group (n = 10). Data presented as mean and pooled standard deviation (SD).

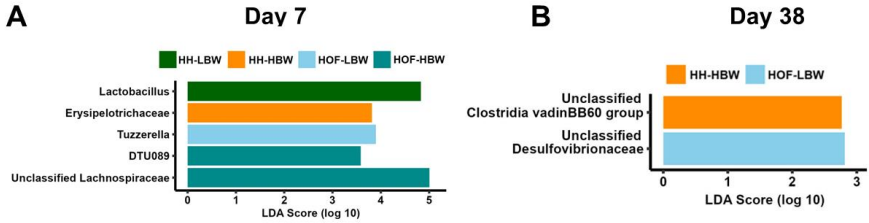

Fig. S2.1 Bacterial genera with significant interaction between hatching systems (HS) and body weight (BW) for differential analysis with LEfSe on day 7 (A) and day 38 (B). Only genera with FDR ≤ 0.05 and with an absolute value of LDA > 2 are presented.

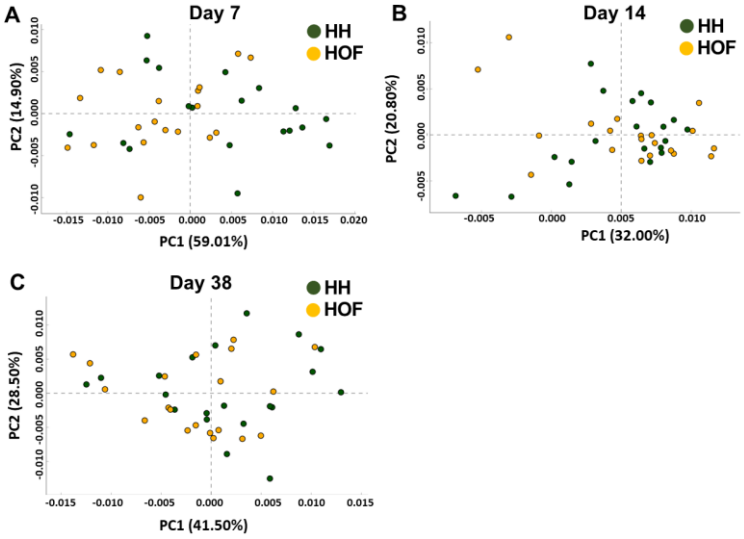

Fig. S2.2 Principal component analysis of predicted pathways of the differential microbiota in chicks hatched in the hatchery (HH) or on-farm (HOF) on day 7 (A), 14 (B), and 38 (C).

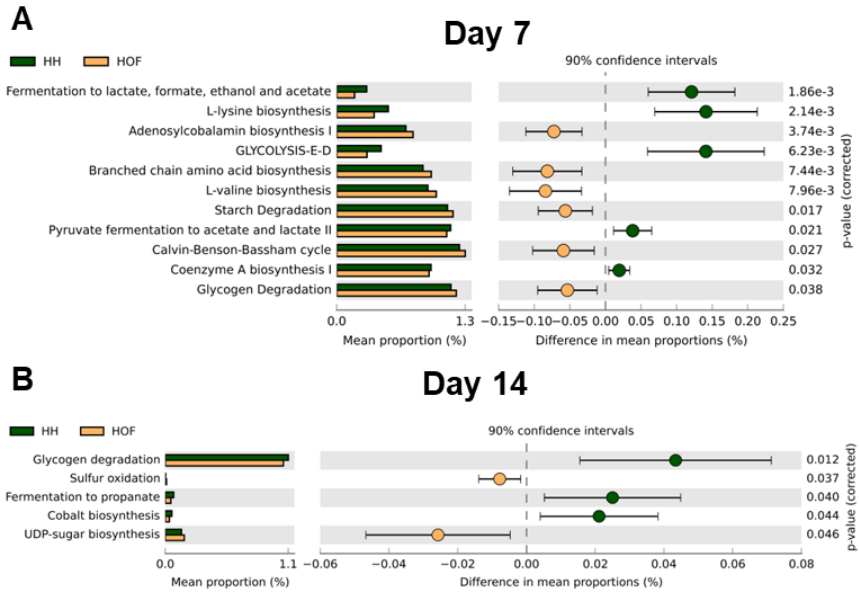

Fig. S2.3 Predicted functions of the cecal microbiota of broilers hatched in hatchery (HH) or on-farm (HOF) on day 7 (A) and day 14 (B). No differences were observed between HH and HOF chicks on day 38.

Supporting information for Chapter 3

Table S3.1 Primers used for high-throughput qPCR, and a brief description of their main function. There are 79 target genes and 13 reference genes in total.

Function	Gene	Description	Primers (5' → 3')	Reference
Barrier function	*CLDN1*	Transmembrane protein of tight junction (TJ)	F:CTTCATCATTGCAGGTCTGTCAG R:AAATCTGGTGTTAACGGGTGTG	[1]
	CLDN2	Transmembrane protein of TJ	F: ACTGCAGCTGCCCTCGGT R: AAGCTTCACCCTGCTGCTGT	This study
	CLDN3	Transmembrane protein of TJ	F: GCCAAGATCACCATCGTCTC R: CACCAGCGGGTTGTAGAAAT	[2]
	CLDN4	Transmembrane protein of TJ	F: CTGTGCCGGGACACTGAATG R: TCCTCCACAGTGGTGTTTGG	This study
	CLDN5	Transmembrane protein of TJ	F: GTCCCAGAAGCGGGAGATAG R: CGAGTACTTGACGGGGAAGG	This study
	OCLN	Protein of TJ involved in both inter-membrane and paracellular diffusion of small molecules	F:ACGGCAGCACCTACCTCAA R: GGGCGAAGAAGCAGATGAG	[1]
	ZO-1	Scaffold proteins that form part of the cytoplasmic plaque of TJ	F: ACCACAAGGAGCCATTCCAG R: GTGAGGCCCACACATTACCA	This study
	ZO-2	Scaffold proteins that form part of the cytoplasmic plaque of TJ	F: GCCCAGCAGATGGATTACTT R: TGGCCACTTTTCCACTTTTC	[2]
	JAM-2	Transmembrane protein of TJ	F:GGTACTTGGGGGTCTTCTGC R:TGTGCTTGCAACTAAGAATAGCC	This study
	JAM-3	Transmembrane protein of TJ	F: CCAGAGTGTTGAGCTGTCCT R: AGAATTTCTGCCCGAGTTGC	[3]
	MUC2	Secretory mucine important in the establishment of the mucus layer	F: CCCTGGAAGTAGAGGTGACTG R: TGACAAGCCATTGAAGGACA	[1]
	MUC13	Transmembrane mucine that plays a role in cell signalling pathways	F: CCAGGCACCAGAAGTGCTAA R: TGCGTACTGATGCACGTAGT	This study
	MUC5ac	Mucin 5ac	F: TGTGGTTGCTATGAGAATGGA R: TTGCCATGGTTTGTGCAT	[4]
	CDX	Intestinal tract development	F: ACAGCTGTCCCCTAATGCAC R: TCCTTTGTCCTCGTCTTGCC	This study
Gut hormone	*GHRL*	Ghrelin: Hunger hormone. Induces	F: AACTGCTCTGGCTGGCTCT R: CTCCCTCTGTTTCATCTGTAT	[5]

