Effects of a multi-genus synbiotic on gut health, microbiome and performance in Broiler Breeders and their progeny

by

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Dedication

Στον Πατέρα μου..

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In memory of my father, whose love for poultry farming led me to devote myself passionately to this sector as a Veterinarian.

Declaration

I declare that this thesis is entirely my own work and that it has not been submitted, in whole or in part, as part of any previous application for a degree. Unless otherwise indicated by references or acknowledgments, this thesis is entirely my own work.

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Zoi Prentza

June 2023

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List of abbreviations

AGPs	**Antibiotic growth promoters**
AMPs	**Antimicrobial peptides**
AMR	**Antimicrobial resistance**
ATP	**Adenosine triphosphate**
BCO	**Bacterial Chondronecrosis with Osteomyelitis**
BWG	**Body weight gain**
E. coli	**Escherichia coli**
FCR	**Feed conversion ratio**
GI	**Gastrointestinal**
GIT	**Gastrointestinal Tract**
IECs	**Intestinal epithelial cells**
IFN-γ	**Interferon gamma**
IgA	**Immunoglobulin A**
IL	**Interleukin**
IP	**Intestinal permeability**
LPS	**Lipopolysaccharide**
MUCs	**Mucins**
NE	**Necrotic Enteritis**
NFκB	**Nuclear factor kappa B**
PRRs	**Pathogen recognition receptors**
PS	**Poultry Star**
rRNA	**ribosomal Ribonucleic Acid**
TLRs	**Toll-like receptors**
VH/CD	**Villus Height to Crypt Depth**

Abstract

Introduction- Scope

Gut health in poultry has far-reaching implications for animal welfare, production efficiency, food safety, and environmental impact. It is well known that the gut microbiota of chickens plays a role in regulating physiological functions and host homeostasis. The application of 16S rRNA gene sequencing has revealed the relationship between gut dysbiosis and disease in poultry. Because antibiotic growth promoters (AGPs) have been critical in controlling dysbacteriosis and enteropathogens, but their use is increasingly restricted, there is growing interest in alternative products to support production performance. The efficacy of synbiotics is based on a synergistic effect between probiotics and prebiotics. The aim of this work, conducted under field conditions, was to evaluate the effects of a multispecies synbiotic PS (PoultryStar*, BIOMIN):

- on the gut health and performance parameters of the Broiler Breeders, during the rearing and laying period.
- on the caecal microbiota of the Broiler Breeders at 15, 25 and 40 weeks of age.
- on the gut health and performance parameters to broilers in three different flocks
- on the caecal microbiota of the Broilers at 28 days of age

Methods

A total of 24761 day-old Ross 308 parent stock chicks were acquired from a single hatchery and housed on the same farm. Female chicks were divided into three groups and raised in different houses (A, B, and C), where males were introduced at the age of mating and observed until 40 weeks of age. The synbiotic was administered to the flocks in houses A and B via drinking water, while house C served as a control. According to the manufacturer's guidelines, the product was administered intermittently once every two weeks, except during the first and twenty-first weeks, when it was administered for three consecutive days. Data on performance parameters, egg quality traits, bacterial enteritis scoring, intestinal morphometry, and histopathology were recorded, and cecal contents were sampled at 15, 25, and 40 weeks of age to examine the caecal microbiota using high-throughput next-generation sequencing.

Subsequently, three Ross 308 broiler flocks representing separate progeny of the breeder flock were treated with the same synbiotic. Day-old chicks from eggs laid at 30, 35, and 40 weeks of age were housed on three different commercial farms (1, 2, and 3) and raised to slaughter at 42 days of age (doa) under typical field conditions. On each farm, chicks were divided between two different houses, and PS was administered in one of the two houses on each of the three broiler farms according to the manufacturer's instructions, while the other served as a control. Data on performance parameters, bacterial enteritis scoring, intestinal morphometry, and histopathology were recorded, and cecal contents were sampled at 38 doa to examine the caecal microbiota using high-throughput next-generation sequencing (NGS).

Results/Conclusions

Hens treated with synbiotics showed significantly higher survivability during production compared to the control group. No clear differences were observed between the treated and control hens in terms of egg production and quality, and the effect of the synbiotic on weight gain also appeared limited. From week 25, the synbiotic-treated chickens performed better in terms of macroscopic lesions and had longer intestinal villi. Significant differences in crypt length and histopathological lesions were also observed at several sampling points. A treatment effect on the bacterial composition of the caecum was noted, with differential abundance of Gastranaerophilales, Lachnospiraceae, Helicobacter, Ruminococcaceae, and Clostridia, among others.

In broiler flocks, administration of synbiotics was found to improve intestinal health even in the absence of challenge, with limited changes in macroscopic intestinal lesions and more marked differences in histopathological scores and villi length. Chickens fed synbiotics consistently performed better in terms of body weight gain, feed conversion, and survivability. Finally, evaluation of the caecal microbiome by next-generation sequencing revealed the effects of synbiotic supplementation on bacterial population composition.

CHAPTER ONE

LITERATURE REVIEW

1.1 INTRODUCTION

Genetic selection for high production rates, along with improved management techniques and nutritional measures, has resulted in higher performance standards on all poultry farms. However, poultry performance may soon reach an upper limit due to genetic and/or physiological limitations. With the goal of further optimizing performance, poultry researchers and producers are now focusing on gut health (Kogut et al., 2017).

The gastrointestinal tract is often referred simply as "the gut" and actually consists of: (1) an epithelium; (2) a diverse and robust immune arm that contains most of the body's immune cells; and (3) the commensal bacteria, which contain more cells than the entire host organism. Understanding the interactions between all of these interrelated components of the gut makes the gut the foundation of animal well-being and the engine of their performance. Optimal gut health is critical to the performance of livestock and is synonymous with animal health in the poultry industry (Kogut et al., 2017).

According to the literature disruption of the commensal microbiota can lead to an imbalanced host-microbe relationship known as "dysbiosis" that causes the weakening of the intestinal barrier. Impaired intestinal barrier function, commonly known as "leaky gut," is a condition in which the mucosa of the small intestine becomes damaged, allowing luminal contents such as bacteria and their constituents, including toxins, to penetrate between the epithelial cells. This results in cellular damage and/or inflammation of the intestine, characterized by increased

concentrations of bacterial endotoxins such as lipopolysaccharide (LPS), into the bloodstream. This inflammatory process consumes significant amounts of nutrients and subsequently negatively affects metabolic responses, particularly immunometabolic and endocrine responses. As a result, animal performance is severely impaired (Shehata et al., 2022).

The commensals in the gut play a key role in preventing the spread of potential pathogens, thus contributing to colonization resistance. The major mechanisms of commensal bacteria involved in colonization resistance are summarized in (Figure 1) and include: (A) commensals provide a direct barrier to pathogen colonization by competing for space and nutrients; (B) commensals continuously stimulate pathogen recognition receptors (PRRs), such as Toll-like receptors (TLRs), on intestinal epithelial cells (IECs) to secrete protective mucins (MUCs) and antimicrobial peptides (AMPs); (C) commensals contribute to adaptive immunity by stimulating secretion of secretory immunoglobulin A (IgA), which provides protection against pathogens by cross-linking pathogenic bacteria and neutralizing bacterial toxins; (D) commensals produce microbial fermentation products such as butyrate, which has trophic effects on the intestinal mucosa, thereby strengthening the intestinal barrier (Ringseis et al., 2022).

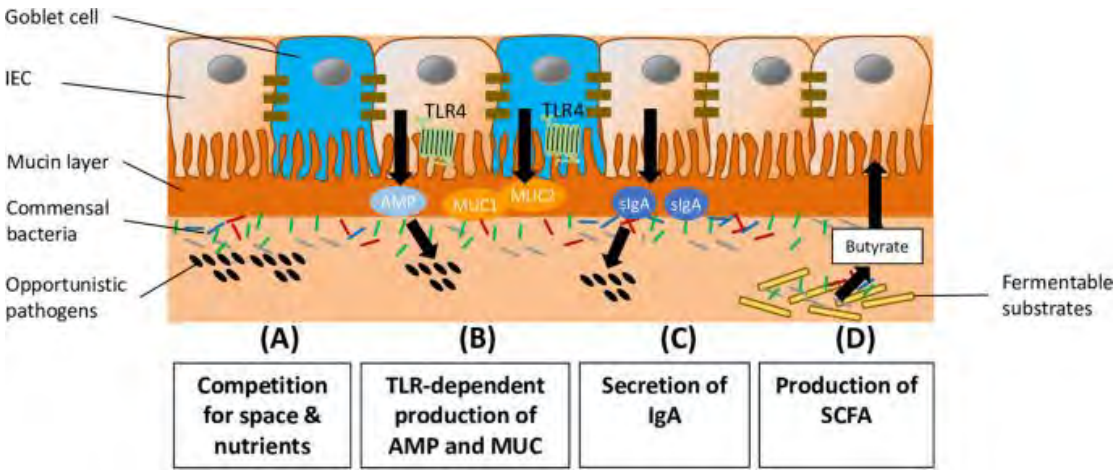

Figure 1. Important mechanisms of commensals involved in colonization resistance

Broiler chickens often have intestinal barrier integrity problems (increased permeability), which makes it easier for toxins, feed antigens, bacteria, and bacterial byproducts to overcome this barrier and spread systemically. This bacterial spread can also be a potential cause of infectious skeletal disease. Bacteria that spread systemically through the bloodstream can colonize various sites in the body, including bone. The bacteria are thought to cross the intestinal barrier, enter the bloodstream, and spread to osteochondritic clefts or microfractures of the growth plates. When the bacteria colonize the growth plates, they are usually inaccessible to antibiotics and the host immune system, so they can cause necrosis (nutrinews.com).

Bacteria that are found in bacterial chondronecrosis with osteomyelitis and lameness (BCO) in chickens are commensal intestinal bacteria that have translocated through the intestinal epithelium and have spread systemically. Bacterial genera and species that are isolated from BCO cases are, amongst others, opportunistic bacteria including staphylococcus, Escherichia coli, and enterococci (nutrinews.com).

Until recently, research on the poultry gastrointestinal GI microbiota relied on conventional microbiological techniques that can only culture a small proportion of the complex community comprising the gut microbiota. 16S rRNA based next

generation sequencing is a powerful tool to investigate the biological and ecological roles of the GI microbiota in chicken. Although several challenges remain in understanding the chicken GI microbiome, optimizing the taxonomic composition and biochemical functions of the GI microbiome is an attainable goal in the post-genomic era (Shang et al., 2018).

1.2 GUT HEALTH

Maintaining "gut health" is considered a priority in commercial chicken farms, although a precise definition of what constitutes gut health and how to evaluate it is still lacking. Gut health is determined by host (immunity, mucosal barrier), nutritional, microbial, and environmental factors. Deficits in gut health are associated with changes in the composition of the gut microbiome (dysbiosis), a leaky mucosal barrier, and/or inflammation (Ducatelle et al., 2018).

Gut health' is a term increasingly used in medical literature and by the food industry. It encompasses several positive aspects of the gastrointestinal tract (GIT), such as effective digestion and absorption of food, absence of GI disease, a normal and stable gut microbiota, effective immune status, and a state of well-being (Bischoff, 2011).

Celi et al. (2019) proposed the definition of gut health as "a stable state in which the microbiome and intestinal tract exist in symbiotic balance and in which the animal's welfare and performance are not compromised by gut dysfunction." This definition combines the most important components of gut health, namely nutrition, effective GIT barrier structure and function, and a normal and stable microbiota, with effective digestion and absorption of feeds and an effective immune status. All of these components play a critical role in GIT physiology, animal health, well-being (including animal behaviour), and performance (Celi et al., 2019).

Maintaining a healthy gut is complex and depends on a fine balance between the immune system and the endogenous microbiota (Oakley et al., 2014).

Therefore, prevention of gut health problems includes reducing stress, promoting a targeted diet, preventing exogenous infections, using antibiotic-free management strategies, and improving rearing conditions (Zhu et al., 2021).

1.2.1 Gut health evaluation

To evaluate intestinal health, candidate biomarkers are currently being investigated by many research groups, but validation will be a major challenge due to the complexity of gut health in the field (Ducatelle et al., 2018).

The most widely used phylogenetic marker is the bacterial 16S small subunit ribosomal RNA gene, which allows the composition of the gut microbial community to be studied in various animal species, including poultry (Ducatelle et al., 2018). Measurements of villus height, crypt depth, and villus/crypt ratio at the level of the duodenum, jejunum, or ileum have become the gold standard for assessing gut health in animals (Ducatelle et al., 2018).

The most promising new biomarkers will be stable molecules that end up in feces and litter and can be quantified, preferably with rapid and simple pen-side assays. However, it is unlikely that a single biomarker will be sufficient to track all aspects of intestinal health. Combinations of multiple biomarkers and/or metabarcoding, metagenomics, metatranscriptomics, metaproteomics and metabolomics approaches will be the way to go in the future (Ducatelle et al., 2018).

In poultry there are Biomarkers allowing non-invasive and/or invasive sampling. Currently, almost all assessment methods of the intestinal barrier function are invasive. However, there are studies aimed to quantify selected proteins as novel

biomarkers in excreta of broiler chickens to facilitate non-invasive assessment of gut barrier function using enzyme-linked immunosorbent assays (ELISA) (Barekatain et al., 2020).

Recent studies list potential biomarkers that could be detected. Fluorescein isothiocyanate dextran (FITC-d) uptake into serum was examined to test intestinal permeability (IP). In addition, other biomarkers included alpha-1 antitrypsin (A1AT), intestinal fatty acid binding protein (I-FABP), lipocalin-2 (LCN2), fibronectin (FN), intestinal alkaline phosphatase (IAP), ovotransferrin (OVT) and superoxide dismutase [CuZn] (SOD1) detected from fresh excreta samples (Barekatain et al., 2020). Other biomarkers that can be measured in blood and provide a good indication of GIT health include, diamine oxidase, D-lactate and lipopolysaccharide (Gilani et al., 2021). In research, monitoring the gut microbiota has attracted considerable attention as shifts in microbial community composition have been linked to gut health and production performance. However, microbial signatures associated with productivity remain elusive because the microbiota of individual birds is highly variable, resulting in diverse and sometimes contradictory profiles associated with poor or high performance (Bindari et al., 2022).

1.3 GASTROINTESTINAL TRACT (GIT)

The main function of the GIT system is to digest food and absorb nutrients. In addition, the GIT tract is responsible for maintaining fluid and electrolyte balance and excretion of waste products. In parallel with these classical functions, we must consider that the GIT tract is also responsible for maintaining a barrier to the external environment, which is the main entry point for pathogens (Khadem et al., 2016), and for this reason the health of the gastrointestinal tract (GIT) has an impact on the productivity of animals (Aruwa et al., 2021).

The crop, proventriculus and gizzard (stomach), duodenum, jejunum and ileum (small intestine), caeca, large intestine, colon and cloaca form the poultry GIT as presented in (Figure 2) (Rychlik, 2020).

Each part plays a different role related to the dynamics of the microbiota. Compared to other livestock, broilers have a short intestinal tract and a much faster passage rate, which limits bacterial populations and growth (Pan and Yu, 2014).

Due to the short digesta transit time, microbial carbohydrate metabolism in the small intestine is limited; therefore, the concentration of short-chain fatty acids (SCFAs) is lower in the ileum compared to the caecum. Pancreatic and bile secretions aid in digestion, ultimately diluting the chyme and limiting the number of bacteria that can colonize the intestinal tract (Rehman et al., 2007). However, when the chyme enters the ileum, digestive enzyme activity decreases and bile acids are deconjugated; both of which facilitate bacterial colonization (Rehman et al., 2007).

Bacterial growth in the small intestine is limited by chemical inhibitors such as acid and bile, nutrient competition with the host, the high passage rate of intestinal contents, constant epithelial cell turnover, and host immune defenses (Apajalahti and Kettunen, 2006).

The cecum contributes to numerous functions in avian physiology, such as water and electrolyte uptake and nitrogen recycling (Svihus, 2014). Microbial populations in the cecum are also capable of fermentation, resulting in metabolites that are utilised by the host. In addition, fermentation occurs in the cecum with numerous microorganisms capable of degrading non-digestible starch and producing SCFAs (Beckmann et al., 2006).

The anaerobic environment, long transit time, and partially digested metabolites that enter the cecum are ideal for microbial fermentation and SCFAs production (Rehman et al., 2007).

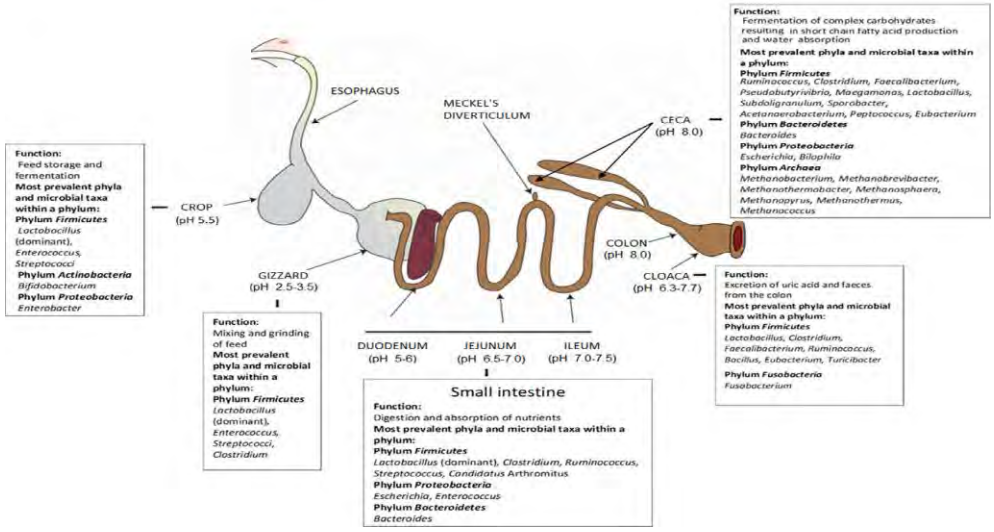

Figure 2. Major organs of the gastrointestinal tract and regional abundance and diversity of gastrointestinal microbiota of chicken (Bindari et al., 2022)

Gut health scoring systems have been used to assess the severity of gut damage associated with coccidiosis (Johnson and Reid, 1970), necrotic enteritis NE (Keyburn et al., 2006), and common inflammatory conditions such as dysbiosis (De Gussem, 2010).

In order to evaluate dysbiosis, a scoring method for gut wall appearance was previously validated (Teirlynck et al., 2011) and many veterinarians employ it for broiler chickens. For this scoring system, 10 parameters are considered in total. These are assessed and given a score that ranges from: 0 when absent or 1 when present under visual inspection at necropsy of the intestinal wall. The animal receives a total score between 0 and 10 where, zero represents a normal gastrointestinal tract and 10 signals the most severe form of dysbiosis.

1.4 MICROBIOTA

The microbiota of the gastrointestinal tract is a complex ecosystem composed predominantly of bacteria, but also includes viruses, archaea, fungi, and protozoa (Smulikowska, 2006; Wei et al., 2013). In studies dealing with the bacterial communities that are part of the gut microbiota, the term "microbiota" is used to refer to the bacterial microbiota for simplicity. The microbiota in the gastrointestinal tract plays a critical role in promoting chicken health and production performance (Yan et al., 2017).

The poultry microbiome has functions ranging from protection against pathogens and nutrient production to maturation of the host immune system. Variations in the microbiome have also been linked to prevailing environmental conditions (Aruwa et al., 2021).

The gut microbiota lies at the barrier between the internal and external environments of the gut and plays an important role in gut dysbiosis. The gut microbiota ferments complex carbohydrates into SCFAs, which are the main source of energy for intestinal epithelial cells. The gut microbiota produces antimicrobial peptides that protect the host from pathogen colonization. The gut microbiota stimulates the

immune system by releasing ligands such as microbial-associated molecular patterns that bind to host cell receptors. Ideally, beneficial bacteria compete with pathogenic bacteria and prevent their colonization. An imbalance between beneficial and harmful gut bacteria results in dysbiosis. Dysbiosis can be caused by non-infectious factors such as altered diet, non-starch polysaccharides (NSP) and mycotoxins or infectious agents such as Clostridium perfringens and coccidia, which are summarized in (Figure 3). Dysbiosis in broilers is characterized by intestinal inflammation and villous atrophy of the small intestine (Fathima et al., 2022).

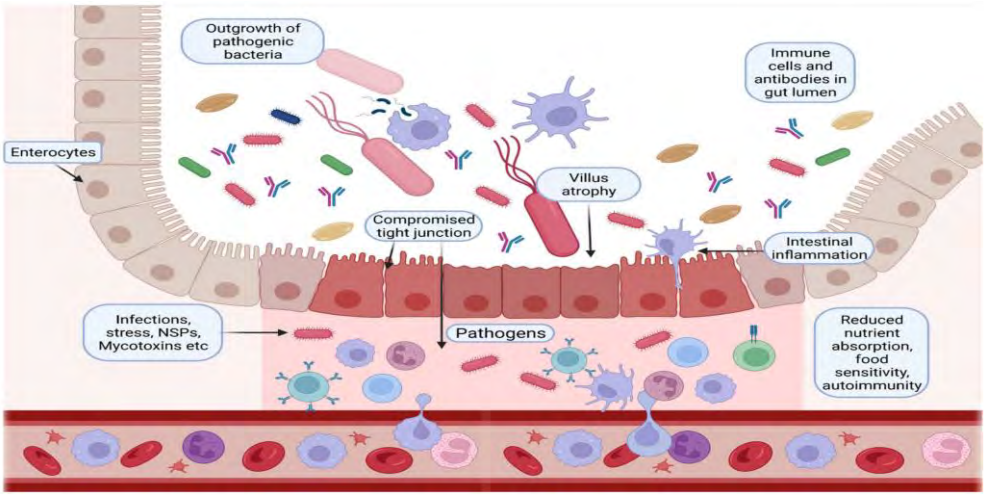

Figure 3. Dysbiosis induced by different factors alters the gastrointestinal homeostasis causing impaired epithelial barrier function and systemic inflammation

The microbiome absorbs nutrients, digests food, triggers mucosal immune response, maintains homeostasis, and regulates bioactive metabolites. An imbalance in the microbiota affects host physiology and gradually leads to disorders and disease (Figure 4). Antibiotic use can cause a shift from dysbiosis with a higher density of pathogens to homeostasis. However, the progressive use of higher doses of antibiotics

proved to be harmful and led to the emergence of multidrug-resistant microbes. As a result, the use of antibiotics as feed additives was banned (Kalia et al., 2022). It is also believed that when antibiotics are included in the feed, the overall dynamics of the GIT microbial community change. Drastic changes to the microbial composition, including a decrease in Lactobacilli and an increase in E. coli abundance could be observed with antibiotics (Feye et al., 2020).

