THE *IN OVO* APPROACH TO OPTIMIZING POULTRY GUT HEALTH AND PERFORMANCE: AN INQUIRY

by

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ABSTRACT

Several proposed alternatives to antibiotic growth promoters (AGP) in poultry have been reported ineffective due to limitations of delivery routes. Hence, an urgent need for new approaches to maintain poultry gut health exists. This thesis thus evaluated the *in ovo* approach to optimizing broiler chicken's gut health and growth performance in five experiments involving three bioactive substances (probiotics, essential oil, and folic acid).

Experiment 1 revealed that the *in ovo* delivery of probiotics product (*Bacillus subtilis* fermentation extract) enhanced gut morphology without compromising hatch and gut homeostasis. Experiment 2 showed that all probiotics (independent of delivery routes and dose) were mostly comparable to the in-feed antibiotics treatment in their ability to ensure gut microbiota homeostasis, enhanced gut morphology, and feed conversion efficiency. Experiment 3 indicated that the *in ovo* delivery of an essential oil blend reduced hatchability and chick length in broiler chickens. However, successive delivery of this essential oil blend via *in ovo* and in-water routes improved broiler chicken's antioxidant status and blood biochemical profile with no adverse effect on growth performance. *In ovo*, delivery of folic acid (0.15 mg /egg) is observed to increase hatchling weight and enhance broiler chicken gut morphology and feed conversion ratio in a similar capacity as the in-feed antibiotic treatment in experiment 4. However, hatchability was reduced by the *in ovo* delivery of folic acid. Finally, experiment 5 was conducted to validate the gut-optimizing potential of these *in ovo*-delivered bioactive substances under a heat stress challenge. Results showed that independent of heat stress, the successive delivery of essential oil via *in ovo* and in-water routes improved broiler chicken gut morphology and intestinal barrier integrity. Under heat stress conditions, the *in ovo* + in-water EO routes induced a numerical increase in feed conversion efficiency (+30%) and as much as a 3.5-fold significant upregulation of amino acid transporter gene, suggesting that the delivery of this bioactive substance offer potential gut thermo-protective functions under heat stress condition. Summarily, results from this thesis reveal that the *in ovo* delivery of probiotics, essential oil, and folic acid shows promising benefits as alternatives to AGP in the post-AGP era.

LIST OF ABBREVIATIONS USED

ABWG	average body weight gain
ADFI	Average daily feed intake
ADG	average daily gain
AFI	average feed intake
AGP	antibiotic growth promoters
ALDH1L2	aldehyde dehydrogenase 1 family member L2
ALP	alkaline phosphatase
ANOVA	analysis of variance
AST	aspartate aminotransferase
ASV	amplicon sequence variants
BMD	bacitracin methylene disalicylate
BVES	blood vessel epicardial substance
BW	body weight
BWG	body weight gain
cAMP	cyclic adenosine monophosphate
CE	competitive exclusion
CEP70	centrosomal protein 70
CFU	colony forming units
cGMP	cyclic guanosine monophosphate
CK	creatine kinase
CLCA1	chloride channel accessory 1
CTD	C-terminal domain
CTRL	control
CUBN	cubilin
DEG	differentially expressed genes
DNA	Deoxyribonucleic acid
ED	embryonic days
ELISA	enzyme-link immunosorbent assay

EO	essential oil
EU	European Union
FA	folic acid
FCR	feed conversion ratio
FDA	US Food and Drug Administration
FI	feed intake
gDNA	Genomic DNA
GIT	gastrointestinal tract
GO	Gene ontology
GOS	galactooligosaccharides
GUCY2C	guanylate cyclase 2C
HS	heat stress
HSD	honest significant difference
IgG	immunoglobulin G
IgM	immunoglobulin M
INTS2	integrator complex subunit 2
KEGG	Kyoto Encyclopedia of Genes and Genome
MC5R	melanocortin 5 receptor
MDA	Malondialdehyde
MED13	mediator complex subunit 13
MOS	mannanoligosachharides
MTMR6	myotubularin related protein 6
NASH	nonalcoholic steatohepatitis
NC	Negative control
NE	necrotic enteritis
OTU	operational taxonomic unit
PANX2	pannexin 2
PCoA	principal coordinate analysis
PDE11A	phosphodiesterase 11A
PFA	phytogenic feed additives
PICRUST2	Phylogenetic Investigation of Communities by Reconstruction of

	Unobserved States
PUFA	polyunsaturated fatty acid
RFO	raffinose family oligosaccharides
RNA	Ribonucleic acid
RNAP II	RNA polymerase II
RNA-seq	RNA sequencing
SCFA	short-chain fatty acid
SEM	standard error of the means
SLC5A10	solute carrier family 5-member 10
snRNA	small nuclear RNA
SOD	superoxide dismutase
STAMP	Statistical Analysis of Metagenomic Profiles
TAC	Total antioxidant capacity
TEER	trans-epithelial resistance values
TFA	trans-fatty acid
TN	thermoneutral room
VFA	volatile fatty acids
WHO	World Health Organisation

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1 CHAPTER 1 GENERAL INTRODUCTION

Food is a basic human necessity. However, feeding a projected human population of 11 billion persons by the end of this century signifies an enormous challenge (The Eat-Lancet Commission, 2019). Expectedly, this projected increase in global population is accompanied by a simultaneous increase in food, especially protein demand. In fact, a 725% increase in poultry demand by 2030 has been predicted for South Asia alone (FAO, 2011). Poultry meat currently accounts for nearly one-third of global meat production and is projected to account for at least half of the increased protein demand in this decade (FAO, 2020).

Much of the success of the global poultry industry, in the last sixty years at least, is anchored on the sub-therapeutic use of antibiotic growth promoters (AGP) to optimize chicken's gut health (Gadde et al., 2017). The gut is a vital organ system integral to ensuring efficient nutrient utilization and immunocompetence in birds (Choct, 2009). Poor gut health has been associated with compromised digestive and nutrient absorption capacity, increased susceptibility to diseases and ultimately poultry performance (Choct, 2009; Sugiharto, 2016). A "healthy gut" may thus be synonymous with a "healthy chicken." The benefits accruing from the use of AGP to modify poultry gut health include the minimal occurrence of subclinical infections, reduced morbidity, and mortality, increased nutrient efficiency, and improved growth performance (Huyghebaert et al., 2011; Chattopadhyay, 2014). Despite these benefits associated with AGP use, evidence now abounds that the sub-therapeutic use of AGP in the livestock industry contributes to the development and spread of antibiotic-resistant bacteria, a public health concern (Asai et al., 2007; Baron et al., 2014; Garcia-Migura et al., 2014). In quantitative terms, about 10 million deaths per year and a cumulative economic loss of 100 trillion USD by 2050 are examples of the global burden associated with continuous sub-therapeutic use of AGP in livestock production (O'Neill, 2016). Consequently, country-specific restrictions regarding AGP use in the poultry industry are now in place. The European Union (EU) member nations banned the sub-therapeutic use of AGP in 2006, following the European Parliament and Council Regulation EC No. 1831/2003. The US and Canada have also instituted policy directions guiding AGP use in livestock production (FDA, 2012; Government of Canada, 2018). These restrictions in the use of AGP is not without consequences; in the absence of alternatives to AGP, poor gut health associated with increased disease incidence, morbidity, mortality, and poor poultry growth performance are imminent. The poultry industry, therefore, faces the challenge of sustainable intensification while reducing and ultimately eliminating the use of AGP.

To solve this challenge, various bioactive substances are investigated as potential alternatives to AGP due to their gut optimizing potentials. These include probiotics (Torshizi et al., 2010; Salim et al., 2013; Khoobani et al., 2020), prebiotics (Kim et al., 2011; Houshmand et al., 2012; Craig et al., 2020), synbiotics (Jung et al., 2008), enzymes (Hooge et al., 2010; Jackson and Hanford, 2014), organic acids (Paul et al., 2007; Banday et al., 2015), antimicrobial peptide (Wen and He, 2012), bacteriophages (Wang et al., 2013), herbs, spices, and essential oils (Lee et al., 2003; Hoffman-Pennesi and Wu, 2010). Although, yet to be researched as an alternative to AGP, folic acid is another bioactive substance with gut-optimizing potential. A potent alternative to AGP should guarantee resilience against all potential immunological or environmental stressors (including heat stress). These potential alternatives have been administered through conventional in-feed and in-water routes. Consequently, the efficacy of these alternatives has been inconsistent in literature, owing to multiple interacting factors that include the age of the bird, immune status of the bird, occurrence of environmental stressors, bird microbiota balance, stability during heat treatment, water quality, dosage, timing, duration, and routes of administration (Gaggìa et al., 2010; Huyghebaert et al., 2011; Ducatelle et al., 2014; Bednarczyk et al., 2016). Additionally, gut-enhancing bioactive substances are reported to be more effective in chickens when introduced as early as possible, preferably at the pre-hatch stage (Sobolewska et al., 2017). Hence, there is a need to investigate new delivery strategies that ensure the optimum efficacy of these bioactive substances.

In ovo technology is one of such delivery strategies that offers scope to develop effective alternatives to AGP for the poultry industry. *In ovo* technology involves the delivery of bioactive substances directly to the developing embryo. It helps to overcome challenges that include nutrient inactivation due to heat treatment of feedstuff and potential water quality risks associated with in-water and in-feed delivery of alternatives to AGP. It also offers a cheaper means of delivering these bioactive substances, as lesser quantities of bioactive substances are required compared to in-water and in-feed delivery routes (Bednarczyk et al., 2016; Tavaniello et al., 2018). Considering that the perinatal period (ranging from the late embryonic stage till few days post-hatch) is critical to gut development and immunity in poultry, a major advantage of *in ovo* technology is that alternatives to AGP can be delivered to the developing embryo to stimulate a healthy gut profile early on, rather than trying to modify an already established gut profile (Roto et al., 2016).

This research, therefore, sought to evaluate the effectiveness of the *in ovo* delivery of selected bioactive substances as a strategy to optimize gut health while developing effective alternatives to

AGP in broiler chickens.

Specific objectives included-

1. To validate the *in ovo* delivery system by comparing it with common delivery methods (in-feed and in-water) of a probiotic product on growth performance, gut morphology, incidence of necrotic enteritis, short-chain fatty acid production and gut microbiota homeostasis.
2. To determine the effect of *in ovo* delivery of essential oil, on growth performance, gut morphology, blood biochemistry, immune and antioxidant status, short-chain fatty acid production, gut microbiota homeostasis and liver transcriptomics.
3. To determine the effect of *in ovo* delivery of folic acid on growth performance, gut morphology, blood biochemistry, immune and antioxidant status.
4. To evaluate the gut-optimizing potential of these *in ovo*-delivered bioactive substances (probiotics, essential oil, and folic acid) under a heat stress challenge model.

2 CHAPTER 2 LITERATURE REVIEW

IN OVO DELIVERY OF BIOACTIVE SUBSTANCES: AN ALTERNATIVE TO THE USE OF ANTIBIOTIC GROWTH PROMOTERS IN POULTRY PRODUCTION – A REVIEW

Contents from this section have been published elsewhere:

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2.1 Summary

For more than 6 decades, the global poultry industry has profited from the advancement in science and technology. Success in animal breeding and genetics has made available fast-growing poultry strains. Similarly, evolution in Medicine and Veterinary science has provided farmers with antibiotics, which have served the dual role of treating poultry diseases and improving growth performance of poultry. Interestingly, these gains from science are not without consequences. Issues such as lameness in fast-growing chickens and the failure of some antibiotics to treat important human infections as a result of the development of antibiotic-resistant bacteria have been reported. With an ever-increasing demand for poultry products, there is an urgent need to solve these identified challenges. Results emanating from research on antibiotics alternatives in poultry have generally been reported inconsistent because of several factors including environmental, animal health, and delivery routes (in-feed and in-water). Once again, innovation in science and technology is being called upon. A recent emerging field of animal biotechnology is the use of *in ovo* technology to deliver bioactive compounds to broiler chickens. *In ovo* delivery of bioactive compounds shows potential to help reduce and ultimately end the use of antibiotics in poultry production.

2.2 Description of Problem

The poultry industry remains a significant source of high-quality protein, vitamins, and essential micro-nutrients in human nutrition. Accounting for 37% of the global meat industry in 2017, the global poultry industry is projected to produce about 331 million tonnes of meat in 2028 (OECD/FAO, 2019). This remarkable growth has been linked with the sub-therapeutic use of

antibiotic growth promoters (AGPs); AGPs often improve growth performance of poultry birds by increasing feed conversion efficiency and reducing disease incidence (Castanon, 2007; Gadde et al., 2017). Despite these benefits, the continuous use of AGPs in poultry production stirs public health concerns relating to antimicrobial resistance and presence of antibiotic residues in the food chain and the environment (Muaz et al., 2018; Wales et al., 2019). World Health Organisation (WHO) forewarned that approximately 10 million deaths could be recorded by 2050 if these public health concerns are left unchecked (WHO, 2019). The poultry industry is thus saddled with the challenge of maintaining increased productions trends, whilst eliminating or reducing the use of AGPs.

As alternatives to AGPs, numerous bioactive substances (compounds interacting with living tissues to yield a variety of effects (Guaadaoui et al., 2014) including probiotics, prebiotics, synbiotics, phtytobiotics, essential oils, organic acids, bacteriophages and antimicrobial peptides are experimented, albeit through the conventional dietary and drinking routes (Lee et al., 2003; Hoffman-Pennesi and Wu, 2010; Wen and He, 2012; Wang et al., 2013; Naito, 2015; Hu et al., 2017; Al-Khalaifa et al., 2019; Araujo et al., 2019; Zhu et al., 2019; Herrero-Encinas et al., 2020). Results emanating from most studies have been inconsistent, owing to multiple interacting factors that include age of the bird, immune status of the bird, occurrence of environmental stressors, bird microbiota balance, stability during heat treatment, water quality, dosage, timing, and duration of administration (Gaggìa et al., 2010; Huyghebaert et al., 2011; Ducatelle et al., 2014; Bednarczyk et al., 2016). Furthermore, conventional delivery routes (i.e., in-feed and in-water) might not often yield positive results because of possible adverse interactions with other feed additives (i.e., antibiotics, oligosaccharides, or coccidiostats), potential prior contamination with pathogenic microorganisms, and differences in chemical structures and composition of alternatives to AGPs (Cheng et al., 2014; Tavaniello et al., 2018). A recently emerging field, *in ovo* technology involving the delivery of bioactives directly to the developing embryo, presents an opportunity to develop effective alternatives to AGP for the poultry industry. This review, therefore, aims to provide an overview of the *in ovo* delivery of alternative to AGPs, as opposed to *in ovo* delivery of nutrients and vaccines, a more researched theme. In addition, practical insight is provided on the factors influencing the efficacy of *in ovo* delivery of these bioactive substances.

2.3 *In ovo* Technology- Basic Concepts

The history of *in ovo* technology is traceable to the works of Sharma and Burmester (1982), which

revealed that embryonic vaccination confers superior immunity against Marek disease in chickens; a novel discovery which was subsequently patented in the United States (Sharma and Burmester, 1984). *In ovo* technology can be defined as the direct inoculation of bioactive substances to the developing embryo to elicit superior lifelong effects while considering the dynamic physiology of the chicken embryo. It is based on the simple concept of supplementing the chick embryo with bioactive substances to establish lifelong phenotypes, including superior performance, immunity, and healthy gut microbiome in the bird (Siwek et al., 2018). It is the only method of external supplementation that influences the development of both the embryo and its neonate (Slawinska et al., 2016). Owing to the success of Sharma and Burmester (1984) patent, the automation and commercialization of *in ovo* technology, especially as an effective vaccine delivery method has been reported (Johnston et al., 1997; Ricks et al., 1999). Automated *in ovo* vaccination offers scope to inoculate an increased number of eggs with minimal human effort and error (Ricks et al., 2003; Peebles, 2018).

In ovo delivery of bioactive substances could also be a more economical route compared to conventional supplementation routes (i.e., in-feed and in-water). Bednarczyk et al. (2016) obtained similar performance-enhancing effect from delivering almost 11 times less prebiotics via *in ovo* administration compared to in-water delivery in broiler chickens (3.5 mg BI/embryo *in ovo* vs 40 mg BI/chick in-water) - a trend confirmed by Tavaniello et al. (2018) as they utilized 10 times less *in ovo* prebiotic supplementation relative to in-water supplementation to achieve similar growth effects. Despite the benefits this technology portends, a lack of procedural standardization impairs its commercial adoption as a viable means of delivering bioactive substances in the poultry industry (except for vaccines). This is not unexpected, as several interrelated factors including but not limited to, hatchery hygiene (Ricks et al., 1999; Williams and Zedek, 2010), degree of automation (manual vs automatic) (Wakenell et al., 2002; Triplett et al., 2018), dosage of bioactives (McGruder et al., 2011; Zhai et al., 2011), time (Ricks et al., 1999; Bednarczyk et al., 2016; Sokale et al., 2017) and site of injection (Avakian et al., 2002; Williams, 2007; Miśta et al., 2017) influences the efficacy of *in ovo* technology. An attempt is made to synthesize standard guidelines regarding these factors later in this review.

2.4 *In ovo* Applications in Poultry

Modern-day poultry is susceptible to nutritional, environmental, and infectious pathogenic stressors. An effective pathogen control strategy is one that induces optimal and protective levels

of humoral and/or cellular immune responses few days after vaccination (Sharma and Burmester, 1982). *In ovo* vaccination has been reported to stimulate an early immune response in young chicks compared with posthatch vaccination (Negash et al., 2004). *In ovo* technology is essentially a biotechnological intervention adopted with the main goal of ensuring early immunological programming in birds. This goal has been successfully achieved via the *in ovo* delivery of vaccines and potentially other bioactives (probiotics, prebiotics, and synbiotics). After the adoption of *in ovo* technology as a viable method of vaccine delivery, its relevance to other potential bioactives is being investigated. *In ovo* delivery of bacteriophages, electrolyte solution, glycerol, hormones, organic acid, peptide, silver nanoparticle, trace elements, amino acids, vitamins, carbohydrates, and plant extracts have all been reported in the literature (summarized in Table 2.1).

Table 2.1 A summary of *in ovo*–delivered bioactive substances (with the exception of vaccines, probiotics, prebiotics, and synbiotics).

S/N	Bioactive substance	References
1	Bacteriophages	Wang et al., 2013
2	Electrolyte solution	McGruder et al., 2011
3	Glycerol	Dal Pont et al., 2019
4	Hormones	Coco et al., 1992
5	Organic acids	Krisnan et al., 2019
6	Peptides	Cuperus et al., 2018
7	Silver nanoparticle	Goel et al., 2017
8	Trace elements	Patric Joshua et al., 2016; Jasim and Al-Qaisy, 2019
9	Amino acids	Ohta and Kidd, 2001; Gao et al., 2018; Zhang et al., 2018; Groff-Urayama et al., 2019; Kop-Bozbay and Ocak, 2019; Omidi et al., 2020
10	Vitamins	Bello et al., 2013; El-Senousey et al., 2018 ; Nouri et al., 2018 ; Zhang et al., 2018; Hayakawa et al., 2019; Hussian et al., 2019
11	Carbohydrates	Tako et al., 2004; Zhai et al., 2011; Bhanja et al., 2015; Zamani et al., 2019; Slawinska et al., 2020
12	Plant extracts	Fazli et al., 2015; Morovat et al., 2016; Coskun et al., 2017; N'nanle et al., 2017

Aside from stimulating favorable immunological responses in birds, *in ovo* technology could also be used to mitigate the perinatal (the period before and after hatch) nutritional deficiencies of the bird—often caused by a transition from embryonic yolk nutrition to exogenous feeding, long hatchery window (24–36 h), and time-consuming hatchery logistics (sorting, sexing, vaccinations, beak trimming, comb dubbing, and chick transport) (Noy and Uni, 2010). In addition, this technology offers the opportunity to stimulate the colonization of the embryonic gut with beneficial microbiome and also the development of the embryonic gastrointestinal tract (GIT) and gut-associated lymphoid tissue (Siwek et al., 2018). More recently, *in ovo* sexing, an application of this technology, has been proposed to solve an important animal welfare and ethics issue—the culling by maceration or suffocation of male chicks in commercial layer production. Male chicks are considered to have minimal commercial value because of their inability to lay eggs. *In ovo* sexing is carried out through several invasive and non-invasive methods. These include the use of specific DNA amplification techniques (polymerase chain reaction and quantitative polymerase chain reaction) (Hou et al., 2015; Clinton et al., 2016), hormone detection (Weissmann et al., 2013), odor analysis (Webster et al., 2015) and several spectroscopic analyses (Fioranelli et al., 2019). Knepper et al. (2019) recently secured patent for a noninvasive *in ovo* sexing system, involving the use of electromagnetic radiation transmitter and detectors.

Based on the modulatory effect of bioactive substances injected, the application of *in ovo* technology in poultry production can be broadly categorized into performance-enhancing substances, immunostimulants, and alternatives to AGPs (Figure 2.1). Although the application of *in ovo* technology as a strategy for early nutritional and immunomodulatory programming in poultry birds has been the subject of other reviews (Uni and Ferket, 2004; Noy and Uni, 2010; Kadam et al., 2013; Hou and Tako, 2018; Retes et al., 2018; Jha et al., 2019), here we reveal that alternatives to AGPs through several mechanisms ultimately fulfill both functions, leading to improved performance and welfare of the bird.

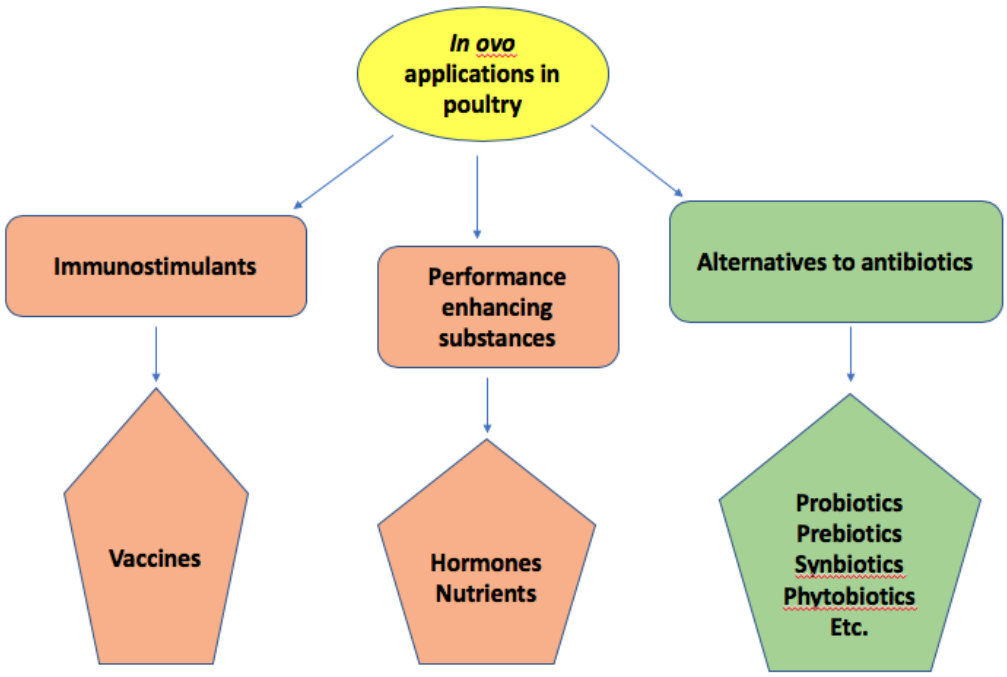


Figure 2.1 Applications of in ovo technology in the poultry industry.

In ovo technology was first applied to the delivery of immunostimulants (vaccines) to confer poultry immunity against economically important diseases including Marek's disease (MD), Coccidiosis, infectious bronchitis (IB) and so on. Subsequently, the technology was applied to the delivery of nutrients (carbohydrates, vitamins, amino acids, trace elements, growth hormones, etc) as an early feeding strategy that guarantees early growth start-off and improved bird performance. More recently, this technology has been applied to the delivery of alternatives to antibiotic growth promoters (probiotics, prebiotics, synbiotics, phytobiotics, and so on), to mitigate limitations such as heat inactivation of active ingredients and potential water quality risks associated with conventional delivery routes (in-feed and in-water). This technology thus has prospect to improve the efficacy of alternatives to antibiotic growth promoters.

2.5 Alternatives to Antibiotic Growth Promoters in Poultry

An effective replacement to AGP is expected to exhibit similar, if not superior growth-promoting effect as AGPs. Hence, understanding of AGPs' mode of action is crucial to characterizing an effective alternative. There is currently no consensus regarding AGP's mode of action in the literature, possibly due to our still-evolving knowledge of the chicken gastrointestinal tract (GIT) microbiome. AGPs have been theorized to act by interacting with immune cells to yield anti-inflammatory response (Niewold, 2007; Yang et al., 2017), increasing nutrient absorption rate via thinning of the intestinal epithelium (Gaskins et al., 2002) and reducing the production of growth-depressing bacteria metabolites (Huyghebaert et al., 2011). Also, the advancement of molecular biological tools (as opposed to culture-dependent methods), alongside studies on gnotobiotic hosts reveals that AGPs influence chicken's intestinal microbiota diversity/balance (intestinal microbiota is defined as the totality of the microbial community of the chicken's gut (Cho et al., 2012; Sender et al., 2016). Consequently, an effective alternative to AGP has been characterized as bioactive substances with a well-defined mode of action, improves feed efficiency, maintain bird intestinal health, exhibit toxicity only to the pathogen and not the host and is easy to use (Collett, 2004; Cheng et al., 2014). As previously stated, a growing body of research work seeks to understand the efficacy of the *in ovo* delivery of these alternatives including probiotics, prebiotics, synbiotics, plant extracts, and so on. A summary of studies involving the *in ovo* delivery of alternatives to AGPs is presented in Table 2.2

Table 2.2 A summary of research results on the in *ovo* delivery of alternatives to antibiotic growth promoters in poultry.

S/N	Class of bioactive substance	Description and quantity	Site of injection	Time of injection	Results	References
1	Probiotics	3.1×10^9 cfu/50 µl of *L. animalis* 5.4×10^6 cfu/50µlof *E. faecium*	amnion	Day 18	Differing mode of action exist between the two probiotics, no negative effect on hatchability with both probiotics.	Beck et al. (2019)
2	Prebiotics	3.5 mg/embryo Galacto-oligosaccharide (GOS) 0.88 mg/embryo DN 1.9 mg/embryo Raffinose family oligosaccharide (RFO)	air cell	Day 12	All prebiotics increased feed intake and feed conversion ratio. RFO showed the highest improvement in performance traits.	Bednarczyk et al. (2016)
3	Prebiotics and synbiotics	Inulin (1.76 mg/embryo GOS (0.528 mg/ embryo synbiotic1- inulin + 1000 cfu *L. lactis subsp. Lactis* synbiotic 2-GOS + 1000 cfu *L. lactis subsp. Cremoris*	air chamber	Day 12	*in ovo* injection of the combination of L. lactis subsp. lactis IBB SL1 and inulin improved the growth, cecal SCFA profile and intestinal morphology of broiler chickens. However, the *in ovo* injection of prebiotic preparations did not produce the expected results	Mista et al. (2017)
4	Prebiotics and synbiotics	1.760 mg inulin 0.528 mg GOS 1.760 mg inulin and 1,000 cfu *Lactococcus lactis spp. lactis* 0.528 *mg Bi2tos and 1,000 cfu Lactococcus lac- tis spp. cremoris IBB*	air chamber	Day 12	No conclusive explanation as to the effect of prebiotics and synbiotics on quality parameters and microstructural features of pectoral muscles in broiler chickens	Dankowiakowska et al. (2019)
5	Probiotics - 7 different commercial	15×10^9 cfu/egg- 32×10^9 cfu/egg	amniotic fluid	Day 17. 5	Performance of all chicks that received probiotic, independent of bacteria, was numerically superior to negative control	De Oliveira et al. (2014)

	probiotics					
6	*Silybum marianum* (milk thistle)	100 and 200 mg/kg *Silybum marianum extract*	amniotic fluid	Day 17.5	Dietary feeding of *Silybum marianum* extract improved performance, immunity and carcass characteristics	Morovat et al. (2016)
7	Probiotics	*Lactobacillus acidophilus, Bacillus subtilis, Bifidobacterium animalis* (10^3-10^6 cfu)	amniotic sac	Day 18	*B. subtilis* negatively impacted almost all facets of hatchability in broiler breeder hatching eggs	Triplett et al. (2018)
8	Probiotics	1.4×10^7 cfu/egg- *Enterococcus faecium*	amniotic fluid	Day 18	*Enterococci* were recovered in high concentration in the yolk sac, caeca and intestinal samples from both 1-day-old and 7-day- old chickens	Skjøt-Rasmussen et al. (2019)
9	Prebiotics and synbiotics	1.9 mg RFO 1.9 mg RFO + 1,000 cfu of *Lactococcus lactis ssp. Lactis* 1.9 mg RFO + 1,000 cfu of Lactococcus lactis ssp. cremoris 1.9 mg RFO + 500 cfu of Streptococcus faecium	air chamber	Day 12	Abdominal fat, ultimate pH, and cholesterol of the pectoral muscle were not affected by *in ovo* administration.	Maiorano et al. (2012)
10	Galactooligosaccharides (GOS)	3.5 mg GOS/egg	air chamber	Day 12	GOS increased overall growth performance, feed efficiency and improved Foot pad dermatitis score.	Slawinska et al. (2020)

11	Prebiotics and symbiotics	1.76 mg/egg inulin 0.528 mg/egg GOS 1.76 mg/egg inulin + 1000 cfu/egg *Lactococcus lactis subsp.* 0.528 mg/egg GOS + 1000 cfu/egg *Lactococcus lactis subsp., cremoris IBB477*	air cell	Day 12	Of four bioactive compounds delivered *in ovo,* GOS proved to be the most potent one in the stimulation of the host– microbiome interactions. The strong bifidogenic effect of GOS triggered a strong down-regulation of immune-related genes and pathways in CT	Slawinska et al. (2016)
12	Prebiotics	3.5 mg/embryo (GOS), 0.88 mg/embryo DN (DiNovo)	air cell	Day 12	Final BW, breast muscle yield and fatty acid profile of broiler chickens was improved upon delivery of prebiotics.	Tavaniello et al. (2018)
13	methanolic root bark extracts of the African Baobab tree	250 mg/ml extract	allantoic route	-	Methanolic root-bark extract of *A. digitata* had direct antiviral activity against ND virus	Sulaiman et al. (2011)
14	Moringa oleifera leaves extract	0.5 µg/ml/ - 50µg/ml	air chamber	Day 18	*In ovo* administration of 0.5 µg/ml moringa oleifera leaves extract at d 18 of incubation improves hatchability rate and day-old chicken weight	N'nanle et al. (2017)

			air sac			
15	Prebiotics	1.5-4.5 mg/ 0.2 mL of a commercial diluent of Raffinose family oligosaccharides (RFO)	14	Day 12	RFO delivered *in ovo* did not significantly improve growth performance and relative organ weight, but ileum mucosa morphology and immune response indicators in the small intestine was significantly improved.	Berrocoso et al. (2017)
16	Probiotics	10^7 cfu concentration (B. *subtilis, Pediococcus acidilactici, Enterococcus faecium*)	amniotic fluid	Day 18	*In ovo* feeding of probiotic bacteria strains had a positive effect on ileal MUC2 gene expression in the late-term embryo and beneficiary microbial colonisation only during the first week post-hatch	Majidi-Mosleh et al. (2017)
17	Prebiotics	0.88 mg/egg (DINOVO)	air chamber	Day 12	Injection of DiNovo® prebiotic significantly influenced histomorphological parameters on day 21 of rearing without negatively affecting productivity in chickens at the end of rearing.	Sobolewska et al. (2017b)
18	Prebiotics and synbiotics	1.76 mg of inulin 0.528 mg of Bi2tos 1.76 mg inulin + 1000 cfu *L. lactis subsp. Lactis* IBB 528 mg of Bi2tos + 1000 CFU of *L. lactis subsp. cremoris IBB.*	air cell	Day 12	Prebiotics and synbiotic may temporarily modulate not only the production/maturation of leukocytes but also their reactivity.	Stefaniak et al. (2019)
19	Prebiotics	Bi2tos- 3.50 mg/embryo DiNovo -0.88 mg/embryo RFO- 1.90 mg /embryo	air cell	Day 12	Total tissue factor (TF) levels increased with age in all experimental groups with prebiotics.	Buzala et al. (2016)

			air cell			
20	Prebiotics and symbiotics	1.76 mg/egg inulin 0.528 mg/egg GOS 1.76 mg/egg inulin + 1000 cfu/egg *Lactococcus lactis subsp. Lactis* 0.528 mg/egg GOS +1000 cfu/egg *Lactococcus lactis subsp., cremoris*	15	Day 12	GOS proved to be the most potent in the stimulation of the host microbiome interactions. The strong bifidogenic effect of GOS triggered a strong down-regulation of immune-related genes and pathways in cecal tonsils.	Slawinska et al. (2016)b
21	Synbiotics	*L. salivarius* IBB3154 + Bi2tos *L. plantarum* IBB3036 + lupin RFO	air chamber	Day 12	*L. salivarius* IBB3154 has a potential for modulation of the GIT microbiota due to its adherence ability.	Aleksandrza-Piekarczyk et al. (2019)
22	Probiotics	5×10^7 cfu/mL- DFM culture- two *Bacillus amyloliq- uefaciens* and one *Bacillus subtilis*	Amnion	Day 18	*Bacillus*-based probiotic can reduce the severity of virulent *E. coli* horizontal transmission and infection in broiler chickens.	Arreguin-Nava et al. (2019)
23	Probiotics	*Lactobacillus acidophilus* ($1x10^6$-$1x10^{12}$ cfu)	amnion	Day 18	*Lactobacillus acidophilus* at the dose of $1x10^6$ significantly increased the concentration of probiotic bacteria *Lactobacillus spp.* and lowered the concentration of harmful microbes in the jejunal contents of broilers compared to other *in ovo* treated groups	Kanagaraju et al. (2019)

24	Probiotics	*Bifidobacterium bifidum and Bifidobacterium longum* (200 µl)	yolk sac	Day 17	A significant improvement in live body weight, body weight gain, feed conversion ratio, haematological parameters, and villi height was observed without negative effect on carcass traits, liver and renal function indication parameters.	El-Moneim et al. (2019)
25	Probiotics	*Lactobacillus bacteria* (1×10^5 - 10^7 cfu)	amniotic fluid	Day 18	*In ovo* inoculation of *lactobacilli* downregulated cytokine gene expression in the cecal tonsils, indicating the anti- inflammatory capacity of this bacteria in the intestine.	Alizadeh et al. (2020)
26	Probiotics	*Lactobacillus salivarius* (10^9 cfu of/ 0.1 mL)	air cell	Day 17.5	*In ovo* probiotic supplementation caused a long-lasting benefit on jejunum morphology in terms of villus length.	Khaligh et al. (2018)
27	Probiotics	undefined cecal microbiota-($5.0x10^7$ /ml) undefined cecal microbiota (5.0×10^4 CFU / ml) *L. salivarius* culture (1.5×10^7 cfu / ml)	air chamber	Day 18	*In ovo* inoculation with *Lactobacillus salivarius* prevented liver infection in chicks by challenged with *Salmonella Enteritidis*.	Khaligh et al. (2018)
28	Prebiotics	GOS (3.5 mg/embryo)	air chamber	Day 12	GOS reduced the incidence of intestinal lesions and oocyst excretion in tropical Kuroiler chickens exposed to natural coccidiosis challenge.	Angwech et al. (2019)

29	Prebiotics	BI (3.5 mg/embryo) DN (0.88 mg/embryo)	air chamber	Day 12	Prebiotics were associated with significant improvements in a number of parameters, including, body weight, carcass yield, and breast muscle weight.	Maiorano et al. (2017)

Abbreviations: BI, commercial trans-GOS; DN, a commercial extract of beta-glucans; GIT, gastrointestinal tract; MUC2, Mucin 2; SCFA, short-chain fatty acid.

2.5.1 Probiotics

Several definitions have been proposed for bioactive substances tagged "probiotics" in literature, but FAO/WHO (2001) proposition might be well-grounded. They define probiotics as "live microorganisms which, when administered in adequate amounts, confer a health benefit on the host", taking into cognizance a dose-effect. By inoculating an undefined anaerobically grown competitive exclusion (CE) culture obtained from adult chicken ceca into the air cell region of incubated eggs on day 17, Cox et al. (1992) was the first study to establish the immunomodulatory effect of *in ovo* administered probiotics. Inoculated chicks exhibited better resistance to *Salmonella typhimurium* challenge compared to uninoculated ones in their study. While Greenberg (1969) was the first to utilize the CE concept to depict the exclusion of *Salmonella typhimurium* by specific bacterium species in maggots. Nurmi and Rantala (1973) extended its use to avian species. CE (also referred to as Nurmi concept) was considered as causing the resistance to Salmonella seen in birds orally administered with adult chickens' intestinal bacteria population. As opposed to the prevailing belief, it is now evident that the avian embryonic GIT is not sterile at hatch (Deeming, 2005; Pedroso, 2009; Bogucka et al., 2016), the perinatal period (the period before and after hatch) therefore presents a window of opportunity not only to colonize the avian GIT microbiome with beneficial microbes but to also prevent pathogenic microbial colonies by CE. This is the opportunity the poultry industry needs to harness by delivering alternatives to AGPs through the *in ovo* route.

There are over 57 strains of probiotics available for use in animal nutrition (Bajagai et al., 2016). By utilizing dosage, hatchability, and biometric results (chick length and relative asymmetry) obtained from their study, De Oliveira et al. (2014) were able to select out *E. faecium* and *B. subtilis* from 7 other commercial probiotic products *in ovo* delivered to the amniotic fluid on day 17.5 of embryonic development. These selected probiotics had the highest number ($P<0.05$) of recovered bacteria both in the gizzard and ceca at 48 hours post-hatch; further validating their selection. *In ovo* delivery of these probiotics had no effect on hatchability. Bearing in mind that the jeopardization of hatchability is one of the factors impairing the commercialization of this technology for delivery of bioactives. *E. faecium* and *B. subtilis* from De Oliveira et al. (2014) study had no effect on hatchability rate, though B. subtilis had numerically higher hatch percentage (96.11 vs. 81.67%). *B. subtilis* was thus suggested to offer better CE benefits (De Oliveira et al., 2014). Similarly, Majidi-Mosleh et al. (2017) observed no significant effect of *in ovo* delivery of *Pediococcus acidilactic, E. faecium* and *B. subtilis* (all at

10^7 CFU) on hatchability. Despite *in ovo* injection, the hatchability (%) obtained from their study (95-98.5%) was similar to what is obtainable in commercial hatcheries. These amnion-delivered probiotics, especially *B. subtilis*, increased expression of the intestinal MUC2 (Mucin 2) gene at day 21 of incubation and at day 3 post-hatch. On day 3 after hatch, probiotics were reported to increase the intestinal population of lactic acid bacteria, while decreasing *Eschericha coli* population in the same study. They reported no effect of probiotic inoculation on bird immune response. Contrary to previously cited studies, Triplett et al. (2018) found that *B. subtilis* (10^3 - 10^6 CFU/50 µl diluent) injected (using commercial inovoject system) to the amnion sac on day 18 negatively affected hatchability, which was attributed to the energy-sapping sporulating activities of the *B. subtilis* specie. Indeed, additional research that takes into cognizance all likely factors influencing the efficacy of *in ovo* delivery is needed to rationalize this occurrence.

Using the same commercial inovoject system, Beck et al. (2019) successfully inoculated *Lactobacillus animalis* and *E. faecium* combinations into the amnion at day 18 of incubation with no negative effect on hatchability, suggesting that other factors (including probiotic strain, volume, and dosage) other than injection method can potentially affect hatchability. In addition, probiotic treatments in the study of Beck et al. (2019) yielded higher feed conversion ratios and intestinal (jejunum and ileum) weights on day 14 after hatch. Skjøt-Rasmussen et al. (2019) have recently validated the viability of *E. faecium* (M74) strain for probiotic injection on day 18 via the amniotic fluid, as both visual examination of colonies and DNA genotypic fingerprinting (PFGE) revealed high recovery of enterococci in the yolk sac, caecal tonsils, and the rest of the intestinal tract. El-Moneim et al. (2019) just recently utilized a novel delivery route- the yolk sac route- to deliver *Bifidobacterium bifidum* ATC 29521 and *Bifidobacterium longum* ATTC 15707 (1×10^7 - 5×10^9 CFU), achieving a 100% hatchability rate. The *in ovo* delivered *bifidobacteria* strains significantly improved overall bird performance as evidenced by increased live body weight, body weight gain, and feed conversion ratio and villus height.

The immunomodulatory effect of probiotics has also been affirmed by Andreatt-Filho et al. (2006), the inoculation of *Lacillus salivarius* (1.5×10^7 CFU / ml) through the air chamber at day 18 (of incubation) prevented *Salmonella enterica* infection in the liver of broiler chickens challenged with *S. enterica sorovar Enteritidis*. Conversely, Yamawaki et al. (2013) showed that *Salmonella Enteritidis* infection could not be prevented by the *in ovo* inoculation of *Lactobacillus spp.* (*Lactobacillus acidophilus, L. fermentum*, and *L. salivarius*) through the air cell at embryonic day 18. Yamawaki et al. (2013) harvested bird ceca samples 5 days after hatch to confirm the

incidence of Salmonella enteritidis infection, while liver and cecum samples were harvested 5 days after the challenge (7 days after hatch) by Andreatti Filho et al. (2006), implying that time and other factors may play a role in competitive exclusion. Indeed, more studies are needed to further expound on the CE concept. Recently, Arreguin-Nava et al. (2019), showed that the *in ovo* delivery of vegetative *Bacillus spp.* strains (contained in Norum) reduced the severity of virulent *E. coli* cross-infection in broiler chickens in their study. This was further evidenced by metagenomic analysis (beta diversity) revealing a favorable microbial balance. Using molecular tools, Alizadeh et al. (2020) also reported the anti-inflammatory capacity of *in ovo* delivered *Lactobacillus spp.*, which downregulated cytokine gene expression in the cecal tonsils.

Overall, only organisms validated by *in vitro* analysis to be non-pathogenic to future host and tolerant to acidity should be considered for *in ovo* delivery (Bajagai et al., 2016). While it is recognized that *in ovo* delivered probiotics operate through some sort of CE mechanism, it is yet to be fully known if specie-specific mode of action does exist. The performance and immunomodulatory effect of probiotics might need to be considered on a case-by-case basis.

2.5.2 Prebiotics

Since Gibson and Roberfroid (1995) proposed the "prebiotic" concept, several definitions have been in use. Most definitions limit prebiotics to a few carbohydrate compounds. The International Scientific Association of Probiotics and Prebiotics (ISAPP) has recently offered a more encompassing definition of prebiotics as "a selectively fermented ingredient that results in specific changes in the composition and/or activity of the gastrointestinal microbiota, thus conferring benefit(s) upon host health" (Scott et al., 2016). A bioactive substance will qualify as a prebiotic if it exhibits high substrate fermentability to yield a beneficial gut microbiota, while also displaying high resistance to gut acidity and hydrolysis (Gibson and Fuller, 2000; Roberfroid, 2007). Examples of prebiotics utilized in the poultry industry include Fructans (inulin and fructooligosaccharide (FOS)), galactooligosaccharides (GOS), mannanoligosachharides (MOS), arabinoxylan oligosaccharides and raffinose family oligosaccharides (RFOs) (Ducatelle et al., 2014; Adhikari and Kim, 2017). Prebiotics are known to stimulate a healthy gut microflora in birds, hence their utilization in the poultry industry. Delivering such beneficial bioactives early enough is expected to yield performance-enhancing outcomes. These effects are reflected in literature.

After dose optimization and inoculation of RFO, DN (a commercial extract of beta-

glucans) and BI (commercial trans-GOS) into embryonic air cell at day 12, Bednarczyk et al. (2016) observed RFO (1.9 mg/embryo) to have the best effect on bird feed intake and feed conversion ratio. This study included an in-water delivery comparison, which result was not different from *in ovo* delivery. Contrarily, Miśta et al. (2017) observed that the *in ovo* delivery of inulin (1.76 mg/embryo) and Bi2tos (commercial trans-GOS; 0.528 mg/embryo) did not yield positive performance effect (body weight gain, saturated fatty acid concentration and intestinal morphology) over a 35-day trial period. On a large scale (using 25,000 chickens), Sobolewska (2017b) substantiated the positive effect of prebiotic DiNOVO (BioAtlantis Ltd., Bioatlantis Ltd., Tralee, Co., Kerry, Ireland; an extract of Laminaria spp. containing laminarin and fucoidan). Intestinal morphological parameters (duodenal villi width and crypt depth) were positively improved, suggesting improved intestinal secretion and absorption rates. Similar performance-enhancing effect was confirmed by Maiorano et al. (2017) under field conditions, birds inoculated with prebiotics DiNOVO and Bi2tos expressed a significant increase in body weight, carcass weight, carcass yield, and breast muscle weight compared to controls in their study.

The immunomodulatory effect of prebiotics has also been confirmed by Angwech et al. (2019) who observed that Bi2tos (3.5 mg/embryo) reduced the incidence of intestinal lesions and oocyst excretion in tropical Kuroiler chickens exposed to natural Coccidiosis challenge. It has also been recently shown that *in ovo* -delivered GOS (3.5 mg GOS/egg) had a tendency to cushion heat-triggered growth reduction in heat- challenge broilers (Slawinska et al., 2020).

It is inferable that prebiotics executes these beneficial effects through both direct and indirect mechanisms. A direct mechanism will be by direct interaction with epithelial and immune cells of the GIT, followed by partial absorption into the intestine (Seifert and Watzl, 2007). A reduction of intestinal pH and harvest of energy derived from short-chain fatty acid fermentation are equally associated with this direct mechanism (Dankowiakowska et al., 2019). An indirect mechanism of stimulating the growth of beneficial gut microbiota is such that the *in ovo* technology offers scope to better accomplish; it is based on the evidence of the non-sterility of the avian embryo. Hence, prebiotics delivered early enough can help stimulate a healthy gut microbiome (Dankowiakowska et al., 2019). Practically, only prebiotics with high solubility (to aid easy diffusion into the embryo GIT) should be considered for *in ovo* delivery, at dosage that does not compromise hatchability (Bednarczyk et al., 2016).

2.5.3 Synbiotics

Synbiotics are defined as bioactives comprising an admixture of probiotics and prebiotics acting synergistically to improve host's gut health (Huyghebaert et al., 2011). The application of synbiotics is based on the concept that their use stimulates the growth and/or metabolism of microbial colonies in the GIT. It has been hypothesized that the use of synbiotics is more effective than probiotics or prebiotics alone (Dankowiakowska et al., 2019). Results on *in vivo* evaluation of *in ovo* delivered synbiotics in literature are encouraging; however, more studies are needed to substantiate these results. Mista et al. (2017) observed that the *in ovo* delivery of synbiotics (inulin + *lactis subsp. lactis IBB SL1*; BI + *Lactococcus lactis subsp. cremoris* IBB SC1) improved bird performance over a 35-day trial. This was evident by increased body weight, cecal short-chain fatty acid profile and jejunal villus length: crypt depth ratio. Conversely, Maiorano et al. (2012) have previously reported low impact of *in ovo* delivery of synbiotics (S1-1.9 mg of RFO + 1,000 CFU of *Lactococcus lactis ssp. lactis* SL1, S2-1.9 mg of RFO + 1,000 CFU of *Lactococcus lactis ssp. cremoris* IBB SC1, S3-Duolac + 500 cfu of *Lactobacillus acidophilus* + 500 CFU of *Streptococcus faecium* + lactose (0.001 mg/embryo)). While only synbiotic (S3) reduced carcass yield percentage, all other synbiotics had no effect on abdominal fat, ultimate pH, and pectoral muscle cholesterol in their study. Dunislawska et al. (2017) shed light on synbiotics mode of action in their study involving the *in ovo* delivery of synbiotics (S1)-*Lacillus salivarius* with GOS-and S2 Lactob - *Lactobacillus plantarum* with RFO. Both synbiotics recorded a different gene expression pattern of immune-related genes. The different probiotic components of the synbiotics were considered a cause of this effect. S1 was referred to as synergistic to inoculated probiotic, while S2 was thought to be more synergistic to host GIT; this is expected to influence their affinity to membrane receptors and ultimately the response they trigger in host cells. This trend was further confirmed by Sobolewska et al (2017) where a synergy existed between *L. salivarius* IBB3154 and Bi2tos (Clasado Ltd. Reading, United Kingdom) while *L. plantarum* IBB3036 + lupin RFOs was more synergistic to the host. Bogucka et al. (2016) also showed the differential effects of two synbiotics on bird intestinal morphology. While inulin + *Lactococcus lactis subsp. lactis* IBB SL1 increased jejunum villi length on posthatch day 1 and 4, Bi2tos + *Lactococcus lactis subsp. cremoris* IBB SC1 caused a reduction in the height of villi on both days. This result emphasizes the peculiarities associated with different synbiotics and the need to optimize by *in vitro* analysis, hatchability, and microbiological screening before *in ovo* delivery. Recently, Dankowiakowska et al. (2019) found birds inoculated with synbiotics (1.760 mg inulin and 1,000 CFU *Lactococcus*

lactis spp. lactis IBB SL1) had higher body weight relative to prebiotic (0.760 mg inulin, 0.528 mg Bi2tos) and control birds. No negative effect on hatchability (89-98%) was recorded in their study. Considering the variability in results that exist in the literature, it is practically recommended to utilize *in vitro* assays and quantitative prebiotic parameters (prebiotic index and prebiotic activity score) to select best probiotic-substrate combinations (Figueroa-González et al., 2019).

2.5.4 Essential oils

Essential oils are a mix of volatile and aromatic compounds extracted from plant parts (including the seeds, flowers, leaves, buds, twigs, herbs, bark, wood, fruits, and roots) by distillation (Brenes and Roura, 2010). These admixtures of compounds are highly variable in their chemical composition and concentrations. For instance, thyme EO contains thymol and carvacrol (3-60%) as the active component, cinnamon EO contains cinnamaldehyde (60-75%), and oregano oil contains carvacrol (>85%) (Lawrence and Reynolds, 1984; Ultee et al., 1999; Duke et al., 2002). These active components confer EO its bioactive properties that include antioxidative, anti-inflammatory, anti-fungal, antimicrobial, hypocholesterolemic, digestibility-enhancing and gut health-promoting activities (Brenes and Roura, 2010; Gopi et al., 2014; Stevanović et al., 2018). The antibacterial activity of EO is thought to be exerted by membrane perforation or binding principle, which causes distortion of bacteria enzyme systems (Farag et al., 1989a; b). There are about 300 commercially available EO (Brenes and Roura, 2010). Emerging studies suggest that maximum benefits may be derived from blends of EOs and other compounds via synergism rather than using individual EO (Mitsch et al., 2004; Wang et al., 2018a). Results on *in vivo* evaluation of dietary supplemented EO in the literature have been variable (Basmacioğlu et al., 2004; Khattak et al., 2014; Kirkpinar et al., 2014; Peng et al., 2016).

With regards to *in ovo* delivery of EO, available studies are limited. Toosi et al., (2016) reported that the *in ovo* delivery of a blend of essential oils and organic acids had no effect on hatchability. However, *in ovo* delivery of this blend increased humoral immune responses. Similarly, the *in ovo* delivery of clove extract at embryonic day 10 improved chick's hatch and post-hatch performance (El-Kholy et al., 2021). The bird's immune status (IgG) and antioxidant status (Superoxide dismutases activity) were also improved by the *in ovo* delivery of clove extract (El-Kholy et al., 2021). Successive delivery of nano-encapsulated thyme oil via *in ovo* + in-feed routes has also been reported to increase initial chick body weight, as well as final bodyweight

and bodyweight gain at 6 wks. (Yaseen et al., 2022). Additionally, by using three rosemary extract dosages (0.05-0.1 ml), Sulaiman and Tayeb, (2021) were able to report that the highest dosage (0.1ml) recorded a positive effect on hatchability, growth performance, immunity, and blood cholesterol levels. Given the paucity of studies in this regard, more studies are thus needed to standardize guidelines regarding the *in ovo* delivery of EO.

2.5.5 Folic acid

Folic acid, or Pteroylglutamic acid, is a water-soluble B vitamin (Naderi and House, 2018). Compounds in this vitamin (B9) group are generally referred to as folates. Exogenous uptake of folic acid is necessary because humans and livestock lack the enzymes to synthesize folate de novo (Kennedy, 2016). Folate distribution and metabolism take place in the cytosol and mitochondria (Wagner, 1995). Folates also play important roles as cofactor or cosubstrate for several physiological reactions and processes, including nucleic acid synthesis and methionine regeneration (Kennedy, 2016; El-Husseiny et al., 2018). Oilseed meals like soybean meal are important sources of folic acid in animal diets (Perloff and Butrum, 1977). In poultry, factors such as strain, age, production phase, management guidelines and environmental conditions affect folic acid requirement (Terčič and Pestotnik, 2014). Deficiencies of folic acid in poultry include decreased growth performance, bone abnormalities, and oxidative stress (McDowell, 1989; Huang et al., 2001; Hebert et al., 2005). While the results of dietary supplementation of folic acid on animal performance and health are promising, they have been inconsistent. Terčič (2014) reported that a 4-weeks supplementation of 50 mg/kg FA to layer breeders improved hatchability and chick body weight. Similarly, dietary supplementation of 1-2 mg/kg folic breeders increased hatched chick weight and post-hatch performance (Barroeta et al., 2012). Conversely, the supplementation of 5-15 mg/kg folic acid to layer hens for six weeks caused a reduction in feed conversion ratio and egg weight (Bagheri et al., 2019). Jadavji et al. (2015) also reported no effect of as much as 8 mg/kg folic acid dietary supplementation on egg parameters in quail. Gouda et al. (2020) also highlighted the potential of folic acid to mitigate heat stress, as folic acid supplementation increased total antioxidant capacity and the concentration of antioxidant enzymes in their study.

Aside from the paucity of studies involving the *in ovo* delivery of FA, conflicting results on the effect of FA on broiler chicken performance exist in the literature. For instance, varying doses of *in ovo* delivered FA have been reported to improve hatchability in poultry. Li et al.

(2016) reported that 100 and 150 µg folic acid injected on embryonic day 11 improved hatchability. Conversely, Hussian et al. (2019) reported that lower levels of folic acid (10 and 20 µg) could improve hatchability and quality of hatched chicks from Chinese white duck eggs. Ismail et al. (2019) injected a combination of amino acid and folic acid (6µg amino acid+150µg folic acid) on 14th day of incubation and recorded that both treatments improved the hatchability of fertile eggs and subsequent post-hatch growth performance (d1-28). On the other hand, Nouri et al. (2018) reported no effect on hatchability when folic acid (40 -120 µg) was injected into broiler chicken hatching eggs on day 14 of incubation. Notwithstanding, blood biochemistry parameters (blood glucose, phosphorus, calcium, triglycerides, and cholesterol) were all positively modified by folic acid in their study. Despite the reported potential of folic acid to improve poultry performance, interestingly, they are yet to be researched in the context of an alternative to antibiotics.

2.5.6 Other Bioactive Substances

Research on the *in ovo* delivery of other alternatives to AGPs in poultry is relatively scarce. A lot more alternatives to AGPs are currently being researched through dietary means (reviewed by Gadde et al., 2017). The trend is still developing with respect to *in ovo* technology. With regards to *in ovo* technology, Goel et al. (2017) validated the *in ovo* delivery of silver nanoparticles at embryonic day 18 through the air cell; this time does not negatively impart hatchability compared to inoculation at embryonic day 7. The immunological properties of silver nanoparticles were also confirmed in their study, silver nanoparticle- 12.5 µg/egg enhanced cellular immune response while concentrations above 25 and 50 µg/egg improved humoral and adaptive immunity in broilers. Salahi et al. (2011) also reported the performance-enhancing effect of *in ovo* -delivered butyric acid; chick length (at hatch), body weight (10 days after hatch), feed conversion ratio (10 days after hatch) and intestinal morphology were all improved by the inoculation of 1ml (28-32% butyric acid) in broiler chickens. Salmanzadeh et al. (2015) similarly observed an improvement in intestinal morphology which translated to improved body weight and feed conversion ratio, both at day 21 and 42 post-hatch in turkey eggs inoculated with butyric acid through the yolk sac on day 7. On the contrary, Morovat (2016) observed that dietary and not *in ovo* delivery of Silybum marianum extract (from the milk thistle known for its anti-inflammatory and antioxidant properties) resulted in improved performance, immunity, and carcass characteristics. *In ovo* delivery of this plant extract into the amniotic fluid at embryonic day 17.5, had no effect on

hatchability (range-84-86%). Overall, considering the large array of bioactive plants that abound, the possibility of finding effective potential phytobiotics that can be delivered *in ovo* is high.

2.6 Optimal Time Points for *In ovo* Delivery of Bioactive Substances

The efficacy of *in ovo* injection is affected by a host of factors, among which are time of injection, site of injection, dosage of bioactives, hatchery hygiene and so on (Bednarczyk et al., 2016). With reference to time, two optimal time points that ensure effective delivery of bioactive substances into the developing embryo are described in literature. These time points coincide with the type of bioactive substance to be delivered. Both Villaluenga et al. (2004) and Bednarczyk et al. (2016) have validated embryonic day 12 as the appropriate time for delivering prebiotics and synbiotics *in ovo*. The optimal site of inoculation for these bioactives is the air cell because the injected bioactive substance can easily flow from the air cell into the embryonic GIT located in the highly vascularized chorioallantoic membrane at this time. This injection technique is expected to lead to the stimulation of beneficial microflora in the embryo GIT and has been confirmed by an increased presence of bifidobacteria by Villaluenga et al. (2004) and Tako et al. (2014). Gulewicz and Bednarczyk (2008) hold the patent for this technique, which has been termed *in ovo* stimulation.

The success of this procedure is well documented in literature, with no negative effect on hatchability reported (Pilarski et al., 2005; Bednarczyk et al., 2011, 2016; Maiorano et al., 2012). The other time point is at embryonic day 17 or 18, which is appropriate for probiotic inoculation through the amnion. It is referred to as *in ovo* feeding and has been patented by Uni and Ferket (2003). Bioactive substances are to be delivered into the amnion before day 19, which is when the embryo consumes the amniotic fluid. This procedure is also well validated in literature (De Oliveira et al., 2014; Majidi-Mosleh et al., 2017; Beck et al., 2019). These time points are consistent with the dynamic physiology of the developing embryo. Considering that the chick embryo is completely developed at day 17/18 of egg incubation and will rely on yolk nutrition till hatch, *in ovo* feeding at this time is thus a practical nutritional intervention that could mitigate perinatal nutritional deficiencies (Siwek et al., 2018). This time point also coincides with major activities (candling and egg transfer from the incubator setter to the hatcher) in the hatchery, making it convenient for hatchery operators (Siwek et al., 2018). On the other hand, day 12 would be an ideal time to stimulate the still-developing embryonic GIT with native microbiota, to ensure optimal microbiome and ultimately desirable phenotypes throughout life (Siwek et al., 2018).

In ovo stimulation and *in ovo* feeding both represent 2 unique approaches to *in ovo* technology in poultry production. The differences between the 2 approaches are evident in procedures, biological mechanisms, and technical requirements (Siwek et al., 2018). While *in ovo* stimulation aims to induce the growth of native microflora in the embryonic gut via prebiotics or synbiotics metabolism, *in ovo* feeding is majorly a strategic nutritional intervention (as highlighted previously) (Siwek et al., 2018). *In ovo* delivered probiotics are also capable of acting as pioneer colonizers which influence the gut microbiota by altering the gut environment (Pedroso et al., 2016). The difference in sites of injection between both approaches is related to the changing size and structure of the egg at the different time points (Siwek et al., 2018). Technically, both approaches might require modifications to hatchery operations, especially under commercial settings. As stated previously, day 18 of incubation accommodates several hatchery operations, including Marek's vaccination. Nonetheless, the practicality of combining *in ovo* vaccination and *in ovo* feeding at this time point is yet to be known (Teague et al., 2017). Hence, injector systems that uses the air cell on day 12 of egg incubation are needed to ensure easy adoption of this technology.

In ovo delivery of bioactive substances can also be manual or automatic, depending on the number of eggs to be injected, the manual method could be susceptible to human error. The successful adoption and commercialization of the *in ovo* vaccination system largely depends on the automation of injector systems. The success of the *in ovo* delivery of other bioactive substances is likely to depend on the same. Two main types of automated injection systems exist, and they include the automated multiple-head injector system (MIS) and the semi-automated injector system (Schijns et al., 2014). MIS improves the ease and efficiency of *in ovo* delivery while also saving time and labor. Newer models are self-sterilizing and only selectively inoculate viable embryos, hence reducing the risks of pathogenic contamination and vaccine wastage. By using MIS, as much as 35,000–70,000 eggs per hour can be injected by 2 persons (Schijns et al., 2014). Despite these benefits, they come at a great financial cost. The semiautomated machines are more affordable for smaller hatcheries. They are capable of inoculating about 12,000–20,000 eggs (Schijns et al., 2014). In any case, it is practically expedient that the appropriate dose of bioactive substances to be injected be optimized by microbiological and hatchability screening before inoculation.

2.7 Conclusion and Applications

1. This review details a recently emerging niche of *in ovo* application in the poultry industry. Literature reveals that the *in ovo* delivery of bioactive substances eligible as an alternative to AGPs (probiotics, prebiotics, synbiotics, phytobiotics, organic acids etc.) show a promising effect on bird performance and health (Table 2.2).

2. The *in ovo* technology overcomes several challenges associated with in-water and in-feed delivery of alternatives to AGP. These challenges include nutrient inactivation due to heat treatment of feedstuff and potential water quality risks. With the utilization of *in ovo* technology, alternatives to AGP can be delivered to the developing embryo to stimulate a healthy gut microbiome early on.

3. More well-designed studies that include antibiotic positive control are needed to substantiate the efficacy of *in ovo* -delivered bioactive substances replacing AGP. It is still unclear if the positive effects observed for alternatives to AGPs persist throughout a bird's life, trials of long duration are needed to confirm this.

4. The list of bioactive substances that qualify as alternatives to AGP is no way exhaustive, it is probable that the *in ovo* delivery of novel alternatives particularly phytobiotics would be actively researched on in the coming years. *In ovo* technology has great potential to help bring an end to the use of AGP in the poultry industry.

3 CHAPTER 3 PROBIOTICS DELIVERY ROUTES 1

BACILLUS SUBTILIS DELIVERY ROUTE: EFFECT ON GROWTH PERFORMANCE, INTESTINAL MORPHOLOGY, CECAL SHORT-CHAIN FATTY ACID CONCENTRATION AND CECAL MICROBIOTA IN BROILER CHICKENS

This work has been published and presented elsewhere:

- Oladokun, S., Koehler, A., MacIsaac, J., Ibeagha-Awemu, E. M., and Adewole, D. I. 2021. *Bacillus subtilis* delivery route: Effect on growth performance, intestinal morphology, cecal short-chain fatty acid concentration, and cecal microbiota in broiler chickens. Poultry science, 1003:100809. https://doi.org/10.1016/j.psj.2020.10.063

- Oladokun, S., Koehler, A., MacIsaac, J., and Adewole, D.I. 2020. "Does *In ovo* delivery of probiotics affect hatch and growth performance, and intestinal functionality in broiler chickens?" Animal Nutrition Conference of Canada (ANCC), May 26 –June 11, 2020.

3.1 Abstract

As the poultry industry recedes from the use of antibiotic growth promoters, the need to evaluate the efficacy of possible alternatives, and the delivery method that maximizes their effectiveness arises. This study aimed at expounding knowledge on the effect of the delivery method of a probiotic product (*Bacillus subtilis* fermentation extract) on performance and gut parameters in broiler chickens. A total of 450 fertile eggs sourced from Cobb 500 broiler breeders were randomly allotted to 3 groups: *in ovo* probiotic (n = 66), *in ovo* saline (n = 66), and non-injection (n = 200) and incubated for 21 days. On day 18.5 of incubation, 200 µl of either probiotic (10 X 10^6 CFU) or saline was injected into the amnion. At hatch, chicks were reallotted to 6 new treatment groups: *in ovo* probiotic, *in ovo* saline, in-feed antibiotics, in-water probiotic, in-feed probiotic, and control (CTRL; Corn-wheat-soybean diet) in 6 replicate cages and raised for 28 days. Of all hatch parameters evaluated, only percentage pipped eggs were found significant ($P < 0.05$) with the non-injection group having higher percentage pipped eggs than the other groups. Treatments did not affect the incidence of necrotic enteritis on day 28 ($P > 0.05$). Irrespective of the delivery method, the probiotic treatments had no significant effect on growth performance. The ileum villus width of the *in ovo* probiotic treatment was 18% higher than the *in ovo* saline group ($P = 0.05$), but not statistically higher than other groups. The jejunum villus height was 23% higher ($P = 0.000$) in the *in ovo* probiotic group than the control group. There was no effect of treatment on total cecal short-chain fatty acid concentration and cecal gut microbiota

composition and diversity ($P > 0.05$), although few unique bacteria differential abundance were recorded per treatment. Conclusively, although probiotic treatments (irrespective of delivery route) did not affect growth performance, *in ovo* delivery of the probiotic product enhanced intestinal morphology, without compromising hatch performance and gut homeostasis.