		motor activity in the intestinal tract		
	Proglucagone	Precurose of GLP-1	F: CACAAGGCACATTCACCAGT R: TTCTTTGGCAGCTTGACCTT	6
	Proglucagone B	Precursor of GLP-1	F: CACAAGGCACATTCACCAGT R:TGGTATTCTCCCAAAAGGTCTC	6
	PYY	Peptide tyrosine tyrosine. feed intake regulatory hormone	F: AGGAGATCGCGCAGTACTTCT R: TGCTGCGCTTCCCATACC	6
	CCK	Feed intake regulatory hormone	F: GAAGGTAGGGAGCGGCAC R: TCGGAAAAGGGGGAAAACGA	7
Nutrient receptors	*T1R1*	Taste receptor type 1 member 1	F: GTGTCATCCCCACAACCAA R: CACCACTGCCTCAAAGAAGG	5
	T1R3	Taste receptor type 1 member 3	F: CATTACCGTCTTCGCCACTC R: CTCTGTTCAAATCGGGCTTC	5
	GRP43	FFAR2 – activated by short chain fatty acids	F: AGGGAATCCGGGATGGAGAA R: ACGCAGTCAGGTTGGTTCAA	This study
	GPR41	FFAR3 – activated by short chain fatty acids	F: GAAGGTGGTTTGGGAGTGAA R: CAGAGGATTTGAGGCTGGAG	8
	GPR120	FFAR4 – activated by medium-chain and unsaturated long-chain FFAs	F: ACTTCACTGCTTTGCCTCAGT R: CCAGTACAAGTGGAGGGTTCA	This study
Immune response	*IL-4*	Cytokine that induces differentiation of naive helper T cells (Th0 cells) to Th2 cells	F:TTATGCAAAGCCTCCACAATTG R: GTGGGACATGGTGCCTTGAG	9
	IL-6	Humoral immunity related genes	F: CTCGTCCGGAACAACCTCAA R: GGAGAGCTTCGTCAGGCATT	This study
	IL-8	Secreted in response to pathogenic bacteria infection or specific inflammatory cytokines	F: AGATGTGAAGCTGACGCCAA R: GAGCTGAGCCTTGGCCATAA	This study
	IL-10	Anti-inflammatory cytokine produced by activated macrophages and T cell	F:CTGAGGGTGAAGTTTGAGGAAAT R:AGCCAAAGGTCCCCTTAAACTC	9
	IL-18	Pro-inflammatory cytokine. primarily produced by macrophages. targeting T helper type-1 (Th1) cells	F: CTCCTCCACACAGCAACACA R: ATGCAGTTGGCCACTTCTGT	This study
	IL-22	Commonly used as marker of inflammation involved in T-lymphocytes activation	F: ACATCAGGGAGAACAACCGC R: TGCCACATCCTCAGCATACG	This study
	IL-1β	Mediator of the inflammatory response and involved in cellular processes	F: CGCTACACCCGCTCACAGT R:GCAATGTTGAGCCTCACTTTCTG	9

	TLR2	Transmembrane receptor for the recognition of gram positive bacteria	F: AGGCGATCCCAAGAGGTTC R: TTTCCCAAAACATCTGCTGTTG	9
	TLR4	Transmembrane receptor for the recognition of gram negative bacteria	F: CAGTCCGTGCCTGGAGGT R:TTGAGCTTAGCAATTTCAGACT GTTG	9
	TNF-α	Regulation of the host immunity against multiple pathogens	F: TTGCGAGGGGAGAGGAGAAA R: GTCAGTACCGCGTCGTCTTT	This study
	AHSA1	Co-chaperone activator of HSP90	F: GGGGAAGCCTCCATCAACAA R: TCACTCCTGTGGTCGAGGT	This study
	AvBD6	Avian defense involved in antimicrobial functions and protecting the gut epithelium	F:CTTGCTGTGTGAGGAACAGGT G R: TTTGGTAGTTGCAGGCAGGAT	9
	AvBD9	Avian defense with antimicrobial properties and other cellular functions	F: CTGAGACCTCACTGACCACG R: GTGCTCCCAGGACTCTTCAC	9
	HSPA4	Member of HSP proteins and play a prominent role in repair and protection of the intestinal environment	F:TGAGACTAATAAATGAATCAA CTGCAGT R:CCCCATATCCACAAAAACAACA	9
	IFNG	Host defense for combating against the intracellular pathogens including Salmonella	F: ACCTTCCTGATGGCGTGAAG R: CTGAAGAGTTCATTCGCGGC	This study
	NOS2	Induce the development of Th1 type of IR in infections	F:CTCCAGCAGAGCTTCTACC TCAA R:GCCAGGTGCTCTTCTATTT TTAATTC	9
	PTGES	Intestinal inflammatory factor	F: GGCTCTGAGGACAATGCAGA R: CCAGAGGAGAGCACAGCAAA	This study
	Cox-1	Cyclo-oxygenase 1	F: GCGCATCAGTAGACCTAGCC R: TGGTATTGTGACAGTGCGGG	10
	Cox-2	Cyclo-oxygenase 2	F:ATTCCTGACCCACAAGGCAC R: AGTCAACCCCATGGCCGTAA	11
	Lox-12	Lipo-oxygenase	F: CTGATTACGCCGTGCTGGAT R: ATTGGGGCACACAGGAATGT	10
	CYP450	Cytochromes P450	F: ACCACTTCTGGAAGGAGGGA R: CGCTCTCGTAGACACCCAAC	10
Nutrient transport	*SLC15A1*	Peptide transporter-1	F: CAGGATTTCCCTGTGTCAGGT R: GCAGCGTGGACAAGTATGG	This study
	SLC1A1	Excitatory amino acid transporter	F: TGCTGCTTTGGATTCCAGTGT R:AGCAATGACTGTAGTGCAGAA GTAATATATG	12
	SLC1A4	Neutral amino acid transporter by ASC system	F: ACAGCAAGCTGTGGTCAGAA R:TCTCCCAGAATGCAATCACAGT	This study