Thus, research has attempted to find alternatives to antibiotic growth promoters for poultry that do not have adverse effects and meet the challenges associated with these restrictions. Influencing the host gut microbiome by regulating nutritional factors is much easier than manipulating host genetics (Kalia et al., 2022).

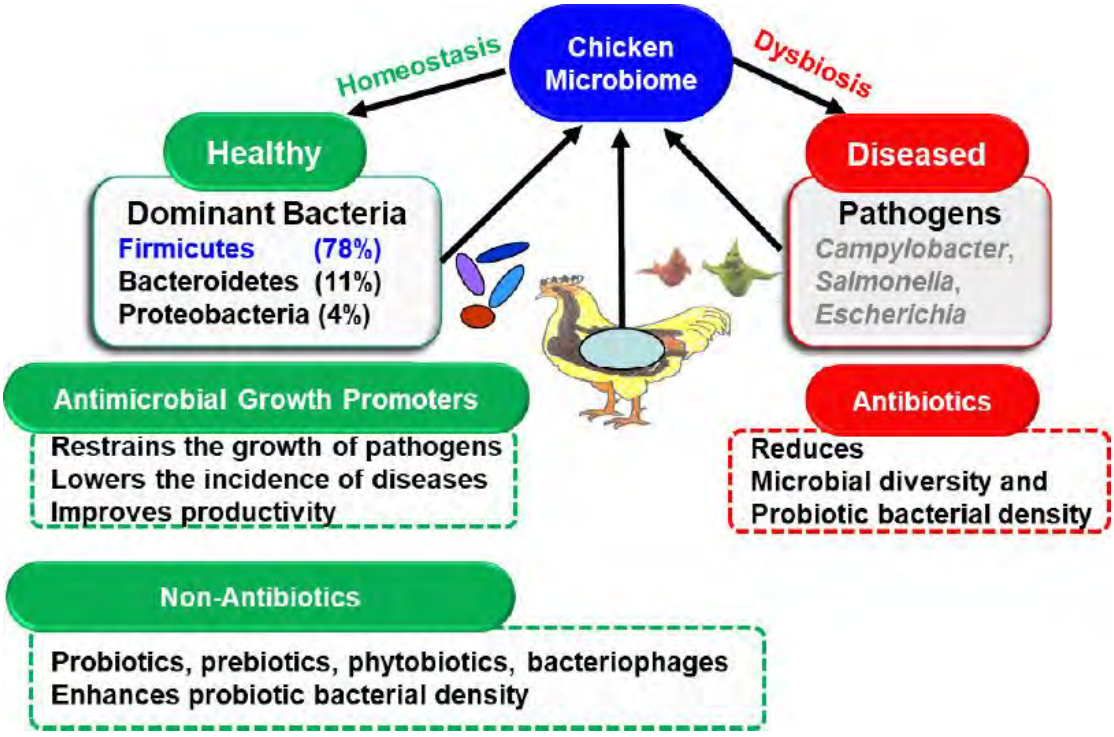

Figure 4. The Chicken Microbiome in Gut homeostasis versus Dysbiosis (Kalia et al., 2022)

The intestine of a newly hatched chick is usually sterile, but is rapidly colonized by microbes from the environment and undergoes various developmental cycles. Various factors such as diet, gastrointestinal tract region, housing, environment, and genetics can influence the microbial composition of an individual bird and give it a characteristic microbiome signature (Fathima et al., 2022). A high diversity of bacterial groups was found in the ileum and cecum, suggesting that colonization was rapid after hatching (Awad et al., 2016).

The most important factors affecting the composition of the gut microbiome are age, sex and breed, diet, and litter conditions (Kers et al., 2018).

At day of hatch, the fecal bacteria community consisted mainly of *Enterococcus* (52%), followed by *Escherichia* (26%), *Clostridium* (14%), *Zea* (5%), and *Lactobacillus* (2%). At 35 day of age, *Lactobacillus* was the most predominant bacteria (72%), followed by *Clostridium* (15%), *Turicibacter* (2%), *Arthomitus* (1%), and other minor bacteria which collectively comprised 10% of the population in the feces (Feye et al., 2020).

The composition of the microbiome found in the gut of avians differed between the younger and older birds. Proteobacteria were found at increased levels during the first few days of life and thereafter decreased. However, in older birds, Firmicutes such as Lachnospiraceae, Ruminococcaceae, Clostridiaceae, and Lactobacillaceae were the most dominant phyla present. The diverse bacterial population found in the chicken caecum increases during the first 6 weeks of life. At the age of 3 weeks, the bacterial population of chickens shifts from Proteobacteria, Bacteroides and Firmicutes to only Firmicutes (Feye et al., 2020).

The maturation of the microbiota occurs with the age of the bird, where the microbial community matures and does not change over time, as shown in several studies. In a

study of commercial broiler operations, phylogenetic diversity in caeca was found to stabilize after 21 days (Kers et al., 2020). However, in a study conducted by Lu et al., (2003), regarding the microbial communities in the cecum suggesting that the microbiota stabilizes after 28 days of chicken age under experimental conditions.

More than 90% of all gut microbiota species in humans and animals belong to the phyla Bacteroidetes, Firmicutes and Actinobacteria, others are Fusobacteria, Proteobacteria, Verrucomicrobia, and Cyanobacteria. In chickens, the phyla Bacteroidetes and Firmicutes are the most predominant representatives in the gut. In human and several animals, the ratio between Firmicutes and Bacteroidetes is a health/metabolism-associated marker. Firmicutes species decompose polysaccharides and produce butyrate, and Bacteroidetes species degrade complex carbohydrates and synthesize mainly propionate (Shehata et al., 2022).

It is known that microbial richness on GIT increases with age and that microbial composition changes, the microbiota becomes more stable with age, and that environment plays a role in when stabilization occurs. However, regardless of age or intestinal tract section, Firmicutes, Bacteroidetes, Actinobacteria, and Proteobacteria are the most abundant phylum in turkeys and chickens. In particular, Firmicutes (70%), Bacteroidetes (12.3%), and Proteobacteria (9.3%) are most abundant in the chicken intestine (Feye et al., 2020).

The highest bacterial density and diversity in the chicken intestinal tract are in the cecum. The most abundant phyla found in the cecum are Firmicutes (44 to 54%) and included the following genera: Ruminococcus, Faecalibacterium, Pseudobutyrivibrio, Subdoligranulum, Acetanaerobacterium, Peptococcus, Sporobacter, Megamonas, Oscillospira, Oscillibacter, Lactobacillus, Blautia, Heliobacterium Eubacterium, and Clostridium. The next most abundant phylum was Bacteroidetes, (23 to 46%) and

consisted predominantly of the genera Bacteroides. Proteobacteria is also a phylum that has been identified in the caeca and ranges from 1 to 16% of the microbiota and includes the Escherichia and Bilophila populations. The minor populations that likely have significant impacts physiologically include Archeans (0.81%), which are primarily represented by Methanobrevibacter, Methanobacterium, Methanosphaera, Methanothermus, Methanothermobacter, Methanopyrus, and Methanococcus (Feye et al., 2020).

Butyrogenic bacteria are strictly anaerobic and oxygen-sensitive saccharolytic bacteria from the phylum Firmicutes, including Ruminococcaceae, Lachnospiraceae, Erysipelotrichaeceae, and Clostridiaceae (clusters IV and XIVa) (Correa et al., 2022). Butyrate is considered an indicator of optimal gut health, stable symbiotic microbial populations, and is linked to reducing foodborne pathogens such as *Salmonella* (Feye et al., 2020)

In humans, terms such as "gut-lung axis," "gut-brain axis," and "gut-kidney axis" have been coined to highlight the importance of this interaction between the gut microbiota and the respiratory, nervous, and renal systems. Butyrate has a significant impact on all of these axes through direct or indirect (i.e., via the immune system) effects in various cell types. SCFAs such as butyrate, once inside cells, can be used in mitochondria to generate ATP, serve mainly as an energy source for enterocytes, or be transported outside cells into the intestinal lamina propria and subsequently into the bloodstream. When SCFAs enter the systemic circulation, they can affect the function of various target tissues, including lung, kidney, and brain (Correa et al., 2022).

Recent literature also describes the immunomodulatory effects of butyrate. Butyrate produced by butyrogenic bacteria in the intestinal lumen acts on immune cells and

regulates their functions. Butyrate induces neutrophil responses to pathogens, reduces cytokine production by mononuclear cells, decreases the activity of mast cells, eosinophils, and innate lymphoid cells, and induces a tolerogenic response in lymphocytes. DCs, dendritic cells; ILC2, innate lymphoid cells type 2; GATA3, GATA binding protein 3; IL-10, interleukin 10; IL-22, interleukin 22; IgA, immunoglobulin A (Correa et al., 2022).

Butyrate exerts anti-inflammatory effects by inhibiting NFκB and inhibiting the expression of the proinflammatory cytokines IFN-γ, IL -6, and IL -1β in LPS-activated macrophages (Fathima et al., 2022).

In poultry, it is considered that the gut microbiota modulates the host physiology via the gut-brain axis, a bi-directional communication system based on neural, endocrine and immunological mechanisms (Figure. 5). Although this phenomenon has been vigorously investigated in mammals, not much work has been conducted on birds.

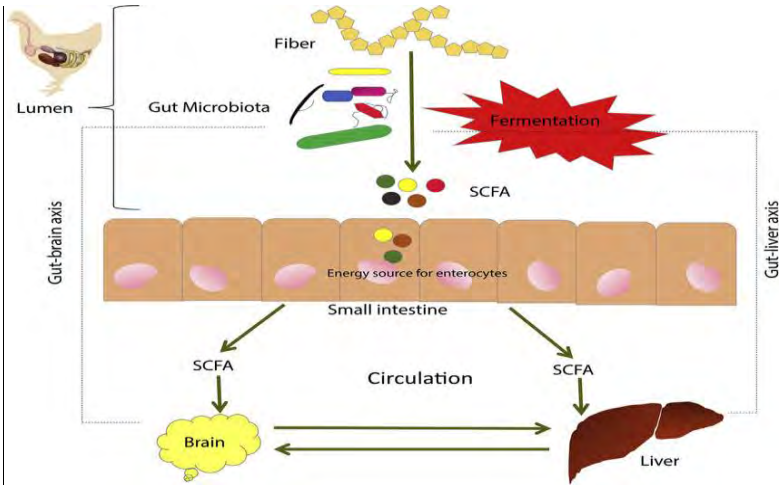

Figure 5. Gut-liver and gut-brain axis of chickens

In addition, the gut-liver axis is likely the most important connection between the resident microbiota and extra-intestinal organs. It represents a close functional and

bidirectional communication between the intestine and the liver. It is well known that the liver is continually exposed not only to the products of digestion and absorption but also to the gut derived factors including bacteria and bacterial components such as lipopolysaccharide (LPS) (Mahmood et al., 2020).

When chickens encounter enteric stress or inflammation, the intestinal epithelium, enteric muscles, and associated immune cells transmit signals to the brain via the central nervous system (vagus nerve). Further, it induces the leucocytes to release cytokines into circulation from the intestine. Cytokines trigger the activation of the central nervous system. Neurotransmitters produced by chicken gut microbes also induce the central nervous system. All these stimuli from the intestine via the vagus nerve activate the hypothalamic– pituitary–adrenal (HPA) axis and increase the serum corticosterone levels. Corticosterone thereby modulates heterophile migration into the inflammation site of the GI tract to attune inflammation. Activation of the HPA axis leads to sickness behavior in chicken, owing to elevated cortisol secretion. (Wickramasuriya S, 2023).

Stress can cause physiological changes including elevated heat shock proteins, acute phase proteins and interleukin IL-6. DEX (Dexamethasone) which mimics the stress in animals has been successfully applied as a model to increase intestinal permeability IP in chickens recently in several studies. These studies confirm that DEX increases IP in Cobb or Ross birds fed corn or wheat-based diets, in the feed or repeated intraperitoneal injections suggesting that stress can induce leaky gut. In modern poultry production is relevant because stress is present in various stages of the bird's life and production, including transportation of chicks to farm, exposure to diseases, environmental temperature and humidity, phase feeding, overcrowding, pecking disorders, catching and transportation to slaughterhouses (Gilani et al., 2021).

Bacteria attached to the epithelium act as a protective barrier. They produce vitamins (vitamins B and K), organic acids, bacteriostatic short-chain fatty acids (SCFAs) such as acetic, propionic and butyric acids, antimicrobial substances (bacteriocins) and trigger beneficial immune responses. These metabolites derived from the gut microbiome play an important role in improving metabolism, nutrient digestion and absorption to improve poultry health, growth and welfare. In chicken, caecal microbiota is involved in the recycling of nitrogen from uric acid with the production of essential amino acids and the digestion of non-starch polysaccharides (NSPs). The bacterial community lowers triglyceride levels and induces non-pathogenic immune responses that can provide nutrition and protection to the host. In addition it has been reported that a low diversity gut microbiome has poor stability and health compared to a highly diverse gut flora (Feye et al., 2020).

Overall, the percentage of human or avian pathogenic bacteria such as Campylobacter, Clostridium, E. coli, and Salmonella is about 1.5% and varies with increasing age of the chicks depending on environmental, health, and nutritional factors (Feye et al., 2020).

The decline of E. coli is associated with changes in microbial community structure that affect pathogen colonization and host immune response.

As mentioned earlier, the predominant bacteria in each section of the intestinal tract are not static, as the microbiome is dynamic and influenced by numerous factors such as rearing practices, age, sex, diet, endemic and episodic disease states, antibiotic use and other growth or health promoters, geographic location, and environmental stress. Therefore, it is critical to consider these factors when comparing microbiome populations between different farms or experimental units (Feye et al., 2020).

Animal species, breed, age, diet, environment, husbandry practices, stocking density, stress, and medications can all affect the delicate composition of the gut microbiota. Factors affecting gut microbiota composition are shown in (Figure 6) (Shehata et al., 2022).

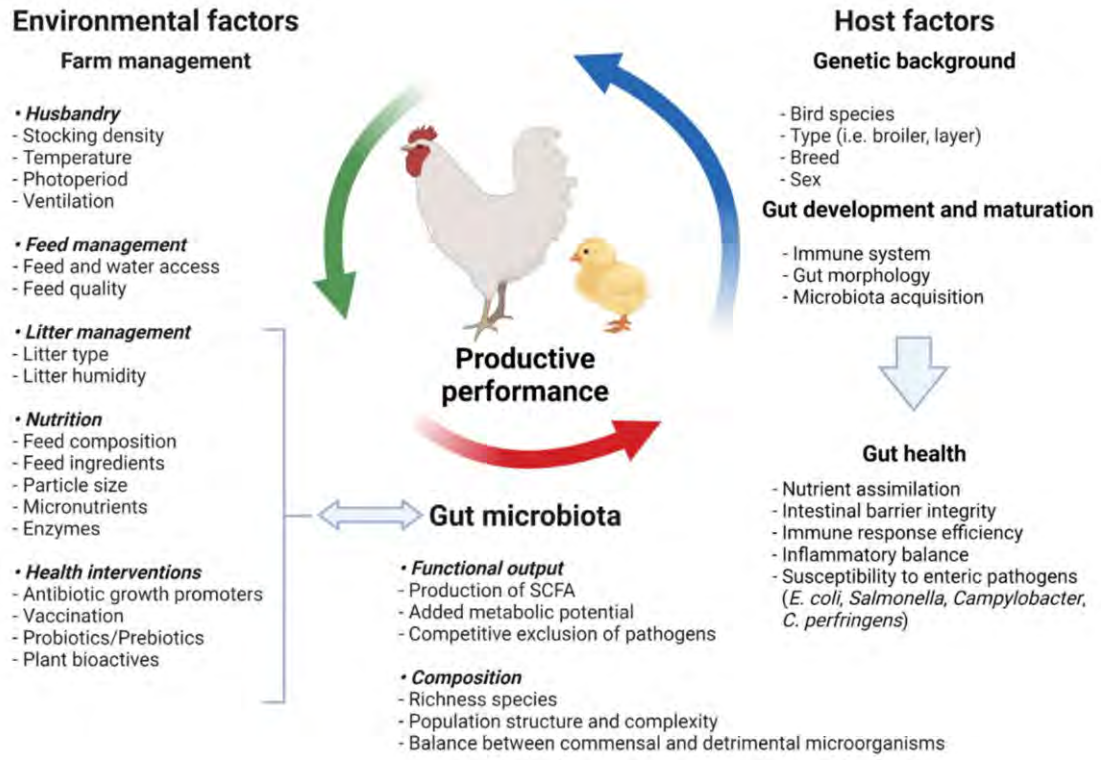

Figure 6. Factors affecting the gut microbiota composition

1.5 PERFORMANCE

Many factors related to health, housing, and management can affect bird performance, including bird origin, light regime, ventilation, stocking density, feed, water quality, and disease (Van Limbergen et al., 2020).

Good performance and growth rate are essential for animal production as long as the host-microbiome relationship remains intact (Aruwa et al., 2021).

As antimicrobial resistance (AMR) remains a major global public health crisis, the poultry production faces increasing challenges to increase productivity while

minimizing AMR (De Mesquita et al., 2022). Field observations in Europe have shown that the poultry industry faced several problems after the ban of antibiotic growth promoters (AGPs), including negative effects on performance, animal welfare aspects, and general health issues (Hafez et al., 2021).

Thus, alternatives to antibiotics are needed to maintain gut health and performance by combating pathogens and improving nutrient digestion and absorption (Dhama et al., 2014a).

1.6 ALTERNATIVES

Nutraceuticals can include everything from isolated nutrients (vitamins, minerals, amino acids, fatty acids) to botanicals (polyphenols, herbs, spices), supplements (probiotics, prebiotics, synbiotics, organic acids, antioxidants, enzymes), and genetically modified foods. These nutraceuticals help in the prevention of infectious diseases of the host (Hailu et al., 2009) and required to reduce the use of antibiotics (Ballou et al., 2019)

Alternatives, should improve performance effectively, have little therapeutic use in human or veterinary medicine, not cause deleterious disturbances of the normal gut flora, not be involved with transferable drug resistance, not be absorbed from the gut into edible tissue, not cause cross-resistance to other antibiotics at actual use level, not promote *Salmonella* shedding, not be mutagenic or carcinogenic, not give rise to environmental pollution, non-toxic to the birds and its human handlers and should be readily biodegradable (Yaday et al., 2016).

In poultry production, such prominent alternatives include the use of probiotic microorganisms, prebiotic substrates that benefit the proliferation of beneficial

bacterial populations, or synbiotic (combinations of prebiotics and probiotics) ensuring better production and maintaining the health of the birds.

1.6.1 PROBIOTICS

As defined by the Food and Agriculture Organization of the United Nations (FAO)/World Health Organization (WHO), probiotics are "live microorganisms that, when administered in sufficient quantity, confer a health benefit to the host" (Hotel, 2014). The possible mechanisms of probiotic action as reported by El-Hack et al. (2020) in (Figure 7) are (1) Competitive exclusion of pathogenic microorganisms. (2) Production of antimicrobial substances. (3) Competition for growth factors nutrients. (4) Enhancement of adhesion to intestinal mucosa. (5) Improvement of epithelial barrier function. (6) Improvement of secretion of IgA.

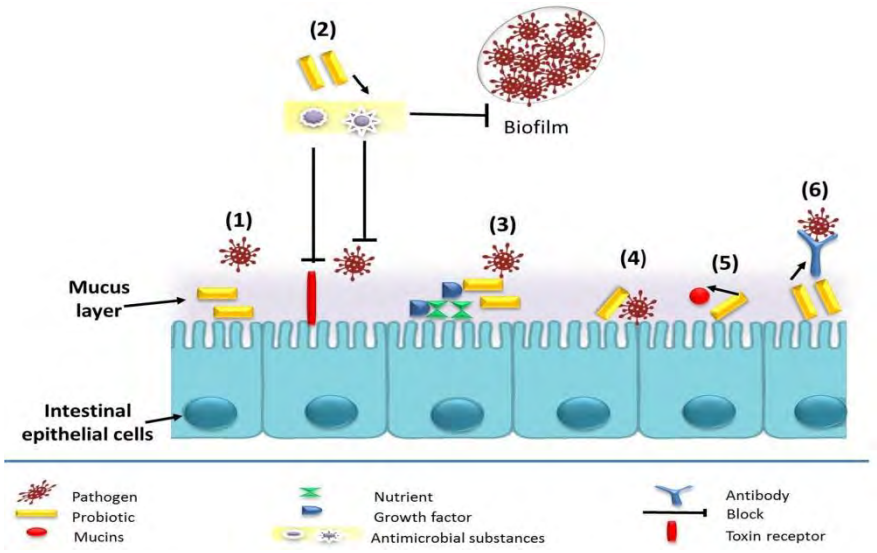

Figure 7. The possible mechanisms of probiotic action

Probiotic preparations include: lactic acid bacteria (LAB) (*Lactobacillus plantarum, Lactobacillus helveticus, Lactobacillus bulgaricus*, *Lactobacillus lactis, Lactobacillus. casei, Lactobacillus acidophilus, Lactobacillus salivarius*), *Bifidobacterium* spp., *Enterococcus faecalis, Enterococcus faecium*, *Streptococcus thermophilus*, *Bacillus, Pediococcus* and yeast such as *Saccharomyces cerevisiae* and *Candida* spp. (Alagawany et al., 2021).

Probiotics may consist of one or more strains and may be administered in combination with other feed additives via feed or water (Yaqoob et al., 2022). Probiotics can be administered in a variety of ways, including feed, water, and spray. In addition, in-ovo administration of selected probiotics into the amniotic sac of embryonated eggs is a potential route that could facilitate early colonization of the gut with beneficial bacteria and mitigate the adverse effects of environmental stressors and delayed access to feed and water (Alizadeh et al., 2021). It is generally accepted that probiotics with multiple strains are more beneficial than single strains because multiple strains have synergistic effects (Yaqoob et al., 2022). Probiotics can also be divided into 'colonizing' species, which consist of Lactobacillus, Streptococcus, and Enterococcus spp. and 'non-colonizing' species, which consist of spores of Bacillus spp. and the yeast Saccharomyces cerevisiae (Huyghebaert et al, 2011). The bacteria of the colonizing species compete for potential binding sites on the intestinal epithelium or mucosa, whereas the non-colonizing species are viable in the intestinal contents but do not colonize the intestinal epithelium (Gauthier, 2008).

The effectiveness of probiotics is related to the viability of the microorganisms used. However, there is evidence that the viability of the microbes is not a prerequisite for probiotics to benefit the host. Nowadays, terms such as 'postbiotics," 'paraprobiotics," 'metabiotics," 'inactivated probiotics," and 'ghost probiotics' suggest that

supplementation of non-viable microbes or microbial products may also provide health benefits to the host (Aquilar-Toala et al., 2018).