3.2 Introduction

Antibiotic growth promoters (AGPs) have been used sub-therapeutically to improve bird performance and health in the poultry industry for almost 8 decades (Fallis, 2013; Gadde et al., 2017). This trend is now receiving strong criticism as a result of concerns of antimicrobial resistance, antibiotic residues, and food safety hazards (Muaz et al., 2018; Wales et al., 2019). In the light of the foregoing, the poultry industry is thus faced with the challenge of developing urgent alternatives to AGPs, potent against economically important poultry diseases like Necrotic enteritis (NE), Colibacillosis, Salmonellosis, and so forth.

Probiotics are one of such alternatives being experimented. Probiotics are defined as "live microorganisms which, when administered in adequate amounts, confer a health benefit on the host" (FAO/WHO, 2001). These organisms help to improve bird performance by modulating a favorable gut microflora in the host (Mountzouris et al., 2009; Cao et al., 2013; Zhang et al., 2014), improving feed conversion and digestive efficiency (Jin et al., 2000; Afsharmanesh and Sadaghi, 2014; Zhang and Kim, 2014), and producing antimicrobial substances (Fayol-Messaoudi et al., 2005; Corr et al., 2007), and several other benefits. Probiotics can achieve these positive effects because they successfully colonize the gastrointestinal (GIT) tract of the host (Lan et al., 2003). Examples of probiotics bacteria in current use in broiler chicken production include *Enterococcus, Lactobacillus, Lactococcus*, *Bifidobacterium,* and *Bacillus species (*Patterson and Burkholder, 2003).

Several probiotics delivery routes exist, but conventional in-feed supplementation is the most commonly used. Other possible delivery routes include in-water supplementation, spray method, litter delivery, and more recently, *in ovo* delivery. The efficacy of probiotics has been inconsistent in the literature because of several limitations that characterize these delivery routes (Applegate et al., 2010; Ajuwon, 2016). During heat treatment, in-feed probiotics could be subjected to potential heat inactivation and instability (Ducatelle et al., 2014). In-water probiotics delivery will depend on the precision of chick watering devices, whilst also posing potential water quality risks. *In ovo* technology which involves the direct inoculation of bioactive substances to

the developing embryo to elicit superior lifelong effects (Oladokun and Adewole, 2020), offers the opportunity to address some of these identified limitations. In additionally, with *in ovo* technology, lesser quantities of bioactive substance are reported to be needed than in conventional delivery routes (Bednarczyk et al., 2016; Tavaniello et al., 2018). Furthermore, *in ovo* technology has been proffered as a solution to the perinatal nutritional stresses associated with a shift from yolk feeding to exogenous feeding, long hatchery window (24–36 h), and time-consuming hatchery activities that chicks often encounter (Noy and Uni, 2010). This technology has also been shown to be useful to stimulate the colonization of the embryonic gut with beneficial microbiota, amongst other potential advantages (reviewed by Oladokun and Adewole, 2020).

This study, therefore, sought to evaluate the effect of delivery route (in-water vs. in-feed vs. *in ovo*) of a probiotics product (*Bacillus subtilis* fermentation extract) on growth performance, intestinal morphology, cecal short-chain fatty acid (SCFA) concentration, and cecal microbiota in broiler chickens.

3.3 Material and Methods

The experiment was carried out at the hatchery facility of the Agricultural Campus of Dalhousie University and the broiler rearing facility of the Atlantic Poultry Research Center, Dalhousie Faculty of Agriculture. All experimental procedures were approved by the Animal Care and Use Committee of Dalhousie University, in accordance with guidelines of the Canadian Council on Animal Care (CCAC, 2009).

3.3.1 Eggs and Incubation

A total of 450 fertile eggs (Cobb 500) with average weight 64 ± 0.2 g (mean ± standard error) were sourced from a commercial breeder flock in Nova Scotia and incubated in a ChickMaster single-stage incubator (ChickMaster G09, Cresskill, NJ) under standard conditions (37.5°C and 55% RH) for 21 days, in the aforementioned hatchery facility. Eggs were arranged in 6 replicate trays inside the incubator, each tray containing 75 eggs. The eggs were candled on day 17 and infertile eggs were disposed of. On day 18.5 of incubation, eggs were randomly allotted to 3 experimental groups: the *in ovo* probiotic group (66 eggs) injected with 200 µl of *Bacillus subtilis* fermentation extract (Strain - *Bacillus subtilis* 10SI) (each egg received 10 X 10^6 colony forming units- **CFU** of the bacterium/200 µl saline diluent), *in ovo* saline group injected with 200 µl of physiological saline (0.9% NaCl) (66 eggs) and the control group – non-injected (200 eggs). Eggs

were placed in a single incubator in such a way that all treatment groups were evenly distributed across all the trays. The probiotic solution was prepared for 100 eggs by dissolving 0.1g of the *Bacillus subtilis* product into 20 mL of 0.9% saline. The *Bacillus subtilis* product was obtained from a commercial source (Probiotech International, St. Hyacinth, QC, Canada) at a concentration of 10 X 10^9 CFU/g.

3.3.2 Injection Procedure

Eggs were injected according to the procedure described by Tako et al. (2004) with some modifications. The amnion was the site of injection. Eggs were disinfected by swabbing the blunt ends with cotton balls soaked in 70% ethanol, a small hole was then punched into the shell at the center of the air-cell (the blunt end) using an 18-gauge needle. The injected bioactive substance was delivered to the amnion of each egg using a self-refiling injector (Socorex ultra-1810.2.05005, Ecublens, Switzerland) equipped with a 22-gauge needle at a 45-degree angle. After *in ovo* injection, eggs were sealed with sterile paraffin. However, *in ovo* delivery of bioactive substances could be manual or automated, with the automated method capable of inoculating as much as 35,000–70,000 eggs per hour (depending on the type) (Schijns et al., 2014), the manual method was employed in the current study only to confirm the efficacy of our inoculated bioactive substance under experimental conditions. In any case, the *in ovo* technology has been reported to offer several advantages over conventional delivery routes (recently reviewed by Oladokun and Adewole, (2020)).

3.3.3 Bird Rearing Conditions and Diets

On day 21, unhatched eggs were counted and opened to check for the cause of embryo death. As presented in Figure 3.1, hatchlings were weighed and randomly re-allotted to 6 new treatment groups. Birds in the initial non-injection group were randomly allocated into four groups (there were 42 birds per group) consisting of (1) chicks fed a basal corn-soybean meal-wheat based diet (CTRL); (2) chicks fed CTRL + 0.05% bacitracin methylene disalicylate (in-feed antibiotics); (3) chicks fed CTRL + *Bacillus subtilis* fermentation extract at a concentration of 0·025 g/L of drinking water (in-water probiotic containing 2.5 x 10^8 CFU of *Bacillus subtilis*/L of drinking water); and (4) chicks fed 0.005 % *Bacillus subtilis* fermentation extract (in-feed probiotic containing 5 x 10^8 CFU/kg of feed). The initial *in ovo* saline and *in ovo* probiotic groups were placed on the control diet to form treatments 5 (*in ovo* saline treatment with 42 birds) and 6 (*in ovo* probiotic treatment with 42 birds), respectively, and raised in the previously mentioned broiler-rearing facility. Birds were allocated to 36 cages with 6 replicate cages of each treatment,

comprising 7 birds per cage. Each cage was 0.93 m x 2.14 m in dimension. Broiler chickens were reared for 28 days under uniform controlled environmental conditions in line with recommendations of Cobb Broiler Management Guide (Cobb-Vantress, 2020). Room temperature was set at 31°C on day 0 and gradually reduced to 23°C on d 28 and relative humidity ranged between 45 and 55%. Dietary treatments, ingredients, and nutritional composition are presented in **Error! Reference source not found.** The probiotic diet was prepared by pre-mixing 0.005 % *Bacillus subtilis* fermentation extract with preground corn and adding the premix with the formulation thereafter. Diets were fed as mash during the starter phase and fed as pellets during the grower phase. All cages were equipped with water troughs, which were being monitored and replenished daily. Diets were formulated to meet Cobb 500 broiler requirements. Birds were provided feed and water *ad libitum* during a starter phase (0-14 days) and grower phase (15-28 days).

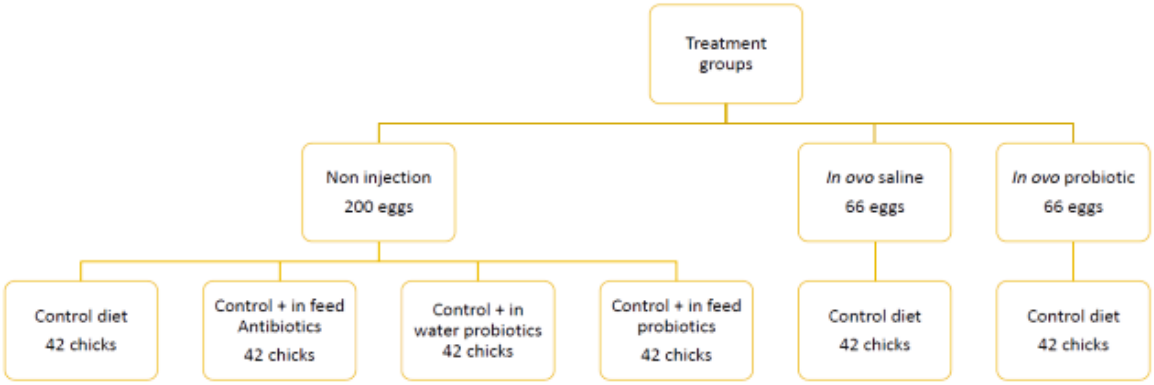


Figure 3.1 Treatment structure in the hatchery and broiler barn.

Table 3.1 Ingredient and composition of experimental diets[1] (as-fed basis, %, unless otherwise stated)

	Starter			Grower		
	Control diet	Antibiotic diet	Probiotic diet	Control diet	Antibiotic diet	Probiotic diet
Corn	51.08	50.97	51.08	44.32	44.22	44.31
Soybean meal-46.5	41.44	41.45	41.44	36.48	36.49	36.48
Animal/Vegetable fat	2.93	2.97	2.93	4.59	4.63	4.60
Wheat	-	-	-	10.00	10.00	10.00
Limestone	1.80	1.80	1.80	1.65	1.65	1.65
Dicalcium phosphate	1.24	1.24	1.24	1.06	1.06	1.06
DL-methionine premix[2]	0.59	0.59	0.59	0.53	0.53	0.53
Lysine HCl	0.01	0.01	0.01	0.00	0.00	0.00
Vitamin-mineral premix[3]	0.50	0.50	0.50	0.50	0.50	0.50
Salt	0.41	0.41	0.41	0.37	0.37	0.37
Pellet binding agent[4]	-	-	-	0.50	0.50	0.50
BMD 110 G[5]	-	0.05	-	-	0.05	-
Bacillus subtilis	-	-	0.005	-	-	0.005
Total	100	100	100	100	100	100
Calculated composition						
ME (kcal/kg)	3000	3000	3000	3100	3100	3100
Crude protein	23.00	23.00	23.00	21.50	21.50	21.50
Calcium	0.96	0.96	0.96	0.87	0.87	0.87
Available phosphorus	0.48	0.48	0.48	0.44	0.44	0.44
Sodium	0.19	0.19	0.19	0.18	0.18	0.18
Digestible lysine	1.28	1.28	1.28	1.16	1.16	1.16
Digestible methionine + cysteine	0.95	0.95	0.95	0.87	0.87	0.87
Analyzed composition						
Dry matter	89.23	90.94	90.85	87.10	88.01	86.83
Crude protein	22.77	22.40	24.16	21.72	21.63	21.87

Crude fat	5.06	5.23	5.17	6.77	6.56	6.35
Calcium	1.13	1.31	1.04	0.89	0.95	0.89
Total Phosphorus	0.65	0.65	0.62	0.55	0.58	0.57
Sodium	0.19	0.20	0.33	0.15	0.17	0.21

[1]Basal diet (NC); antibiotic diet containing NC + 0.05 % bacitracin methylene disalicylate (BMD); probiotic diet containing NC + 0.005 % *Bacillus subtilis*.

[2]Supplied/kg premix: DL-Methionine, 0.5 kg; wheat middlings, 0.5 kg

[3]Starter vitamin-mineral premix contained the following per kg of diet: 9750 IU vitamin A; 2000 IU vitamin D3; 25 IU vitamin E; 2.97 mg vitamin K; 7.6 mg riboflavin; 13.5 mg Dl Ca-pantothenate; 0.012 mg vitamin B12; 29.7 mg niacin; 1.0 mg folic acid, 801 mg choline;0. 3 mg biotin; 4.9 mg pyridoxine; 2.9 mg thiamine; 70.2 mg manganese; 80.0 mg zinc; 25 mg copper; 0.15 mg selenium; 50 mg ethoxyquin; 1543mg wheat middlings; 500 mg ground limestone. Grower vitamin-mineral premix contained the following per kg of diet: 9750 IU vitamin A; 2000 IU vitamin D3; 25 IU vitamin E; 2.97 mg vitamin K; 7.6 mg riboflavin; 13.5 mg Dl Ca-pantothenate; 0.012 mg vitamin B12; 29.7 mg niacin; 1.0 mg folic acid, 801 mg choline;0. 3 mg biotin; 4.9 mg pyridoxine; 2.9 mg thiamine; 70.2 mg manganese; 80.0 mg zinc; 25 mg copper; 0.15 mg selenium; 50 mg ethoxyquin; 1543mg wheat middlings; 500 mg ground limestone

[4]Pel-stik: Uniscope, Inc., Johnstown, CO, USA.

[5]Bacitracin methylene disalicylate (providing 55 mg/kg mixed feed); Alpharma, Inc., Fort Lee, NJ, US

3.3.4 Hatch Parameters and Chick Quality

Hatched chicks were counted and weighed individually. Hatchability was calculated as the percentage of hatched chicks to incubated eggs, per replicate. The stage of egg embryonic death was classified as pipped (death occurring after chick had made the piping hole) and late dead (chicks fully formed, but dead without pipping), the ensuing counts were expressed as a percentage of fertile eggs and recorded. Hatched chick bodyweight/initial egg weight ratio was also determined and recorded. Chick navel quality was evaluated by adapting Reijrink et al. (2009) scoring method. Navel quality was scored 1- when navel was completely closed and clean, scored 2- when navel was discolored (i.e., when navel color differs from chick's skin color) with a 2 mm maximum opening, and scored 3- when navel was discolored and with more than a 2 mm opening.

3.3.5 Growth Performance Parameters and Sampling

Growth performance parameters- feed intake, and average body weight were measured on a pen basis at 7, 14, 21 and 28 days of age. Average daily feed intake (ADFI), average daily gain (ADG), and feed conversion ratio (FCR) were subsequently calculated from obtained data. FCR was calculated as the amount of feed consumed per unit body weight gain. Mortality was recorded daily and used to correct for feed consumption. On day 28, 2 birds per pen (12 replicate birds per treatment group) were randomly selected and euthanized by electrical stunning and exsanguination. After euthanasia of birds, the intestinal segments the jejunum (1.5 cm length midway between the point of entry of the bile ducts and Meckel's diverticulum) and ileum (1.5 cm length midway between the Meckel's diverticulum and ileocecal junction) were excised and fixed in neutral buffered formalin (10%) for further histomorphological processing (Awad et al., 2009). The digesta samples from each pair of the cecum of the euthanized bird were mixed and subsampled, a portion was stored in biofreeze kits (Alimetric Diagnostics, Espoo, Finland) for SCFA concentration measurement and the other held in RNase and DNase free tubes, immediately frozen in liquid nitrogen, and later stored at –80°C for subsequent gut microbiota analysis.

Incidence of NE was evaluated on small intestinal segments of euthanized birds using lesion scoring guide by Shojadoost et al. (2012), with slight modifications. This scoring guide was as follows: NE score 0 - no gross lesions present; NE score 1- no obvious ulcers in the mucosa, but the entire mucosal surface is covered with a layer of loosely adherent fibrin; NE score 2- excavated ulcer of the mucosa with acute, bright red hemorrhage within the ulcer bed and scant crusting of fibrin around the periphery; NE score 3 - excavated ulcer of the mucosa with dark

green-black pigment within the ulcer bed and scant crusting of fibrin over the surface; NE score 4 - excavated ulcers of the mucosa, with periphery covered by thick, tightly adherent layers of fibrin, necrotic tissue, and inflammatory cells; NE score 5 - mucosa covered by large, confluent plaques of fibrin, necrotic tissue, and inflammatory cells to the point of extending over broad regions of the intestinal mucosa.

3.3.6 Gut Morphology Measurement

Fixed jejunum and ileum tissue samples were further subjected to microtomy processing. This involved slicing into 3 sections and dehydration by increasing alcohol concentration from 0 to 100%. Tissue slices were infused with xylene and fixed in paraffin wax. Tissue section (0.5 µm thick) was cut with a microtome (Leica RM 2145, Leica Microsystems, Wetzlar, Germany) and mounted on a glass slide, followed by staining (Drury and Wallington, 1980) and morphometric measurements. Morphometric measurements included villus height (from the base of the intestinal mucosa to the tip of the villus excluding the intestinal crypt), villus width (halfway between the base and the tip), crypt depth (from the base upward to the region of transition between the crypt and villi), and total mucosa thickness (villus height + crypt depth) (Ozdogan et al., 2014). Ten measurements of each component per slide were carried out using an image processing and analysis system (ImageJ, WI, USA).

3.3.7 SCFA and Total Eubacteria Quantification

Cecal samples were submitted to Alimetrics Diagnostics AD19024-1(Espoo, Finland) for both SCFA concentration and total Eubacteria quantification. Acids quantified were acetic, propionic, butyric, valeric, isobutyric, 2-methylbutyric, isovaleric, and lactic acid in 6 replicates per treatment.

3.3.8 Gut Microbiota Analysis

Genomic DNA (gDNA) was extracted from 70-90 mg of cecal digesta samples obtained from 12 replicate birds per treatment group using Quick-DNA Fecal and Soil Microbe 96 Kit (CAT: D6011, Zymo Research, Orange County, CA) with slight modification to manufacturer's protocols. BashingBead™ Buffer (400µl), beta-mercaptoethanol, and Genomic Lysis Buffer (0.5% v/v) were added to cecal samples in a 96-well block/plate bead beater, followed by centrifugation (10,000 x g, 2 x 5 min) to ensure cell lysis. BashingBead™ Lysis Rack (0.1 & 0.5 mm) was also centrifuged (4,700 x g, 5 min), after which 250 µl supernatant was transferred to a 96-well plate. Genomic Lysis Buffer (750 µl) was further added to the filtrate in the 96-well plate,

followed by mixing and centrifugation (4,700 x g for 5 min). 500 µl from each well was transferred to the wells of a Silicon-A™ Plate, followed by centrifugation (4,700 x g for 5 min). Flow through from the collection plate was discarded. 200 µl DNA prewash buffer and 500 µl gDNA wash buffer were added to the wells of the Silicon-A™ plate, this was followed by concurrent centrifugation (3,000 x g for 5 min). 150 µl prep solution was added to the wells of a prepared Silicon-A™ HRC plate mounted on an elution plate; this was then incubated at room temperature for 5 min and centrifuged (3,500 x g for 5 min). Finally, 100 µl of DNA elution buffer was added directly to the matrices on the Silicon-A™ Plate, followed by centrifugation (3,500 x g for 7 min) to elute DNA extract.

The efficiency of the DNA extraction protocol was confirmed by visual assessment on a 1% agarose gel. Extracted DNA concentration and purity were determined by spectrophotometry (Nanodrop ND1000; Thermo Scientific, USA). Universal 16S primers, 515 F (5'-GTGCCAGCMGCCGCGGTAA) and 806R (5'GACTACHVGGGTWTCTAAT) targeting the V4 variable region of the 16S rRNA gene were used to prepare amplicon libraries and sequencing (paired ends 250 bp) was carried out on an Illumina MiSeq system at McGill University and Genome Quebec Innovation Center (Montreal, Canada). Amplicon analysis was carried out following Dada2 analysis methods (Callahan et al., 2016) at the Canadian Centre for Computational Genomics (C3G, Montreal, Canada) (Bourgey et al., 2019).

3.3.9 Statistical Analysis

Data were analyzed as a completely randomized design. The normality of all datasets was ascertained by testing residuals by Anderson-Darling test in Minitab statistical package (v.18.1). Data sets found to be normal including, performance data, navel score, SCFA concentrations, and gut morphology were subjected to one-way analysis of variance (ANOVA) in the same statistical package with experimental treatments as factor and the aforementioned data sets as variables. For hatchability parameters, hatching trays were the experimental units and the pen was the experimental unit for growth performance parameters.

Data sets on total Eubacteria, relative operational taxonomic unit (OTU) taxa abundance (except for phylum *Fimicutes* and genus *Ruminiclostridium*) were natural log-transformed, whereas pipped eggs (%) was cube root transformed. After transformations, the data was equally subjected to ANOVA procedures in the same statistical package, with appropriately back-transformed data presented. Data sets found to be non-normal including late dead eggs (%),

hatched chick BW/initial egg weight, NE scores, and mortality were subjected to a non-parametric Kruskal-Wallis test in the same statistical package, after failed transformation. Differences between significant means were tested using Tukey's honest significant difference (HSD) test in the same statistical package. Analyzed data are presented as means ± SEM and probability values. Values were considered statistically different at $P \leq 0.05$.

3.3.10 Gut Microbiota Statistical and Bioinformatics Analysis

Statistical analysis and visual exploration of bioinformatics data were carried out with the MicrobiomeAnalyst tool (Dhariwal et al., 2017). Data were filtered to minimum count 2 and 10% prevalence in samples. Alpha diversity analysis was calculated based on Shannon Index. Significant differences in alpha diversity among different groups were calculated based on ANOVA, where a significant difference level was set at $P < 0.05$. Beta diversity was calculated based on Bray-Curtis index, and statistical comparisons among groups were performed with permutational multivariate ANOVA. To determine differentially abundant taxa at different groups, MetagenomeSeq (Paulson et al., 2013) that uses zero-inflated Gaussian fit model was used with adjusted *P* value cutoff at 0.05.

3.4 Results

3.4.1 Hatch Performance

In this study, among all hatchability parameters, only percentage pipped eggs were found to be significantly ($P < 0.05$) different among the treatments (Table 3.2). Non-injected eggs recorded 98.75% and 57.84% more pipped eggs (%) compared to *in ovo* saline and *in ovo* probiotic treatments respectively (Table 3.2). No difference ($P > 0.05$) among treatments was found for late dead eggs (%), hatchability, the average chick weight, and hatched chick bodyweight to initial egg weight. Nonetheless, *in ovo* probiotic treatment had numerically higher average chick weight and hatched chick bodyweight to initial egg weight relative to other treatments. In addition, chick navel quality was not significantly different across treatments, although *in ovo* probiotic treatment had the highest percentage of birds with navel score 1 (27.96%) and *in ovo* saline treatment had the highest percentage of birds with navel score range 2-3 (77.63%) (Table 3.3).

Table 3.2 Effect of the in *ovo* delivery of *Bacillus subtilis* fermentation extract on hatch performance in broiler chickens.

Hatch Parameters	Treatments[1]			SEM[2]	*P* value[3]
	Non-injection Control	*In ovo* saline	*In ovo* probiotics		
Pipped eggs (%)	6.38[a]	0.08[b]	2.69[ab]	1.26	0.043
Late dead eggs (%)	1.39	0.00	0.00	4.42[4]	0.584
Hatchability (%)	87.02	90.91	90.91	1.51	0.505
Average chick weight (g)	53.02	52.93	54.27	0.50	0.510
Chick BW[6]/ initial egg weight (%)	82.15	83.64	84.31	2.33[5]	0.196

[1]Treatment groups include- non-injected eggs (Control), *in ovo* saline group injected with 200 µl of physiological saline (0.9% NaCl) and *in ovo* probiotic group injected with 200 µl of *Bacillus subtilis* fermentation extract (10 X 10^6 CFU); n = 6 replicate trays.
[2]SEM = standard error of means.
[3]Means and median not sharing the same superscript differ significantly by Tukey's test ($P \leq 0.05$).
[4,5]Measure of variation about the median represented by the interquartile range.
[6]BW= body weight.

Table 3.3 Effect of the in *ovo* delivery of *Bacillus subtilis* fermentation extract on hatched chick navel quality

Navel quality score (%)[2]	Treatments[1]			SEM[3]	*P* value[4]
	Non-injection Control	*In ovo* saline	*In ovo* probiotics		
Score 1	26.7	22.4	28.0	3.48	0.809
Score 2	70.4	57.2	53.9	4.35	0.274
Score 3	2.9	20.4	18.2	4.06	0.162

[1]Treatment groups include- non-injected eggs (Control), *in ovo* saline group injected with 200 µl of physiological saline (0.9% NaCl) and *in ovo* probiotic group injected with 200 µl of *Bacillus subtilis* fermentation extract (10 X 10^6 CFU) in n=6 replicate trays.
[2]Navel quality was scored 1 when the navel was completely closed and clean, scored 2 when the navel was discolored with a 2mm maximum opening and scored 3 when the navel was discolored with more than a 2mm opening.
[3]SEM = standard error means
[4]Significance was set at $P \leq 0.05$.

3.4.2 Growth Performance

Results on evaluated growth parameters were not statistically significant between treatments (Table 3.4). During the starter phase (0 - 14 days), antibiotic treatment had the highest ADG and the lowest FCR compared with other treatments. *In ovo* probiotic treatment recorded the lowest ADFI and FCR of all treatments, during the grower phase (15 - 28 days).

Table 3.4 Effect of Bacillus subtilis fermentation extract delivery route on growth performance in broiler chicken raised for 28 days.

Growth Performance Parameters	Treatments[1]						SEM[2]	*P* value[3]
	Control	In-feed antibiotics	In-water probiotics	In-feed probiotics	*In ovo* saline	*In ovo* probiotics		
Starter Phase (0 - 14 days)								
ADFI[4] (g/bird)	25.4	23.3	23.2	23.6	26.2	27.7	0.84	0.582
ADG[5] (g/bird)	16.9	20.9	18.0	18.3	19.0	16.0	0.51	0.086
FCR[6]	1.53	1.15	1.33	1.36	1.40	1.75	0.07	0.254
Mortality (%)	0.00	0.00	0.00	0.00	0.00	0.00	0.00[7]	0.818
Grower phase (15 - 28 days)								
ADFI (g/bird)	83.0	91.8	85.1	90.2	91.6	80.5	1.59	0.168
ADG) (g/bird)	62.4	57.6	61.4	67.9	63.4	64.6	1.51	0.529
FCR	1.35	1.52	1.39	1.33	1.46	1.26	0.05	0.254
Mortality (%)	0.00	0.00	0.00	0.00	0.00	0.00	0.00[8]	0.191
Total trial period (0 - 28 days)								
ADFI (g/bird)	56.3	59.7	56.9	58.7	58.9	55.1	0.96	0.739
ADG (g/bird)	49.9	51.6	50.4	54.1	52.6	50.2	0.84	0.709
FCR	1.13	1.09	1.13	1.09	1.12	1.10	0.02	0.935
Mortality (%)	0.00	0.00	0.00	0.00	0.00	0.00	0.00[9]	0.750

[1]Treatment groups include – Control (CTRL), in-feed antibiotics treatment containing CTRL + 0.05 % bacitracin methylene disalicylate (BMD), CTRL diet + in-water probiotic (containing 0.025 g/L of *Bacillus subtilis* fermentation extract), in-feed probiotic containing CTRL + 0.005 % *Bacillus subtilis*, *in ovo* saline group injected with 200 µl of physiological saline (0.9% NaCl) and *in ovo* probiotics group injected with 200 µl of *Bacillus subtilis* fermentation extract (10 X 10^6 CFU) in n=6 replicate pens of 7 birds each.
[2]SEM = standard error means
[3]Significance was set at $P \leq 0.05$
[4]ADFI =Average daily feed intake
[5]ADG = Average daily gain
[6]FCR = Feed Conversion ratio
[7,8,9]-Measure of variation about the median represented by the interquartile range.

Treatments had no significant effect on intestinal NE lesion score in broiler chickens in this study (Table 3.5). Based on the used NE scoring guide, no bird had a NE score of 4. All treatments, except in-water probiotics and *in ovo* probiotic, had 50% of birds with NE score 2. The CTRL treatment had the least number of birds with NE score 0 (25%); 50% of birds in other treatments had NE score 0.

Table 3.5 Effect of *Bacillus subtilis* fermentation extract delivery route on Necrotic enteritis (NE) lesion scores in broiler chickens raised for 28 days.

Necrotic enteritis lesion score (% of birds)[2]	Treatments[1]						IQR[3]	*P* value[4]
	Control	In-feed antibiotics	In-water probiotics	In-feed probiotics	*In ovo* saline	*In ovo* probiotics		
0	25	50	50	50	0	50	50.00	0.219
1	0	0	0	0	0	0	0.00	0.821
2	50	50	25	50	50	25	87.50	0.577
3	0	0	0	0	0	0	0.00	0.671
4	-	-	-	-	-	-	-	-
5	0	0	0	0	0	0	0.00	0.532

[1]Treatment groups include – Control (CTRL), in-feed antibiotics treatment containing CTRL + 0.05 % bacitracin methylene disalicylate (BMD), CTRL diet + in-water probiotic (containing 0.025 g/L of *Bacillus subtilis* fermentation extract), in-feed probiotic containing CTRL + 0.005 % *Bacillus subtilis*, *in ovo* saline group injected with 200 µl of physiological saline (0.9% NaCl) and *in ovo* probiotic group injected with 200 µl of *Bacillus subtilis* fermentation extract (10 X 10^6 CFU) in n=6 replicate pens of 7 birds each.
[2]Scores are described as: score 0 - No gross lesions present; score 1 - No obvious ulcers in the mucosa, but the entire mucosal surface is covered with a layer of loosely adherent fibrin; score 2 - Excavated ulcer of the mucosa with acute, bright red hemorrhage within the ulcer bed and scant crusting of fibrin around the periphery; score 3 - Excavated ulcer of the mucosa with dark green-black pigment within the ulcer bed and scant crusting of fibrin over the surface; score 4 - Excavated ulcers of the mucosae, with periphery covered by thick, tightly-adherent layers of fibrin, necrotic tissue, and inflammatory cells; score 5 - Mucosae covered by large, confluent plaques of fibrin, necrotic tissue, and inflammatory cells to the point of extending over broad regions of the intestinal mucosa. No bird was scored an NE score of 4
[3]IQR = Interquartile range
[4]Significance was set at $P \leq 0.05$.

3.4.3 Gut Morphology

Probiotics delivery route significantly ($P \leq 0.05$) influenced the jejunum and ileum morphology of broiler chickens in this study (Table 3.6). In the jejunum, the villus height of the *in ovo* probiotics treatment was significantly higher ($P < 0.001$) than in-water probiotic, antibiotics,

and CTRL treatments. The *in ovo* probiotic villus height was 23% higher ($P < 0.001$) than the CTRL treatment. *In ovo* probiotic villus width in the ileum was also 18% wider ($P < 0.001$) than the in-feed treatment. Total mucosa thickness in the ileum of *in ovo* probiotic treatment was also 21% higher ($P < 0.001$) than the CTRL treatment. This was significantly different ($P < 0.001$) from in-water, antibiotic, and CTRL treatment. In the ileum, the villus height of the *in ovo* probiotic treatment was found highest; this was 18% higher ($P \leq 0.05$) than the *in ovo* saline treatment, but not statistically different from other treatments.

Table 3.6 Effect of *Bacillus subtilis* fermentation extract delivery route on ileum and jejunum morphology in broiler chickens raised for 28 days.

Gut morphology parameters (Measured in mm)	Treatments[1]						SEM[2]	*P* value[3]
	Control	In-feed antibiotics	In-water probiotics	In-feed probiotics	*In ovo* saline	*In ovo* probiotics		
Jejunum								
Villi height	0.960^{c}	1.008^{bc}	1.087^{bc}	1.156^{ab}	1.154^{ab}	1.253^{a}	0.02	0.000
Villi width	0.220^{a}	0.221^{a}	0.223^{a}	0.178^{b}	0.192^{ab}	0.218^{a}	0.00	0.001
crypt depth	0.140	0.127	0.130	0.154	0.132	0.147	0.00	0.070
Villi height: crypt depth	8.115	9.681	9.365	9.967	9.843	11.023	0.32	0.203
Total mucosa thickness	1.100^{d}	1.135^{cd}	1.217^{bcd}	1.310^{ab}	1.286^{abc}	1.399^{a}	0.02	0.000
Ileum								
Villi height	0.560	0.533	0.555	0.593	0.596	0.574	0.01	0.080
Villi width	0.196^{ab}	0.205^{ab}	0.193^{ab}	0.199^{ab}	0.174^{b}	0.213^{a}	0.00	0.052
Crypt depth	0.141	0.132	0.136	0.145	0.132	0.130	0.00	0.268
Villi height: crypt depth	4.320	4.270	4.379	4.461	4.799	4.731	0.09	0.352
Total mucosa thickness	0.701	0.665	0.692	0.738	0.728	0.704	0.01	0.087

[1]Treatment groups include – Control (CTRL), in-feed antibiotics treatment containing CTRL + 0.05 % bacitracin methylene disalicylate (BMD), CTRL diet + in-water probiotic (containing 0.025 g/L of *Bacillus subtilis* fermentation extract), in-feed probiotic containing CTRL + 0.005 % *Bacillus subtilis*, *in ovo* saline group injected with 200 µl of physiological saline (0.9% NaCl) and *in ovo* probiotic group injected with 200 µl of *Bacillus subtilis* fermentation extract (10 X 10^{6} CFU) in n = 10 observations per treatment.
[2]SEM = standard error means. [3]Means not sharing the same superscript differ significantly by Tukey's test ($P \leq 0.05$).

3.4.4 Cecal SCFA Concentration

No significance ($P > 0.05$) was found for cecal SCFA concentration (micromolar) in this study (Table 3.7). Nonetheless, *in ovo* probiotic treatment had the numerically highest concentration of total SCFA and volatile fatty acids (VFA) compared to other treatments.

Table 3.7 Effect of *Bacillus subtilis* fermentation extract delivery route on cecal short-chain fatty acids concentrations in broiler chickens raised for 28 days.

Short-chain fatty acids concentration (μM)	Treatments[1]						SEM[2]	P value[3]
	Control	In-feed antibiotics	In-water probiotics	In-feed probiotics	*In ovo* saline	*In ovo* probiotics		
Acetic acid	47.4	55.0	43.5	48.7	49.5	52.7	2.44	0.832
Propionic acid	1.98	1.69	3.16	2.57	2.10	2.73	0.24	0.535
Butyric acid	13.7	12.4	11.5	7.15	10.1	13.4	0.82	0.184
Valeric acid	0.28	0.16	0.44	0.12	0.19	0.30	0.04	0.107
Lactic acid	2.05	3.23	4.93	2.52	2.13	4.66	0.63	0.664
Branched-chain fatty acids	0.40	0.07	0.30	0.33	0.16	0.22	0.04	0.321
Volatile fatty acids	63.8	69.4	58.9	58.9	62.0	69.4	3.05	0.865
Total short-chain fatty acids	65.8	72.6	63.8	61.4	64.2	74.1	2.92	0.790

[1]Treatment groups include – Control (CTRL), in-feed antibiotics treatment containing CTRL + 0.05 % bacitracin methylene disalicylate (BMD), CTRL diet + in-water probiotic (containing 0.025 g/L of *Bacillus subtilis* fermentation extract), in-feed probiotic containing CTRL + 0.005 % *Bacillus subtilis*, *in ovo* saline group injected with 200 μl of physiological saline (0.9% NaCl) and *in ovo* probiotic group injected with 200 μl of *Bacillus subtilis* fermentation extract (10 X 10^6 CFU) in n= 6 replicates per treatment.
[2]SEM = standard error means
[3]Significance was set at $P \leq 0.05$

3.4.5 Cecal Microbiota

A total of 5,286,777 quality read counts were obtained, at an average of 73,427 counts per sample after quality filtering and demultiplexing. Information on the sequencing quality profile is presented in Figure 3.2. A total of 805 OTU were identified at the 97% similarity level, belonging to a total of 5 phyla, 6 classes, 8 orders, 17 families, 57 genera, and 12 species. The relative abundance (percentage abundance) of different phyla and genera across treatment groups are presented in Figure 3.3. Bacteria composition at the family taxa is shown in Figure 3.4. Treatment effects on major phyla and genera are presented in Table 3.8. Taxonomic analysis by ANOVA showed no difference for total *Eubacteria* counts across treatments (Table 3.8). *Firmicutes* represented > 98% of identified phyla. No significant difference was recorded for all major phyla

within treatments (Table 3.8). At the genus level, *Ruminococcaceae_UCG-014* in the in-feed probiotic treatment tended ($P = 0.07$) to be 38% higher than the antibiotic treatment.

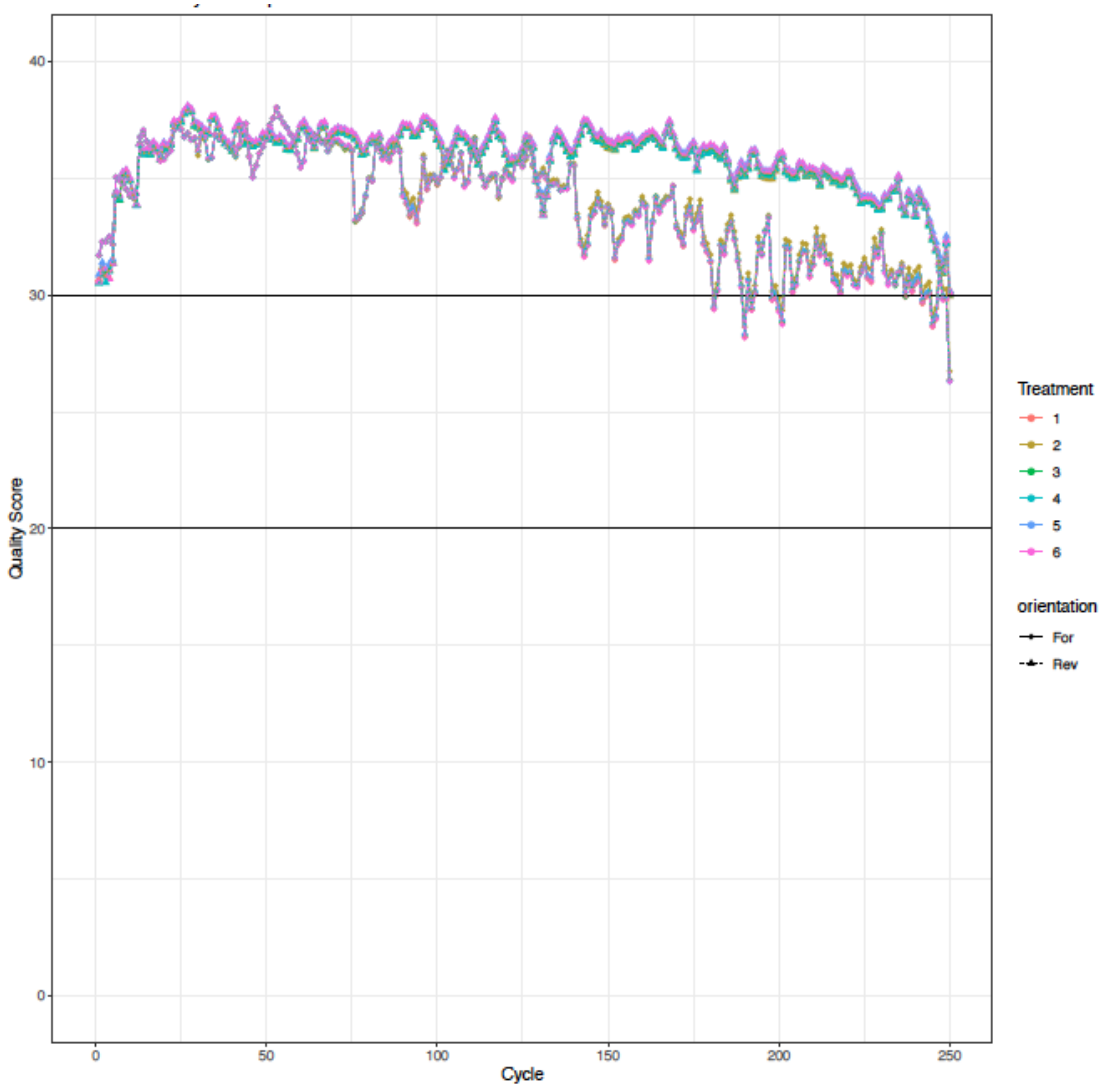
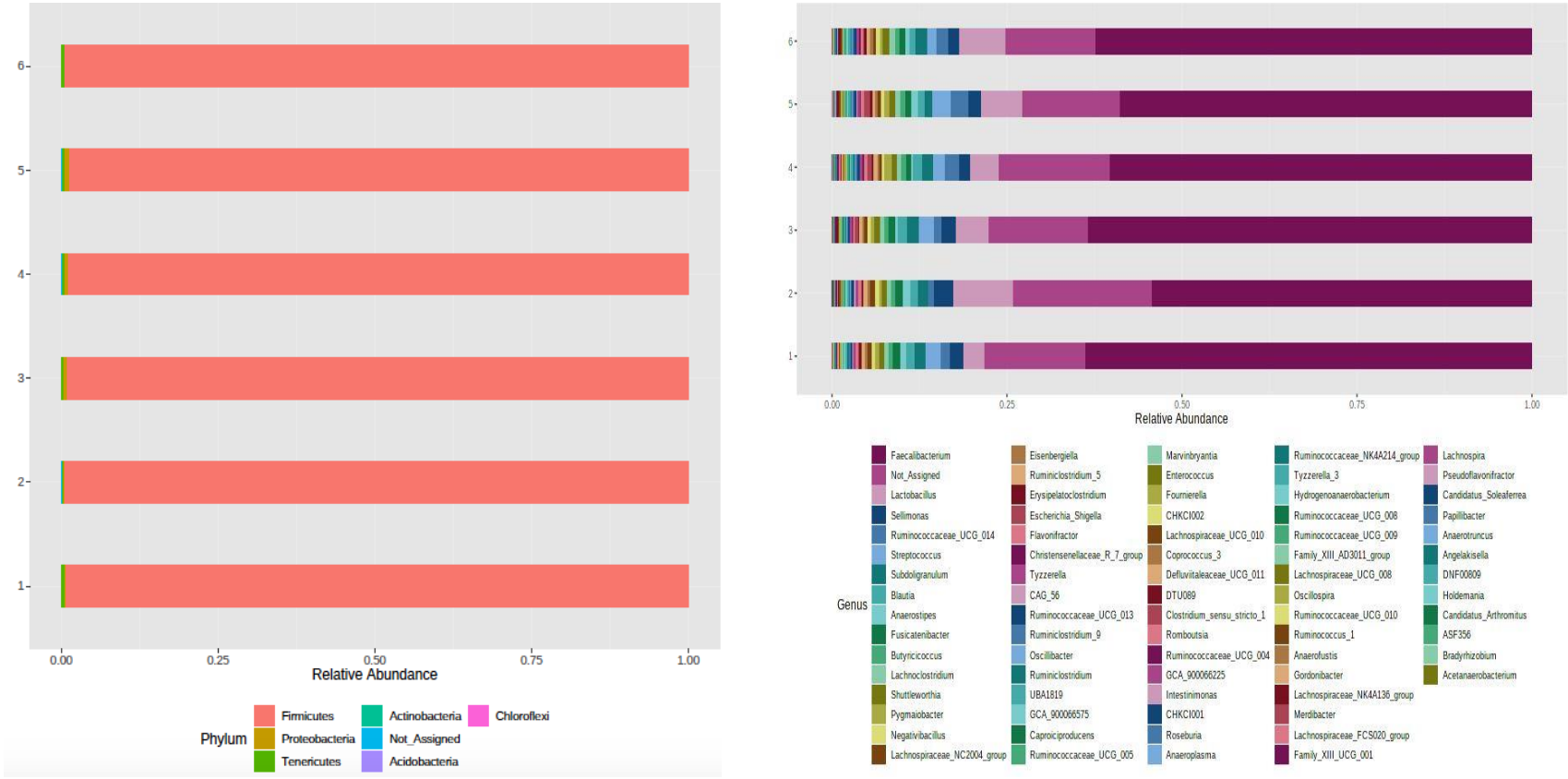


Figure 3.2 The mean quality score per treatment for forward and reverse reads.

Treatment groups which include 1- Control (CTRL), 2-in-feed antibiotics treatment containing CTRL + 0.05 % bacitracin methylene disalicylate, 3- CTRL diet + in-water probiotic containing 0.025 g/L of *Bacillus subtilis* fermentation extract), 4- in-feed probiotic containing CTRL + 0.005 % *Bacillus subtilis*, 5-*in ovo* saline group injected with 200 µl of physiological saline (0.9% NaCl) and 6-*in ovo* probiotic group injected with 200 µl of *Bacillus subtilis* fermentation extract (10 X 10^6 CFU), are presented in different colors. Treatments were in 12 replicates (72 samples) (Bourgey et al., 2019).



(A) (B)

Figure 3.3 Bacteria composition at the A) phylum and B) genus levels of broiler chickens.

Treatments groups 1–control (CTRL), 2–in-feed antibiotics treatment containing CTRL + 0.05% bacitracin methylene disalicylate, 3–CTRL diet + in-water probiotic (containing 0.025 g/L of *Bacillus subtilis* fermentation extract), 4–in-feed probiotic containing CTRL + 0.005% *Bacillus subtilis*, 5–*in ovo* saline group injected with 200 µl of physiological saline (0.9% NaCl) and 6–*in ovo* probiotic group injected with 200 µl of *Bacillus subtilis* fermentation extract (10 X 10^6 CFU). The

cecal content was collected from 28-day-old chickens. DNA was extracted from the cecal content, and relative abundances are shown as determined by Illumina sequencing and visualized with the web-based tool MicrobiomeAnalyst.

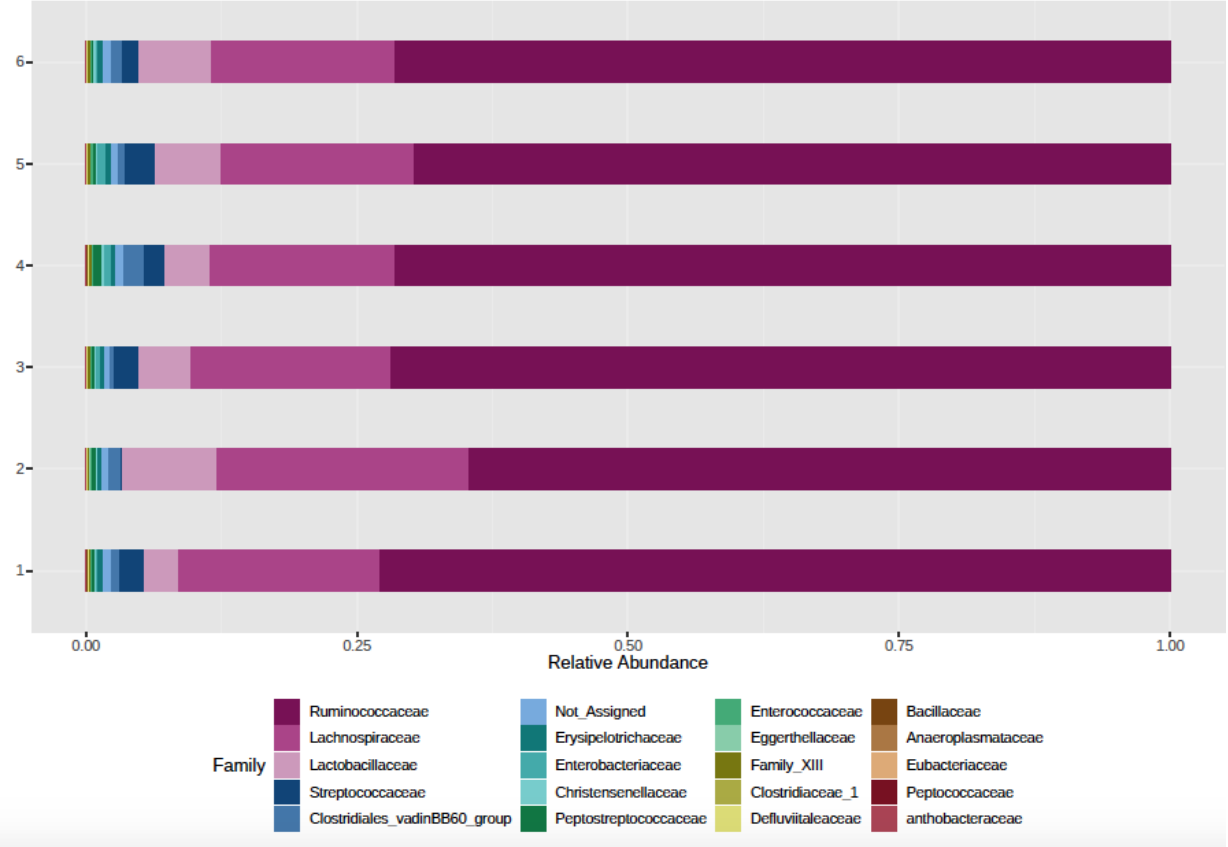


Figure 3.4 Bacteria composition at the family taxa of broiler chickens.

Treatments groups 1- Control (CTRL), 2-in-feed antibiotics treatment containing CTRL + 0.05 % bacitracin methylene disalicylate, 3- CTRL

diet + in-water probiotic (containing 0.025 g/L of *Bacillus subtilis* fermentation extract), 4- in feed probiotic containing CTRL + 0.005 % *Bacillus subtilis*, 5-*in ovo* saline group injected with 200 µl of physiological saline (0.9% NaCl) and 6-*in ovo* probiotic group injected with 200 µl of *Bacillus subtilis* fermentation extract (10 X 10^6 CFU). Cecal content was collected from 28-day-old chickens. DNA was extracted from the cecal content, and relative abundances are shown as determined by Illumina sequencing and visualized with the web-based tool MicrobiomeAnalyst.

Table 3.8 Effect of Bacillus subtilis fermentation extract delivery route on relative OTU abundance (specific phyla, genera and Total Eubacteria) in broiler chickens raised for 28 days.

Item	Treatments[1]						SEM[2]	*P* value[3]
	Control	In-feed antibiotics	In-water probiotics	In-feed probiotics	*In ovo* saline	*In ovo* probiotics		
Phylum (OTUs)								
Firmicutes	70853	75313	74532	70603	74231	71784	1363	0.875
Actinobacteria	58.9	92.5	63.0	77.5	77.5	68.5	1.10	0.170
Proteobacteria	55.3	53.4	89.1	175.9	73.8	36.4	1.20	0.118
Tenericutes	105	34.3	74.4	72.5	67.4	96.7	1.20	0.295
Genus (OTUs)								
Ruminococcaceae_UCG-014	1230	1099	1111	1783	1694	1601	1.10	0.073[4]
Ruminiclostridium_5	498	616	421	611	553	637	34.0	0.425
Lactobacillus	1272	3744	2436	1659	1893	2649	1.20	0.389
Faecalibacterium	45528	41070	47618	42992	44195	44954	1501	0.878
Subdoligranulum	524	813	851	708	582	923	1.10	0.531
Total Eubacteria (Absolute copy number)	1.71E+12	1.37E+12	1.96E+12	2.01E+12	1.73E+12	2.66E+12	1.10E+00	0.483

[1]Treatment groups include – Control (CTRL), in-feed antibiotics treatment containing CTRL + 0.05 % bacitracin methylene disalicylate (BMD), CTRL diet + in-water probiotics (containing 0.025 g/L of *Bacillus subtilis* fermentation extract), in-feed probiotics containing CTRL + 0.005 % *Bacillus subtilis*, *in ovo* saline group injected with 200 µl of physiological saline (0.9% NaCl) and *in ovo* probiotics group injected with 200 µl of *Bacillus subtilis* fermentation extract (10 X 10^6 CFU) in n= 12 observations per treatment, with the exceptio

of Total Eubacteria where n=10 observations per treatment.
[2]SEM = standard error means
[3]Significance was set at $P \leq 0.05$.
[4]marginal significance at $P < 0.07$.

The differential abundance at different taxonomic levels by MetagenomeSeq ($P < 0.05$) are presented in Table 3.9. Order *Rhizobiales* and family *Xanthobacteraceae* were differentially significant ($P < 0.001$) in the CTRL treatment. Phylum *Actinobacteria*, class *Coriobacteriia*, order *Coriobacteriales,* and family *Eggerthellaceae* were all differentially significant ($P < 0.001$) in the in-feed antibiotic treatment. Family *Streptococcaceae*, genus *Streptococcus,* and an unknown specie DNF0089 were significantly differentiated ($P < 0.001$) in the in-water probiotic treatment.

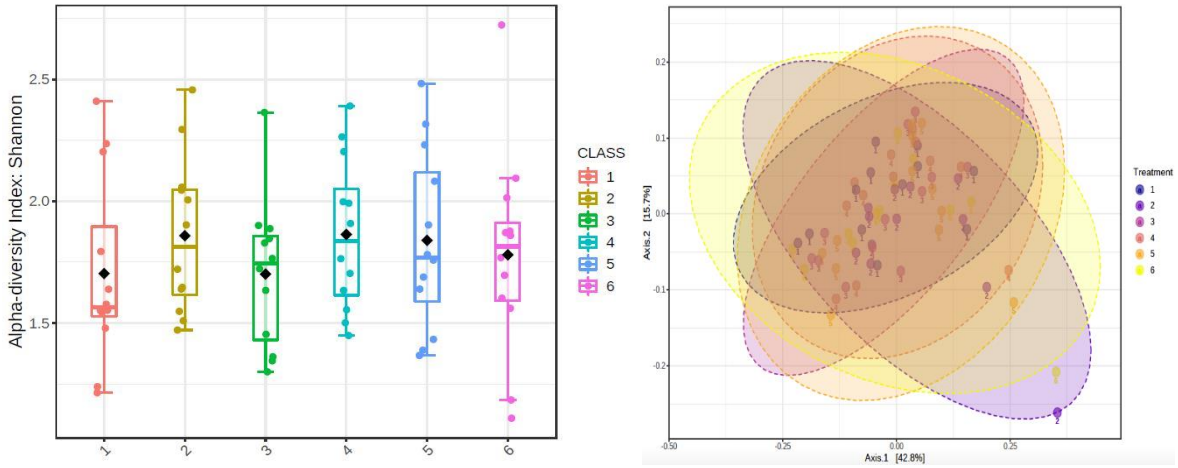
Table 3.9 Effect of *Bacillus subtilis* fermentation extract delivery route on differentially abundant bacterial taxa between treatment groups.

Taxa (Log-transformed counts)	Treatments[1]						*P* value[2]	False Discovery Rate (FDR)
	Control	In-feed antibiotics	In -water probiotics	In-feed probiotics	*In ovo* saline	*In ovo* probiotics		
Phylum		*Actinobacteria*					0.002	0.013
Class		*Coriobacteriia*					0.001	0.014
Order		*Coriobacteriales*					0.002	0.009
	Rhizobiales						0.002	0.009
Family			*Streptococcaceae*				0.000	0.000
		Eggerthellaceae					0.002	0.002
	Xanthobacteraceae						0.007	0.045
Genus			*Streptococcus*				0.000	0.000
			DNF0089				0.000	0.000

[1]Treatment groups include – Control (CTRL), in-feed antibiotics treatment containing CTRL + 0.05 % bacitracin methylene disalicylate (BMD), CTRL diet + in-water probiotics (containing 0.025 g/L of *Bacillus subtilis* fermentation extract), in-feed probiotics containing CTRL 0.005 % *Bacillus subtilis*, *in ovo* saline group injected with 200 µl of physiological saline (0.9% NaCl) and *in ovo* probiotics group injected with 200 µl of *Bacillus subtilis* fermentation extract (10 X 10^6 CFU) in n= 12 observations per treatment.
[2]Significance was set at $P \leq 0.05$.

Analyzing the alpha diversity (specie richness) of cecal content expressed as the number of observed OTU by Shannon Index showed similarity between treatments (Figure 3.5A). Numerically, the highest average Shannon index was in the antibiotic treatment 1.81 ± 0.09 (Mean ± SE), whereas the lowest was 1.65 ± 0.11 in the CTRL treatment.

Beta diversity of cecal bacteria communities of the treatment groups are illustrated in the Principal Coordinates Analysis (**PCoA**) plot based on the Bray-Curtis dissimilarity index (Figure 3.5B). Permutational multivariate (ANOVA) showed no significant differences in microbial community structure between treatments (R-squared 0.09, $P > 0.05$).



A) B)

Figure 3.5 Alpha and Beta diversity measure.

A) Alpha diversity Index showed no significant difference among treatments (ANOVA, P = 0.7619). The cecal content was collected from 28-day-old broiler chickens. The diamond shape represents the mean value in each group and the whiskers indicate the minimum/maximum value. B) Beta diversity measure of the effect of *Bacillus subtilis* fermentation extract delivery route on cecal bacteria communities of broiler chickens raised for 28 days. Treatment groups include the following: 1—control (CTRL), 2—in-feed antibiotics treatment containing CTRL + 0.05% bacitracin methylene disalicylate, 3–CTRL diet + in-water probiotic (containing 0.025 g/L of *Bacillus subtilis* fermentation extract), 4— in-feed probiotic containing CTRL + 0.005% *Bacillus subtilis*, 5—*in ovo* saline group

injected with 200 µl of physiological saline (0.9% NaCl) and 6-*in ovo* probiotic group injected with 200 µl of *Bacillus subtilis* fermentation extract (10 X 10^6 CFU) (PERMANOVA; P value < 0.128, F value = 1.3787, R-squared: 0.09457).

3.5 Discussion

In this study, the *in ovo* delivery of probiotics has been validated in broiler chickens, by comparing it with in-water and in-feed delivery routes. The probiotic used in this study was a *Bacillus subtilis* fermentation extract. *Bacillus subtilis* is a spore-forming bacterium, with high resistance to temperature and harsh conditions (AFRC, 1989). These qualities make it a suitable probiotic candidate in poultry production.

Hatchability remains one of the most significant indicators of successful *in ovo* injection. In this study, we have successfully validated the *in ovo* delivery of *Bacilllus subtilis* fermentation extract through the amnion on day 18.5 of incubation, with no negative effect on embryo viability. All *in ovo* –injected eggs in our study recorded 91% hatchability and was not significantly different from the non-injected eggs which had 87% hatchability. These hatchability values are well in line with what is obtainable in commercial hatcheries. The patent of Uni and Ferket (2003) has previously proved that the inoculation of enteric modulators between day 17 and 19 of incubation through the amnion had no negative effect on hatchability because the injected substance is orally swallowed by the embryo in the amnion, after which it is made available to enteric tissues and other gut microbiota cells (Torshizi et al., 2010). Our results on hatchability are in conformation with the findings of Majidi-Mosleh et al. (2017), Edens et al. (1997), Pender et al. (2016), Alizadeh et al. (2020), Beck et al. (2019), Skjøt-Rasmussen et al. (2019) and Khaligh et al. (2018); which all reported no negative effect of the *in ovo* delivery of probiotics on hatchability. Contrarily, El-Moneim et al. (2019) and Tripplet et al. (2018) have both recently reported a negative effect of *in ovo* delivery of probiotics on hatchability. The disparity in the literature on the effect of *in ovo* delivery of probiotics on hatchability is attributable to several factors including the *in ovo* injection procedure, site of injection (air cell vs amnion), inoculated dose, hatchery hygiene, and differing probiotics strain (Johnston et al., 1997; Bednarczyk et al., 2011; De Oliveira et al., 2014; Beck et al., 2019). In addition, non-injected eggs in this study recorded the highest percentage of pipped eggs (6.38), whereas *in ovo* probiotic treatment was intermediate (2.69) and the *in ovo* saline

treatment had the least (0.08) percentage of pipped eggs. Previous researchers have reported no effect of probiotics inoculation on percentage pipped eggs (De Oliveira et al., 2014; Pender et al., 2016; Triplett et al., 2018). Factors affecting percentage pipped eggs include deficient hatching conditions (insufficient humidity and poor ventilation) (Willemsen et al., 2010), poor hatchery hygiene, and embryonic malposition within a particular region of the incubator, in response to gravity (Byerly and Olsen, 1937). Furthermore, treatments had no effect on navel quality in this study (Table 3.3). Chick navel quality is often influenced by the rate of nutrient metabolism and yolk absorption during the late incubation period (Hamburger and Hamilton, 1951). Except for percentage pipped eggs, other hatchability parameters evaluated in this study, including, percentage late dead eggs, hatchability, average chick weight, hatched chick bodyweight to initial egg weight and chick navel quality elicited no significant treatment effect. These results suggest that the *in ovo* delivery of *Bacilllus subtilis* fermentation extract does not negatively impair embryo viability and hatch performance.

Furthermore, the use of probiotics, especially in the diet, as enteric gut modulators that ultimately elicit superior bird performance, continues to gain momentum in the poultry industry. No significant effect of treatment on all post-hatch growth performance was recorded in this study. In conformation with our results, Majidi-Mosleh et al. (2017) have previously recorded no significant effect of amnion delivered *Bacillus subtilis* on performance parameters in a 42-day trial with broiler chickens, suggesting that probiotics supplementation in the late embryonic stage might not be sufficient enough to elicit superior performance effects. Subsequent in-feed supplementation of probiotics inoculated chicks to stimulate significant post-hatch performance, is an area that warrants further investigation. Knap et al. (2011) also reported no significant effect of orally delivered *B. subtilis* DSM17299 on ADG and FCR in their study. Similarly, Santoso et al. (1999) found no significant effect of in-feed delivered *B. subtilis* on feed intake, body weight gain (BWG), and FCR. Olnood et al. (2015) also reported no significant effect of L. *joshnsonii* either delivered in-feed, in-water, sprayed on litter or orally gavaged on feed intake, BWG, and FCR in broiler chickens. Chen et al. (2009) supplemented broiler feed with *B. subtilis* (10^6 CFU/g) and also recorded no significant effect on growth performance. On the contrary, other studies have reported positive effect of *B. subtillis* delivery on growth performance. Sen et al. (2012) reported a linear increase in feed intake, BWG and FCR with increasing in-feed delivery of *B. subtilis* LS 1-2. Jeong et al. (2014) also confirmed that the inclusion of *B.*

subtilis spores significantly enhanced ADG both in starter and overall experimental period, in their study. These inconsistencies in *B. subtilis* performance effect across several routes could be due to a variety of factors including viability, dosage, environmental stressors (Huang et al., 2004; Mountzouris et al., 2007), and sample size. Irrespective of delivery route, probiotic treatments in this study (despite being non-significant) had at least 0.5% higher ADG, compared to the CTRL treatment, over the 28-day trial. This insignificant performance effect might portend considerable economical gains, especially for large scale commercial broiler producers. Indeed, more studies on the effect of probiotics delivery route on broiler performance, especially in commercial context, are needed.

In addition, no treatment effect on mortality and incidence of NE was found in this study. Several predisposing factors are reported to contribute to the growth and proliferation of *Clostridium perfringens,* the etiological agent of NE in broiler intestine. These include management conditions (including stress, alteration in feeding regimes, hatchery hygiene) and diet composition (especially barley- or wheat- containing diets as offered in the current study (Craven, 2000; Craven et al. 2001; Annet et al. 2002). Similarly, antibiotics withdrawal has also been associated with an increased incidence of necrotic enteritis (Wade, 2015). Asides from horizontal transmission (via contaminated feed and litter) of *Clostridium perfringens* spores, vertical transmission from parent to progeny is also possible (Williams, 2002; Thanissery et al. 2010). These reasons make our assessment of NE in birds unchallenged with NE relevant, although we acknowledge that the bacteria distribution might not be uniform across treatments. Most experimented alternatives to AGP including organic acids, essential oils, synbiotics, prebiotics, and probiotics have all been reported to exhibit varying levels of pathogen exclusion activities, which often results in reduced incidence of NE (Finucane et al., 1999; Kaldhusdal, 2000). These activities are either direct or indirect via immunity boosting (Ao et al., 2012). With the CTRL treatment having the least number of birds with a desirable NE score of 0, it is plausible that all supplementations conferred birds with some sort of protection against NE.

Post-hatch changes are more evident in the chicken's intestinal segments, compared to other parts (Prabakar et al., 2016). In this study, beneficial effects of *in ovo* delivered probiotic were observed both in the ileum and jejunum. The villus height, villus width, and total mucosa thickness were all numerically and, in most cases, significantly higher in the *in ovo* probiotic

treatment. Intestinal morphological parameters, including villus height, villus width, crypt depth, and villus length–to–crypt depth ratio are good indicators of gut health and the functional capacity of the intestine (Fasina and Olowo, 2013). The increased villus height, villus height–to–crypt depth ratio, and a decreased crypt depth are correlated with an increased epithelial turnover and increased digestive and absorptive functions (Fan et al., 1997; Xu et al., 2003; Munyaka et al., 2012; Shang et al., 2015). In agreement with our results, Sen et al. (2012) showed that the supplementation of *B. subtilis* LS 1-2 in broiler diets resulted in increased villus height and villus height: crypt depth ratio in duodenum and ileum at day 35. Li et al. (2018) also demonstrated that dietary co-supplementation of AGP and *B. subtilis* improved intestinal morphology during the first 3 weeks in pullets. A recent meta-analysis of 25 controlled trials also concluded that the supplementation of direct-fed microbials was associated with increased villus height of the small intestine in broiler chickens (Heak et al., 2017). Improved digestive capacity, as evidenced by improved intestinal morphometric characteristics, would be expected to translate into improved feed conversion efficiency and ultimately significant improvement in growth performance. The smaller sample size utilized in the present study could have contributed to the lack of significant improvement in growth performance. Future studies on this type of product should utilize a larger sample size. In addition, the present study was conducted under a well-controlled management system with no sanitary challenge to disturb the intestinal health of the chickens.