	SLC3A1	Protein related to neutral amino acid transporter	F: CCCGCCGTTCAACAAGAG R:AATTAAATCCATCGACTCCTTTGC	[12]
	SLC7A9	Na+-independent neutral/cysteine. cationic amino acid exchanger	F:CAGTAGTGAATTCTCTGAGTGTGAAGCT R:GCAATGATTGCCACAACTACCA	[12]
	SLC6A19	Na+-dependent neutral amino acid transporter	F: CCAGAGGGCAATGTAACCCA R: AAGGCTAAGCCGGTTCCTTC	This study
	SLC7A1	Transport lysine. arginine. and histidine	F:CAAGAGGAAAACTCCAGTAATTGCA R:AAGTCGAAGAGGAAGGCCATAA	[12]
	SLC7A2	Transport lysine. arginine. and histidine	F: TGCTCGCGTTCCCAAGA R: GGCCCACAGTTCACCAACAG	[12]
	SLC7A5	Transport hydrophobic amino acids	F: ACGTGCAAGCTCACACCTAA R: CGAGGCCTCCTCAACTCTCA	This study
	SLC7A6	Na+-dependent neutral/cationic amino acid exchanger	F:GCCCTGTCAGTAAATCAGACAAGA R:TTCAGTTGCATTGTGTTTTGGTT	[12]
	SLC7A7	L amino acid transporter 2	F:CAGAAAACCTCAGAGCTCCCTTT R: TGAGTACAGAGCCAGCGCAAT	[12]
	SLC2A1	Glucose transporter-1	F: GCAAGATGACAGCTCGCCT R: GCTCCTCATATCGGTACAGCC	This study
	SLC2A2	Glucose transporter-2	F: CAGGAACGTTGGTCCTCTCC R: GCGCCCATAGTGTGCTTCTA	This study
	SLC5A1	Sodium glucose transporter 1	F: GCCATGGCCAGGGCTTA R:CAATAACCTGATCTGTGCACCAGTA	[12]
	SLC2A5	Transport fructose	F: AAAGAGCTGTAGGTGTGGGC R: CTTTTGCCTGGTTGCCTTCC	This study
	SLC5A9	Sodium glucose transporter-4	F:ATACCCAAGGTCATAGTCCCAAAC R: TGGGTCCCTGAACAAATGAAA	[12]
	FABP	Liver fatty acid binding protein	F: TGAATGTGGCTGGCTCGATTT R: CAGGTTGACCCCTCCTGTACG	This study
	FABP1	Fatty acid binding protein	F: CATCTTCTCTTGTGTTGGGAGC R: TGATCATCAGGAAGCCCGAG	This study
	FABP2	Related with epithelial cell content and occurrence	F: ATGGAAGCAATGGGCGTGAA R: TTCGATGTCGATGGTACGGA	This study
	FABP6	Necessary for the transport of bile acids in the gut and it is associated with bacterial presence and inflammation	F: CGGTCTCCCTGCTGACAAGA R: CCACCTCGGTGACTATTTTGC	[13]
	SLC34A2	Intestinal phosphate absorption and phosphate homeostasis	F:TGGGGAGAAAGAAGTGTCACAGA R: GTGAAGCCACGTTGCCTTTGT	This study

	VDR	Transcription factor that mediates the vitamin D3. involved in signalling intestinal calcium and phosphate absorption	F:GCAAAAGGCCGAGAAATGGG R: GAACACCCGTGGCAGATTCA	This study
	ATP1A1	ATPase Na+/K+ transporting subunit alpha 1 (Calcium transporter)	F:TGCAAATCCATCAGAATCTCGT R- TCCTCATCCAAGGGTTGCAC	This study
	SLC30A1	Efflux of Zn2+	F: TGGGTGATATGAAGGAC R: AACCTAAGGCATCTCCA	This study
	CALB1	Calcium transporter	F: GGCAGGCTTGGACTTAACACC R: GTCGGCAACACCTGAGCAAG	[1]
Metabolism	*Cox-16*	Enzyme involved in the generation of energy by the mitochondria	F:CCTGCTTTGAAGGAAAAATTGAAG R:CCAAGTCAGATTGTTCCAATTTCTC	[9]
	EIF4EBP1	mTOR pathway proteins—protein synthesis and cell proliferation	F:ATTGAGAACAACCATGTCCAGAAC R:ATGTCAAACTGCTCTTCTTCACCT	[9]
	mTOR	mTOR pathway proteins—protein synthesis and cell proliferation	F:TGCTGACAAACGCTATGGAGGT R:AGCCATGACACTGTCCTTATGCT	[9]
	RPS6KB1	mTOR pathway proteins—protein synthesis and cell proliferation	F:ACACCTGTTGATAGCCCAGATGA R:GCCACATACGTAAAACCCAGAAA	[9]
Oxidation	*GPX7*	Intracellular antioxidant. and plays a great role in the detoxification of various peroxides	F: GGTGCCTCCTTTCCTATGTTCA R:GTTGGTTCTTCTCCAGTAGAATCAA	[9]
	HIF1A	Transcription factor that regulates genes involved in inflammation and cell death	F:CACTTTTTCAGGCAGTTGGAATTG R: TTTTGCACGCCTTTACACGTT	[9]
	HMOX2	Oxidative stress marker	F: TCCAGTCCACGATGGGAAA R: GCATTGCCTGCTAGCTTGTCT	[9]
	SOD1	Antioxidant enzyme	F: CCGGCTTGTCTGATGGAGAT R: CTGCGCTGGTACACCCATTT	[9]
	XDH	Enzyme associated to the synthesis of reactive oxygen species and is member of cellular defense system	F:GAAGCCATTCCATTACTTCAGTTATG R:AATGTCTGTGCGGATGTTCTTG	[9]
Reference genes	*LBR*	Reference gene	F: CTAACCGTCGCTCAGGGC F: TCCAAAAGCAATACCTGGCG	This study
	NDUFA	Reference gene	F:TGTGCAGAAACTACAGGACAAACTG	[9]