1.6.1.1 Probiotic effects

The positive effects of probiotics can be observed directly in the gastrointestinal tract and indirectly in the immunomodulation of the poultry immune system (Krysiak et al., 2021). Probiotics produce organic acids and SCFAs such as lactate, propionate, acetate, and isovalerate to lower the pH of the gut to inhibit colonization and growth of pathogenic microbes (Immerseel et al., 2004). Lactobacillus and Bacillus spp. secrete bacteriocins to inhibit E.coli, Vibrio spp, Salmonella, Proteus, Campylobacter, C.perfringens, Staphylococcus aureus and Shigella (AL Allaf et al., 2011). Probiotics also compete with pathogenic bacteria for nutrients and attachment to the gastrointestinal epithelium and may act as an adjuvant to stimulate the immune system to inhibit pathogen colonization and reduce mortality (Fathima et al., 2022).

Probiotics enhance the epithelial barrier integrity by increasing the production of mucus while enhance mucin secretion by the upregulation of MUC2 in goblet cells and regulate tight junction permeability through upregulating zonulin, a protein that regulates tight junction permeability (Fathima et al., 2022).

Probiotic supplementation increases the ratio of villus height to crypt depth resulting in increased intestinal absorption of nutrients (Awad et al., 2008). Thus, the effect of probiotics on villus height and crypt depth can be correlated with the increased FCR and BWG in the probiotic supplemented birds. Some probiotic bacteria such as Bifidobacterium and *Lactobacillus* spp. synthesize vitamins such as vitamin A, vitamin K, folate, nicotinic acid, pyridoxine, riboflavin, thiamine, cobalamin, pantothenic acid, biotin, and increase the activity of antioxidant enzymes such as

glutathione peroxidase, catalase, superoxide dismutase, and glutathione S-transferase which adds to the nutritional value of the probiotic (Fathima et al., 2022).

Jiang et al. (2021) demonstrated that probiotic supplementation increased femur and tibia mineralization, resulting in significantly greater bone strength and improved gait score and attenuated heat stress by restoring gut microbial balance and preventing dysbiosis associated with heat stress.

Probiotics increased egg laying performance and quality, increased daily gains, and improved feed conversion ratio (FCR) and meat quality (Krysiak et al., 2021). Administration of probiotics to laying hens subjected to heat stress had a positive effect on hen performance, increasing eggshell thickness, eggshell strength, and albumen height. In addition, these probiotics were shown to improve gut microflora by reducing pathogenic microorganisms and increasing gut integrity (Zhang et al., 2016).

Administration of L. salivarius showed effective prevention of Campylobacter jejuni colonization in broiler intestines (Saint-Cyr et al., 2017) and supplementation of the direct-fed microbial strain B. subtilis DSM improved pathology and performance losses associated with NE (Sokale et al., 2019). L. acidophilus could alleviate gut inflammation and impairment, improve gut morphology and gut barrier integrity, and modulate gut microflora. Consequently, the addition of L. acidophilus positively affected intestinal health and reduced mortality of broiler chickens infected with C. perfringens (Li et al., 2018). Dietary supplementation with probiotics from multiple strains improved broiler growth performance, ileal amino acid digestibility, and humoral immunity. In addition, probiotics reduced the cecal numbers of E. coli and the NH3 content of excreta (Zhang et al., 2014).

De Oliveira et al. (2014) demonstrated that in ovo colonization with probiotic could become an important method to reduce *Salmonella* and other intestinal bacterial infections in poultry.

A combination of Enterococcus faecium, Pediococcus acidilactici, Bacillus animalis, Lactobacillus salivarius and Lactobacillus reuteri decreased the colonisation of Campylobacter jejuni and Salmonella Enteritidis in the gastro-intestinal tract (GIT) of broilers, whereas Bacillus subtilis improved feed conversion, intestinal morphology, stimulated the immune system and inhibited the colonisation of Campylobacter jejuni, Escherichia coli and Salmonella Minnesota (Neveling and Dicks, 2021).

Lactobacillus salivarius and Pediococcus parvulus improved weight gain, bone characteristics, intestinal morphology and immune response, and decreased the colonisation of S. Enteritidis. Lactobacillus crispatus, L. salivarius, Lactobacillus gallinarum, Lactobacillus johnsonii, Enterococcus faecalis and Bacillus amyloliquefaciens decreased the Salmonella count and led to an increase in lysozyme and T lymphocytes. Probiotics may also improve feed digestion through production of phytases, lipases, amylases and proteases or stimulate the GIT to secrete digestive enzymes (Neveling and Dicks, 2021).

It is reported that daily application of probiotic strain E. faecium prior to hatching reduces S. Enteritidis colonization in broilers and daily application improves intestinal integrity (Olsen et al., 2022). The beneficial effects of probiotics could be attributed to improvement of intestinal morphology and microbial diversity in the caecum of broilers, which are different from the effects of antibiotics (Qiu et al., 2022).

Dietary supplemented with B. subtilis or B. licheniformis improved growth performance, immune status, and antioxidant capacity, increased SCFAs production, and modulated cecal microbiota in chickens (Xu et al., 2021).

In recent study, the supplementation with B. methylotrophicus SY200 had the ability to diminish the depth of crypts, boost villus growth, and increase VH/CD ratio in the ileum, which is beneficial for the digestion and absorption of nutrients and led to growth performance improvement of broilers (Xiao et al., 2022).

Studies showed that Necrotic Enteritis induced body weight loss, intestinal lesions, and histopathological inflammation, as well as intestinal-cell apoptosis. These symptoms were alleviated following the administration of probiotic *E. faecium* NCIMB 11181 (Wu et al., 2019). Nevertheless, studies have reported decreased growth and performance parameters associated with the supplementation of probiotics and synbiotics (Rehman et al., 2020, Nyamagonda et al., 2011). Studies showed a range of variation in the performances produced due to differences in the methodology of the experiments. This was hypothesised to be due to several factors, such as the probiotic strains, probiotic dose, age, breed of birds, species, pathogen inoculation levels, and external factors (Jha et al., 2020).

It was shown that the addition of *E. faecium* to broiler diet increased the ileal villus height and enhanced broiler performance with respect to weight gain and FCR (Samli et al., 2007) and addition of a probiotic containing lactobacilli, *B. thermophilum*, and *E. faecium* to the broiler diet increased the jejunal villus height (Chichlowski et al., 2007).

Supplementation of probiotic in the presence of *Eimeria* infection increased the abundance of some commensal genera, included *Clostridium sensu stricto*

1, *Corynebacterium*, *Enterococcus*, *Romboutsia*, *Subdoligranulum*, *Bacillus*, *Turicibacter* and *Weissella*, with roles in butyrate production, anti-inflammation, metabolic reactions and the modulation of protective pathways against pathogens (Memon et all., 2022).

Wang et al. (2016) reported that the supplementation of probiotics in the feed promoted higher biodiversity of the intestinal microbiome in poultry (Wang et al., 2016).

The early establishment of probiotics in the GIT can serve as a barrier to foodborne pathogen colonization. Probiotics are a potential feed additive for reducing and eliminating the colonization of *Campylobacter* in the GIT of poultry. Probiotics that limit *Campylobacter* colonization in the GIT rely on different mechanistic strategies such as competitive exclusion, antagonism, and immunomodulation (Deng et al., 2020). The competitive exclusion (CE) concept was first developed by Nurmi and Rantala (1973) when they attempted to limit the *Salmonella* proliferation in broiler flocks and this procedure and has since been applied to control salmonellosis and campylobacteriosis in poultry (Stern et al., 2001). Shanmugasundaram et al. (2020) found that probiotic supplementation during *Salmonella* challenge significantly reduced the cecal *Salmonella* load (Shanmugasundaram et al., 2020).

Finally, Bacillus spp. Direct-fed microbial (DFM) has shown beneficial effects in preventing and controlling the toxic effects of Aflatoxin B1 (AFB1) (Hernandez-Patlan et al., 2019).

1.6.2 PREBIOTICS

Prebiotics are known as non-digestible carbohydrates that selectively stimulate the growth of beneficial bacteria, thus improving the overall health of the host (Ricke et al., 2020)**.** Prebiotics are metabolized by the commensal microbiota of the gut. The gut microbiota can ferment prebiotics to SFCAs, mainly acetate, propionate and butyrate. SCFAs lower luminal pH, provide energy sources for epithelial cells, and have profound effects on inflammatory modulators and metabolic regulation. A balanced bacterial community can also improve the structure of the intestinal mucosa. Some bacterial strains produce antimicrobial factors or stimulate the immune system by signaling dendritic cells. Oligosaccharides and monosaccharides can reduce pathogen colonization by blocking receptor sites used by pathogens to attach to the epithelial cell surface (Figure 8) (Pourabedin and Zhao, 2015).

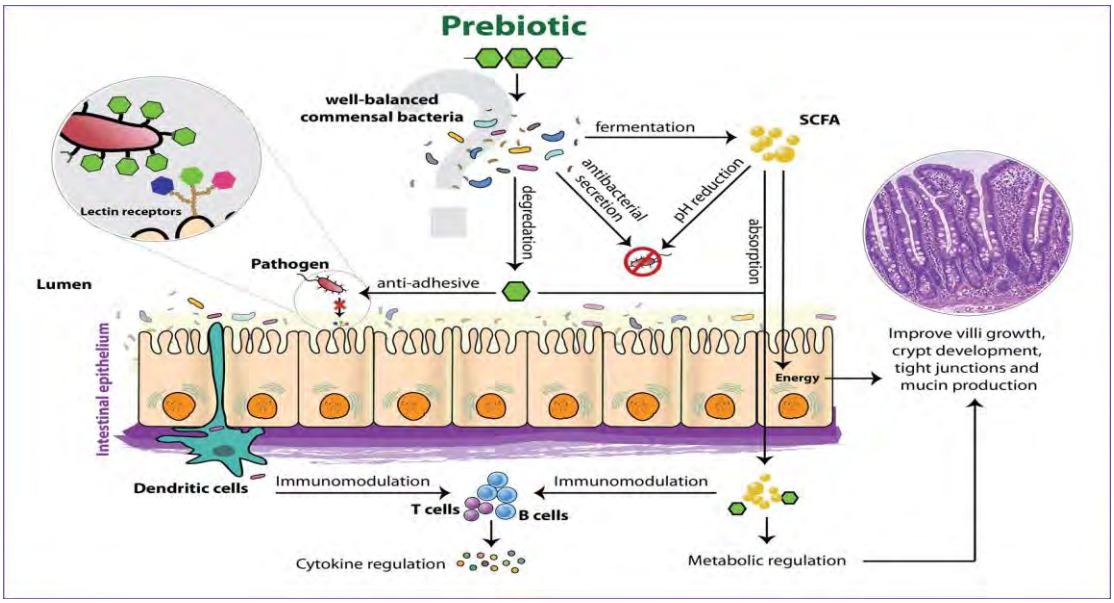

Figure 8. Potential mechanisms of action of prebiotics

A key characteristic of prebiotics is that they are non-digestible and non-absorbable compounds. Prebiotics commonly used in broiler production include fructo-oligosaccharides (FOS), galactooligosaccharides (GOS), mannanoligosaccharides (MOS), oligochitosan, inulin, Pyrodextrins, stachyose, maltooligosaccharides, isomaltooligosaccharides (IOS), xylooligosaccharides (XOS), glucooligosaccharides, soyoligosaccharides, lactitol, lactulose, pectin, and arabinoxylan. Non-carbohydrate compounds such as polyphenols, certain lipids, peptides, and proteins also qualify as prebiotics. These macromolecules are either synthesized by microorganisms or extracted from plants (Fathima et al., 2022).

1.6.2.1 Prebiotic effects

Fructooligosaccharides (FOS) and their longer-chain variant, inulin, are among the most studied prebiotics for humans and animals. FOS are not hydrolyzed by mammalian or avian digestive enzymes and thus enter the colon undigested, where they can be fermented by the gut microbiota indicated that FOS increases the population of Lactobacillus while limiting the growth of C. perfringens and E. coli in broilers (Roberfroid et al., 2010, Kim et al., 2011).

Mannanoligosaccharides (MOS) are mannose-based oligomers linked by β-1,4-glycosidic bonds. They occur naturally in certain plants, beans, and the mannoprotein portion of the cell wall of the yeast Saccharomyces cerevisiae. Because birds lack enzymes to break down the mannan backbone, this oligosaccharide is thought to enter the lower GI tract undigested (Pourabedin et al., 2015).

Kim et al. (2011) showed that MOS reduced *C. perfringens* and *E. coli*, and increased the relative population of *Lactobacillus*. MOS supplementation modified the cecal

microbial composition, and increased the number of species within the phylum *Bacteroidetes*, particularly after 35 days (Corrigan et al., 2015).

Studies on broilers and turkeys have found an increase in the phylum Firmicutes and a decrease in Bacteroidetes and Proteobacteria as a result of supplementation with MOS (Corrigan et al., 2012).

In a study with broiler chickens kept under suboptimal environmental conditions, MOS increased cecal bacterial diversity, and promoted growth of *Lactobacillus* and *Bifidobacterium* species in the cecum (Pourabedin et al., 2014).

MOS may bind with pathogen lectins and prevent its attachment to the epithelial surface. Mannose-bound pathogens therefore pass through the GI tract without colonization (Pourabedin & Zhao, 2015).

Xylooligosaccharides (XOS) are unusual oligosaccharides whose main constituent is xylose linked by β 1-4 bonds, produced by partial hydrolytic degradation of lignocellulosic materials, commonly arabinoxylans, which are found in abundance in the cereal grains found that the administration of two different doses of wheat-bran derived arabino-XOS (AXOS) significantly reduced cecal colonization and translocation of *S.* Enteritidis to the spleen at 3 and 7 day postinfection (Carvalho et al., 2013, Eeckhaut et al., 2008).

Prebiotics selectively enrich microorganisms such as Lactobacillus spp. and Bifidobacteria spp. that are able to utilize the non-digestible but fermentable substrates. In addition, prebiotics inhibit the attachment of pathogens such as Salmonella, E. coli and Campylobacter to receptors on host intestinal cells by acting as decoy receptors, thus not only contributing to better growth and performance, but

also improving the microbiological quality and safety of poultry products (Fathima et al., 2022).

1.6.3 SYNBIOTICS

Synbiotics are the combination of prebiotics and probiotics that have the ability to further enhance the viability of probiotics. Synbiotics consisting of probiotics and prebiotics have been shown to act synergistically when used together (Song et al., 2022). The multiple roles of synbiotics on digestive physiology are summarized in (Figure 9) (Fathima et al., 2022).

Synbiotics provide a live culture and feed it to improve the survival, persistence, and better growth of beneficial organisms in the intestines of birds with a specific substrate for fermentation (Rafiq et al., 2022).

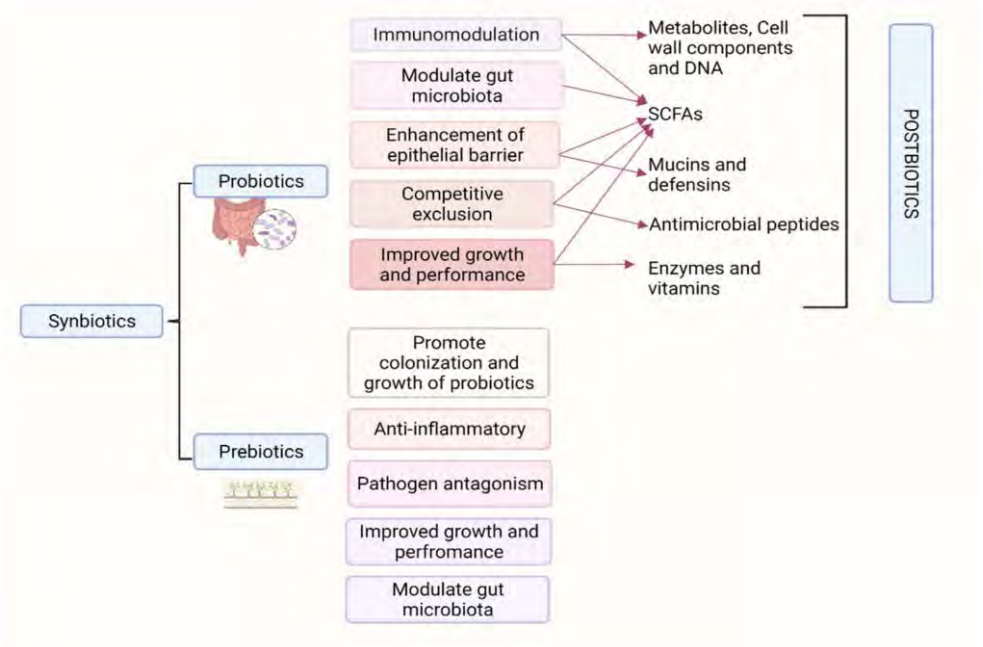

Figure 9. The role of synbiotics on digestive physiology

Postbiotics have emerged to denote that non-viable microbial cells, microbial fractions, or cell lysates might also offer physiological benefits to the host by providing additional bioactivity (Aguilas-Toala et al., 2018).

The addition of synbiotic had a positive effect on growth, immune system, antioxidant parameters, and digestibility of calcium and phosphorus, suggesting that it can serve as a substitute for antibiotics in broiler feeding (Song et al., 2022). Similarly, the dietary inclusion of synbiotic increased the growth performance and improved intestinal morphology and nutrient absorption (Awad et al., 2008).

A study conducted by Villagran de la Mora et al. (2019) found that Synbiotic promoted longer villi, fewer deep crypts, and a better villus-to-crypt ratio. Broilers treated with synbiotic, whether infected with pathogens or not, had healthier mucosa, and the synbiotic group had higher numbers of lactic acid bacteria than the control group on day 39, and the isolation frequency of S. Typhimurium was lower in the Synbiotic-treated groups (Villagran de la Mora et al., 2019).

Results of a recent study show that growth performance of broilers fed synbiotics is better, carcass yield increases, and villus height and goblet cells significantly increase as indicators of gut health. Broilers fed synbiotics had higher caecal Lactobacillus content and lower numbers of Salmonella, E. coli and Clostridia than those fed control diets (Khalid et al., 2021).

Recent studies have shown that when Synbiotic was used, the predominant bacterial families found in the cecum belonged to the Clostridiales. As for these families, the largest population of Lachnospiraceae showed (Rodrigues et al., 2020).

Synbiotics can mitigate the negative effects of heat stress HS on chicken health, as shown by changes in gut architecture and levels of the heat shock protein 70 HSP70.

Broiler chickens treated with synbiotics had greater villus height in the duodenum and a greater ratio of villus height to crypt height in the ileum compared to controls. Therefore, supplementation with synbiotics could be a viable nutritional strategy for the poultry industry to improve the health and welfare of chickens exposed to hot ambient temperatures (Jiang et al., 2020).

A study analyzed the inhibitory effects of a synbiotic product contained 4 probiotic bacterial strains (*Lactobacillus reuteri*, *Enterococcus faecium*, *Bifidobacterium animalis*, and *Pediococcus acidilactici*) and a prebiotic fructooligosaccharide in laying hens with and without a *Salmonella* challenge, concluded that supplementation of the synbiotic product could be beneficial to layer diets as a growth promoter, performance enhancer, and for protection against Salmonella Enteritidis (SE) infection (Luoma et al., 2017).

Another study highlights the positive effect of the synbiotic approach to reduce C. jejuni in broilers, which is essential for the safety of poultry meat consumers. The concentration of C. jejuni in poultry feces was significantly reduced in chickens administered the synbiotic mixture (Baffoni et al, 2012). Similarly, synbiotic supplementation decreased Clostridium perfringens (CP) proliferation while improving production parameters during a necrotic enteritis (NE) infection in broilers (Shanmugasundaram et al., 2020).

The administration of synbiotics in the form of gel drops in the hatchery in combination with dietary supplementation throughout the growth cycle positively affects feed efficiency and welfare of broiler chickens. These results may be attributed to significant variations of caecal microbiota, such as a higher ratio of Firmicutes to Bacteroidetes (with beneficial effects on host energy recovery potential from the diet)

and a more favorable microbial consortium that presumably supports eubiosis (Brugaletta et al., 2020).

Performance parameters such as daily cumulative mortality rate, feed conversion ratio (FCR), and European Production Efficiency Factor (EPEF) were improved by the use of synbiotics and also resulted in an increase in the count of beneficial bacteria and limitation of the growth of potential pathogens in the gastrointestinal tract. Synbiotics led to an increase in the concentration of lactic acid and SCFA and a decrease in the concentration of BCFA in the broiler's excreta (Slizewska et al., 2020).

Finally, the beneficial effect of supplementation with synbiotics was noted by a significant increase in the number of Bifidobacterium spp. and Lactobacillus spp. in the intestinal contents and excreta of the animals. On the other hand, administration of the elaborated synbiotics and commercial probiotics to broiler chickens had no statistically significant effect on the numbers of Enterococcus spp. and Bacteroides spp. in the intestinal contents and excreta of the animals (Slizewska et al., 2020).

1.7 CONCLUSION

It can be concluded that the substances used as alternatives are of particular interest in poultry production due to their beneficial effects on gut health and performance parameters. The control of dysbiosis is crucial in poultry production because the use of antimicrobials has led to the emergence of antibiotic resistance, which is a significant problem for human and animal health. Therefore, the prophylactic use of probiotics, prebiotics and synbiotics, and other by-products in conjunction with a holistic approach on the farm, such as effective cleaning and disinfection measures,

biosecurity measures, avoidance of stress, clean water and better-formulated feed, etc., can be a promising approach for better productivity, profitability, and sustainability in the future.