The SCFA are the by-products of microbial fermentation in the cecum. They play important roles in bird's energy metabolism, intestinal functionality, and gut pathogen reduction (Van Der Wielen et al., 2000; Meimandipour et al., 2010). In the present study, no effect of treatment was recorded for the concentrations of total SCFA and individual fatty acids, although the *in ovo* probiotic treatment consistently recorded the highest concentration of total SCFA, VFA, and propionic acid concentrations. Meimandipour et al. (2010) have shown that the supplementation of *Lactobacillus salivarius* ssp. *salicinius* JCM 1230 and *Lactobacillus agilis* JCM 1048 can significantly increase propionate and butyrate concentrations using a 24–h simulated chicken cecum. Fujiwara et al. (2009) have also reported that 2% *B. subtilis var. natto*–fermented soybean supplementation tended to increase total VFA and acetic acid concentration in chicks, especially when fed from day old, suggesting a linear age effect of *B. subtilis* supplementation on SCFA concentration. Because SCFA concentrations are associated with gut microbiota colonization, it is important to note that both *Lactobacillus* and *Bacillus spp.* differ in

their capacities to colonize the gut. Although *Lactobacillus* and *Enterococcus spp.* are considered to be colonizing species, *Bacillus spp.* are considered free flowing and do not colonize the gut (Huyghebaert et al., 2011). To the best of our knowledge, this is the first study to report on the effect of *in ovo* delivered *B. subtilis* on SCFA concentrations in broiler chickens, more studies are thus needed to fully understand these effects.

The chicken's gut is inhabited by numerous species of microorganisms, whose continuous interaction, influences host performance and well-being. This is particularly true for the cecum, the posterior gut section with the highest bacteria diversity (Oakley et al., 2014). In this study, we observe that broiler chicken cecum microbiota is mainly composed of >95% members of phyla *Firmicutes*, *Proteobacteria*, *Tenericutes,* and *Actinobacteria*; irrespective of treatments (Figure 3.3A). This is to an extent consistent with results reported for breeder fecal microbiota (Trudeau et al., 2020), probiotics supplemented chicken ceca (Wang et al., 2017), and *Bacillus* direct-fed microbial supplemented broiler chicken ceca (Hernandez-Patlan et al., 2019a). Although our results might be consistent with the relative percentage of microbes reported in these studies, it does not necessarily conform with the order of abundance reported. In addition, we did not record the presence of bacteria in the phylum *Bacteroidetes*. The resolution of the V4 region of the 16S rRNA gene sequenced in this study could have influenced this outcome. García-López et al. (2020) have recently shown that both the V3 and V3V4 hypervariable regions capture a broader spectrum of microbiota diversity compared to the V4 region. Although the V3 region offers the advantage of faster sequencing time and lower cost, the V3V4 region offers a higher taxonomic resolution at an increased cost (García-López et al., 2020). Increased abundance of F*irmicutes* has been associated with increased nutrient absorption and energy harvest from diets (Jumpertz et al., 2011). Phylum *Proteobacteria* on the other hand is made up of gram-negative bacteria implicated in some metabolic diseases, including gut-brain alterations and intestinal inflammation in rats (Maharshak et al., 2013; Vaughn et al., 2017). *Tenericutes* are also implicated in mycoplasma infection. In contrast, *Actinobacterial* species are reported to combat bacteria diseases and at the same time help convert feedstuff into fermentable microbial biomass (Anandan et al., 2016). It is important to note that, the relative abundance of phylum *Proteobacteria* and *Tenericutes* ranged from 0.2-1.02 % of total OTU identified, justifying the homeostatic gut environment, as evidenced by non-compromised bird performance and health, across treatments, observed in our study. Order *Rhizobiales* and family *Xanthobacteraceae* were differentially significant in the CTRL treatment

as compared to other treatments. Both bacteria are rarely found in animal species and have been reported in host fed nitrogen-deficient feedstuff (Stoll et al., 2007; Ikeda-Ohtsubo et al., 2018). This observation is surprising as our basal diet met or exceeded NRC (1994) crude protein requirement. Phylum *Actinobacteria*, class *Coriobacteriia*, order *Coriobacteriales,* and family *Eggerthellaceae* were all differentially significant in the in-feed antibiotic treatment as compared to other treatments. The functional roles of these bacteria communities include lipid metabolism and cholesterol metabolism (Martínez et al., 2013). They have also been linked to the pathologies of periodontitis, bacteremia, and other zoonotic diseases; especially *Coriobacteriaceae and Eggerthella* (Clavel et al., 2014; Pandit et al., 2018). This further emphasizes the cost-benefit effects of antibiotic use in poultry production. In addition, family *Streptococcaceae*, genus *Streptococcus,* and an unknown specie DNF0089 were significantly differentiated in the in-water probiotics treatment; although *Streptococcus* has been associated with infections in poultry (Sekizaki et al., 2008), they are also capable of reducing gut pathogen load through competitive exclusion (Roto et al., 2015). However, more information on the specific strain of *Streptococcus* is needed, as the 2 main *Streptococcus* strains have been reported to have different functions (Fåk and Bäckhed, 2012). We also recorded no significant difference in bacterial alpha diversity among treatments (Figure 3.5A). Thibodeau et al. (2015) demonstrated that only extreme events that modify the number of ecological niches in different bacterial species can alter the alpha diversity. However, the ability of *B. subtilis* to enhance bacteria species richness has been reported (Li et al., 2016; Oh et al., 2017). Similarly, we recorded no significant effect of treatment on beta-diversity in this study (Figure 3.5B). This suggests phylogenetic similarities between treatments. Except for treatment effect, which is nutrition; other possible factors or conditions shared by the birds could have influenced beta-diversity. These shared factors include local gastrointestinal condition, gut pH, and chick-rearing environment (Cisek and Binek, 2014; Oakley et al., 2014; Choi et al., 2015). Taken together, it is obvious that our probiotics treatment, irrespective of delivery routes, did not inhibit microbiota-mediated homeostasis.

3.6 Conclusions

This study has successfully established the procedure for the *in ovo* delivery of *Bacillus subtilis* in broiler chickens, recording 91% hatchability rate. Although, *Bacillus subtilis* treatment (irrespective of delivery route) had no significant effect on growth performance, *in ovo* delivery of the probiotic product enhanced intestinal morphology, without compromising hatch and gut

homeostasis.

4 CHAPTER 4 PROBIOTICS DELIVERY ROUTES 2

THE EFFECT OF *BACILLUS SUBTILIS* AND ITS DELIVERY ROUTE ON HATCH AND GROWTH PERFORMANCE, BLOOD BIOCHEMISTRY, IMMUNE STATUS, GUT MORPHOLOGY, AND MICROBIOTA OF BROILER CHICKENS

This section has been presented and submitted for publication elsewhere:

- Oladokun, S., and D. Adewole. 2022. Effect of *Bacillus subtilis* and its delivery route on hatch and growth performance, blood biochemistry, and immune status of broiler chickens. Oral presentation. Poultry Science Association (PSA) Annual Meeting, July 11-14, San Antonio, Texas, USA.

- Oladokun, S., and D. Adewole. 2022. The effect of *Bacillus subtilis* and its delivery route on hatch and growth performance, blood biochemistry, immune status, gut morphology, and microbiota of broiler chickens. Poultry Science (submitted).

4.1 Abstract

This study evaluated the effect of probiotics (*Bacillus subtilis* fermentation extract) and its delivery route (in-feed or *in ovo*) on hatch and growth performance, blood biochemistry, immune status, gut morphology, and microbiota of broiler chickens. Hatching eggs were incubated for 21 days. On d 12, viable eggs were randomly allotted to 4 groups: the non-injected, *in ovo* saline (**S**), *in ovo Bacillus subtilis* 1 (P1), and *in ovo Bacillus subtilis* 2 (P2). On d 18, S, P1, and P2 groups received 0.2 mL saline diluent, 10×10^6, and 20×10^6 CFU of the bacterium via the amnion, respectively. At hatch, chicks were re-allotted to 5 new treatment groups: P1, P2, 0.005% in-feed *Bacillus subtilis* extract (P3), 0.05% in-feed bacitracin methylene disalicylate (BMD,), and corn-wheat-soybean diet negative control (NC) in 9 replicate pens (22 birds/pen) and raised for 35 d. Hatch parameters were assessed on d 0, and growth performance indices measured weekly. On d 25, 1 bird/cage was euthanized, and samples collected for further analysis. Data were analyzed by generalized linear model. Treatments S and P2 recorded higher ($P = 0.01$) chick BW/ Egg Weight values compared to the non-injected eggs. P3 and P2 reduced ($P = 0.02$) FI at week 5 compared to the NC treatment. However, no change in average body weight gain (ABG) and feed conversion ratio (FCR) were observed during the same period. At d35, while BMD treatment showed a tendency ($P = 0.09$) to increase FI compared to the NC treatment, ABG and FCR were similar for all treatments. Blood sodium and chloride levels were increased ($P < 0.05$) by the BMD treatment

compared to the NC treatment. Compared to other treatments, BMD and P3 treatments increased ($P < 0.001$) jejunal and ileal villus height to crypt depth ratios, respectively. However, P1 and P2 increased ($P < 0.001$) villus height to crypt depth ratio in the duodenum compared to NC treatment. Treatments did not affect gut microbial diversity; however, BMD treatment increased ($P < 0.05$) the proportion of bacteria in the genus *Enterococcus* in the ileum and reduced ($P < 0.05$) the proportion of bacteria in the genus *Streptococcus* in the ceca. All probiotics treatments (irrespective of route and dose) reduced ($P < 0.001$) the levels of serum IgG compared to the NC treatment. However, P1 and P2 had the lowest numerical decrease in serum IgG concentrations, suggesting that *Bacillus subtilis* (especially *in ovo* delivered) might provide broiler chickens with better immunological protection by neutralizing pathogenic organisms that could result in the production of natural antibodies.

4.2 Introduction

In a bid to meet the increasing food demands of the growing global population, agriculture continues to be intensified. One such intensification effort led to the adoption of antimicrobial compounds to promote growth in the livestock industry. Interestingly, the livestock industry currently represents the largest user of antimicrobials produced globally (Van Boeckel et al., 2019). The use of antibiotic growth promoters (AGP) sub-therapeutically for growth promotion and disease prevention remains a critical part of intensive poultry production (Castanon, 2007; Hedman et al., 2020). In spite of the benefits that AGP use poses to the poultry industry, there is also the risk of the development of antimicrobial resistance, which has undesirable consequences for human and animal health (Van Den Bogaard and Stobberingh, 2000; Diarra and Malouin, 2014; Lekshmi et al., 2017). Hence, it is unsurprising that several country-specific regulatory measures against AGP use in poultry production, as well as increased consumer demands for AGP-free poultry products now exist (Muaz et al., 2018; Oladokun et al., 2021b). As the poultry industry recedes from using AGP, the challenge going forward is finding suitable alternatives and the delivery routes that maximize their effectiveness.

Several bioactive substances, including phytobiotics, prebiotics, essential oils, and probiotics, are thus currently being researched as potential alternatives to AGP in the poultry industry (reviewed by Gadde et al., 2017). Probiotics, defined as "live microorganisms which, when administered in adequate amounts, confer a health benefit on the host" (FAO/WHO, 2001) continue to receive growing interest as an alternative to AGP in poultry production as a result of

its immunomodulating properties (Pender et al., 2016). Evidence abounds in the literature of the potential of probiotics to improve the growth performance of poultry (Torres-Rodriguez et al., 2007; Sen et al., 2012; Bai et al., 2013), improve nutrient digestibility (Mountzouris et al., 2010; Nawaz et al., 2016; Opoola et al., 2021), improve gut health (Oladokun et al., 2021a; Zeng et al., 2021; Gyawali et al., 2022), stimulate immunity (Zulkifli et al., 2000; Nawaz et al., 2016; Hedayati et al., 2022) and positively modulate gut microbiota profile (Mountzouris et al., 2007a, 2010; Hedayati et al., 2022). Popular probiotic strains utilized in poultry include *Lactobacillus*, *Bifidobacterium*, *Enterococcus*, and *Bacillus* (Bajagai et al., 2016).

The use of *Bacillus* species continues to gain interest in animal production, especially from a commercial standpoint (Kim et al., 2018). This is because spore-based probiotic strains like *Bacillus* are highly resilient to environmental stressors (Cartman et al., 2008). The use of several *Bacillus* strains to promote gut health, immunity, and growth of poultry is well documented (Gadde et al., 2017a; Grant et al., 2018; Oladokun et al., 2021a). Despite these reported results, probiotics (including *Bacillus* strains) have also been reported to not affect growth performance indices like feed intake, weight gain, and feed conversion ratio in broiler chicken studies (Cavazzoni et al., 1998; Li et al., 2019). Other reports have also documented a reduced feed conversion ratio in broiler chickens supplemented with dietary *Bacillus subtilis* (Knap et al., 2011; Lee et al., 2014). Although popular theories on probiotics mode of action will include bacterial antagonism, immunostimulation, and competitive exclusion (Ohimain and Ofongo, 2012), it is possible that a complete delineation of probiotics mode of actions is yet to be elucidated. Several other factors, including strain-specific mode of action, the health state of the host, housing and environmental conditions, supplemented dose, time of supplementation, and delivery routes, may contribute to the inconsistencies in probiotics results observed in the literature (Yang et al., 2009; Cox and Dalloul, 2015; Untoo et al., 2018).

As a solution to the challenges that characterize the conventional delivery routes in poultry (i.e., in-feed and in-water; summarized in Oladokun et al., (2021a)), the *in ovo* delivery routes continue to gain considerable interest. Asides from other benefits that the *in ovo* technology affords (documented in Oladokun and Adewole (2020) and Oladokun et al., (2021a)), it also offers the opportunity to colonize the embryonic gut with beneficial microbiota very early on, considering that contact between chick and hen which use to be status quo mode of gut colonization has been eliminated in the present-day poultry industry. Oladokun et al., (2021a) have previously reported that the *in ovo* delivery of 10×10^6 CFU *of Bacillus subtilis* improved

broiler chicken gut morphology and microbiota profile but with no significant effect on growth performance. As a follow-up to this study, it was hypothesized that modifying the supplemented dose (i.e., 10×10^6 CFU vs. 20×10^6 CFU), rearing period (28 days vs. 35 days), and housing conditions (battery cages vs. floor pens) might influence observed results. Consequently, the objective of this study was to evaluate the effect of the supplementation of two doses of *Bacillus subtilis* fermentation extract (i.e., 10×10^6 CFU and 20×10^6 CFU), and its delivery routes (*in ovo* vs. in-feed) on hatch and growth performance, blood biochemistry, immune status, gut morphology, and gut microbiota profile of broiler chickens, compared to in-feed antibiotics.

4.3 Materials and Methods

4.3.1 Ethics declarations

The experiment was carried out at the hatchery facility of the Agricultural Campus of Dalhousie University and the broiler rearing facility of the Atlantic Poultry Research Center, Dalhousie Faculty of Agriculture. The experiment was conducted following guidelines recommended by the Canadian Council on Animal Care (Rowsell, 1990). All methods were approved by the Animal Care and Use Committee of Dalhousie University (Protocol number: 2021-032).

4.3.2 Egg incubation and *in ovo* Injection Procedure

Hatching broiler eggs (Cobb 500, 52-week-old breeders, average weight=63g ± 1.27, n=1,860) were obtained from a commercial hatchery (Cox Atlantic Chick hatchery, Nova scotia) and incubated in a ChickMaster single-stage incubator (ChickMaster G09, Cresskill, NJ, USA), under standard conditions (37.5°C, 55% relative humidity) from embryonic days (EDs) 1 to 19, and then to an average of 32°C and 68% from EDs 19 to 21. Eggs were candled on ED12 to determine viability. Viable eggs were subsequently assigned to one of four experimental groups: a) non-injected eggs (control; 166 eggs); b) *in ovo* saline group (38 eggs; injected with 0.2 mL of physiological saline, i.e., 0.9% NaCl, Baxter Corporation, ON, Canada); c) *in ovo* probiotic group 1 (53 eggs; injected with 0.2 mL of *Bacillus subtilis* fermentation extract, each egg received 10×10^6 CFU of the bacterium/0.2 mL saline diluent) and d) *in ovo* probiotic group 2 (53 eggs; injected with 0.2 mL of *Bacillus subtilis* fermentation extract, each egg received 20×10^6 CFU of the bacterium/0.2 mL saline diluent). The described treatments were replicated in six similar incubators operated under similar conditions. The *Bacillus subtilis* product (Strain - *Bacillus subtilis* 10SI) injected in this experiment was obtained from a commercial source (Probiotech International, St. Hyacinth, QC, Canada) at a concentration of 10×10^{10} CFU/g. The *Bacillus subtilis* product was injected on ED18. The injection procedure utilized in this study have been

previously described by Oladokun et al. (2021a). Briefly, eggs were disinfected by cleaning with of 70% alcohol swabs (BD alcohol swabs-catalog 326910, ON, Canada), followed by careful punching of the air cell (the blunt end of the egg) using an 18-gauge needle. The injected probiotics treatments were then delivered to the amnion using a self-refilling injector (Socorex ultra-1810.2.05005, Ecublens, Switzerland) equipped with a 22-gauge needle (injection needle length—3 cm) at a 45-degree angle. After *in ovo* injection, the injection sites were sealed with sterile medical tapes (Nexcare™ Flexible Clear Tape-7100187758, 3M, MN, USA). The non-injected eggs were also taken out and returned to the incubator simultaneously with other injected treatment groups.

4.3.3 Birds, Housing, and Diets

As presented in Figure 4.1, hatchlings were weighed and randomly assigned to 5 new treatment groups. Chicks from the initial non-injection group were randomly allocated into 3 new treatment groups consisting of (1) chicks fed a basal corn-soybean meal-wheat–based diet (Negative control treatment; NC); (2) chicks fed NC + 0.05% bacitracin methylene disalicylate (in-feed antibiotics); and (3) chicks fed NC + 0.005% *Bacillus subtilis* containing 1 x 10^8 CFU/kg of feed. The *in ovo* probiotics treatments were placed on the control diet to form treatments (4) *in ovo* probiotics group 1 and (5) *in ovo* probiotics group 2. Chicks (mixed sex, n = 22) were weighed and assigned to 9 replicate floor pens (0.93 m × 2.14 m)/treatment at a stocking density of 0.076 m^2/bird. Two broiler production rooms were utilized. The temperature in the broiler rooms was monitored daily and was gradually reduced from 32 to 22.5 °C from d 0 to 35. The lighting program was set to produce 18 h of light and 6 h of darkness throughout the experimental period, and illumination was gradually reduced from 20 lx on d 0 to 5 lx on d 35. Dietary treatments, ingredients, and nutritional composition are presented in Table 4.1. Birds were provided with feed and water *ad libitum*; diets were fed as mash in the starter (0–14 days) phase and pellets in the grower (15–25 days) and finisher (26-35 days) phases. Diets were formulated to meet Cobb 500 broiler chicken requirements.

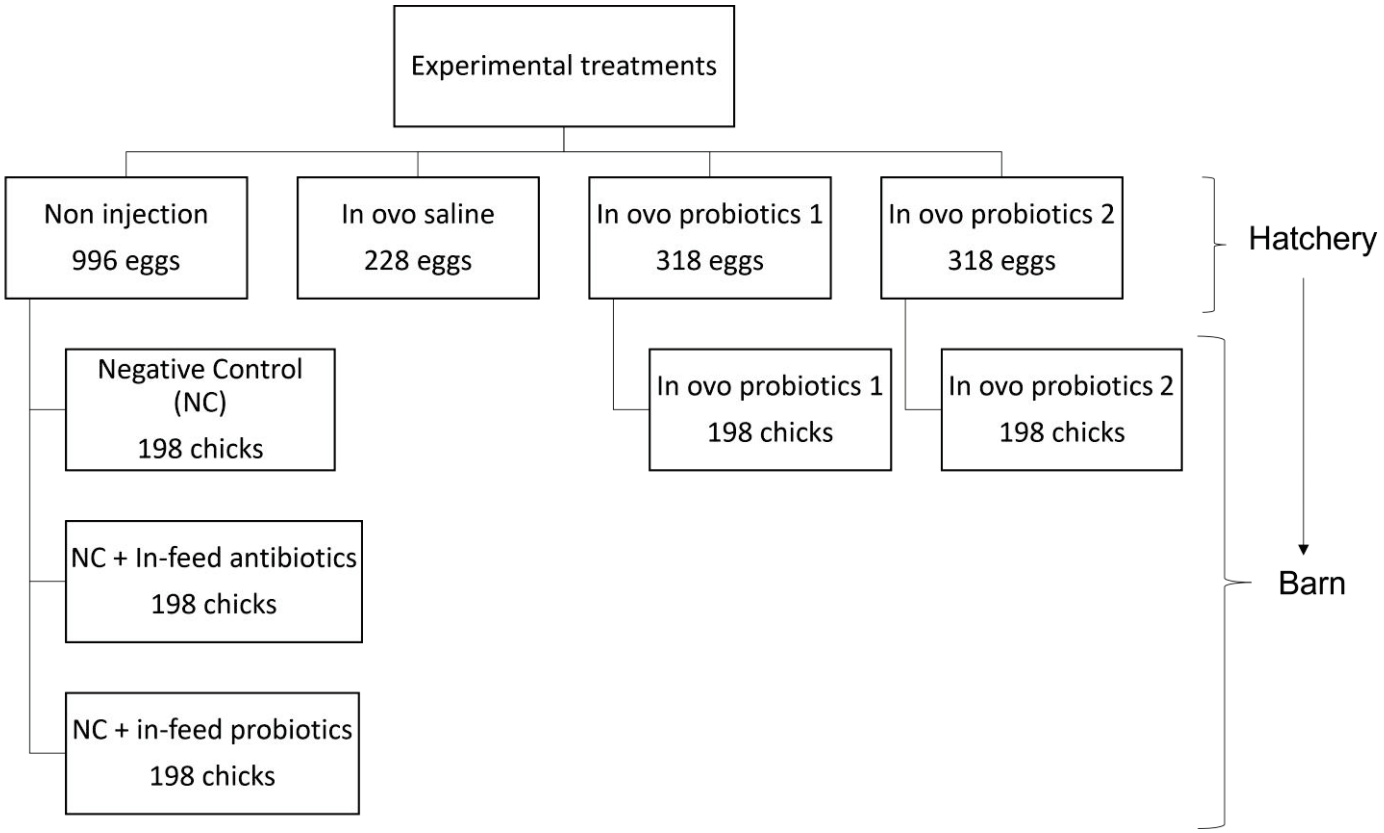


Figure 4.1 Schematic presentation of experimental structure in the hatchery and barn. In ovo probiotics group 1- eggs injected with 10×10^6 CFU of Bacillus subtilis fermentation extract /0.2 mL saline diluent; in ovo probiotics group 2- eggs injected with 20×10^6 CFU of Bacillus subtilis fermentation extract /0.2 mL saline diluent; in-feed antibiotics- chicks fed NC + 0.05% bacitracin methylene disalicylate; in-feed probiotics- chicks fed 0.005% Bacillus subtilis containing 1×10^8 CFU/kg of feed; and NC-Negative Control treatment- chicks fed a basal corn-soybean meal-wheat–based diet.

Table 4.1 Ingredients, calculated, and analyzed compositions of experimental diets[1] (as-fed basis, percentage, unless otherwise stated).

Ingredients	Phases								
	Starter (0-14 d)			Grower (15-25 d)			Finisher (26-35 d)		
	Negative Control	In-feed Antibiotics	In-feed probiotics	Negative Control	In-feed Antibiotics	In-feed probiotics	Negative Control	In-feed Antibiotics	In-feed probiotics
Ingredient Composition									
Corn (ground)	46.63	46.53	46.62	51.16	51.06	51.15	53.63	53.53	53.62
Soybean meal-46.5	37.12	37.14	37.13	31.87	31.89	31.88	29.2	29.22	29.21
Wheat	10.0	10.0	10.0	10.0	10.0	10.0	10.0	10.0	10.0
Soybean Oil (young or mature)	1.80	1.83	1.795	2.18	2.21	2.175	2.75	2.78	2.75
Limestone	1.37	1.37	1.37	1.30	1.30	1.30	1.19	1.19	1.19
Dicalcium Phosphate 21 P	1.45	1.45	1.45	1.35	1.35	1.35	1.18	1.18	1.18
DL Methionine premix[2]	0.58	0.58	0.58	0.57	0.57	0.57	0.52	0.52	0.52
Vitamin/Mineral Premix or MCB10 [3, 4]	0.50	0.50	0.50	0.50	0.50	0.50	0.50	0.50	0.50
Salt	0.38	0.38	0.38	0.36	0.36	0.36	0.36	0.36	0.36
Lysine HCl	0.17	0.17	0.17	0. 21	0.21	0.21	0.17	0.17	0.17

Pellet Binding Agent	-	-	-	0.50	0.50	0.50	0.50	0.50	0.50
BMD 110G[5]	-	0.05	-	-	0.05	-	-	0.05	-
Bacillus subtilis	-	-	0.005	-		0.005	-	-	0.005
Total	100	100	100	100	100	100	100	100	100
Nutrient	Calculated composition								
Metabolizable energy (kcal/kg)	2,975	2,975	2,975	3,025	3,025	3,025	3,100	3,100	3,100
Crude protein	22.0	22.0	22.0	20.0	20	20.0	19.0	19.0	19.0
Calcium	0.90	0.90	0.90	0.84	0.84	0.84	0.76	0.76	0.76
Available phosphorus	0.45	0.45	0.45	0.42	0.42	0.42	0.38	0.38	0.38
Sodium	0.18	0.18	0.18	0.17	0.17	0.17	0.17	0.17	0.17
Digestible lysine	1.22	1.22	1.22	1.12	1.12	1.12	1.02	1.02	1.02
Digestible methionine + cysteine	0.91	0.91	0.91	0.85	0.85	0.85	0.80	0.80	0.80
Digestible Tryptophan	0.24	0.24	0.24	0.22	0.22	0.22	0.20	0.20	0.20
Digestible Threonine	0.84	0.84	0.84	0. 76	0.76	0.76	0.72	0.72	0.72

	Analyzed composition								
Dry Matter	92.2	92.2	92.2	91.5	92.1	91.4	91.7	91.8	91.8
Crude protein	24.5	24.7	23.9	21.3	21.2	21.8	19.3	20.9	21.0
Crude fat	4.05	4.31	4.17	4.86	4.69	3.63	4.81	4.25	4.17
Calcium	0.81	0.80	1.03	0.89	0.90	0.83	0.83	0.75	0.75
Potassium	1.05	1.00	0.98	0.94	0.91	0.95	0.84	0.92	0.89
Phosphorus	0.62	0.65	0.72	0.66	0.65	0.65	0.57	0.60	0.59
Sodium	0.14	0.15	0.20	0.17	0.17	0.17	0.16	0.15	0.15

[1]Basal diet (NC); In-feed antibiotic diet containing NC + 0.05% bacitracin methylene disalicylate (BMD); In-feed probiotics diet containing NC + 0.005% *Bacillus subtilis* containing- 1 x 10^8 CFU/kg of feed. [2] Supplied/kg premix: DL-Methionine, 0.5 kg; wheat middling, 0.5 kg. [3] Starter vitamin-mineral premix contained the following per kg of diet: 9750 IU vitamin A; 2000 IU vitamin D3; 25 IU vitamin E; 2.97 mg vitamin K; 7.6 mg riboflavin; 13.5 mg Dl Ca-pantothenate; 0.012 mg vitamin B12; 29.7 mg niacin; 1.0 mg folic acid, 801 mg choline; 0.3 mg biotin; 4.9 mg pyridoxine; 2.9 mg thiamine; 70.2 mg manganese; 80.0 mg zinc; 25 mg copper; 0.15 mg selenium; 50 mg ethoxyquin; 1543 mg wheat middling's; 500 mg ground limestone. [4] Grower and Finisher vitamin-mineral premix contained the following per kg of diet: 9750 IU vitamin A; 2000 IU vitamin D3; 25 IU vitamin E; 2.97 mg vitamin K; 7.6 mg riboflavin; 13.5 mg Dl Ca-pantothenate; 0.012 mg vitamin B12; 29.7 mg niacin; 1.0 mg folic acid, 801 mg choline; 0.3 mg biotin; 4.9 mg pyridoxine; 2.9 mg thiamine; 70.2 mg manganese; 80.0 mg zinc; 25 mg copper; 0.15 mg selenium; 50 mg ethoxyquin; 1543 mg wheat middling's; 500 mg ground limestone. [5] Bacitracin methylene disalicylate (providing 55 mg/kg mixed feed); Alpharma, Inc., Fort Lee, NJ, USA.

4.3.4 Measurements

4.3.4.1 Hatch Parameters and Chick Quality

Hatched chicks were counted and weighed individually. Hatchability was calculated as the percentage of hatched chicks to fertile incubated eggs per replicate. The BW/initial egg weight ratio of hatched chicks was also determined and recorded. Chick navel quality was evaluated by adopting the scoring method by Reijrink et al. (2009). Navel quality was scored 1—when the navel was completely closed and clean; scored 2—when the navel was discolored (i.e., when the navel color differs from the chick's skin color) with a maximum 2 mm opening; and scored 3—when the navel was discolored and with more than a 2 mm opening. Chick length was obtained by placing the chick on its ventral side and measuring from the tip of the beak to the middle toe on the right leg.

4.3.4.2 Growth Performance Parameters

Growth performance parameters, including feed intake and average body weight (BW) were measured on a pen basis weekly. Subsequent calculations, including the average feed intake (AFI), average body weight gain (ABWG), and feed conversion ratio (FCR) were then obtained from the recorded data. Mortality was recorded daily and used to correct for FCR.

4.3.4.3 Sampling

On day 25, 1 bird (male) per cage (9 replicate birds per treatment group) was randomly selected, weighed, and euthanized by electrical stunning and exsanguination. After euthanasia of the bird, blood samples were collected from each bird into 10 mL blood serum collection tubes (BD Vacutainer™ Serum Tubes, fisher scientific- BD366430) for further serum assays and into 10 mL heparinized tubes (BD Vacutainer™ Glass Blood Collection Tubes with Sodium Heparin, fisher scientific- BD366480) for further blood plasma assays. Blood serum and plasma were centrifuged at 1,200 g x 10 minutes x 18 °C. The resulting supernatants were stored in aliquots at -80 °C until further analysis. The weights of bursa of Fabricius and spleen were also determined by trained personnel. The small intestinal segments, including the duodenum (region from the gizzard junction to the pancreatic and bile ducts), jejunum (1.5-cm length midway between the point of entry of the bile ducts and Meckel's diverticulum) and ileum (1.5-cm length midway between Meckel's diverticulum and the ileocecal junction), were also excised and fixed in neutral buffered formalin (10%) for further histomorphology processing. Ceca and ileal digesta samples were also collected in RNase and DNase-free tubes, and immediately snap frozen in liquid nitrogen, and later stored at −80°C for subsequent gut microbiota analysis.

4.3.4.4 Relative Weight of Organs

The weights of the bursa of Fabricius and the spleen were recorded and reported as a percentage of the live BW of the slaughtered chicken (g/Kg BW).

4.3.4.5 Serum Immunoglobulins

Chicken-specific immunoglobulins enzyme-link immunosorbent assay (ELISA) quantitation kits (Bethyl Laboratories, Montgomery, TX, USA; Catalog No. E33-104-200218 and E33-102-180410, respectively) were used to measure the concentrations of immunoglobulins (IgG and IgM) following manufacturer instructions. Absorbance values were read on a microplate reader (Bio-Tek Instrument Inc., Wonooski, VT, USA) using a software program (KC4, version #3.3, Bio Tek Instruments), and immunoglobulins concentration was extrapolated using the four-parameter logistic model.

4.3.4.6 Blood Biochemistry

Samples for blood biochemical analysis were shipped on ice to Atlantic Veterinary College, University of Prince Edward Island Pathology Laboratory, and analyzed using cobas® 6000 analyzer series (Roche Diagnostics, Indianapolis, IN, USA).

4.3.4.7 Gut Morphology

The procedure for intestinal morphometric analysis has previously been reported by Oladokun et al. (2021a). Briefly, fixed intestinal tissues were embedded in paraffin, sectioned (0.5 µm thick), and stained with hematoxylin and eosin for morphological examinations. In each cross-sectioned tissue, ten morphometric measurements including the villus height (from the base of the intestinal mucosa to the tip of the villus excluding the intestinal crypt), villus width (halfway between the base and the tip), crypt depth (from the base upward to the region of transition between the crypt and villi) (Ozdogan et al., 2014) per slide were carried out using Leica 1CC50 W microscope at 4× Magnification (Leica Microsystems, Wetzlay, Germany) and an image processing and analysis system (Leica Application Suite, Version 3.4.0, Leica Microsystems, Wetzlay, Germany).

4.3.4.8 DNA Extraction, Quantification, Library Preparation, and Sequencing

Following manufacturer's instructions, DNA was extracted from the ileal and ceca digesta contents using the Qiagen DNeasy® PowerSoil Pro Kit (50) (catalog number 47014, Qiagen GmbH, Hilden, Germany). The concentration and purity of extracted DNA were subsequently determined by spectrophotometry (Nanodrop ND1000; Thermo Scientific). Extracted DNA samples (volume-50 µL, concentration-10-200 ng/µL) were then sent to Genome Quebec Innovation Center (Montreal, Canada) for amplicon library preparation and sequencing (primers,

V3V4, 341F-CCTACGGGNGGCWGCAG and 805R-GACTACHVGGGTATCTAATCC).

4.3.4.9 *Statistics and Bioinformatic Analysis*

Hatch data were analyzed as a randomized complete block design, with the incubator considered as the blocking factor. Datasets from the grow-out trial were also analyzed in a randomized complete block design, with broiler production rooms being the blocking factor. The normality of all data sets was ascertained by testing residuals with the Anderson-Darling test in Minitab statistical package (v.18.1). Data were analyzed using the generalized linear model in the same statistical package. Significant means were separated using Tukey's honest significant difference test in the same statistical package. Analyzed data were presented as means ± SEM and probability values. Values were considered statistically different at $P \leq 0.05$ and considered a statistical trend at $P < 0.1$.

Bioinformatic analysis of the microbiota data was performed by the Canadian Centre for Computational Genomics at McGill University. The GenPipes version 4.0.0 (Bourgey et al., 2019) amplicon-seq pipeline was used to perform analyses. This pipeline is based on the DADA2 package in R environment. First, the trimming was done using Trimmomatic [Bolger et al., 2014], taking off 16 bp from the start of the reads. Then, 8,455,050 paired-end reads passed the quality-filtering parameters applied [truncLen=c(284,176); maxN = 0; maxEE=c(2,2); truncQ = 2] with an average value of 93,945 reads/sample and thus were merged (minimum overlap of 20 bp) and subjected to de novo chimera removal. Taxonomy was assigned to the resulting amplicon sequence variants (ASVs) using Silva database version 123. Visual exploration of the data was then performed in the MicrobiomeAnalyst tool (Dhariwal et al., 2017). Alpha and Beta diversity were calculated based on Shannon and Bray-Curtis indices, respectively, with statistical significance set at $P < 0.05$.

4.4 Results

4.4.1 Hatch performance and chick quality

Results on hatch performance and chick quality are presented in Table 4.2. The chick BW/ Egg Weight recorded treatment differences. Both the *in ovo* saline and the *in ovo* probiotics 2 treatment groups recorded higher (*P = 0.01*) chick BW/ Egg Weight values compared to the non-injected eggs. The *in ovo* probiotics 1 treatment group recorded statistically intermediate chick BW/ Egg Weight value compared to other treatment groups. There was no effect of treatment on average navel score, average chick length, average chick weight, and hatchability in this study.

4.4.2 Growth performance

Results on growth performance indexes are presented in Table 4.3. Compared to other treatments, the in-feed probiotics treatment showed a tendency ($P = 0.07$) to increase ABG by at least 23.6% in week 1. However, this tendency soon disappeared in subsequent weeks. Further treatment differences were only recorded in week 5. The AFI of the in-feed antibiotic treatment was higher ($P = 0.02$) than the in-feed probiotics and the *in ovo* probiotics 2 treatment groups. Other treatments had statistically similar AFI as the in-feed antibiotic treatment. Both the ABWG and FCR values were similar ($P > 0.05$) for all treatment groups from week 2 to week 5. At the end of the entire trial period (d0-35), the in-feed antibiotic treatment showed a tendency ($P = 0.09$) to increase AFI by at least 17.6%, compared to other treatments. However, no corresponding change in ABWG nor FCR were recorded across treatment groups. Furthermore, in order to evaluate if treatment effects on ABWG were sex-linked, ABWG was calculated on a sex basis (males and females separately) at weeks 4 and 5, when visual sexual distinction and weighing of birds could be carried out. However, no difference ($P > 0.05$) in ABWG for males and females was recorded at this time.

Table 4.2 Effect of in *ovo* delivery of *Bacillus subtilis* on hatch performance and chick quality.

Hatch Parameters	Treatments[1]				SEM[2]	*P* value[3]
	Non-injected	*In ovo* Saline	*In ovo* Probiotics 1	*In ovo* Probiotics 2		
Hatchability (%)	96.1	95.2	96.8	96.9	0.51	0.711
Average chick weight (g)	43.1	43.8	43.4	43.7	0.13	0.118
Average chick length (cm)	18.8	18.2	19.2	18.9	0.18	0.202
Chick BW/ egg weight (%)	68.1^{b}	69.5^{a}	68.9^{ab}	69.2^{a}	0.17	0.005
Average navel score	1.40	1.38	1.47	1.36	0.07	0.79

[1] Treatments include— (1) non-injected eggs; (2) *in ovo* saline group- injected with 0.2 mL of physiological saline (0.9% NaCl); (3) *in ovo* probiotics group 1- eggs injected with 10×10^6 CFU of *Bacillus subtilis* fermentation extract /0.2 mL saline diluent; and (4) *in ovo* probiotics group 2- eggs injected with 20×10^6 CFU of *Bacillus subtilis* fermentation extract /0.2 mL saline diluent. [2] SEM = Standard error of means. [3]Means within a row with different superscripts [a,b] significantly differ.

Table 4.3 Effect of *Bacillus subtilis* and its delivery routes on the growth performance of broiler chickens raised for 35 days

Growth Performance Parameters	Treatments[1]					SEM[2]	P value[3]
	Negative Control	In-feed Antibiotics	In-feed probiotics	*In ovo* probiotics 1	*In ovo* probiotics 2		
			Week 1				
Average feed intake (g)	155	153	159	160	154	1.52	0.859
Average body weight gain (g)	85.7	87.4	91.6	88.7	74.1	1.00	0.071
FCR[4]	1.81	1.76	1.73	1.81	2.07	0.02	0.336
			Week 2				
Average feed intake (g)	184	190	148	163	168	3.94	0.237
Average body weight gain (g)	213	235	212	200	168	3.09	0.102
FCR[5]	0.87	0.81	0.69	0.81	0.99	0.02	0.193
			Week 3				
Average feed intake (g)	449	499	416	466	409	13.4	0.552
Average body weight gain (g)	483	555	480	476	441	9.07	0.188
FCR[4]	0.93	0.89	0.87	0.99	0.93	0.03	0.912
Week 4							
Average feed intake (g)	839	917	812	859	853	16.6	0.222
Average body weight gain (g)-Mixed sex	714	608	695	773	846	15.2	0.415
Average body weight gain (g)-	761	722	902	954	918	17.7	0.351

Males							
Average body weight gain (g)- Females	635	483	524	677	820	21.9	0.293
FCR[4]	1.31	1.24	1.43	1.38	1.39	0.09	0.516
Week 5							
Average feed intake (g)	1,378[ab]	1,329[a]	1,146[b]	1,102[ab]	970[b]	14.8	0.024
Average body weight gain (g)- Mixed sex	1,030	1,181	906	789	710	143	0.324
Average body weight gain (g)- Males	970	1157	936	926	857	504	0.850
Average body weight gain (g)- Females	977	1,019	831	679	625	42.1	0.412
FCR[4]	1.37	1.14	1.27	1.43	1.48	0.04	0.655
Total Trial Period (1-35 d)							
Average feed intake (g)	2,974	3,051	2,656	2,753	2,595	34.4	0.087
Average body weight gain (g)	2,578	2,655	2,385	2,353	2,217	139	0.574
FCR[4]	1.16	1.16	1.13	1.20	1.20	0.02	0.830

[1] Treatments include— (1) Negative Control treatment- chicks fed a basal corn-soybean meal-wheat–based diet; (2) In-feed antibiotics- chicks fed NC + 0.05% bacitracin methylene disalicylate; (3) In-feed probiotics- chicks fed NC + 0.005% *Bacillus subtilis* containing 1 x 10^8 CFU/kg of feed; (4) *In ovo* probiotics group 1- eggs injected with 10×10^6 CFU of *Bacillus subtilis* fermentation extract /0.2 mL saline diluent; and (5) *In ovo* probiotics group 2- eggs injected with 20×10^6 CFU of *Bacillus subtilis* fermentation extract /0.2 mL saline diluent. [2] SEM = Standard error of means. [3] Means within a row with different superscripts [a,b] significantly differ. [4]FCR = Feed Conversion Ratio.

4.4.3 Organ weight and serum immunoglobulin concentration

According to Table 4.4, no significant treatment effect on the relative weight of the bursa of Fabricius and spleen was recorded in this study. Conversely, of the two immunoglobulins evaluated, the serum IgG concentration was reduced ($P < 0.001$) both in the *in ovo* probiotics 1 and *in ovo* probiotics 2 treatments, compared to both the NC and the in-feed antibiotics treatment. However, the serum IgG concentration in the in-feed probiotics treatment was statistically similar to that of the *in ovo* probiotics 1 treatment. Nevertheless, the highest reduction in serum IgG concentration was recorded in the *in ovo* probiotics 2 treatment, being at least 38% lower than other treatment groups.

Table 4.4 Effect of Bacillus subtilis and its delivery routes on relative weight of immune organs and serum immunoglobulin concentrations in broiler chickens

Parameters	**Treatments[1]**					**SEM[2]**	**P value[3]**
	Negative Control	**In-feed Antibiotics**	**In-feed probiotics**	***In ovo* probiotics 1**	***In ovo* probiotics 2**		
Bursa weight (g/Kg BW)	1.81	1.80	1.64	1.76	1.93	0.06	0.645
Spleen weight (g/Kg BW)	0.70	0.76	0.73	0.73	0.69	0.02	0.920
Immunoglobulin G (Mg/mL)	10.3^{a}	4.75^{ab}	2.71^{bc}	0.96^{cd}	0.06^{d}	0.31	<0.001
Immunoglobulin M (Mg/mL)	2.37	0.61	0.35	0.19	0.15	0.05	0.333

[1]Treatments include— (1) Negative Control treatment- chicks fed a basal corn-soybean meal-wheat–based diet; (2) In-feed antibiotics- chicks fed NC + 0.05% bacitracin methylene disalicylate; (3) In-feed probiotics- chicks fed NC + 0.005% *Bacillus subtilis* containing 1 x 10^8 CFU/kg of feed; (4) *In ovo* probiotics group 1- eggs injected with 10×10^6 CFU of *Bacillus subtilis* fermentation extract /0.2 mL saline diluent; and (5) *In ovo* probiotics group 2- eggs injected with 20×10^6 CFU of *Bacillus subtilis* fermentation extract /0.2 mL saline diluent. [2] SEM = Standard error of means. [3] Means within a row with different superscripts ^{a,b,c,d} significantly differ.

4.4.4 Blood biochemistry

Results on blood biochemistry are presented in Table 4.5. Only the concentrations of plasma

sodium and chloride showed significant treatment effect in this study. Both electrolytes' minerals recorded similar trend. In both cases, the in-feed antibiotics treatment recorded higher ($P < 0.05$) concentrations of both minerals compared to the NC treatment. Other treatment groups recorded intermediate statistical values for the concentrations of both minerals (sodium and chloride).

4.4.5 Gut morphology

Table 4.6 shows the results on the morphology of the three gut sections (duodenum, jejunum, and ileum). Both doses of the *in ovo* delivered probiotics treatment increased ($P < 0.001$) duodenal villus height compared to NC treatment. The *in ovo* probiotics 2 treatment increased ($P = 0.023$) compared to the in-feed probiotics and NC treatment. Duodenal crypt depth was reduced by all treatments compared to the control treatment, with the exception of the *in ovo* probiotics 2 treatment which recorded a statistical intermediate crypt depth value. Conversely, jejunal villus height was increased ($P = 0.001$) by all treatments compared to the *in ovo* probiotics 2 treatment. The in-feed probiotics treatment recorded statistically intermediate jejunal villus height values. On the contrary, the *in ovo* probiotics 2 treatment recorded increased ($P < 0.001$) villus width compared to other treatments (except for the negative control treatment). Jejunal crypt depth was also reduced ($P < 0.001$) by the in-feed antibiotics and *in ovo* probiotics 2 treatments compared to other treatments. In terms of jejunal villus height to crypt depth ratio, the in-feed antibiotic treatment was better ($P < 0.001$) than all other treatments. Treatments had no effect ($P = 0.115$) on villus height in the ileum. The in-feed probiotics treatment also recorded the least ($P < 0.001$) ileal crypt depth, but this was only different from the NC treatment. Similarly, the NC and *in ovo* probiotics 1 treatment recorded the least ($P < 0.001$) villus height: crypt depth values in the ileum.

Table 4.5 Effect of *Bacillus subtilis* and its delivery routes on broiler chicken plasma biochemistry indices.

Parameters	Treatments[1]					SEM[2]	*P* value[3]
	Negative Control	In-feed Antibiotics	In- feed probiotics	*In ovo* probiotics 1	*In ovo* probiotics 2		
	Electrolytes (mmol·L^{-1})						
Sodium	149.4^{b}	152.0^{a}	151.7^{ab}	151.2^{ab}	151.6^{ab}	0.52	0.031
Potassium	6.96	6.62	6.64	6.85	6.87	0.07	0.416
Sodium: Potassium	21.49	23.04	23.04	22.18	22.07	0.25	0.090
Chloride	109^{b}	113^{a}	111^{ab}	111^{ab}	110^{ab}	0.5	0.022
Calcium	3.16	2.80	2.98	3.03	3.11	0.06	0.148
Phosphorus	2.47	2.18	2.29	2.38	2.34	0.05	0.317
Magnesium	0.87	0.80	0.78	0.80	0.84	0.01	0.063
	Metabolites (mmol·L^{-1})						
Urea	0.31	0.30	0.35	0.29	0.28	0.01	0.318
Glucose	15.5	15.3	15.6	16.	15.4	0.14	0.542
Cholesterol	3.49	3.53	3.51	3.43	3.63	0.05	0.859
Iron	18.5	20.6	21.1	19.9	19.7	0.01	0.772
Bile acids	22.5	24.4	24.4	20.7	25.2	0.94	0.584
Uric acid	364	375	424	384	396	0.01	0.498
Creatinine	1.99	1.69	2.00	1.55	6.42	0.06	0.083
	Enzymes (U·L^{-1})						

Amylase	606	703	726	795	782	38.6	0.579
Lipase	22.1	23.7	24.3	20.4	20.7	0.03	0.895
Creatine kinase	6,496	8,291	7,562	5,111	4,598	0.04	0.411
Alkaline Phosphatase	10,205	7,775	11,986	13,378	9,264	1000	0.438
Alanine transaminase	2.62	2.25	3.56	2.00	2.38	0.04	0.287
Aspartate Aminotransferase	166	192	184	159	162	0.01	0.318
Gamma-Glutamyl Transferase	9.17	10.72	9.50	9.50	10.72	0.26	0.140
			Proteins ($g \cdot L^{-1}$)				
Total Proteins	28.6	27.2	28.2	29.4	29.1	0.001	0.596
Albumin	11.8	11.9	11.7	11.9	11.6	0.14	0.973
Globulin	16.7	15.3	16.3	17.5	17.4	0.003	0.421
Albumin: Globulin	0.71	0.78	0.73	0.69	0.67	0.02	0.429

[1]Treatments include— (1) Negative Control treatment- chicks fed a basal corn-soybean meal-wheat–based diet; (2) In-feed antibiotics- chicks fed NC + 0.05% bacitracin methylene disalicylate; (3) In-feed probiotics- chicks fed NC + 0.005% *Bacillus subtilis* containing 1 x 10^8 CFU/kg of feed; (4) *In ovo* probiotics group 1- eggs injected with 10×10^6 CFU of *Bacillus subtilis* fermentation extract /0.2 mL saline diluent; and (5) *In ovo* probiotics group 2- eggs injected with 20×10^6 CFU of *Bacillus subtilis* fermentation extract /0.2 mL saline diluent. [2] SEM = Standard error of means. [3] Means within a row with different superscripts [a,b,] significantly differ.

Table 4.6 Effect of *Bacillus subtilis* and its delivery routes on broiler chicken intestinal morphology.

Parameters	Treatments[1]					SEM[2]	P value[3]
	Negative Control	In-feed Antibiotics	In-feed probiotics	*In ovo* probiotics 1	*In ovo* probiotics 2		
Duodenum							
Villus height (mm)	2.05^{cd}	2.15^{bc}	1.99^{ab}	2.22^{ab}	2.30^{a}	0.02	< 0.001
Villus width (mm)	0.22^{b}	0.24^{a}	0.22^{b}	0.23^{ab}	0.24^{a}	0.00	0.023
Crypt depth (mm)	0.16^{a}	0.14^{b}	0.14^{b}	0.145^{ab}	0.16^{a}	0.00	<0.001
Villus height: Crypt depth	12.5^{b}	14.9^{a}	13.9^{ab}	15.1^{a}	14.2^{a}	0.19	< 0.001
Jejunum							
Villus height (mm)	1.16^{a}	1.19^{a}	1.11^{ab}	1.19^{a}	1.09^{b}	0.01	0.001
Villus width (mm)	0.24^{ab}	0.20^{c}	0.21^{c}	0.22^{bc}	0.26^{a}	0.00	< 0.001
Crypt depth (mm)	0.11^{a}	0.10^{bc}	0.11^{a}	0.11^{a}	0.10^{bc}	0.00	< 0.001
Villus height: Crypt depth	10.4^{b}	12.2^{a}	9.77^{b}	10.8^{b}	10.5^{b}	0.15	< 0.001
Ileum							
Villus height (mm)	0.74^{ab}	0.76^{ab}	0.80^{a}	0.71^{b}	0.82^{a}	0.01	0.001
Villus width (mm)	0.19	0.18	0.17	0.19	0.19	0.00	0.115
Crypt depth (mm)	0.15^{a}	0.134^{abc}	0.127^{c}	0.133^{bc}	0.14^{abc}	0.37	<0.001
Villus height: Crypt depth	4.93^{b}	5.67^{ab}	6.39^{a}	5.41^{b}	5.74^{ab}	0.11	<0.001

[1]Treatments include — (1) Negative Control treatment- chicks fed a basal corn-soybean meal-wheat–based diet; (2) In-feed antibiotics- chicks fed NC + 0.05% bacitracin methylene disalicylate; (3) In-feed probiotics- chicks fed NC + 0.005% *Bacillus subtilis* containing 1 x 10^8 CFU/kg of feed; (4) *In ovo* probiotics group 1- eggs injected with 10×10^6 CFU of *Bacillus subtilis* fermentation extract /0.2 mL saline diluent; and (5) *In ovo* probiotics group 2- eggs injected with 20×10^6 CFU of *Bacillus subtilis* fermentation extract /0.2 mL saline diluent. [2] SEM = Standard error of means. [3] Means within a row with different superscripts [a,b,c] significantly differ.

4.4.6 Gut microbiota

Sequencing analysis yielded a total of 1,712 operational taxonomic units (OTUs) with ≥ 2 counts after quality filtering and demultiplexing. The % of taxon assigned at the Genus level was ~ 60%. Rarefaction curve showing specie richness is presented in Figure 4.2. Alpha diversity (Shannon index) showed significant ($P < 0.001$) diversity between the ileal and cecal samples but not between treatment groups (Figures 4.3 a, b, and c). Similarly, Beta diversity determined by ordination analysis based on Bray-Curtis Index showed unique cluster separation between the ileal and cecal microbiota but not between treatment groups in both gut sections (Figure 4.4 a, b, and c).

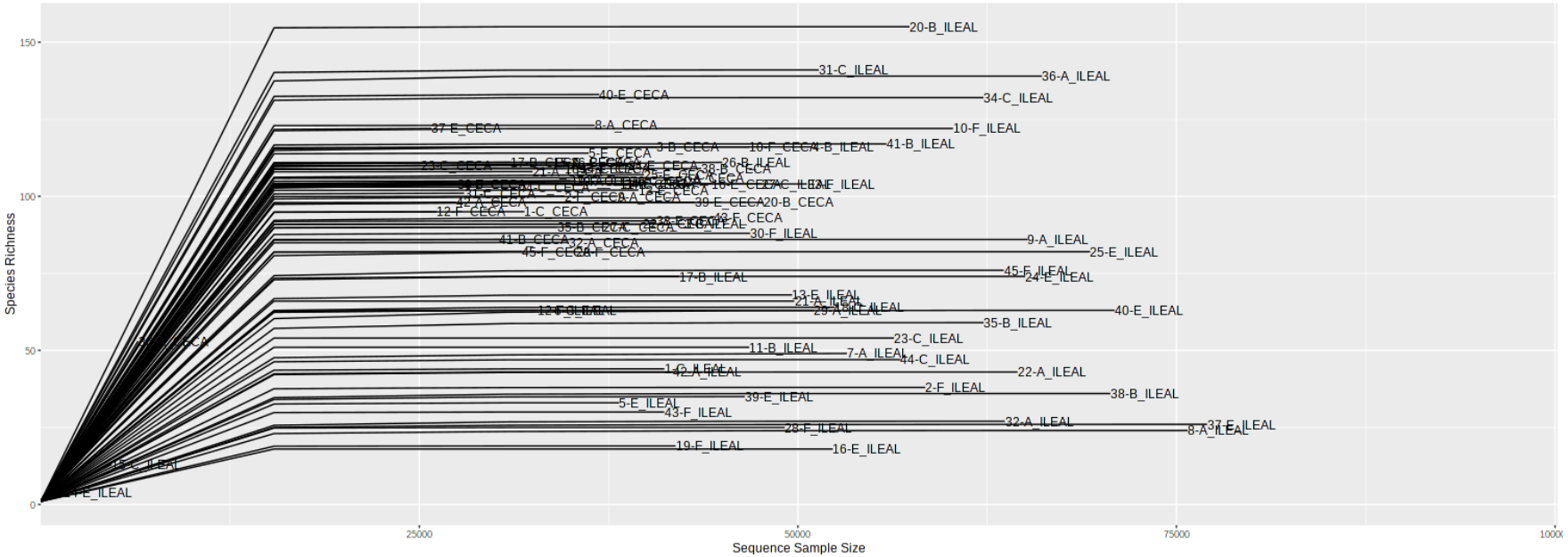
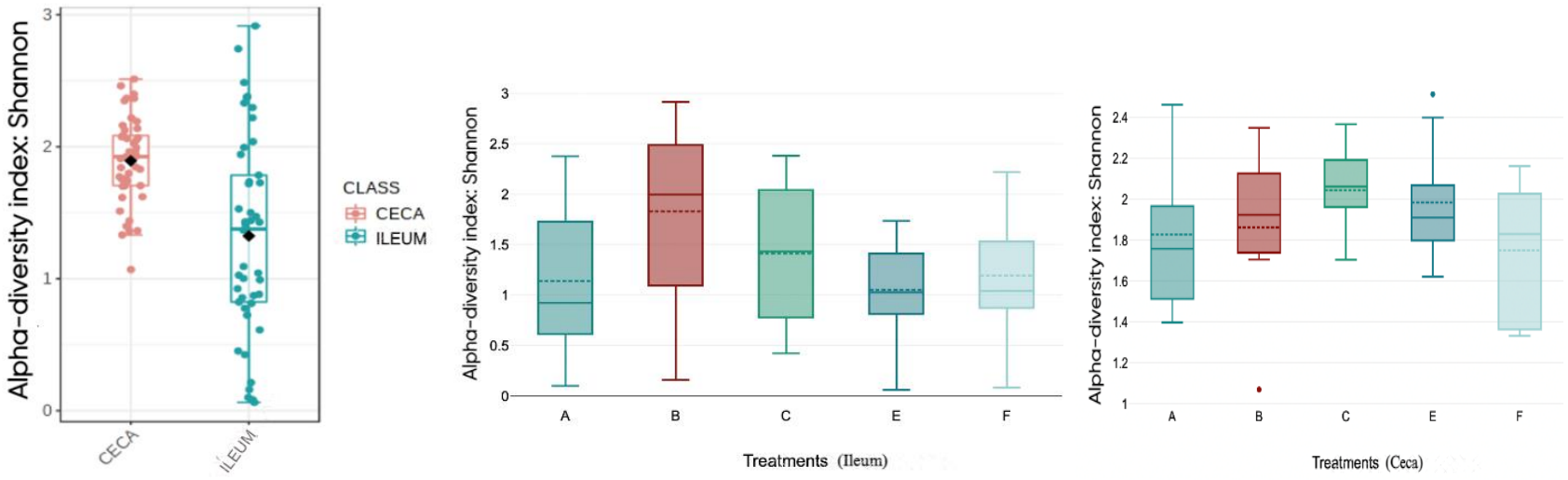


Figure 4.2 Rarefaction curves showing specie richness obtained from 16S rRNA gene V3V4 sequences.

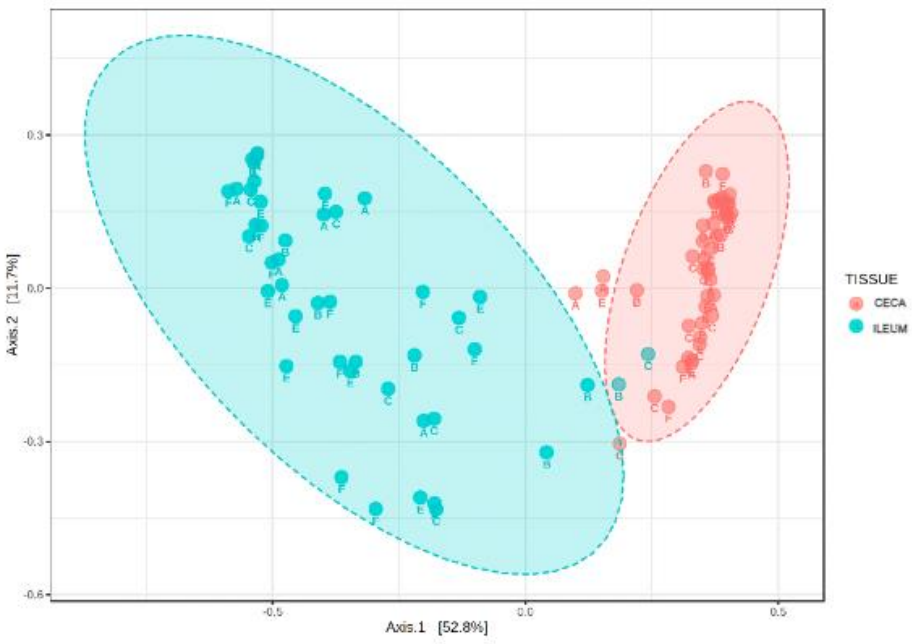
Based on (a) microbiota source - ceca and ileum and (b) treatment - (A) Negative Control treatment- chicks fed a basal corn-soybean meal-wheat–based diet; (B) In-feed antibiotics- chicks fed NC + 0.05% bacitracin methylene disalicylate; (C) In-feed probiotics - chicks fed NC + 0.005% *Bacillus subtilis* containing 1 x 108 CFU/kg of feed; (E) *In ovo* probiotics group 1- eggs injected with 10 × 106 CFU of *Bacillus subtilis* fermentation extract /0.2 mL saline diluent; and (F) *In ovo* probiotics group 2- eggs injected with 20 × 106 CFU of *Bacillus subtilis* fermentation extract /0.2 mL saline diluent.



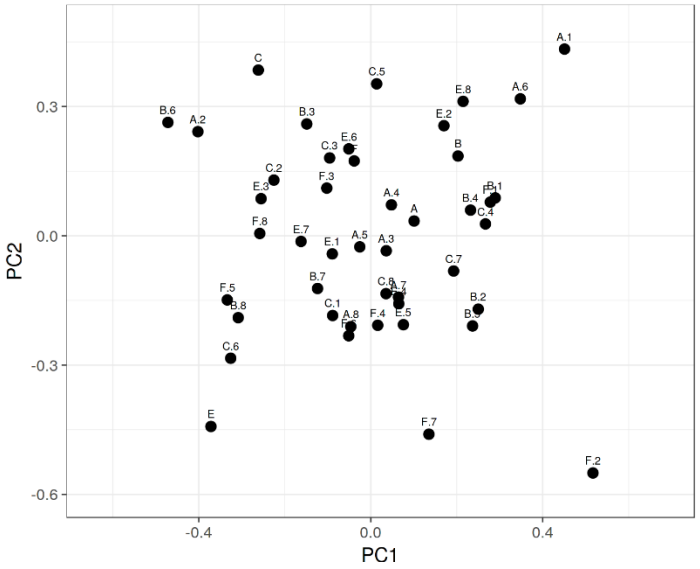
(a) (b) (c)

Figure 4.3 Alpha diversity (Shannon's index) box plots.

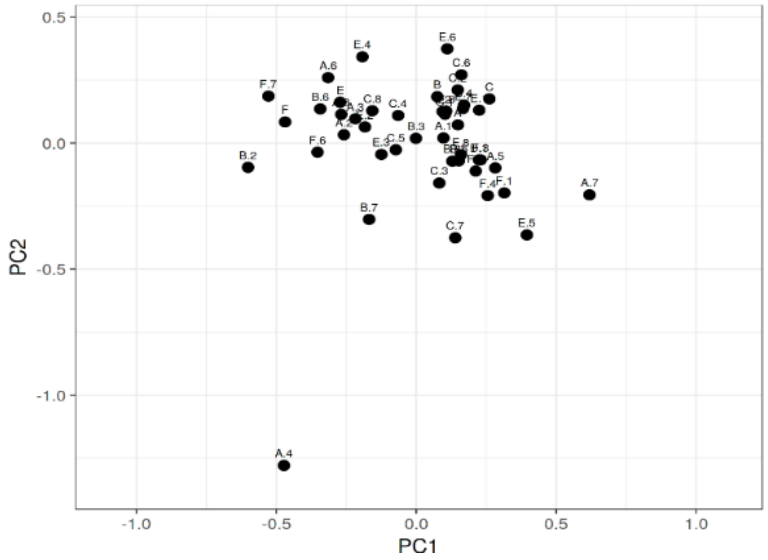
Shows (a) significant difference between ileal and cecal microbiota (T-test, $P >$ 0.001), (b) no significant effect of treatments on ileal microbiota diversity (ANOVA, $P = 0.180$), (c) no significant effect of treatment on ceca microbiota (ANOVA, $P = 0.320$). Treatments include— (A) Negative Control treatment- chicks fed a basal corn-soybean meal-wheat–based diet; (B) In-feed antibiotics- chicks fed NC + 0.05% bacitracin methylene disalicylate; (C) In-feed probiotics- chicks fed NC + 0.005% *Bacillus subtilis* containing 1 x 10^8 CFU/kg of feed; (E) *In ovo* probiotics group 1- eggs injected with 10×10^6 CFU of *Bacillus subtilis* fermentation extract /0.2 mL saline diluent; and (F) *In ovo* probiotics group 2- eggs injected with 20×10^6 CFU of *Bacillus subtilis* fermentation extract /0.2 mL saline diluent. Boxes in the boxplots denote interquartile range, solid middle line in the boxes denote the median, and dotted lines denote the means, all symbols outsides the boxes represent outliers.



(a)



(b)



(c)

Figure 4.4 Beta diversity (based on analysis based on Bray-Curtis Index) principal coordinate plots.