			R:AGGGAAAGCTCATTTTCAGCCT	
	YWHAZ	Reference gene	F:GCAAGCAGAAAGCAAAGTTTTCT R: TGTGATTGCTCCACAATCCCT	[9]
	GAPDH	Reference gene	F: CGTGCAGCAGGAACACTA R: CAGATCGATGAAGGGATC	This study
	18S	Reference gene	F: ATTCCGATAACGAACGAGACT R: GGACATCTAAGGGCATCACA	[13]
	B-Actin	Reference gene	F:TGACTGACCGCGTTACT R: GACCCACGATAGATGGGAA	This study
	UB	Reference gene	F: GGGATGCAGATCTTCGTGAAA R:CTTGCCAGCAAAGATCAACCTT	[2]
	RPS7	Reference gene	F: GGCGCTGAGCGAGAAAGG R: CTCCAGGAGAGCCTGGGATA	This study
	B2M	Reference gene	F: TACTCCGACATGTCCTTCAACG R: TCAGAACTCGGGATCCCACTT	[2]
	GUSB	Reference gene	F: GGCAGACTGGTCCTGTTGTTG R:GGGTCCTGAGTGATGTCATTGA	[2]
	TBP	Reference gene	F: AGCTCTGGGATAGTGCCACAG R:ATAATAACAGCAGCAAAACGCTTG	[2]
	TUBAT	Reference gene	F:CAAGCATGAATGCCAACTCTCC R: TCACGCATGGTTCGTCCT	This study
	r28s	Reference gene	F: GGCGAAGCCAGAGGAAACT R: GACGACCGATTTGCACGTC	[2]

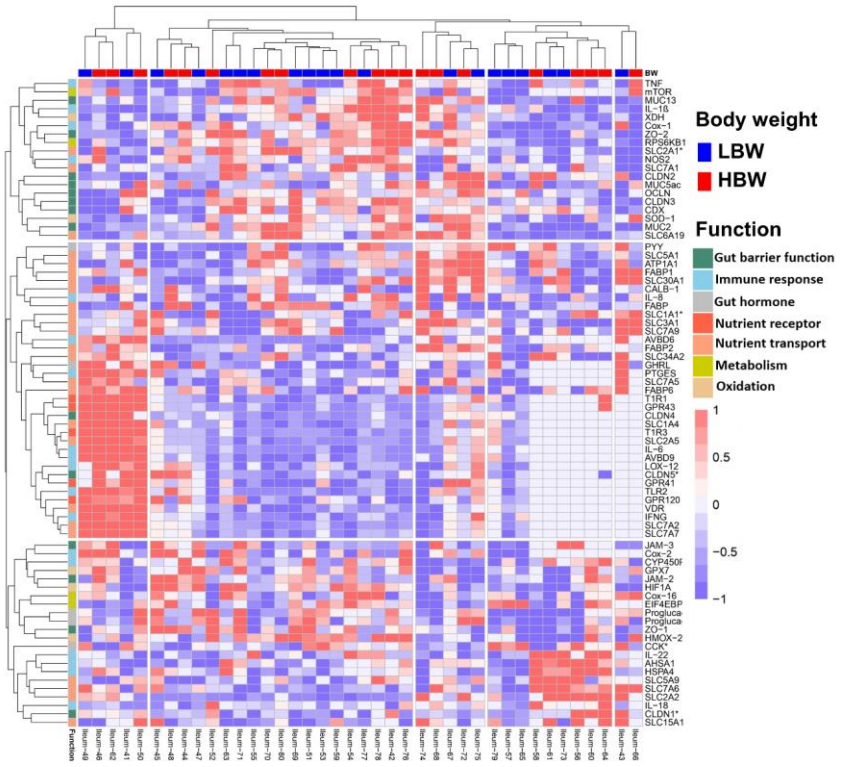

Fig. S3.1 Two-way hierarchical cluster analysis showing the expression level of the genes analyzed in the ileum between low (LBW, n = 20) and high (HBW, n = 20) body weight groups on day 14. Samples are represented on the x-axis and genes on the y-axis. The red color indicates high expression while blue indicates low expression. Gene functions (y-axis) were labeled with different colors. The dendrogram on the left of the heatmap clusters genes with similar expression patterns, while the dendrogram on the top groups samples with similar gene expression profiles. Genes with (*) indicate significant differences between BW groups based on the univariate analysis (Student's t-test).