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CHAPTER TWO

EFFECTS OF A MULTI-GENUS SYNBIOTIC (PoultryStar® sol) ON GUT HEALTH AND PERFORMANCE OF BROILER BREEDERS

2.1 Introduction

The poultry industry is a crucial source of high-quality protein worldwide, with 199 million tonnes of chicken meat produced in 2020 (more than any other meat type) and egg production also accounting for 86 million tonnes (FAOSTAT, 2022). The unceasing growth of the sector is built upon production efficiency, pursued through genetic selection and rigorous health, nutrition, and production management. These measures became even more important in recent years due to the emergence of significant new challenges to the profitability and sustainability of the poultry supply chain (Mottet and Tempio, 2017; Hafez et al., 2020). One of the key areas of interest for the poultry industry is the optimum utilization of available feed ingredients and improvements in nutrient availability (Carré et al., 2008). The intestinal health of poultry plays a role not only in the uptake of nutrients, but also in many aspects of physiology and immune response, with broad implications for animal wellbeing, production efficiency, food safety, and environmental impact (Oviedo-Rondon, 2019). Chicken gut microbiota is known to play a role in the modulation of the host's physiological functions and homeostasis, mainly through the competitive exclusion of detrimental microorganisms and pathogens (Diaz Carrasco et al., 2019). The application of 16S rRNA gene sequencing also revealed the association between enteric dysbiosis and diseases in poultry (Yang et al., 2022). For these reasons, and to cope with the increasing restrictions on the use of antibiotics, a rising interest is paid

to nutraceuticals, which are seen as a potential alternative to support production performance (Alagawany et al., 2021). In particular, an ever-growing literature has been produced on probiotics, and their combinations, defined as synbiotics (Awad et al., 2009; Madej et al., 2016; Alagawany et al., 2021). The efficacy of synbiotics relies on a synergistic effect between probiotics and prebiotics, selectively favoring the survival, implantation, and growth of beneficial bacteria populations in the gut (Awad et al., 2009; Babazadeh et al., 2011; Papatsiros et al., 2013; Nikpiran et al., 2013; Vahdatpour and Babazadeh, 2016; Alizadeh et al., 2017; Syed et al., 2020). Their capacity to improve body weight (BW) gain and feed efficiency (Mousavi et al., 2015; Luoma et al., 2017; Kridtayopas et al., 2019), modulate the immune system and stimulate the development of the gut-associated lymphoid tissue (GALT) and other lymphoid organs (Madej et al., 2015; Madej and Bednarczyk, 2016), and increase the resistance to heat stress (Yan et al., 2019; Jiang et al., 2020; Hu et al., 2022) has been consistently documented. In addition, synbiotics may help to decrease the intestinal and carcass load of various harmful bacteria, including *Campylobacter* (Baffoni et al., 2017), *Clostridium perfringens* (Abd El-Ghany et al., 2010; Shanmugasundaram et al., 2020) and *Salmonella enterica* serovar *Enteriditis* (Markazi et al., 2018; Shanmugasundaram et al., 2019; Sobotik et al., 2021). Since most of the experiments on synbiotics have been conducted in broilers, less is known about their possible applications in other productive categories, whose different genetic features and farming systems entail a different set of challenges and requirements. For this reason, this work aims to evaluate the benefits induced by a multi-species synbiotic product on broiler breeders, by assessing its effects in terms of performance and gut health during the rearing and laying periods.

2.2 Materials and Methods

2.2.1 Ethical approval

Ethical review and approval were waived for this study since animals were sampled during commercial activities in the farm regulated by national and international laws.

2.2.2 Experimental design

The present field study was conducted in a private broiler breeder farm located in the region of Ioannina, Greece, and covered the first 40 weeks of age of the chickens. A total of 24761 day-old Ross 308 parent stock chicks were supplied from the same hatchery and placed in separate houses on the same farm. In detail, 6200, 6264, and 8937 females were placed in houses A, B, and C, respectively. The synbiotic was administered to houses A and B, while house C acted as a control group. A total of 3360 males were raised in a separate house and were introduced in houses A, B and C at the age of mating (19 weeks) with a ratio of one male to 10 females.

2.2.3 Management

To ensure flock welfare and achieve high performance, management conditions followed the official guidelines for parent stocks (Aviagen, 2018). Chickens were placed on a floor covered with straw (deep litter system) and were fed *ad libitum* for the first 2 weeks. Restricted daily feeding was observed from the second to the fourth week; then, starting from week 4, the feed was supplied on a skip-a-day regimen. Feed allocation followed the recommendations for breeders, weighing the chickens weekly and adjusting the dose accordingly (Aviagen, 2018). The light period was 20 hours in the first week, 12 hours in the second week, and 8 hours from the third to week 21. From week 21 onwards, the light period was increased from 8 hours up to

14 hours based on average BW and weight uniformity. The temperature was set according to official guidelines, starting at 30°C at the chicks' arrival and decreasing by 1°C every three days until day 27, then keeping it at 20°C for the rest of the productive cycle. The relative humidity was kept at 60-70% (Aviagen, 2018). Stocking densities were seven female chickens/m^2 and five male chickens/m^2, as indicated by EFSA (2010). The diet was formulated in accordance with the official genetic line guidelines (Aviagen, 2016), implementing a seven-phase feeding system (starter 1, 0-21 days; starter 2, 22-35 days; grower, 36-105 days; pre-breeder, 106 days to 5% production; breeder 1, 5% production to 245 days; breeder 2, 246-350 days; breeder3, after 351 days). The exact nutrient specifications are provided in Table 1. Water was provided *ad libitum.* Chickens were vaccinated at the hatchery against infectious bursal disease (IBD) and Marek's disease (MD). The full vaccination protocol was administered throughout the cycle, including vaccines against infectious bronchitis (IB), Newcastle disease (ND), avian rhinotracheitis (ART), chicken infectious anemia (CIA), infectious avian encephalomyelitis, *Escherichia coli*, salmonellosis, and coccidiosis (Table 2). No antibiotics were administered throughout the considered period.

Table 1. Nutrient composition of the seven-phase feeding system observed to raise the Ross 308 broiler breeders used in the experiment

Diet	**Starter 1 (days 1-21)**		**Starter 2 (days 22-35)**		**Grower (days 36-105)**		**Pre-Breeder (day 106 to 5% production)**		**Breeder 1 (5% production to day 245)**		**Breeder 2 (days 246-350)**		**Breeder 3 (after day 351)**	
Energy	2800 kcal/kg		2800 kcal/kg		2600 kcal/kg		2700 kcal/kg		2800 kcal/kg		2800 kcal/kg		2800 kcal/kg	
Amino acids (%)	**Total**	**Digest**	**Total**	**Digest**	**Total**	**Digest**	**Total**	**Digest**	**Total**	**Digest**	**Total**	**Digest**	**Total**	**Digest**
Lysine	1.06	0.95	0.74	0.67	0.58	0.52	0.58	0.52	0.67	0.60	0.62	0.56	0.58	0.52
Methionine + Cysteine	0.84	0.74	0.67	0.59	0.59	0.52	0.58	0.51	0.67	0.59	0.65	0.57	0.59	0.54
Methionine	0.51	0.46	0.41	0.37	0.36	0.33	0.35	0.32	0.41	0.37	0.40	0.36	0.36	0.35
Threonine	0.75	0.66	0.60	0.53	0.50	0.44	0.47	0.41	0.55	0.49	0.53	0.47	0.51	0.47
Valine	0.80	0.71	0.70	0.63	0.49	0.44	0.51	0.45	0.63	0.56	0.60	0.53	0.57	0.51
IsoLeucine	0.70	0.62	0.62	0.55	0.45	0.40	0.47	0.41	0.56	0.50	0.54	0.48	0.51	0.45
Arginine	1.17	1.05	0.93	0.83	0.71	0.64	0.74	0.67	0.88	0.79	0.86	0.77	0.80	0.72
Tryptophan	0.19	0.16	0.18	0.15	0.14	0.12	0.15	0.13	0.16	0.14	0.15	0.13	0.14	0.12
Leucine	1.23	1.11	0.93	0.83	0.77	0.69	0.80	0.72	1.04	0.94	1.00	0.90	0.96	0.86
Crude Protein	19.00		17.00		13.00-14-00		14.00		15.00		14.00		13.00	
Minerals (%)														
Calcium	1.00		1.00		0.90		1.20		3.00		3.20		3.40	
Available Phosphorus	0.45		0.45		0.42		0.35		0.35		0.33		0.32	
Sodium	0.18-0.23		0.18-0.23		0.18-0.23		0.18-0.23		0.18-0.23		0.18-0.23		0.18-0.23	
Chloride	0.18-0.23		0.18-0.23		0.18-0.23		0.18-0.23		0.18-0.23		0.18-0.23		0.18-0.23	
Potassium	0.40-0.90		0.40-0.90		0.40-0.90		0.60-0.90		0.60-0.90		0.60-0.90		0.60-0.90	
Added trace minerals (mg/kg)														
Copper	16								10					
Iodine	1.25								2.00					
Iron	40								50					
Manganese	120								120					
Selenium	0.30								0.30					
Zinc	110								110					
Minimum specifications														
Choline (mg/kg)	1400		1400		1300		1200		1200		1050		1050	
Linoleic acid (%)	1.00		1.00		1.00		1.00		1.25		1.25		1.25	
Added Vitamins/Kg	Wheat-based feed				Maize based feed				Wheat-based feed				Maize based feed	
Vitamin A (IU)	11000				10000				12000				11000	

Vitamin D3 (IU)	3500	3500	3500	3500
Vitamin E (IU)	100	100	100	100
Vitamin K (mg)	3	3	5	5
Thiamin (B1) (mg)	3	3	3	3
Riboflavin (B2) (mg)	6	6	12	12
Nicotinic Acid (mg)	30	35	50	55
Pantothenic Acid (mg)	13	15	13	15
Pyridoxine (B6) (mg)	4	3	5	4
Biotin (mg)	0.20	0.15	0.30	0.25
Folic Acid (mg)	1.50	1.50	2.00	2.00
Vitamin B12 (mg)	0.02	0.02	0.03	0.03

Table 2. Vaccination protocol administered at the hatchery and throughout the production cycle on the Ross 308 broiler breeders used in the experiment

Age (day)	Vaccine(s)	Disease(s)	Route
day 18 of incubation	Cevac® MD HVT+Rispens	Marek's disease	*In ovo* injection
Hatch day	Cevac® Transmune IBD	Infectious bursal disease virus	Subcutaneous injection
1	Nobilis® IB H120 + Cevac® IBird + Poulvac® E. coli	Infectious bronchitis + colibacillosis	Spray
2	Gallivac® Se + AviPro Salmonella VAC T	Salmonellosis (*Salmonella enteriditis and Typhimurium*)	Water
6	Paracox®	Coccidiosis	Spray/Water
10	Avinew®	Newcastle disease	Spray/Water
18	Nobilis® IB 4/91	Infectious bronchitis	Spray/Water
28	Nobilis® IB Ma5+ Nobilis® ND Clone 30	Infectious bronchitis + Newcastle disease	Spray/Water
35	Nemovac	Avian rhinotracheitis	Spray
50	Gallivac® Se + AviPro Salmonella VAC T	Salmonellosis (*Salmonella Enteriditis* and *Typhimurium*)	Water
55	Avinew®	Newcastle disease	Spray/Water
70	Nemovac	Avian rhinotracheitis	Spray
78	Nobilis® IB Ma5 + Nobilis® ND Clone 30	Infectious bronchitis + Newcastle disease	Spray/Water
88	Nobilis® IB 4/91	Infectious bronchitis	Spray/Water
92	AviPro Thymovac®	Chicken infectious anemia	Water
100	Nobilis® ND Clone 30+ Poulvac® E. coli	Newcastle disease + colibacillosis	Spray
107	AviPro AE®	Infectious avian encephalomyelitis	Water
125	Gallimune® 303 + Gumboriffa® + Gallimune® SE+ST + Hiprapox®	Newcastle disease + infectious bronchitis + avian rinotracheitis + infectious bursal disease + salmonellosis (*Salmonella Enteriditis* and *Typhimurium*)+ fowlpox	Intramuscular injection-wing web stab
154	Avinew®	Newcastle disease	Water
224	Nobilis® IB Ma5 + Avinew®	Infectious bronchitis, Newcastle disease	Water

2.2.4 Synbiotic administration

The synbiotic product PoultryStar® sol (BIOMIN GmbH, Getzersdorf, Austria), containing patented probiotic strains plus prebiotic fructooligosaccharides, was applied in houses A and B by drinking water based on a protocol planned with the manufacturer's guidance. In detail, a daily dosage of 20 g/1,000 chickens was supplied for three consecutive days during weeks 1 and 21 (the first administration after males were introduced) and for one day every two weeks during the rest of the cycle of the product.

2.2.5 Sample collection

Ten randomly selected chickens per treatment group were euthanized by cervical dislocation at 15, 25, and 40 weeks of age to collect specimens for histopathological analysis and lesion scoring. About 3 g of caecal content was also collected to evaluate the microbial composition.

2.2.6 Performance parameters

Live BW and mortality were recorded on a weekly and daily basis, respectively, and egg production was expressed on a hen-day basis from the beginning of the production period (23 weeks) up to 40 weeks. Egg fertility and hatchability were recorded as a percentage of total settable eggs throughout the laying period.

2.2.7 Egg quality traits

At week 30, from the beginning of the laying period, 20 eggs per group were randomly collected every 2 weeks up to week 40 to assess several external and internal egg traits. Individual eggs were weighed to the nearest 0.01 g accuracy with a

digital balance, and the egg length and breadth were measured using digital calipers. A shape index was then calculated by dividing the breadth by the length and multiplying by 100. The shell strength was measured using TA.HD plus Texture Analyser (Stable Micro Systems Limited, Godalming, UK). Shell weight was measured after removing the inner shell membrane and keeping it dry for 24 hours. Shell thickness was evaluated using the Egg Shell Thickness Measure Model 25-5 (B.C. Ames Incorporation, Melrose, Massachusetts) by considering the average of three equidistant points on the equator. The albumen height was measured with the Egg Quality Micrometers S-8400 spherometer (B.C. Ames Incorporation, Melrose, Massachusetts) at 3-4 locations and averaged. The yolk and albumen were weighed on a digital balance to the nearest 0.01 g accuracy. The Haugh unit (HU) was calculated using the formula HU = 100 logs (H+7.57-1.7 W37), where H is the height of the albumen in millimeters and W is the egg weight in grams.

2.2.8 Bacterial enteritis scoring

A macroscopic lesion scoring system was applied to evaluate the chickens' intestinal health in each group at three different time points. Specifically, ten parameters (De Gussem, 2010) were assessed by visual inspection of the intestinal wall during the necropsy. Each parameter was scored 0 when absent and 1, summed and divided by 2.5, resulting in a total score between 0 (normal gastrointestinal tract) and 4 (severe dysbacteriosis) (De Gussem, 2010; Teirlynck et al., 2011).

2.2.9 Histology

Segments of 3 cm were collected from the duodenum, jejunum, ileum, and caecum, keeping the collection sites consistent for each tract. All samples were placed in

individually labeled flasks containing 10% neutral buffered formalin as described by Hoerr (2001). Transversal sections approximately 1 mm thick of each sample were then cut after 48 hours. Sections of 3-5 μm were taken, stained with hematoxylin and eosin, and evaluated. The histopathological and morphometrical evaluation of specimens was performed blindly. The scoring system proposed by Kraieski et al. (2017) was adopted to assess the degree of inflammation in each section. Specifically, the severity of the lesions was graded on a 0-3 scale: 0 corresponded to absent or rare leukocytic infiltration, 1 to leukocytic infiltration up to 5% of a field at x400, 2 to approximately 25% leukocytic infiltration of a field at the same magnification, 3 to leukocytic infiltration in the range of 50%. The morphometry of the intestinal villi and crypts was examined, using optical capture and measurement with Image Pro-Plus version 6.0 software (Media Cybernetics, Silver Spring, MD). The selection of the villi for the morphometrical analysis was conducted according to Gava et al. (2015), considering only those that had their bases clearly embedded in the submucosa, without any discontinuity or folds in their length, and with intact epithelium at the tip.

2.2.10 Evaluation of enteric microbiota

High-throughput sequencing was performed on a total of 64 samples, consisting of 10 caecal content from each of the treatment groups and for each sampling point, two meconium samples from the breeders' grandparents (sequencing controls) and two water samples (contamination controls). The analysis was performed on an Illumina MiSeq System (Illumina, San Diego, California) at BioLizard (Ghent, Belgium), LGC genomics (Berlin, Germany) targeting the V3 region of the 16S rRNA gene, and generated 2 x 300 paired-end sequences. Following a preliminary evaluation of the read quality of unmerged sequences with FastQC 0.11.9, the forward reads were

trimmed at 195 bp, and the reverse reads at 220, ensuring a minimal Phredscore of 28. The amplicon sequence variants (ASVs) that most accurately describe the data were inferred with DADA2 (Callahan et al., 2016), and then the forward and reverse reads were merged, setting the minimal overlap to 12 bp. After removing chimeric sequences from the dataset, the SILVA 138 reference database (Quast et al., 2013; Yilmaz et al., 2014) was used to classify ASVs as taxons. Four diversity indexes (Simpson, Shannon, Chao1, and Observed species index) were used to calculate the alpha diversity. For significance testing between groups, permutational ANOVAs were performed on the euclidean distances between samples. Since these tests require an adequate homogeneity of the separate group dispersions, this assumption was first verified with the betadisper function from the vegan R package (Dixon, 2003). To verify the presence of no systematic biases or confounding effects, the Spearman correlation of the treatment effect with other variables (such as age, weight, bacterial enteritis score, histological lesion scores, crypts, villi length, etc.) was run. Differential abundance analysis was then performed with DESeq2 to evaluate the isolated effect of the treatment and the other factors.

2.2.11 Statistical analysis

Data were organized and analyzed in R version 3.3.2 (R Core Team, 2013). For each considered variable, the statistical significance of between-treatment differences was evaluated at each time point using a Student t-test or, if relative assumptions were violated, the non-parametric Mann-Whitney test. Differences between the three houses were evaluated using ANOVA or, in case the relative assumptions were not met, with the Kruskal-Wallis test followed by post-hoc Mann-Whitney test with Bonferroni correction. Survival analysis was performed using the survival library in

R. Kaplan-Meier cumulative survival curves were calculated, and the significance of the difference between treatment groups in the survival curves was assessed using the Log-rank (M-H). The significance level was set to $p < 0.05$. The statistical evaluation of sequencing data was performed independently at BIOLIZARD NV (Ghent, Belgium). For differential abundance analysis, the significance level was set to $p < 0.01$.

2.3 Results

2.3.1 Bacterial enteritis and histopathological lesion scores

The BE score measured in the control group was higher than in the treated chickens at every time point, with a statistically significant difference ($p = 0.049$) observed at week 25 (Graph 1). No significant differences were found between houses. As for the histopathological lesion score, lower and statistically significant scores were found in the synbiotic-treated chickens than in control ones at week 25 in the caecum ($p = 0.025$), and at week 40 at caecum ($p = 0.021$) and ileum ($p = 0.002$). Conversely, the control group showed a lower score than treated chickens in the jejunum at week 25 ($p = 0.032$, Graph 2). No significant differences ascribable to the house effect were found at between the two treatment houses at duodenum level at week 15 ($p = 0.42$), week 25 ($p = 0.6$) and week 40 ($p = 0.18$); at jejunum level at week 15 ($p = 0.42$), week 25 ($p = 0.6$) and week 40 ($p = 1$); at ileum level at week 15 ($p = 0.42$), week 25 ($p = 1$) and week 40 ($p = 0.27$); and at caecum level at week 15 ($p = 0.42$), week 25 ($p = 0.27$) and week 40 ($p = 0.42$).

2.3.2 Evaluation of intestinal villi and crypts

As shown in Graph 3, several differences could be observed between treated and control animals in terms of gut morphometric parameters. Considering only significant differences, synbiotic-treated chickens showed longer villi than control chickens at week 15 in the ileum ($p = 0.004$), at week 25 at duodenum ($p < 0.0001$), jejunum ($p < 0.0001$), ileum ($p = 0.001$) and caecum ($p < 0.0001$) level, and again at week 40 in all four tracts (all with $p < 0.0001$). Less consistent differences were observed when measuring the crypts, which were significantly deeper in synbiotic-treated than in control chickens in the duodenum at week 25 ($p < 0.0001$) and in the jejunum tract at week 15 ($p < 0.0001$) and week 40 ($p= 0.0004$), but less deep in the caecum at week 25 ($p = 0.002$). The house effect on villi length was significant in the duodenum at week 15 ($p = 0.005$), in the jejunum at week 25 ($p < 0.0001$) and week 40 ($p = 0.009$), in the ileum at week 40 ($p = 0.006$) and in the caeca at week 25 ($p = 0.007$). In terms of crypt length, houses A and B differed significantly at week 25 at the duodenum ($p < 0.0001$) and jejunum level ($p = 0.006$, Graph 4).

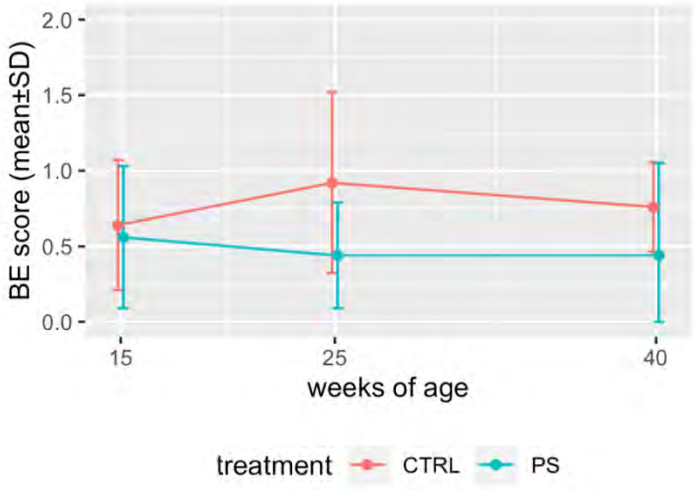
2.3.3 Performance

There was a significant between synbiotic-treated chickens and control group in terms of live BW, (Graph 5a, $p = 0.05$). However, the house effect seemed far more relevant in determining the observed differences ($p < 0.0001$), as house C (control) performed better than house B but worse than house A. In particular, the biggest difference was observed in the BW of males, which was remarkably higher for house A ($p < 0.0001$ when compared to both house B and C). On the other hand, the BW of producing hens was less heterogeneous, and better performance was observed in house C than in the treated houses ($p < 0.001$ for both comparisons) (Graph 5b). A significant difference

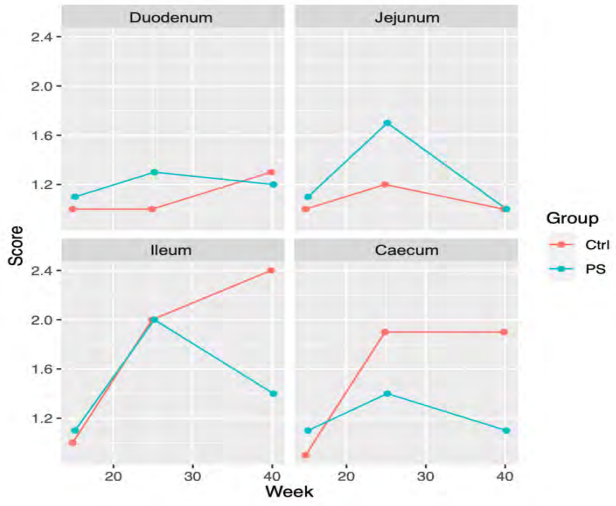
in terms of survivability throughout the production period (23-40 weeks) was observed between the treated and control groups ($p < 0.001$) (Graph 6a). Significant differences were also observed when considering the three houses separately ($p < 0.001$), with both treatment houses scoring better than the control (Graph 6b). No significant differences were found in terms of egg fertility and hatchability, neither between synbiotic-treated and control chickens ($p = 0.12$ for egg fertility, $p = 0.67$ for hatchability) nor between treated houses ($p = 0.1$ for egg fertility, $p = 0.47$ for hatchability).