Show (a) significant difference between ileal and cecal microbiota (PCOA, ANOSIM, $P > 0.05$), (b) no significant effect of treatments on ileal beta diversity (PCA, ANOSIM, $P > 0.05$), (c) no significant effect of treatment on ceca beta diversity (PCA, ANOSIM, $P > 0.05$). Treatments include— A) Negative Control treatment- chicks fed a basal corn-soybean meal-wheat–based diet; (B) In-feed antibiotics- chicks fed NC + 0.05% bacitracin methylene disalicylate; (C) In-feed probiotics- chicks fed NC + 0.005% *Bacillus subtilis* containing 1 x 10^8 CFU/kg of feed; (E) *In ovo* probiotics group 1- eggs injected with 10×10^6 CFU of *Bacillus subtilis* fermentation extract /0.2 mL saline diluent; and (F) *In ovo* probiotics group 2- eggs injected with 20×10^6 CFU of *Bacillus subtilis* fermentation extract /0.2 mL

In terms of microbiota composition, the relative abundance of the predominant bacteria phyla and genera in the ileum and ceca are shown in Figures 4.5 and 4.6. Ileal phyla were dominated by >89% phylum Firmicutes across all treatment groups. The relative abundance of other dominant phyla followed the trend Actinobacteria (range of 0.5-9.8% across treatments) > Cyanobacteria (range of 0.4-2.5%) > Proteobacteria (range of 0.4-1.2%) > Bacteroidetes (range of 0-0.03%). Conversely, ceca phyla were dominated by >96% Firmicutes. Phylum Actinobacteria (range of 0.2-3.4% across treatments) and Proteobacteria (range of 0.1-3.1% across treatments) together accounted for the remainder of the ceca phyla microbiota composition. Phylum Bacteroidetes were not reported in the ceca. At the genus taxa, the ileal microbiota was dominated by ~ 54% *Lactobacillus*, with a 43-65% relative abundance across treatment groups. Other predominant genera in the ileum included *Streptococcus* > *Enterococcus* > *Romboutsia* > *Clostridium sensu_stricto_1* > *Lachnospiraceae Sp*. > *Candidatus Arthromitus* > *Faecalibacterium* > *Peptostreptococcaceae Intestinibacter.* Unlike the ileum, the ceca were dominated by ~ 48% genus *Ruminococcaceae Faecalibacterium,* with a 39-53% relative abundance across treatment groups. Other predominant genera in the ceca followed the order *Lachnospiraceae Sp*. > *Streptococcus* > *Romboutsia* > *Ruminococcaceae Sp.* > *Lactobacillus* > *Peptostreptococcaceae Intestinibacter* > *Clostridium sensu_stricto_1* > *Enterococcus.* Concurrently, significant differences in the cumulative proportions of bacteria in the genus *Enterococcus* in the ileum were observed (Figure 4.7a). While the in-feed antibiotic treatment increased ($P = 0.02$) the proportion of this bacteria compared to the in-feed probiotics and *in ovo* probiotic 1 treatment, other treatments recorded statistically intermediate proportions of bacteria in this genus. Similarly, in the ceca, significant differences in the cumulative proportion of bacteria were only detected in the genus *Streptococcus* (Figure 4.7b). The in-feed antibiotic treatment reduced ($P = 0.03$) the proportion of bacteria in this genus compared to the in-feed probiotics treatment. Other treatments recorded statistically intermediate proportions of bacteria in this genus.

Figure 4.5 Gut microbiota composition at the phylum taxa.

For both (a) ileal and (b) cecal digesta in broiler chickens with treatment groups- A) Negative Control treatment- chicks fed a basal corn-soybean meal-wheat–based diet; (B) In-feed antibiotics- chicks fed NC + 0.05% bacitracin methylene disalicylate; (C) In-feed probiotics - chicks fed NC + 0.005% *Bacillus subtilis* containing 1 x 10^8 CFU/kg of feed; (E) *In ovo* probiotics group 1- eggs injected with 10 × 10^6 CFU of *Bacillus subtilis* fermentation extract /0.2 mL saline diluent; and (F) *In ovo* probiotics group 2- eggs injected with 20 × 10^6 CFU of *Bacillus subtilis* fermentation extract /0.2 mL saline diluent.

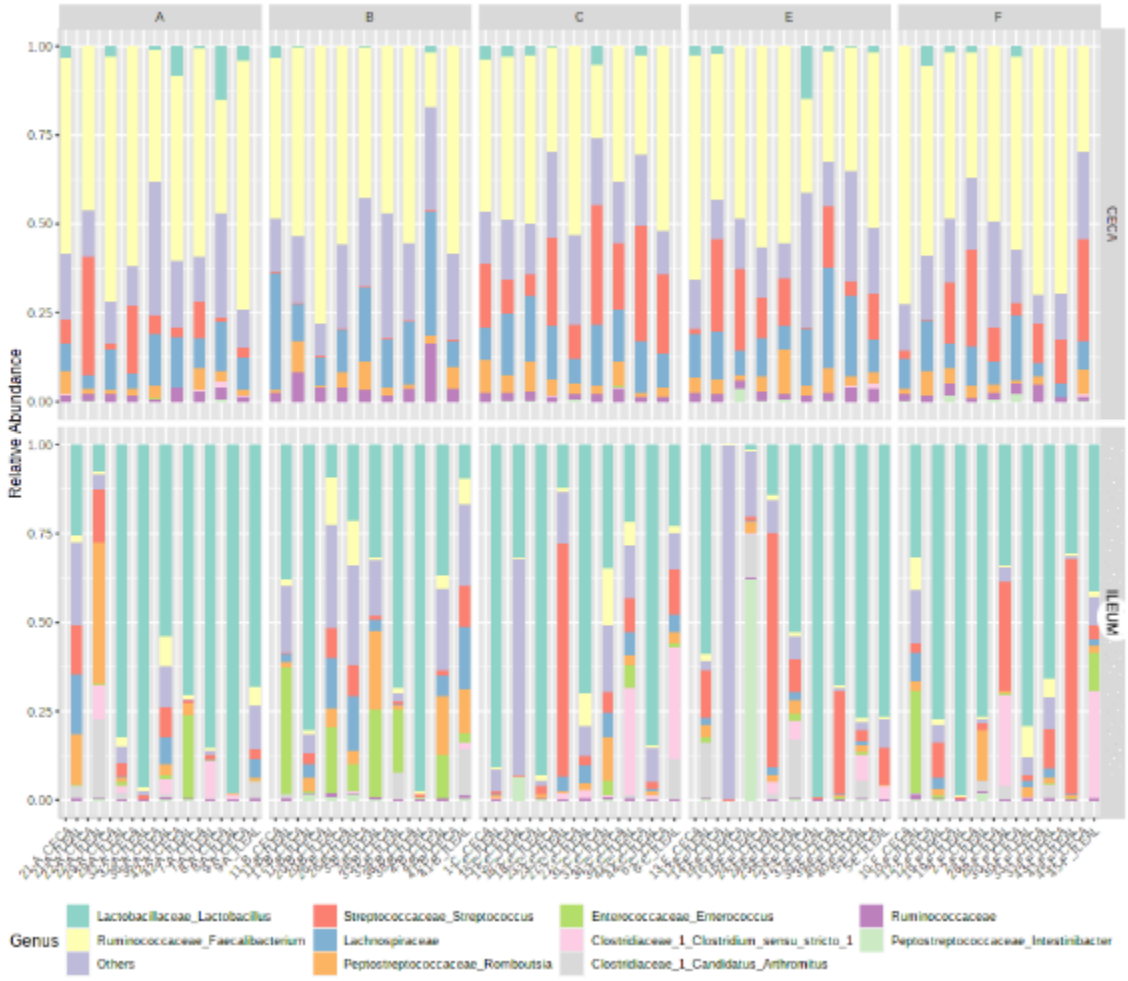


Figure 4.6 Gut microbiota composition at the genus taxa

For both (a) ileal and (b) ceca digesta in broiler chickens with treatment groups- A) Negative Control treatment- chicks fed a basal corn-soybean meal-wheat–based diet; (B) In-feed antibiotics- chicks fed NC + 0.05% bacitracin methylene disalicylate; (C) In-feed probiotics- chicks fed NC + 0.005% *Bacillus subtilis* containing 1 x 10^8 CFU/kg of feed; (E) *In ovo* probiotics group 1- eggs injected with 10 × 10^6 CFU of *Bacillus subtilis* fermentation extract /0.2 mL saline diluent; and (F) *In ovo* probiotics group 2- eggs injected with 20 × 10^6 CFU of *Bacillus subtilis* fermentation extract /0.2 mL saline diluent.

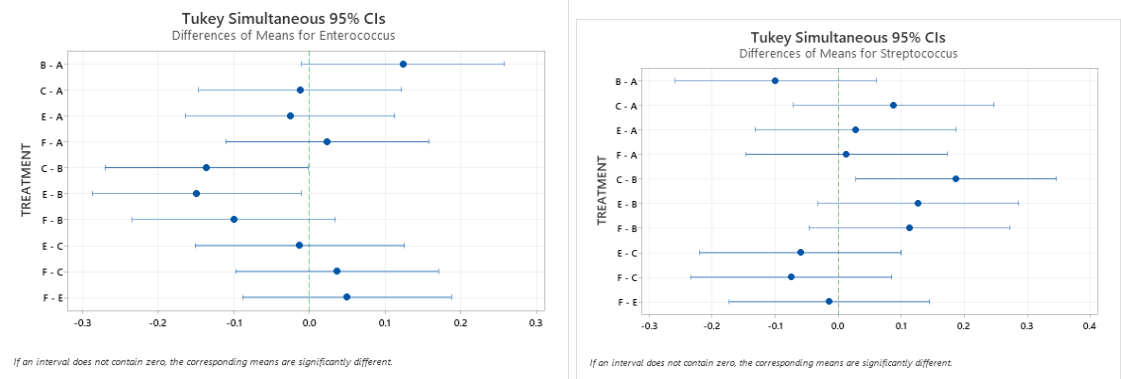


Figure 4.7 Significant differences in cumulative proportions of bacteria.

In the genera (a) *Enterococcus* in the ileum (ANOVA, $P = 0.023$) and (b) *Streptococcus* in the ceca of broiler chickens (ANOVA, $P = 0.031$) under different treatment groups. Treatment groups include A) Negative Control treatment- chicks fed a basal corn-soybean meal-wheat–based diet; (B) In-feed antibiotics- chicks fed NC + 0.05% bacitracin methylene disalicylate; (C) In-feed probiotics- chicks fed NC + 0.005% *Bacillus subtilis* containing 1 x 10^8 CFU/kg of feed; (E) *In ovo* probiotics group 1- eggs injected with 10×10^6 CFU of *Bacillus subtilis* fermentation extract /0.2 mL saline diluent; and (F) *In ovo* probiotics group 2- eggs injected with 20×10^6 CFU of *Bacillus subtilis* fermentation extract /0.2 mL saline diluent.

4.5 Discussion

The use of *Bacillus subtilis* probiotic strains as prospective alternatives to AGPs due to their spore-forming, immunomodulatory and antibacterial properties continue to gain momentum in the poultry industry (Duc et al., 2004; Griggs and Jacob, 2005). Nonetheless, like other competitive exclusion cultures, strain-specific properties like proteolytic activity, toxin-producing capacity, inoculation dose, and delivery routes are potential factors that could limit their efficacy (Edens et al., 1997; Peebles, 2019). Using selected parameters and direct comparison to an AGP (Bacitracin); this study thus attempts to validate the optimum dose (10×10^6 CFU vs. 20×10^6 CFU) and delivery route (*in ovo* vs. in-feed) of *Bacillus subtilis* that qualify it as an effective alternative to AGP.

This study revalidates previous reports from our laboratory (Oladokun et al., 2021a) that showed that amniotic delivery of *Bacillus subtilis* fermentation extract at embryonic day 18 had no negative effect on embryo viability and hatchability. Both *in ovo* probiotics treatments in this study recorded ~ 96% hatchability, similar to the non-injected eggs. Consistent with the result reported here, other studies (Edens et al., 1997; Pender et al., 2016, 2017; Majidi-Mosleh et al., 2017a; b; Castañeda et al., 2020; Alizadeh et al., 2020) have also affirmed no adverse effect of *in ovo* delivered probiotics on hatchability. Contrastingly, although dependent on the broiler chicken strain, probiotic strain, injection site, and injection dose (De Oliveira et al., 2014; El-Moneim et al., 2020; Leão et al., 2021), a few studies (Meijerhof and Hulet, 1997; Triplett et al., 2018) have reported reduced hatchability following *in ovo* delivery of probiotics. Besides, Uni and Ferket (2003) patent has previously recommended that amniotic delivery of enteric modulators between embryonic days 17 and19 does not impair hatchability, as the developing embryo maximizes the *in ovo* delivered substances at this time-point.

Additionally, chicks hatched from the non-injected eggs treatment in this study recorded reduced ratio of chick bodyweight to egg weight compared to the *in ovo* saline and *in ovo* probiotics 2 treatments. Several factors including egg size (Wilson, 1991; Tahir et al., 2011), length of egg storage (Lapão et al., 1999), post-hatch chick-holding time (Pinchasov, 1991; Reis et al., 1997), and age of breeder flock (Leão et al., 2021) are reported to influence the ratio of chick body weight to egg weight. Older breeder flocks are known to lay heavier eggs, and heavier eggs usually undergo less dehydration leading to a high chick body weight to egg weight ratio. Considering that all eggs in this study were sourced from the same source and underwent similar

incubation and post-hatch conditions, the observed result might not be attributed to the variabilities associated with egg source, egg storage, or post-hatch handling condition. Additionally, although randomly allotted, the average weight of non-injected eggs in this study was at least 0.2% heavier than other treatments (data not shown), suggesting that egg size could also not have influenced the observed result. Nonetheless, chick body weight to egg weight ratio recorded for all treatments in this study were within the normal range (62-76%) for broiler chickens reported by Kumar et al. (1994). Despite the foregoing, it would be important to limit the use of small-sized eggs in current hatchery practice, as this has practical implications on bird hatch weight and subsequent market weights.

Furthermore, in this study, at the end of the total trial period (d35) and week 5 especially, all probiotics treatments (irrespective of delivery routes) recorded similar feed conversion efficiency ($P > 0.05$) as the antibiotic treatment, with similar or less feed intake ($P < 0.1$). Several studies have affirmed the role of AGP (especially BMD) in improved growth performance (especially via increased AFI) in poultry (Gadde et al., 2017a; N. Karthikeyan et al., 2017; Walters et al., 2019). On the other hand, probiotics (whether in-feed or *in ovo*) are theorized to improve growth performance in poultry by positively modulating the gut microbiota in favour of host's nutrient utilization and energy uptake (Furuse and Yokota, 1985). Notwithstanding, variable results on the effect of probiotics (especially *Bacillus subtilis*) supplementation on growth performance are reported in the literature. Consistent with the results presented here, a number of studies (Knap et al., 2011; E Malik et al., 2016; Majidi-Mosleh et al., 2017b; a; Duneska and Bustillo, 2020; Castañeda et al., 2021) have reported no significant effect of *Bacillus subtilis* delivered across several routes (in-feed, in-water, or *in ovo*) on ABWG in broiler chickens. Conversely, improved ABWG following probiotics supplementation across several routes has also been reported in poultry (Aliakbarpour et al., 2012; Sen et al., 2012; Jeong and Kim, 2014; Gadde et al., 2017b; Hayashi et al., 2018). A plethora of factors, including probiotic viability, diet interaction, bird's genetic potential, and environmental or stress status, could account for the inconsistency in probiotics effect on growth performance recorded across the literature (Patterson and Burkholder, 2003; Mountzouris et al., 2007b; Flint and Garner, 2009). Additionaly, it has been speculated that a single time point delivery of *Bacillus subtilis* via the *in ovo* route might only guarantee a transient beneficial effects in the chicken gut (Latorre et al., 2014; Bernardeau et al., 2017). Both Patterson and Burkholder (2003) and Nunes et al. (2012) have submitted that significant improvement in growth performance following probiotics supplementation is mostly

feasible in evironmental or imunological challenged birds producing below their genetic potential. Although, the birds utilized in this study have been genetically selected for high growth performance, it is interesting to note that all probiotics treatments recorded at most 3-point less FCR values at d35, compared to the performance objectives metric recommended by the breeders (Cobb, 2018).

Similar to the results on growth performance, all probiotic treatments (regardless of delivery routes) in this study reduced ($P < 0.001$) the concentration of serum IgG compared to the control treatment. Serum immunoglobulins are reflective of the humoral immune status of the bird. Despite the considerable number of reports in the literature that have reiterated the immunomodulatory role of probiotics (Haghighi et al., 2006; Bai et al., 2017; Pender et al., 2017; Royan, 2017), a complete mechanistic insight on the specific mode (s) of action is yet to be fully elucidated. A few of the prevailing rationale for the immunomodulatory role of probiotics in the literature will include increased antimicrobial peptide production (Royan, 2017), neutralizing dysbiosis (Cisek and Binek, 2014), mucosal immunostimulation (Nava et al., 2005), and increased antibody production against infectious antigens (Lee et al., 2007). Consistent with the report in the literature, Kabir et al. (2004) and Elkhouly et al. (2016) reported increased antibody production in broiler chickens exposed to sheep red blood cells and pathogenic antigen challenges. On the contrary, considering that birds in this study were raised under experimental conditions and were not subjected to any form of challenge, it is rational to speculate that the reduced levels of serum IgG might be a result of probiotics elimination of pathogenic agents that could have resulted in increased production of natural antibodies. This is corroborated by the report of Munyaka et al. (2012), a similar unchallenged study with broiler chickens. Nothwithstanding, more studies are needed to provide a broader understanding of the immunomodulatory mechanisms of probiotics in poultry.

With regards to blood biochemistry indices, the in-feed antibiotics treatment recorded increased ($P < 0.05$) levels of blood plasma sodium and chloride, compared to the negative control treatment. The blood is often considered a window to the health status of the bird. This report's findings are consistent with recent data from our laboratory (Oladokun and Adewole, 2022a), which also demonstrates that the use of in-feed antibiotics raises the levels of both electrolyte minerals. While all evaluated blood biochemical indices are in the range of published values for healthy broiler chickens (Ilo et al., 2019), both electrolyte minerals were within the upper limit of those ranges (Leeson and Summers, 2001). The effect of antibiotics on the levels of these blood

minerals is largely unreported in the literature. However, excessive levels of these minerals in the blood have been linked with the maladies of acidosis, immunosuppression, and poor bone health (Oviedo-Rondón et al., 2001; Pohl et al., 2013). This study may thus offer even another reason to promote the cessation of AGP use in poultry.

In terms of gut morphology, treatment effects were quite variable in this study. Broiler chicken growth rate has been correlated with its gut morphological development (Smith et al., 1990), as the gut is predicted to account for about 1.5% of body weight (Faruq et al., 2019). In this study, both *in ovo* probiotic treatments and in-feed antibiotics treatment improved duodenal morphology, compared to the negative control treatment, as evidenced by increased ($P < 0.001$) villus width and villus height to crypt depth ratio. In the jejunum, the in-feed antibiotic treatment recorded the highest ($P < 0.001$) villus height to crypt depth ratio compared to other treatments. In the ileum, all treatments except the *in ovo* probiotics 1 treatment recorded higher ($P < 0.001$) villus height to crypt depth ratio than the negative control treatment. Nonetheless, in terms of improved ileal morphology, as evidenced by villus height to crypt depth ratios, both levels of *in ovo* delivered probiotics displayed statistical similarity. The almost identical growth performance indices observed in this study could be potentially explained by the statistical comparability for evaluated gut morphological indicators demonstrated by most treatments. Although the jejunum is thought to be the primary location of nutrient absorption in the intestine (Zeinali et al., 2017), broiler chickens' duodenum and ileum also play important roles in the digestion and absorption of protein, lipids, fat-soluble vitamins, and starch (Svihus, 2014). Increased villus height and villus height to crypt depth ratio are indicators of higher epithelial cell turnover and a well-differentiated intestinal mucosa, usually suggestive of increased digestive and absorptive ability (Jeurissen et al., 2002). Numerous studies (Viveros et al., 2011; Khodambashi Emami et al., 2012; Adewole and Akinyemi, 2021; Akinyemi and Adewole, 2022a) have already documented the beneficial effects of AGP (particularly BMD) on the gut, which are frequently linked to their antibacterial and gut microbiota-modulating capabilities. In agreement with the result presented here, probiotics have also been shown to have a positive effect on broiler chicken gut morphological indices, in numerous studies (Awad et al., 2008, 2010; Aliakbarpour et al., 2012; Deng et al., 2012; Xiang et al., 2019; Castañeda et al., 2020; El-Moneim et al., 2020; Bogusławska-Tryk et al., 2021). Neverthelesss, it is inferable from these studies that this beneficial effect might be dependent on probiotic strains and delivery routes, with lactic acid-based probiotics and *in ovo* delivery routes affording the most benefits. Probiotics are thought to exert this beneficial effect through

competitive exclusion of pathogens (which occurs early enough in the case of *in ovo* delivery) (Vieco-Saiz et al., 2019; Castañeda et al., 2020).

As highlighted in the introductory section, one of the benefits derivable from *in ovo* delivery of probiotics is the advantage of colonizing the gut microbiota with beneficial microbes very early on, rather than trying to alter an already established microbiota in later life. In this study, the different evaluated gut sections (i.e., ileum and caecum) revealed distinct microbial diversity (alpha-Shannon index), with the ceca recording higher diversity compared to the ileum. However, treatments had no significant effect on alpha diversity index across both gut sections in this study. This result is in conformation with prevailing knowledge in the literature that microbial diversity is higher in the ceca compared to the ileum as a result of higher fermentation activity (Yeoman and White, 2014; Mohd Shaufi et al., 2015; Qin et al., 2018; Oladokun et al., 2022). Similarly, other studies (Chang et al., 2020; Oladokun et al., 2021a; Zhang et al., 2021; Guo et al., 2021; Deng et al., 2022; Memon et al., 2022) involving probiotics supplementation have also reported no significant effect of probiotics (irrespective of delivery routes) on alpha diversity indices. According to Thibodeau et al. (2015), only extreme events that distort the number of ecological niches across bacterial species can modify alpha diversity indices. Beta diversity analysis also showed no variation in microbial community structure between treatments at the ileum and caecum, but there were clear differences in bacterial community profile across both gut sections. Oladokun et al. (2021a) have previously reported that *Bacillus subtilis* supplementation across several routes does not cause a shift in beta diversity. Asides from differences in gut sections and nutrition, other potential factors that could cause a shift in beta diversity include broiler chicken age, breed, and environmental/stress condition (Stanley et al., 2014; Oakley et al., 2014; Choi et al., 2015). Regarding microbiota composition, phylum Firmicutes, Actinobacteria and Proteobacteria were the dominant taxa across both ileum and caecum. Similar findings have been reported in ileal and cecal samples from probiotic-supplemented broiler chickens (Wang et al., 2017; Oladokun et al., 2021a; Memon et al., 2022).

Furthermore, the in-feed antibiotics treatment increased the abundance of bacteria in the genus *Enterococcus* in the ileum compared to the in-feed probiotics and *in ovo* probiotic 1 treatment. The genus *Enterococcus* potentially consists of harmful and beneficial species. For instance, Tortuero (1973) has previously reported that the implantation of probiotics *Lact. acidophilli* to leghorn chicks could promote bacterial antagonists that would subsequently inhibit the abundance of bacteria in the genus *Enterococci*, dubbed to cause a “fat malabsorption

syndrome". Contrarily, *Enterococcus faecium* is an important lactic acid-producing bacteria famous for its use as probiotics in poultry production (Yu, 2018; Zheng et al., 2020). Beneficial effects associated with *Enterococcus faecium* includes pathogen exclusion, improved host immunocompetence, improved feed conversion ratio, and weight gain, and enhanced antioxidant status (Capcarova et al., 2011; Kreuzer et al., 2012; Wu et al., 2019). Feed additives, including probiotics, antibiotics, and anticoccidials, have all been reported to enhance the abundance of bacteria in the genus *Enterococcus* in healthy broiler chickens (Lu et al., 2003). Additionaly, in the cecum, the proportion of bacteria in the genus *Streptococcus* was reduced by the in-feed antibiotic treatment compared to the in-feed probiotics treatment. Similar to the genus *Enterococcus*, the activities of bacteria in the genus *Streptococcus* might also be species-specific. *Streptococcus jaecalis* has been implicated in the incidence of "fat malabsorption syndrome", which was counteracted with antibiotics supplementation (Huhtanen and Pensack, 1965). Conversely, a few studies have also reported the capacity of *Streptococcus thermophilus* to enhance gut integrity (Briskey et al., 2016; Zeng et al., 2017). Consistent with the result observed here, Bauer et al. (2019) have reported that oregano supplementation (1% w/v) on microbial cell cultures obtained from the cecum of broiler chickens significantly reduced bacteria in the genus *Streptococcus*. Given the healthy state of the flock in this study, it is probable that treatments in this study might have enhanced the abundance of beneficial species of both genera.

4.6 Conclusions

This study has successfully revalidated that amniotic delivery of *Bacillus subtilis* fermentation extract at embryonic day 18 has no adverse effect on embryo viability and hatchability. *In ovo* delivered *Bacillus subtilis* in this study recorded ~ 96% hatchability. All *Bacillus subtilis* treatments (independent of delivery routes and dose) were mostly comparable to the in-feed antibiotics treatment in their ability to ensure gut microbiota homeostasis, enhanced gut morphology, and feed conversion efficiency, even while consuming similar or less feed. Similarly, all *Bacillus subtilis* treatments reduced serum IgG concentrations compared to the negative control treatment. However, the *in ovo* delivered *Bacillus subtilis* treatments showed the lowest numerical decrease in serum IgG concentrations, suggesting that *Bacillus subtilis* (especially *in ovo* delivered) might provide broiler chickens with better immunological protection by neutralizing pathogenic organisms that could result in the production of natural antibodies, without adversely

affecting hatch and growth performance. As the results obtainable for both *in ovo Bacillus subtilis* delivered treatments were mostly comparable for most of the evaluated parameters, the *in ovo* probiotics 1 (10×10^6 CFU) treatment might thus be a more practical option from an economic standpoint. Nonetheless, it would be important for further research to determine if indeed immunological protection is conferred on broiler chickens supplemented with this treatment under some sort of immunological or environmental challenge conditions.

5 CHAPTER 5 ESSENTIAL OIL DELIVERY ROUTES

ESSENTIAL OIL DELIVERY ROUTE: EFFECT ON BROILER CHICKEN'S GROWTH PERFORMANCE, BLOOD BIOCHEMISTRY, INTESTINAL MORPHOLOGY, IMMUNE, AND ANTIOXIDANT STATUS

This section has been published and presented elsewhere:

- Oladokun, S., MacIsaac, J., Rathgeber, B. and Adewole, D., 2021. Essential Oil Delivery Route: Effect on Broiler Chicken's Growth Performance, Blood Biochemistry, Intestinal Morphology, Immune, and Antioxidant Status. Animals, 11:3386. https://doi.org/10.3390/ani11123386

- Oladokun, S., MacIsaac, J., Rathgeber, B. and Adewole. 2021. Successive delivery of essential oil via *in ovo* and in-water route improves broiler chicken blood biochemical and antioxidant status without altering growth performance. Oral Presentation. Poultry Science Association (PSA) Annual Meeting, July 19-22.

5.1 Abstract

This study evaluated the effect of an essential oil (EO) blend and its delivery routes on broiler chicken growth performance, blood biochemistry, intestinal morphology, and immune and antioxidant status. Eggs were incubated and allotted to 3 groups: non-injected group, *in ovo* saline group, and *in ovo* essential oil group. On day 18 of incubation, essential oil in saline or saline alone was injected into the amnion. At hatch, chicks were assigned to post-hatch treatment combinations (1) *in ovo* essential oil + in-water essential oil (*in ovo* + in-water EO); (2) *in ovo* essential oil (*in ovo* EO); (3) *in ovo* saline; (4) in-water essential oil; (5) in-feed antibiotics (Bacitracin methylene disalicylate) and (6) a negative control (NC; corn-wheat-soybean diet) in 8 replicate cages (6 birds/cage) and raised for 28 day. The *in ovo* EO group reduced ($P < 0.05$) chick length and hatchability, all groups recorded no difference in growth performance at 0–28 day. The *in ovo* + in-water EO treatment reduced ($P < 0.05$) blood creatine kinase and aspartate aminotransferase levels whilst increasing ($P < 0.05$) total antioxidant capacity in birds. The *in ovo* + in-water delivery of EO might represent a potential antibiotic reduction strategy for the poultry industry but more research is needed to address the concern of reduced hatchability.

5.2 Introduction

The poultry meat industry is growing fast and is the cheapest source of animal protein for humans (Stiborova et al., 2020). This substantive growth in the poultry industry has, over the years, been facilitated by the sub-therapeutic use of antibiotic growth promoters (AGPs) (Mohebodini et al., 2021). The supplementation of AGPs at sub-therapeutic levels is broadly used to improve the growth rate, feed efficiency, and reduce morbidity and mortality in poultry birds (Zeng et al., 2015). However, the continuous use of AGPs in the poultry industry has come under scrutiny due to public health concerns bordering on the emergence of antibiotic resistance (Diarra and Malouin, 2014). Consequently, a few countries have instituted restrictions against the use of AGPs in the poultry industry. For example, the European Union (EU) banned AGPs as far back as 2006 (Castanon, 2007). The US Food and Drug Administration (FDA) also issued industry guidance on the prohibition of voluntary labeling of medically important animal drugs for animal growth promotion in 2013 (FDA, 2013). Canadian poultry producers eliminated the preventive use of category 1 and 2 antibiotics in 2014 and 2018, respectively (Chicken Farmers of Canada, 2021), while China also banned the use of AGPS in 2020 (Su et al., 2021). To preserve the potency of medically important antibiotics for human use, prevent the emergence of public health risks associated with the use of AGPs, satisfy increased consumer demands for antibiotic-free poultry products, and to sustain increased poultry production trends, there is a dire need for the development of safe, cost-effective, eco-friendly, and effective alternatives to AGPs for the poultry industry.

Several bioactive substances are being evaluated as potential alternatives to AGPs as reviewed by Gadde et al. (2017). These bioactive substances include probiotics, prebiotics, symbiotics, organic acids, enzymes, and several phytogenic feed additives (PFAs). The solid, dried, ground form or extracts from plants constitute these PFAs. Based on the extraction procedures, PFAs can be broadly classified as oleoresins (extracts derived by non-aqueous solvents) and essential oils (EOs; extracts obtained by cold, steam, or alcohol distillation) (Windisch et al., 2008; Van Der Klis and Vinyeta-Punti, 2014). Although the major component of most EOs, such as thymol, carvacrol, and eugenol, are phenolic compounds (terpenoids and phenylpropanoids) (Bassolé and Juliani, 2012), EOs vary in individual chemical compositions and concentrations. For example, as low as 3% and as high as 60%, thymol and carvacrol have been reported as the total EO in thyme (Lawrence and Reynolds, 1984) and a cinnamaldehyde range of 60% to 75% in cinnamon EOs (Duke, 2002). The activity of EOs is strongly associated with their

chemical composition, functional groups, and synergistic interactions between components (Nazzaro et al., 2013; Adaszyńska-Skwirzyńska and Szczerbińska, 2017). Common aromatic oils utilized in poultry production include oils from garlic (*Allium sativum*), oregano (*Origanum vulgare*) turmeric, (*Curcuma longa*), lemon balm (*Melissa officinalis*), peppermint (*Mentha piperita*), star anise fruit (*Illicium verum*), cinnamon (*Cinnamomum zeylanicum*), rosemary (*Rosmarinus officinalis*), and thyme (*Thymus vulgaris*) (Bolukbasi, 2008; Faramarzi et al., 2013; Drăgan et al., 2014; Feizi et al., 2014; Reyer et al., 2017).

To explore a synergistic effect, commercial combinations, or a blend of several EO types is becoming increasingly popular. Across the literature, several in vitro studies have highlighted the antibacterial, antiviral, antifungal, antimycotic, antiparasitic, insecticidal, antioxidant, anti-inflammatory, anti-toxigenic, anti-quorum-sensing, and immune-regulating properties of EOs (Devi et al., 2010; Gopi et al., 2014; Swamy et al., 2016; Stevanović et al., 2018). Contrastingly, in vivo results reported in the literature on the effect of EOs on poultry performance are somewhat inconsistent. While a few studies have reported the positive effect of EOs on poultry performance, digestive function, immune response, antioxidant capacity, and meat quality (Jamroz et al., 2003; Murugesan et al., 2015; Barbarestani et al., 2020), other studies have equally recorded poor (Khosravinia, 2016) or no effect of EOs on poultry production parameters (Case et al.,1995; Botsoglou et al., 2002; Lee et al., 2003; Hernandez et al., 2004; Shanmugavelu et al., 2004; Jang et al., 2007; Akbarian et al., 2015; Wan et al., 2017). These inconsistencies in the efficacy of EOs have been associated with the limitations that characterize their mode of delivery (Balia et al., 2014; Heydarin et al., 2020), as most EOs are conventionally supplied via feed or water to poultry birds. These conventional routes limit the efficacy of EOs because EOs are extremely volatile, easily degradable, and sensitive to environmental variables (Zeng et al., 2015; Heydarin et al., 2020). For example, when supplied in the diet, pelleting temperature of 58 °C have been reported to cause considerable loss of EO activity (Maenner et al., 2011). Additionally, EOs may potentially interact with the composition of basal diets, hence limiting their efficacy (Botsoglou et al., 2004; Basmacioğlu Malayoğlu et al., 2010; Mountzouris et al., 2011). On the other hand, when supplied via the in-water route, the efficacy of EOs will depend on the quality of the water, and the quality of chick watering devices.

To overcome the identified challenges that characterize conventional delivery routes and, by extension, the efficacy of EOs; *in ovo* delivery has been proposed. *In ovo* technology has been defined as “the direct inoculation of bioactive substances to the developing embryo to elicit

superior lifelong effects, while considering the dynamic physiology of the chicken embryo" (Oladokun and Adewole, 2020). This mode of delivery offers a few advantages over conventional delivery routes. These advantages include an economic benefit, as fewer bioactive substances are reportedly needed to elicit similar performance-enhancing effects as conventional routes (Bednarczyk et al., 2016; Tavaniello et al., 2018). Additionally, *in ovo* delivery also offers scope for early immunomodulatory programming and nutritional intervention in chicks (Oladokun and Adewole, 2020). Interestingly, research on the *in ovo* delivery of EOs in poultry is relatively scarce in the literature.

Accordingly, the objective of this study was to evaluate the effect of the *in ovo* delivery of a commercial EO blend (containing star anise, cinnamon, rosemary, and thyme oil) on hatch and growth performance, immune and antioxidant status, blood biochemistry, and intestinal morphometric properties in broiler chickens, compared to conventional delivery routes. This is the first study evaluating the efficacy of *in ovo* delivered EOs compared to AGPs within the limits of the available literature. This study also sought to evaluate if an additive benefit exists from the successive delivery of EOs via the *in ovo* and continuous in-water delivery routes. From available knowledge, this is also the first study seeking to evaluate such an effect.

5.3 Materials and Methods

The experiment was carried out at the hatchery facility of the Agricultural Campus of Dalhousie University and the broiler rearing facility of the Atlantic Poultry Research Center, Dalhousie Faculty of Agriculture. All experimental procedures were approved by the Animal Care and Use Committee of Dalhousie University (Protocol number: 2020-035), following guidelines of the Canadian Council on Animal Care (CCAC, 2020).

5.3.1 Egg Incubation and *In ovo* Injection Procedure

A total of 670 hatching eggs with an average weight of 77.87 ± 2.43 g (mean ± SE) from 41-week-old Cobb 500 broiler breeders were sourced from a commercial hatchery (Synergy hatchery) in Nova Scotia, Canada. Eggs were incubated in a ChickMaster single-stage incubator (ChickMaster G09, Cresskill, NJ, USA) under standard conditions (37.5 °C and 55% relative humidity) from embryonic days (EDs) 1 to 17, and then to an average of 32 °C and 68% from EDs 18 to 21. Incubators were preheated for 24 h prior to setting eggs to ensure that proper temperature and humidity were stable. Egg trays were turned on a 90° arc four times an hour from the time of set until ED 18. Eggs were arranged in 6 replicate trays inside the incubator, each tray containing 96

eggs. On ED 12, eggs were candled, and infertile eggs were disposed of, leaving a total of 576 eggs for the trial. The remaining eggs were subsequently assigned to one of three treatment groups: (1) non-injected eggs (control; 288 eggs); (2) *in ovo* saline group (96 eggs; injected with 0.2 mL of physiological saline, i.e., 0.9% NaCl); (3) *in ovo* essential oil group (192 eggs; injected with 0.2 mL of a saline + essential oil blend mixture at a dilution ratio of 2:1). The essential oil utilized in this study is a commercial blend (Probiotech International Inc., St Hyacinthe, Quebec, Canada) containing phytonutrients star anise, cinnamon, rosemary, and thyme oil. The EO blend is registered by Health Canada as a Veterinary Health Product (VHP). On ED 18, eggs were injected according to the procedure described by Oladokun et al. (2021), with slight modifications. Briefly, this involved disinfecting the eggs with 70% ethanol-dipped swabs and using an 18-gauge needle to carefully punch the shell at the center of the air cell (the blunt end). The injected EO was then delivered to the amnion using a self-refilling injector (Socorex ultra-1810.2.05005, Ecublens, Switzerland) equipped with a 22-gauge needle (injection needle length—3cm) at a 45-degree angle. After *in ovo* injection, the injection sites were sealed with sterile paraffin, and eggs were placed back in the incubator. The non-injected eggs were also taken out and returned to the incubator simultaneously as other injected treatment groups.

5.3.2 Birds, Housing, and Diets

As presented in Figure 5.1, hatchlings were weighed and randomly assigned to 6 new treatment groups. Chicks from the initial non-injection group were randomly allocated into four new treatment groups consisting of (1) chicks fed a basal corn-soybean meal-wheat–based diet (Negative Control treatment; NC); (2) chicks fed NC + 0.05% bacitracin methylene disalicylate (in-feed antibiotics); and (3) chicks supplied the same commercial blend of EOs as earlier described via the water route (in-water essential oil) at the recommended dosage of 250 mL/1000 L of drinking water. The initial *in ovo* saline and *in ovo* essential oil groups were placed on the control diet to form treatments (4) (*in ovo* saline treatment) and (5) (*in ovo* essential oil treatment), respectively. The last treatment group, (6) consisted of chicks from the *in ovo* essential oil treatment group also supplied EO via the water route (*in ovo* + in-water essential oil treatment). All treatment groups had 48 birds each. Birds were placed in battery cages (0.93 m × 2.14 m), there were 6 birds per cage and 8 replicate cages per treatment. To minimize variability, only the top-tier cages were used; each treatment group was evenly represented across a tier. Birds were reared for 28 d under uniform controlled environmental conditions in line with Cobb Broiler Management Guide recommendations. Room temperature was set at 31 °C on day 0 and gradually

reduced to 23 °C on day 28, and relative humidity ranged between 45 and 55%. Dietary treatments, ingredients, and nutritional composition are presented in Table 5.1. Birds were provided with feed and water ad libitum; diets were fed as mash throughout the rearing period, including the starter (0–14 d) and grower (15–28 d) phases. Diets were formulated to meet Cobb 500 broiler chicken nutrient requirements.

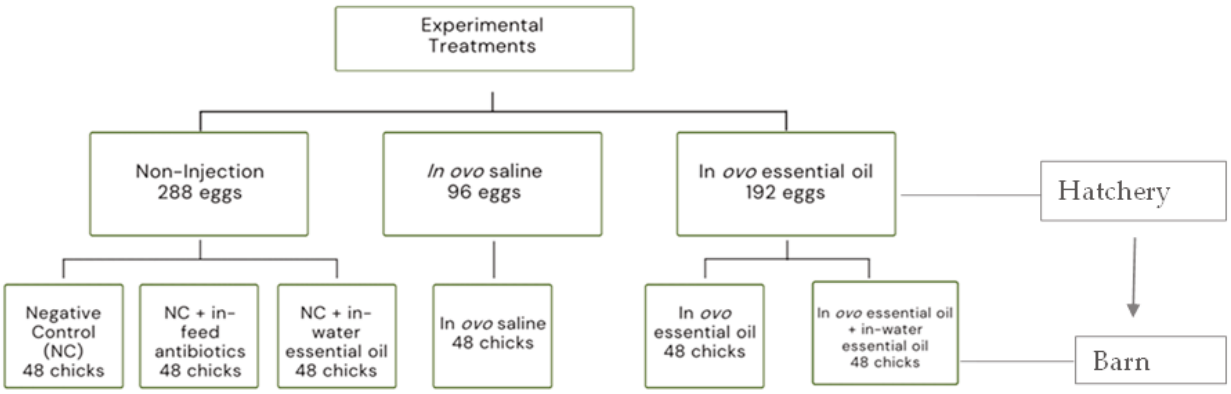


Figure 5.1 Schematic presentation of experimental structure in the hatchery and barn.

Table 5.1 Ingredients, calculated, and analyzed compositions of experimental diets1 (as-fed basis, percentage (%), unless otherwise stated).

Ingredients	Phases			
	Starter (0–14 d)		Grower (15–28 d)	
	Control Diet	Antibiotic Diet	Control Diet	Antibiotic Diet
Ingredient Composition				
Corn (ground)	51.08	50.98	45.36	45.25
Soybean meal-46.5	41.44	41.45	36.31	36.33
Wheat	-	-	10	10
Animal/vegetable fat	2.93	2.97	4.22	4.26
Limestone	1.80	1.80	1.65	1.65
Dicalcium Phosphate	1.24	1.24	1.06	1.06
DL Methionine premix [2]	0.59	0.59	0.53	0.53
Vitamin/Mineral Premix [3,4]	0.50	0.50	0.50	0.50
Salt	0.41	0.41	0.37	0.37
Lysine HCl	0.01	0.01	0.00	0.00
BMD 110G [5]	-	0.05	-	0.05
Total	100	100	100	100
Nutrient	Calculated composition			
Metabolizable energy (kcal/kg)	3000	3000	3100	3100
Crude protein	23	23	21.5	21.5
Calcium	0.96	0.96	0.87	0.87
Available phosphorus	0.48	0.48	0.44	0.44
Sodium	0.19	0.19	0.18	0.18
Digestible lysine	1.28	1.28	1.15	1.16
Digestible methionine + cysteine	0.95	0.95	0.87	0.87
Digestible Tryptophan	0.25	0.25	0.23	0.23
Digestible Threonine	0.89	0.89	0.82	0.82
	Analyzed composition			
Dry Matter	90.7	90.8	93.2	93.5
Crude protein	24.8	25	22.5	23.8
Crude fat	5.50	5.79	6.84	6.85
Calcium	1.06	1.13	1.00	0.96
Potassium	1.14	1.16	0.99	1.04
Phosphorus	0.69	0.70	0.67	0.62
Sodium	0.19	0.21	0.21	0.16

[1]Basal diet (NC); antibiotic diet containing NC + 0.05% bacitracin methylene disalicylate (BMD). [2] Supplied/kg premix: DL-Methionine, 0.5 kg; wheat middling, 0.5 kg. [3] Starter vitamin-mineral premix contained the following per kg of diet: 9750 IU vitamin A; 2000 IU vitamin D3; 25 IU vitamin E; 2.97 mg vitamin K; 7.6 mg riboflavin; 13.5 mg Dl Ca-pantothenate; 0.012 mg vitamin B12; 29.7 mg niacin; 1.0 mg folic acid, 801 mg choline; 0. 3 mg biotin; 4.9 mg pyridoxine; 2.9 mg thiamine; 70.2 mg manganese; 80.0 mg zinc; 25 mg copper; 0.15 mg selenium; 50 mg ethoxyquin; 1543 mg wheat middling's; 500 mg ground limestone. [4] Grower and Finisher vitamin-mineral premix contained the following per kg of diet: 9750 IU vitamin A; 2000 IU vitamin D3; 25 IU vitamin E; 2.97 mg vitamin K; 7.6 mg riboflavin; 13.5 mg Dl Ca-pantothenate; 0.012 mg vitamin B12; 29.7 mg niacin; 1.0 mg folic acid, 801 mg choline; 0. 3 mg biotin; 4.9 mg pyridoxine; 2.9 mg thiamine; 70.2 mg manganese; 80.0 mg zinc; 25 mg copper; 0.15 mg selenium; 50 mg ethoxyquin; 1543 mg

wheat middling's; 500 mg ground limestone. [5] Bacitracin methylene disalicylate (providing 55 mg/kg mixed feed); Alpharma, Inc., Fort Lee, NJ, USA.

5.3.3 Hatch Parameters and Chick Quality

Hatched chicks were counted and weighed individually. Hatchability was calculated as the percentage of hatched chicks to fertile incubated eggs per replicate. Hatched chick BW/initial egg weight ratio was also determined and recorded. Chick navel quality was evaluated by adopting the scoring method by Reijrink et al. (2009). Navel quality was scored 1—when the navel was completely closed and clean; scored 2—when the navel was discolored (i.e., when the navel color differs from the chick's skin color) with a maximum 2 mm opening; and scored 3—when the navel was discolored and with more than a 2mm opening. Chick length was obtained by placing the chick on its ventral side and measuring from the tip of the beak to the middle toe on the right leg.

5.3.4 Growth Performance Parameters and Sampling

Growth performance parameters—feed intake and average body weight (BW) were measured on a cage basis weekly. The average feed intake (AFI), average body weight gain (ABWG), and feed conversion ratio (FCR) were subsequently calculated from the obtained data. The FCR was calculated as the amount of feed consumed per unit of body weight gain. Cages were checked for mortality daily; dead birds were subsequently weighed and sent to the Nova Scotia Agriculture, Animal Health Laboratory for necropsy. Mortality weight was then used to correct the FCR.

On day 21, 1 bird per cage (8 replicate birds per treatment group) was randomly selected, weighed, and euthanized by electrical stunning and exsanguination. After euthanasia of the bird, blood samples were collected from each bird into 10 mL blood serum collection tubes (BD Vacutainer™ Serum Tubes, fisher scientific- BD366430) for serum immunoglobulins assay. After slaughter, the weights of the bursa of Fabricius and liver were also determined by trained personnel.

On day 28, 2 birds per cage (16 replicate birds per treatment group) were randomly selected and euthanized by electrical stunning and exsanguination. After euthanasia of the bird, blood samples were collected from each bird into 10 mL heparinized tubes (BD Vacutainer™ Glass Blood Collection Tubes with Sodium Heparin, fisher scientific- BD366480) for blood biochemistry and plasma total antioxidant assays. After slaughter, the small intestinal segments, including the jejunum (1.5-cm length midway between the point of entry of the bile ducts and Meckel's diverticulum) and ileum (1.5-cm length midway between Meckel's diverticulum and the

ileocecal junction) were excised and fixed in neutral buffered formalin (10%) for further histomorphological processing (Awad et al., 2009).

5.3.4.1 Relative Weight of Organs

The weights of the bursa of Fabricius and liver were recorded and then specified as a percentage of the live BW of the slaughtered chicken (g/Kg BW).

5.3.4.2 Serum Immunoglobulins

Serum samples were used to measure concentrations of immunoglobulins (IgG and IgM) using chicken-specific immunoglobulins enzyme-link immunosorbent assay (ELISA) quantitation kits (Bethyl Laboratories, Montgomery, TX, USA; Catalog No. E33-104-200218 and E33-102-180410, respectively) following manufacturer instructions. The values were determined on a microplate reader (Bio-Tek Instrument Inc., Wonooski, VT, USA) using a software program (KC4, version #3.3, Bio Tek Instruments). The four-parameter logistic model was used to extrapolate immunoglobulins concentration and absorbance readings.

5.3.4.3 Blood Biochemistry

Samples for blood biochemical analysis were centrifuged at 5,000× g at 4 °C for 10 min and shipped on ice to Atlantic Veterinary College, University of Prince Edward Island Pathology Laboratory, where samples were analyzed using cobas® 6000 analyzer series (Roche Diagnostics, IN, USA).

5.3.4.4 Total Antioxidant Capacity (TAC)

Total antioxidant capacity (TAC) in plasma was analyzed using the Oxiselect Total Antioxidant Capacity assay kit (STA360; Cell BioLabs Inc., San Diego, USA) following the manufacturer's instructions (Silva-Guillen et al., 2020). Absorbance was measured at 490 nm on a microplate reader (Bio-Tek Instrument Inc., Wonooski, VT, USA) using a software program (KC4, version #3.3, Bio Tek Instruments). Results were expressed as mM Uric acid equivalent.

5.3.4.5 Intestinal Morphology

The procedure for intestinal morphometric analysis was as described by Oladokun et al. (2021a). Briefly, fixed intestinal tissues were embedded in paraffin, sectioned (0.5 μm thick), and stained with hematoxylin and eosin for morphological examinations. In each cross-sectioned tissue, ten morphometric measurements including the villus height (from the base of the intestinal mucosa to the tip of the villus excluding the intestinal crypt), villus width (halfway between the base and the tip), crypt depth (from the base upward to the region of transition between the crypt and villi) (Ozdogan et al., 2014), per slide were carried out using Leica 1CC50 W microscope at 4×

Magnification (Leica Microsystems, Wetzlay, Germany) and an image processing and analysis system (Leica Application Suite, Version 3.4.0, Leica Microsystems, Wetzlay, Germany). The total mucosa thickness (villus height + crypt depth) was subsequently calculated from the obtained data.

5.3.5 Statistical Analysis

Hatch data were analyzed as a completely randomized design, while other datasets obtained were analyzed as a randomized complete block design, with cage-tiers being the blocking factor. The normality of all data sets was ascertained by testing residuals by the Anderson-Darling test in Minitab statistical package (v.18.1). Data sets found to be normal were subjected to one-way ANOVA in the same statistical package with experimental treatments as a factor and the relevant data sets as variables. Data sets found not to be normal, including plasma protein, globulin, and bile acids, were transformed using the reciprocal function. Data on plasma potassium, chloride, and magnesium were transformed using the reciprocal cube function, while plasma glucose and chloride were transformed by the square reciprocal function. Data on plasma alkaline phosphatase (ALP), uric acid, serum IgG were transformed using the natural log function. Data on plasma aspartate aminotransferase (AST), creatine kinase (CK), and urea were transformed using the logarithm base ten functions. Following data transformations, the transformed data were equally subjected to ANOVA procedures in the same statistical package, with appropriately back-transformed data presented. For hatchability parameters, hatching trays were the experimental units, and the pen was the experimental unit for growth performance parameters. Significant means were separated using Tukey's honest significant difference test in the same statistical package. Analyzed data were presented as means ± SEM and probability values. Values were considered statistically different at $P \leq 0.05$ and considered a statistical trend at $P < 0.1$.

5.4 Results

5.4.1 Hatch Performance and Chick Quality

The results on hatch performance and chick quality are presented in Table 5.2. No effect of treatment was recorded for the average chick weight and average navel score parameters. However, both hatchability and average chick length were significantly ($P < 0.05$) affected by treatments. The *in ovo* essential oil treatment recorded an 18.1 and 19.5% reduction in hatchability compared to the non-injected and *in ovo* saline treatment, respectively. Similarly, the *in ovo* essential oil treatment also recorded a 3.7 and 3.2% reduction in average chick length compared

to the non-injected and *in ovo* saline treatment, respectively. At the pretrial stage of this experiment, the ovo delivery of EO + saline increased chick weight and had no effect on chick length (Table 5.3).

Table 5.2 Effect of essential oil delivery route on hatch performance and chick quality.

Hatch Parameters	Treatments [1]			SEM [2]	*P* Value [3]
	Non-Injected	*In ovo* Saline	*In ovo* Essential Oil		
Hatchability (%)	95.3[a]	97.0[a]	78.1[b]	4.10	0.001
Average Chick Weight (g)	52.3	49.7	47.4	2.00	0.590
Average Chick Length (cm)	18.9[a]	18.8[a]	18.2[b]	0.11	0.002
Chick BW/ Egg Weight (%)	56.7	70.2	64.4	3.00	0.183
Average Navel Score	1.67	1.51	1.77	0.10	0.299

[1]Treatments include— (1) non-injected eggs; (2) *in ovo* Saline group- injected with 0.2 mL of physiological saline (0.9% NaCl); (3) *in ovo* essential oil group- injected with 0.2 mL of a saline + essential oil blend mixture at a dilution ratio of 2:1. [2] SEM = Standard error of means. [3] Means within a row with different superscripts [a, b] differs ($P < 0.05$).

Table 5.3 Effect of essential oil delivery route on hatch performance and chick quality at the pretrial stage.

Hatch Parameters	Treatments[1]				SEM[2]	*P* Value[3]
	Non-Injected	*In ovo* Saline	*In ovo* Essential oil	*In ovo* Essential oil + Saline		
Hatchability (%)	100[a]	81.5[ab]	79.5[b]	74.6[b]	3.18	0.008
Average Chick Weight (g)	42.1[ab]	35.1[bc]	31.7[c]	43.8[a]	1.52	<0.001
Average Chick Length (cm)	19.7	18.6	18.7	19.2	0.20	0.216

[1] Treatments include- 1) non-injected eggs; 2) *in ovo* Saline group- injected with 0.2 mL of physiological saline (0.9% NaCl); 3) *in ovo* essential oil group- injected with 0.2mL essential oil blend mixture containing phytonutrients star anise, cinnamon, rosemary, and thyme oil; 4) *in ovo* essential oil + saline group- injected with 0.2mL essential oil blend mixture (containing phytonutrients star anise, cinnamon, rosemary, and thyme oil) and saline (0.9% NaCl) solution at a dilution ratio of 1:1. Each treatment groups had 60 eggs each (average weight 78.4 ± 2.73 g; mean ± SE), sourced from Ross 308 broiler breeders. [2] SEM = Standard error of means. [3] Means within a row with different superscripts [a,b,c] differ ($P < 0.05$).

5.4.2 Growth Performance

Results on evaluated growth parameters are presented in Table 5.4. At the starter phase (d 0–14), antibiotic treatment recorded higher ($P < 0.05$) ABWG compared to all *in ovo*-delivered treatments (*in ovo* saline, *in ovo* EO, *in ovo* + in water EO). At the grower phase (d 15–28) and for the entire length of study (d 0–28), there was no treatment effect on evaluated growth performance parameters.

Table 5.4 Effect of essential oil delivery route on broiler chicken growth performance

Growth Performance Parameters	Treatments [1]						SEM [2]	*P* Value [3]
	Negative Control	In-Feed Antibiotics	In-Water Essential Oil	*In ovo* Saline	*In ovo* Essential Oil	*In ovo* Essential Oil + In-Water Essential oil		
				Average Feed Intake (g/bird)				
D 1–14	305	292	308	272	296	294	27.5	0.845
D 15–28	1297	1303	1497	1182	1166	1139	67.6	0.386
D 1–28	1599	1604	1790	1447	1461	1439	92.7	0.449
				Average Body weight gain (g/bird)				
D 1–14	294^{ab}	307^{a}	287^{abc}	246^{c}	253^{bc}	250^{bc}	7.68	0.040
D 15–28	950	994	931	869	852	857	26.7	0.345
D 1–28	1243	1301	1217	1110	1104	1104	32.9	0.161
				Feed conversion ratio				
D 1–14	1.07	0.97	1.09	1.14	1.22	1.23	0.11	0.116
D 15–28	1.38	1.30	1.53	1.43	1.43	1.39	0.09	0.574
D 1–28	1.31	1.23	1.43	1.38	1.38	1.37	0.09	0.463
				Average water intake (l)				
D 1–14	1.09	1.24	1.14	1.08	1.04	1.13	0.03	0.448
D 15–28	2.68	2.69	2.74	2.51	2.49	2.94	0.08	0.527
D 1–28	3.75	3.90	3.86	3.62	3.53	4.05	0.07	0.232

[1] Treatments include- (1) Negative Control treatment- chicks fed a basal corn-soybean meal-wheat–based diet; (2) In-feed antibiotics- chicks fed NC + 0.05% bacitracin methylene disalicylate and (3) In-water essential oil- chicks supplied the essential oil via the water route at the recommended dosage of 250 mL/1000 L of drinking water; (4) *In ovo* saline treatment- eggs injected with 0.2 mL of physiological saline (0.9% NaCl); (5) *In ovo* essential oil treatment- eggs injected with 0.2 mL of a saline + essential oil blend mixture at a dilution ratio of 2:1, (6) *In ovo* + in-water essential oil treatment- chicks offered the essential oil blend via the *in ovo* and in water route, successively. [2] SEM = Standard error of means. [3] Means within a row with different superscripts [a, b, c] differs ($P < 0.05$).

5.4.3 Relative Weight of Organic and Serum Immunoglobulins

Treatments recorded no significant effect on serum IgG and IgM levels in broiler chickens in this study (Table 5.5). Results on evaluated organ weights are also presented in Table 5.5. The relative weight of the bursa was equally not affected by treatments in this study. However, the *in ovo* + in-water essential oil treatment recorded a tendency ($p = 0.07$) to increase the relative weight of the liver compared to other treatments. The relative liver weight of birds in this treatment was 15% heavier than the NC treatment and at least 6% heavier than other treatments.

Table 5.5 Effect of essential oil delivery route on the relative weight of broiler chicken organs and serum immunoglobulins levels.

Parameters	Treatments [1]						SEM [2]	*P* Value
	Negative Control	**In-Feed Antibiotics**	**In-Water Essential Oil**	***In ovo* Saline**	***In ovo* Essential Oil**	***In ovo* Essential Oil + In-Water Essential oil**		
Bursa weight (g/Kg BW)	1.84	2.03	1.79	1.80	1.91	1.72	0.07	0.826
Liver weight (g/Kg BW)	27.5	27.5	29.3	30.0	29.2	31.8	0.51	0.066
Immunoglobulin G (Mg/mL)	0.74	0.44	0.78	1.29	0.76	0.68	0.31	0.189
Immunoglobulin M (Mg/mL)	0.11	0.07	0.12	0.15	0.11	0.08	0.02	0.289

[1]Treatments include- (1) Negative Control treatment- chicks fed a basal corn-soybean meal-wheat–based diet; (2) In-feed antibiotics- chicks fed NC + 0.05% bacitracin methylene disalicylate and (3) In-water essential oil- chicks supplied the essential oil via the water route at the recommended dosage of 250 mL/1000 L of drinking water; (4) *In ovo* saline treatment- eggs injected with 0.2 mL of physiological saline (0.9% NaCl); (5) *In ovo* essential oil treatment- eggs injected with 0.2 mL of a saline + essential oil blend mixture at a dilution ratio of 2:1, (6) *In ovo* + in-water essential oil treatment- chicks offered the essential oil blend via the *in ovo* and in water route, successively. [2]SEM = Standard error of means.

5.4.4 Blood Biochemistry

The effects of treatments on blood plasma biochemical characteristics are presented in Table 5.6. Blood enzymes— CK and AST, were significantly affected by treatments. *In ovo* saline and *in ovo* + in-water essential oil treatments, both significantly reduced ($p < 0.05$) plasma CK levels, compared to the in-feed antibiotics treatment. Nonetheless, the highest reduction in plasma CK levels was observed in the *in ovo* + in-water essential oil treatment; this was as much as about a 3-fold reduction, compared to the in-feed antibiotics treatment. The NC, in-water, and *in ovo* essential oil treatments recorded intermediate plasma CK levels. Compared to the NC and in-feed antibiotics treatments, blood plasma AST levels were significantly reduced ($p < 0.05$) by the in-water, *in ovo* saline, and *in ovo* + in-water essential oil treatments. Nevertheless, the *in ovo* + in-water essential oil treatment recorded the highest reduction in plasma AST level, 29.6% lower than the in-feed antibiotics treatment. Further-more, the *in ovo* + in-water essential oil treatment recorded a tendency ($p = 0.07$) to increase plasma calcium level by as much as 12.6%, relative to the NC treatment. All other evaluated blood plasma characteristics evaluated in this study were not affected by treatments.

Table 5.6 Effect of essential oil delivery route on plasma biochemical characteristics in broiler chickens.

Parameters	Treatments [1]						SEM [2]	*P* Value [3]
	Negative Control	In-Feed Antibiotics	In-Water Essential Oil	*In ovo* Saline	*In ovo* Essential Oil	*In ovo* Essential Oil + In-Water Essential Oil		
Electrolyte minerals ($mmol \cdot L^{-1}$)								
Sodium	150	142	143	145	145	151	1.96	0.561
Potassium	5.93	5.79	6.12	6.66	5.90	6.36	0.22	0.497
Sodium: Potassium	25.9	24.8	23.8	23.0	24.9	24.0	0.61	0.556
Chloride	110	105	106	106	107	112	1.28	0.565
Calcium	2.68	3.01	2.69	2.65	2.67	3.04	0.05	0.072
Phosphorus	1.78	1.66	2.01	1.92	1.73	1.78	0.07	0.690
Magnesium	0.87	0.86	0.78	0.82	0.77	0.84	0.03	0.519
Metabolites ($mmol \cdot L^{-1}$)								
Urea	0.29	0.30	0.26	0.31	0.22	0.32	0.02	0.352
Glucose	14.1	13.4	13.5	13.6	14.0	14.1	0.22	0.809
Cholesterol	3.34	2.91	2.72	3.08	3.18	3.42	0.09	0.233
Iron	15.8	14.1	14.4	16.6	15.1	17.9	0.71	0.589
Bile acids	15.7	17.7	13.8	14.0	18.8	19.2	3.79	0.393
Uric acid	328	327	342	383	364	396	16.3	0.622
Enzymes ($U \cdot L^{-1}$)								
Amylase	459	405	376	657	572	626	39.6	0.221
Alkaline phosphatase	4506	2614	4263	4879	4055	5157	398	0.173
Creatine kinase	4873^{ab}	7903^{a}	3100^{ab}	2541^{b}	4309^{ab}	2408^{b}	609	0.022
Aspartate Aminotransferase	180^{a}	186^{a}	144^{b}	143^{b}	165^{ab}	138^{b}	5.33	0.028
Gamma-Glutamyl Transferase	9.7	9.5	10.0	10.0	9.3	9.9	0.38	0.993
Lipase	24.5	22.2	21.4	21.5	19.5	27.7	1.46	0.661
Proteins ($g \cdot L^{-1}$)								
Total Proteins	28.1	23.7	24.1	25.9	24.7	27.8	0.80	0.336
Albumin	12.6	11.0	10.5	11.3	11.4	11.8	0.28	0.459
Globulin	15.7	12.9	13.5	14.5	13.5	16.0	0.58	0.268

Albumin: Globulin	0.76	0.84	0.78	0.78	0.83	0.75	0.02	0.547

[1]Treatments include- (1) Negative Control treatment- chicks fed a basal corn-soybean meal-wheat–based diet; (2) In-feed antibiotics- chicks fed NC + 0.05% bacitracin methylene disalicylate and (3) In-water essential oil- chicks supplied the essential oil via the water route at the recommended dosage of 250 mL/1000 L of drinking water; (4) *In ovo* saline treatment- eggs injected with 0.2 mL of physiological saline (0.9% NaCl); (5) *In ovo* essential oil treatment- eggs injected with 0.2 mL of a saline + essential oil blend mixture at a dilution ratio of 2:1, (6) *In ovo* + in-water essential oil treatment- chicks offered the essential oil blend via the *in ovo* and in water route, successively. [2]SEM = Standard error of means. [3]Means within a row with different superscripts [a, b] differs ($P < 0.05$).

5.4.5 Total Antioxidant Capacity (TAC)

The result on TAC is presented in Figure 5.2. The *in ovo* + in-water essential oil treatment significantly increased ($P < 0.05$) TAC in birds compared to the NC treatment. This increase in TAC in the *in ovo* + in-water essential oil treatment was as much as 5-fold the NC treatment. Other treatments recorded intermediate TAC values.

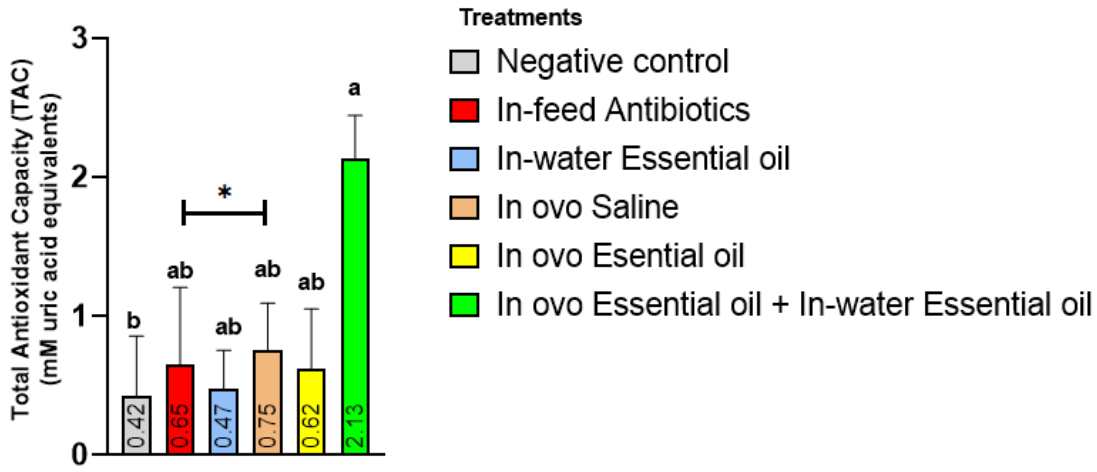


Figure 5.2 Effect of essential oil delivery route on broiler chicken's total antioxidant capacity (TAC). Bar charts with different letters a, b differ ($P < 0.05$).