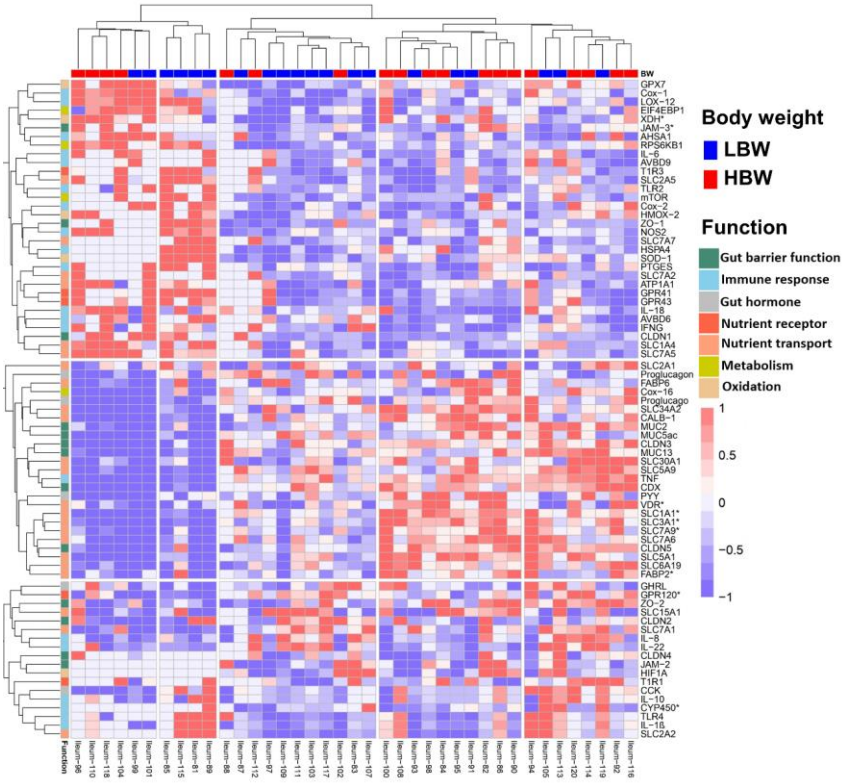

Fig. S3.2 Two-way hierarchical cluster analysis showing the expression level of the genes analyzed in the ileum between low (LBW, n = 20) and high (HBW, n = 19) body weight groups on day 38. Samples are represented on the x-axis and genes on the y-axis. The red color indicates high expression while blue indicates low expression. Gene functions (y-axis) were labeled with different colors. The dendrogram on the left of the heatmap clusters genes with similar expression patterns, while the dendrogram on the top groups samples with similar gene expression profiles. Genes with (*) indicate significant differences between BW groups based on the univariate analysis (Student's t-test).

Supporting information for Chapter 4

Table S4.1 Genes excluded from the statistical analysis due to lack of expression readouts.

Days	Gene names
Day 7	*CLDN4, JAM 3, T1R1, TLR4, SLC5A9, FABP, and FABP1*
Day 14	*IL-4, IL-10, and TLR4*
Day 38	*OCLN, IL-4, FABP, and FABP1*

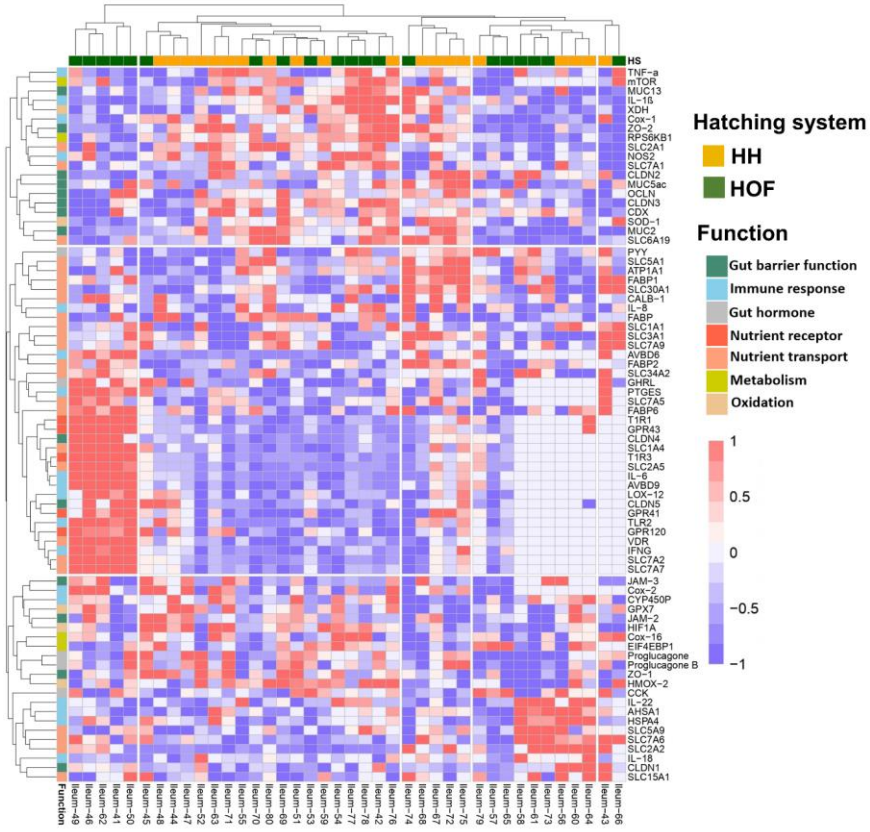

Fig. 4.1 Heatmap of ileal gene expression levels on day 14 of chickens hatched in hatchery (HH, n = 20) or on-farm (HOF, n = 20). The x-axis represents individual samples, while the y-axis shows the genes. Expression levels are color-coded, with red corresponding high expression and blue indicating low. Gene functions are denoted by different colors on the y-axis. The left dendrogram clusters genes with similar expression patterns, and the top dendrogram groups samples with similar gene expression profiles.