2.3.4 Egg quality traits

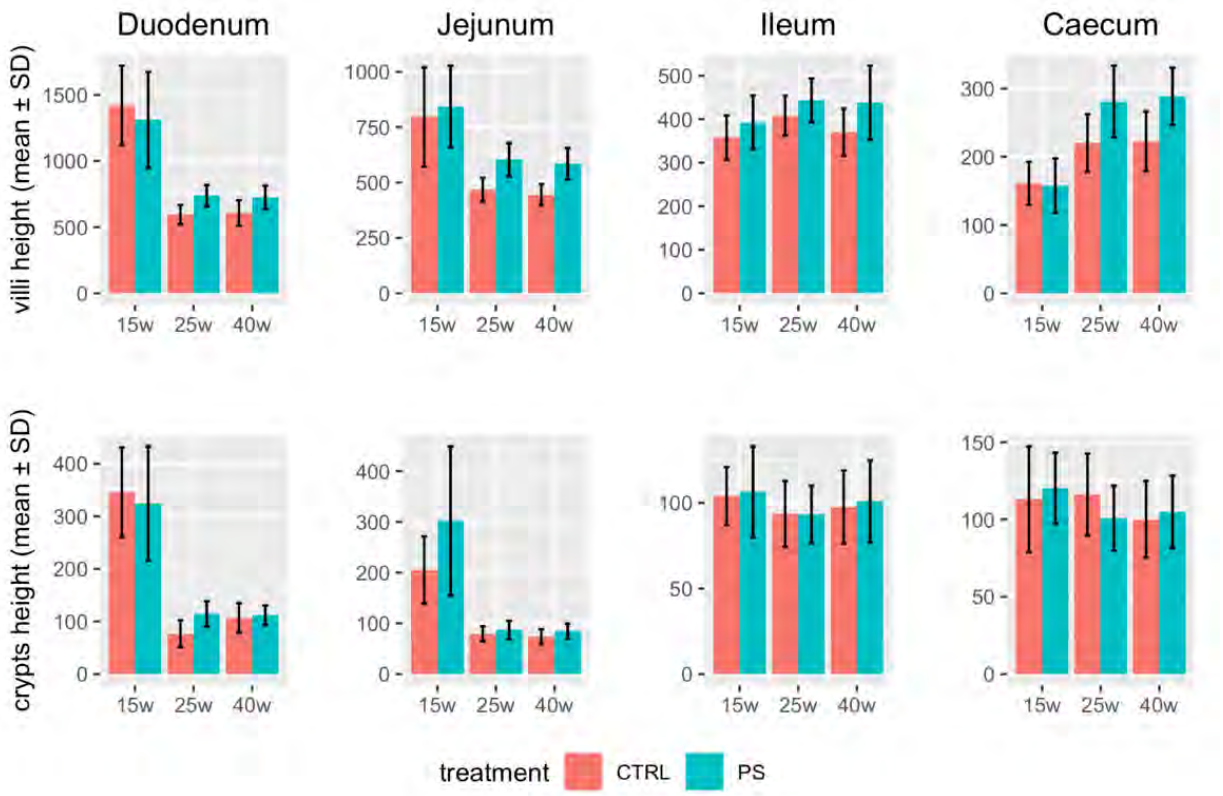
There were no significant differences in terms of eggshell strength, shell thickness and shape index, but some were found at limited time points in egg weight, shell weight and combined albumen and yolk weight between treatments and, more limitedly, between houses. In particular, the egg weight was higher in synbiotic-treated chickens than in control ones at week 30 ($p = 0.009$) but lower at week 40 ($p = 0.032$). Shell weight was higher in synbiotic-treated chickens than in control ones at week 30 ($p = 0.018$). The combined weight of yolk and albumen was higher in control chickens than synbiotic-treated ones at week 40 ($p = 0.026$). Overall, no clear trends that could be ascribable to the synbiotic treatment were identified (Graph 7).



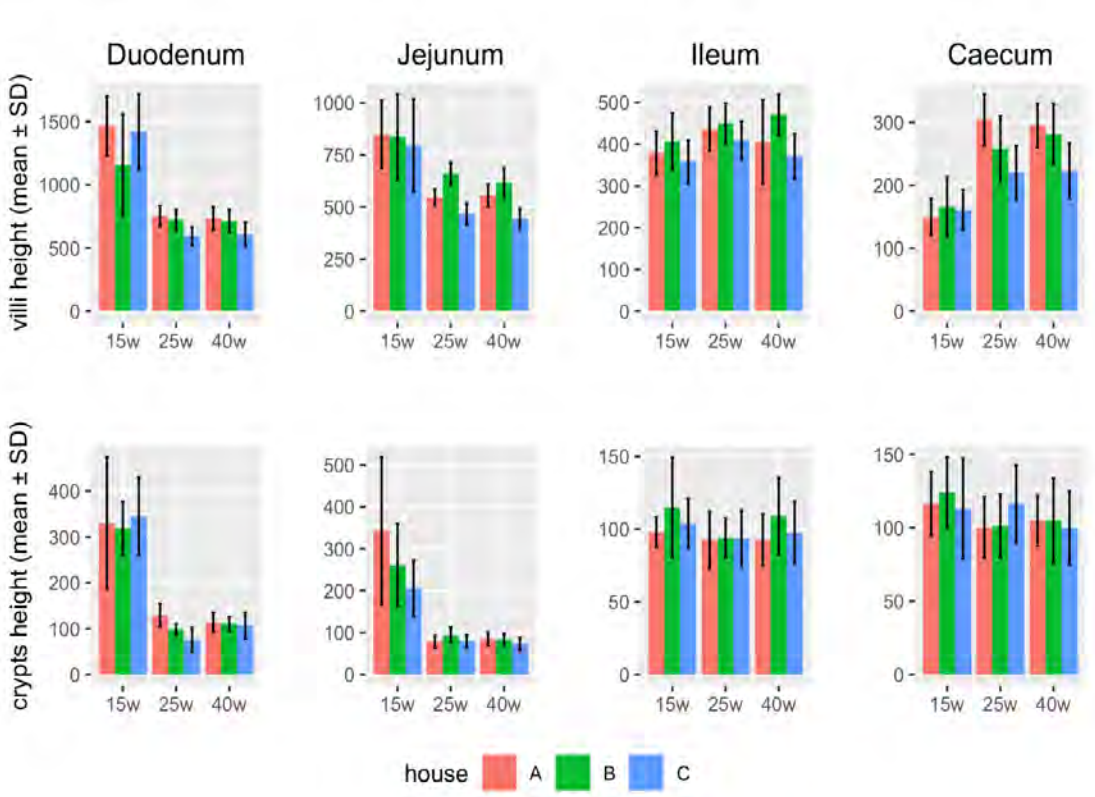
Graph 1. Bacterial enteritis score measured in synbiotic-treated and control broiler breeders



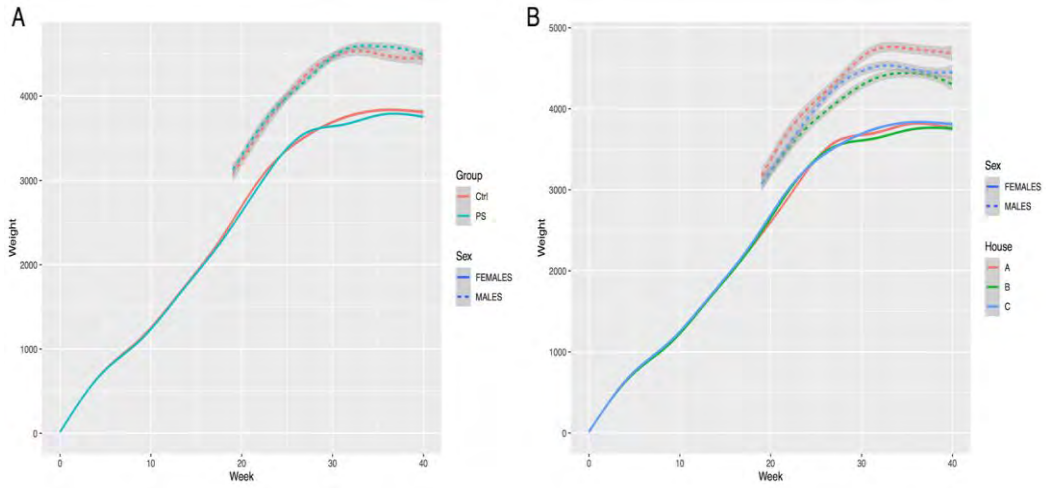
Graph 2. Histopathological lesion scores measured in different intestinal tracts in synbiotic-treated and control broiler breeders



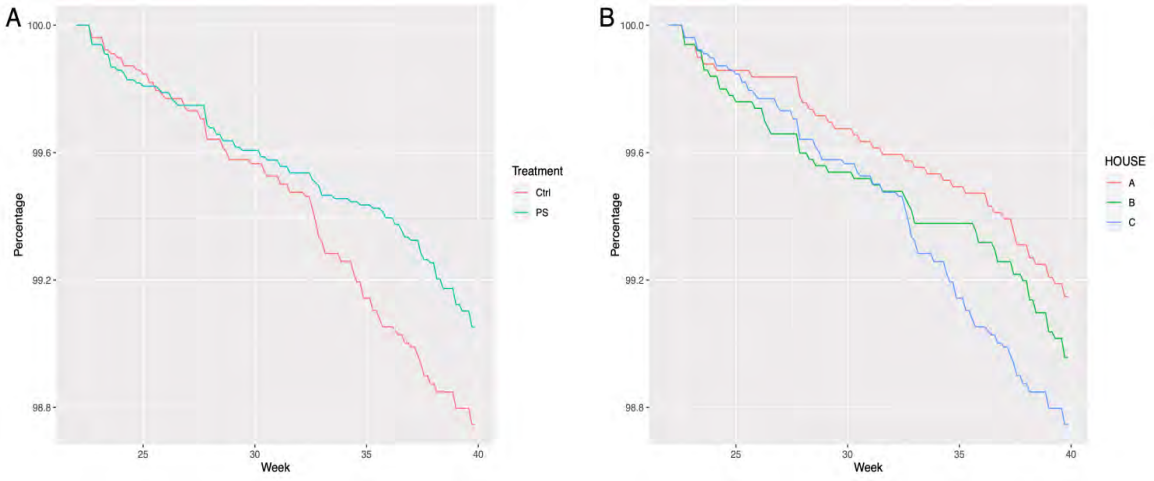
Graph 3. Gut morphometric parameters measured in different enteric tracts in synbiotic-treated and control chickens



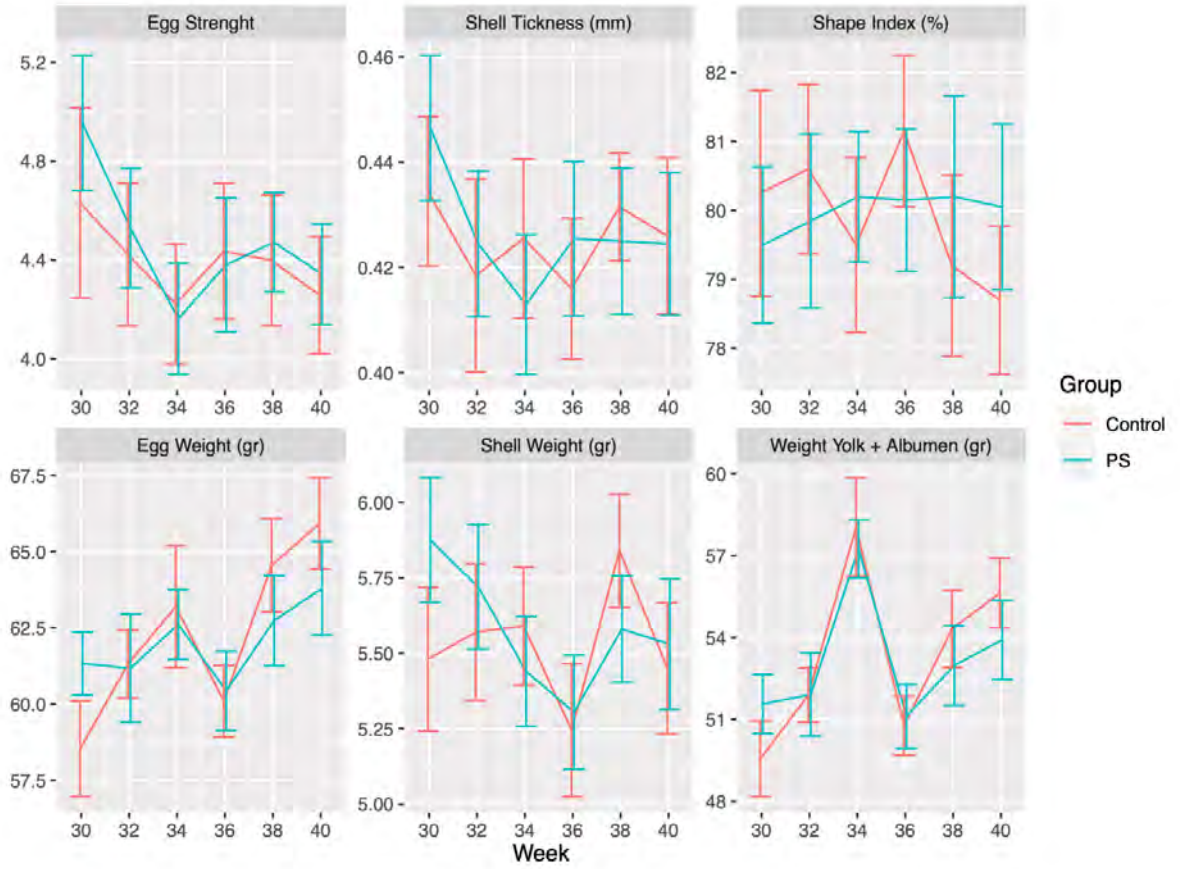
Graph 4. Gut morphometric parameters measured at 15, 25, and 40 weeks of age in different enteric tracts of the broiler breeders raised in the three houses. The synbiotic was administered in houses A and B, while house C acted as the control group



Graph 5. Growth curves comparison between synbiotic-treated and control broiler breeders (a) and between the three houses (b). The synbiotic was administered in houses A and B, while house C acted as the control group

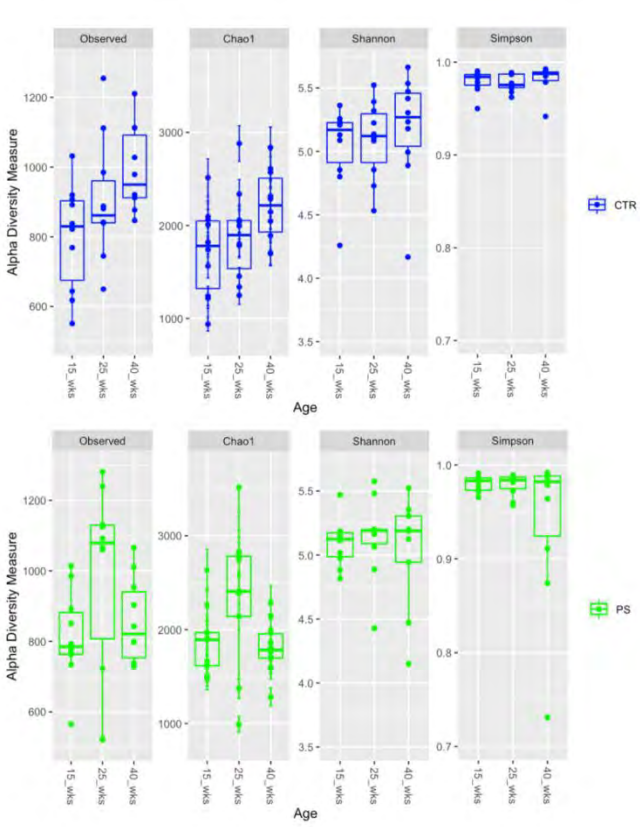


Graph 6. Comparison of survivability rates during the production period (23-40 weeks) between synbiotic-treated and control female broiler breeders (a) and between the three houses (b). The synbiotic was administered in houses A and B, while house C acted as the control group

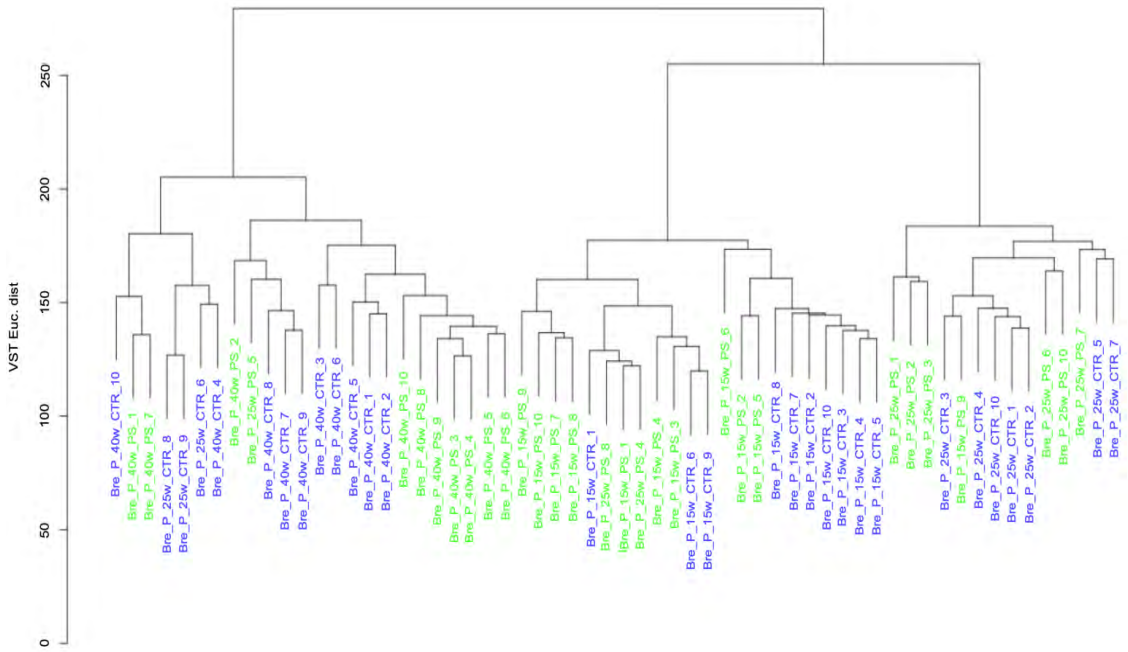


Graph 7. Comparison of egg traits between synbiotic-treated and control broiler breeder chickens

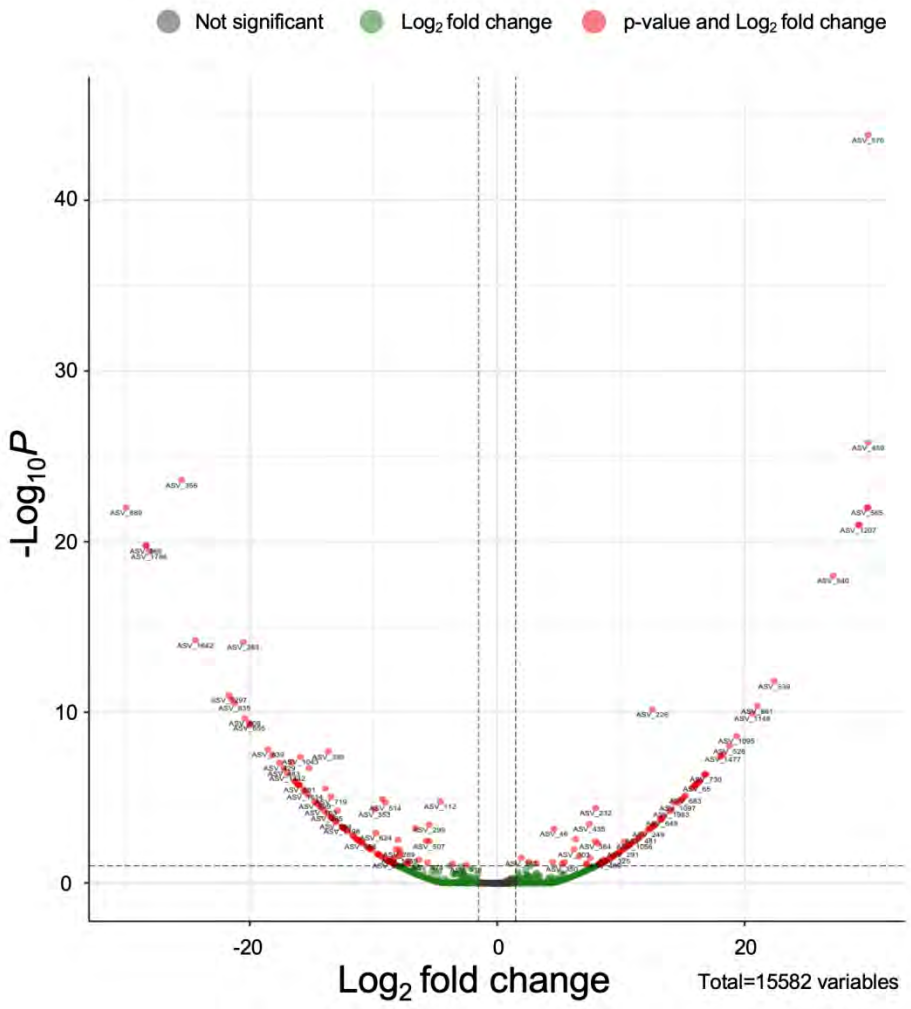
Graph 8. Relative microbial composition of caecal content of synbiotic-treated and control broiler breeder chickens, shown at Phylum (top), Order (centre) and Family (bottom) level. Phylum (top), Order (centre) and Family (bottom) level



Graph 9. Alpha-diversity indexes measured in synbiotic-treated (PS) and control (CTR) broiler breeder chickens and divided per age group



Graph 10. Dendrogram of the broiler breeder caecum samples, clustered on the Euclidean distance between their count data. Sample names are colored green for synbiotic-treated chickens and blue for control chickens. The age at sampling (15, 25 and 40 weeks) is indicated in the code of each sample



Graph 11. Volcano plot showing the differential abundance of amplicon sequence variants in the caecal microbiota of broiler breeders due to the synbiotic treatment effect. The statistical significance value was set to $p < 0.01$ (horizontal line), while, to be considered biologically significant, the effect size expressed in terms of Fold Change (FC) should have had an absolute value of 3 (vertical lines at $\log_2$ FC = 1.5)

2.3.5 Evaluation of enteric microbiota

According to sequencing results, the overall diversity in the caecum samples was rather high, with a total of 15582 different ASVs. The relative microbial abundance of

each caecal content is shown in Graph 8. According to the measured diversity indexes, the richness of different bacterial species was rather high in most of the samples and generally increased between weeks 15 and 25. A less evident trend was observed from week 25 to 40, when the bacterial diversity in the synbiotic-treated chickens was even shown to decrease (Graph 9). Hierarchical clustering on euclidean distance showed that samples tended to cluster based on treatment and age, with clear segregation between 15-week-old and 40-week- old chickens and only a slight overlap of 25-week-old chickens with both groups (Graph 10). A significant treatment effect was found by comparing the microbial composition of samples from synbiotic-treated and control chickens ($p = 0.025$). When the comparisons were between same-age chickens, the treatment effect was significant at week 15 ($p < 0.001$) and week 40 ($p = 0.03$), but not significant at week 25 ($p = 0.064$). The age effect was confirmed significant by comparing samples taken at different ages, both among treated and control chickens ($p < 0.001$ in both cases). Since synbiotic-treated chickens were reared in two separate houses, the possible house effect was also investigated but was found to be non-significant ($p = 0.083$). Inter-correlation analysis revealed no significant Spearman correlation of any variables to the treatment, indicating a proper experimental setup. When isolating the treatment effect, significant differences were detected in the abundance of 119 out of a total of 15582 ASVs (after Benjamini-Hochberg multiple testing correction, Graph 11). In particular, 45 ASVs were more abundant in the treated breeders, while 74 were less abundant. Among others, the treatment effect seems to have affected the relative abundance of *Gastranaerophilales*, *Helicobacter*, *Ruminococcaceae*, *Lachnospiraceae,* and *Clostridia* (Table 3).