5.4.6 Intestinal Morphology

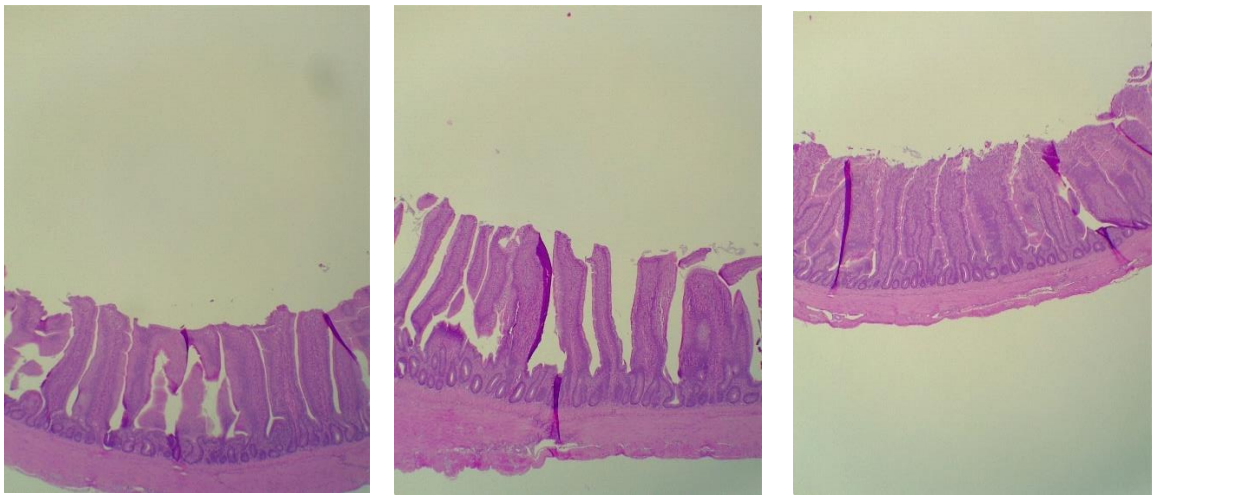
The morphology of the duodenum and ileum was significantly influenced by treatments in this study (Table 5.7). No effect of treatment on jejunum morphology was found. In the duodenum, the *in ovo* essential oil treatment recorded the longest ($P < 0.001$) villus compared to other treatments, except for the *in ovo* + in-water essential oil treatment. The *in ovo* + in-water essential oil treatment recorded intermediate duodenal villus length. The duodenal villus width of birds in the *in ovo* treatment group was only wider ($P = 0.01$) than those in the *in ovo* saline treatment group; all other treatments recorded intermediate duodenal villus width. Similarly, total mucosa thickness was also highest ($P < 0.001$) in the *in ovo* essential oil treatment compared to the in-feed antibiotic, in-water and *in ovo* saline treatments. Representative ileal histology images are presented in Figure 5.3. In the ileum, the *in ovo* essential oil treatment and the NC treatment recorded significantly longer ($P < 0.001$) villus height than the in-water essential treatment. Other treatments recorded intermediate villus height values. Similarly, total mucosa thickness in the ileum was significantly enhanced ($P < 0.001$) by the *in ovo* essential oil treatment compared to the in-water essential oil treatment. Statistical intermediate total mucosa thickness was recorded

for other treatments in the ileum.

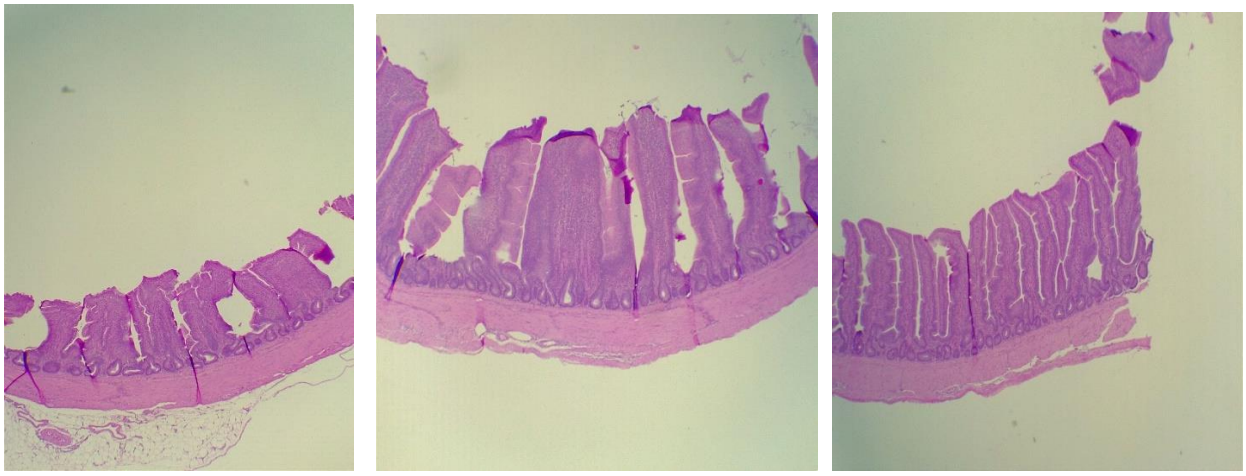
Table 5.7 Effect of essential oil delivery route on broiler chicken intestinal morphology.

Parameters / Intestinal Segment (Measured in mm)	Treatments [1]						SEM [2]	*P* Value [3]
	Negative Control	In-Feed Antibiotics	In-Water Essential Oil	*In ovo* Saline	*In ovo* Essential Oil	*In ovo* Essential Oil + In-Water Essential Oil		
Duodenum								
Villus Height	1.56^{bc}	1.54^{bc}	1.53^{bc}	1.50^{c}	1.63^{a}	1.59^{ab}	0.01	<0.001
Villus width	0.15^{ab}	0.15^{ab}	0.15^{ab}	0.14^{b}	0.17^{a}	0.1^{ab}	0.00	0.010
Crypt depth	0.14	0.14	0.13	0.13	0.14	0.14	0.00	0.29
Villus height: Crypt depth	10.96	10.66	11.69	10.90	11.48	11.64	0.18	0.307
Total mucosa thickness	1.70^{abc}	1.68^{bc}	1.66^{bc}	1.63^{c}	1.78^{a}	1.73^{ab}	0.01	<0.001
Jejunum								
Villus Height	0.90	0.88	0.89	0.87	0.93	0.92	0.01	0.192
Villus width	0.16	0.16	0.16	0.16	0.15	0.16	0.00	0.825
Crypt depth	0.13	0.13	0.13	0.13	0.13	0.14	0.00	0.431
Villus height: Crypt depth	6.47	6.19	6.75	6.25	7.16	6.51	0.14	0.126
Total mucosa thickness	1.05	1.03	1.02	1.02	1.07	1.07	0.01	0.240
Ileum								
Villus Height	0.48^{a}	0.45^{ab}	0.43^{b}	0.45^{ab}	0.49^{a}	0.46^{ab}	0.01	<0.001
Villus width	0.15	0.16	0.16	0.15	0.16	0.16	0.00	0.756
Crypt depth	0.10	0.11	0.09	0.10	0.10	0.10	0.38	0.135
Villus height: Crypt depth	4.91	4.24	4.72	4.77	4.84	4.80	0.09	0.147
Total mucosa thickness	0.58^{ab}	0.57^{ab}	0.52^{c}	0.54^{bc}	0.60^{a}	0.56^{abc}	0.38	<0.001

[1]Treatments include- (1) Negative control treatment- chicks fed a basal corn-soybean meal-wheat–based diet; (2) In-feed antibiotics- chicks fed NC + 0.05% bacitracin methylene disalicylate and (3) In-water essential oil- chicks supplied the essential oil via the water route at the recommended dosage of 250 mL/1000 L of drinking water; (4) *In ovo* saline treatment- eggs injected with 0.2 mL of physiological saline (0.9% NaCl); (5) *In ovo* essential oil treatment- eggs injected with 0.2 mL of a saline + essential oil blend mixture at a dilution ratio of 2:1, (6) *In ovo* + in-water essential oil treatment- chicks offered the essential oil blend via the *in ovo* and in water route, successively. [2]SEM = Standard error of means. [3]Means within a row with different superscripts $^{a, b, c}$ differs ($P < 0.05$).



(1) (2) (3)



(4) (5) (6)

Figure 5.3 Representative ileal histology images presented on a treatment basis.

Treatments include- (1) Negative control treatment- chicks fed a basal corn-soybean meal-wheat–based diet; (2) In-feed antibiotics- chicks fed NC + 0.05% bacitracin methylene disalicylate and (3) In-water essential oil- chicks supplied the essential oil via the water route at the recommended dosage of 250 mL/1000 L of drinking water; (4) *In ovo* saline treatment- eggs injected with 0.2 mL of physiological saline (0.9% NaCl); (5) *In ovo* essential oil treatment- eggs injected with 0.2 mL of a saline + essential oil blend mixture at a dilution ratio of 2:1, (6) *In ovo* + in-water essential oil treatment- chicks offered the essential oil blend via the in ovo and in water route, successively.

5.5 Discussion

As the search for effective alternatives to AGPs for the poultry industry continues, there is also a need for the urgent development of delivery strategies that optimize their effectiveness. The potential of several EOs extracted from herbs and spices as alternatives to AGPs continues to be recognized due to their biological properties. For instance, EOs derivable from the star anise plant has been reported to have growth-promoting (Al-Kassie, 2008), antioxygenic (Padmashree, 2007), antibacterial, and digestion-enhancing properties (Singh et al., 2002). Similarly, cinnamon EO has cinnamaldehyde (3- phenyl-2-propenal) as its major component, conferring it antimicrobial (Lee and Ahn, 1998), cardio-protective (Ensminger, 1986), antidiabetic (Babu et al., 2007), and hypocholesterolemic properties (Sang-Oh et al., 2013). Rosemary extract has carnosic acid, carnosol, rosmanol, rosmariquinone and rosmaridiphenol, ursolic acid, and caffeic acid as major phenolic components (Basaga et al., 1997). The antimicrobial, anti-inflammatory, antidiabetic, anticancer, and antioxidant properties of rosemary EOs have also been documented (Dorman and Deans, 2000; Moreno et al., 2006; Khalil et al., 2012; Bozkurt et al., 2012a; Karadas et al., 2013; Moore et al., 2016; Sethiya, 2016; Attia et al., 2017; Al-hijazeen, 2021). Thyme EO also has thymol, carvacrol, and linalool as its main active compounds (Lee et al., 2003; Attia et al., 2017). The antimicrobial, antioxidant, and digestion-enhancing properties of thyme EO are also well documented (Dorman and Deans, 2000; Bozkurt et al., 2012b; Seithiya, 2016). Despite the beneficial biological properties that each of these EO can exhibit, an accurate blend of these EOs can manifest greater responses via a synergistic mode of action (Isabel and Santos, 2009; Karadas et al., 2013; Bravo et al., 2014). Accordingly, the first comparison of an essential oil blend with an AGP across several delivery routes in the literature is thus presented herein.

In this study, *in ovo* delivery of EOs reduced hatchability and chick length in broiler chickens. Conversely, most of the very limited studies on *in ovo* delivered EOs have recorded no effect (Saki and Salary, 2015; Toosi et al., 2016) or increased hatchability (Nadia et al., 2008; Sulaiman and Tayeb, 2020). Compared to this study, the differences in injected EO nature, concentration, volume, injection site, and dosage might potentially explain the observed result. These factors have previously been highlighted as critical to the success of *in ovo* delivery (Oladokun and Adewole, 2020). The impaired hatchability recorded by the *in ovo* delivery route at this injection dosage (0.2 mL of a saline + essential oil blend mixture at a dilution ratio of 2:1) is considered a significant limitation that could prevent the adoption of this delivery route for EO delivery by poultry producers. More research is thus needed to optimize standard guidelines

regarding these factors in order to guarantee successful *in ovo* delivery of EOs. In principle, the antioxidant capacity of injected EOs is expected to mitigate the overproduction of free radicals, and in consequence, cause increased hatchability (Galobart et al., 2001; Surai et al., 2003; Nadia et al., 2008). Chick length is often used to predict chick growth potential (Hill, 2001). As this is the first study on *in ovo* delivery of EOs to evaluate this parameter, there was no basis for comparison with other studies. Thus, it can only be speculated that the *in ovo* delivered EOs dose was unfavorable to the hatched chicks. Ideally, *in ovo* delivered EOs after day 18 are expected not to influence chick length. This is because any biological substances delivered to the embryo after day 18 are devoted to providing energy for the hatching process and not for organogenesis; *in ovo* delivery of nutrients is the perfect candidate for this time-point. On the other hand, biological substances provided to the embryo before the first 18 days of incubation are devoted to embryo organogenesis and growth (Pearson et al., 1996; De Smit et al., 2006). It is also important to state that the commercial EO blend utilized in this study is not dedicated to being *in ovo* administered, per default, and contained emulsifiers as prepared for in-water administration. Therefore, bioactive substances intended for *in ovo* administration might require specific formulation. As the concept of *in ovo* delivery of bioactive substances other than vaccines in poultry is relatively new, a need for specially formulated commercially available bioactive substances thus exists. Furthermore, besides injection dosage, egg source and egg quality might also potentially impair hatch performance and chick quality. Following *in ovo* delivery of EO + saline, increased chick weight and no effect on chick length were recorded during the pretrial stage of this study.

At the end of the trial (d 28), all treatments recorded no significant effect on the evaluated post-hatch growth performance parameters (AFI, ABWG, and FCR) in this study. Only in the starter phase (d 0–14) did the antibiotic treatment record a significant increase in ABWG. The growth-enhancing properties of AGPs are well substantiated across the literature (Butaye et al., 2003; Castanon, 2007; Gadde et al., 2017). Generally, the effect of EOs on bird performance is observed to be inconsistent across the literature. While the result presented here are consistent with other studies that have reported no effect of EOs on bird post-hatch growth performance (Botsoglou et al., 2002; Lee et al., 2003; Hernandez et al., 2004; Shanmugavelu et al., 2004; Florou-Paneri et al., 2005; Jang et al., 2007; Cross et al., 2007, Ali et al., 2007; Demir et al., 2008; Mathlouthi et al., 2012; Ma, 2013; Feizi et al., 2014; Celikbilek, 2014; Olgun and Yidiz, 2014;Abdel-Ghaney et al., 2017; Wade et al., 2018; El-Kholy et al., 2021). Contrastingly, other studies have recorded increased (Alcicek et al., 2004; Mathlouthi et al., 2012; Sulaiman and

Tayeb, 2020; El-Kholy et al., 2021) and decreased growth performance on EO supplementation (Kirkpinar et al., 2011). The variability in the growth performance effect of EOs is attributable to several intrinsic and extrinsic factors that include the physiological status of the bird, housing type (cage vs. floor pens), housing hygiene (clean vs. unclean), challenge acuity index, basal diet composition, and time of rearing (Botsoglou et al, 2002; Windisch et al., 2008; Khattak et al., 2014; Farouk et al., 2020; Mathlouthi et al., 2021). It would be reasonable to speculate that the short timing of this study (28 days) and housing type (battery cages) largely influenced the obtained results on growth performance. Moreover, digestive enzyme secretion capacity is reportedly limited in young chicks (Lilja, 1983) while nutrient requirements decrease with increasing age (NRC, 1994). Additionally, due to a well-developed digestive tract and organs, older birds are thus able to utilize the finisher diets better (Mast et al., 2000).

Immunoglobulins are synthesized by the B cells to regulate humoral immunity. They are often synthesized in response to immune stressors such as infection and oxidative stress (Alp et al., 2012). This study recorded no effect of EOs and their delivery routes on serum immunoglobulins G and M levels. This result was not surprising as birds were raised under experimental and controlled conditions with no strain on their immune system. Previous studies have also reported no effect of EOs on the level of immunoglobulins G and M in broiler chickens (Aami-Azghadi et al., 2010, Liu et al., 2020; Adaszyńska-Skwirzyńska et al., 2021), laying hens (El-Gogary et al., 2018), and rabbits (Movahhedkhah et al., 2019). While a few studies have recorded increased immunoglobulins G and M levels following EO supplementation (Nadia et al., 2008; Abdel-Ghaney et al., 2017; Sulaiman and Tayeb, 2020). Movahhedkhah et al. (2019) have suggested an age-dependent immune response on EOs supplementation exists in birds. The authors suggested evaluating this parameter at the later stage of growth in broiler chickens. In any case, the exact mechanism by which EOs stimulate an immunological response in birds is not fully known, thus warranting further investigation. Furthermore, a tendency for increased relative weight of the liver in the *in ovo* + in-water EO treatment was observed in this study. An increase in the relative weight of the liver in birds receiving thymol (Lee et al., 2003), oregano oil (Al-Kassie et al., 2009), EO blend containing thyme and cinnamon (Bozkurt et al., 2012b), and EO blend containing oregano and sage oil (Clinical Diagnostic Division, 1990) have been reported. The observed tendency for increased liver weight exerted by the *in ovo* + in-water EO treatment could be attributed to decreased apoptosis of liver tissues resulting from the antioxidant properties of the delivered EO blend (Bozkurt et al., 2012b).

Blood biochemistry indices are valuable indicators of the health and wellbeing of the bird. The plasma biochemistry indices observed in this study were all within the normal physiological range for broiler chickens (Khattak et al., 2014; Perai et al., 2015; Ilo et al., 2019). The highest reduction in the level of blood enzyme CK was observed in the *in ovo* + in-water EO treatment in this study. The blood enzyme CK is an intracellular enzyme whose plasma concentration is usually used as an indicator of skeletal muscle damage (Salehifar et al., 2017). Skeletal muscle damage is inducible by congenital myopathies, nutritional myopathies, or oxidative stress. In agreement with the result presented here, Salehifar et al. (2017), have reported the efficacy of lemon pulp powder to decrease the activities of CK in heat-stressed broilers. Similarly, EO blends containing rosemary oil (Hosseini et al., 2016) and *Curcuma xanthorrhiza* oil (Zhu et al., 2014) have all been reported to reduce CK levels in broiler chickens significantly. The reduction in blood CK levels could be associated with the antioxidant capacities of the delivered EO blends, which was enhanced by the *in ovo* + in-water EO delivery route. This same delivery route (*in ovo* + in-water EO) also recorded the highest reduction in plasma AST level. Increased levels of AST in the blood are often indicative of increased permeability of liver cells and liver damage (Zhang, 2011; Tayeb et a., 2019). Consistent with the results from this study *in ovo* delivered cinnamon, thyme, and clove oil have all been reported to individually reduce serum AST levels in broiler chickens (El-Kholy et al., 2021). Several supplemented EOs have equally reported similar AST lowering effects (Zhang, 2011; Osman et al., 2010; Yadegari et al., 2019; Abd El-Latif et al., 2013). The hepatoprotective effect provided by the *in ovo* + in-water EO delivery route is possibly due to the rich antioxidant compounds present in the supplemented EO blend. Additionally, this hepatoprotective effect is also effectuated by the induction of endogenous interferon (Olgun, 2016). A tendency for increased plasma calcium level was also recorded in the *in ovo* + in-water EO delivery route. Blends of EO containing thyme, black cumin, fennel, anise, and rosemary have been reported to increase the calcium concentration in the tibia of laying hens (Amad et al., 2011). The same EO blend has also been reported to decrease calcium excretion in breeder quails (Olgun and Yidiz, 2014). Ileal calcium bioavailability has also been enhanced by the supplementation of EO in broilers (Mountzouris et al., 2011; Alagawany et al., 2021). This tendency for increased plasma calcium level in the *in ovo* + in-water EO delivery route is possibly induced by increased mobilization of calcium-binding protein in the mucosa, activating the calcium-activated tenderization complex. This observation has implications for improving bone strength and bird leg health (Baratta et al., 1998).

An apparent increase in TAC was also observed in the *in ovo* + in-water EO treatment, buttressing that this delivery route clearly enhanced the antioxidant potential of the delivered EO blend. The values of TAC are indicative of the overall antioxidant defense systems, both enzymatic and non-enzymatic. Several in vitro studies have reported the antioxidant properties of several plant extracts and EOs (Lee and Shibamoto, 2002; Pizzale et al., 2002; Yu et al., 2018). Increased levels of TAC resulting from supplementation of star anise EO in laying hens (Alhajj et al., 2017) and broilers (Ri et al., 2017), oregano powder in broiler chickens (Bozkurt et al., 2016), oregano EO in broiler chickens (Ryzner et al., 2013), *Satureja officinalis* EO in broiler chickens (Estevez et al., 2007), and rosemary EO in rabbits (Movahhedkhah et al., 2019) have been reported. Varying antioxidant capacities are reported for most evaluated EOs in the literature, justifying the need for research evaluating various EO combination types and delivery routes in order to ensure EO efficacy either by synergistic or additive mechanisms. For instance, rosemary is regarded as the plant with the highest antioxidant capacity (Avila-Ramos et al., 2012), while the antioxidant capacity of oregano EO is also reported to be greater than vitamin E (Yanishlieva et al., 1999). Thymol is also reported to exhibit greater antioxidant capacity than carvacrol, possibly because thymol has greater stearic inhibition of the phenolic group than carvacrol (Svihus, 2014). The antioxidant properties of these EOs are due to the presence of phenolic OH groups in their chemical structure; this acts as a hydrogen donor interacting with peroxyl radicals during the initial process of lipid oxidation and thereby inhibiting the formation of hydroxy peroxide (Lee et al., 2003). Another potential mode of action that requires further research is via the upregulation of antioxidant-related genes.

It is well documented that the structure and morphology of a bird's small intestine influence its functionality. The *in ovo* EO delivery route enhanced duodenal and ileal morphology in this study. While the villus length, width, and total mucosa thickness were enhanced by *in ovo* delivery of this treatment in the duodenum, only the villus height and total mucosa thickness were enhanced by this delivery route in the ileum. The duodenum is an important site for chemical digestion, while the ileum plays a vital role in starch digestion and absorption, especially in fast-growing broiler chickens (Munyaka et al., 2012). Increased villus height, villus width, and total mucosa thickness are generally associated with improved digestive and absorptive functions in the bird (Shang et al., 2015; Yang et al., 2019). To our knowledge, this is the first study to evaluate the effect of *in ovo* delivered EOs on broiler chicken's intestinal morphology, thus providing limited scope to compare obtained results. Nonetheless, several aromatic plants and their extracts

are reported to enhance the intestinal morphology of broiler chickens (Jang et al., 2007; Hong et al., 2012). A blend of EOs containing star anise and oregano oil is also reported to increase the height of duodenal villi in broiler chickens (Chowdhury et al., 2018). Similarly, dietary supplementation of 300 mg cinnamon bark oil kg–1 also reportedly increased villus height in the duodenum and ileum of broiler chickens (Chiang et al., 2009). Blend of EOs containing basil, caraway, laurel, lemon, oregano, sage, tea, and thyme is also reported to significantly increase the width and surface area of the small intestine (Khattak et al., 2014). Similarly, both (El-Katcha et al., 2017; Masood et al., 2020) have also reported increased villus length with in-water EO supplementation in quails and broiler chickens, respectively. The beneficial effect of the *in ovo* EO route on intestinal morphology in this study might be attributed to the early time of delivery. Moreover, the development of the small intestine in chicks has been described to be synonymous with the mammalian neonates, with the greatest morphological change occurring within the first 24 h post-hatch. The *in ovo* technology has been recognized to be a means to stimulate the development of the embryonic gastrointestinal tract (GIT) (Oladokun and Adewole, 2020). The potential of the active ingredients in the EO blend to stimulate the secretion of endogenous digestive enzymes while also ensuring a balanced gut microbial diversity could also have contributed to the observed effect (Ghazanfari et al., 2014; Du et al., 2016; Adewole et al., 2021). The antimicrobial, anti-inflammatory, and antioxidant properties of the supplied EO blend also play an important role in gut morphometric development (Du et al., 2016; Ding et al., 2020). Improved intestinal digestion and absorption due to improved intestinal morphology facilitated by the *in ovo* delivery route would be expected to translate into improved growth performance in the birds. However, this was not the case in this study. It has been previously speculated that the length of this study and the housing type could have contributed to the observed results on growth performance.

5.6 Conclusions

This study revealed that the *in ovo* delivery of EO blends containing star anise, cinnamon, rosemary, and thyme oil reduced hatchability and chick length in broiler chickens. However, successive delivery of this EO blend via *in ovo* and in-water route improved broiler chicken's antioxidant status and blood biochemical profile, with no adverse effect on growth performance. Additionally, *in ovo* delivery of this EO blend also improved intestinal morphometric properties of the bird. Based on observed hatchability and chick length results, it would be essential to optimize injected EO dose through further studies. Furthermore, considering that the supplied EO

blend has reported antioxidant and immune-enhancing properties, it would be interesting to evaluate the efficacy of the *in ovo* + in-water EO delivery route under a heat stress challenge model. Heat stress could potentially induce oxidative stress and immunosuppression in birds. Conclusively, subject to further research with favorable hatchability outcomes, this novel delivery strategy might be a potential alternative to the use of antibiotics in the poultry industry.

6 CHAPTER 6 MICROBIOTA AND TRANSCRIPTOMIC EFFECT OF ESSENTIAL OIL DELIVERY ROUTES

MICROBIOTA AND TRANSCRIPTOMIC EFFECTS OF AN ESSENTIAL OIL BLEND AND ITS DELIVERY ROUTE COMPARED TO AN ANTIBIOTIC GROWTH PROMOTER IN BROILER CHICKENS

This section has been published and presented elsewhere:

- Oladokun, S., Clark, K.F. and Adewole, D.I., 2022. Microbiota and Transcriptomic Effects of an Essential Oil Blend and Its Delivery Route Compared to an Antibiotic Growth Promoter in Broiler Chickens. Microorganisms. 10:861. https://doi.org/10.3390/microorganisms10050861
- Oladokun, S., and Adewole, D.I. 2022. Does essential oil delivery route affect broiler chickens' gut microbiota profile and ceca short-chain fatty acid concentration? International Poultry Scientific Forum (IPSF), Atlanta, Georgia, USA. January 24-27.

6.1 Abstract

This study evaluated the effect of the delivery of a commercial essential oil blend containing the phytonutrients star anise, cinnamon, rosemary, and thyme oil (via different routes) on broiler chickens' ileal and ceca microbiota and liver transcriptome compared to an antibiotic growth promoter. Eggs were incubated and allocated into three groups: non-injected, *in ovo* saline, and *in ovo* essential oil. On day 18 of incubation, 0.2 mL of essential oil in saline (dilution ratio of 2:1) or saline alone was injected into the amnion. At hatch, chicks were assigned to post-hatch treatment combinations: (A) a negative control (corn-wheat-soybean diet), (B) in-feed antibiotics, (C) in-water essential oil (250 mL/1000 L of drinking water), (D) *in ovo* saline, (E) *in ovo* essential oil, and (F) *in ovo* essential oil plus in-water essential oil in eight replicate cages (six birds/cage) and raised for 28 days. On days 21 and 28, one and two birds per cage were slaughtered, respectively, to collect gut content and liver tissues for further analysis. Alpha and beta diversity differed significantly between ileal and ceca samples but not between treatment groups. In-feed antibiotic treatment significantly increased the proportion of specific bacteria in the family *Lachnospiraceae* while reducing the proportion of bacteria in the genus *Christensenellaceae* in the ceca, compared to other treatments. Sex-controlled differential expression of genes related to cell signaling and tight junctions were recorded. This study

provides data that could guide the use of these feed additives and a foundation for further research.

6.2 Introduction

Over the years, the sub-therapeutic supplementation of antibiotic growth promoters (AGPs) has been used to preserve gut health, intestinal microbiota balance, and growth performance in the poultry industry (Mahmood and Guo, 2020). This trend has now triggered both consumer and public health concerns bordering on the emergence of antibiotic resistance and antibiotic residues in the food chain (Ma et al., 2021; Thakur et al., 2019). Accordingly, a few country-specific restrictions on the use of AGPs are already in place, including in the EU (Castanon, 2007), the US (FDA, 2013), and Canada (Chicken Farmers of Canada, 2021). The potential elimination of AGPs could exacerbate the risks of intestinal dysbiosis and bacterial diseases in poultry (Paiva and McElroy, 2014). In the post-AGP era, understanding the complex interplay between the host and its intestinal microbiome signifies a critical step to achieving optimum gut health and intestinal microbiota balance in poultry.

The gastrointestinal tract (GIT) of poultry, populated by microorganisms in constant interaction with the host and digesta, is known to play a critical role in the host's growth and health. It is now evident that the proliferation of a balanced and beneficial gut microbiota population is vital to ensuring host protection against pathogenic bacteria and enhancing gut integrity and immunity (Rinttilä and Apajalahti, 2013; Rowland et al., 2018; Yadav and Jha, 2019). Several factors, including environmental stressors (Burkholder et al., 2008, Kers et al., 2018; Shi et al., 2019), bird age (Lu et al., 2003), and nutrition (Thompson et al., 2008; Oviedo-Rondón et al., 2010; Shang et al., 2018), can modify the gut microbiota profile. Of all these factors, nutrition (including the type of diet and time of feeding) has been regarded as the main factor influencing poultry gut microbiota dynamics (Yadav and Jha, 2019). Given this modulatory role, several feed additives, including probiotics, prebiotics, organic acids, exogenous enzymes, and essential oils, are being investigated as potential alternatives to AGPs in the poultry industry (Yadav and Jha, 2019).

Essential oils (EOs) are mostly plant extracts with mixtures of phytochemical compounds like thymol, carvacrol, and eugenol (Bassolé and Juliani, 2012). To explore a synergistic effect, commercial combinations and blends of several EO types are becoming increasingly popular. Several in vitro studies have highlighted the antibacterial, antiviral, antifungal, antimycotic, antiparasitic, antioxidant, anti-inflammatory, anti-toxigenic, and immune-regulating properties of

EOs (Gopi et al., 2014; Swamy et al., 2016; Stevanović et al., 2018). However, in vivo results on the effect of EO on chicken microbiota are somewhat inconsistent. While EO blends have been reported to reduce the relative abundance of pathogenic bacteria like *Escherichia coli* (Cho et al., 2014; Hashemipour et al., 2016), Salmonella (Pathak et al., 2017), and *Clostridium perfringens* (Mitsch et al., 2004) in broiler chickens, a few studies have equally reported no effect of EO supplementation on gut commensal bacteria (Hong et al., 2012; Pathak et al. 2017; Paraskeuas and Mountzouris, 2019). These inconsistencies in the efficacy of EOs could be associated with the limitations that characterize their mode of delivery (Bilia et al., 2014; Heydarian et al., 2020), as most EOs are conventionally supplied via feed or water to poultry birds. These conventional routes expose EOs to potential thermal instability, especially during feed milling processes like pelleting (Maenner et al., 2011) and negative interaction with other feed additives like oligosaccharides and coccidiostats (Malayoğlu et al., 2010; Mountzouris et al., 2011). The success of in-water EO supplementation will depend on the water quality and the quality of the chick watering device. In-water EO delivery also has the potential to promote wet feather risks and other welfare issues.

The delivery of EO via the *in ovo* route presents a viable means to overcome the identified challenges that characterize conventional delivery routes (i.e., in feed and in water). *In ovo* delivery of bioactive substances has been defined as "the direct inoculation of bioactive substances to the developing embryo to elicit superior lifelong effects, while considering the dynamic physiology of the chicken embryo" (Oladokun and Adewole, 2020). The *in ovo* delivery route offers an economic advantage, as low doses of bioactive substances are required to initiate long-term performance effects in the birds (Slawinska et al., 2016; Oladokun and Adewole, 2020). It offers the opportunity to stimulate the colonization of the embryonic gut with beneficial microbiota very early on, rather than trying to alter an already established microbiota community in later life (Roto et al., 2016). Additionally, it is yet to be known if an additive benefit exists from the successive delivery of EOs via the *in ovo* and continuous in-water delivery routes. This study is thus interested in evaluating if such an effect exists in the broiler chicken microbiota and liver transcriptome.

Studies have also suggested that microbial community might vary depending on the segment of the small intestine considered (Gong et al., 2002a; Gong et al., 2007; Glendinning et al., 2019). In addition to the reported microbiota modifying effect, EOs can also influence the expression of several genes involved in de novo fat synthesis and deposition (Sabino et al., 2018)

as well as antioxidant activity (Li et al., 2020) Studies involving the liver transcriptome of EO-fed birds have also reported the enrichment of Gene Ontology Consortium (GO) terms associated with performance and metabolism (Sabino et al., 2018), as well as a higher expression of antioxidant genes (Bastos et al., 2017). The liver remains a good candidate tissue to study the transcriptomic effect of EO supplementation, as it is involved in several metabolic functions, including carbohydrate, protein, and lipid metabolism; bile secretion; and immune defense, among others (Li et al., 2015). Additionally, the combination of modern molecular biological techniques, such as 16S ribosomal RNA (16S rRNA) gene sequencing and RNA sequencing (RNA-seq) technology, could help unravel the precise mechanism underpinning the delivery of EOs. Most studies on EO delivery have mainly focused on low throughput gene expression analysis and bird performance evaluation (Lillehoi et al., 2011; Akbarian et al., 2013). Accordingly, the objective of this study was to evaluate the effect of a commercial EO blend containing star anise, cinnamon, rosemary, and thyme oil delivered via in-water and *in ovo* routes on broiler chickens' ileal and ceca microbiota, ceca short-chain fatty acid concentration, and liver transcriptome as compared to an in-feed antibiotic growth promoter.

6.3 Materials and Methods

6.3.1 Ethics Statement

The experiment was carried out at the hatchery facility of the Agricultural Campus of Dalhousie University and the broiler rearing facility of the Atlantic Poultry Research Center, Dalhousie Faculty of Agriculture. All experimental procedures were approved by the Animal Care and Use Committee of Dalhousie University (Protocol number: 2020-035), in accordance with the guidelines of the Canadian Council on Animal Care (2021).

6.3.2 Egg Incubation and *in ovo* Injection Procedure

A total of 670 hatching eggs with an average weight 77.87 ± 2.43 g (mean ± SE) from 41-week-old Cobb 500 broiler breeders were sourced from Synergy hatchery, Nova Scotia, Canada. Eggs were incubated in a ChickMaster single-stage incubator (ChickMaster G09, Cresskill, NJ, USA) under standard conditions (37.5 °C and 55% relative humidity) from embryonic days (EDs) 1 to 17, and then to an average of 32 °C and 68% from EDs 18 to 21. Incubators were preheated for 24 h prior to setting eggs to ensure that proper temperature and humidity were stable. Egg trays were turned on a 90° arc four times an hour from the time of setting until ED 18. Eggs were arranged in 6 replicate trays inside the incubator, with each tray containing 96 eggs. On ED 12, eggs were candled, and in-fertile eggs were disposed of, leaving a total of 576 eggs for the trial.

The remaining eggs were subsequently assigned to one of three treatment groups: (1) non-injected eggs (control, 288 eggs), (2) *in ovo* saline group (96 eggs, injected with 0.2 mL of physiological saline, i.e., 0.9% NaCl), (3) *in ovo* essential oil group (192 eggs, injected with 0.2 mL of a saline and essential oil blend mixture at a dilution ratio of 2:1).

The essential oil utilized in this study is a commercial blend (Probiotech International Inc., St Hyacinthe, QC, Canada) containing the phytonutrients star anise, cinnamon, rosemary, and thyme oil. The EO blend is registered by Health Canada as a veterinary health product (VHP). On ED 18, eggs were injected according to the procedure described by Oladokun et al. (2021a) with slight modifications. Briefly, this involved disinfecting the eggs with 70% ethanol-dipped swabs and using an 18-gauge needle to carefully punch the shell at the center of the air cell (the blunt end). The injected EO was then delivered to the amnion using a self-refilling injector (Socorex ultra-1810.2.05005, Ecublens, Switzerland) equipped with a 22-gauge needle (injection needle length—3 cm) at a 45-degree angle. After *in ovo* injection, the injection sites were sealed with sterile paraffin and eggs were placed back in the incubator. The non-injected eggs were also taken out and returned to the incubator simultaneously as other injected treatment groups.

6.3.3 Birds, Housing, and Diets

Hatchlings were weighed and randomly assigned to 6 new treatment groups (Figure 6.1). Chicks (straight run) from the initial non-injection group were randomly allocated into 3 new treatment groups consisting of (A) chicks fed a basal corn-soybean meal-wheat–based diet (negative control treatment, NC), (B) chicks fed NC + 0.05% bacitracin methylene disalicylate (in-feed antibiotics), and (C) chicks supplied the same commercial blend of EOs as earlier described via the water route (in-water essential oil) at the recommended dosage of 250 mL/1000 L of drinking water. The initial *in ovo* saline and *in ovo* essential oil groups were placed on the control diet to form treatments (D) (*in ovo* saline treatment) and (E) (*in ovo* essential oil treatment), respectively. The last treatment group, (F), consisted of chicks from the *in ovo* essential oil treatment group also supplied EO via the water route (*in ovo* + in-water essential oil treatment). All treatment groups had 48 birds each. Birds were placed in battery cages (0.93 m × 2.14 m), there were 6 birds per cage, and 8 replicate cages per treatment. Birds were reared for 28 d under uniform controlled environmental conditions in line with Cobb Broiler Management Guide recommendations. The room temperature was set at 31 °C on day 0 and gradually reduced to 23 °C on day 28, and relative humidity ranged between 45 and 55%. The ingredient and nutritional

compositions of the basal diet used in the study are available in Oladokun et al. (2021b) and Table 6.1. Birds were provided with feed and water ad libitum and diets were fed as mash throughout the rearing period which included the starter (0–14 d) and grower (15–28 d) phases. Diets were formulated to meet Cobb 500 broiler chicken nutrient requirements.

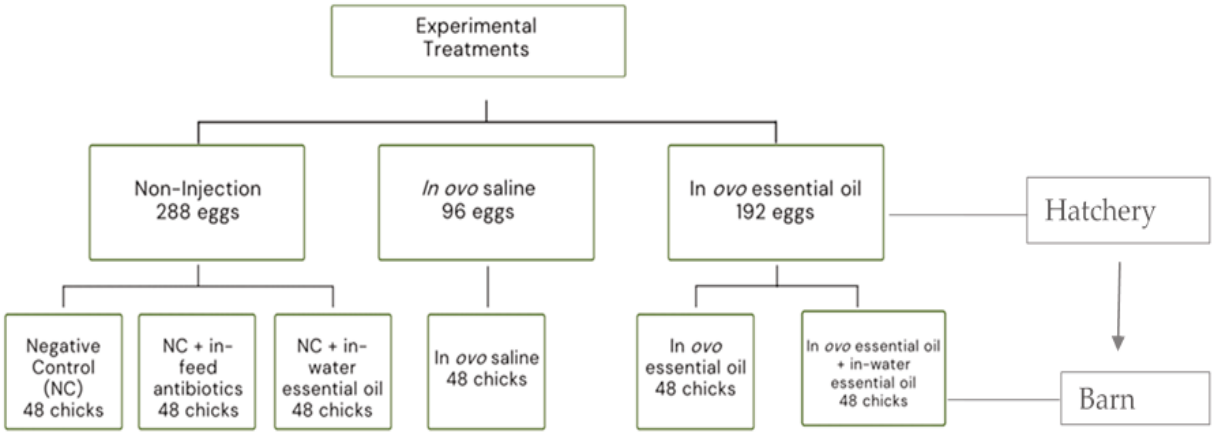


Figure 6.1 Schematic presentation of experimental structure in the hatchery and barn.

Table 6.1 Ingredients, calculated, and analyzed compositions of experimental diets1 (as-fed basis, percentage (%), unless otherwise stated).

Ingredients	Phases			
	Starter (0-14 d)		Grower (15-28 d)	
	Control diet	Antibiotic diet	Control diet	Antibiotic diet
Ingredient Composition				
Corn (ground)	51.08	50.98	45.36	45.25
Soybean meal-46.5	41.44	41.45	36.31	36.33
Wheat	-	-	10	10
Animal/vegetable fat	2.93	2.97	4.22	4.26
Limestone	1.80	1.80	1.65	1.65
Dicalcium Phosphate	1.24	1.24	1.06	1.06
DL Methionine premix[2]	0.59	0.59	0.53	0.53
Vitamin/Mineral Premix [3, 4]	0.50	0.50	0.50	0.50
Salt	0.41	0.41	0.37	0.37
Lysine HCl	0.01	0.01	0.00	0.00
BMD 110G[5]	-	0.05	-	0.05
Total	100	100	100	100
Nutrient	Calculated composition			
Metabolizable energy (kcal/kg)	3,000	3,000	3,100	3,100
Crude protein	23	23	21.5	21.5
Calcium	0.96	0.96	0.87	0.87
Available phosphorus	0.48	0.48	0.44	0.44
Sodium	0.19	0.19	0.18	0.18
Digestible lysine	1.28	1.28	1.15	1.16
Digestible methionine + cysteine	0.95	0.95	0.87	0.87
Digestible Tryptophan	0.25	0.25	0.23	0.23
Digestible Threonine	0.89	0.89	0.82	0.82
Analyzed composition				
Dry Matter	90.7	90.8	93.2	93.5
Crude protein	24.8	25	22.5	23.8
Crude fat	5.50	5.79	6.84	6.85
Calcium	1.06	1.13	1.00	0.96

Potassium	1.14	1.16	0.99	1.04
Phosphorus	0.69	0.70	0.67	0.62
Sodium	0.19	0.21	0.21	0.16

[1] Basal diet (NC); antibiotic diet containing NC + 0.05% bacitracin methylene disalicylate (BMD). [2] Supplied/kg premix: DL-Methionine, 0.5 kg; wheat middling, 0.5 kg. [3] Starter vitamin-mineral premix contained the following per kg of diet: 9750 IU vitamin A; 2000 IU vitamin D3; 25 IU vitamin E; 2.97 mg vitamin K; 7.6 mg riboflavin; 13.5 mg Dl Ca-pantothenate; 0.012 mg vitamin B12; 29.7 mg niacin; 1.0 mg folic acid, 801 mg choline; 0.3 mg biotin; 4.9 mg pyridoxine; 2.9 mg thiamine; 70.2 mg manganese; 80.0 mg zinc; 25 mg copper; 0.15 mg selenium; 50 mg ethoxyquin; 1543 mg wheat middling's; 500 mg ground limestone. [4] Grower and Finisher vitamin-mineral premix contained the following per kg of diet: 9750 IU vitamin A; 2000 IU vitamin D3; 25 IU vitamin E; 2.97 mg vitamin K; 7.6 mg riboflavin; 13.5 mg Dl Ca-pantothenate; 0.012 mg vitamin B12; 29.7 mg niacin; 1.0 mg folic acid, 801 mg choline; 0.3 mg biotin; 4.9 mg pyridoxine; 2.9 mg thiamine; 70.2 mg manganese; 80.0 mg zinc; 25 mg copper; 0.15 mg selenium; 50 mg ethoxyquin; 1543 mg wheat middling's; 500 mg ground limestone. [5] Bacitracin methylene disalicylate (providing 55 mg/kg mixed feed); Alpharma, Inc., Fort Lee, NJ, USA.

6.3.4 Sample Collection

On day 21, 1 bird per cage (8 replicate birds per treatment group) was randomly selected, weighed, and euthanized by electrical stunning and exsanguination. After slaughter, the small intestinal segment—the ileum (1.5-cm length mid-way between Meckel's diverticulum and the ileocecal junction)—was longitudinally opened, and digesta content was collected into microcentrifuge tubes. Aside from being the most studied small intestinal segments, the ileum microbiota was evaluated because reported trends suggest increasing microbial density in the distal region of the small intestine compared to the proximal regions as a result of longer digesta transit times (Gong et al., 2007; Shang et al., 2018).

Similarly, on day 28, 2 birds per cage (16 replicate birds per treatment group) were randomly selected and euthanized by electrical stunning and exsanguination. After euthanasia of the birds, digesta content from the pair of ceca was mixed and divided into two subsamples. One part was stored in plastic RNase- and DNase-free tubes placed in liquid nitrogen to analyze gut microbiota. The other part was placed in bio-freeze kits (Alimetric Diagnostics, Espoo, Finland) for the determination of short-chain fatty acids following published protocols (Oladokun et al., 2021b). Liver tissues (50–100 mg) using 1 mL TRIzol™ (Qiagen, Hilden, Germany) from 8 replicate birds/treatment were also rapidly collected on day 28 and promptly frozen in liquid nitrogen. All samples were stored at −80 °C until further analysis.

6.3.5 DNA Extraction, Qualification, Library Preparation, and Sequencing

The Qiagen DNeasy® PowerSoil Pro Kit (50) (Cat. No./ID: 47014) was used to extract DNA from both ileal and ceca digesta contents. Digesta contents were allowed to thaw briefly at room temperature before subsequent DNA extraction, following the manufacturer's protocol. Briefly, 250 mg of digesta content was added to PowerBead Pro Tubes and then subjected to cell lysing steps involving vortexing and centrifugation. The retrieved lysate was then captured onto an MB Spin Column, followed by a series of purification and centrifugation steps.

The MB Spin Column was then carefully placed into the provided 1.5 mL elution tubes from which extracted DNA was recovered. The concentration and purity of extracted DNA were subsequently determined by spectrophotometry (Nanodrop ND1000, Thermo Scientific). Extracted DNA samples (volume—50 µL, concentration—10–200 ng/µL) were then sent to the Integrated Microbiome Resource (IMR), located at Dalhousie University in Halifax, Nova

Scotia, for library preparation and sequencing. Libraries of the V4–V5 hypervariable region of the 16S rRNA gene were prepared using universal primers 515 F (Illumina adapters + 5′GTGYCAGCMGCCGCGGTAA3′) and 926 R (Illumina adapters + 5′CCGYCAATTYMTTTRAGTTT3′) following protocols described by Comeau et al. (2017). Each sample was amplified with a different combination of index barcodes to allow for sample identification after multiplex sequencing. Library preparation and sequencing for all samples were performed with the Illumina MiSeq at the Integrated Microbiome Resource (http://imr.bio/, accessed on 15 July 2021) of Dalhousie University.

6.3.6 Short-Chain Fatty Acid Concentration and Total Bacteria Density

Ceca samples were collected using BioFreeze™ sampling kits (Alimetrics Diagnostics Ltd., Espoo, Finland) following the manufacturer's protocol. Samples were then subsequently submitted to Alimetrics Diagnostics 20007-1 (Espoo, Finland) for both SCFA concentration and total bacterial density quantification. The SCFA profiles were analyzed by gas chromatography (Agilent Technologies, Santa Clara, CA, USA) using pivalic acid as an internal standard. The acids quantified included acetic, propionic, butyric, valeric, and lactic acids. To quantify the total bacteria density, submitted samples were initially washed to remove solid particles and complex polysaccharides that may disturb subsequent DNA purification processes and downstream qPCR applications. The liquid phase was subjected to differential centrifugation for collecting the bacterial cells. The cell walls of the microbial cells were disrupted, and the chromosomal DNA was quantitatively extracted and purified using optimized protocol (Alimetrics Diagnostics 20007-1, Espoo, Finland). All measurements were performed with 16 replicates per treatment group.

6.3.7 RNA Extraction, Qualification, Library Preparation, and Sequencing

Total RNA in liver tissues was extracted on a QIAcube Connect using RNeasy Plus Universal Mini Kit (Qiagen, Cat. No. ID: 73404) following the manufacturer's instructions after disruption and homogenization were performed with a TissueLyser system. RNA elution volume was 30 µL. Total RNA was quantified, and its integrity was assessed on a LabChip GXII (PerkinElmer). Libraries were generated from 250 ng of total RNA and mRNA enrichment was performed using the NEBNext Poly(A) Magnetic Isolation Module (New England BioLabs). cDNA synthesis was achieved with the NEBNext RNA First-Strand Synthesis and NEBNext Ultra Directional RNA

Second Strand Synthesis Modules (New England BioLabs). The remaining library preparation steps were performed using the NEBNext Ultra II DNA Library Prep Kit for Illumina (New England BioLabs). Adapters and PCR primers were purchased from New England BioLabs. Libraries were quantified using the Kapa Illumina GA with Revised Primers-SYBR Fast Universal kit (Kapa Biosystems). Average fragment size was determined using a LabChip GXII (PerkinElmer) instrument. The libraries were normalized, pooled, and then denatured in 0.05 N NaOH and neutralized using HT1 buffer. The pool was loaded at 225 pM on an Illumina NovaSeq S4 lane using Xp protocol as per the manufacturer's recommendations. The run was performed for 2 × 100 cycles (paired-end mode). A phiX library was used as a control and mixed with libraries at 1% level. Base calling was performed with RTA v3.4.4. The program bcl2fastq2 v2.20 was then used to demultiplex samples and generate fastq reads.

6.3.8 Bioinformatics and Statistical Analysis

The analysis of microbiota data was carried out using the Microbiome Helper pipeline (https://github.com/LangilleLab/microbiome_helper/wiki accessed on 29 July 2021,), based on QIIME2. This uses amplicon sequence variants (ASVs) created with Deblur. Primer sequences were removed from sequencing reads using cutadapt (v 1.14) (Martin et al., 2011), and primer-trimmed files were imported into QIIME2 (v. 2019.4.0) (Bolyen et al., 2019). Reads (forward and reverse paired ends) were joined using VSEARCH (v 2.9.0) (Rognes et al., 2016) and inputted into Deblur (Amir et al., 2017) to correct reads and obtain amplicon sequence variants (ASVs). Taxonomic assignment was performed with the SILVA database (v.1.3.2) using a naive Bayes approach implemented in the scikit learn Python library (Comeau et al., 2017). Rarefaction curves were used to examine the individual alpha diversity for all samples (with the default observed OTUs as the metric). Alpha diversity comparisons for the treatments were explored using boxplots and the Kruskal–Wallis statistical test set at $p < 0.05$. Beta diversity was visualized using weighted UniFrac PCoA plots. The relative abundance at different taxonomic levels was visualized using stacked bar charts, while significant microbiota proportions were determined in the Statistical Analysis of Metagenomic Profiles (STAMP) software (Parks et al., 2014) with an ANOVA test using the Benjamin–Hochberg false discovery rate as multiple test correction and then sorting by Corrected *p*-value ($p < 0.05$). Data on SCFA concentrations and total bacteria density were subjected to one-way ANOVA analysis in the Minitab statistical package (v.18.1). Data were analyzed in a completely randomized design and the analyzed data are presented as means ± SEM and probability values. Values were considered statistically different at $p \leq 0.05$.

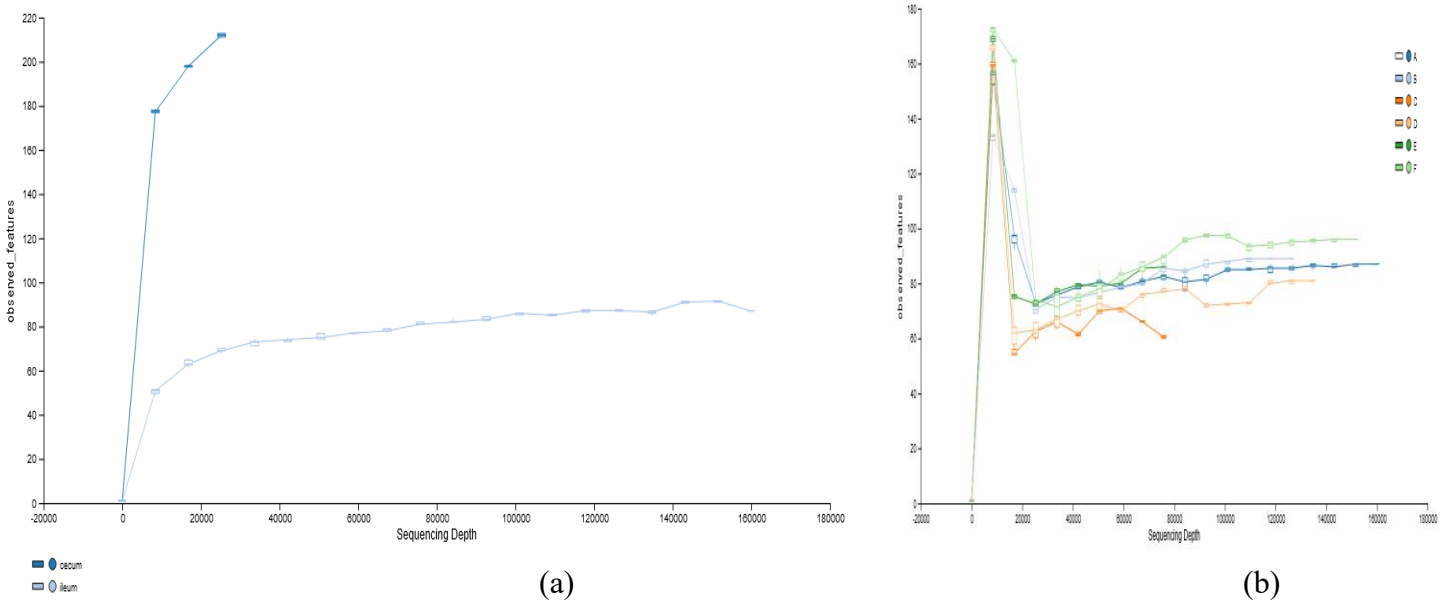
For the RNA-Seq analysis, adaptor sequences and low-quality scores containing bases (Phred score < 30) were trimmed from reads using Trimmomatic (Bolger et al., 2014). The resulting reads were aligned to the GRCg6 genome using STAR (Dobin et al., 2013). Read counts were obtained using HTSeq (Anders et al., 2015). The R package DESeq2 (Love et al., 2014) was used to identify differentially expressed genes between the groups. Nominal *p*-values were corrected for multiple testing using the Benjamini–Hochberg method. Gene ontology (GO) enrichment analysis was performed using the R package GOSeq (Young et al., 2010). Kyoto Encyclopedia of Genes and Genome (KEGG) Pathway Enrichment analyses of differentially expressed genes were performed on the PANTHER platform (http://pantherdb.org accessed on 26 September 2021,) (Mi et al., 2021).

6.4 Results

The 16S rRNA V4–V5 sequencing resulted in 8,774,523 quality read counts at an average of 60,934 counts per sample after quality filtering and demultiplexing. A total of 554 operational taxonomic units (OTUs) at the 97% sequence similarity level were obtained from all samples.

6.4.1 Microbiota Diversity

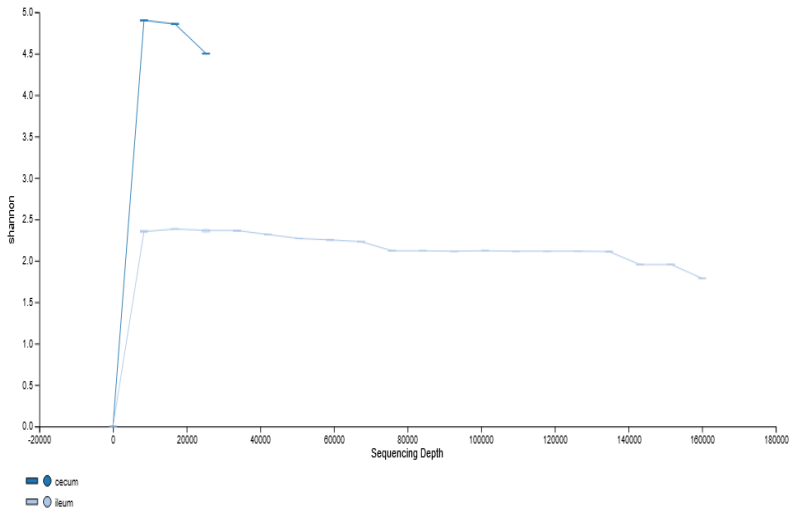
Internal sample α-diversity was estimated using the number of observed features (richness) and Shannon's index (diversity). Rarefaction curves of observed features and Shannon's index values reached a plateau in all samples, demonstrating that sequencing depth was adequate to cover the bacterial diversity in both ceca and ileal samples (Figures 6.2 and 6.3).



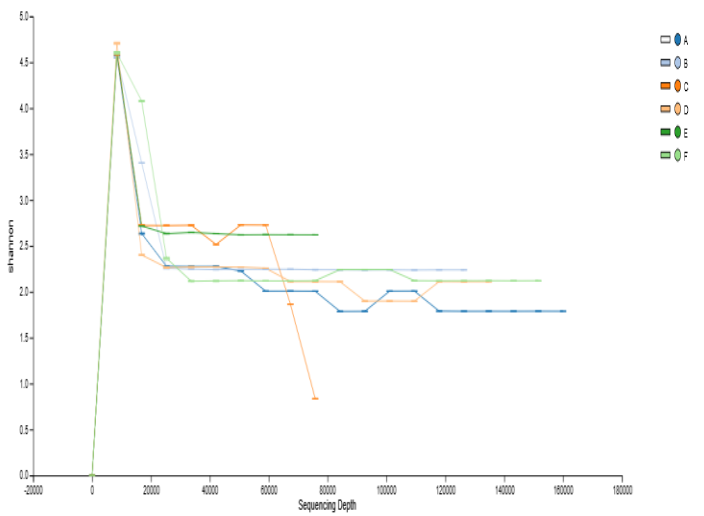
(a) (b)

Figure 6.2 Rarefaction curves of observed features obtained from 16S rRNA gene V4–V5 sequences.

On the basis of (a)microbiota source- cecum and ileum and (b) treatment- A) chicks fed a basal corn-soybean meal-wheat–based diet (Negative Control treatment; NC); B) chicks fed NC + 0.05% bacitracin methylene disalicylate (in-feed antibiotics); C) chicks supplied the same commercial blend of EOs via the water route (in-water essential oil) at the recommended dosage of 250 ml / 1000 L of drinking water; D) *in ovo* saline treatment; E) *in ovo* essential oil treatment; and F) chicks supplied EO via the *in ovo* and water route (*in ovo* + in-water essential oil treatment).



(a)

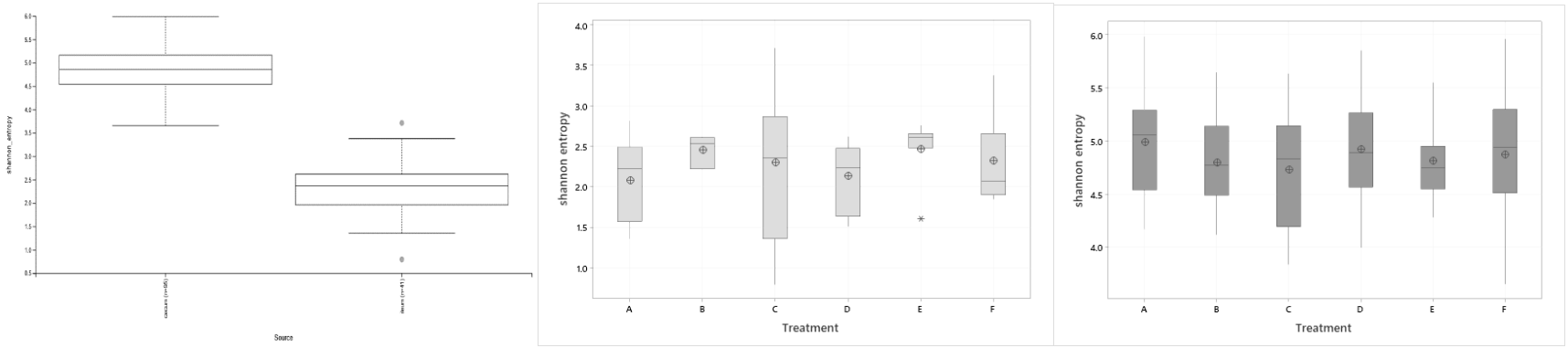


(b)

Figure 6.3 Rarefaction curves of Shannon's index obtained from 16S rRNA gene V4–V5 sequences.

On the basis of (a) Microbiota source- cecum and ileum and (b) Treatments, which include- A) Negative Control treatment- chicks fed a basal corn-soybean meal-wheat–based diet; B) In-feed antibiotics- chicks fed NC + 0.05% baci-tracin methylene disalicylate and C) In-water essential oil- chicks supplied the essential oil via the water route at the recommended dosage of 250 ml / 1000 L of drinking water; D) *In ovo* saline treatment- eggs injected with 0.2 mL of physiological saline (0.9% NaCl); E) *In ovo* essential oil treatment- eggs injected with 0.2mL of a saline + essential oil blend mixture at a dilution ratio of 2:1, F) *In ovo* + in-water essential oil treatment- chicks offered the essential oil blend via the *in ovo* and in water route, successively.

Alpha diversity inspection revealed significant ($p < 0.001$) diversity between the ileal and ceca samples but not between treatment groups (Figure 6.3a–c). Ceca samples recorded a higher Shannon diversity index compared to ileal samples. While Shannon's diversity index showed a similar profile between the treatment groups in both ceca and ileal tissues, the *in ovo* EO treatment recorded numerically higher alpha diversity in the ileum, the same as the NC treatment in the ceca.



(a) (b) (c)

Figure 6.4 Alpha diversity (Shannon's index) box plots.

Show (a) significant difference between ileal and ceca microbiota (Kruskal–Wallis, $p < 0.001$), (b) no significant effect of treatments on ileal microbiota (Kruskal–Wallis, $p > 0.05$), (c) no significant effect of treatment on ceca microbiota (Kruskal–Wallis, $p > 0.05$). Treatments include (A) negative control treatment—chicks fed a basal corn-soybean meal-wheat-based diet; (B) in-feed antibiotics—chicks fed NC + 0.05% bacitracin methylene disalicylate and (C) in-water essential oil—chicks supplied the essential oil via the water route at the recommended dosage of 250 mL/1000 L of drinking water; (D) in ovo saline treatment—eggs injected with 0.2 mL of physiological saline (0.9% NaCl); (E) in ovo essential oil treatment—eggs injected with 0.2 mL of a saline + essential oil blend mixture at a dilution ratio of 2:1; and (F) in ovo + in-water essential oil treatment—chicks offered the essential oil blend via the in *ovo* and in water route, successively. Boxes in the boxplots denote interquartile range, solid middle line in the boxes denote the median, and ⊕ denote the means.

To determine beta diversity, a principal coordinate analysis (PCoA) based on unweighted UniFrac distances was conducted. The PCoA plot showed unique cluster separation between the ileal and ceca microbiota; contrastingly, no difference in microbial community structure between treatments in both the ileum and ceca was observed (Figures 6.4).