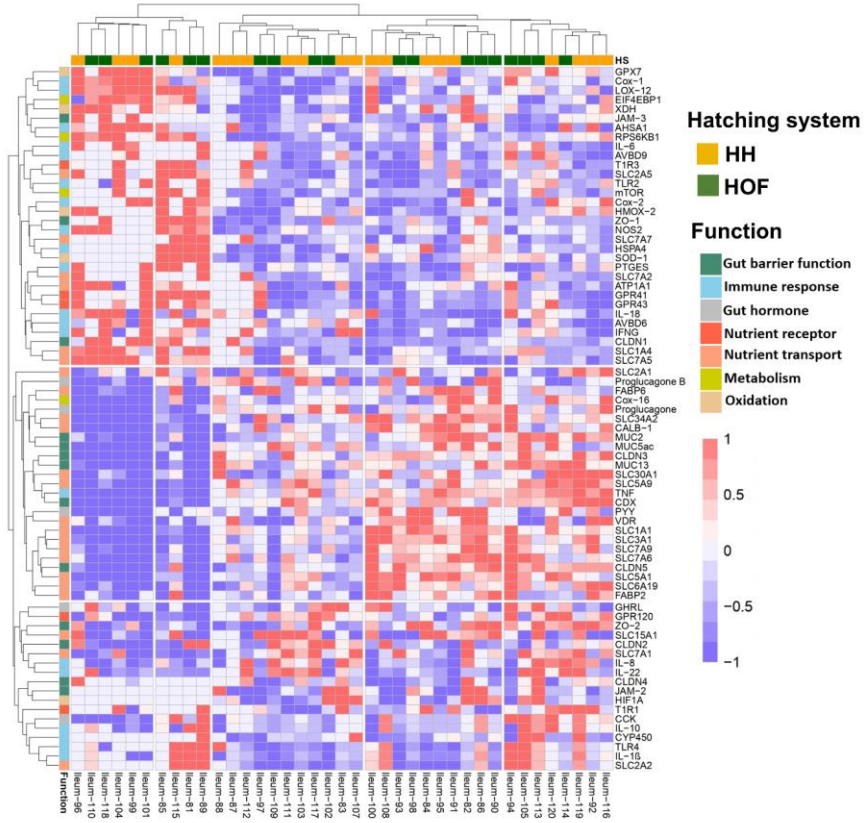

Fig. 4.2 Heatmap of ileal gene expression levels on day 38 of chickens hatched in hatchery (HH, n = 20) or on-farm (HOF, n = 19). The x-axis represents individual samples, while the y-axis shows the genes. Expression levels are color-coded, with red corresponding high expression and blue indicating low. Gene functions are denoted by different colors on the y-axis. The left dendrogram clusters genes with similar expression patterns, and the top dendrogram groups samples with similar gene expression profiles.

Supporting information for Chapter 5

Table S5.1 Primers used for gene expression and a brief description of their functions.

[1]Gene	Function	Primer sequences (5' to 3')	Reference
CLDN1	Barrier integrity and permeability regulation	F: TCTTCATCATTGCAGGTCTGTC R: AACGGGTGTGAAAGGGTCAT	[14]
TJP1	Regulates cellular tight junctions	F: AGGAAGCGATGAATCCCTGTT R: TCACTCAGATGCCAGATCCAA	[14]
MUC6	Produces protective mucus	F: TTCAACATTCAGTTCCGCCG R: TTGATGACACCGACACTCCT	[14]
IL-10	Anti-inflammatory cytokine: Interleukin 10	F: CATGCTGCTGGGCCTGAA R:CGTCTCCTTGATCTGCTTGATG	[14]
IL-1B	Pro-inflammatory cytokine: interleukin 1 beta	F: GGAGGTTTTTGAGCCCGTC R: TCGAAGATGTCGAAGGACTG	[14]
IL-12p40	Pro-inflammatory cytokine: interleukin-12 subunit p40	F: TTGCCGAAGAGCACCAGCCG R: CGGTGTGCTCCAGGTCTTGGG	[14]
ACTB	Reference gene: Actin beta	F:CACAGATCATGTTTGAGACCTT R: CATCACAATACCAGTGGTACG	[14]
G6DPH	Reference gene: Glucose-6-phosphate dehydrogenase	F: CGGGAACCAAATGCACTTCGT R: GGCTGCCGTAGAGGTATGGGA	[14]

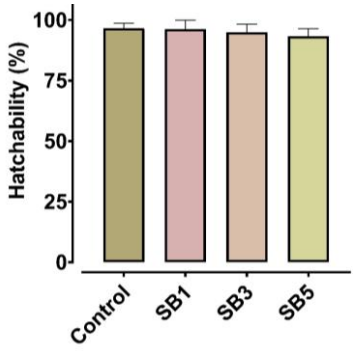

Fig. 5.1 Hatchability (%) of eggs subjected to in ovo injection of different sodium butyrate (SB) doses. The data was analysed by one-way ANOVA (n = 10 repetitions per treatment group). Control group received normal saline, while other groups received SB level of either 0.1% (SB1), 0.3% (SB3) or 0.5% (SB5).

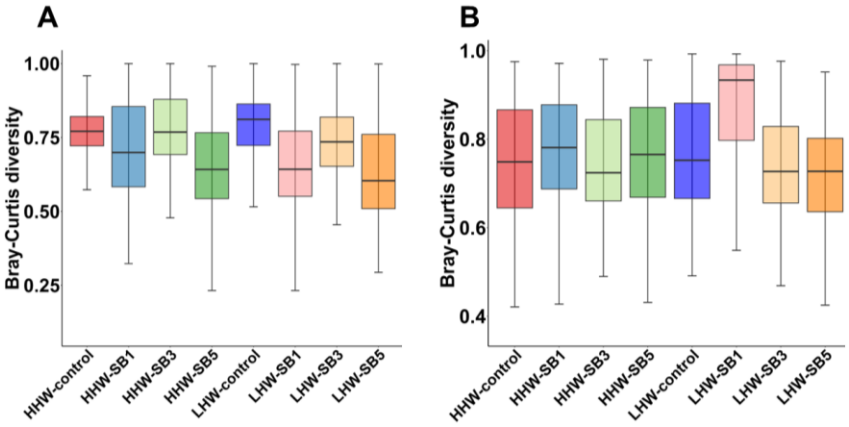

Fig. S5.2 Boxplot showing the pairwise Bray–Curtis dissimilarity between groups of high (HHW) and low (LHW) hatch weight (HW) chickens on day 14 (A) and day 42 (B) that had received three levels of in ovo sodium butyrate (SB1: 0.1%, SB3: 202 0.3%, SB5: 0.5%) or 0.9% NaCl (control). Individually sampled chickens were considered experimental units (n = 6 birds/group).

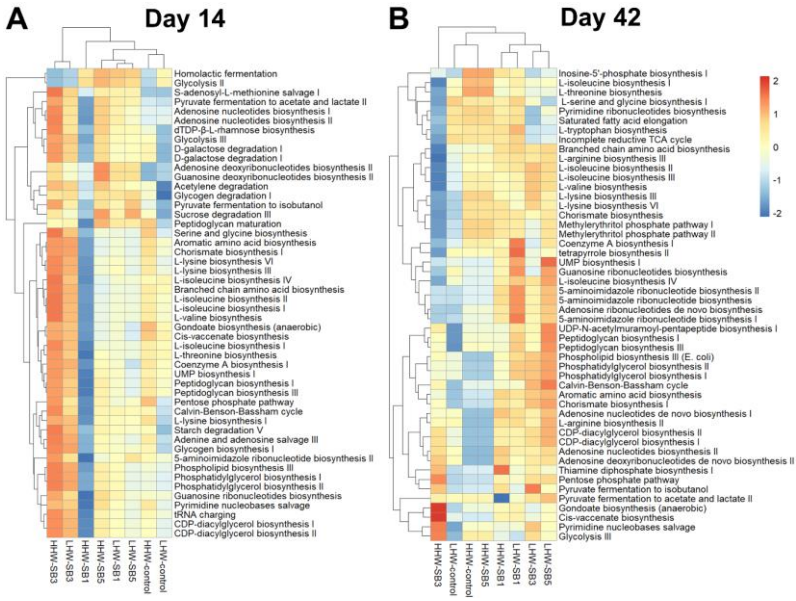

Fig. S5.3 Heat map of the top 50 predicted metabolic pathways by MetaCyc database in high (HHW) and low (LHW) hatch weight chickens on day 14 (A) and day 42 (B) that had received three levels of sodium butyrate (SB1: 0.1%, SB3: 0.3%, SB5: 0.5%) or 0.9% NaCl (control) in ovo. The red color indicates high relative abundance while blue indicates low relative abundance of metabolic pathways.