Table 3. Top 10 differentially abundant amplicon sequence variants for the treatment effect ranked on the adjusted p-value. The direction of differential abundance can be inferred from the sign of the Log2 Fold Change

Amplicon sequence variant	Log2 fold change	Standard error	Adjusted p-value	Lowest resolved taxon
ASV_576	30.000000	2.057181	1.4620e-44	Gastranaerophilales
ASV_459	30.000000	2.643062	1.4979e-26	*Helicobacter*
ASV_356	-25.517909	2.349552	2.3997e-24	*Ruminococcaceae*
ASV_565	29.919828	2.863515	1.0061e-22	*Lachnospiraceae*
ASV_797	30.000000	2.870802	1.0061e-22	Bacteria
ASV_889	-29.994918	2.864174	1.0061e-22	Gastranaerophilales
ASV_1207	29.208433	2.864828	1.0554e-21	Clostridia UCG-014
ASV_1822	29.286145	2.872488	1.0554e-21	Clostridia UCG-014
ASV_966	-28.400792	2.867760	1.7165e-20	Clostridia UCG-014
ASV_1298	-28.383064	2.867419	1.7165e-20	Clostridia

2.4 Discussion

The present results comprehensively depict the effects of the considered synbiotic product on the performance and gut health of broiler breeders. Following a protocol devised with the manufacturer's guidance, PoultryStar® sol was administered for three consecutive days of weeks 1 and 21, as recommended for newly hatched poultry and around stressful periods and changes, such as the introduction of males. An intermittent schedule was observed throughout the rest of the cycle, which is recommended to support gut eubiosis continuously. Regarding the obtained results, it is useful to compare them to those obtained in previous trials of other synbiotics, bearing in mind that the outcomes may differ depending on each product's

composition, dosage, administration route, and timing, along with environmental and host-related factors. The effect of PoultryStar® sol administration on BW gain appeared limited, and the observed heterogeneity between the different groups seemed more easily ascribable to the house effect. Several synbiotics, mostly tested on broilers, were shown to increase BW gain and feed conversion ratio (Mohammed et al., 2018; Kridtayopas et al., 2019; Abdel-Wareth et al., 2019), while others had no impact on BW or feed conversion ratio (Chang et al., 2019; Dankowiakowska et al., 2019; Shanmugasundaram et al., 2020). Ultimately, it should also be considered that breeders' feeding programs are targeted at maintaining high weight uniformity and keeping close to BW targets, rather than maximizing growth and feed efficiency (Aviagen, 2018). Any overperformance compared to target BW during both rearing and production periods, may be compensated with feed restrictions (EFSA, 2010), thus masking any potential increase in feed efficiency related to synbiotic administration. Egg production and quality were also evaluated, as several synbiotics were shown to improve them. Luoma et al. (2017) found that administering a multi-species synbiotic increased egg production between 19 and 28 weeks of age, even after the chickens were challenged with *Salmonella enterica* serovar Enteritidis. Similar results were obtained by Radu-Rusu et al. (2010), Abdel-Wareth (2016), and Tang et al. (2017), who also reported a positive effect on egg quality, resulting in heavier, larger eggs with thicker shells. According to Buyarov and Metasova (2019), synbiotic-fed broiler parent stocks also showed an increase in egg production and hatchability. On the other hand, other tested probiotics and synbiotics had limited or no effect on laying performance (Tang et al., 2015; Liu et al., 2019; Sjofjan et al., 2021). In the present study, no significant differences were found in terms of egg fertility, hatchability, and morphology, except for specific sampling points in terms of

egg weight, shell weight, and combined albumen and yolk weight. Based on these findings, the tested synbiotic did not seem to affect egg production. A significant treatment effect was found in terms of survivability during the laying period, with both treated groups exhibiting lower mortality than the control one. The decision to focus on the production phase was taken because mortality rates in the rearing phase may be easily altered by culling procedures, which are often due to factors unrelated to the breeders' health, such as chickens not meeting selection criteria or sexing errors (EFSA, 2010). The observed differences suggest that PoultryStar® sol can effectively reduce mortality in field conditions, as already reported for other synbiotics (Awad et al., 2009; Abdel-Wareth et al., 2019; Rodrigues et al., 2020). Although the ultimate goal of synbiotic administration is to have healthier and, thus, more productive chickens, the evaluation of performance parameters only offers a partial and indirect assessment of their effect on gut health. Ringenier et al. (2021) noted that a healthier intestinal tract does not always correspond to an increase in production parameters, as birds can cope with a certain degree of gut lesions before their performance is affected. For this reason, gut health scores and intestinal morphometry were also considered to assess the effect of PoultryStar® sol in preventing any unfavorable state of inflammation or dysbacteriosis which could negatively alter the integrity of the intestinal mucosa and thus its absorption and immune functions (Willing and Van Kessel, 2009; Teirlynck et al., 2011). The BE score was lower in treated chickens than in control ones at all time points, with a statistically significant difference at 25 weeks of age. The histopathological lesion score was also significantly lower in the treated groups in the caecum (at 25 and 40 weeks) and ileum (at 40 weeks), while the control group scored better only at a single point at the jejunum level. According to these results, synbiotic-treated chickens exhibited better intestinal health even in the

absence of a challenge. This conclusion is supported by the evaluation of gut morphometric parameters, which showed that synbiotic-treated chickens had longer villi consistently along all intestinal tracts from 25 weeks of age onwards. Synbiotic trials often report an increase in villus height in different intestinal tracts, indicating a larger surface for nutrient absorption (Samanya and Yamauchi, 2002) throughout different intestinal tracts (Kridtayopas et al., 2019; Villagrán-de la Mora et al., 2019; Jiang et al., 2020). The effect of PoultryStar® sol on crypts, whose depth is related to the mucosal proliferative activity (Prakatur et al., 2019), appeared less evident and consistent, with deeper crypts being reported in the jejunum and duodenum while caecal crypts were less deep at 25 weeks of age. Similar findings are reported in previous studies, in which different synbiotic formulations were shown to increase (Villagrán-de la Mora et al., 2019), decrease (Sobolewska et al., 2017), or have no effects (Awad et al., 2009, Sobotik et al., 2021) on crypts depth. It should be noted that the interpretation of the obtained data was complicated by the fact that the two treated houses also exhibited significant differences in villi and crypts length. Nonetheless, the existence of an actual beneficial effect of the synbiotic treatment on intestinal morphology is supported by the overall agreement between the two treated houses compared to the control one, and by the general increase seen in the ratio between villi and crypts length. The use of high-throughput sequencing provided useful insights into the composition of the caecal bacterial population. However, exactly defining a healthy intestinal microbiota is not an easy task, as it is influenced by a multitude of environmental and host-related factors, such as litter, housing, climate and the chickens' age, sex and breed (Kers et al., 2018). The overall bacterial diversity was rather high and was shown to increase with age, in agreement with previous studies (Videnska et al., 2014; Ocejo et al., 2019). A highly diverse bacterial

community is indicative of good intestinal health, while a reduced heterogeneity could signal intestinal disease states (Ocejo et al., 2019; Madlala et al., 2021). The observed caecum composition was in agreement with what was expected in poultry, exhibiting a clear predominance of *Firmicutes,* and, in particular, of families belonging to the class *Clostridia*, such as *Lachnospiraceae*, *Methanobacteriaceae* and *Ruminococcaceae* (Clavijo and Florèz, 2018; Such et al., 2021). *Firmicutes* are associated with butyrate production, while *Bacteroidetes*, which represent a small fraction of the caecal microbiota, are involved in the production of propionate. Their ratio is commonly accepted as an indicator of the efficiency of energy harvesting in both humans and animals (Zhu et al., 2019). Videnska et al. (2014) studied the development of the caecal microbiota in laying hens over the entire production cycle and reported that, while *Firmicutes* were predominant during the first month of age, the relative abundance of *Bacteroidetes* increased between the second and the sixth month, leading to an even ratio between the two phyla in adult hens. Several studies also reported *Firmicutes* to be predominant in broiler chickens and young hens (Bjerrum et al., 2006; Nordentoft et al., 2011; Videnska et al., 2013), while members of *Bacteroidetes* seem more abundant in older chickens (Callaway et al., 2009). While this shift has not been observed in the present study, with *Firmicutes* being by far the predominant phyla even at 40 weeks of age, it should be considered that the F/B ratio is heavily determined by the administered feed (Nordentoft et al., 2011) and that it has never been investigated before in broiler breeders, thus preventing comparisons with chickens sharing the same genetic features and producing conditions.

The treatment effect on bacterial composition was confirmed to be statistically significant and led to a differential abundance of 119 ASVs. Among the most impacted were members of the families *Lachnospiraceae* and of the genus

Helicobacter, which were overrepresented in treated chickens, and of *Ruminococcaceae*, which in turn were underrepresented. More puzzlingly, members of *Gastranaerophilales* and *Clostridia* were found among both the most over and underrepresented ASVs in treated chickens. All these bacteria are common inhabitants of the caecal microbiome (Aruwa et al., 2021; Gilroy et al., 2021; Xiao et al., 2021), and their abundance was already proven to be modulated by several nutraceuticals. Díaz Carrasco et al. (2018) found that tannins administration increased the relative abundance of *Helicobacter* and, more importantly, of members of both *Lachnospiraceae* and *Ruminococcaceae* (and decreased other members of the two families), possibly shifting the short-chain fatty acids caecal profile towards butyrate production. Li et al. (2020) reported that the supplementation of fermented soybean meal in broilers led to an increased abundance of *Gastranaerophilales*, which in turn was positively correlated to an improved average daily gain and serum immunity. Previous studies relying on high-throughput sequencing already investigated the effect of synbiotics with different compositions on chickens' intestinal microbiota, but, to the authors' knowledge, this is the first time this technique is carried out in broiler breeders, not allowing a comparison with chickens with similar genetic traits and raised under the same production system. Pineda-Quiroga et al. (2019) found that treating laying hens with a synbiotic product based on dry whey powder and *Pediococcus acidilactici* increased the caecal abundance of *Actinobacteria*, *Olsenella* spp. and *Lactobacillus crispatus*, among others. The double administration of a multi-species synbiotic, both by spray at the hatchery and in the feed throughout the broiler cycle, caused an increased abundance of *Actinobacteria* and *Lactobacillus* spp. as well, along with several members of *Clostridia*, and also led to a higher *Firmicutes* to *Bacteroidetes* ratio (Brugaletta et al., 2020). Another trial conducted in broiler

chickens found that a synbiotic containing *Bacillus subtilis*, yeast, and inulin did not affect the caecal microbiota (Such et al., 2021). The diversity in the results obtained by these studies can be easily justified by the many variables at play (experimental design, synbiotic composition and dosage, productive type, breed, age at sampling, feed, rearing conditions) and by the inherent complexity of the caecal ecosystem, which hosts the largest (and partially unculturable) bacterial population out of all intestinal tracts (Aruwa et al., 2021). On the other hand, this adds value to the herein reported data, which are among the first to provide a longitudinal perspective on the enteric microbiome of broiler breeders.

2.5 Conclusion

Based on the reported results, the synbiotic product PoultryStar® sol appears fully applicable to broiler breeders by intermittent administration via drinking water. Histopathological and morphometrical findings support its beneficial effect on gut health, and higher survivability was also observed in treated chickens during the production phase. In addition, the synbiotic treatment had a modulating effect on several bacterial populations hosted in the caeca, whose actual impact will require further investigations to be fully elucidated.

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CHAPTER THREE

ADMINISTRATION OF A MULTI-GENUS SYNBIOTIC TO BROILERS: EFFECTS ON GUT HEALTH, MICROBIAL COMPOSITION AND PERFORMANCE

3.1 Introduction

For decades, antibiotics have been utilized in the poultry industry to prevent and treat diseases and promote growth. Despite the significant improvements in technology and hygienic practices made at all stages of poultry production, bacterial diseases remain a persistent threat not only to animal health, but also to humans, with recent reports showing that *Salmonella* spp. and *Campylobacter* spp. are the most common causes of human foodborne bacterial diseases linked to poultry (Hafez and Adawy, 2019, Thames and Sukumaran, 2020). Due to the increasing awareness towards antimicrobial resistance, stricter regulations have been imposed on antibiotic use, and antibiotic-free poultry production has grown more and more popular. Nonetheless, several issues related to food safety and chicken welfare still need to be addressed to ensure the viability of such production systems, and there is an increasing need for alternative strategies to support production efficiency (Haque et al., 2020).

Since antibiotic growth promoters (AGPs) were crucial to controlling dysbacteriosis and enteropathogens (Dibner and Richards., 2005), novel alternatives must be found following their ban to support gut health, which has several implications for the birds' overall health, production efficiency, food safety and environmental impact (Oviedo-Rondon, 2019). Promoting eubiosis and minimizing enteric diseases is therefore essential to ensure the sustainability of poultry production. Several feed additives

have been explored as natural alternatives to AGPs, including probiotics, prebiotics, synbiotics, organic acids, essential oils, enzymes, immunostimulants and phytobiotics (Abd El-Hack et al., 2022). Many of these products demonstrated beneficial effects similar to antibiotics in modulating the gut microbiome and improving the health and growth of the animals (Khomavezi and Adewole, 2022). The studies conducted on synbiotics, which rely on a synergism between probiotics and prebiotics, have shown particularly promising results. The supplementation of different synbiotics to broilers was demonstrated to improve body weight (BW) gain and feed efficiency (Awad wt al., 2009), reduce mortality (Mohnl et al., 2007), increase the resistance to heat stress (Hu et al., 2022), stimulate the development of the gut-associated lymphoid tissue (GALT) (Madej and Bednarczyk, 2016), and decrease the intestinal and carcass load of coliforms (Dibaji et al., 2014), *Clostridium perfringens* (Abd El-Ghany, 2010), *Campylobacter* spp. (Baffoni et al., 2017) and *Salmonella* spp. (Sobotik et al., 2021). The benefits of synbiotics in terms of performance and intestinal health, along with their modulatory action on the microbial enteric composition (Fathima et al., 2022), appear therefore well documented. Nonetheless, different formulations may have diverse features and modes of action and require a dedicated assessment of their efficacy and safety, including the risk of carrying antimicrobial resistance and producing deleterious metabolites (Applegate et al., 2010). In the present study, a commercial multi-species synbiotic product was administered to three broiler flocks, which were progenies of a breeder flock treated with the same synbiotic and were reared in three separate farms in typical field conditions, evaluating its effects on productive performance, gut health and caecal microbiota.

3.2 Materials and Methods

3.2.1 Experimental setup

The present study was conducted on the broiler progenies of a broiler breeder flock that was treated with the synbiotic product PoultryStar® sol (PS) (BIOMIN GmbH, Herzogenburg, Austria), as detailed in Prentza et al. (2022). Day-old chicks from eggs laid at 30, 35 and 40 weeks of age were placed into three different commercial farms (named 1, 2 and 3) and raised under typical field conditions until slaughter at 42 days of age (doa). In each farm, the chicks were divided between two different houses, observing a stocking density of 15 birds/m^2. PS was administered in one of the two, while the other acted as control. In detail, depending on the house size, 8160 chicks were housed in the treatment house of farm 1, while 14,280 were placed in the control house; for farm 2, 11,730 and 10,200 birds were set up in the treatment and control houses, respectively; in farm 3, 15,198 chicks were placed in each of the houses.

3.2.2 Management

To ensure flock health and welfare and achieve good flock performance, management conditions followed the official guidelines for broiler birds (Ross Broiler Handbook, 2018). Wheat straw and rice hulls were used as litter materials in cleaned and disinfected houses. The basal diet was formulated using maize, wheat and soybean meal in accordance with the official genetic line guidelines (Ross Broiler Nutrition Specifications, 2022). Feed and water were provided *ad libitum*. The lighting program started at 7 days of age, providing 4 hours of darkness and 20 hours of light. Birds were vaccinated against infectious bursal disease (IBD), infectious bronchitis (IB) and Newcastle disease (ND) at the hatchery following the local vaccination program.

3.2.3 Synbiotic administration

PS, which is a synbiotic containing patented probiotic strains plus prebiotic fructooligosaccharides, was administered in one of the two houses in each of the three broiler farms, based on the manufacturer's guidance. Specifically, a daily dosage of 20 g/1000 birds was administered in clean drinking water consecutively for the first three days of age, as recommended following the chicks' placement, and then once a week till slaughter age was reached.

3.2.4 Bacterial enteritis (BE) scoring

To evaluate the chickens' intestinal health and the presence of dysbiosis, the integrity of the intestinal wall was visually evaluated at three different time points (10, 28 and 38 doa) on ten birds randomly picked from different points of each house of each farm by applying a macroscopic lesion scoring system consisting of ten different parameters, which were scored 0 when absent and 1 when present. The individual scores were summed and divided by 2.5, yielding a total score ranging between 0 and 4 (De Gussem, 2010, Teirlynck et al., 2011).

3.2.5 Histology

Specimens from different intestinal tract segments were collected at 38 doa from ten birds randomly picked from different points of each house of each farm for histopathological and morphometrical evaluations. In detail, 3 cm long segments were collected from the duodenum, jejunum, ileum and caecum, keeping the collection sites consistent for each tract, and placed in 10% neutral buffered formalin as described by Hoerr (2001). One mm thick transversal sections were cut after 48 hours, then sections of 3–5 μm were taken, stained with hematoxylin and eosin and evaluated. The histopathological scoring system proposed by Kraieski et al. (2017)

was adopted to assess the degree of inflammation in each section, grading the severity of the lesions on a 0–3 scale (0: absent or rare leukocytic infiltration; 1: leukocytic infiltration up to 5% of a ×400 field; 2: approximately 25% leukocytic infiltration of a ×400 field; 3: leukocytic infiltration in the range of 50% or more of a ×400 field).

The morphometry of the intestinal villi and crypts were examined in each section, performing optical capture and measurement with Image Pro-Plus v.6.0 software (Media Cybernetics, Silver Spring, MD, USA). The selection of the villi followed the criteria proposed by Gava et al. (2015), namely the embedment of the base into the submucosa, the absence of any discontinuity or folding in the length of the villus, and the presence of intact epithelium at the tip.

3.2.6 Recording of performance parameters

To evaluate potential growth differences, 100 randomly selected chickens from each house of each farm were weighed longitudinally from 0 to 30 doa. The final BW was recorded when birds were loaded onto the trucks at 42 doa, and the feed conversion ratio (FCR) was calculated by dividing feed intake by the total BW gain. In addition, the carcass weight of 100 randomly selected birds from each group was measured at the slaughterhouse. Mortality was recorded daily throughout the whole cycle.

3.2.7 Evaluation of enteric microbiota

To evaluate the caecal microbial composition, Next Generation Sequencing (NGS) was performed on 60 caecal content samples taken at 38 doa from 10 randomly selected birds of each of the two houses of the three farms. The analyses, conducted using an Illumina MiSeq System (Illumina, San Diego, CA, USA) at LGC Genomics GmbH (Berlin, Germany), targeted the V3 region of the 16s rRNA gene, generating 2 × 300 paired-end sequences. Following a preliminary quality evaluation with FastQC

0.11.9, the forward and reverse sequences were trimmed at 195 bp and 220 bp, guaranteeing a minimal Phred score of 28 and allowing for a maximum expected error of 2 bases for each read. DADA2 (Callahan et al., 2016) was used to infer the best fitting Amplicon Sequence Variants (ASVs), then the forward and reverse sequences were merged, and chimeric sequences were discarded. Finally, ASVs were converted to taxa using the SILVA 138 database (Quast et al., 2013, Yilmaz et al., 2014) as a reference. Alpha diversity was evaluated using the Simpson, Shannon, Chao1, and Observed species indexes. Permutational ANOVAs on the euclidean distances among samples were performed for significance testing between groups, after verifying that group dispersions were adequately homogeneous using the *betadisper* function of the *vegan* R package (Dixon, 2003). The absence of systematic biases was also confirmed by calculating the Spearman correlation between the treatment effect and all the other variables. Finally, the isolated treatment effect, along with the potential effect of other factors, was assessed by performing a differential abundance analysis with DESeq2.

3.2.8 Statistical analyses

The existence of significant differences in terms of BE score, histopathological lesion score, villi and crypts height which may have been ascribed to treatment, farm effect or sampling age (in case of longitudinal sampling) were investigated using the non-parametric Kruskal–Wallis test followed by post-hoc Mann–Whitney test with Bonferroni correction. The treatment and farm effects on the carcass weight was investigated with a two-way ANOVA followed by post-hoc Tukey's test. Log-rank test was used to compare the Kaplan–Meier survival curves with the *survival* package. All statistical analyses were performed in R (version 3.3.2) (R Core Team, 2017) setting the significance level to $p < 0.05$, with the sole exception of the differential

abundance analysis of microbial populations, for which the significance level was set to $p < 0.01$.

3.3 Results

3.3.1 Bacterial enteritis and histopathological lesion scores

The results obtained in terms of BE score (Figure 1) showed that the macroscopic signs of dysbacteriosis were limited in all treatment and control groups, whose scores were always below or around 1 on a scale from 0 to 4, where 0 corresponds to a normal gastrointestinal tract and 4 to a status of severe dysbacteriosis. However, the BE score was shown to increase significantly with age ($p < 0.0001$). Significant differences between PS-fed and control birds were found at 10 ($p = 0.0228$) and 38 doa ($p = 0.0495$) and when considering all ages together ($p = 0.0162$). Since the farm effect was also shown to be significant ($p = 0.009$), each farm was also assessed individually. The differences between synbiotic-treated and control groups were found to be limited to farm 3, where they were again significant at 10 ($p = 0.0077$) and 38 doa ($p = 0.0108$) and when considering all ages together ($p = 0.0009$). No statistically significant differences were observed in farm 1 and 2.