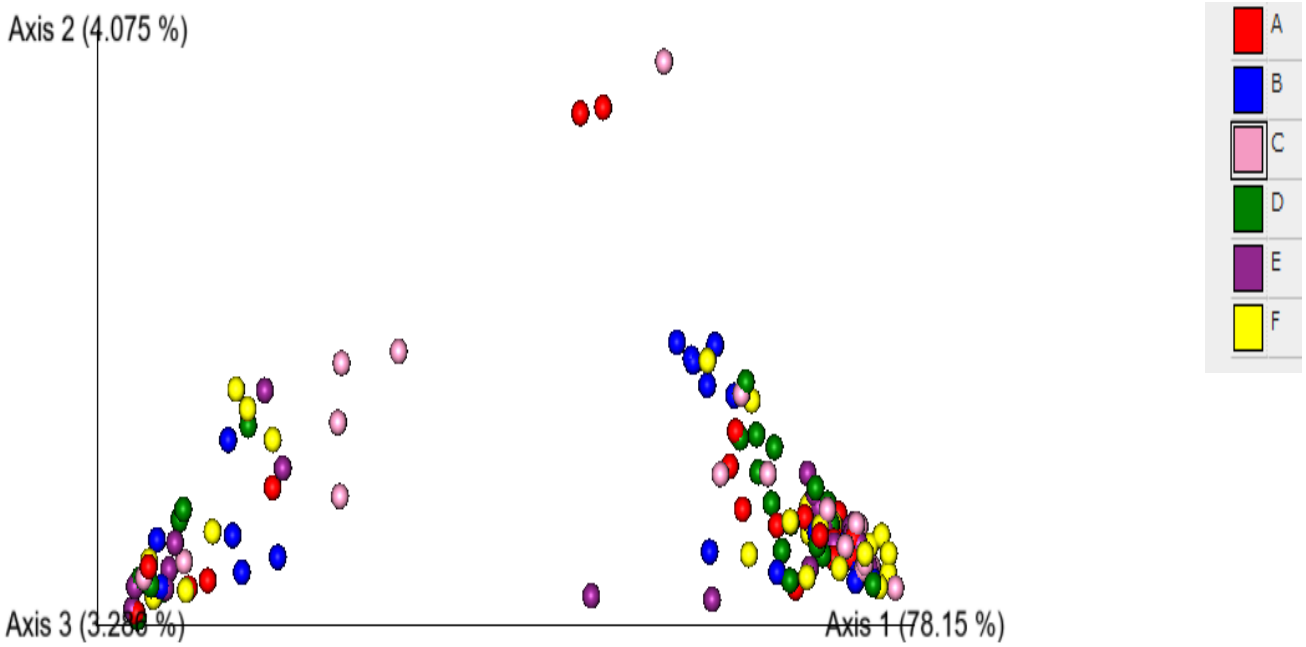
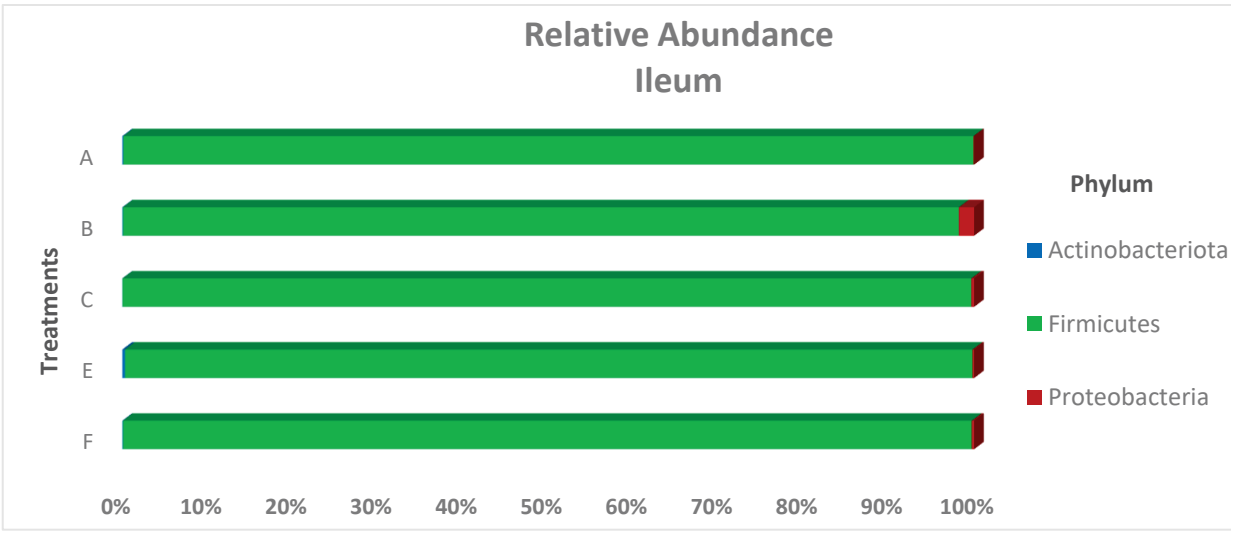


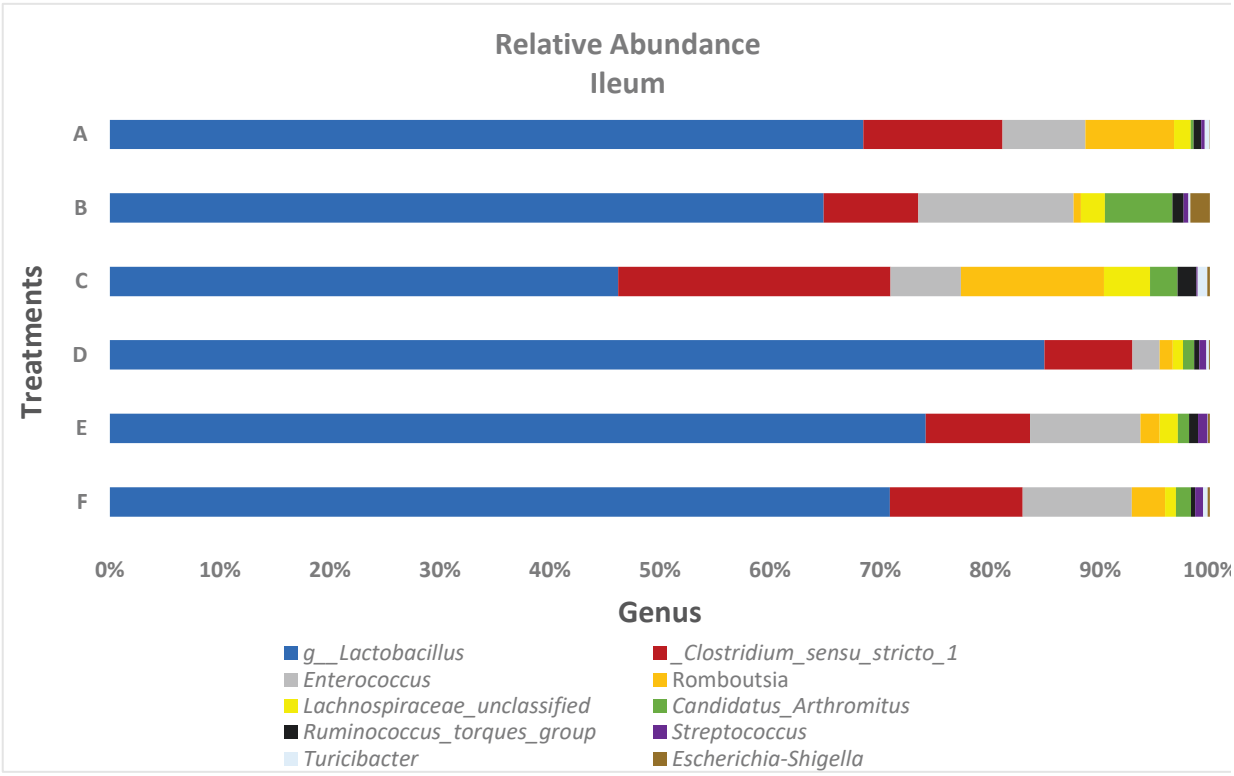
Figure 6.5 PCoA plots based on unweighted UniFrac metric. Each color represents a different comparison of interest. Ileum samples clustered to the left and ceca samples clustered to the right. Treatments include- A) Red: Negative Control treatment- chicks fed a basal corn-soybean meal-wheat–based diet; B) Blue: In-feed antibiotics- chicks fed NC + 0.05% bacitracin methylene disalicylate and C) Pink: In-water essential oil- chicks supplied the essential oil via the water route at the recommended dosage of 250 ml / 1000 L of drinking water; D)Green: In *ovo* saline treatment- eggs injected with 0.2 mL of physiological saline (0.9% NaCl); E) Purple: In *ovo* essential oil treatment- eggs injected with 0.2mL of a saline + essential oil blend mixture at a dilution ratio of 2:1, F) Yellow: In *ovo* + in-water essential oil treatment- chicks offered the essential oil blend via the in *ovo* and in water route, successively

6.4.2 Microbiota Composition

The relative abundance of the predominant bacteria phyla and genus in both the ileum and ceca are presented in Figures 6.4 and 6.5, respectively. At the phyla level, ileum microbiota was dominated by Firmicutes (range of 99.5–99.8%), Proteobacteria (range of 0.03–1.79%), and Actinobacteria (range of 0.03–0.12%) for all treatments. Ceca microbiota phyla taxa showed a similar trend as the ileal microbiota, as the relative abundance of Firmicutes (range of 98.3–99.6%) was found higher than Proteobacteria (range of 0.38–0.81%), which was also higher than Actinobacteria (range of 0.01–0.24%) across the treatment groups. At the genus taxa, the ileal microbiota was 96% dominated by *Lactobacillus*, *Clostridium sensu_stricto_1*, *Enterococcus*, *Romboutsia*, and *Lachnospiraceae_unclassified* species, with *Lactobacillus* species being the prevalent species (occurring > 64% in all treatments, except for the in-water EO treatment, which recorded a 46.2% *Lactobacillus* relative abundance). *Faecalibacterium* was the most abundant genus in the ceca, recording at least 40% abundance across treatment groups.



(**a**)

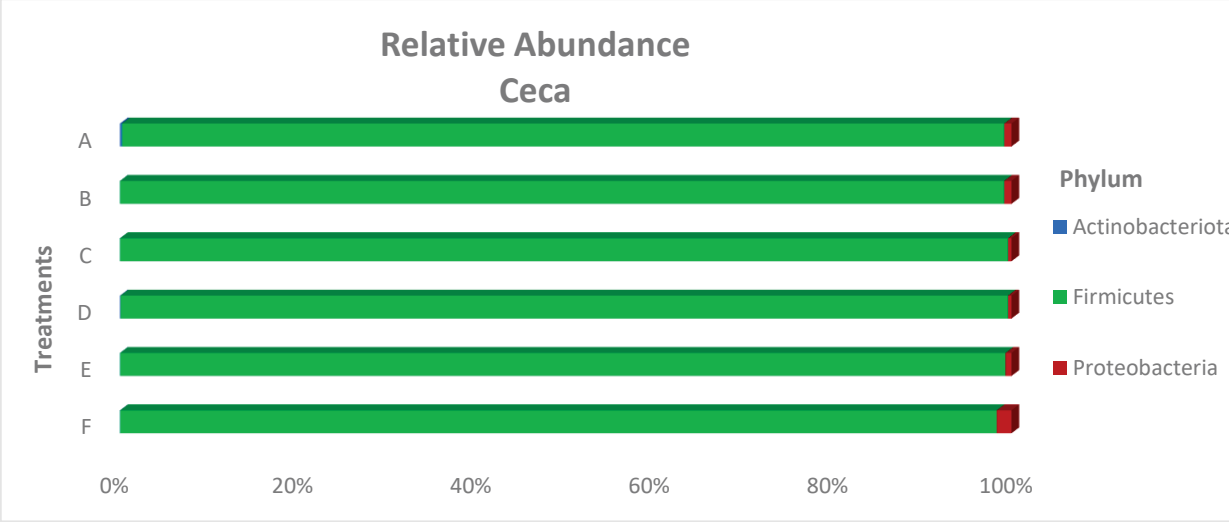


(**b**)

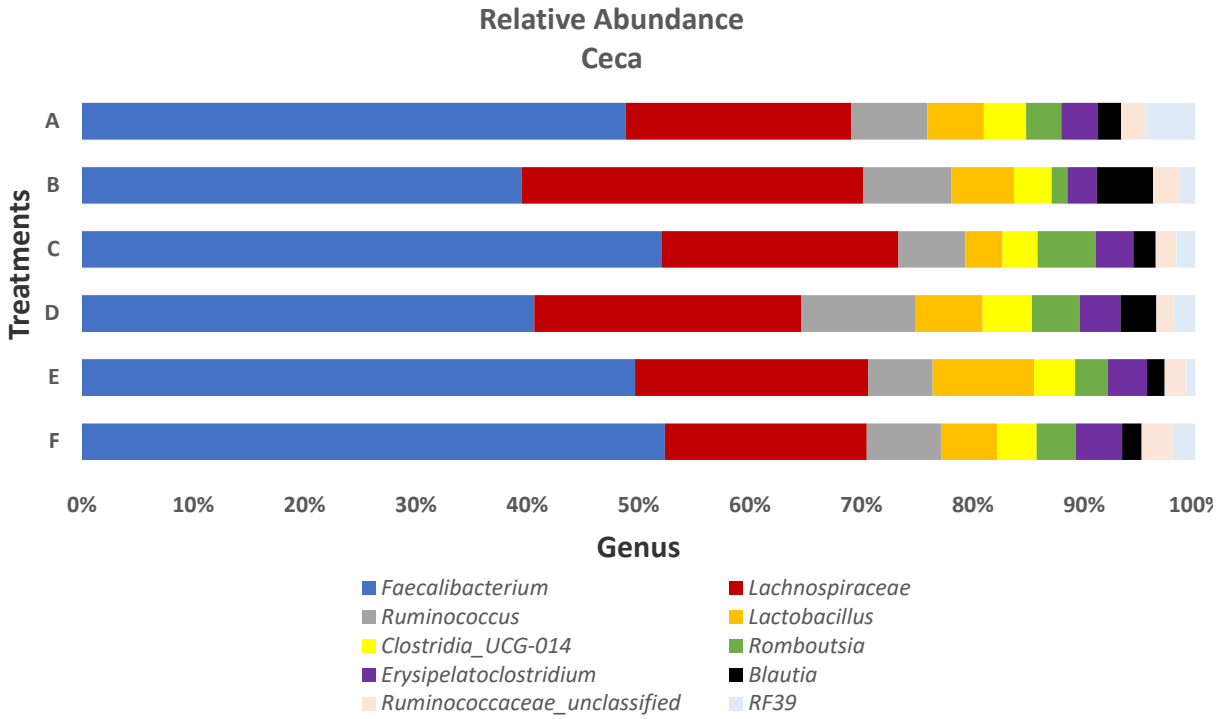
Figure 6.6 Ileal microbiota bacteria composition at the (**a**) phylum and (**b**) genus levels of broiler chickens subjected to different treatments groups.

Treatments include (A) negative control treatment—chicks fed a basal corn-soybean meal-wheat-based diet; (B) in-feed antibiotics—chicks fed NC + 0.05% bacitracin methylene disalicylate; (C) in-water essential oil—chicks supplied the essential oil via the water route at the recommended dosage of 250 mL/1000 L of

drinking water; (D) *in ovo* saline treatment—eggs injected with 0.2 mL of physiological saline (0.9% NaCl); (E) *in ovo* essential oil treatment—eggs injected with 0.2 mL of a saline + essential oil blend mixture at a dilution ratio of 2:1l; and (F) *in ovo* + in-water essential oil treatment—chicks offered the essential oil blend via the *in ovo* and in water route, successively.



(a)

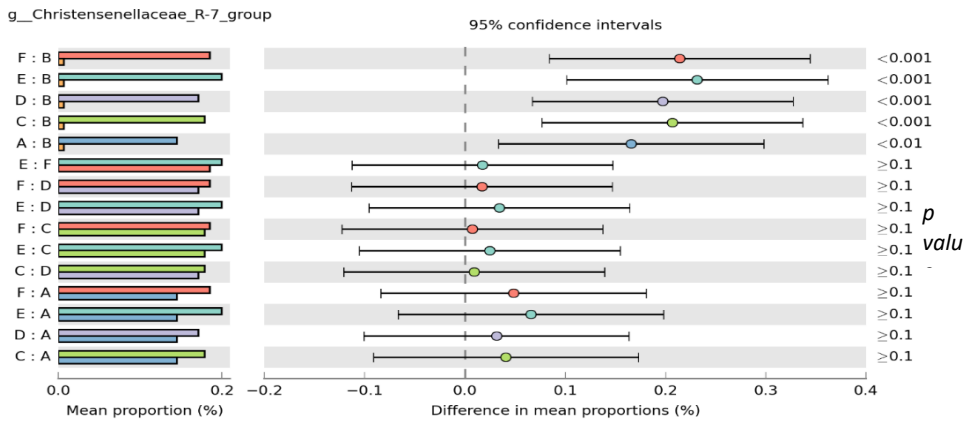


(**b**)

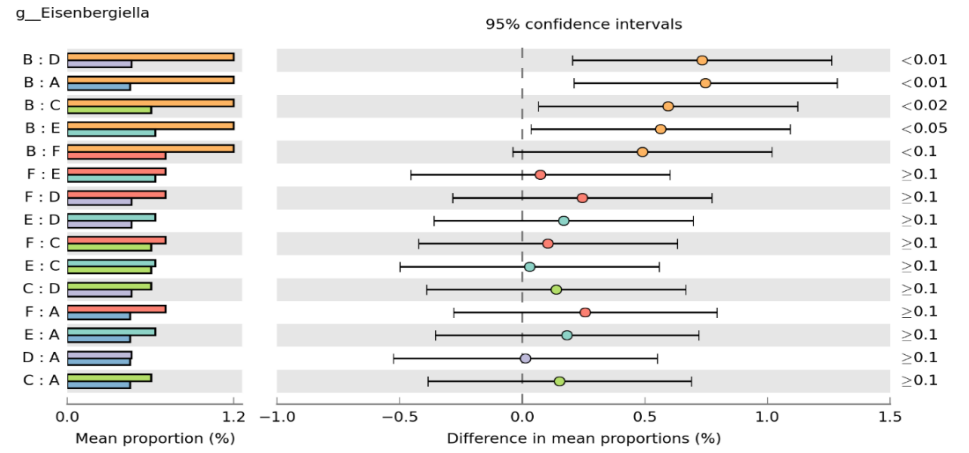
Figure 6.7 Ceca microbiota bacteria composition at the (**a**) phylum and (**b**) genus levels of broiler chickens subjected to different treatments groups.

Treatments include (A) negative control treatment—chicks fed a basal corn-soybean meal-wheat-based diet; (B) in-feed antibiotics—chicks fed NC + 0.05% bacitracin methylene disalicylate; (C) in-water essential oil—chicks supplied the essential oil via the water route at the recommended dosage of 250 mL/1000 L of drinking water; (D) *in ovo* saline treatment—eggs injected with 0.2 mL of physiological saline (0.9% NaCl); (E) *in ovo* essential oil treatment—eggs injected with 0.2 mL of a saline + essential oil blend mixture at a dilution ratio of 2:1; and (F) *in ovo* + in-water essential oil treatment—chicks offered the essential oil blend via the *in ovo* and in water route, successively.

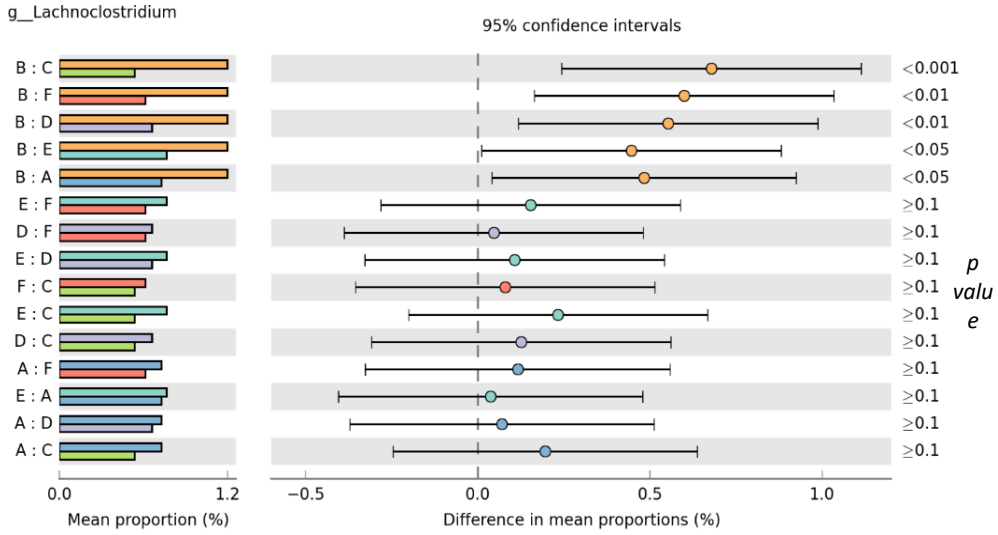
Similar to the ileal microbiota, genus *Lactobacillus* and *Romboutsia* were also found in the ceca, although at lower relative abundance. Contrastingly, the genus *Lachnospiraceae* was higher in the ceca (22.39%) compared to the ileum (1.91%). Significant differences in the cumulative proportions of bacteria in the genera *Christensenellaceae_R-7_group*, *Elsenbergiella*, *Lachnoclostridium*, and *Shuttleworthia* were recorded between treatments in the ceca (Figure 6.5). Compared to other treatments, the in-feed antibiotic treatment significantly ($p < 0.05$) increased the proportion of *Eisenbergiella*, *Lachnoclostridium*, and *Shuttleworthia*. Contrastingly, the proportion of bacteria *Christensenellaceae_R-7_group* ($p < 0.01$) in the ceca was reduced by the in-feed antibiotic treatment when compared to other treatments. No significant differences in the microbiota proportion between treatments were recorded in the ileum at both the phylum and genus levels.


g__Christensenellaceae_R-7_group
95% confidence intervals
F : B
E : B
D : B
C : B
A : B
E : F
F : D
E : D
F : C
E : C
C : D
F : A
E : A
D : A
C : A
<0.001
<0.001
<0.001
<0.001
<0.01
≥0.1
≥0.1
≥0.1
≥0.1
≥0.1
≥0.1
≥0.1
≥0.1
≥0.1
≥0.1
p valu
0.0
0.2
Mean proportion (%)
−0.2
−0.1
0.0
0.1
0.2
0.3
0.4
Difference in mean proportions (%)

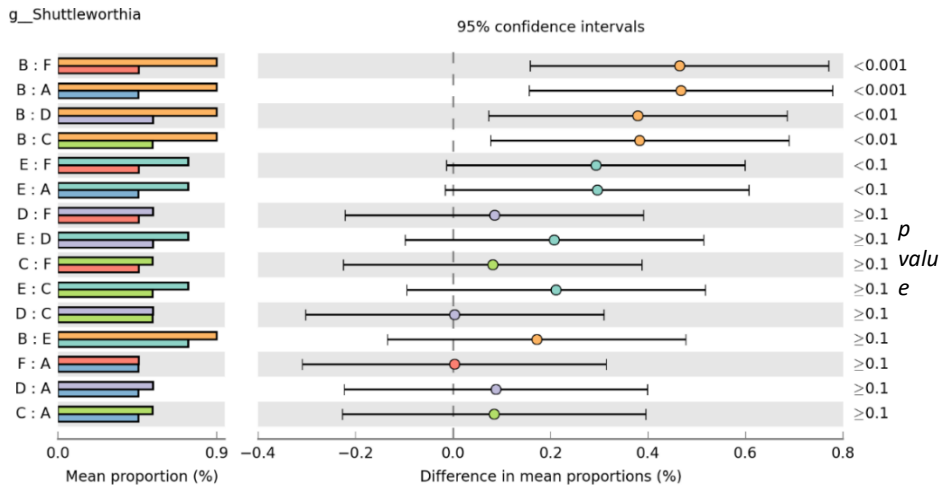
(a)


g__Eisenbergiella
95% confidence intervals
B : D
B : A
B : C
B : E
B : F
F : E
F : D
E : D
F : C
E : C
C : D
F : A
E : A
D : A
C : A
<0.01
<0.01
<0.02
<0.05
<0.1
≥0.1
≥0.1
≥0.1
≥0.1
≥0.1
≥0.1
≥0.1
≥0.1
≥0.1
≥0.1
0.0
1.2
Mean proportion (%)
−1.0
−0.5
0.0
0.5
1.0
1.5
Difference in mean proportions (%)

(b)


g__Lachnoclostridium
95% confidence intervals
B : C
B : F
B : D
B : E
B : A
E : F
D : F
E : D
F : C
E : C
D : C
A : F
E : A
A : D
A : C
<0.001
<0.01
<0.01
<0.05
<0.05
≥0.1
≥0.1
≥0.1
≥0.1
≥0.1
≥0.1
≥0.1
≥0.1
≥0.1
≥0.1
p valu e
0.0
1.2
Mean proportion (%)
−0.5
0.0
0.5
1.0
Difference in mean proportions (%)

(c)



(a)

Figure 6.8 Significant differences (ANOVA, B–H FDR corrected *P* value: $P < 0.05$) in cumulative proportions of genus (a) *Christensenellaceae*_R-7_group, (b) *Elsenbergiella*, (c) *Lachnoclostridium*, and (d) *Shuttleworthia* in ceca microbiota of broiler chickens subjected to different treatments groups. Treatments include (A) negative control treatment—chicks fed a basal corn-soybean meal-wheat-based diet; (B) in-feed antibiotics—chicks fed NC + 0.05% bacitracin methylene disalicylate; (C) in-water essential oil—chicks supplied the essential oil via the water route at the recommended dosage of 250 mL/1000 L of drinking water; (D) in ovo saline treatment—eggs injected with 0.2 mL of physiological saline (0.9% NaCl); (E) in *ovo* essential oil treatment—eggs injected with 0.2 mL of a saline + essential oil blend mixture at a dilution ratio of 2:1; and (F) in *ovo* + in-water essential oil treatment—chicks offered the essential oil blend via the in *ovo* and in water route, successively.

6.4.3 Ceca SCFA Concentration

The resulting concentrations of ceca SCFA are presented in Table 6.2. Only the concentration of butyric acid recorded a statistical trend towards significance ($P = 0.09$) in the in-water EO treatment, compared to other treatments. All other acids that were quantified recorded no statistical significance between treatment groups ($P > 0.05$). Nonetheless, the in-water essential oil treatment equally recorded numerically higher concentrations of acetic, lactic, volatile, and total fatty acids. Total bacteria (copies/gram of sample) were also found to be higher ($P > 0.05$) in the in-water EO treatment when compared to other treatments.

Table 6.2 Effect of essential oil delivery route on ceca short-chain fatty acid concentration (SCFA) and total eubacteria (copies/gram of sample) in broiler chickens.

Short-Chain Fatty Acid Concentration (mmol/kg)	Treatments [1]						SEM [2]	*P* Value
	Negative Control	In-Feed Antibiotics	In-Water Essential Oil	*In ovo* Saline	*In ovo* Essential Oil	*In ovo* Essential Oil + In-Water Essential Oil		
Acetic acid	51.9	50.5	57.2	55.4	50.9	55.8	1.73	0.82
Propionic acid	4.24	3.79	4.31	4.43	3.81	4.36	0.18	0.86
Butyric acid	13.6	15.7	19.3	18.8	13.4	17.9	0.77	0.09
Valeric acid	1.05	0.79	0.87	1.17	1.08	1.07	0.07	0.67
Lactic acid	0.60	0.73	1.40	1.26	1.09	0.83	0.86	0.49
Total SCFA	74.1	74.2	89.7	84.1	74.4	82.4	2.65	0.41
Branched-chain fatty acids	2.25	1.63	1.94	2.28	1.77	1.91	0.11	0.46
Volatile fatty acids	73.1	72.4	83.6	82.1	70.9	81.0	2.40	0.49
Total eubacteria (copies/gram of sample)	2.3×10^{12}	1.9×10^{12}	3.0×10^{12}	2.6×10^{12}	2.5×10^{12}	2.2×10^{12}	2.06×10^{12}	0.72

[1]Treatments include (1) negative control treatment—chicks fed a basal corn-soybean meal-wheat-based diet; (2) in-feed antibiotics—chicks fed NC + 0.05% bacitracin methylene disalicylate; (3) in-water essential oil-chicks supplied the essential oil via the water route at the recommended dosage of 250 mL/1000 L of drinking water; (4) *in ovo* saline treatment—eggs injected with 0.2 mL of physiological saline (0.9% NaCl); (5) *in ovo* essential oil treatment—eggs injected with 0.2 mL of a saline + essential oil blend mixture at a dilution ratio of 2:1; (6) *in ovo* + in-water essential oil treatment—chicks offered the essential oil blend via the *in ovo* and in water route, successively. [2] SEM = pooled standard error of means. Mean values from n = 16 birds/treatment group are presented.

6.4.4 Transcriptome Analysis

In this study, to identify differentially expressed mRNAs in the liver of broiler chickens, a total of 6,360,427,350 raw reads were generated from 48 samples (Table 6.3). After the trimming step, 6,357,176,660 clean reads were obtained, and the clean reads were aligned to the whole genome of Gallus gallus domesticus (Fasta: Gallus_gallus. GRCg6a.fa, Annotation: Gallus_gallus.GRCg6a.Ensembl98.gtf, source: Ensembl98). An average of 95.56% of clean reads were mapped to the genome. To determine the statistical significance of differentially expressed genes (DEGs) between treatments, the genes with the parameter of false discovery rate (FDR < 0.05) were considered differentially expressed genes/transcripts. Only a limited number of DEGs were observed (6: 3 up-regulated, and 3 down-regulated) to be differentially influenced by the treatments (Table 6.4). The *in ovo* + in-water EO recorded the highest number (2) of DEGs in this category, as it down-regulated the expression of both cubilin (CUBN) and aldehyde dehydrogenase 1 family member L2 (ALDH1L2) genes. A heatmap illustrating the top 100 most variable genes is presented in Figure 6.6.

Table 6.3 Sequencing data quality control metrics for 48 liver samples for 6 treatment groups[1]

Sample	Raw Reads #	Surviving Reads #	Surviving %	Mapped Reads #	Mapped %
10D	128931886	128864410	99.95	123496072	95.83
11F	123815424	123749336	99.95	117546419	94.99
12E	100217620	100171610	99.95	95675887	95.51
13A	91263880	91218332	99.95	87621362	96.06
14E	102874686	102803518	99.93	98365003	95.68
15F	89610956	89558286	99.94	85844381	95.85
16B	103696522	103649450	99.95	99566563	96.06
17D	102727142	102673518	99.95	97718554	95.17
18C	102658094	102589816	99.93	97517267	95.06
19E	114613450	114541804	99.94	109055322	95.21
1B	111989480	111935440	99.95	107217188	95.78
20B	105128716	105083912	99.96	101140738	96.25
21D	86558618	86518732	99.95	82682287	95.57
22A	86201504	86143930	99.93	82400996	95.66
23F	97189216	97135594	99.94	92538383	95.27
25F	129747010	129692014	99.96	124312086	95.85
26D	98609268	98564006	99.95	94445790	95.82
27A	103167822	103122160	99.96	98995073	96.00
28E	119433662	119384804	99.96	113827077	95.34
29C	71048832	71015376	99.95	67880932	95.59
2E	122294110	122221882	99.94	116442856	95.27
30B	100435658	100385796	99.95	96299990	95.93
31D	110590420	110541296	99.96	106048032	95.94
32C	157571810	157485346	99.95	150219923	95.39
33A	103717506	103676040	99.96	99480220	95.95
34E	114963742	114911374	99.95	109633108	95.41
35F	99621346	99571764	99.95	94015694	94.42
36B	89037488	88999280	99.96	85651443	96.24
37E	93691904	93644694	99.95	89307326	95.37
38A	74565050	74529310	99.95	71237815	95.58
39F	96777946	96716112	99.94	92174462	95.30
3D	115361710	115292412	99.94	110464518	95.81
40D	81332670	81290328	99.95	77473448	95.30
41B	78153658	78115760	99.95	74718506	95.65
42C	89136784	89093766	99.95	85224056	95.66
43B	84600692	84556594	99.95	80853617	95.62
44E	106835414	106789686	99.96	102394089	95.88
45A	95541636	95493314	99.95	91460060	95.78
46F	76636156	76598132	99.95	73012433	95.32
47C	112641994	112573638	99.94	107374095	95.38
48D	94773314	94702008	99.92	90015678	95.05
4A	93142656	93092202	99.95	88266669	94.82
5C	102491666	102433822	99.94	97796357	95.47
6F	98781198	98720274	99.94	94109603	95.33

7B	834450506	833996014	99.95	798312414	95.72
8A	851554080	851180034	99.96	812162674	95.42
9C	123838248	123780280	99.95	118300543	95.57
24C	88404200	88369454	99.96	84734829	95.89

[1] Treatments include- A) Negative Control treatment- chicks fed a basal corn-soybean meal-wheat–based diet; B) In-feed antibiotics- chicks fed NC + 0.05% bacitracin methylene disalicylate and C) In-water essential oil- chicks supplied the essential oil via the water route at the recommended dosage of 250 ml / 1000 L of drinking water; D) *In ovo* saline treatment- eggs injected with 0.2 mL of physiological saline (0.9% NaCl); E) *In ovo* essential oil treatment- eggs injected with 0.2mL of a saline + essential oil blend mixture at a dilution ratio of 2:1, F) *In ovo* + in-water essential oil treatment- chicks offered the essential oil blend via the *in ovo* and in water route, successively.

Table 6.4 Differentially expressed genes in the liver of broiler chickens as influenced by treatment groups [1].

Treatments	Gene Symbol	Gene Description	Expression Level	log2FoldChange	*P* Value
B vs. A	ALDH1L2	aldehyde dehydrogenase 1 family member L2	Down	−0.6	<0.01
C vs. A	BTN1A1	butyrophilin subfamily 1 member A1-like	Up	0.4	0.02
D vs. A	AVD	Avidin	Up	0.5	<0.01
E vs. A	ST8SIA6	ST8 alpha-N-acetyl-neuraminide alpha-2,8-sialyltransferase 6	Up	0.6	<0.01
	CUBN	Cubilin	Down	−0.7	0.01
F vs. A	ALDH1L2	aldehyde dehydrogenase 1 family member L2	Down	−0.6	0.01

[1]Treatments include (A) negative control treatment—chicks fed a basal corn-soybean meal-wheat-based diet; (B) in-feed antibiotics-chicks fed NC + 0.05% bacitracin methylene disalicylate; (C) in-water essential oil—chicks supplied the essential oil via the water route at the recommended dosage of 250 mL/1000 L of drinking water; (D) *in ovo* saline treatment—eggs injected with 0.2 mL of physiological saline (0.9% NaCl); (E) *in ovo* essential oil treatment—eggs injected with 0.2 mL of a saline + essential oil blend mixture at a dilution ratio of 2:1; (F) *in ovo* + in-water essential oil treatment-chicks offered the essential oil blend via the *in ovo* and in water route, successively. Each comparison is specified in the format "B vs. A", where group B is compared to group A, with group A being the denominator for the comparison. Liver tissues (50–100 mg) were sampled from 8 replicate birds/treatment (independent of sex) using 1 mL TRIzol™ (Qiagen, Hilden, Germany).

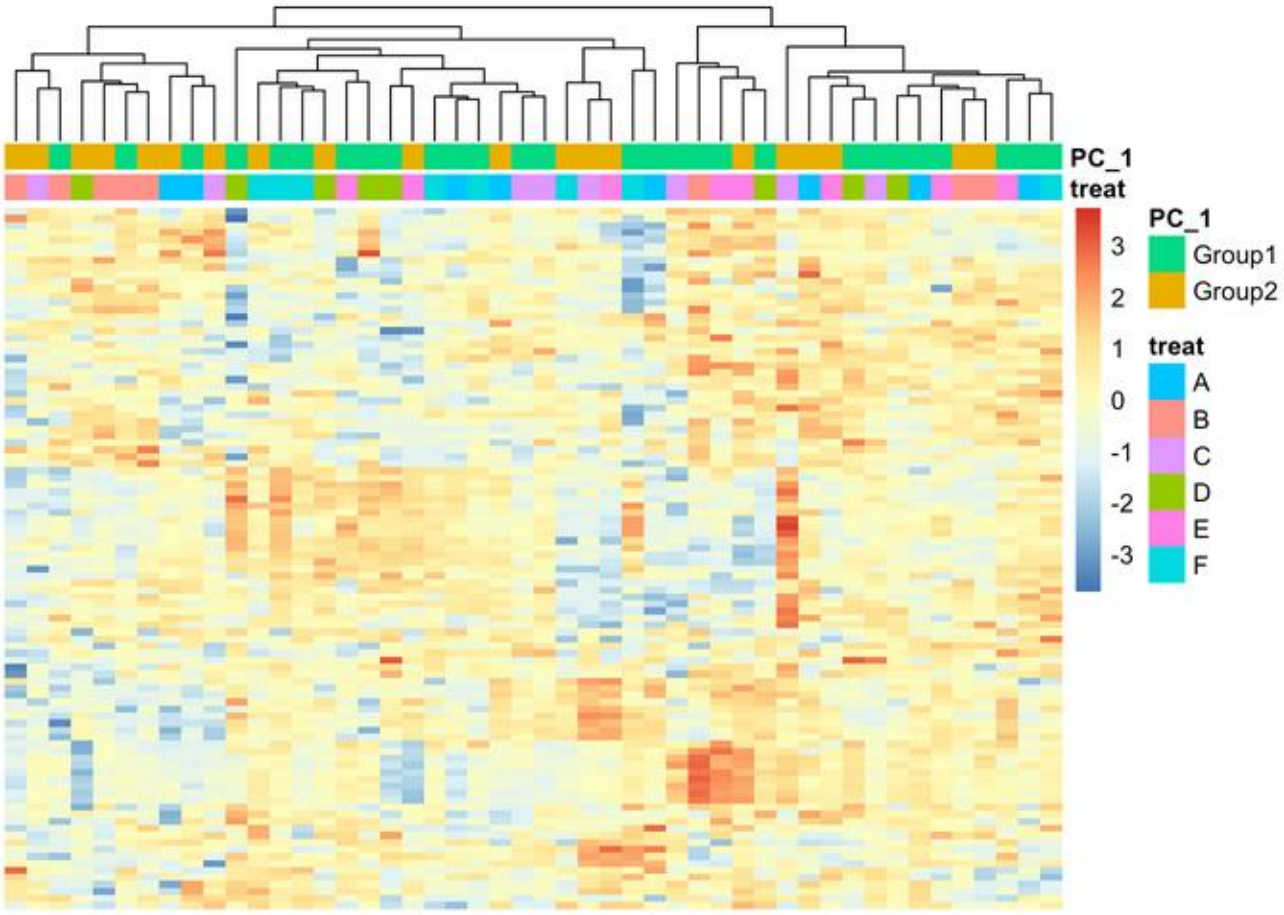


Figure 6.9 Heatmaps of the top 100 most variable genes.

Orange = low expression, blue = high expression Treatments include- A) Negative Control treatment- chicks fed a basal corn-soybean meal-wheat–based diet; B) In-feed antibiotics- chicks fed NC + 0.05% bacitracin methylene disalicylate and C) In-water essential oil- chicks supplied the essential oil via the water route at the rec-ommended dosage of 250 ml / 1000 L of drinking water; D) *In ovo* saline treatment- eggs injected with 0.2 mL of physiological saline (0.9% NaCl); E) *In ovo* essential oil treatment- eggs injected with 0.2mL of a saline + essential oil blend mixture at a dilution ratio of 2:1, F) *In ovo* + in-water essential oil treatment- chicks offered the essential oil blend via the *in ovo* and in water route, successively.

6.5 Discussion

The supplementation of phytogenic feed additives, especially essential oil, is reported to promote lipid and cholesterol metabolism (Sabino et al., 2018), as well as enhance immunity (Kim et al., 2010), leading to improved poultry performance. These favorable effects are thought to be exerted through the modulation of gut microbiota and the expression of several unique genes (Kim et al., 2010; Lillehoj et al., 2011; Bastos et al., 2017; Sabino et al., 2018; Li et al., 2020). However, in vivo results on the effect of EO on chicken microbiota are somewhat inconsistent. While EO blends have been reported to reduce the relative abundance of pathogenic bacteria like *Escherichia coli* (Cho et al., 2014; Hashemipour et al., 2016), *Salmonella* (Pathuk et al., 2017), *and Clostridium perfringens* (Mitsch et al., 2004) in broiler chickens, a few studies have equally reported no effect of EO supplementation on gut commensal bacteria (Hong et al., 2012; Pathak et al., 2017; Paraskeuas and Mountzouris et al., 2019). This study utilized 16S rRNA gene sequencing in combination with transcriptomic analysis to investigate the effect of essential oil and its delivery routes (in water, *in ovo,* and *in ovo* + in water) and 0.05% bacitracin on both the composition and diversity of ileal and ceca microbiota and liver transcriptomics. Additionally, ceca short-chain fatty acid concentration was also evaluated. Bacitracin, the positive control in this study, is an extensively used antibiotic growth promoter in the poultry industry (Huyghebaert et al., 2011). There is no doubt that the detailed delineation of the effect of a classic AGP like bacitracin and an alternative to AGP and its delivery routes, as this study presents, is key to understanding the molecular mechanisms underlying growth promotion in poultry. Accordingly, this study provides insight into the microbiota-mediated mode of action of antibiotics growth promoters, as well as preliminary transcriptomic evidence suggesting sex-controlled hepatic differential gene expression in broiler chickens offered antibiotics and essential oil (via water, *in ovo*, and *in ovo* + in-water delivery routes).

The gut microbiota plays an important role in host health, immune modulation, nutrient absorption, and pathogen control (Oakley et al., 2014; Wang et al., 2016). Although no treatment effect was recorded, this study revealed higher alpha (Shannon index) diversity in broiler chicken ceca compared to the ileum. This agrees with other studies (Gong et al., 2008; Owens et al., 2008; Choi et al., 2014), which have also recorded higher microbial diversity in the ceca. Higher microbial richness and stability observed in the ceca compared to the ileum in broiler chicken have been correlated with the higher number of obligate anaerobic microbes present therein, compared

to aerobes or facultative anaerobes (Wang et al., 2016). Consistent with the results of this study, both Abdelli et al. (2020) and Pham et al. (2020) have equally reported no effect of EO on alpha diversity. Thibodeau et al. (2015) have shown that only extreme events (dysbiosis and disease inclusive) which modify the number of ecological niches in different bacterial species can alter the alpha diversity.

Furthermore, beta diversity analysis revealed no difference in microbial community structure between treatments at both the ileum and ceca; however, the bacteria communities clearly differed across both gut sections. Other studies involving AGP or EO supplementation in broiler chickens have also observed similar results (Choi et al., 2018; Bauer et al., 2019; Yang et al., 2020). Conversely, Pham et al. (2020) have recently highlighted the potential of EO to modulate the gut bacterial community structure. Aside from differences in intestinal sections, time of sampling, and supplemented additives, other factors, including broiler chicken breed, age, environmental condition, and disease status, can potentially cause shifts in beta diversity (Stanley et al., 2014; Wang et al., 2016). Diseases accompanied by intestinal dysbiosis like necrotic enteritis and *Eimeria* infection are reported to cause a significant change in gut microbiota community structure (Xu et al., 2018; Proszkowiec-Weglarz et al., 2020), buttressing the healthy state of the flock in this study.

Furthermore, in-feed antibiotic treatment significantly ($p < 0.05$) increased the proportion of *Eisenbergiella*, *Lachnoclostridium*, and *Shuttleworthia*, while decreasing ($p < 0.01$) the proportion of *Christensenellaceae_R-7_*group in the ceca, as compared to other treatments in this study. Interestingly, all of the bacteria with an increased proportion belong to the family *Lachnospiraceae*. The abundance of bacteria in the family *Lachnospiraceae* has been associated with improved weight gain (Lee et al., 2017), feed conversion ratio (Stanley et al., 2016), and butyrate production in broiler chickens (Meehan and Beiko, 2014; Yacoubi et al., 2018). The performance result from this study published in Oladokun et al. (2021b) shows the increased weight gain recorded by the in-feed antibiotic treatment in the early period (d 1–14), compared to the *in ovo* treatments group, supports this result. Consistent with our results, Zhong et al. (2021) reported the increased abundance of bacteria in the genus *Eisenbergiella* in neonates offered probiotics and antibiotics concurrently. An increased abundance in bacteria of the genus *Eisenbergiella* has been associated with reduced incidence of gastrointestinal disorders linked to metabolic and microbiota changes (functional dyspepsia), resulting in improved nutrient metabolism in broiler chickens (McKenna et al., 2020). Nonetheless, a few studies have also

associated the abundance of this genera with the incidences of subclinical enteritis and *Eimeria* infection in broiler chickens (Yang et al., 2018; Wang et al., 2021), emphasizing the cost-benefit effects of antibiotic use in poultry production and the need for more studies in this regard. *Lachnoclostridium* has been positively correlated with increased butyrate production with attendant gut health protection and pathogen control effects (Eeckhaut et al., 2011; Polansky et al., 2016). Probiotics (Jacquier et al., 2019) prebiotics (wheat bran) (Shang et al., 2020), and antibiotics, but not essential oil, have all been reported to enrich the abundance of *Lachnoclostridium* in broiler chicken ceca (Xue et al., 2020).

Similar to other enriched genera in the ceca in this study, the genus *Shuttleworthia* has also been associated with increased weight gain and growth performance resulting from a possible role in lipid and carbohydrate metabolic pathways (Lee et al., 2017). Furthermore, disease conditions like avian leukosis virus (Ma et al., 2017), coccidia infection (Chen et al., 2020), and *Salmonella* infection have all been reported to decrease the abundance of bacteria in the genus *Shuttleworthia* in broiler chicken ceca (Khan et al., 2020). Similar to the results presented here, Hung et al. (2019) have also reported a reduced abundance of members of the genus *Christensenellaceae* in the feces of weaned piglets offered the antibiotic bacitracin. Although the functional role of bacteria in this genus in the chicken microbiota is not fully known, their abundance has been associated with the colonization *of Campylobacter jejuni*, a foodborne zoonotic pathogen (Thibodeau et al., 2015). While the results presented here suggest that antibiotic growth promoters might give the birds a better growth advantage than EOs under this experimental condition, such growth advantage might come with some metabolic costs, deducible from the metabolic functions of bacteria species enhanced by this treatment. Hence, more research is needed on potent alternatives to antibiotic growth promoters with no reported adverse effects on the poultry industry. Nonetheless, the results on gut microbiota presented here provide a critical perspective on microbiota-mediated mode of action of antibiotics growth promoters in broiler chickens.

The fermentation of dietary fibers to yield SCFA constitutes an important function of the ceca commensal microbiota. No significant effect of evaluated treatments on ceca SCFA concentration was recorded in this study. Only the in-water EO treatment showed a statistical trend ($p = 0.09$) of enhancing ceca butyric acid concentration relative to other treatments. Butyric acid serves as an important energy substrate for the maintenance and proliferation of gut colonic cells and structures (Kulshreshtha et al., 2017; Bauer et al., 2019). Essential oils (in feed or in water) have been reported to increase the concentrations of acetic, butyric, propionic, and lactic

acids and total SCFA in quail breeders (Aydın and Yıldız, 2020) and broiler chickens (Tiihonen et al., 2010; Mašek et al., 2014; Ren et al., 2019). The positive effect of EO on ceca SCFA concentration could be related to the capacity of their phytogenic formulations to enhance bacteria proliferation in the lower gut. Several variable factors that potentially influence SCFA concentrations in broiler chickens, including the microbiota composition, bird age, and the amount and type of available fermentable substrates, explain the observed results in this study (Cho et al., 2014; Sun et al., 2020; Adewole et al., 2021).

Transcriptomic analysis in this study suggests unique sex-controlled gene expression in broiler chicken livers. To evaluate the similarities and dissimilarities between samples in an unsupervised manner, principal component exploratory analysis (PCA) was carried out. The PCA showed that samples were not segregated by treatments (Figure 6.7) but instead showed modest segregation based on an unknown variable, probably sex. To confirm the hypothesis that treatments indeed clustered based on sex, the expression of five highly expressed genes on the W chromosome was examined. The results showed that all five genes showed much higher expression in group two than group one, indicating that group two samples were probably female (group two were samples with PC1 score > 0, group one were samples with PC1 scores < 0) (Figure 6.8).

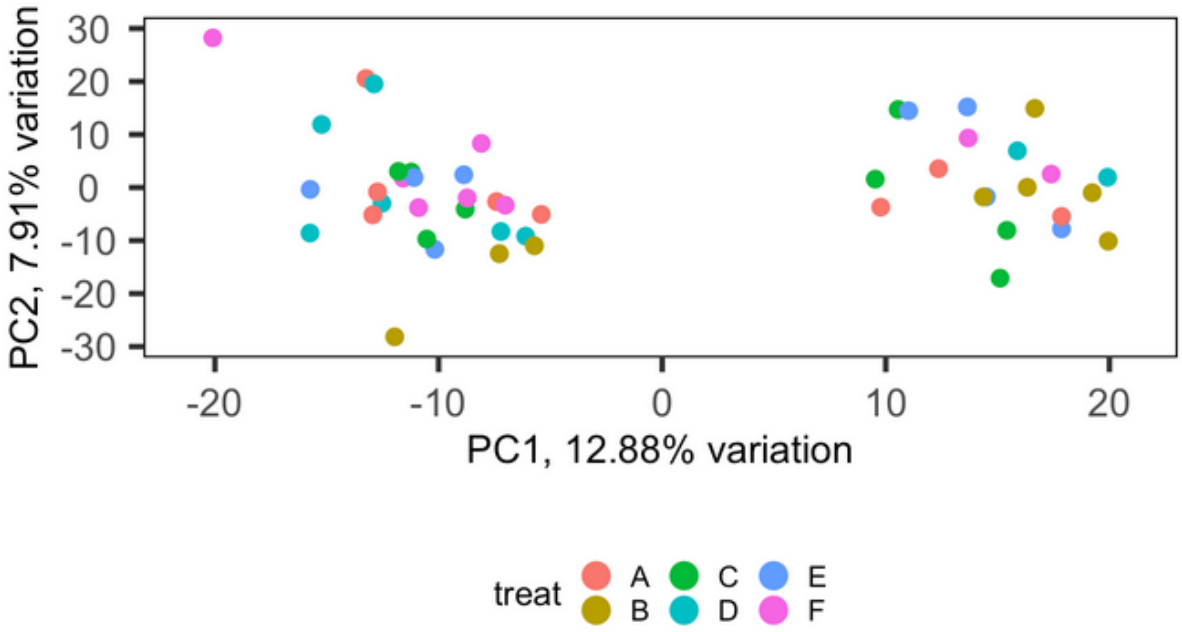


Figure 6.7 Principal component analysis (PCA) was performed with R package gmodels.

The more similar the treatment, the closer the distance reflected in PCA. Treatments include- A) Negative Control treatment- chicks fed a basal corn-soybean meal-wheat–based diet; B) In-feed antibiotics- chicks fed NC + 0.05% bacitracin methylene disalicylate and C) In-water essential oil- chicks supplied the essential oil via the water route at the recommended dosage of 250 ml / 1000 L of drinking water; D) *In ovo* saline treatment- eggs injected with 0.2 mL of physiological saline (0.9% NaCl); E) *In ovo* essential oil treatment- eggs injected with 0.2mL of a saline + essential oil blend mixture at a dilution ratio of 2:1, F) *In ovo* + in-water essential oil treatment- chicks offered the essential oil blend via the *in ovo* and in water route, successively.

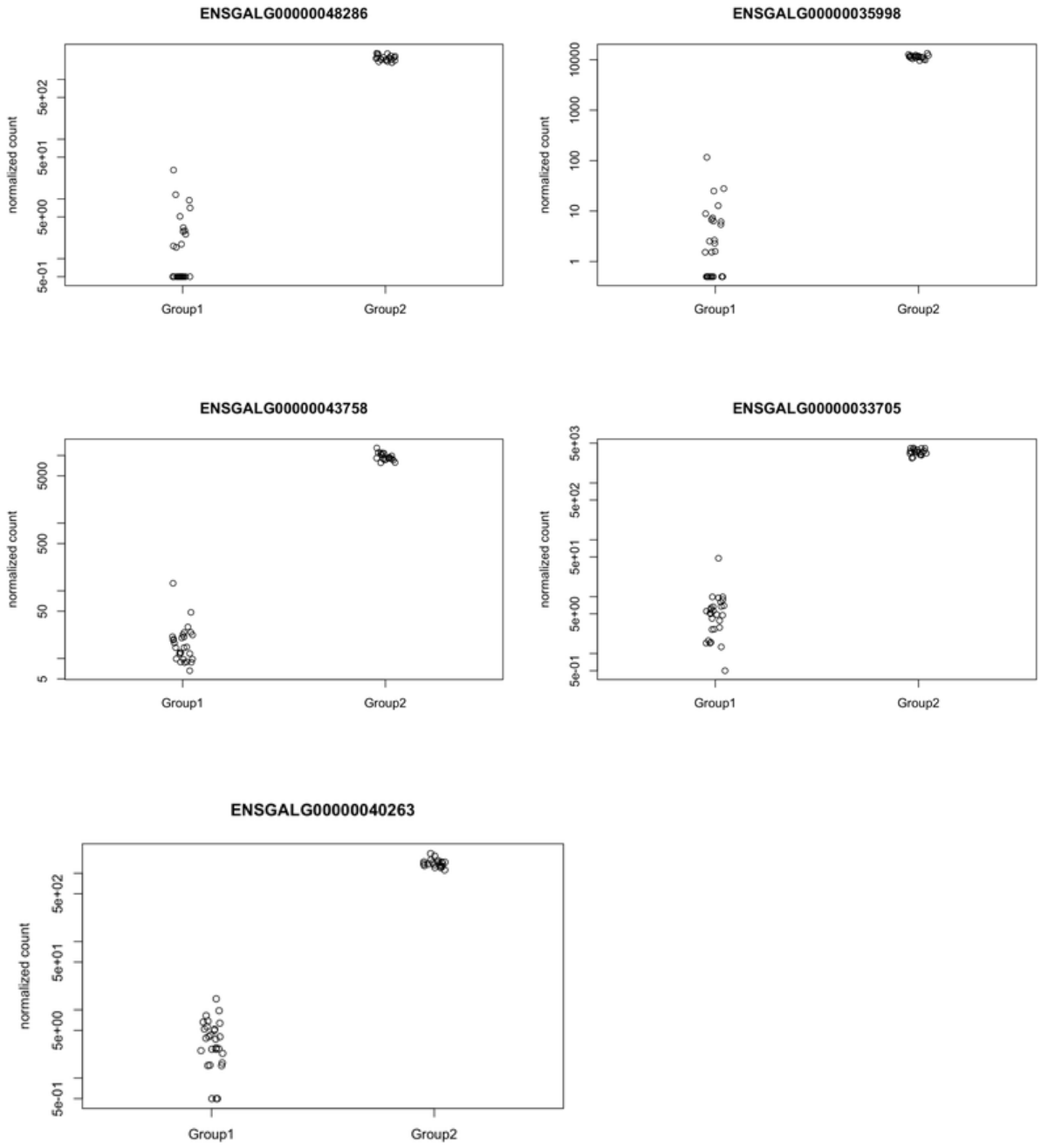


Figure 6.8 Gene expression of chrW gene shows much higher expression in Group2 than Group1, indicating that Group2 samples are probably females (n=20) and Group1 samples are probably males (n=28). Group2 are samples with PC1 score > 0, Group 1 are samples with PC1 scores < 0.

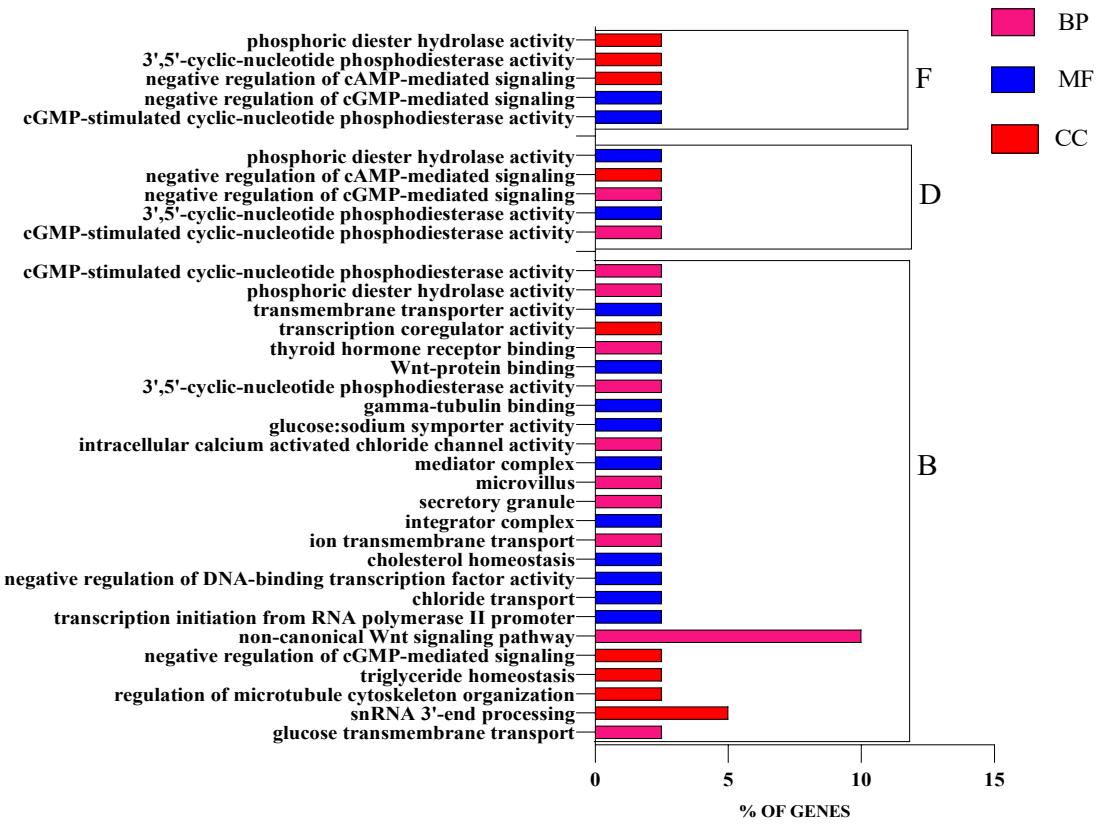
Based on these PCA gene expression plots, samples were thus assigned as male or female according to their PC1 score. A total of 14 DEGs were found to be influenced by the treatment and sex (Table 6.5). Of these DEGs, six genes were up-regulated (fold change range from 0.4 to 1.1) and eight genes were down-regulated (fold change range from −0.5 to −0.9). Sex-based analysis revealed that in male transcripts, antibiotic treatments recorded the highest number of DEGs (seven genes: four upregulated and three downregulated) compared to other treatments. Similarly, in male transcripts, the BVES (blood vessel epicardial substance) gene was significantly downregulated in both antibiotics, in-water essential oil, and *in ovo* essential oil treatments. In female birds, only four DEGS (two upregulated, two downregulated) were recorded amongst treatment groups. To understand the functional roles of identified DEGs, GO and KEGG pathway analysis was carried out. The GO analysis showed that a total of 33 significant GO categories were enriched ($P < 0.05$) compared to the negative control treatment (Figure 6.9a, b). The GO terms included both biological process (BP), cellular component (CC), and molecular function (MF).

Table 6.5 Differentially expressed genes in the liver of broiler chickens as influenced by sex and treatment groups[1].

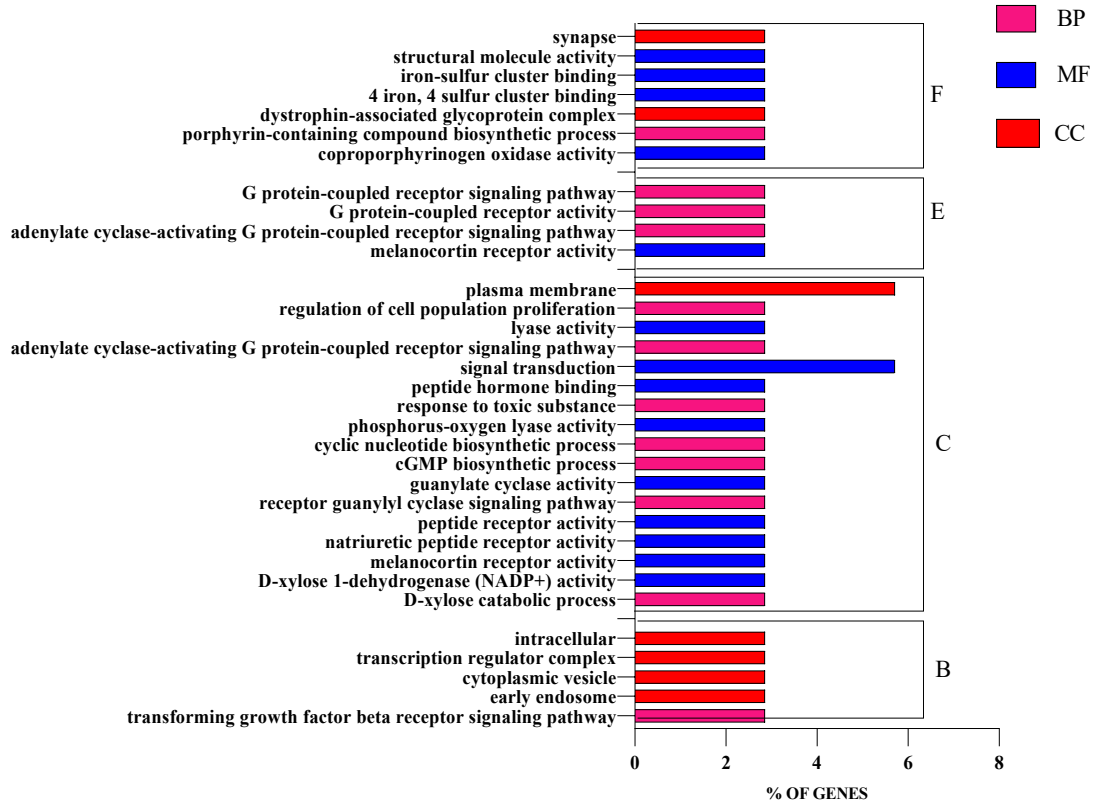
Sex / Treatments	Gene Symbol	Gene Description	Expression level	log2FoldChange	*P* Value	GO Terms	KEGG Pathways
Males (n=28)							
B vs A	INTS2	integrator complex subunit 2	Up	0.5	<0.01	snRNA 3'-end processing,	Genetic information processing (Spliceosome)
	SLC5A10	solute carrier family 5-member 10	Up	0.7	0.01	glucose transmembrane transport	Signaling and cellular processes (Sodium glucose cotransporter)
	MED13	mediator complex subunit 13	Up	0.6	0.01	mediator complex	Transcription machinery (RNA polymerase II system
	CEP70	centrosomal protein 70	Up	0.4	0.01	gamma-tubulin binding	Chromosome and associated proteins
	BVES	blood vessel epicardial substance	Down	-0.5	0.01	regulation of microtubule cytoskeleton organization	Tight junction
	PDE11A	phosphodiesterase 11A	Down		0.02	3',5'-cyclic-nucleotide phosphodiesterase activity	Phosphoric-diester hydrolases

	CLCA1	chloride channel accessory 1	Down	-0.5	<0.01	intracellular calcium activated chloride channel activity	Signaling and cellular processes (Ion channels)
C VS A	BVES	blood vessel epicardial substance	Down	-0.6	0.01	regulation of microtubule cytoskeleton organization	Tight junction
E VS A	BVES	blood vessel epicardial substance	Down	-0.6	<0.01	regulation of microtubule cytoskeleton organization	Tight junction
Females (n=20)							
B VS A	PANX2	pannexin 2	Up	1.1	<0.01	plasma membrane	signaling and cellular processes (Pores ion channels)
C VS A	GUCY2C	guanylate cyclase 2C	Up	1.1	0.02	peptide hormone binding	Purine metabolism
	MC5R	melanocortin 5 receptor	Down	-0.9	0.02	melanocortin receptor activity	Neuroactive ligand-receptor interaction (Signalling molecules and interaction)
F VS A	MTMR6	myotubularin related protein 6	Down	-0.6	0.02	peptidyl-tyrosine dephosphorylation	Phosphatidylinositol signaling system (Inositol phosphate metabolism)

1 Treatments include- A) Negative Control treatment- chicks fed a basal corn-soybean meal-wheat–based diet; B) In-feed antibiotics- chicks fed NC + 0.05% bacitracin methylene disalicylate and C) In-water essential oil- chicks supplied the essential oil via the water route at the recommended dosage of 250 ml / 1000 L of drinking water; D) *In ovo* saline treatment- eggs injected with 0.2 mL of physiological saline (0.9% NaCl); E) *In ovo* essential oil treatment- eggs injected with 0.2mL of a saline + essential oil blend mixture at a dilution ratio of 2:1, F) *In ovo* + in-water essential oil treatment- chicks offered the essential oil blend via the *in ovo* and in water route, successively. Each comparison is specified in the format "B VS A", where group B is compared to group A, with group A being the denominator for the comparison. Liver tissues (50–100 mg) was sampled from 8 replicate birds/treatment (independent of sex) using 1 mL TRIzol™ (Qiagen, Hilden, Germany).


BP
MF
CC
phosphoric diester hydrolase activity
3',5'-cyclic-nucleotide phosphodiesterase activity
negative regulation of cAMP-mediated signaling
negative regulation of cGMP-mediated signaling
cGMP-stimulated cyclic-nucleotide phosphodiesterase activity
F
phosphoric diester hydrolase activity
negative regulation of cAMP-mediated signaling
negative regulation of cGMP-mediated signaling
3',5'-cyclic-nucleotide phosphodiesterase activity
cGMP-stimulated cyclic-nucleotide phosphodiesterase activity
D
cGMP-stimulated cyclic-nucleotide phosphodiesterase activity
phosphoric diester hydrolase activity
transmembrane transporter activity
transcription coregulator activity
thyroid hormone receptor binding
Wnt-protein binding
3',5'-cyclic-nucleotide phosphodiesterase activity
gamma-tubulin binding
glucose:sodium symporter activity
intracellular calcium activated chloride channel activity
mediator complex
microvillus
secretory granule
integrator complex
ion transmembrane transport
cholesterol homeostasis
negative regulation of DNA-binding transcription factor activity
chloride transport
transcription initiation from RNA polymerase II promoter
non-canonical Wnt signaling pathway
negative regulation of cGMP-mediated signaling
triglyceride homeostasis
regulation of microtubule cytoskeleton organization
snRNA 3'-end processing
glucose transmembrane transport
B
0
5
10
15
% OF GENES

(a)


BP
MF
CC
synapse
structural molecule activity
iron-sulfur cluster binding
4 iron, 4 sulfur cluster binding
dystrophin-associated glycoprotein complex
porphyrin-containing compound biosynthetic process
coproporphyrinogen oxidase activity
F
G protein-coupled receptor signaling pathway
G protein-coupled receptor activity
adenylate cyclase-activating G protein-coupled receptor signaling pathway
melanocortin receptor activity
E
plasma membrane
regulation of cell population proliferation
lyase activity
adenylate cyclase-activating G protein-coupled receptor signaling pathway
signal transduction
peptide hormone binding
response to toxic substance
phosphorus-oxygen lyase activity
cyclic nucleotide biosynthetic process
cGMP biosynthetic process
guanylate cyclase activity
receptor guanylyl cyclase signaling pathway
peptide receptor activity
natriuretic peptide receptor activity
melanocortin receptor activity
D-xylose 1-dehydrogenase (NADP+) activity
D-xylose catabolic process
C
intracellular
transcription regulator complex
cytoplasmic vesicle
early endosome
transforming growth factor beta receptor signaling pathway
B
0
2
4
6
8
% OF GENES

(b)

Figure 6.9 Gene ontology (GO) classifications of differentially expressed genes (DEGs) between treatment groups in (a) male and (b) female transcripts, respectively. Treatments groups include- A) Negative Control treatment- chicks fed a basal corn-soybean meal-wheat–based diet; B) In-feed antibiotics- chicks fed NC + 0.05% bacitracin methylene disalicylate and C) In-water essential oil- chicks supplied the essential oil via the water route at the recommended dosage of 250 ml / 1000 L of drinking water; D) *In ovo* saline treatment- eggs injected with 0.2 mL of physiological saline (0.9% NaCl); E) *In ovo* essential oil treatment- eggs injected with 0.2mL of a saline + essential oil blend mixture at a dilution ratio of 2:1, F) *In ovo* + in-water essential oil treatment- chicks offered the essential oil blend via the *in ovo* and in water route, successively. Gene Ontology terms included both biological process (BP), cellular component (CC), and molecular function (MF).