Supporting information for Chapter 6

Table S6.1 Relative abundance (%) of phyla in caecal samples of broiler chickens on d 14, 21 and 38.

Phylum	HBWC (*n* =12)	LBWC (*n* =12)	LBW+CC (*n* =12)	LBW+OH (*n* =12)	LBW+CO (*n* =12)	SD
d 14						
Firmicutes	95.49	95.38	95.75	96.51	95.81	0.442
Bacteroidota	1.98	1.53	1.09	1.54	1.10	0.371
Proteobacteria	1.22	2.50	0.95	1.28	2.39	0.720
Cyanobacteria	0.90	0.20	1.75	0.34	0.42	0.634
Actinobacteriota	0.33	0.27	0.27	0.20	0.17	0.065
Desulfobacterota	0.08	0.11	0.18	0.13	0.10	0.037
Other	0.01	0.01	0.01	0.00	0.01	0.003
d 21						
Firmicutes	94.29	93.56	95.90	94.87	91.59	1.618
Bacteroidota	1.23	2.44	1.20	1.18	2.23	0.624
Proteobacteria	1.14	1.11	1.68	1.00	1.22	0.265
Cyanobacteria	0.55	1.19	0.80	0.97	1.26	0.291
Actinobacteriota	2.62	1.46	0.22	1.84	3.60	1.269
Desulfobacterota	0.17	0.22	0.17	0.12	0.10	0.046
Other	0.01	0.03	0.03	0.02	0.00	0.012
d 38						
Firmicutes	94.49	92.47	94.61	94.24	94.38	0.888
Bacteroidota	2.26	1.31	1.93	1.57	1.74	0.359
Proteobacteria	0.64	2.08	0.39	0.83	0.32	0.717
Cyanobacteria	0.57	1.08	0.61	1.03	1.17	0.282
Actinobacteriota	1.82	2.79	2.17	2.08	2.13	0.357
Desulfobacterota	0.21	0.26	0.27	0.23	0.23	0.023
Other	0.02	0.01	0.03	0.01	0.03	0.009

Abbreviations: HBWC: high body weight chickens fed commercial broiler feed with fine corn; LBWC: low body weight chickens fed commercial broiler feed with fine corn; LBW+CC: low body weight chickens fed commercial broiler feed with 7% coarse corn; LBW+OH: low body weight chickens fed commercial broiler feed with 3% oat hulls; LBW+CO: low body weight chickens fed commercial broiler feed with 7% coarse corn and 3% oat hulls.

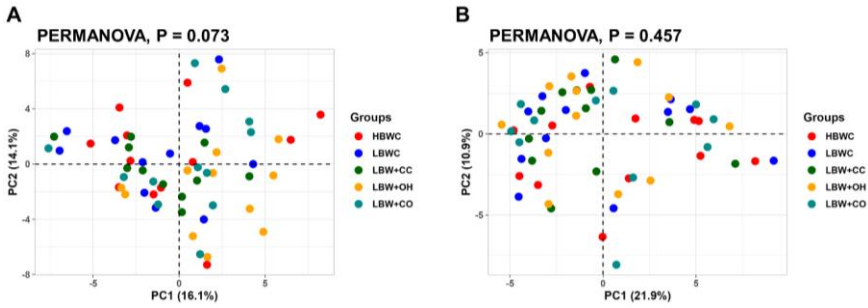

Fig. S6.1 Principal component analysis (PC1 and PC2) based on the gene expression in the ileum of high body weight broilers fed a commercial broiler diet with 10 % fine corn (HBWC, *n*=12), low body weight chickens fed a commercial broiler diet with 10 % fine corn (LBWC, *n*=12), and LBW broilers fed diets with 7% coarse corn (LBW+CC, *n*=12), 3% oat hulls (LBW+OH, *n*=12), or a CC+OH combination (LBW+CO, *n*=12) on d 14 (A) and 38 (B).

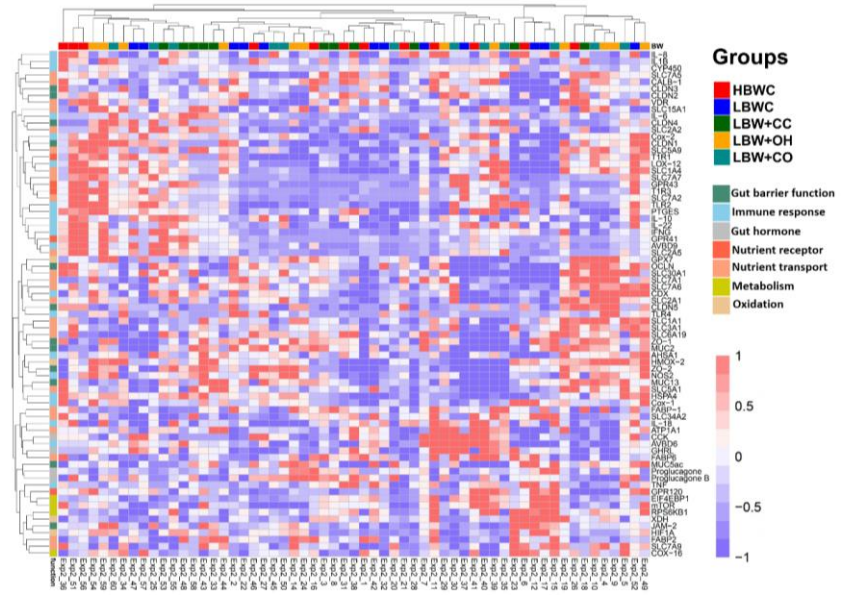

Fig. S6.2 Heatmap of ileal gene expression levels on d14 of high body weight broilers fed a commercial broiler diet with 10 % fine corn (HBWC, *n*=12), low body weight chickens fed a commercial broiler diet with 10 % fine corn (LBWC, *n*=12), and LBW broilers fed diets with 7% coarse corn (LBW+CC, *n*=12), 3% oat hulls (LBW+OH, *n*=12), or a CC+OH combination (LBW+CO, *n*=12). The x-axis represents individual samples, while the y-axis shows the genes. Expression levels are color-coded, with red corresponding high expression and blue indicating low. Gene functions are denoted by different colors on the y-axis. The left dendrogram clusters genes with similar expression patterns, and the top dendrogram groups samples with similar gene expression profiles.