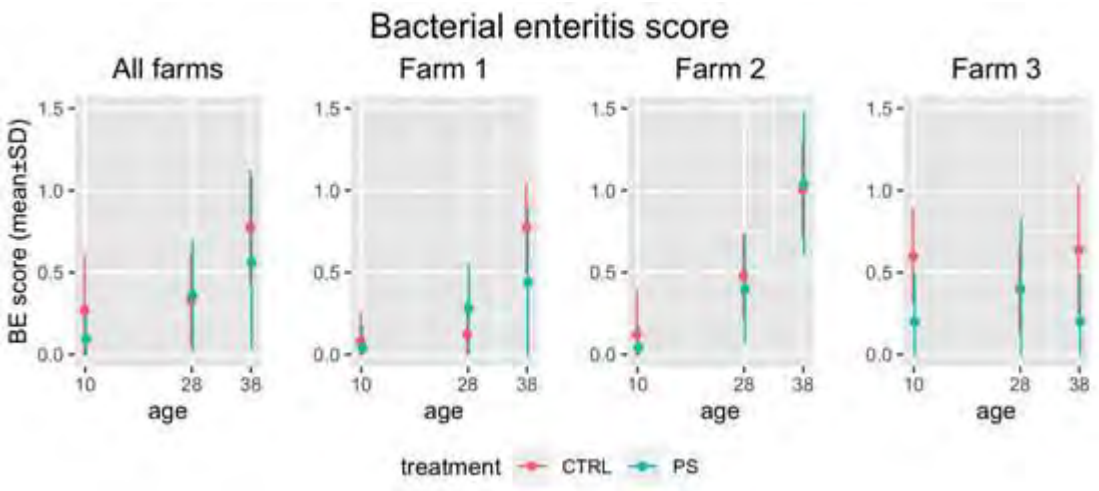

Figure 1. BE score measured in treatment (PS) and control (CTRL) groups at different time points by considering all farms together and then each one separately. BE scores are expressed on a scale from 0 (normal gastrointestinal tract) to 4 (severe dysbacteriosis)

More overt differences were found in terms of histopathological lesions scores (Table 1). The average scores measured in each group mostly corresponded to a mild to moderate grade of inflammation in all intestinal tracts, with treated birds scoring significantly better in most of the comparisons. Significant between-farm differences were found at jejunum level ($p = 0.0023$), with farm 2 scoring worse than both farm 1 ($p = 0.0005$) and farm 3 ($p = 0.04265$), and at caecum level ($p = 0.01499$), which seemed mostly ascribable to differences between farm 2 and farm 3 ($p = 0.03127$).

Table 1. Mean ± standard deviation of the histopathological lesion scores measured at duodenum, jejunum, ileum and caecum level of treated (PS) and control (CTRL) birds. Scores are reported on a scale from 0 (absent or rare leukocytic infiltration) to 3 (leukocytic infiltration in the range of 50% of a ×400 field). *P*-values below 0.05, marking the statistical significance of a difference observed when comparing treatment and control groups across all farms or in each farm individually, are underlined.

Farm	**Intestinal tract**	**Histopathological lesion score**		
		CTRL	**PS**	***p*-value**
All farms	Duodenum	1.667 ± 0.480	1.367 ± 0.490	0.0211
	Jejunum	1.833 ± 0.379	1.3 ± 0.466	<0.0001
	Ileum	1.8 ± 0.407	1.233 ± 0.430	<0.0001
	Caecum	2.067 ± 0.691	1.5 ± 0.630	0.0081
Farm 1	Duodenum	1 ± 0.000	1.6 ± 0.516	0.005
	Jejunum	1.5 ± 0.527	1.1 ± 0.316	0.06362
	Ileum	1.5 ± 0.527	1 ± 0.000	0.0137
	Caecum	2.3 ± 0.675	1.2 ± 0.483	0.0017
Farm 2	Duodenum	2 ± 0.000	1.3 ± 0.483	0.0016
	Jejunum	2 ± 0.000	1.7 ± 0.483	0.0767
	Ileum	1.9 ± 0.316	1.4 ± 0.516	0.0251
	Caecum	1.6 ± 0.516	1.5 ± 0.527	0.6934
Farm 3	Duodenum	2 ± 0.000	1.2 ± 0.133	0.0004
	Jejunum	2 ± 0.000	1.1 ± 0.100	<0.0001
	Ileum	2 ± 0.000	1.3 ± 0.157	0.0016
	Caecum	2.3 ± 0.213	1.8 ± 0.249	0.1557

3.3.2 Evaluation of intestinal villi and crypts

The average villi and crypts lengths measured at 38 doa in PS-treated and control flocks, along with the villi/crypts (V/C) ratio, are shown in Table 2. Significant differences in villi length were observed in most of the enteric tracts, both at overall level and when considering each farm separately. On the other hand, the differences in terms of crypt length appeared more limited (Table 2). The farm effect proved significant for both villi ($p < 0.0001$ for all intestinal tracts) and crypts ($p < 0.00001$ for all intestinal tracts) length.

Table 2. Mean ± standard deviation of villi and crypts length (measured in μm) in each intestinal tract of synbiotic-treated (PS) and control (CTRL) birds. *P*-values below 0.05, marking the statistical significance of a difference observed when comparing treatment and control groups across all farms or in each farm individually, are underlined.

Farm	Intestinal tract	Villi length			Crypt length		
		CTRL	PS	*p*-value	CTRL	PS	*p*-value
All farms	Duodenum	725.7± 150.2	760.6± 125.6	0.0221	160.8± 62.7	155.9± 70.2	0.1343
	Jejunum	446.7± 117.0	498.0± 109.0	<0.0001	114.8± 50.0	121.9± 57.0	0.5289
	Ileum	255.7± 90.2	304.4± 85.9	<0.0001	107.2± 55.6	109.4± 43.6	0.2507
	Caecum	114.3± 46.2	146.0± 81.8	0.0113	91.7± 34.7	94.0± 38.1	0.764
Farm 1	Duodenum	709.8± 136.2	710.9± 178.7	0.9478	216.4± 73.1	234.9± 63.1	0.2385
	Jejunum	518.4± 94.8	525.6± 98.0	0.6969	165.1± 53.2	184.4± 50.4	0.0589
	Ileum	345.3± 64.6	382.9± 94.0	0.046	158.4± 67.9	163.2± 29.9	0.0189
	Caecum	152.0± 42.2	224.3± 93.4	0.0002	123.1± 29.2	132.2± 37.3	0.2482
Farm 2	Duodenum	791.6± 109.9	804.0± 72.3	0.8795	147.4± 31.0	118.3± 28.4	<0.0001
	Jejunum	344.0± 87.9	484.6± 92.1	<0.0001	88.5± 18.9	88.2± 22.3	0.5372
	Ileum	199.0± 46.5	276.9± 40.3	<0.0001	80.5± 17.8	82.5± 13.9	0.5259
	Caecum	78.8± 29.4	112.3± 31.9	<0.0001	81.2 ± 23.7	70.6± 19.8	0.0329
Farm 3	Duodenum	659.7 ± 170.2	766.8 ± 79.4	0.0025	118.7 ± 22.4	114.6 ± 24.8	0.6075
	Jejunum	477.8 ± 88.9	483.8 ± 129.9	0.5837	90.87 ± 23.3	93.0 ± 29.0	0.855
	Ileum	222.6 ± 76.3	254.3 ± 49.9	0.0383	82.9 ± 21.5	82.3 ± 16.1	0.9341
	Caecum	112.1 ± 33.2	101.5 ± 34.3	0.0926	70.5 ± 25.7	79.5 ± 19.1	0.0141

3.3.3 Performance parameters

Throughout the cycle, the average live BW measured in synbiotic-treated flocks was consistently higher than in control ones (Figure 2). The comparison of average carcass weights and FCRs, shown in Table 3, proves a significant treatment effect at overall level ($p < 0.0001$) and in each individual farm, while the farm effect and the interaction between treatment and farm effect were not.

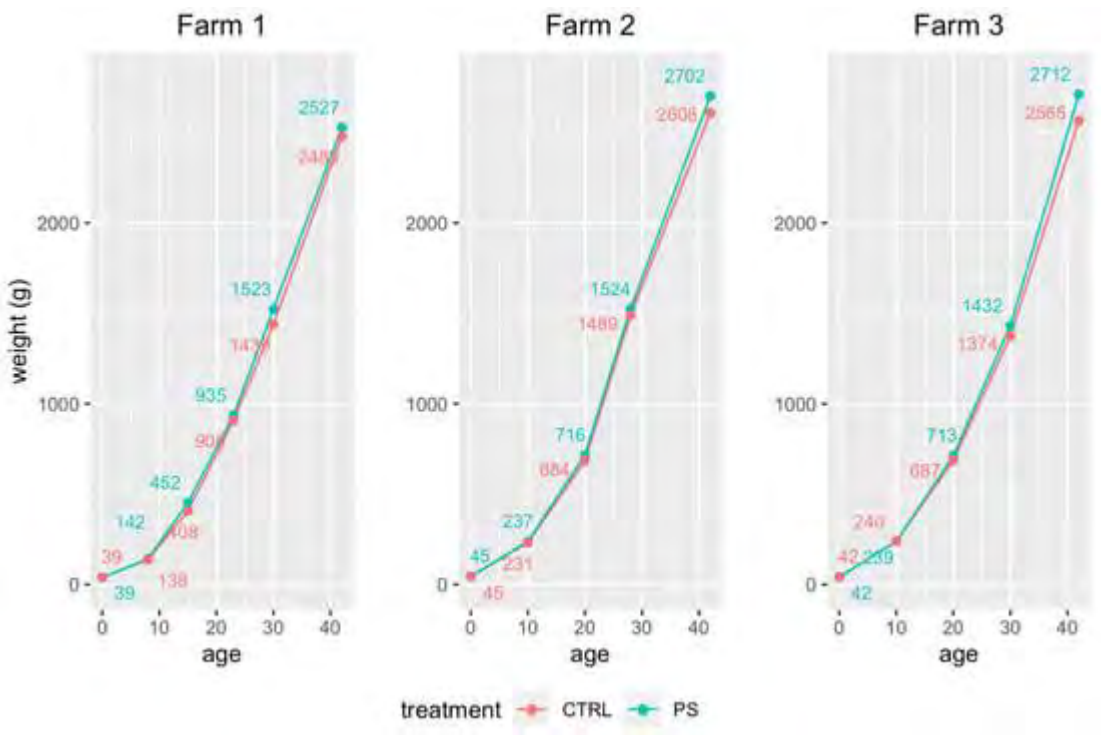

Figure 2. Live BW progression in synbiotic-treated (PS) and control chickens (CTRL)

Table 3. Average carcass weights and feed conversion ratios (FCRs) measured in treated and control houses of the three farms. P-values below 0.05, marking the statistical significance of a difference observed when comparing treatment and control groups, are underlined.

Farm	Average Carcass Weight (FCR)		
	CTRL	PS	*p*-value
Farm 1	1948 g (1.85)	2021 g (1.79)	0.0094
Farm 2	2001 g (1.70)	2095 g (1.64)	0.0052
Farm 3	1979 g (1.76)	2087 g (1.70)	0.0079

Mortality rates observed in treated and control birds at overall level were 3.5% and 5.3%, respectively ($p < 0.0001$). Since the farm effect was found to be significant ($p < 0.0001$), each farm was also considered separately. In detail, mortality was 4.8% in the control group and 2.5% among treated birds in farm 1 ($p < 0.0001$); 3.7% and 2.8% in farm 2 ($p = 0.0002$); 6.9% and 4.7% in farm 3 ($p < 0.0001$) (Figure 3).

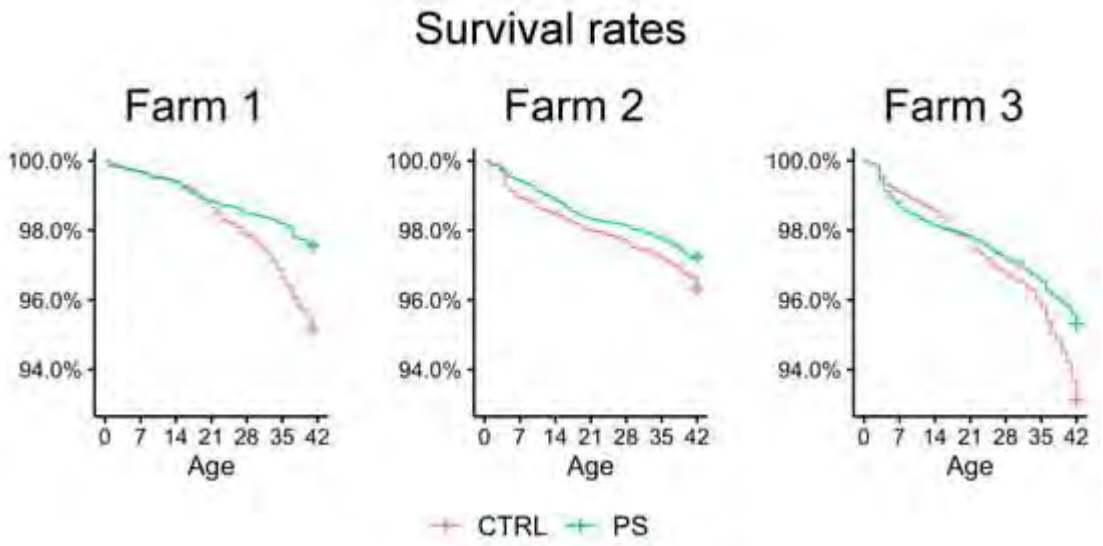

Figure 3. Kaplan–Meier survival estimates showing the mortality rates observed throughout the productive cycle in treated (PS) and control (CTRL) groups of each of the three farms

3.3.4 Evaluation of enteric microbiota

As shown in the dendrogram based on euclidean distances provided in Figure 4, samples clustered according to the farm in which they were collected. Samples from farm 2, which housed the progeny from eggs from 35-week-old layers, clustered more closely with farm 3 (progeny from 40-week-old layers) than with farm 1 (progeny from 30 weeks-old layers). Within farms, samples tended to cluster according to treatment, albeit with a few exceptions.

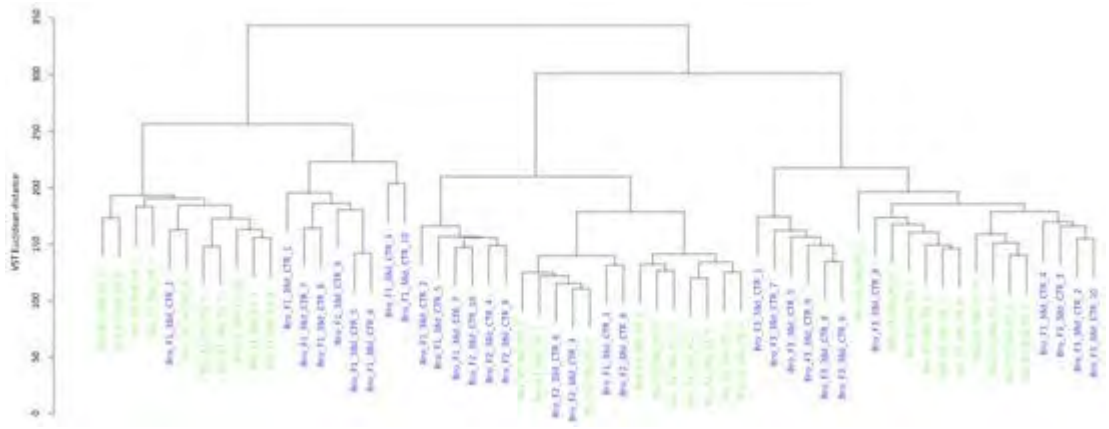

Figure 4. Dendrogram of caecal content samples, clustered on the euclidean distance between their taxonomic count data. Samples are color-coded by treatment (green for synbiotic-treated chickens, blue for control ones)

The differences between treatments within the same farm was also made visible by some of the alpha diversity measures, namely the Observed species and the Chao1 indexes, although were less evident according to the Shannon and Simpson indexes (Figure 5). The effect appeared particularly noticeable in samples from farm 2, with synbiotic-treated chickens showing a lower species richness than control birds.

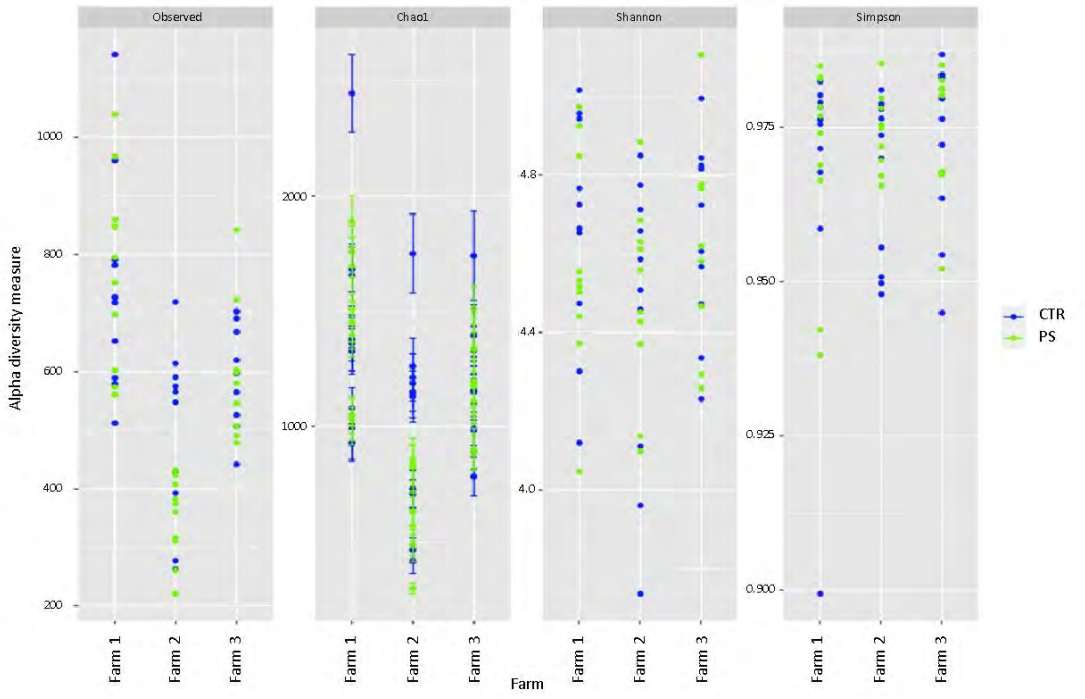

Figure 5. Alpha diversity measures for all caecal content samples divided by farm and color-coded based on treatment (green for synbiotic-treated chickens, blue for control ones)

A more comprehensive overview of diversity is provided by Figure 6, which shows the bacterial composition of each sample split on phylum, order and family level.

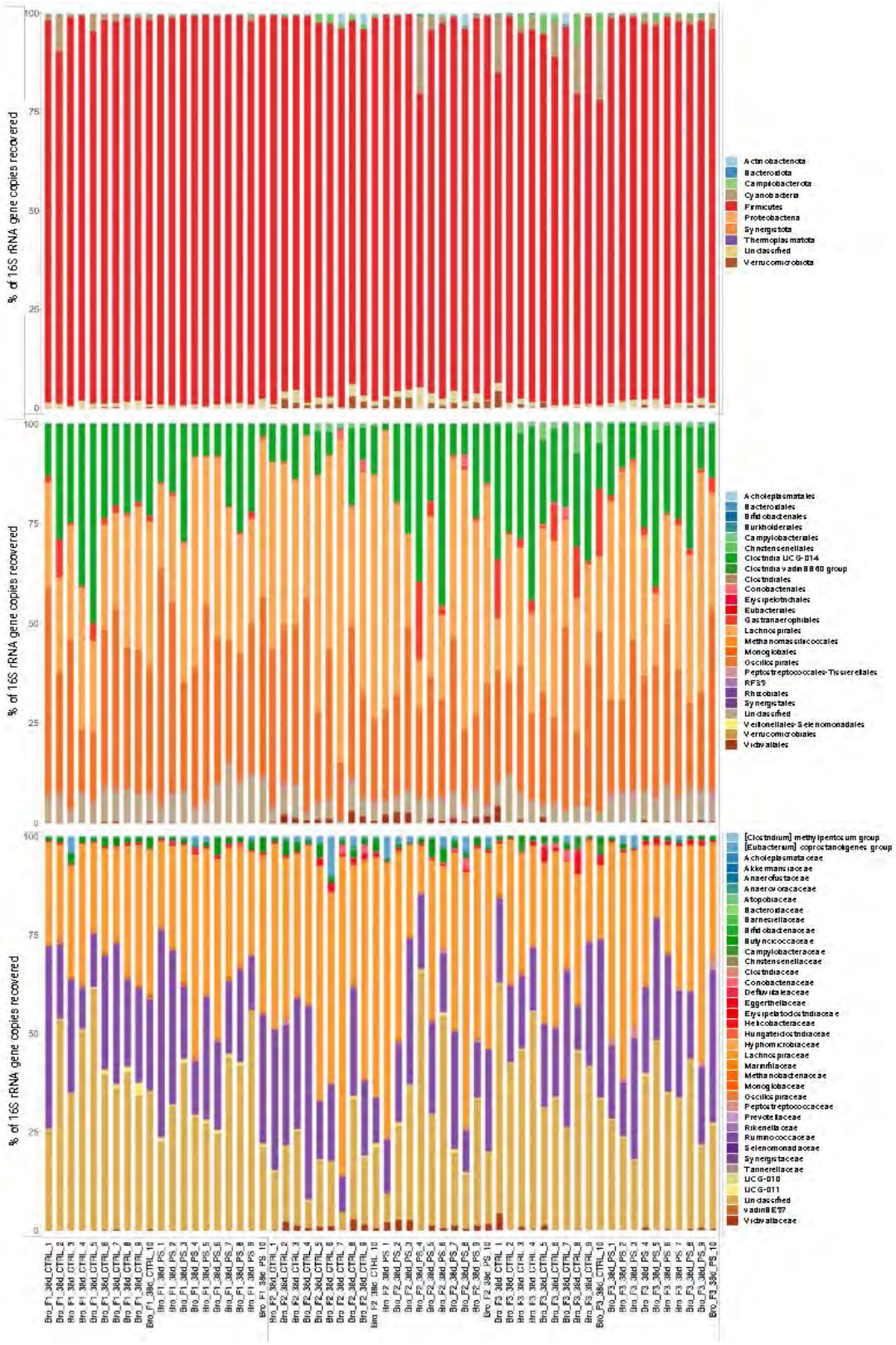

Figure 6. Relative microbial composition measured in individual caecal content samples, shown at Phylum (**top**), Order (**centre**) and Family (**bottom**) level

Since the beta dispersion within treatment group proved adequately homogeneous ($p = 0.228$) a permutational ANOVA test was subsequently performed, revealing siginificant differences between synbiotic-treated and control chickens at overall level ($p = 0.002$) and in each of the three farms ($p < 0.001$ in all three cases).