However, a vast majority (75.8%) of the significant GO terms in the male transcript were observed in the antibiotic treatment, with GO terms in the BP category including “non-canonical Wnt signaling pathway” and “snRNA 3′-end processing” being the principal terms. In the female transcript, the vast majority (51.5%) of the significant GO terms were observed in the in-water EO treatment, with GO terms in the CC category including “signal transduction” and “plasma membrane” being the principal terms. The KEGG pathway analysis results, also shown in Table 6.5, provides predictions of differentially regulated pathways across treatments and sex. The results revealed that the main enriched pathways were cell signaling- (acting in sodium-glucose transporter, ion channels, exosome, and inositol phosphate metabolism) and tight-junction-related pathways. Other highlighted enriched pathways include genetic information processing, transcription machinery, spindle formation proteins, phosphoric diester hydrolases, and purine metabolism. Several factors, including the threshold of significance levels, but certainly not the number of animals or sample number utilized in this study, could have contributed to the low number of DEGs observed in this study (n = 14). Compared to this study, other broiler chicken RNA-Seq experiments (You et al., 2019; Bajagai et al., 2021; Hong et al., 2021) have utilized lower animal or sample numbers to detect a higher number of DEGs.

The BVES gene with the cellular GO term category “regulation of microtubule cytoskeleton organization and tight junction related pathways” was found to be ubiquitously downregulated in male transcripts in this study irrespective of treatments, suggesting that this gene plays a vital metabolic function in the cell. Since first identified in 2001, the BVES gene has mostly been functionally correlated with the maintenance of epithelial integrity and tight junctions (Wada et al., 2001; González-Mariscal et al., 2003; Osler et al., 2005; Russ et al., 2010); this has been validated by decreased trans-epithelial resistance values (TER, a measure of tight junction integrity) (Olser et al., 2005). Nonetheless, the exact mechanisms of its role in tight junction maintenance are yet to be fully elucidated (A Hager et al., 2009). Osler et al. (2005) have proposed that BVES’s role in tight junction maintenance might indeed be a secondary effect, with more primary roles likely related to cell signaling, and structural support, among others. Besides the intestine, where tight junction proteins are noted to ensure gut barrier integrity, the BVES gene is also reported to be highly expressed in cardiac and skeletal muscle (DiAngelo et al., 2001; Andrée et al., 2002; Vasavada et al., 2004). More recently, Gu et al. (2020) reported the detection of BVES following whole-genome resequencing of the autochthonous Niya chicken breed and associated its function to the regulation of heart rate and heart development (Torlopp et al., 2006; Froese et

al., 2012). Considering that ischemic hepatic necrosis, which is linked to heart failure, could occur in broiler chickens (Aengwanich and Simaraks, 2004) and the healthy state of flocks in this study, it is hypothesized that the downregulation of the BVES gene in broiler chicken liver observed in male transcripts in this study might be functionally related to the regulation of heart rate. Moreover, male embryos and adult chickens are reported to exhibit slower heart rates as compared to females (Ringer et al., 1957; Glahn et al., 1987). More studies are thus needed to validate the relationship between BVES expression in the liver and heart rate regulation in broiler chickens.

Furthermore, antibiotic treatment upregulated the expression of INTS2 (integrator complex subunit 2), SLC5A10 (solute carrier family 5-member 10), CEP70 (centrosomal protein 70), and MED13 (mediator complex subunit 13) genes, while downregulating the expression of PDE11A (phosphodiesterase 11A) and CLCA1 (chloride channel accessory 1) genes in male transcripts in this study. Only in female transcripts did the antibiotic treatment upregulate PANX2 (pannexin 2) gene expression. INTS2 is a subunit of the integrator complex, which interacts with the C-terminal domain (CTD) of RNA polymerase II (RNAP II) large subunit and modulates 3-prime end processing of small nuclear RNAs (snRNAs) U1 and U2 (Baillat et al., 2005). The snRNAs are components of the spliceosome involved with the processing of pre-mRNA while also modulating the expression of other genes (Will and Lührmann, 2011). The modulation of snRNAs has also been reported to impact the innate immune system (Tsalikis et al., 2015). Slc5a10 (encoding SGLT5) is a mannose, fructose, and to a less degree, a glucose and galactose transporter (Wright, 2013; Chittka et al., 2018). Although glucose transporter 2 (GLUT2) is considered the main sugar transporter relevant to liver function (Leturque et al., 2005), Fukuzawa et al. (2013) have reported exacerbated hepatic steatosis induced by diminished sodium-dependent fructose uptake in SGLT5-deficient mice, suggesting the potential use of this gene as an indirect biomarker of liver health. Moreover, while the liver is the main site of ingested fructose metabolism, the occurrence of excess fructose beyond the liver's metabolic capacity triggers GLUT5 transporter upregulation to ensure fructose absorption into the epithelial cells (Gaby, 2005).

Similar to the BVES gene with predicted cardioprotective effect, the upregulation of the MED13 gene by the antibiotic treatment is also thought to exert a cardioprotective effect in the birds. The MED13 gene is a component of the mediator complex, working in synchronization with RNA polymerase II to direct transcription (Boles et al., 2009). Its mutation has been implicated in lethal cardiac defects (Ito et al., 2000; Ito et al., 2002; Wolton et al., 2014). Similarly, upregulated CEP70 expression, as induced by antibiotic treatment in this study, has been

implicated in the pathophysiology of numerous cancers (Kim et al., 2020). It is a centrosomal-associated protein that has been linked with the regulation of microtubule nucleation in animal cells (Shi et al., 2012; Yang et al., 2014; Shi et al., 2015). Centrosome dysfunction has been linked to the incidences of liver diseases and other non-apparent cell cycle defects in humans (Nigg and Raff, 2009). The downregulated PDE11A is a dual-specificity phosphodiesterase that catalyzes the breakdown of the cyclic nucleotides cyclic adenosine monophosphate (cAMP) and cyclic guanosine monophosphate (cGMP) (Libe et al., 2011). Although mainly expressed in the prostate, it also finds expression to a lower degree in the pituitary gland, heart, and liver (Faucz et al., 2011).

Although CLCA1 is noted for its role in the activation of calcium-activated chloride channels, its downregulation is reported to enhance pro-inflammatory cytokine release in both mice mucus cells (Dietert et al., 2014) and human small airway epithelial cells (Mamber et al., 2020). Increased innate immune responses are usually associated with increased energy demands; this suggests that antibiotic use might be an energy-intensive means of growth promotion. Similar to this study, Farmahin et al. (2019) have reported the differential expression of PANX2 in the liver of female, but not male, Fischer rats. PANX2 is functionally known for its potential to create gap junctions that facilitate ion exchange between cells and their role as a potential tumor suppressor in the human brain, skin, and liver tissues (Tang et al., 2008; Kwon et al., 2014; Xie et al., 2015; Jiang and Penuela, 2016).

In a like manner, in-water EO treatment equally downregulated the expression of the MC5R (melanocortin 5 receptor) gene in female transcripts in this study. MC5R encodes a protein receptor for melanocyte-stimulating hormone and adrenocorticotropic hormone. It has been functionally designated as a candidate gene for obesity and fatness in humans and domestic animals (Anand et al., 2021). Consistent with the results presented here, Ren et al. (2017) have previously reported that its expression in the chicken liver might be estrogen activated, further buttressing its differential expression in female transcripts in this study. Similarly, Blankenship et al. (2016) have reported that the downregulation of MC5R was critical to achieving feed efficiency phenotype in first-generation female, but not male, quails in their study. This is likely achieved directly by fatty acid metabolism or indirectly by glucose homeostasis. This result is not unexpected, considering that the broiler chickens utilized in this study have been bred for high feed efficiency. Conversely, the in-water EO treatment up-regulated the expression of the GUCY2C (guanylate cyclase 2C) gene, which encodes guanylate cyclase belonging to the membrane guanylyl cyclase family (Wilson et al., 2014). Mice deficient in GUCY2C have been

reported to have reduced inflammatory response due to reduced expression of pro-inflammatory molecules (Steinbrecher et al., 2011). In contrast, higher expression of GUCY2C in the liver of milk-restricted lambs has been associated with increased pro-inflammatory response (Santos et al., 2018). This is likely the molecular basis of the antibacterial properties of essential oils, especially as it relates to pro-inflammatory hepatic stimulus. This also has an energy trade-off, as more energy might be directed towards countering systemic inflammation and not growth. Higher expression of GUCY2C in human females as compared to males has also been reported (Erwin et al., 2021). Furthermore, while the *in ovo* EO treatment downregulated the expression of the cubilin (CUBN) gene, the *in ovo* + in-water EO treatment downregulated the expression of the MTMR6 (myotubularin related protein 6) gene in male and female transcripts, respectively. Although the functional relevance of CUBN in chickens is not fully understood, CUBN is generally noted to play a role in the uptake of vitamin, iron, and lipoprotein endocytosis (Christensen et al., 2002; Shaik et al., 2013). Lee et al. (2015) have reported the downregulation of the CUBN gene in chicken lines with high residual feed intake, suggesting a possible role in amino acid metabolism and molecular transport network. Sun et al. (2015) have also alleged that the downregulation of this gene could be induced by stressors, particularly heat stress. More research is thus needed to fully elucidate the functionality of this gene in chickens. Overexpression of the downregulated MTMR6 by the *in ovo* + in-water EO treatment has been reported to inhibit the Ca^{2+}-activated potassium channel (Srivastava et al., 2005; Balla, 2013). Given the physiological function of the $Ca2^{+}$-activated potassium channel, which is to regulate cellular membrane potential and calcium signaling, this is considered to be a beneficial effect of essential oil delivery via this route. Moreover, hypoxia has been reported to increase mitoBKCa channel activity (big conductance potassium channel) of rat liver (Cheng et al., 2008). Furthermore, in this study, both in-feed antibiotic and *in ovo* + in-water EO treatments downregulated the expression of ALDH1L2 in the liver of broiler chickens. Similar to this study, (Li et al. 2014) have previously reported the downregulation of ALDH1L2 in a nonalcoholic steatohepatitis (NASH) rat model by analyzing the liver proteome, suggesting that ALDH1L2 may be involved in NASH progression. Contrary to the result presented here, Bajagai et al. (2021) have reported upregulation of the ALDH1L2 gene with continuous EO (2% oregano powder) supplementation in the liver of male broiler chickens. Differences in routes of EO supplementation and length of study may have possibly influenced reported results. Although little is known about the BTN1A1 gene in chickens, which was upregulated by the in-water EO treatment in this study, Huang et al. (2021) reported that this

gene might play a role in immune response via inhibition of T-cell activation using lipopolysaccharide-challenged broiler chickens (Smith et al., 2010; Yamazaki et al., 2010). Low hepatic expression of this gene has also been reported in water buffalo (Wu et al., 2014).

In addition, while the ST8SIA6 gene has been reported to be downregulated in the liver of apolipoprotein E-knockout (apo E-KO) mice offered phytosterol treatment for 14 weeks (Xu et al., 2008), an upregulation of this gene in birds of the *in ovo* EO treatment was observed in this study. Phytosterols are of plant origin and have reported cholesterol-lowering effects (Moghadasian, 2000; Wolfs et al., 2006). In humans, high expression of the ST8SIA6 gene has been attributed an oncogenic function, including tumor cell proliferation, invasion, and migration (Zhang et al., 2021). As little is known of this gene in chickens, an overt attribution of a high expression of this gene to an increased likelihood of hepatic steatosis or liver cancer might be farfetched. More studies are needed in this regard to enable a more definite prognosis. On the other hand, the CUBN gene is reported to play an important role in the metabolism and transport of the active form of vitamin D (1,25-dihydroxy vitamin D) in the liver. This has been confirmed in transcriptomics studies involving mice supplemented with cholecalciferol (Nykjaer et al., 2001; Bonnet et al., 2018). Although this gene is reported to be downregulated by the *in ovo* EO treatment in this study, Collision et al. (2009) have previously reported its upregulation in mice liver under a trans-fatty acid (TFA)-induced non-alcoholic fatty liver disease challenge. Overall, the results presented here provide transcriptomic evidence on the possibility of "natural" phytobiotics (including essential oil) having side effects depending on the length of use, dosage, and administration routes, an important concept to be considered in the development of potent human and animal pharmacotherapeutic strategies.

6.6 Conclusions

Summarily, while treatments yielded no difference in alpha and beta bacteria diversity in this study, clear differences in ileal and ceca microbiota distribution and structure were recorded. In-feed antibiotic treatment is also reported to significantly increase the proportion of specific beneficial bacteria in the family *Lachnospiraceae* while reducing the proportion of bacteria in the genus *Christensenellaceae*, all in the ceca. No significant effect of the evaluated treatments on ceca SCFA concentration was recorded in this study. Only the concentration of butyric acid recorded a statistical trend towards significance in the in-water essential oil treatment when compared to other treatments. The study also suggests unique sex-controlled gene expression in broiler chicken liver. Compared to the negative control treatment, the differential expression of the INTS2, SLC5A10, MED13, CEP70, PDE11A, and CLCA1 genes functionally associated with genetic information processing, glucose transport, mediator complex, spindle formation proteins, phosphoric-diester hydrolases, and ion channel activity, respectively, were all regulated by the antibiotic treatment in male transcripts. Only the BVES and CUBN gene sets, functionally associated with tight junctions and cholesterol homeostasis, were regulated by the in-water and *in ovo* EO treatments in male transcripts, respectively, compared to the negative control treatment.

Conversely, in female transcripts, while the antibiotic treatment regulated the expression of the PANX2 gene functionally associated with ion exchange, the in-water and *in ovo* + in-water treatments regulated the differential expression of GUCY2C, MC5R, and MTMR6 genes functionally associated with peptide hormone binding, melanocortin receptor activity, and peptidyl-tyrosine dephosphorylation, respectively, all compared to the negative control treatment. Taken together, the results presented here provide mechanistic insights on the microbiota-mediated mode of action of antibiotics growth promoters by modulating the abundance of specific bacteria communities, as well as preliminary transcriptomic evidence suggesting sex-controlled hepatic differential gene expression in broiler chickens offered antibiotics and essential oil (via water, *in ovo,* and *in ovo* + in-water delivery routes). To our knowledge, this is the first study to suggest such sex-controlled hepatic differential gene expression in broiler chickens offered these treatments. There is thus a need for well-designed in vivo studies that take sex into consideration in order to fully validate the results presented herein. Nonetheless, the data presented here not only provide guidance on antibiotics and essential oil application in the poultry industry; they also provide a solid framework for further research in the field.

7 CHAPTER 7 FOLIC ACID DELIVERY ROUTES

AN INVESTIGATION OF THE EFFECT OF FOLIC ACID AND ITS DELIVERY ROUTES ON BROILER CHICKENS' HATCH AND GROWTH PERFORMANCE, BLOOD BIOCHEMISTRY, ANTIOXIDANT STATUS, AND GUT MORPHOLOGY.

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- Oladokun, S., and Adewole, D.I. 2022. An Investigation of the Effect of Folic Acid and its Delivery Routes on Broiler Chickens' Hatch and Growth Performance, Blood Biochemistry, and Antioxidant Status. Journal of Animal Science (submitted).

7.1 Abstract

This study investigated the effect of folic acid (FA) and its delivery routes (in-feed or *in ovo*) on broiler chicken's hatch and growth performance, blood biochemistry, antioxidant status, and intestinal morphology. A total of 1,860 Cobb 500 hatching eggs were incubated for 21 days. On d 12 of incubation, viable eggs were randomly allotted to four groups: the non-injected group, *in ovo* saline (injected with 0.1 mL/egg of saline solution), *in ovo* FA 1 (injected with 0.1 ml FA containing 0.1 mg/egg; FA1), and *in ovo* FA 2 (injected with 0.1 ml FA containing 0.15 mg/egg). All *in ovo* treatments were delivered via the amnion. At hatch, chicks were re-allotted to 5 new treatment groups: FA1, FA2, in-feed FA (FA 3; 5mg/kg of feed), in-feed bacitracin methylene disalicylate (BMD; 55 mg/kg of feed), and negative control (NC; corn-wheat-soybean diet) in 6 replicate pens (22 birds/pen) and raised in starter (d 0 -14), grower (d 15-24) and finisher (d 25-35) phases. Hatch parameters were assessed on d 0, and body weight and feed intake (FI) were determined weekly. On d 25, 1 bird/cage was euthanized, immune organs weighed, and intestinal tissues harvested. Blood samples were collected for biochemistry and antioxidant (Superoxide

dismutase-SOD and Malondialdehyde-MDA) analysis. Data were analyzed in a randomized complete block design. While FA1 and FA2 decreased ($P < 0.001$) hatchability in a dose-dependent manner, FA2 caused a 2% increase ($P < 0.05$) in average chick weight compared to the non-injected group. Compared to the BMD treatment, FA3 decreased ($P < 0.05$) average FI across all feeding phases. At the end of the trial on d35, FA2 had similar feed conversion ratio as the BMD treatment while recording less ($P < 0.001$) FI. FA1 and FA2 recorded a tendency ($P < 0.1$) to increase MDA levels and SOD activity by 50% and 19%, respectively, compared to the NC treatment. Compared to NC treatment, FA2 increased ($P < 0.01$) villus height, width, and villus height to crypt depth ratio in the duodenum, and villus width in the jejunum. Besides its negative effect on hatchability, FA2 may help improve embryonic development and antioxidant status in broiler chickens.

7.2 Introduction

Antibiotic growth promoters (AGP) are renowned in animal production, especially poultry production, for their roles in disease prevention and growth promotion. However, issues related to the emergence of antibiotic-resistant bacteria and increased consumer demand for antibiotic-free poultry product have stirred public outcry against AGP use in poultry production. This public outcry has necessitated the search for potent alternatives that ensure disease prevention and growth promotion in the poultry industry. For the poultry industry to successfully depart from AGP use, it is pertinent that all strategies that ensure bird growth promotion and disease prevention are employed to meet the rising demand for poultry products.

Moreover, the avian embryonic development is unique compared to their mammalian counterparts, as a continuous maternal supply of nutrients is lacking. This limits the embryo's nutritional requirements to what can be supplied by the egg alone. Several indicators suggest the inadequacies of avian embryo nutrition via the egg. For instance, Ohta et al. (2001) have reported that only 25-30% of nutrients, including vitamins supplied to breeder diets, are incorporated into their eggs, suggesting that the embryo might require an external supply of nutrients for optimum growth. Additionally, an imbalance in available nutrients is triggered by excessive metabolic heat produced by the growing embryo during the late incubation phase (Janke et al., 2004). Interestingly, these nutrient deficiencies occur at a time (especially the late stage of incubation) when embryo energy requirements are usually high. Hence, it is unsurprising that a reduction in embryo growth rate and increased mortality due to nutrient and energy deficiencies at this time

point have been recorded in broiler chickens (Zhai et al., 2011; Ebrahimi et al., 2017). Furthermore, under current commercial poultry settings, chicks often encounter other perinatal nutritional deficiencies that include delayed access to feed that could last about 24-36 hours due to long hatch window and other time-consuming hatchery activities that could include chick sexing, sorting and transportation. Hatchlings are thus unable to meet the energy and thermoregulation nutritional requirements, making them predisposed to immunosuppression, dysbiosis and reduced growth rate (Gholami et al., 2015; Momeneh and Torki, 2018; Nouri et al., 2018). As the late incubation period is critical for enteric embryo development and post-hatch growth development (Uni and Ferket, 2003; Luqman et al., 2019), an optimal supply of nutrients at this time-point must be ensured.

In ovo delivery technology, defined as "the direct inoculation of bioactive substances to the developing embryo to elicit superior lifelong effects, while considering the dynamic physiology of the chicken embryo," offers an opportunity to mitigate the perinatal nutritional deficiencies that chicks encounter (Oladokun and Adewole, 2020). The *in ovo* delivery of nutrients has been established as one possible way to enhance hatchability and post-hatch performance in poultry (Najih Jabir Al-Shamery and Mohammed Baqur S. Al-Shuhaib, 2015; Joanna et al., 2017; Bhattacharyya et al., 2018; Zhang et al., 2018). Oladokun and Adewole (2020) have also recently revealed the potential application of *in ovo* technology to deliver several bioactive substances with immunomodulatory properties as alternatives to AGP. To substantiate the efficacy of *in ovo*-delivered bioactive substances replacing AGP, the *in ovo* delivery of several nutrients, including trace elements, amino acids, and vitamins, continues to gain interest across several poultry studies (Bakyaraj et al., 2012; Hou and Tako, 2018; Oladokun and Adewole, 2020).

Although scarcely researched, folic acid (**FA**) is one of several vitamins whose *in ovo* delivery is currently being researched. Folic acid belongs to the water-soluble vitamin B-complex group. It is physiologically important for its role in Deoxyribonucleic acid (**DNA**), Ribonucleic acid (**RNA**), and protein methylation, as well as acting as a coenzyme involved in nucleic and amino acids synthesis and metabolism (Bailey and Gregory, 1999; Choi and Mason, 2000; Leung et al., 2013; El-Azeem et al., 2014). It also plays a crucial role in embryo development and is essential for embryo brain and nerve cell development (Viera, 2007; Hussian et al., 2019). Moreover, breeder hens are reported to have higher FA requirement compared to laying hens (Viera, 2007). The role of FA in red blood cell synthesis and immunocompetence in domestic animals have also been reported (Feng et al., 2011; Asif, 2016). Folate deficiency has been

associated with cardiovascular disease, intra-uterine growth retardation in humans (Boushey et al., 1995), short bones, curved tibia, and beak defects in poultry (Ezzat and Shoeib, 2011). Long-term storage of hatching eggs has been implicated as a possible cause of FA deficiency (Whitehead et al., 1985; Liu et al., 2016).

A few studies have highlighted the potential of the *in ovo* delivery of FA to improve embryo growth and organ development, growth performance indices, immune status, and blood biochemical properties, including plasma cholesterol, glucose, and phosphorus levels in broiler chickens (Bekhet, 2013; El-Azeem et al., 2014; Liu et al., 2016; Nouri et al., 2018; Ismail et al., 2019; Gouda et al., 2022; Tufarelli et al., 2022). Aside from the paucity of studies involving the *in ovo* delivery of FA, conflicting results on the effect of FA on broiler chicken performance exist in the literature. For instance, varying doses of *in ovo* delivered FA have been reported to improve hatchability in poultry (Robel, 2002; Li et al., 2016; Hussian et al., 2019; Ismail et al., 2019). Also, some studies have reported that hatchability and growth performance were not affected by FA supplementation (Robel, 1993a; Nouri et al., 2018; Tufarelli et al., 2022). Therefore, this study sought to investigate the effect of the supplementation of two doses of FA (0.1 and 0.15 mg per egg) and its delivery routes (*in ovo* vs in-feed) on hatch and growth performance, intestinal morphology, blood biochemistry, immune, and antioxidant status of broiler chickens, compared to in-feed antibiotics. To our knowledge, this is the first direct comparison of *in ovo* and in-feed delivered FA in poultry studies.

7.3 Materials and Methods

7.3.1 Ethics statement

The experiment was carried out at the hatchery facility of the Agricultural Campus of Dalhousie University and the broiler rearing facility of the Atlantic Poultry Research Center, Dalhousie Faculty of Agriculture. The experiment was conducted following guidelines recommended by the Canadian Council on Animal Care (Rowsell, 1990) and approved by the Animal Care and Use Committee of Dalhousie University (Protocol number: 2021-032).

7.3.2 Egg incubation and *in ovo* injection procedure

Hatching broiler eggs (Cobb 500, 52 wk old breeders, average weight = 63 ± 1.27 g, n = 1860) were obtained from a commercial hatchery (Cox Atlantic Chick hatchery, Nova scotia) and incubated in a ChickMaster single-stage incubator (ChickMaster G09, Cresskill, NJ, USA), under standard conditions (37.5°C, 55% relative humidity) from embryonic days (**ED**) 1 to 19, and then

to an average of 32°C and 68% from ED 19 to 21. Eggs were candled on ED12, and unviable eggs were discarded. Viable eggs were subsequently assigned to one of four experimental groups: a) non-injected eggs (control; 166 eggs); b) *in ovo* saline eggs (38 eggs; injected with 0.2 mL of physiological saline, i.e., 0.9% NaCl, Baxter Corporation, ON, Canada); c) *in ovo* FA group 1 (53 eggs; injected with 0.1 mL FA (FA; ≥97%; Sigma, USA) at 0.1 mg per egg) and d) *in ovo* FA group 2 (53 eggs; injected with 0.1 mL FA at 0.15 mg per egg). The injection of FA on ED 12 was via the amnion. Treatments were replicated in six similar incubators operated under similar conditions. The injection procedure utilized in this study has been previously described by Oladokun et al. (2021). Briefly, all eggs were disinfected by cleaning with 70% alcohol swabs (BD alcohol swabs-catalogue 326910, ON, Canada), followed by careful punching of the air cell (the blunt end of the egg) using an 18-gauge needle. The injected FA treatments were then delivered to the amnion using a self-refilling injector (Socorex ultra-1810.2.05005, Ecublens, Switzerland) equipped with a 22-gauge needle (injection needle length—3 cm) at a 45-degree angle. After injection, the injection sites were sealed with sterile medical tapes (Nexcare™ Flexible Clear Tape-7100187758, 3M, MN, USA). The non-injected eggs were taken out and returned to the incubator simultaneously with other injected treatment groups.

7.3.3 Birds, Housing, and Diets

Hatchlings were weighed and randomly assigned to 5 new treatment groups (Figure 7.1). Chicks from the initial non-injection group were randomly allocated into 3 new treatment groups consisting of (1) chicks fed a basal corn-soybean meal-wheat–based diet (Negative Control treatment; NC); (2) chicks fed NC + 0.05% bacitracin methylene disalicylate (in-feed antibiotics); and (3) chicks fed NC + 5 mg/kg FA (in-feed FA). The *in ovo* FA treatments were placed on the control diet to form treatments (4) *in ovo* FA group 1 and (5) *in ovo* FA group 2. Chicks (mixed sex, n=22) were weighed and assigned to 6 replicate floor pens (0.93 m × 2.14 m)/treatment at a stocking density of 0.076 m^2/bird. There were two broiler production rooms. The temperature in the broiler room was monitored daily and was gradually reduced from 32 to 22.5 °C from d 0 through d35. The lighting program was set to 18 h of light and 6 h of darkness throughout the experimental period, and illumination was gradually reduced from 20 lx on d 0 to 5 lx on d 35. Dietary treatments, ingredients, and nutritional composition are presented in Table 7.1. Birds were provided with feed and water ad libitum; diets were fed as mash in the starter (0–14 d) phase and pellets in the grower (15–25 d) and finisher (26-35 d) phases. Diets were formulated to meet Cobb 500 broiler chicken nutrient requirements.

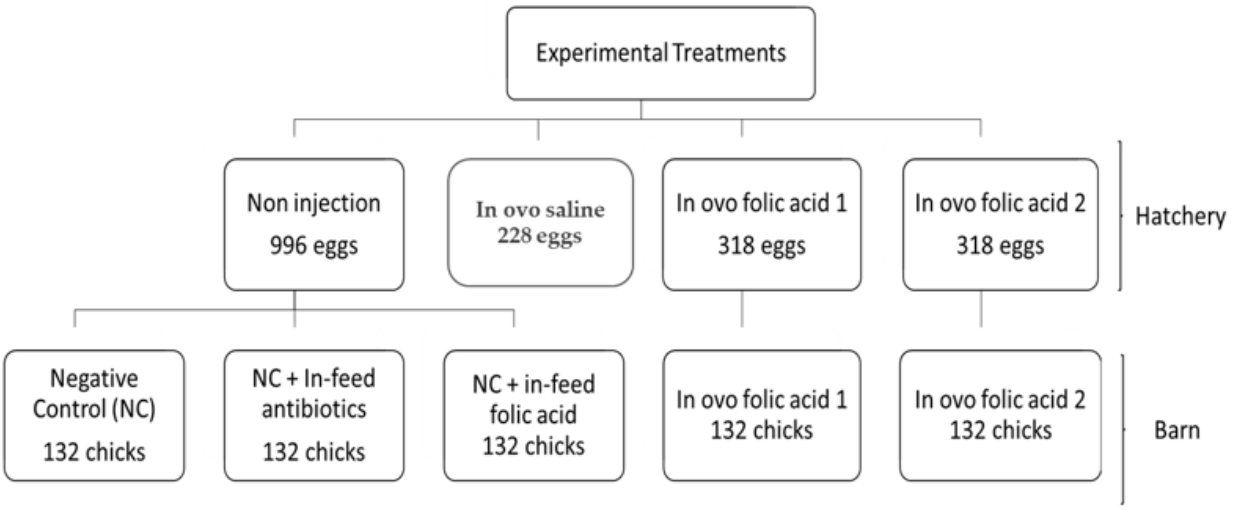


Figure 7.1 Schematic presentation of experimental structure in the hatchery and barn.

In ovo folic acid group 1- eggs injected with 0.1 mg folic acid per egg; *In ovo* folic acid group 2- eggs injected with 0.15 mg folic acid per egg; *In ovo* saline group- injected with 0.2 mL of physiological saline (0.9% NaCl); In-feed antibiotics- chicks fed NC + 0.05% bacitracin methylene disalicylate; In-feed folic acid- chicks fed NC + 5 mg/kg folic acid; NC- Negative Control treatment- chicks fed a basal corn-soybean meal-wheat–based diet.

Table 7.1 Composition and nutritional contents of experimental diets1 (as-fed basis, percentage (%), unless otherwise stated).

Ingredients	Phases								
	Starter (0-14 d)			Grower (15-25 d)			Finisher (26-35 d)		
	Negative Control	In-feed Antibiotics	In-feed folic acid	Negative Control	In-feed Antibiotics	In-feed folic acid	Negative Control	In-feed Antibiotics	In-feed folic acid
	Ingredient Composition								
Corn (ground)	46.63	46.53	46.63	51.16	51.06	51.16	53.63	53.53	53.62
Soybean meal-46.5	37.12	37.14	37.13	31.87	31.89	31.87	29.2	29.22	29.21
Wheat	10.0	10.0	10.0	10.0	10.0	10.0	10.0	10.0	10.0
Soybean Oil (young or mature)	1.80	1.83	1.80	2.18	2.21	2.18	2.75	2.78	2.75
Limestone	1.37	1.37	1.37	1.30	1.30	1.30	1.19	1.19	1.19
Dicalcium Phosphate 21 P	1.45	1.45	1.45	1.35	1.35	1.35	1.18	1.18	1.18
DL	0.58	0.58	0.58	0.57	0.57	0.57	0.52	0.52	0.52

Methionine premix[2]									
Vitamin/Mineral Premix [3, 4]	0.50	0.50	0.50	0.50	0.50	0.50	0.50	0.50	0.50
Salt	0.38	0.38	0.38	0.36	0.36	0.36	0.36	0.36	0.36
Lysine HCl	0.17	0.17	0.17	0.21	0.21	0.21	0.17	0.17	0.17
Pellet Binding Agent	-	-	-	-	0.50	0.50	0.50	0.50	0.50
BMD 110G[5]	-	0.05	-	-	0.05	-	-	0.05	-
Folic acid Sigma	-	-	0.0005	-		0.0005	-	-	0.0005
Total	100	100	100	100	100	100	100	100	100
Nutrient	Calculated Composition								
Metabolizable energy,	2,975	2,975	2,975	3,025	3,025	3,025	3,100	3,100	3,100

kcal/kg									
Crude protein	22	22	22	20	20	20	19	19	19
Calcium	0.90	0.90	0.90	0.84	0.84	0.84	0.76	0.76	0.76
Available phosphorus	0.45	0.45	0.45	0.42	0.42	0.42	0.38	0.38	0.38
Sodium	0.18	0.18	0.18	0.17	0.17	0.17	0.17	0.17	0.17
Digestible lysine	1.22	1.22	1.22	1.12	1.12	1.12	1.02	1.02	1.02
Digestible methionine + cysteine	0.91	0.91	0.91	0.85	0.85	0.85	0.80	0.80	0.80
Digestible Tryptophan	0.24	0.24	0.24	0.22	0.22	0.22	0.20	0.20	0.20
Digestible Threonine	0.84	0.84	0.84	0.76	0.76	0.76	0.72	0.72	0.72

Analyzed Composition

Dry Matter	92.2	92.2	91.6	91.5	92.1	91.4	91.7	91.8	
Crude protein	24.5	24.7	24.3	21.3	21.2	21.6	19.3	20.9	91.6
Crude fat	4.05	4.31	4.09	4.86	4.69	3.67	4.81	4.25	21.3
Calcium	0.81	0.80	1.08	0.89	0.90	0.83	0.83	0.75	4.92
Potassium	1.05	1.00	1.07	0.94	0.91	0.95	0.84	0.92	0.83
Phosphorus	0.62	0.65	0.72	0.66	0.65	0.65	0.57	0.60	0.95
Sodium	0.14	0.15	0.19	0.17	0.17	0.17	0.16	0.15	0.63

[1]Basal diet (NC); In-feed antibiotic diet containing NC + 0.05% bacitracin methylene disalicylate (BMD); In-feed folic acid diet containing NC + 5 mg/kg FA. [2] Supplied/kg premix: DL-Methionine, 0.5 kg; wheat middling, 0.5 kg. [3] Starter vitamin-mineral premix contained the following per kg of diet: 9750 IU vitamin A; 2000 IU vitamin D3; 25 IU vitamin E; 2.97 mg vitamin K; 7.6 mg riboflavin; 13.5 mg Dl Ca-pantothenate; 0.012 mg vitamin B12; 29.7 mg niacin; 1.0 mg folic acid, 801 mg choline; 0.3 mg biotin; 4.9 mg pyridoxine; 2.9 mg thiamine; 70.2 mg manganese; 80.0 mg zinc; 25 mg copper; 0.15 mg selenium; 50 mg ethoxyquin; 1543 mg wheat middling's; 500 mg ground limestone. [4] Grower and Finisher vitamin-mineral premix contained the following per kg of diet: 9750 IU vitamin A; 2000 IU vitamin D3; 25 IU vitamin E; 2.97 mg vitamin K; 7.6 mg riboflavin; 13.5 mg Dl Ca-pantothenate; 0.012 mg vitamin B12; 29.7 mg niacin; 1.0 mg folic acid, 801 mg choline; 0.3 mg biotin; 4.9 mg pyridoxine; 2.9 mg thiamine; 70.2 mg manganese; 80.0 mg zinc; 25 mg copper; 0.15 mg selenium; 50 mg ethoxyquin; 1543 mg wheat middling's; 500 mg ground limestone. [5] Bacitracin methylene disalicylate (providing 55 mg/kg mixed feed); Alpharma, Inc., Fort Lee, NJ, USA.

7.3.4 Measurements

7.3.4.1 Hatch Parameters and Chick Quality

Hatched chicks were counted and weighed individually. Hatchability was calculated as the percentage of hatched chicks to fertile incubated eggs per replicate. Chick navel quality was evaluated by adopting the scoring method by Reijrink et al. (2009). Navel quality was scored 1—when the navel was completely closed and clean; scored 2—when the navel was discolored (i.e., when the navel color differs from the chick's skin color) with a maximum 2 mm opening; and scored 3—when the navel was discolored and with more than a 2 mm opening. Chick length was also determined by placing the chick on its ventral side and recording its length from the tip of the beak to the middle toe on the right leg.

7.3.4.2 Growth performance parameters

Feed intake and average body weight (BW) were measured on a pen basis weekly. The obtained data was then used to calculate the average feed intake (AFI), average body weight gain (ABWG), and feed conversion ratio (FCR). The FCR was calculated as the amount of feed consumed per unit of body weight gain. Mortality was recorded daily, and dead birds were subsequently weighed and sent to the Nova Scotia Agriculture, Animal Health Laboratory for necropsy. Mortality was then used to correct the FCR.

7.3.4.3 Sampling

On day 25, 1 bird per pen (6 replicate birds per treatment group) was randomly selected, weighed, and euthanized by electrical stunning and exsanguination. After euthanasia of the bird, blood samples were collected from each bird into 10 mL blood serum collection tubes (BD Vacutainer™ Serum Tubes, fisher scientific- BD366430) for further serum assays and 10 mL heparinized tubes (BD Vacutainer™ Glass Blood Collection Tubes with Sodium Heparin, fisher scientific- BD366480) for further blood plasma assays. Blood serum and plasma were centrifuged at 1200 g x 10 minutes x 18 degrees °C. The resulting supernatants were stored in aliquots at -80 °C until further analysis.

After slaughter, the weights of the bursa of Fabricius and the spleen were also determined by trained personnel. The small intestinal segments, including the duodenum (region from the gizzard junction to the pancreatic and bile ducts), jejunum (1.5-cm length midway between the point of entry of the bile ducts and Meckel's diverticulum) and ileum (1.5-cm length midway between Meckel's diverticulum and the ileocecal junction), were excised and fixed in neutral buffered formalin (10%) for further histomorphological processing.

7.3.4.4 Relative Weight of Organs

The weights of bursa of Fabricius and spleen were recorded and then specified as a fraction of the live BW (g/Kg BW) of the slaughtered chicken.

7.3.4.5 Serum Immunoglobulins

The concentrations of immunoglobulins (IgG and IgM) in the serum were quantified using chicken-specific immunoglobulins enzyme-link immunosorbent assay (**ELISA**) kits (Bethyl Laboratories, Montgomery, TX, USA; catalog numbers E33-104-200218 and E33-102-180410, respectively) following manufacturer instructions. The values were determined on a microplate reader (Bio-Tek Instrument Inc., Wonooski, VT, USA) using a software program (KC4, version #3.3, Bio Tek Instruments). The four-parameter logistic model was used to extrapolate immunoglobulins concentration from absorbance readings.

7.3.4.6 Blood Biochemistry

Samples for blood biochemical analysis were shipped on ice to Atlantic Veterinary College, University of Prince Edward Island Pathology Laboratory, for analysis using cobas® 6000 analyzer series (Roche Diagnostics, Indianapolis, IN, USA).

7.3.4.7 7.3.4.7 Antioxidant Indexes

The activity of superoxide dismutase (**SOD**), and the concentration of Malondialdehyde (**MDA**) in the serum were measured according to the manufacturer's instructions using Cayman's SOD assay kit (catalog number 706002, Cayman Chemical, Ann Arbor, MI, USA) and chicken MDA ELISA kit (catalog number MBS260816, MyBioSource, San Diego, CA, USA) respectively. The Total antioxidant capacity (**TAC**) in blood plasma was analyzed using the Oxiselect Total Antioxidant Capacity assay kit (catalog number STA360; Cell BioLabs Inc., San Diego, CA, USA) according to the manufacturer's instructions. Absorbance for all analysis was measured at recommended wavelengths on a microplate reader (Bio-Tek Instrument Inc., Wonooski, VT, USA) using a software program (KC4, version #3.3, Bio Tek Instruments).

7.3.4.8 Gut morphology

The procedure for intestinal morphometric analysis was as described by Oladokun et al. (2021). Briefly, fixed intestinal tissues were embedded in paraffin, sectioned (0.5 μm thick), and stained with hematoxylin and eosin for morphological examinations. In each cross-sectioned tissue, ten morphometric measurements including the villus height (from the base of the intestinal mucosa to the tip of the villus excluding the intestinal crypt), villus width (halfway between the base and the tip), crypt depth (from the base upward to the region of transition between the crypt and villi)

(Ozdogan et al., 2014) per slide were carried out using Leica 1CC50 W microscope at 4× Magnification (Leica Microsystems, Wetzlay, Germany) and an image processing and analysis system (Leica Application Suite, Version 3.4.0, Leica Microsystems, Wetzlay, Germany).

7.3.5 7.3.5 Statistical Analysis

Hatch data were analyzed as a randomized complete block design, with the incubator as the blocking factor. Datasets from the grow-out trial were also analyzed in a randomized complete block design, with broiler production rooms being the blocking factor. The normality of all data sets was ascertained by testing residuals by the Anderson-Darling test in Minitab statistical package (v.18.1). Data were analyzed using the generalized linear model in the same statistical package. Significant means were separated using Tukey's honest significant difference test in the same statistical package. Analyzed data were presented as means ± SEM and probability values. Values were considered statistically different at $P \leq 0.05$ and considered a statistical trend at $P < 0.1$.

7.4 Results

7.4.1 Hatch performance and chick quality

The results on hatch performance and chick quality are presented in Table 7.2. The *in ovo* delivered FA reduced ($P < 0.001$) hatchability in a dose-dependent manner, with the highest reduction observed in the *in ovo* FA group 2 treatment, having 43% reduction in hatchability compared to the non-injected eggs. In contrast, chicks in the *in ovo* FA group 2 treatment had at least 2% heavier weight ($P = 0.02$) than those in the non-injected eggs group. All other evaluated parameters, including average chick length and average navel score, were observed to be similar across all treatment groups.

Table 7.2 Effect of in *ovo* delivery of folic acid on hatch performance and chick quality.

Hatch Parameters	Treatments[1]				SEM[2]	*P* value[3]
	Non-injected	*In ovo* saline	*In ovo* folic acid 1	*In ovo* folic acid 2		
Hatchability, %	96.1[a]	95.2[a]	75.2[b]	54.5[c]	3.43	<0.001
Average chick weight, g	43.1[b]	43.8[ab]	43.8[ab]	44.0[a]	0.14	0.023
Average chick length, cm	19.0	18.9	19.1	19.0	0.18	0.872
Average navel score	1.45	1.38	1.51	1.45	0.07	0.739

[1]Treatments include— (1) non-injected eggs; (2) *in ovo* saline group- injected with 0.2 mL of physiological saline (0.9% NaCl); (3) *in ovo* folic acid group 1 injected with 0.1 mg folic acid per egg and (4) *in ovo* folic acid group 2 injected with 0.15 mg folic acid per egg. [2] SEM = Standard error of means. [3]Means within a row with different superscripts [a,b,c] significantly differ.

7.4.2 Growth performance

Table 7.3 highlights the results observed for growth performance parameters across all feeding phases. In the starter phase (d 0-14), only the birds in the in-feed antibiotics treatment consumed more feed (27.5%) ($P = 0.01$) than the in-feed FA treatment; other treatments were statistically similar to the in-feed antibiotics treatment. No differences in ABWG and FCR amongst treatment groups were recorded in the starter phase. In the grower (d 15-25) and finisher (d 26-35) phases, the in-feed antibiotic treatment recorded higher ($P < 0.001$) AFI compared to the in-feed FA and *in ovo* FA group 2 treatments. Other treatments recorded statistically similar AFI values compared to the in-feed antibiotic treatment. The in-feed antibiotic treatment also recorded higher ($P = 0.05$) FCR compared to the in-feed FA treatment; other treatments recorded statistically similar FCR values. At the end of the total trial period (d 0-35), only the AFI values were found to be significantly different among treatment groups. A similar trend of the in-feed antibiotic treatment recording higher ($P < 0.001$) AFI compared to the in-feed FA and *in ovo* FA group 2 treatments was also observed. All treatment groups had similar values for ABWG and FCR.

Table 7.3 Effect of folic acid and its delivery routes on the growth performance of broiler chickens raised for 35 days.

Growth Performance Parameters	Treatments[1]					SEM[2]	*P* value[3]
	Negative Control	In-feed Antibiotics	In-feed folic acid	*In ovo* folic acid 1	*In ovo* folic acid 2		
Starter (0-14d)							
Average feed intake, g	353^{ab}	348^{a}	273^{b}	336^{ab}	340^{ab}	6.16	0.013
Average body weight gain, g	291	314	284	309	250	4.57	0.155
FCR[4]	1.21	1.11	0.97	1.10	1.34	0.02	0.058
Grower (15-25d)							
Average feed intake, g	1,479^{abc}	1,643^{a}	1,246^{bc}	1,415^{ab}	1,072^{c}	22.8	<0.001
Average body weight gain, g	1,376	1,331	1,220	1,166	1,067	15.6	0.265
FCR[4]	1.07^{ab}	1.19^{a}	1.03^{b}	1.21^{ab}	1.02^{ab}	0.02	0.045
Finisher (26-35d)							
Average feed intake, g	2,310^{abc}	2,348^{a}	1,922^{bc}	2,117^{ab}	1,780^{c}	25.1	<0.001
Average body weight gain, g	826	908	797	1068	1000	294	0.922
FCR[4]	2.69	2.49	2.41	2.03	1.77	0.11	0.981

Total Trial Period (0-35d)

Average feed intake, g	3,252[abc]	3,332[a]	2,610[bc]	2,930[ab]	2,418[c]	38	<0.001
Average body weight gain, g	2,566	2,643	2,349	2,548	2,300	292	0.683
FCR[4]	1.24	1.24	1.13	1.20	1.06	0.03	0.305

[1] Treatments include— (1) Negative Control treatment- chicks fed a basal corn-soybean meal-wheat–based diet; (2) In-feed antibiotics- chicks fed NC + 0.05% bacitracin methylene disalicylate; (3) In-feed folic acid- chicks fed NC + 5 mg/kg folic acid; (4) *In ovo* folic acid group 1- eggs injected with 0.1 mg folic acid per egg and (4) *In ovo* folic acid group 2- eggs injected with 0.15 mg folic acid per egg. [2] SEM = Standard error of means. [3] Means within a row with different superscripts [a,b,c] significantly differ. [4]FCR = Feed Conversion Ratio.

7.4.3 Immune organ weight, serum immunoglobulin concentration, and antioxidant indexes

Results on the relative weight of immune organs (Bursa of Fabricius and spleen), serum immunoglobulin concentration and antioxidant (SOD, MDA and TAC) indexes are presented in Table 7.4. Of the two immune organs evaluated, only the relative weight of the Bursa of Fabricius (g/Kg BW) recorded a tendency to be increased by the *in ovo* FA group 1 treatment. The relative weight of the Bursa of Fabricius in the *in ovo* FA group 1 was at least 37.8% higher ($P = 0.08$) than those of other treatment groups. In terms of antioxidant indexes, while the *in ovo* FA group 2 treatment recorded a tendency to increase ($P = 0.08$) serum SOD activity by at least 50%, compared to other treatments; a marginal increase ($P = 0.07$) in serum MDA concentration was observed in the *in ovo* FA group 1 treatment, compared to other treatments. There was no effect of treatment on the concentrations of IgG and IgM in the serum.

Table 7.4 Effect of folic acid and its delivery routes on relative weight of immune organs, serum immunoglobulin concentrations, and antioxidant indexes.

Parameters	Treatments[1]					SEM[2]	*P* value
	Negative Control	**In-feed Antibiotics**	**In-feed folic acid**	***In ovo* folic acid 1**	***In ovo* folic acid 2**		
Bursa weight, g/Kg BW	1.86	1.85	2.00	2.55	2.22	0.09	0.076
Spleen weight, g/Kg BW	0.72	0.78	0.81	0.83	0.69	0.03	0.474
Immunoglobulin G, Mg/mL	10.9	2.53	1.18	0.80	1.71	0.32	0.120
Immunoglobulin M, Mg/mL	0.86	0.34	0.24	0.30	0.17	0.05	0.589
SOD[3] activity, U/ml	0.34	0.36	0.36	0.35	0.51	0.02	0.081
MDA[4], ng/ml	20.9	11.9	13.1	24.8	20.7	1.90	0.068
TAC[5], mM uric acid equivalents (UAE)	0.80	0.68	0.84	0.89	0.76	0.04	0.353

[1] Treatments include— (1) Negative Control treatment- chicks fed a basal corn-soybean meal-wheat–based diet; (2) In-feed antibiotics- chicks fed NC + 0.05% bacitracin methylene disalicylate; (3) In-feed folic acid- chicks fed NC + 5 mg/kg folic acid; (4) *In ovo* folic acid group 1- eggs injected with 0.1 mg folic acid per egg and (4) *In ovo* folic acid group 2- eggs injected with 0.15 mg folic acid per egg. [2] SEM = Standard error of means. [3]SOD = Superoxide dismutase. [4]MDA = Malondialdehyde. [5]TAC = Total antioxidant capacity.

7.4.4 Blood biochemistry

Table 7.5 shows the results on blood plasma biochemistry indexes. Only the concentrations of plasma electrolyte minerals - sodium and chloride were affected by the evaluated treatment groups. Sodium concentration (mmol. L^{-1}) in the in-feed antibiotics treatment was higher (P = 0.04) than those of the NC treatment. Other treatment groups recorded statistically intermediate values for sodium concentration in the blood plasma. Conversely, chloride concentration (mmol. L^{-1}) in the in-feed antibiotics treatment was higher (P = 0.001) than in the NC and in-feed FA treatment groups. Both levels of *in ovo* delivered FA treatment groups recorded statistically intermediate values for chloride concentration in the blood plasma.

Table 7.5 Effect of folic acid and its delivery routes on broiler chicken plasma biochemistry indices.

Parameters	Treatments[1]					SEM[2]	*P* value[3]
	Negative Control	In-feed Antibiotics	In- feed folic acid	*In ovo* folic acid 1	*In ovo* folic acid 2		
Electrolytes, mmol·L^{-1}							
Sodium	149^{b}	152^{a}	150^{ab}	152^{ab}	151^{ab}	0.58	0.036
Potassium	6.96	6.62	6.86	6.62	6.78	0.09	0.666
Sodium: Potassium	21.4	23.0	22.1	23.0	22.5	0.30	0.382
Chloride	109^{b}	113^{a}	110^{b}	111^{ab}	112^{ab}	0.50	0.001
Calcium	3.06	2.76	3.05	2.99	3.01	0.01	0.373
Phosphorus	2.46	2.16	2.41	2.42	2.37	0.06	0.221
Magnesium	0.87	0.80	0.82	0.80	0.81	0.01	0.206
Metabolites, mmol·L^{-1}							
Urea	0.30	0.29	0.32	0.28	0.29	0.10	0.692
Glucose	15.5	15.3	15.5	16.2	15.1	0.14	0.231

Cholesterol	3.48	3.51	3.61	3.46	3.91	0.06	0.215
Iron	18.5	20.6	20.2	19.7	20.2	0.01	0.784
Bile acids	22.2	24.0	21.6	19.3	20.5	0.96	0.716
Uric acid	361	371	371	424	365	0.01	0.641
Creatinine	1.95	0.95	1.82	1.17	1.56	0.09	0.117
Enzymes, $U \cdot L^{-1}$							
Amylase	589	686	715	889	758	49.4	0.540
Lipase	21.0	22.5	18.3	21.4	20.4	0.03	0.839
Creatine kinase	6,218	7,937	5,229	8,218	4,791	0.04	0.414
Alkaline Phosphatase	9,813	7,383	10,506	17,443	10,841	1104	0.112
Alanine transaminase	2.56	2.21	2.60	2.40	2.88	0.04	0.888
Aspartate Aminotransferase	164	190	166	173	164	0.01	0.388
Gamma-Glutamyl Transferase	9.07	10.63	10.52	8.67	9.67	0.27	0.128
Proteins, $g \cdot L^{-1}$							

Total proteins	28.6	27.3	29.2	28.8	29.2	0.001	0.508
Albumin	11.8	11.9	12.1	11.7	12.2	0.14	0.82
Globulin	16.8	15.4	17	17	17	0.002	0.343
Albumin: Globulin	0.70	0.78	0.71	0.69	0.71	0.01	0.310

[1] Treatments include— (1) Negative Control treatment- chicks fed a basal corn-soybean meal-wheat–based diet; (2) In-feed antibiotics- chicks fed NC + 0.05% bacitracin methylene disalicylate; (3) In-feed folic acid- chicks fed NC + 5 mg/kg folic acid; (4) *In ovo* folic acid group 1- eggs injected with 0.1 mg folic acid per egg and (4) *In ovo* folic acid group 2- eggs injected with 0.15 mg folic acid per egg. [2] SEM = Standard error of means. [3] Means within a row with different superscripts [a,b] significantly differ.

7.4.5 Gut morphology

Table 7.6 shows the effect of FA and its delivery routes on the three intestinal segments. All FA treatments (irrespective of delivery routes) seemed to improve duodenal morphology in this study, as evidenced by increased ($P < 0.01$) villus height, villus width, villus height to crypt depth ratio, and reduced ($P = 0.04$) crypt depth, compared to the control treatment. Nonetheless, the *in ovo* FA 2 group recorded the highest increase in villus width, at least 13.6 % wider than other treatments and only statistically comparable to the antibiotic treatment.

Conversely, in the jejunum, the antibiotic treatment recorded the least ($P < 0.001$) crypt depth and highest villus height to crypt depth ratio compared to the control treatment. Interestingly, the antibiotic treatment equally recorded reduced ($P < 0.001$) jejunal villus width compared to the control treatment. All other treatments mostly recorded statistically intermediate values for villus width, crypt depth and villus height to crypt depth ratio in the jejunum. No difference in the jejunal villus height was recorded for all treatments in this study.

In the ileum, results on villus height and width were variable. Significant differences were recorded between the in-feed FA group and the *in ovo* FA 1 group for both parameters. While the former increased ($P = 0.001$) ileal villus height compared to the latter, the latter increased ($P = 0.007$) the villus width compared to the former. Nonetheless, the antibiotic treatment recorded the lowest ($P = 0.002$) crypt depth and the highest ($P = 0.03$) villus height to crypt depth ratio compared to the control treatment. Other treatments had statically intermediate crypt depth and villus height to crypt depth ratios.

Table 7.6 Effect of folic acid and its delivery routes on broiler chicken intestinal morphology.

Parameters	Treatments[1]					SEM[2]	*P* value[3]
	Negative Control	In-feed Antibiotics	In-feed folic acid	*In ovo* folic acid 1	*In ovo* folic acid 2		
Duodenum							
Villus height, mm	2.04^{b}	2.14^{ab}	2.15 ^{ab}	2.28^{a}	2.21^{a}	0.02	< 0.001
Villus width, mm	0.22^{b}	0.24^{ab}	0.22^{b}	0.22^{b}	0.25^{a}	0.00	0.003
Crypt depth, mm	0.16^{a}	0.14^{b}	0.14^{b}	0.15a^{b}	0.15^{ab}	0.00	0.004
Villus height: Crypt depth	12.5^{b}	15.0^{a}	14.7^{a}	15.5^{a}	14.4^{a}	0.19	< 0.001
Jejunum							
Villus height, mm	1.15	1.17	1.12	1.16	1.11	0.01	0.497
Villus width, mm	0.25^{a}	0.20^{b}	0.23^{a}	0.24^{a}	0.26^{a}	0.00	< 0.001
Crypt depth, mm	0.11^{ab}	0.10^{c}	0.10^{bc}	0.12^{a}	0.11^{abc}	0.00	< 0.001
Villus height: Crypt depth	10.2^{bc}	11.8^{a}	10.7^{ab}	9.30^{bc}	9.88^{c}	0.18	< 0.001
Ileum							
Villus height, mm	0.76^{ab}	0.77^{ab}	0.82^{a}	0.70^{b}	0.74^{ab}	0.01	0.001

Villus width, mm	0.18^{ab}	0.18^{ab}	0.17^{b}	0.20^{a}	0.19^{ab}	0.00	0.007
Crypt depth, mm	0.15^{a}	0.13^{bc}	0.14^{ab}	0.13^{bc}	0.14^{abc}	0.00	0.002
Villus height: Crypt depth	5.01^{b}	5.74^{a}	5.68^{ab}	5.47^{ab}	5.21^{ab}	0.10	0.028

[1]Treatments include— (1) Negative Control treatment- chicks fed a basal corn-soybean meal-wheat–based diet; (2) In-feed antibiotics- chicks fed NC + 0.05% bacitracin methylene disalicylate; (3) In-feed folic acid- chicks fed NC + 5 mg/kg folic acid; (4) *In ovo* folic acid group 1- eggs injected with 0.1 mg folic acid per egg and (4) *In ovo* folic acid group 2- eggs injected with 0.15 mg folic acid per egg. [2] SEM = Standard error of means. [3] Means within a row with different superscripts [a,b,c] significantly differ.

7.5 Discussion

While a few studies have reported the potential of the *in ovo* delivery of FA to improve embryo growth, bird growth performance, and immune and blood biochemical indexes (El-Azeem et al., 2014; Li et al., 2016; Nouri et al., 2018; Ismail et al., 2019; Gouda et al., 2022), we present here the first comparison of FA (across both *in ovo* and in-feed delivery routes) with a classic antibiotic (bacitracin methylene disalicylate) in broiler chicken study, to our knowledge.

This study reports contrasting results on chick hatchability and hatchling weight. While *in ovo* delivered FA reduced hatchability with increasing dosage, the highest dose (0.15 mg/egg) recorded the highest hatchling weight compared to the non-injected treatment. Conflicting results on the effect of *in ovo* delivered FA on hatchability are reported in the few available literature. While a few studies have reported improved hatchability with *in ovo* delivered FA (Li et al., 2016; Liu et al., 2016; Gouda et al., 2021, 2022), other studies have recorded no effect of *in ovo* delivered FA on hatchability (Nouri et al., 2018; Ismail et al., 2019). Abdel-Halim et al. (2020) observed decreased hatchability (91% vs 76%) with *in ovo* injected eggs (0.2 mg FA/egg via the albumen), compared to non-injected eggs in their study. Similarly, Abdel-Fattah and Shourrap (2013) recorded decreased hatchability values following *in ovo* delivery of FA (1 mg FA/egg via the air cell). Several factors, including the dosage of injected substance, form or solubility of injected substance, the volume of injection, site of injection, injection needle length, and time of injection, have been reported to influence hatchability outcomes across *in ovo* studies (Ohta and Kidd, 2001; Zhai et al., 2011; Abdel-Halim et al., 2020; Oladokun and Adewole, 2020). Of all these factors, the form of injected substance, injection needle length and time of injection are speculated to be the key factors that could have contributed to the hatchability outcome observed in this study. While we utilized commercially available FA in its powder form in this study, it would be important for further studies to consider evaluating liquid FA forms, if available. This might aid easy embryo absorption and utilization of this bioactive substance. Also, Ohta and Kidd (2001) have earlier documented that a 13-mm needle length, as opposed to a 19-mm, is optimum for *in ovo* delivery of amino acids. Our laboratory has successfully established the procedure for the *in ovo* delivery of probiotics (*B. subtilis*) in broiler chickens using a 3-cm (30-mm) injection needle length, recording 91% hatchability rate (Oladokun et al., 2021). While we utilized a similar needle length (3 cm) in this study, it is probable that a shorter needle length might be more relevant for the *in ovo* delivery of FA to ensure increased chances of embryo viability. Regarding the time of injection, Sunde et al. (1950) pointed out that embryo mortality would not be affected by FA

deficiency until embryonic d17, suggesting that d17 onwards might be an ideal time for the *in ovo* delivery of FA. Furthermore, our result on increased hatchling weights following *in ovo* delivery of FA is consistent with previous reports on *in ovo*-delivered FA (Abdel-Fattah and Shourrap, 2013; El-Azeem et al., 2014; El Said, 2017; Gouda et al., 2022). Li et al. (2016) equally recorded increased hatchling weight with a similar *in ovo* delivered FA dose as this study. This positive effect of *in ovo*-delivered FA on hatchling weight has been attributed to the critical role folate plays in DNA and RNA synthesis, cell replication, blood protein synthesis, thyroid activity, hepatic expression of Insulin-like growth factor 2 (IGF2), antioxidant activities, and nutrient utilization (Robel, 1993b; Abdel-Fattah and Shourrap, 2013; El-Azeem et al., 2014; Liu et al., 2016; Ismail et al., 2019).

The growth performance dataset from this study shows the in-feed antibiotic treatment consistently recording higher AFI values across all feeding phases. Even more interesting, birds in the *in ovo* folic acid 2 treatment (0.15 mg/egg) were observed to record similar FCR values as the in-feed antibiotic treatment while consistently consuming less quantity of feed at the grower, finisher, and overall feeding phases, suggesting that this treatment possessed some sort of nutrient utilization efficiency. The growth promotion potential of AGP (especially BMD) to improve AFI and FCR values are well substantiated across the literature (Ao and Choct, 2013; Murugesan et al., 2015; Gadde et al., 2017; Manafi et al., 2017; Crisol-Martínez et al., 2017; Walters et al., 2019). Several theories including inhibition of the synthesis of bacteria cell wall (Smith and Weinberg, 1962; Butaye et al., 2003), improved feed intake resulting from enhanced nutrient digestibility by virtue of improved gut microflora structure (Dibner and Richards, 2005; N. Karthikeyan et al., 2017), and an anti-inflammatory effect via reduced production of cytokines and chemokines resulting in reduced incidence of anorexia (Niewold, 2007) have been posited to explain AGP growth-promoting effect. Similar to the result reported here, Gamboa et al. (2022) observed improved FCR *in ovo* FA injected birds (0.15 mg/egg) at d1-42 compared to control treatment in their study, with similar AFI values and no change in ABWG values. Other studies have also affirmed the capacity of *in ovo* delivered FA to improve FCR in poultry (El-Azeem et al., 2014; Li et al., 2016; Nouri et al., 2018). This nutrient conversion efficiency afforded by higher dosage of *in ovo* delivered FA is traceable to folate's antioxidant and thyroid activity, as well as its effect on muscular metabolism (Joshi et al., 2001; El-Azeem et al., 2014; Nouri et al., 2018).

Furthermore, no treatment effect on serum immunoglobulins G and M levels was recorded in this study. Although immunoglobulins are a critical component of humoral response to infection

and oxidative stress, the results recorded were not surprising as birds were raised under experimentally controlled conditions with no strain on their immune system. Nonetheless, one study (Li et al., 2016) reported that *in ovo* delivered (100 and 150 µg/egg) FA increased plasma IgG and IgM concentration in broiler chickens at d21 and 42. Based on the limited and conflicting results observed, it would be worthwhile for more studies to research the effect of *in ovo* delivered FA on immunoglobulin concentrations and other factors, including sampling time and differences in quantification assays that could potentially affect detectable immunoglobulin concentrations. Contrary to the result recorded on serum immunoglobulins concentration, a tendency for the *in ovo* FA 1 treatment to increase the relative weight of the bursa was also observed. This is consistent with the result of El Said (2017), which showed that *in ovo* delivery of FA (10%, 0.1 ml/egg) increased bursa weight (%). The positive effect of *in ovo*-delivered FA on lymphoid organ hypertrophy has been linked to the antioxidant properties of FA (Joshi et al., 2001; Akinyemi and Adewole, 2022a). Consistent with this result, we also record a tendency for a higher dosage of *in ovo* delivered FA to increase serum SOD activity, alongside a tendency to reduce lipid peroxidation product (MDA), compared to a lower dosage of the *in ovo* delivered FA. Despite the reported antioxidant potential of FA (Joshi et al., 2001; Gliszczyńska-Świgło, 2007), it is interesting to note that this is the first study to actually evaluate the effect of *in ovo* delivered FA on antioxidant indexes, to our knowledge. Several other studies (El-Din et al., 2008; Gouda et al., 2020; Li et al., 2021; Savaram et al., 2022) have affirmed that in-feed supplementation of FA improves various antioxidant indexes.

Blood sampling continues to be an important diagnostic approach in both human and avian research. In this study, all observed blood biochemical parameters were within the normal physiological range for broiler chickens (Ilo S U et al., 2019). Nonetheless, blood plasma sodium and chloride level in the in-feed antibiotic treatment was observed to be significantly increased compared to the control treatment. Both electrolyte minerals were observed to be within the upper limit of recommended physiological ranges for broiler chickens (Leeson and Summers, 2001). Conditions involving high concentrations of sodium and chloride in the blood are referred to as hypernatremia and hyperchloremia, respectively. Considering the role of these minerals in maintaining acid-base balance and osmotic pressure in body fluids, excessively high levels of these electrolyte minerals have been implicated in the incidences of dehydration, edema, acidosis, poor bone development (tibial dyschondroplasia), and decreased humoral immunity (Pimentel and Cook, 1987; Ruíz-López et al., 1993; Oviedo-Rondón et al., 2001; Pohl et al., 2013). The effect

of antibiotics on the concentrations of these blood minerals is relatively unreported in the literature; this study perhaps provides another justification to encourage the discontinued use of AGP in poultry production.

Additionally, a higher dosage (0.15 mg/egg) of the *in ovo* delivered FA enhanced duodenal, jejunal and ileal morphology in this study; in most cases, as comparable to or even better than the in-feed antibiotic treatment. Considering that an increased villus height, villus width, and reduced crypt depth are frequently linked to improved nutrient absorptive functions, these results could explain the similar trend in growth performance observed for both treatments. Despite the paucity of studies that have evaluated the effect of *in ovo*-delivered FA on the intestinal morphology of poultry, Li et al. (2020) have previously reported enhanced intestinal morphology in lamb's offspring with increasing maternal FA supplementation. A similar positive effect of improved intestinal morphology with antibiotics (especially BMD) supplementation is also well documented in the literature (Viveros et al., 2011; Khodambashi Emami et al., 2012; Adewole and Akinyemi, 2021; Akinyemi and Adewole, 2022a). This positive effect of antibiotics on intestinal morphology is theorized to occur as a result of their antibacterial and gut microbiota modulating properties (Marković et al., 2009; Khodambashi Emami et al., 2012). Given that new cells possess shorter villus height and higher crypt depth, by virtue of a shift in gut microbiota, destruction and the subsequent renewal of gut cells are thus reduced.