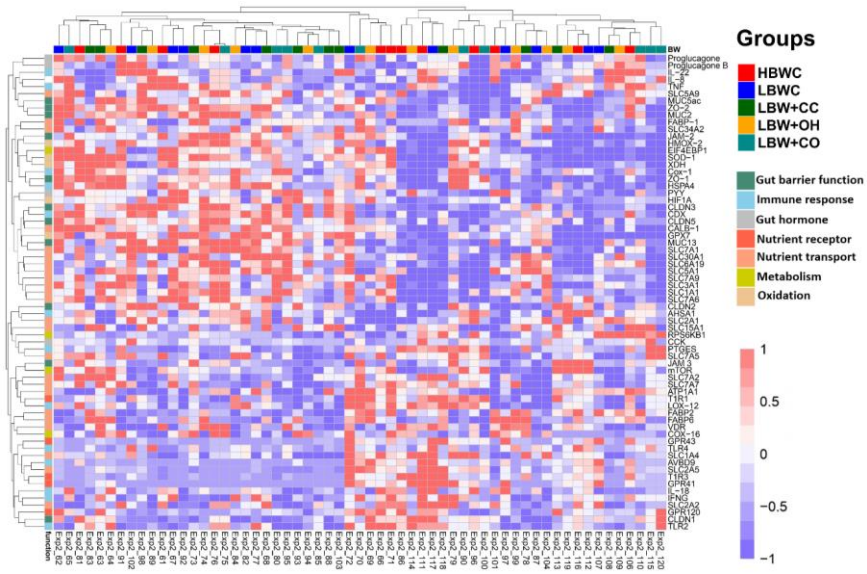

Fig. S6.3 Heatmap of ileal gene expression levels on d 38 of high body weight broilers fed a commercial broiler diet with 10 % fine corn (HBWC, *n*=12), low body weight chickens fed a commercial broiler diet with 10 % fine corn (LBWC, *n*=12), and LBW broilers fed diets with 7% coarse corn (LBW+CC, n=12), 3% oat hulls (LBW+OH, *n*=12), or a CC+OH combination (LBW+CO, *n*=12). The x-axis represents individual samples, while the y-axis shows the genes. Expression levels are color-coded, with red corresponding high expression and blue indicating low. Gene functions are denoted by different colors on the y-axis. The left dendrogram clusters genes with similar expression patterns, and the top dendrogram groups samples with similar gene expression profiles.

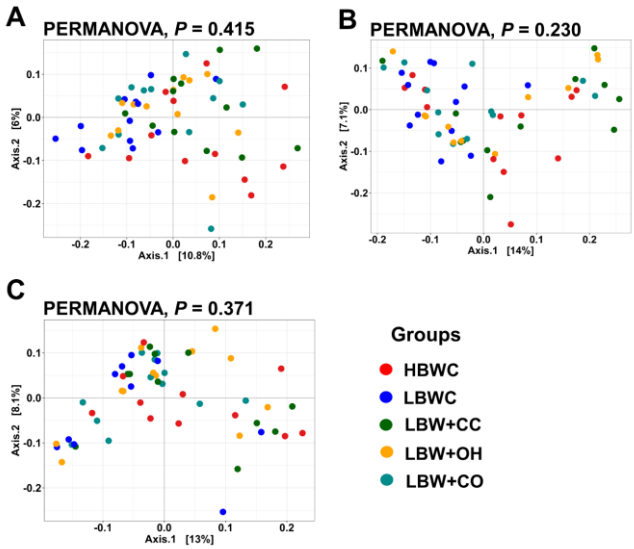

Fig. S6.4 Principal coordinate analysis (PCoA) for Bray–Curtis dissimilarity matrices of the cecal microbiota of high body weight broilers fed a commercial broiler diet with 10 % fine corn (HBWC, n=12), low body weight chickens fed a commercial broiler diet with 10 % fine corn (LBWC, n=12), and LBW broilers fed diets with 7% coarse corn (LBW+CC, n=12), 3% oat hulls (LBW+OH, n=12), or a CC+OH combination (LBW+CO, n=12) on d 14 (A), d 21 (B), and d 38 (C). The multivariate effects of dietary treatments on β diversity were evaluated via nonparametric permutational multivariate analysis of variance (PERMANOVA), and significant differences were indicated as $P < 0.05$.

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Scientific output

Journal articles

Akram, M. Z., Sureda, E. A., Comer, L., Corion, M., & Everaert, N. (2024). Assessing the impact of hatching system and body weight on the growth performance, caecal short-chain fatty acids, and microbiota composition and functionality in broilers. Animal Microbiome, 6(1), 41.

Akram, M. Z., Sureda, E. A., Corion, M., Comer, L., & Everaert, N. (2024). Linking gastrointestinal tract structure, function, and gene expression signatures to growth variability in broilers: a novel interpretation for flock uniformity. Poultry Science, 103(10), 104158.

Akram, M. Z., Sureda, E. A., Corion, M., Comer, L., & Everaert, N. (2025). Effects of on-farm hatching versus hatchery hatching on growth performance, gut development, and intestinal health and function in broiler chickens. Poultry Science, 104(2), 104770.

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Akram, M.Z., Everaert, N. (2024). Differences between low and high body weight broilers and strategies for catch up growth of low performing chickens. Presented at the 75th EAAP Annual Meeting, Florence, Italy, 01 Sep 2024-05 Sep 2024.

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