Intercorrelation analysis revealed a significant Spearman correlation between the treatment and the length of caecal villi ($\rho = -0.6195$; $p < 0.0001$), but not with any other considered parameter. Considering the design of the study, these results allowed us to isolate the effect of the synbiotic treatment on bacterial composition. Out of 9530 ASVs, 65 had a significant differential abundance on an alpha level of 0.01 (after Benjamini–Hochberg multiple testing correction). By setting out the obtained adjusted *p*-values for each ASV against the respective fold changes (Figure 7), 40 ASVs were shown to be less abundant in synbiotic-treated than in control chickens, while 25 ASVs were overrepresented. The top 10 ASVs with the lowest adjusted *p*-values are shown in Table 4.

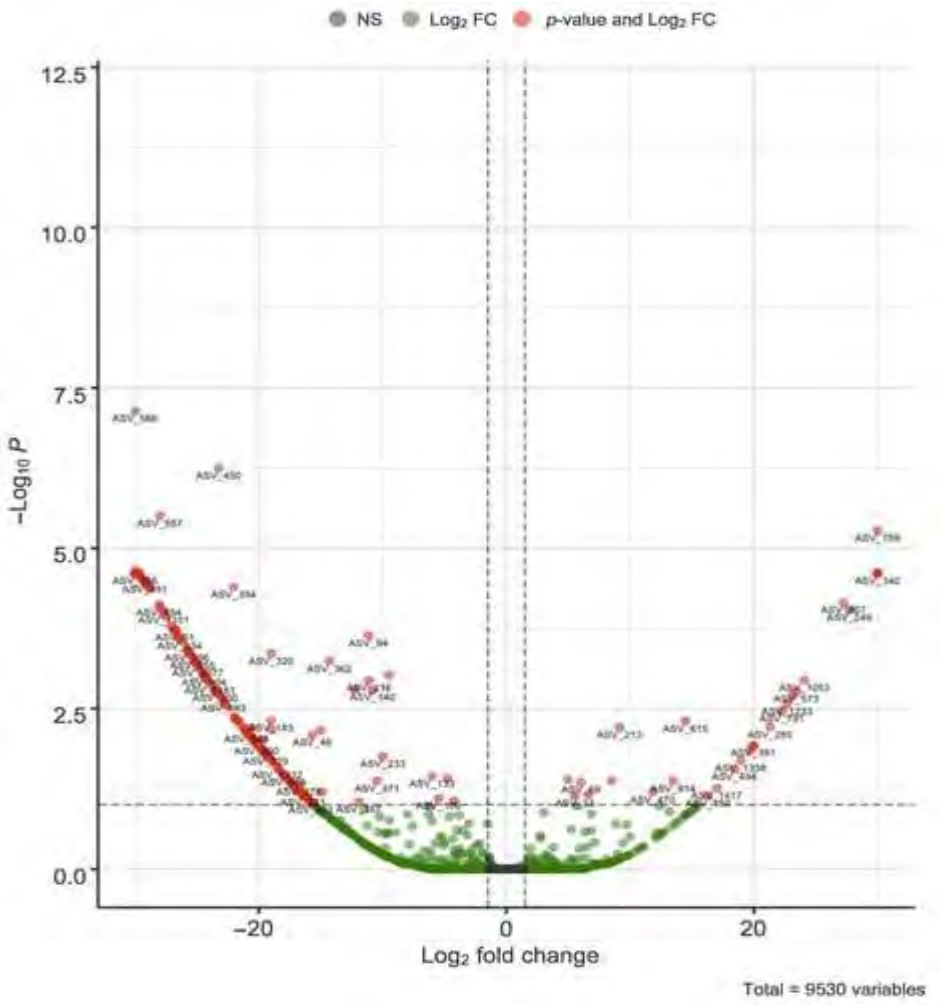

Figure 7. Volcano plot showing the differential abundance of ASVs due to the treatment effect. The statistical significance value was set to p-value < 0.01 (horizontal line), while, to be considered biologically significant, the effect size in terms of Fold Change (FC) should have had an absolute value higher than 3 (vertical lines at $\log_2 FC = 1.5$)

Table 4. Top ten differentially abundant ASVs for the treatment effect based on the adjusted *p*-value. The direction of differential abundance is indicated by the sign of the Log_2 Fold Change.

ASV	Log2 Fold Change	Standard Error	Adjusted p-Value	Lowest Resolved Taxon
ASV_566	−30.000000	4.494941	2.4864×10^{-11}	Faecalibacterium
ASV_450	−23.252888	3.715454	$3.8889e \times 10^{-10}$	Monoglobus
ASV_557	−27.994668	4.727891	3.1965×10^{-9}	Clostridia UCG-014
ASV_788	30.000000	5.187864	7.3500×10^{-9}	Lachnospiraceae
ASV_326	−30.000000	5.454703	3.8013×10^{-8}	Clostridia UCG-014
ASV_156	−30.000000	5.827878	2.6374×10^{-7}	Clostridia UCG-014
ASV_159	−30.000000	5.839874	2.7902×10^{-7}	Clostridia UCG-014
ASV_275	−29.839008	5.840154	3.2338×10^{-7}	Clostridia UCG-014
ASV_340	30.000000	5.832041	2.6895×10^{-7}	Clostridia
ASV_395	−29.762335	5.834230	3.3727×10^{-7}	Clostridia UCG-014

3.4 Discussion

The evaluation of macroscopic intestinal features and histological measurements allowed us to assess the chickens' gut health, and how it was impacted by the synbiotic treatment. Although the BE and histopathological lesion scores suggested good intestinal health in all treatment and control groups, likely due to the absence of a challenge, some differences in favor of the treated chickens could still be noted. In particular, significantly lower BE scores were observed in synbiotic-fed birds only in farm 3, while more overt differences were found in terms of histopathological lesions in most intestinal tracts of chickens raised in all farms. These results were in line with those observed after administering PS to the group of broiler breeders that birthed the investigated progenies, which also pointed at a general intestinal eubiosis in both treatment and control groups, with the former scoring better overall (Prentza et al., 2022). The BE scores measured in this study allowed us to demonstrate a significant age effect, showing a biologically plausible increase in subsequent time points. In

addition, between-farm differences were present according to both scores. Such an effect, which was observed also for other evaluated parameters, could easily be ascribed to environmental factors related to the individual farms, such as differences in management, stockmanship, housing, farm location and others. These variations should be considered inherent to the field conditions in which the study was conducted to reproduce the real-life application of the tested synbiotic. The evaluation of villi and crypts length at 38 doa provided additional insights on the effect of PS. Longer villi are considered an indicator of a greater surface area and thus a greater adsorption capability (Fan et al., 1997, Samanya and Yamauchi, 2002), while shorter villi and deeper crypts may lead to poor nutrient absorption, increased secretions in the gastrointestinal tract, and poorer performance (Xu et al., 2003). Reduced villi length also appears to be associated to macroscopic lesions suggestive of dysbacteriosis (Teirlynck et al., 2011). The villi of the treated broilers were significantly longer across most intestinal tracts and in all three farms, in agreement with other studies which reported the same beneficial effect for other synbiotics (Calik et al., 2017, Markazi et al., 2018, Villagran de la Mora et al., 2019, Kridtayopas et al., 2019, Jiang et al, 2020). On the other hand, the differences in terms of crypt depth were less marked and consistent. Conflicting evidence on the effect of synbiotic supplementation on intestinal crypts may also be found in the literature, with different formulations leading to an increase (Villagran de la Mora et al., 2019), decrease (Sobolewska et al., 2017) or not affecting their depth (Sobotik et al., 2021). Trends similar to the present study were found in the previous experiment conducted on breeders (Prentza et al., 2022), consolidating the knowledge about the effects of PS administration on gut morphology.

By promoting good intestinal health and preventing any undesired condition of dysbacteriosis or inflammation, synbiotic administration should ultimately promote production efficiency. In this study, synbiotic-treated flocks performed significantly better according to several parameters, including feed conversion ratio, carcass BW and daily cumulative mortality rate. These results agree with several other studies in which other synbiotics were tested (Awad et al., 2009, Kridtayopas et al., 2019, Mohammed et al., 2018, Abdel et al., 2019, Slizewska et al., 2020), and further support the promising application of synbiotics to poultry production. The impact of PS administration on caecal bacteriome was also investigated through high-throughput sequencing. Caeca represent the enteric tract with the highest microbial density, mainly composed of obligate anaerobes belonging to the phyla Firmicutes and Bacteroidetes (Flickinger et al., 2003). The studied flocks showed a large predominance of Firmicutes, particularly members of the class Clostridia, as expected in broilers (Zhu et al., 2002, Bjerrum et al., 2006). In comparison, the microbial composition of the broiler breeders which birthed the broiler progenies used for this study was similarly dominated by Firmicutes but, coherently with the older age at sampling, the overall diversity was higher (Prentza et al., 2022). A clear treatment effect was visible in all farms, significantly impacting the abundance of 65 ASVs. Among the most influenced were representatives of *Lachnospiraceae*, a family of cellulolytic bacteria capable of metabolizing non-starch polysaccharides that are among the earliest colonizers of the caecum of broiler chickens (Richards et al., 2019, Fisinin et al., 2016), which were overrepresented in synbiotic-fed flocks compared to control ones. On the other hand, ASVs belonging to the genera *Faecalibacterium*, which are common inhabitants of the caeca involved in butyrate production and in the anti-inflammatory response (Luo et al., 2013, Liu et al., 2021), and *Monoglobus*, a

less abundant component of the intestinal microbiome capable of degrading pectin (Lysko et al., 2021), were underrepresented, along with others classifiable as *Clostridia UCG-014*. Overall, based on alpha diversity measures, PS chickens exhibited a lower species richness than control ones. Considering the complexity and variability of the gut microbiota, it is hard to ascertain the implications of the observed changes. An increasing number of studies on synbiotics are using molecular assays to investigate their effects on gut bacterial populations, reporting different results. For instance, Baffoni et al. (2017) and Pineda-Quiroga et al. (2019) found that, similar to the present study, species richness was decreased by synbiotic treatment, but the opposite was reported by other authors (Such et al., 2021, Song et al., 2022). The taxa affected by synbiotic supplementation were widely variable (Pineda-Quiroga et al., 2019, Such et al., 2021, Brugaletta et al., 2020), further complicating the interpretation of these findings. This diversity can be easily motivated by considering the many variables ascribable to environmental, nutritional, and host factors (Fathima et al., 2022) as well as to the formulation and administration protocol of different synbiotic products. Nonetheless, these data are still valuable to obtain a more complete picture of the mechanism through which a specific synbiotic acts, and also add to the existing general knowledge on synbiotics and nutraceuticals for future comparisons.

3.5 Conclusion

The presented results support the benefits achieved by the administration of PoultryStar® sol in broilers, which improved the productive performance in terms of FCR, carcass weight and mortality rate. Treated chickens also exhibited a better intestinal health, having lower histopathological lesion scores and longer villi across

most intestinal tracts. In addition, synbiotic supplementation was shown to influence the caecal microbial ecosystem, causing some taxa to be more or less abundant in synbiotic-fed flocks. The exact implications of these changes will, however, require further studies to be better understood.

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CHAPTER FOUR

FUTURE PERSPECTIVES

The results of our study confirm the positive effects of administering the synbiotic product PoultryStar® sol on the intestinal health of broiler breeders and their offspring. The synbiotic product appears to be a safe product for use during the laying period, improving the intestinal health of the birds and enhancing their performance, thereby maximizing the profitability of production.

Since the birds were from BB -synbiotic-treated breeders, further studies are needed to clarify the potential cumulative effects compared to broilers from untreated breeders.

Although the synbiotic has shown gut health benefits without challenge, further studies need to investigate the synergistic effects of the synbiotic with other alternative products and vaccines that act primarily in the gastrointestinal tract GIT.

In addition, the benefit of administering synbiotics in poultry production in conjunction with the use of non-invasive biomarkers to assess gut health under field conditions is another interesting aspect of the research.

ANNEX

a

b

Figure 1 (a, b). Broiler Breeder, chick placement

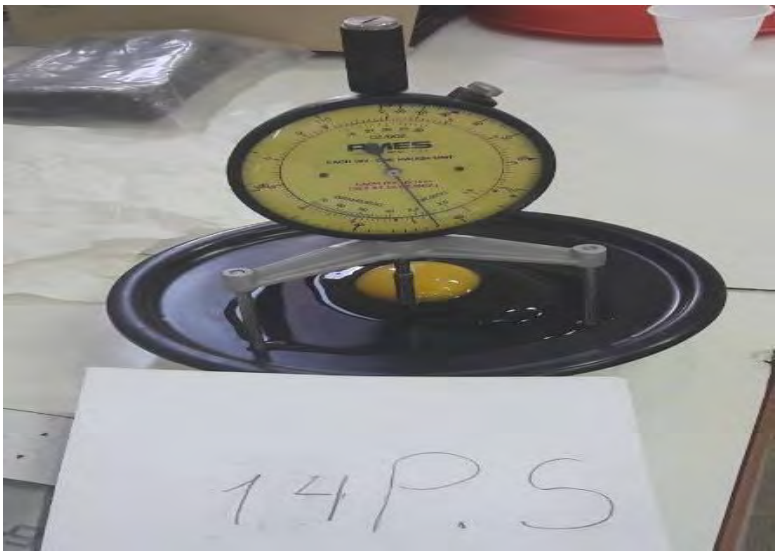
a

b

Figure 2 (a, b). Broiler Breeder rearing period

a

b



c

Figure 3 (a, b, c). Evaluation of Egg Quality traits (eggshell strength, shell thickness, albumen height)

a **b**

Figure 4 (a, b). Broiler Farm 1, PS group and Control group

Figure 5 (a, b). Broiler Farm 2, PS group and Control group

Figure 6 (a, b). Broiler Farm 3, PS group and Control group

Figure 7. Necropsy

Figure 8. Macroscopic lesion scoring

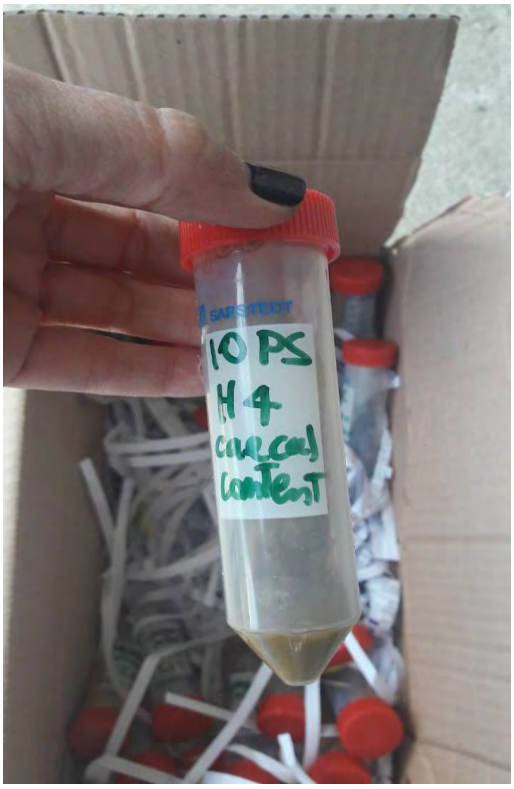


Figure 9. Caecal content samples

Figure 10. Collecting specimens from the gut

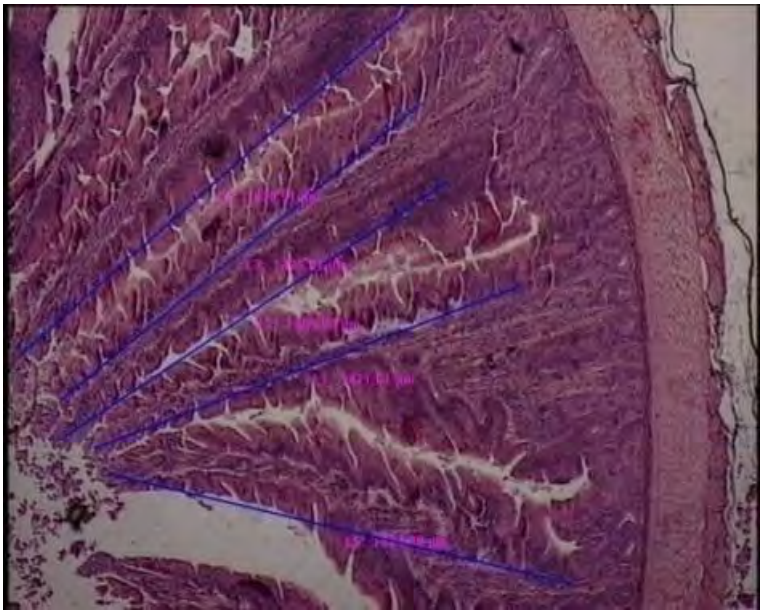


Figure 11. Evaluation of villus height

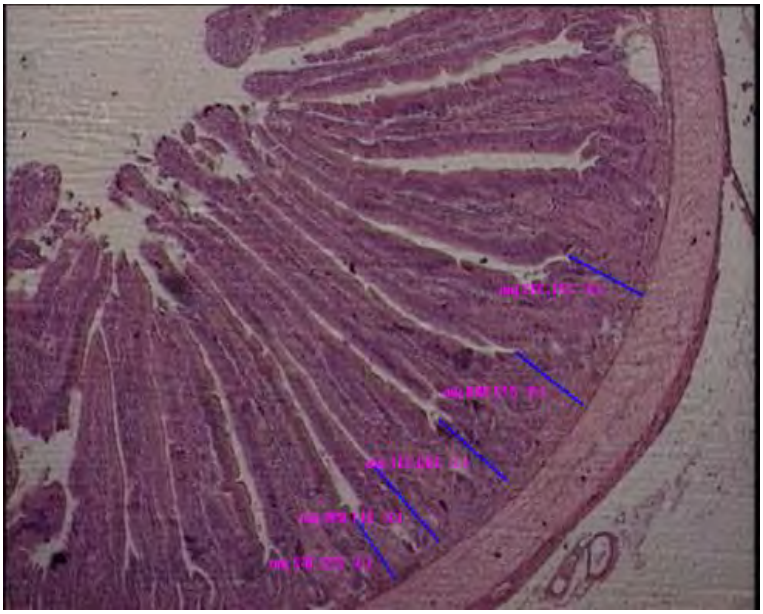


Figure 12. Evaluation of crypt depth

CURRICULUM VITAE

Personal information	
Name	**ZOI PRENTZA**
Address	
Telephone	
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Nationality	
Date of birth	
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Basic degree	
Education	
2017-	PhD candidate in Poultry Pathology-Faculty of Veterinary Medicine in Karditsa – Department of Avian Pathology, University of Thessaly – PhD thesis: *Effects of a multi-genus synbiotic on gut health, microbiome and performance in Broiler Breeders and their progeny*
2012	Master's Degree in ~~–~~Breeding and Pathology of Pigs and Poultry" Laboratory of Animal Husbandry, School of Veterinary Medicine, Faculty of Health Sciences, Aristotle University of Thessaloniki,

	Thessaloniki 54124, Greece. Master Dissertation: *Study of the incidence of deep pectoral myopathy in broiler chicken farms*
2002-2008	Doctor of Veterinary Medicine degree (DVM) Faculty of Veterinary Medicine, University of Bari, Italy Thesis Title: *Innocuita ed efficacacia protettiva di un ceppo di Salmonella Gallinarum nel pollo*
Publications/Presentations	**Prentza Z,** Castellone F, Legnardi M, Antlinger B, Segura-Wang M, Kefalas G, Papaioannou N, Stylianaki I, Papatsiros VG, Franzo G, Cecchinato M, Koutoulis K (2023). Administration of a Multi-Genus Synbiotic to Broilers: Effects on Gut Health, Microbial Composition and Performance. Animals., 13(1):113. doi: 10.3390/ani13010113 **Prentza Z,** Castellone F, Legnardi M, Antlinger B, Segura-Wang M, Kefalas G, Fortomaris P, Papaioannou AAN, Stylianaki I, Franzo G, Cecchinato M, Papatsiros V, and Koutoulis K (2022). Effects of a Multi-Genus Synbiotic (PoultryStar® sol) on Gut Health and Performance of Broiler Breeders. J. World Poult. Res., 12 (3): DOI: https://dx.doi.org/10.36380/jwpr.2022 Dimitrios Koutsianos., Labrini V. Athanasiou., Myrto

Spyropoulou., **Zoe Prentza.,** Anna Dedousi., Zoe Polizopoulou., Dimitris Mossialos & Konstantinos Koutoulis (2021) Evaluation of hematological variables in layer pullets after vaccination and challenge with *E. coli Comparative Clinical Pathology* volume 30, pages113–118.

Franzo G., **Prentza Z**., Paparounis T., Tsiouris V., Centonze G., Legnardi M., Catelli E., Tucciarone C.M., Koutoulis K. & Cecchinato M., (2020) Molecular epidemiology of Fowl adenoviruses (FAdV) in Greece, *Poultry Science*, doi: https://doi.org/10.1016/j.psj.2020.07.019.

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Aleksandar Zocevic, Fabien Vorimore, Cvetka Marhold, Danijela Horvatek, Dongying Wang, Brigita Slavec, **Zoi Prentza**, Grigorios Stavianis, Estella Prukner-Radovcic, Alenka Dovc, Victoria I.

Siarkou and Karine Laroucau (2012) Molecular characterization of atypical *Chlamydia* and evidence of their dissemination in different European and Asian chicken flocks by specific real-time PCR Environmental Microbiology Volume 14, Issue 8, Pages 2212–2222

XXIst WVPAC Congress in Bangkok-Thailand (2019) Poster presentation: Evaluation of a synbiotic product in broiler breeders during the laying period

XXIst WVPAC Congress in Bangkok-Thailand (2019) Poster presentation: Broiler breeders and its progeny supplemented with a synbiotic product: a promising approach to enhance health and performance

58° Italian meeting *SIPA*, *Forlì* (2019) Oral presentation: Field experience on the use of a multi-species synbiotic on gut health and production parameters in broiler breeders and their progeny

20th ECVCP-ESVCP Meeting (2018) Poster presentation: Evaluation of hematological changes in layer chickens after vaccination and challenge with e. coli

14th Panhellenic Veterinary Congress (2018) Oral presentation: Molecular and serologic test to evaluate vaccine efficacy with vectormune® ND in broiler

XX WVPA, Edinburgh (2017) Poster presentation: Effects of increased dark hours on performance and immunological response in

broiler flocks affected by inclusion body hepatitis (IBH)

13th Panhellenic Veterinary Congress (2015) Oral presentation: Effect of three lighting programs in immunological response of broiler

12th Panhellenic Veterinary Congress (2012) Oral presentation: Study of the incidence of deep pectoral myopathy in broiler chicken farms