7.6 Conclusions

This study showed that both dosages (0.1 and 0.15 mg FA/egg) of *in ovo* delivered FA reduced hatchability in a dose-dependent manner. However, *in ovo*-delivered FA at a higher dosage (0.15 mg FA/egg) afforded heavier hatchling weight. The same dosage of *in ovo*-delivered FA also enhanced broiler chicken intestinal morphology and FCR in a similar capacity as the in-feed antibiotic treatment, with birds consuming less quantity of feed. A marginal tendency to increase serum SOD activity was equally observed in the same *in ovo*-delivered FA treatment. Taken together, the results presented here suggest that *in ovo* delivered FA at 0.15 mg/egg could offer a similar growth-promoting effect as antibiotics in broiler production. Notwithstanding, it would be important to optimize all possible injection and incubation conditions through further research in order to yield favorable hatchability outcomes.

8 CHAPTER 8 *IN OVO* DELIVERED BIOACTIVE SUBSTANCES AND HEAT STRESS

SUCCESSIVE DELIVERY OF ESSENTIAL OIL VIA *IN OVO* AND IN-WATER ROUTES IMPROVES BROILER CHICKEN'S GUT INTEGRITY.

8.1 Abstract

Mitigating the negative effects of heat stress (**HS**) is a critical challenge for the global poultry industry. This study thus evaluated the thermoregulatory potential of three *in ovo* delivered bioactive substances using selected gut health parameters. Eggs were incubated and allotted to 5 groups, and respective bioactive substances delivered. These groups included- the non-injected group, *in ovo* saline group, *in ovo* folic acid (**FA**), *in ovo* probiotics (**P**), and *in ovo* essential oil (**EO**) group. At hatch, chicks were assigned to post-hatch treatment combinations, including - (A) a negative control (**NC**; corn-wheat-soybean diet); (B) antibiotics (Bacitracin methylene disalicylate), (C) *In ovo* FA, (D) *In ovo* probiotics and (E) *In ovo* + in-water EO groups in 8 replicate cages (6 birds/cage) and raised for 28 d. Birds were exposed to either a thermoneutral (24ºC ± 0.2) or HS challenge (31ºC) condition from d 21 - d 28. The *in ovo* delivered FA and EO treatments reduced *(P* < 0.001) hatchability by at least 26 % compared to NC. Induced HS reduced *(P* < 0.001) average body weight gain, total plasma protein, total antioxidant capacity, and villus width in the duodenum and jejunum. The relative mRNA expression of intestinal barrier-related genes (Claudin4, Claudin10 and Mucin2) was also reduced by ($P \leq 0.05$) HS. Independent of HS and compared to NC, the *in ovo* + in-water EO treatment recorded (P < 0.05) at least a 15% increase in villus height: crypt depth across the three gut sections. The *in ovo* + in-water EO treatment also increased the relative mRNA expression of intestinal barrier-related genes (Claudin1,3,4, Occludin, Zona occludens-2, and Mucin 2). Under HS, the *in ovo* + in-water EO treatment recorded a 3.5-fold upregulation of amino acid transporter gene (SLC1A1), compared to NC. Subject to further hatchability optimization, the *in ovo* + in-water delivery of EO shows great potential to afford broiler chicken thermotolerance.

8.2 Introduction

According to OECD-FAO, (2018) projection, poultry will be the most consumed animal protein globally in this decade. This is likely due to its affordability and acceptability across several

cultures and religions. Despite the dampened sales that characterized the food sector during the COVID-19 pandemic, poultry meat recorded a meteoric output of 137 million tonnes in 2020 (FAO, 2020). Poultry production also contributes significantly to sustaining the economic livelihoods of millions of rural dwellers, especially in developing countries (De Bruyn et al., 2015; Wong et al., 2017). Despite the poultry industry's importance, the welfare and performance of poultry birds can be impaired by disease incidences and environmental factors. Heat stress has explicitly been regarded as "the main environmental factor" negatively impacting the poultry sector (Zhang et al., 2012).

Heat stress (**HS**) has been defined as "a continuous adaptive response to thermal perturbations outside the thermal comfort zone of a bird" (Oladokun and Adewole, 2022b). The thermal comfort zone of poultry has been stated to be within 18–25 °C (Blas and Scanes, 2015). At temperatures outside this thermal comfort zone, HS has been shown to have adverse effects on poultry wellbeing. Asides from inducing oxidative stress, HS could also cause cellular damage, leading to endocrine disorders, immunosuppression, increased inflammatory conditions, altered microbial ecology, poor gut health, poor meat quality, and the possible onset of enteric diseases in poultry (He et al., 2020; Tavaniello et al., 2020). The economic impact of HS on the poultry industry was recorded to be up to $165 million in annual losses and up to a 7.2% decrease in egg production in the US alone almost two decades ago (St-Pierre et al., 2003). The annual heat-related losses incurred in the broiler sector alone were estimated at $51.8 million [(St-Pierre et al., 2003). It is reasonable to infer that these predictions would be much higher today given current climate change realities and the rising demand for poultry. Moreover, IPCC (2021) has predicted a global temperature increase of about 2.5 °C during the next two decades. Current HS mitigation measures have also been deemed "partially efficacious", given that poultry production systems, particularly in the tropics, continue to suffer the negative effect of HS during hot seasons (Green and Xin, 2009). It is thus important that timely and effective HS mitigation options are made available to the poultry industry.

Across the literature, several HS amelioration strategies, including environmental, genetic, and nutritional supplementation, have been reported. Environmental and management approaches will include infrastructural investment in air ventilation and sprinkling systems in poultry houses (Vandana et al., 2021). Marker-assisted selective breeding for thermotolerance in poultry birds is a genetic option to mitigate HS (Chen et al., 2013). Nutritional supplementation of bioactive substances with HS mitigation potential seems to be the most feasible of these options. The

potential of several bioactive substances, including minerals, amino acids, prebiotics, symbiotics, vitamins, probiotics and essential oils (EO), have been reported (Sahin and Kucuk, 2003; Sohail et al., 2011; Tang et al., 2018; Alagawany et al., 2021; Salem et al., 2022). Antibiotics have also been reported to mitigate HS in broiler chickens (Teeter, 1996; Zulkifli et al., 2000). Notwithstanding, using antibiotics to mitigate HS-related symptoms in poultry may drive continuous antibiotic use (Cole and Desphande, 2019), a public health concern. Therefore, it is crucial to research other bioactive substances that mitigate HS whilst potentially replacing antibiotics-use in poultry production.

By acting through differing modes of action, a few researched alternatives to antibiotics have been reported to possess HS mitigating potential, asides from other beneficial performance-enhancing properties (Tavaniello et al., 2020). For instance, several studies have reported the potential of probiotics (defined as "live microorganisms which, when administered in adequate amounts, confer a health benefit on the host" (FAO/WHO, 2001)) to enhance the growth performance, immune status, and gut health of heat-stressed poultry (Sugiharto et al., 2017; Salem et al., 2022). This positive effect is theorized to be exerted as a result of probiotics' ability to ensure homeostatic gut microbial diversity (Sugiharto, 2016). Similarly, plant essential oils like oregano, rosemary, thyme, etc. are also reported to improve nutrient digestibility, antioxidant capacity, immune status, and ultimately growth performance of poultry under HS challenge conditions (Ghazi et al., 2015; Khafar et al., 2020; Büyükkılıç Beyzi et al., 2020). These EO contain carvacrol and thymol as active bioactive components; this consequently affords them their beneficial antioxidant properties and ability to reduce gut leakage by upregulating tight-junction related genes in poultry (Patra, 2020; Büyükkılıç Beyzi et al., 2020). Folic acid (FA), a vitamin B-complex with potent radical scavenging activity, is another bioactive substance that shows promising HS mitigating potential. Although scarcely researched, a few studies have reported the increase in feed intake, feed efficiency, egg production, growth performance and health status of birds supplemented with FA under HS (Tolba et al., 2007; El-Din et al., 2008; Gouda et al., 2020; Onuoha and Udo, 2022). Despite the reported benefits of these bioactive substances, several other reports have equally documented their ineffectiveness in mitigating HS in poultry(Sohail et al., 2013, 2015; Dardeer et al., 2018). A possible rationale for the recorded inefficiencies of these substances could be related to differences in dose, form, and delivery routes. Conventionally in the poultry industry, these bioactive substances are either delivered through the feed or the water. These conventional routes suffer a few limitations, including the possibility of negative interaction with other feed additives,

heat instability, and potential water quality issues (Gadde et al., 2017; Oladokun and Adewole, 2020). The *in ovo* delivery of bioactive substances (reviewed by Oladokun and Adewole, 2020) offers the opportunity to surmount all the identified challenges, amongst other advantages. The *in ovo* route may thus guarantee the efficacy of these bioactive substances against HS. Using several independent studies, our laboratory has been able to report that *in ovo* delivery of *Bacillus subtilis* enhanced the intestinal morphology and immune status in broiler chickens without compromising hatch and gut homeostasis under thermoneutral conditions (Oladokun et al., 2021a; Oladokun and Adewole, 2022a). Similarly, our previous study also validated that successive delivery of a blend of essential oils via the *in ovo* and in-water route improves broiler chicken's antioxidant status and blood biochemical profile with no adverse effect on growth performance (Oladokun et al., 2021b). We also recently determined that the *in ovo* delivery of FA yields heavier hatchling weight, enhanced gut morphology and improved nutrient conversion efficiency in broiler chickens (Oladokun and Adewole, 2022c). Also, it has been speculated that most alternatives to antibiotics utilized in poultry production are usually more potent under immunological or environmental challenge conditions (Ferket et al., 2002; Patterson and Burkholder, 2003; Yadav et al., 2016). Furthermore, while the antioxidant and gut-enhancing properties of these bioactive substances are well reported (Habibi et al., 2014; Song et al., 2014; Gouda et al., 2020), the molecular basis of their thermo-protective capacity in broiler chickens, especially via the *in ovo* route, remains largely unknown. Consequently, based on the rationale above, it is hypothesized that the beneficial properties of these bioactive substances across these delivery routes might be more apparent, especially under HS conditions.

Accordingly, by integrating enzyme-link immunosorbent assays, blood biochemistry assays, histology and molecular biology techniques, this study sought to gain a holistic insight into the thermoregulatory capacities of selected *in ovo* delivered bioactive substances (FA, probiotics, and EO). The effect of the delivery of these bioactive substances on hatch and growth performance, blood biochemistry, antioxidant, and immune status in heat-stressed broiler chickens compared to a classic antibiotic was thus specifically evaluated. Additionally, the relative expression of splenic-immune-related genes, cellular stress proteins, jejunal nutrient transporter, and intestinal barrier-related genes were also evaluated. This is perhaps the first study seeking to highlight the molecular responses of major *in ovo* delivered bioactive substances with HS mitigating potential. This study thus has the potential to provide novel insight into the mode of action of these bioactive substances and provide information on the biological mechanisms underpinning thermoregulation

in broiler chickens.

8.3 Methods

8.3.1 Ethics statement

This study was conducted at Dalhousie University, Faculty of Agriculture Hatchery facility and the Atlantic Poultry Research Center. All experimental procedures were approved by Dalhousie University Animal Use and Care Committee (File No- 1035699) and were in accordance with the standards set out by the Canadian Council on Animal Care (CCAC, 2009).

8.3.2 *In ovo* procedures

A total of 1,252 hatching eggs with an average weight of 65.07 ± 0.30 g (mean ± SE) from Synergy hatchery, Nova Scotia, Canada were incubated in a ChickMaster single-stage incubator (ChickMaster G09, Cresskill, NJ, USA) under standard conditions (37.5 °C and 55% relative humidity) from embryonic days (EDs) 1 to 17, and then to an average of 32 °C and 68% from EDs 18 to 21. Prior to setting eggs, incubators were preheated for 24 hours to maintain the right temperature and humidity levels. From the time of set until ED 18, egg trays were rotated four times an hour on a 90° arc. Eggs were candled on ED12, and infertile eggs were disposed of, leaving a total of 1,216 eggs for the trial. Afterwards, the remaining eggs were divided into one of five treatment groups: (1) non-injected eggs (control; 98 eggs); (2) *in ovo* saline group (50 eggs; injected with 0.2 mL of physiological saline, i.e., 0.9% NaCl, Baxter Corporation, ON, Canada); (3) *in ovo* FA group (50 eggs, injected with 0.1 mL FA (FA; 95-102%; Thermo Scientific, USA) at 0.15 mg per egg; (4) *in ovo* probiotics group (50 eggs, injected with 0.2 mL of *Bacillus subtilis* fermentation extract, each egg received 10×10^6 CFU of the bacterium/0.2 mL saline diluent); and (5) *in ovo* essential oil group (50 eggs; injected with 0.2 mL of a saline + essential oil blend mixture at a dilution ratio of 2:1). All treatments were replicated in four similar incubators operated under the same condition. FA was injected on ED12, while the probiotics and essential oil group were injected on ED18. The *Bacillus subtilis* product (Strain - *Bacillus subtilis* 10SI) (concentration of 10×10^{10} CFU/g) and the commercial essential oil blend (containing phytonutrients star anise, cinnamon, rosemary, and thyme oil) used in this study were obtained from Probiotech International Inc., St Hyacinthe, Quebec, Canada. The EO blend is registered by Health Canada as a Veterinary Health Product. Detailed information on the injection procedure used in this investigation have previously been detailed by Oladokun et al.(2021a). Summarily, eggs were cleaned with 70% alcohol swabs (BD alcohol swabs-catalog 326910, ON, Canada), and then an 18-gauge needle was used to carefully puncture the air cell (the blunt end of the egg). A

self-refilling injector (Socorex ultra-1810.2.05005, Ecublens, Switzerland) fitted with a 22-gauge needle at a 45-degree angle was then used to provide FA and probiotics to the amnion and EO to the air cell. After *in ovo* injection, sterile medical tapes (NexcareTM Flexible Clear Tape-7100187758, 3M, MN, USA) were used to close the injection sites. The non-injected eggs were also removed and put back in the incubator at the same time as the other treatment groups that received injections.

8.3.3 Experimental design, animal husbandry and diets

A schematic presentation of this study's experimental layout is presented in Figure 8.1. At hatch, hatchlings (n = 480) were weighed and randomly allotted to five new treatment groups. The non-injected eggs gave rise to treatment (A) Negative control treatment- chicks were offered a basal corn-soybean meal-wheat–based diet, and (B) Antibiotics diet- chicks were offered NC + 0.05% bacitracin methylene disalicylate (BMD). The eggs injected with FA, probiotics and EO were used to create treatments- (C) *In ovo* FA, (D) *In ovo* probiotics and (E) *In ovo* + in-water EO groups, respectively. The *in ovo* + in-water EO treatments included hatchlings from the *in ovo* EO group also supplied EO at the recommended dosage of 250 mL/1000 L of drinking water via the water route. The experiment was conducted as a 5 x 2 factorial design, with 5 *in ovo* treatments and 2 temperature challenge models as main factors. Chicks-mixed sex (6 birds/cage) were randomly allotted to either a thermoneutral room or an environmentally controlled room, consisting of 40 battery cages each (dimension - 50cm × 60cm; stocking density - 0.076m^2/bird), with each treatment group having 8 replicate cages. All treatment groups were evenly represented across both tiers of the cages. Throughout the experiment, the lighting was programmed to generate 18 hours of light and 6 hours of darkness, and illumination was gradually decreased from 20 lux on day 0 to 5 lux on day 28. Ambient temperature in the experimental rooms was monitored daily and gradually decreased from 32ºC on d0 to 24ºC on d 21. At 11 am on d21, the temperature was increased to 31ºC in the environmentally controlled heat challenge room. This temperature was retained for only 8 hr./day from d21 – d28 in the HS group in a bid to mimic a typical summer heatwave. The thermoneutral (TN) or unchallenged group temperature was maintained at 24ºC ± 0.2. Relative humidity was between 20 and 62% for both rooms. Temperature and relative humidity were continuously monitored and recorded using Extech Instruments (Nashua, USA) for both thermal groups. All birds were offered feed and water ad libitum throughout the trial, which lasted 28 days. Diets were formulated to meet Cobb 500 broiler chicken nutrient requirements.

Diets were offered in mash form during the starter (0-14d) and grower (15-28d) phases. The ingredient, and nutritional composition of the experimental diets are presented in Table 8.1.

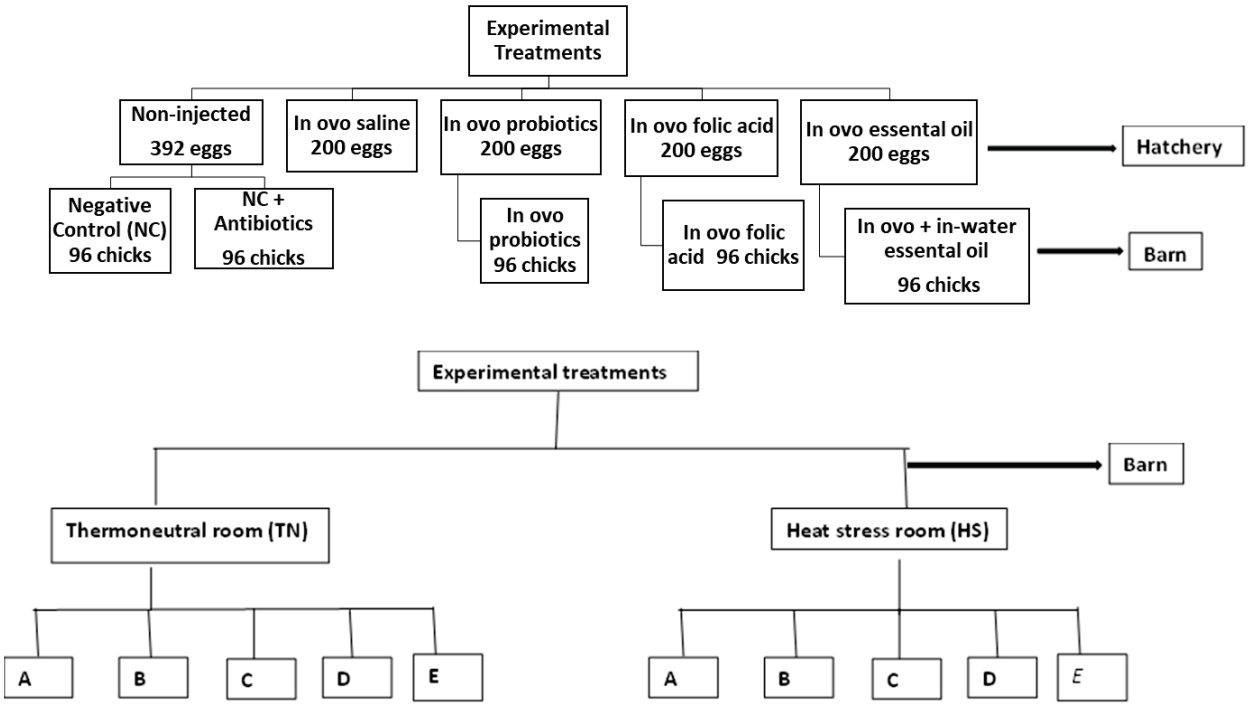


Figure 8.1 Schematic presentation of experimental layout in the hatchery and barn.

At the hatchery - *in ovo* saline eggs were injected with 0.2 mL of physiological saline, i.e., 0.9% NaCl, Baxter Corporation, ON, Canada); *in ovo* probiotics eggs were injected with 0.2 mL of *Bacillus subtilis* fermentation extract (each egg received 10 × 106 CFU of the bacterium/0.2 mL saline diluent); *in ovo* folic acid eggs were injected with 0.1 mL folic acid (95-102%; Thermo Scientific, USA; at 0.15 mg per egg); and *in ovo* essential oil eggs were injected with 0.2 mL of a saline + essential oil blend mixture at a dilution ratio of 2:1). At the barn- the non-injected eggs gave rise to treatment (A) Negative control treatment - chicks were offered a basal corn-soybean meal-wheat–based diet, and (B) Antibiotics diet - chicks were offered NC + 0.05% bacitracin methylene disalicylate (BMD). The eggs injected with folic acid, probiotics and essential oil were used to create treatments - (C) *In ovo* folic acid, (D) *In ovo* probiotics and (E) *In ovo* essential oil groups, respectively

Table 8.1 Effect of *in ovo* delivery of folic acid, probiotics and essential oil on hatch performance and chick quality.

Hatch Parameters	Treatments[1]					SEM[2]	*P* value[3]
	Non-injected	*In ovo* Saline	*In ovo* Folic acid	*In ovo* Probiotics	*In ovo* Essential oil		
Hatchability (%)	94.5[a]	93.2[a]	77.3[b]	92.8[a]	70.0[b]	2.78	<0.001
Average chick weight (g)	46.2	45.9	44.9	46.4	47.4	0.38	0.116
Average chick length (cm)	19.1	19.2	19.0	19.2	19.0	0.06	0.338
Chick BW/ egg weight (%)	0.70	0.71	0.70	0.71	0.72	0.004	0.411
Average navel score	1.32[b]	1.42[ab]	1.46[ab]	1.60[a]	1.64[a]	0.05	0.002

8.3.4 Measurement

8.3.4.1 Hatch Parameters and Chick Quality

The ratio of hatched chicks to viable incubated eggs was used to determine % hatchability on a replicate basis. Each hatched chick was weighed on an individual basis in order to determine the average chick weight per treatment. The BW/initial egg weight ratio of hatched chicks was also determined and recorded. To determine chick length, measurement was taken from the tip of the chick's beak to the middle toe on the right leg. Chick navel quality was also assessed by adapting Reijrink et al. (2009) navel scoring method. Navel quality was scored 1—when the navel was completely closed and clean; scored 2—when the navel was discolored (i.e., when the navel color differs from the chick's skin color) with a maximum 2 mm opening; and scored 3—when the navel was discolored and with more than a 2 mm opening.

8.3.4.2 Growth performance parameters

Feed intake and average body weight (ABW) were measured on a cage basis weekly. The obtained data was then used to calculate the average feed intake (AFI), average body weight gain (ABWG), and feed conversion ratio (FCR). The FCR was calculated as the amount of feed consumed per unit of body weight gain. Mortality was recorded daily and used to correct the FCR. Dead birds were weighed and sent to the Nova Scotia Agriculture, Animal Health Laboratory for necropsy.

8.3.4.3 Sample collection

On d28, 1 bird/cage (males) was randomly selected, weighed, and humanely euthanized by electrical stunning and exsanguination. After euthanasia of the bird, blood samples were collected from each bird into 10 mL blood serum collection tubes (BD Vacutainer™ Serum Tubes, fisher scientific - BD366430) for further serum assays and 10 mL heparinized tubes (BD Vacutainer™ Glass Blood Collection Tubes with Sodium Heparin, fisher scientific- BD366480) for further blood plasma assays. Blood serum and plasma were centrifuged at 1,200 g x 10 minutes x 18 °C. The resulting supernatants were stored in aliquots at -80 °C until further analysis. The Bursa of Fabricius, spleen, liver, and intestinal tissues (duodenum, jejunum, and ileum) were also harvested for further analysis.

8.3.4.4 Relative weight of Bursa of Fabricius

At the point of slaughter, the weights of the bursa of Fabricius were recorded and expressed as a percentage of the live BW of the slaughtered chicken (g/Kg BW).

8.3.4.5 8.3.4.5 Rectal temperature

To confirm that the HS protocol induced HS in the birds, rectal temperatures from 2 birds in each

replicate cage were measured using digital thermometer (Accuflex®5; A.M.G. Medical Inc., Montreal, CA) on days 21, 23, 25 and 27 (during HS) in both HS and TN groups. The digital thermometer probe was inserted 4 cm into the cloaca for 5s.

8.3.4.6 Plasma Biochemistry

Plasma samples for blood biochemical analysis were shipped on ice to Atlantic Veterinary College, University of Prince Edward Island Pathology Laboratory, and analyzed using cobas® 6000 analyzer series (Roche Diagnostics, Indianapolis, IN, USA).

8.3.4.7 Antioxidant indices

The activities of superoxide dismutase (SOD), and the concentration of Malondialdehyde (MDA) in the serum were measured according to the manufacturer's instructions using Cayman's SOD assay kit (catalog number 706002, Cayman Chemical, Ann Arbor, MI, USA) and chicken MDA ELISA kit (catalog number MBS260816, MyBioSource, San Diego, CA, USA) respectively. The Total antioxidant capacity (TAC) in blood plasma was analyzed using the Oxiselect Total Antioxidant Capacity assay kit (catalog number STA360; Cell BioLabs Inc., San Diego, CA, USA) according to the manufacturer's instructions. Absorbance for all analysis was measured at recommended wavelengths on a microplate reader (Bio-Tek Instrument Inc., Wonooski, VT, USA) using a software program (KC4, version #3.3, Bio Tek Instruments).

8.3.4.8 Gut morphology

The procedure for gut morphology analysis has previously been described by Oladokun et al. (2021a). Summarily, fixed intestinal tissues were sectioned (0.5 m thick), embedded in paraffin, and stained with hematoxylin and eosin. Morphometric measurements (n = 6 measurements per replicate slide) were then carried out on each cross-sectioned tissue using a Leica 1CC50 W microscope at 4× Magnification (Leica Microsystems, Wetzlay, Germany) and an image processing and analysis system (Leica Application Suite, Version 3.4.0, Leica Microsystems, Wetzlay, Germany). Morphometric measurements included the villus height (from the base of the intestinal mucosa to the tip of the villus excluding the intestinal crypt), villus width (halfway between the base and the tip), crypt depth (from the base upward to the region of transition between the crypt and villi).

8.3.4.9 RNA Isolation, Reverse Transcription, and Real-Time Quantitative PCR

Total RNA was extracted from broiler chicken (n=6 per treatment) jejunum, spleen, and liver samples using Trizol reagent (Life Technologies, Carlsbad, CA) according to the manufacturer's instructions. The concentration and quality of the extracted RNA were then determined using the

Take3 micro-volume plate in a Synergy HT multimode microplate reader (BioTek, Winooski, VT) and the obtained ratio of absorbance (A260/280). Verified pure RNA was then reverse transcribed using qScript cDNA Synthesis Supermix (Quanta Biosciences, Gaithersburg, MD), and amplified by qPCR (Applied Biosystems 7500 Real Time System) with SYBR green master mix (Life Technologies, Carlsbad, CA) as previously described by Dhamad et al. (2020) and Flees et al. (2021). Relative expression of the target genes was then determined using the $2^{-\Delta\Delta CT}$ method (Schmittgen and Livak, 2008) with 18S rRNA as the housekeeping gene. Chicken-specific oligonucleotide primers used are summarized in 8.2.

Table 8.2 List of qPCR chicken-specific oligonucleotide primers.

Gene	Accession number	Primer sequence (5′ → 3′)	Orientation	Product size (bp)
CLDN1	NM_001013611	CCCACGTTTTCCCCTGAAA	Forward	61
		GCCAGCCTCACCAGTGTTG	Reverse	
CLDN3	NM_204202	CACAGGGTGGTTTCGGTCAG	Forward	70
		GCCCCATAGACATGGTGTCC	Reverse	
CLDN4	XM_040687592.2	CGAGGTGAGATCCCCGAAA	Forward	71
		GGGCGTTTGGTGCTCTTCT	Reverse	
CLDN10	XM_046908153.1	CCGCTGTCTGTCTGGGTTTC	Forward	59
		TGTGCACTTCATCCCAACCA	Reverse	
OCLN	NM 205128	CGCAGATGTCCAGCGGTTA	Forward	59
		GTAGGCCTGGCTGCACATG	Reverse	
ZO-1	XM_015278975	GGGAACAACACACGGTGACTCT	Forward	80
		AGGATTATCCCTTCCTCCAGATATTG	Reverse	
ZO-2	NM 204918	AGGATTATCCCTTCCTCCAGATATTG	Forward	69
		GCAATTGTATCAGTGGGCACAA	Reverse	
ZO-3	XM_015299757	CAAAGCAAGCCGGACATTTAC	Forward	63
		GTCAAAATGCGTCCGGATGTA	Reverse	
MUC2	XM_046942297.1	GATCTTCCTTGACAGCTTTTGAACT	Forward	145
		AAATGATCCATAGGTGTATGCAACTC	Reverse	
GLUT1	NM 205209.1	TCCTGATCAACCGCAATGAG	Forward	60
		TGCCCCGGAGCTTOTTG	Reverse	
GLUT2	NM 207178.1	GAAGGTGGAGGAGGCCAAA	Forward	61
		TTTCATCGGGTCACAGTTTCC	Reverse	
GLUT5	XM 040689119.1	CCTCAGCATAGTGTGTGTCATCATT	Forward	62
		GGATCGGACTGGCTCCAA	Reverse	
SLC1A1	XM 424930.7	GGTGAAGGCGGACAGGAA	Forward	68
		TGCTGAGCAGGAGCCAGTT	Reverse	
SLC15A1	NM 204365.1	GACAACTTTTCTACAGCCATCTACCA	Forward	65
		CCCAGGATGGGCGTCAA	Reverse	
IL-1β	NM_204524	CGAGGAGCAGGGACTTTGC	Forward	71
		GAAGGTGACGGGCTCAAAAA	Reverse	
IL-2	NM_204153.2	CGAGCTCTACACACCAACTGAGA	Forward	62
		CCAGGTAACACTGCAGAGTTTGC	Reverse	
IL-4	NM 0010079.1	GCTCTCAGTGCCGCTGATG	Forward	60
		GAAACCTCTCCCTGGATGTCAT	Reverse	

IL-6	NM 204628.1	GCTTCGACGAGGAGAAATGC GGTAGGTCTGAAAGGCGAACAG	Forward Reverse	63
IL-12A	NM_001398447.1	AAACGAGGCACTCCTGAAGGT ACCTCTTCAAGGGTGCACTCA	Forward Reverse	66
IL-12B	NM_213571.2	TGCCCAGTGCCAGAAGGA TCAGTCGGCTGGTGCTCTT	Forward Reverse	57
IFN-γ	NM_205149.2	CTGACAAGTCAAAGCCGCAC CTTCACGCCATCAGGAAGGT	Forward Reverse	80
SCD	NM 204890	CAATGCCACCTGGCTAGTGA CGGCCGATTGCCAAAC	Forward Reverse	52
SCAP	XM 001231539	TGGCCCAGAGACTCATCATG GCAGGATCCGTATAAACCAGGAT	Forward Reverse	67
FAS	J03860	ACTGTGGGCTCCAAATCTTCA CAAGGAGCCATCGTGTAAAGC	Forward Reverse	70
SBREP1	AY029224	CATCCATCAACGACAAGATCGT CTCAGGATCGCCGACTTGTT	Forward Reverse	82
SBREP2	AJ414379	GCCTCTGATTCGGGATCACA GCTTCCTGGCTCTGAATCAATG	Forward Reverse	63
HSP70	J02579	GGGAGAGGGTTGGGCTAGAG TTGCCTCCTGCCCAATCA	Forward Reverse	55
HSP90	X07265.1	TGACCTTGTCAACAATCTTGGTACTAT CCTGCAGTGCTTCCATGAAA	Forward Reverse	68
18S	AF173612	TCCCCTCCOGTTACTTGGAT GCGCTCGTCGGCATGTA	Forward Reverse	60

8.3.5 Statistical analysis

Hatch data were analyzed as a randomized complete block design, with the incubator as the blocking factor. Datasets from the grow-out trial were analyzed as a 5 x 2 factorial analysis of variance (ANOVA) design. All datasets were analyzed in the Minitab statistical package (v.18.1) using the generalized linear model. When significant main effects and interaction were detected, means were compared using Tukey's honest significant difference test in the same statistical package. Analyzed data were presented as means ± SEM and probability values. Values were considered statistically different at $P \leq 0.05$.

8.4 Results

8.4.1.1 Hatch performance and chick quality

The effect of *in ovo* delivered treatments on hatch performance, and chick quality is presented in Table 8.3. Compared to the non-injected eggs, the *in ovo* delivered FA and EO treatments reduced ($P < 0.001$) hatchability by 18 and 26 % respectively. Similarly, the *in ovo* probiotics and EO treatments recorded higher ($P = 0.002$) average navel score values compared to the non-injected eggs. Other treatments had statistically intermediate average navel score values. *In ovo* delivery of evaluated treatments recorded no effect on average chick weight, chick length and the ratio of Chick BW to incubated egg weight in this study.

Table 8.3 Effect of in ovo delivery of folic acid, probiotics and essential oil on hatch performance and chick quality.

Hatch Parameters	Treatments[1]					SEM[2]	*P* value[3]
	Non-injected	*In ovo* Saline	*In ovo* Folic acid	*In ovo* Probiotics	*In ovo* Essential oil		
Hatchability (%)	94.5[a]	93.2[a]	77.3[b]	92.8[a]	70.0[b]	2.78	<0.001
Average chick weight (g)	46.2	45.9	44.9	46.4	47.4	0.38	0.116
Average chick length (cm)	19.1	19.2	19.0	19.2	19.0	0.06	0.338
Chick BW/ egg weight (%)	0.70	0.71	0.70	0.71	0.72	0.004	0.411
Average navel score	1.32[b]	1.42[ab]	1.46[ab]	1.60[a]	1.64[a]	0.05	0.002

[1]Treatments include— non-injected eggs; *in ovo* saline group- eggs injected with 0.2 mL of physiological saline; *in ovo* folic acid (FA) group- eggs injected with 0.1 mL FA (FA; 95-102%; Thermo Scientific, USA) at 0.15 mg per egg; *in ovo* probiotics group- eggs injected with 0.2 mL of *Bacillus subtilis* fermentation extract (each egg received 10 × 106 CFU of the bacterium/0.2 mL saline diluent); and *in ovo* essential oil group- eggs injected with 0.2 mL of a saline + essential oil blend mixture at a dilution ratio of 2:1. 2 SEM = Standard error of means.

8.4.1.2 Growth performance

Treatment effect on growth performance indices for the entire trial period is shown in Table 8.4. In the starter phase (d0-14), while treatment had no effect on bird's AFI and FCR, the antibiotics treatment increased ($P < 0.01$) ABWG compared to other treatments, with the exception of the *in ovo* + in water EO treatment. At the grower phase (d15-28) and throughout the entire trial period (d0-28), no differences in AFI, ABWG and FCR were recorded between treatments.

Table 8.5 reports the effect of treatments and thermal challenge on growth performance indices of birds exposed to cyclic HS for 1 week. Although the antibiotic treatment showed a tendency to increase ($P = 0.08$) AFI compared to other treatments, no interaction effect of treatment and temperature was recorded for AFI in this study. Conversely, a significant interaction effect of treatment and temperature was observed for ABWG. While the antibiotic treatment recorded the highest ($P < 0.001$) ABWG values compared to other treatments under TN condition, the *in ovo* + in-water EO treatment recorded the highest numerical increase in ABWG values, which was at least 17% higher than other treatments under HS condition. Nonetheless, under HS condition, ABWG values in the *in ovo* + in-water EO treatment were higher ($P < 0.001$) than the *in ovo* probiotics treatment. When both factors were considered independently, HS was observed to clearly decrease ($P < 0.001$) ABWG in birds, while the antibiotic treatment increased ($P < 0.001$) ABWG in comparison to other treatments, while still being statistically comparable to the *in ovo* + in-water EO treatment. Regarding FCR, the antibiotic treatment recorded a lower ($P = 0.03$) FCR than the *in ovo* probiotic treatment; other treatments recorded statistically intermediate FCR values. FCR was also observed to be higher ($P = 0.03$) under TN condition. This study also detected a significant interaction effect between treatment and temperature model for FCR. Under TN condition, the antibiotic treatment recorded lower ($P < 0.001$) FCR values than all *in ovo* treatments. However, under HS condition, the *in ovo* + in-water treatment had at least 19% lower ($P > 0.05$) FCR values than other treatments. Notwithstanding, the FCR values recorded by the *in ovo* + in-water treatment was significantly lower ($P < 0.001$) than those of the *in ovo* probiotics treatment under HS condition.

Table 8.4 Effect of *in ovo* delivered folic acid, probiotics and *in ovo* + in-water essential oil on broiler chicken growth performance.

Performance Parameters	Treatments[1]					SEM[2]	*p* value[3]
	Negative Control	Antibiotics	*In ovo* folic acid	*In ovo* probiotics	*In ovo* + in-water EO		
Starter (d1-14)							
Average feed intake (g/bird)	253	322	255	262	261	57.8	0.23
Average body weight gain (g/bird)	192[b]	254[a]	189[b]	186[b]	212[ab]	6.30	<0.01
Feed conversion ratio	1.28	1.25	1.34	1.37	1.25	0.41	0.90
Grower (d15-28)							
Average feed intake (g/bird)	1387	1459	1405	1346	1341	66.5	0.92
Average body weight gain (g/bird)	498	538	530	525	532	17.8	0.98
Feed conversion ratio	1.48	1.14	1.09	1.02	1.02	0.19	0.47
Total trial period(d1-28)							
Average feed intake (g/bird)	1540	1667	1524	1493	1536	85.4	0.84
Average body weight gain (g/bird)	1037	1142	1028	1012	1071	18.4	0.18
Feed conversion ratio	1.45	1.46	1.48	1.45	1.42	0.09	1.00

[1]Treatments groups include- Negative control treatment- chicks were offered a basal corn-soybean meal-wheat–based diet, Antibiotics treatment- chicks were offered NC + 0.05% bacitracin methylene disalicylate, *In ovo* folic acid (FA) treatment- eggs injected with 0.1 mL FA (FA; 95-102%; Thermo Scientific, USA) at 0.15 mg per egg, *In ovo* probiotics treatment- eggs injected with 0.2 mL of *Bacillus subtilis* fermentation extract, each egg received 10 × 106 CFU of the bacterium/0.2 mL saline diluent, and *In ovo* + in-water essential oil (EO) treatment- eggs injected with 0.2 mL of a saline + EO blend mixture at a dilution ratio of 2:1 and

supplied the EO via the in-water route at the recommended dosage of 250 mL/1000 L of drinking water. 2 SEM = Standard error of means. 3Means that do not share the same superscript are significantly different.

Table 8.5 Effect of in ovo delivered folic acid, probiotics and in ovo + in-water essential oil on the growth performance of broiler chicken's exposed to 1-week (d21-28) cyclic heat stress.

Main effects		Performance Parameters		
		Average feed intake (g/bird)	**Average body weight gain (g/bird)**	**Feed conversion ratio**
Treatments[1]	Negative Control	739	467^{b}	1.58^{ab}
	Antibiotics	881	653^{a}	1.35^{b}
	In ovo folic acid	837	498^{b}	1.68^{ab}
	In ovo probiotics	765	426^{b}	1.79^{a}
	In ovo + in-water EO	815	530^{ab}	1.54^{ab}
	SEM[2]	20.5	18.1	0.05
Temperature[3]	TN	806 ± 17.1	570^{a} ± 20.0	1.46^{b} ± 0.08
	HS	777 ± 37.6	476^{b} ± 27.5	1.71^{a} ± 0.07
Interaction				
Treatments	Temperature			
Negative Control	TN	714 ± 25.3	512^{bc} ± 34.0	1.41^{abc} ± 0.21
	HS	757 ± 35.6	434^{bc} ± 22.3	1.78^{ab} ± 0.08
Antibiotics	TN	926 ± 21.9	860^{a} ± 25.7	1.08^{c} ± 0.08
	HS	817 ± 61.5	498^{bc} ± 21.0	1.69^{ab} ± 0.07
In ovo folic acid	TN	810 ± 22.6	516^{bc} ± 45.9	1.60^{ab} ± 0.09
	HS	836 ± 24.3	499^{bc} ± 29.8	1.76^{ab} ± 0.13
In ovo probiotics	TN	769 ± 35.7	494^{bc} ± 21.0	1.58^{ab} ± 0.12
	HS	746 ± 45.3	374^{c} ± 40.0	2.03^{a} ± 0.13
In ovo	TN	840 ± 22.6	505^{bc} ± 31.3	1.69^{ab} ± 0.17

+ in-water EO	HS	740 ± 148	586[b] ± 49.0	1.37[bc] ± 0.21
***p* value[4]**				
Treatments		0.08	<0.001	0.03
Temperature		0.39	<0.001	0.03
Treatments*Temperature		0.51	<0.001	<0.001

1Treatments groups include- Negative control treatment- chicks were offered a basal corn-soybean meal-wheat–based diet, Antibiotics treatment- chicks were offered NC + 0.05% bacitracin methylene disalicylate, *In ovo* folic acid (FA) treatment- eggs injected with 0.1 mL FA (FA; 95-102%; Thermo Scientific, USA) at 0.15 mg per egg, *In ovo* probiotics treatment- eggs injected with 0.2 mL of *Bacillus subtilis* fermentation extract, each egg received 10 × 106 CFU of the bacterium/0.2 mL saline diluent, and *In ovo* + in-water essential oil (EO) treatment- eggs injected with 0.2 mL of a saline + EO blend mixture at a dilution ratio of 2:1 and supplied the EO via the in-water route at the recommended dosage of 250 mL/1000 L of drinking water. 2 Pooled SEM = Standard error of means. 3Temperature model include- Thermoneutral group (T)- birds were housed at 24ºC ± 0.2 from d 21 – 28, and Heat stress group (H)-birds were housed at 31ºC for 8hrs/day from d 21 – 28. 4Means that do not share the same superscript are significantly different.

8.4.1.3 Relative weight of Bursa of Fabricius

Treatment, temperature nor their interaction recorded no effect on the relative weight of the Bursa of Fabricius (g/kg BW) in this study (Table 8.6).

8.4.1.4 Rectal temperature

Rectal temperature measurements revealed that the utilized HS protocol clearly induced HS in the birds, as there was an obvious difference in rectal temperature values between TN and HS birds at each of the four-time points (Figure 8.2). Across the four different time-points, the HS birds recorded higher ($P < 0.001$) average rectal temperature compared to birds raised under TN condition (HS- 42.0 °C vs TN- 41.2 °C).

Table 8.6 Effect of *in ovo* delivered folic acid, probiotics and in ovo + in-water essential oil and temperature on the relative weight of Bursa of Fabricius (g/kg BW) of broiler chicken on day 28.

Main effects		**Relative weight of Bursa of Fabricius (g/kg BW)**
Treatments[1]	Negative Control	2.32
	Antibiotics	2.02
	In ovo folic acid	2.04
	In ovo probiotics	1.94
	In ovo + in-water EO	1.79
	SEM[2]	0.13
Temperature[3]	TN	2.03 ± 0.09
	HS	2.01 ± 0.09
Interaction		
Treatments	Temperature	
Negative Control	TN	2.52 ± 0.19
	HS	2.12 ± 0.20
Antibiotics	TN	1.70 ± 0.19
	HS	2.33 ± 0.19

In ovo folic acid	TN	2.06 ± 0.19
	HS	2.01 ± 0.19
In ovo probiotics	TN	1.88 ± 0.19
	HS	2.01 ± 0.19
In ovo + in-water EO	TN	1.99 ± 0.19
	HS	1.58 ± 0.19
***p* value**		
Treatments		0.11
Temperature		0.87
Treatments*Temperature		0.06

[1]Treatments groups include- Negative control treatment- chicks were offered a basal corn-soybean meal-wheat–based diet, Antibiotics treatment- chicks were offered NC + 0.05% bacitracin methylene disalicylate, *In ovo* folic acid (FA) treatment- eggs injected with 0.1 mL FA (FA; 95-102%; Thermo Scientific, USA) at 0.15 mg per egg, *In ovo* probiotics treatment- eggs injected with 0.2 mL of *Bacillus subtilis* fermentation extract, each egg received 10×10^6 CFU of the bacterium/0.2 mL saline diluent, and *In ovo* + in-water essential oil (EO) treatment- eggs injected with 0.2 mL of a saline + EO blend mixture at a dilution ratio of 2:1 and supplied the EO via the in-water route at the recommended dosage of 250 mL/1000 L of drinking water._[2] SEM-Standard error of the means; Temperature model include- Thermoneutral group (TN)- birds were housed at 24ºC ± 0.2 from d 21 – 28, and Heat stress group (HS)-birds were housed at 31ºC for 8hrs/day from d 21 – 28.

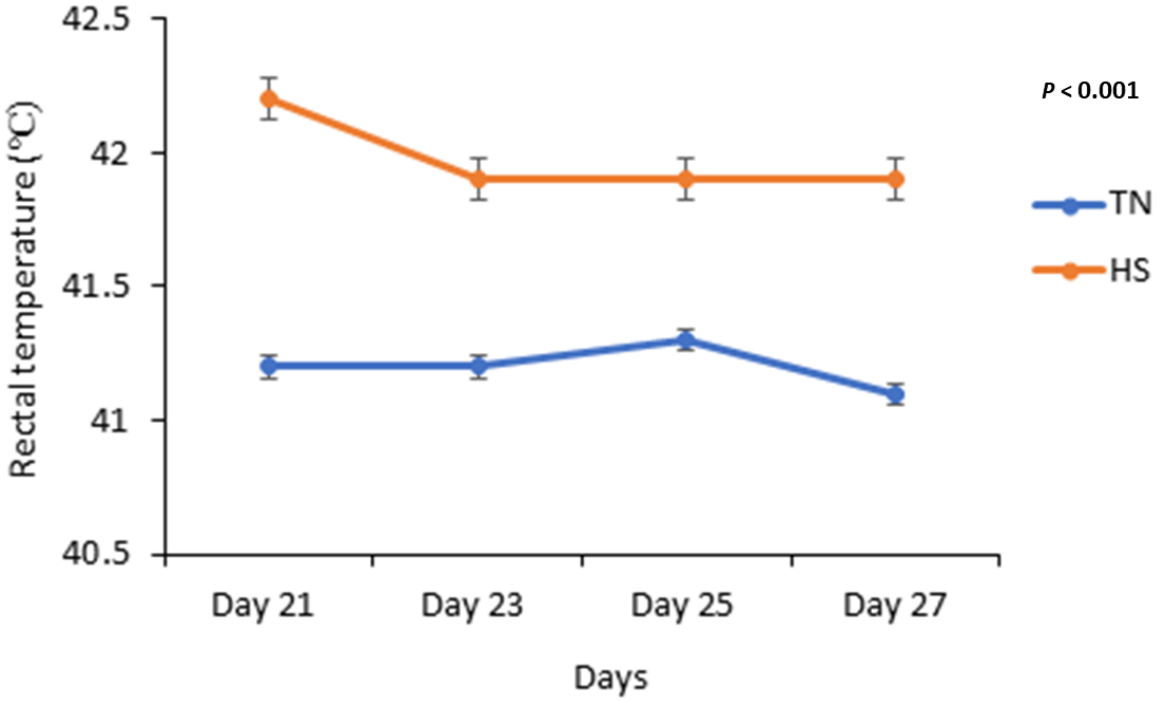


Figure 8.2 Rectal temperature measurements during heat stress.

Temperature models include- Thermoneutral group (TN)- birds were housed at 24ºC ± 0.2 from d 21 – 28, and Heat stress group (HS)-birds were housed at 31ºC for 8hrs/day from d 21 – 28. Data are presented as mean ± SEM (n = 40/thermal group; two birds/cage).

8.4.1.5 Blood plasma Biochemistry

The result of treatment, temperature and their interaction on blood plasma biochemistry indices are shown in Table 8.7. While no interaction effect for both main factors was recorded in this case, significant but independent effects of both treatments and temperature were noted. Birds in the *in ovo* probiotics treatment recorded higher ($P = 0.02$) plasma magnesium levels only compared to the NC treatment; other treatments had statistically intermediate plasma levels. Similarly, the *in ovo* probiotics treatment also had higher ($P = 0.03$) plasma phosphorus levels compared to other treatment groups, except for the *in ovo* FA treatment. Contrarily, the plasma level of Gamma-glutamyl Transferase (GGT) was reduced ($P = 0.05$) in the *in ovo* probiotics treatment compared to the *in ovo* FA and NC treatment. Compared to other treatments, higher ($P \leq 0.01$) plasma Aspartate Transferase (AST) and Albumin to Globulin (A:G) levels were afforded by the antibiotics treatment, with the exception of the *in ovo* FA treatment for AST levels. Additionally, plasma globulin levels were reduced ($P = 0.02$) by the antibiotics treatment compared to the *in ovo* FA and *in ovo* + in-water treatment. With regards to temperature effect, HS is observed to reduce ($P \leq 0.02$) the plasma levels of sodium, glucose, AST, albumin, globulin, and total protein in broiler chickens in this study. In contrast, elevated levels ($P \leq 0.05$) of plasma phosphorus and bile acids were recorded in heat-stressed birds.

Table 8.7 Effect of in ovo delivered folic acid, probiotics and in ovo + in-water essential oil and temperature on broiler chicken's plasma biochemistry indices.

Plasma parameters	Treatments[1]					Temp. Model[2]		Interaction										SEM	*P* value		
								TN					HS								
	NC	BMD	*In ovo* F	*In ovo* P	*In ovo* + In-water EO	TN	HS	NC	BMD	*In ovo* F	*In ovo* P	*In ovo* + In-water EO	NC	BMD	*In ovo* F	*In ovo* P	*In ovo* + In-water EO		Trt.	Temp	Trt. * Temp
								Electrolytes (mmolL^{-1})													
Na[3]	146	145	147	146	147	147[a]	145[b]	147	146	147	147	148	144	144	147	144	145	1.19	0.62	0.01	0.90
K[4]	5.72	5.57	5.71	6.10	5.69	5.80	5.69	5.52	5.55	5.90	6.05	5.55	5.97	5.59	5.55	6.14	5.86	0.20	0.32	0.48	0.48
Na:K[5]	25.4	25.9	25.6	24.6	25.6	25.7	25.1	26.5	26.4	24.8	24.1	26.6	24.1	25.4	26.3	25.2	24.5	0.46	0.84	0.41	0.28
Cl[6]	109	110	109	109	111	110	109	107	110	110	110	111	110	110	108	107	111	0.91	0.21	0.73	0.28
P[7]	2.11[bc]	2.03[c]	2.29[ab]	2.39[a]	2.06[bc]	2.09[b]	2.25[a]	1.96	1.99	2.222	2.23	2.07	2.28	2.07	2.37	2.56	2.05	0.05	0.03	0.05	0.64
Mg[8]	0.73[b]	0.75[ab]	0.76[ab]	0.84[a]	0.75[ab]	0.76	0.77	0.70	0.76	0.78	0.83	0.75	0.76	0.75	0.75	0.83	0.75	0.03	0.02	0.89	0.55
								Metabolites (umolL^{-1})													
Glucose[9]	13.5	13.5	13.2	13.1	13.5	13.7[a]	13.0[b]	13.9	13.8	13.4	13.5	13.8	12.9	13.2	13.0	12.6	12.1	0.15	0.34	<0.001	0.73
Cholesterol[9]	2.73	2.88	2.99	2.90	2.96	2.99[a]	2.79[b]	2.82	3.04	2.97	3.08	3.03	2.64	2.71	3.01	2.69	2.89	0.05	0.52	0.05	0.66
Uric acid	289	324	313	313	293	311	301	295	348	333	273	313	283	302	294	358	275	8.66	0.63	0.55	0.10
Bile acid	18.3	18.7	17.7	17.7	19.8	16.5[b]	20.5[a]	16.5	17	15.5	15.6	17.7	20.2	20.5	20.1	19.9	21.9	0.62	0.80	0.001	1
Iron	17.9	16.3	17.7	18.0	17.7	18.1	17.0	18.5	17.4	18.2	18.4	18.1	17.3	15.4	17.2	17.7	17.4	0.35	0.39	0.08	0.91

Urea[9]	0.34	0.32	0.36	0.34	0.29	0.33	0.34	0.35	0.33	0.37	0.029	0.3	0.34	0.32	0.36	0.40	0.29	0.01	0.14	0.54	0.22
											Enzymes (UL^{-1})										
Amylase	515	609	569	400	697	573	532	632	504	616	402	739	411	723	524	398	638	38.6	0.15	0.58	0.45
ALP[10]	4921	3978	4243	4646	4868	4558	4474	4876	4094	4357	5013	4514	4966	3865	4133	4306	5248	344	0.81	0.89	0.96
AST[11]	154[b]	194[a]	167[qb]	158[b]	154[b]	176[a]	155[b]	161	226	171	158	167	146	165	163	158	141	4.44	0.01	0.01	0.19
ALT[12]	2.17	3.26	2.28	2.91	2.47	3.10	2.16	2.46	3.0	2.72	4.38	3.27	1.92	3.56	1.92	1.93	1.87	0.36	0.65	0.07	0.58
GGT[13]	11.2[a]	10.0[ab]	11.1[a]	9.10[b]	10.1[ab]	10.1	10.5	10.4	10.2	11.4	8.40	10.3	12.0	9.90	10.9	9.80	9.90	0.26	0.05	0.46	0.46
CK[14]	3847	6463	4200	4315	3420	4661	4043	4102	9187	3970	3400	4325	3609	4548	4443	5476	2705	532	0.12	0.36	0.14
											Proteins (UL^{-1})										
T. protein[15]	25.4	24.1	27.4	26.1	26.2	26.9[a]	24.8[b]	26.9	26.1	27.5	27.0	27.3	24.0	22.1	27.3	25.3	25.1	0.40	0.10	<0.001	0.30
Albumin	11.5	11.8	12.1	11.8	11.5	12.1[a]	11.2[b]	12.0	12.8	12.2	12.2	11.9	10.9	10.7	12.0	11.4	10.9	0.16	0.56	<0.001	0.30
Globulin	13.9[ab]	12.3[b]	15.2[a]	14.0[ab]	14.6[a]	14.7[a]	13.3[b]	14.8	13.3	15.3	14.7	15.3	13.0	11.4	15.0	13.4	14.0	0.29	0.02	0.02	0.84
A:G[16]	0.83[b]	0.96[a]	0.80[b]	0.85[b]	0.80[b]	0.85	0.84	0.81	0.99	0.80	0.84	0.82	0.85	0.93	0.81	0.85	0.78	0.01	<0.001	0.69	0.66

[1]Treatments groups include- NC- chicks were offered a basal corn-soybean meal-wheat–based diet, BMD- chicks were offered NC + 0.05% bacitracin methylene disalicylate, *In ovo* F- eggs injected with 0.1 mL folic acid (FA; 95-102%; Thermo Scientific, USA) at 0.15 mg per egg, *In ovo* P- eggs injected with 0.2 mL of *Bacillus subtilis* fermentation extract, each egg received 10 × 106 CFU of the bacterium/0.2 mL saline diluent, and *In ovo* + in-water EO- eggs injected with 0.2 mL of a saline + essential oil (EO) blend mixture at a dilution ratio of 2:1 and supplied the EO via the in-water route at the recommended dosage of 250 mL/1000 L of drinking water. [2] Pooled SEM = Standard error of means. [3]Temperature model include- Thermoneutral group (TN)- birds were housed at 24ºC ± 0.2 from d 21 – 28, and Heat stress group (HS)-birds were housed at 31ºC for 8hrs/day from d 21 – 28. 3 Na-

sodium; [4]K-potassium; [5]Na:K-sodium potassium ratio; [6]Cl-chloride; [7]P-phosphorus; [8]Mg-magnesium; [9]Unit- mmolL-1; [10]ALP- alkaline phosphatase; [11]AST- Aspartate transaminase; [12]ALT-alanine transaminase; [13]GGT- gamma-Glutamyl Transferase; [14]CK-creatine kinase; [15]T.protein-total protein; [16]A:G-albumin to globulin ratio; Means that do not share the same superscript are significantly different.

8.4.1.6 Antioxidant indices

Table 8.7 shows the results obtained for evaluated antioxidant parameters in this study. Birds in the *in ovo* probiotics treatment recorded the lowest TAC values, which were only significantly lower ($P = 0.03$) than those of the antibiotics treatment. Furthermore, while HS reduced ($P \leq 0.03$) the plasma levels of SOD and TAC, MDA levels were increased ($P < 0.001$) in heat-stressed birds compared to birds raised under TN condition. The *in ovo* + in-water EO treatment recorded at least 10% lower ($P > 0.05$) MDA concentration than all other *in ovo* treatments, under HS condition.

Table 8.8 Effect of in ovo delivered folic acid, probiotics and in ovo + in-water essential oil and temperature on broiler chicken's antioxidant indices.

Main effects		Antioxidant parameters		
		Superoxide dismutase Activity (U/ml)	**Malondialdehyde concentration (nmol/mL)**	**Total antioxidant activity (UAE)**
Treatments[1]	Negative Control	0.21	11.1	0.52^{ab}
	Antibiotics	0.20	11.5	0.64^{a}
	In ovo folic acid	0.21	11.6	0.62^{ab}
	In ovo probiotics	0.23	10.9	0.40^{b}
	In ovo + in-water EO	0.22	11.1	0.52^{ab}
	SEM[2]	0.01	0.40	0.03
Temperature[3]	TN	$0.23^{a} \pm 0.01$	$9.4^{b} \pm 0.46$	$0.70^{a} \pm 0.03$
	HS	$0.20^{b} \pm 0.01$	$13.10^{a} \pm 0.46$	$0.38^{b} \pm 0.04$
Interaction				
Treatments	Temperature			
Negative Control	Negative	0. 21 ± 0.03	$9.68^{abc} \pm 1.08$	0.69 ± 0.08

	Control			
Antibiotics	HS	0.22 ± 0.03	12.6^{ab} ± 1.08	0.35 ± 0.08
	Antibiotics	0.23 ± 0.03	11.4^{abc} ± 1.01	0.72 ± 0.08
In ovo folic acid	HS	0.18 ± 0.03	11.6^{abc} ± 1.01	0.55 ± 0.08
	In ovo folic acid	0.26 ± 0.03	8.98^{bc} ± 1.01	0.79 ± 0.08
In ovo probiotics	HS	0.16 ± 0.03	14.3^{a} ± 1.01	0.45 ± 0.08
	In ovo probiotics	0.24 ± 0.03	7.34^{c} ± 1.01	0.62 ± 0.08
In ovo + in-water EO	HS	0.21 ± 0.03	14.5^{a} ± 1.01	0.19 ± 0.08
	In ovo + in-water EO	0.23 ± 0.03	9.49^{bc} ± 1.01	0.69 ± 0.08
	HS	0.21 ± 0.03	12.8^{ab} ± 1.01	0.34 ± 0.08
***p* value**[4]				
Treatments		0.94	0.96	0.03
Temperature		0.03	<0.001	<0.001
Treatments*Temperature		0.50	0.001	0.58

[1]Treatments groups include- Negative control treatment- chicks were offered a basal corn-soybean meal-wheat–based diet, Antibiotics treatment- chicks were offered NC + 0.05% bacitracin methylene disalicylate, *In ovo* folic acid (FA) treatment- eggs injected with 0.1 mL FA (FA; 95-102%; Thermo Scientific, USA) at 0.15 mg per egg, *In ovo* probiotics treatment- eggs injected with 0.2 mL of *Bacillus subtilis* fermentation extract, each egg received 10 × 10^6 CFU of the bacterium/0.2 mL saline diluent, and *In ovo* + in-water essential oil (EO) treatment- eggs injected with 0.2 mL of a saline + EO blend mixture at a dilution ratio of 2:1 and supplied the EO via the in-water route at the recommended dosage of 250 mL/1000 L of

drinking water. [2]SEM-pooled standard error of the means, [3]Temperature model include- Thermoneutral group (TN)- birds were housed at 24ºC ± 0.2 from d 21 – 28, and Heat stress group (HS)-birds were housed at 31ºC for 8hrs/day from d 21 – 28. [4]Means that do not share the same superscript are significantly different.

8.4.1.7 Gut morphology

The effect of treatments, temperature, and their interaction on gut morphometric indices of evaluated gut sections (duodenum, jejunum, and ileum) is presented in Table 8.8. All *in ovo* delivered bioactive substances (probiotics, FA and EO) increased ($P < 0.001$) the villus width in the duodenum, however, duodenal villus width was reduced ($P < 0.001$) by HS. The *in ovo* + in-water treatment also increased ($P = 0.008$) villus height to crypt depth ratio in the duodenum, compared to the antibiotics and NC treatment. In the jejunum, HS is observed to reduce ($P < 0.001$) villus height, width, and crypt depth. Conversely, the *in ovo* + in-water EO treatment consistently increased ($P < 0.001$) villus height and width in the jejunum, compared to the antibiotics and NC treatments. Other treatments recorded statistically intermediate villus height and width values in the jejunum. Similarly, asides the clear reduction ($P = 0.001$) in crypt depth exhibited by the antibiotics treatment when compared to the NC treatment, every other treatment had statistically similar crypt depth values in the jejunum. Only treatment differences were observed in the ileum, with the *in ovo* + in-water treatment increasing ($P < 0.001$) villus height and villus height to crypt depth ratio in the ileum, compared to all other treatments. The ileal crypt depth was reduced ($P = 0.003$) by both the *in ovo* + in-water and probiotics treatment compared to the NC treatment.

Table 8.9 Effect of *in ovo* delivered folic acid, probiotics and *in ovo* + in-water essential oil and temperature on broiler chicken's gut morphology.

Main effects		Duodenum				Jejunum				Ileum			
		Villus height (mm)	Villus width (mm)	Crypt depth (mm)	Villus height: Crypt depth	Villus height (mm	Villus width (mm)	Crypt depth (mm)	Villus height: Crypt depth	Villus height (mm)	Villus width (mm)	Crypt depth (mm)	Villus height: Crypt depth
Treatments[1]	Negative Control	1.18	0.14^{b}	0.12	9.48^{b}	0.64^{b}	0.15^{bc}	0.11^{a}	5.63^{b}	0.36^{c}	0.14	0.10^{a}	$3.72^{c} \pm 0.14$
	Antibiotics	1.17	0.14^{b}	0.11	10.1^{ab}	0.59^{b}	0.14^{c}	0.09^{b}	6.94^{a}	0.41^{b}	0.14	0.09^{ab}	4.74^{b}
	In ovo folic acid	1.18	0.16^{a}	0.12	9.73^{b}	0.74^{a}	0.17^{ab}	0.10^{ab}	7.40^{a}	0.38^{bc}	0.15	0.09^{ab}	4.39^{b}
	In ovo probiotics	1.17	0.16^{a}	0.12	9.74^{ab}	0.6^{ab}	0.16^{abc}	0.10^{ab}	6.71^{ab}	0.37^{c}	0.15	0.08^{b}	4.49^{b}
	In ovo + in-water EO	1.22	0.16^{a}	0.11	10.9^{a}	$0.75^{a} \pm$	0.18^{a}	0.10^{ab}	7.81^{a}	0.45^{a}	0.16	0.08^{b}	5.64^{a}
	SEM	0.01	0.01	0.004	0.13	0.01	0.004	0.003	0.41	0.004	0.002	0.003	0.10
Temperature[2]	TN	1.19 ± 0.01	$0.16^{a} \pm 0.009$	0.12 ± 0.01	10.12 ± 0.24	$0.77^{a} \pm 0.02$	$0.17^{a} \pm 0.01$	$0.11^{a} \pm 0.01$	7.10 ± 0.31	0.40 ± 0.01	0.15 ± 0.003	0.09 ± 0.005	4.45 ± 0.12
	HS	1.18 ± 0.01	$0.15^{b} \pm 0.01$	0.12 ± 0.01	9.84 ± 0.32	$0.60^{b} \pm 0.01$	$0.15^{b} \pm 0.005$	$0.09^{b} \pm 0.003$	6.63 ± 0.17	0.39 ± 0.01	0.15 ± 0.003	0.08 ± 0.004	4.68 ± 0.17
Treatments	Temperature												
Negative Control	Negative Control	1.20 ± 0.03	0.15 ± 0.01	0.12 ± 0.01	9.78 ± 0.42	0.75 ± 0.05	0.17 ± 0.01	0.12 ± 0.02	6.10 ± 0.32	0.36 ± 0.01	0.13 ± 0.005	0.10 ± 0.02	3.55 ± 0.19
	HS	1.17 ± 0.02	0.14 ± 0.004	0.13 ± 0.02	9.19 ± 0.40	0.55 ± 0.02	0.14 ± 0.02	0.11 ± 0.01	5.20 ± 0.35	0.36 ± 0.01	0.14 ± 0.008	0.09 ± 0.02	3.91 ± 0.21

Antibiotics	Antibiotics	1.20 ± 0.03	0.15 ± 0.02	0.12 ± 0.005	10.1 ± 0.34	0.65 ± 0.04	0.14 ± 0.02	0.09 ± 0.01	7.12 ± 0.33	0.40 ± 0.01	0.14 ± 0.007	0.09 ± 0.02	4.59 ± 0.22
	HS	1.14 ± 0.02	0.14 ± 0.006	0.11 ± 0.005	10.1 ± 0.55	0.53 ± 0.02	0.14 ± 0.01	0.08 ± 0.01	6.77 ± 0.33	0.42 ± 0.01	0.14 ± 0.006	0.09 ± 0.003	4.90 ± 0.51
In ovo folic acid	*In ovo* folic acid	1.16 ± 0.02	0.16 ± 0.01	0.13 ± 0.005	9.13 ± 0.36	0.88 ± 0.05	0.19 ± 0.01	0.11 ± 0.01	7.12 ± 1.06	0.38 ± 0.01	0.15 ± 0.008	0.09 ± 0.004	4.20 ± 0.20
	HS	1.20 ± 0.02	0.15 ± 0.02	0.12 ± 0.02	10.3 ± 0.61	0.62 ± 0.03	0.15 ± 0.01	0.09 ± 0.004	6.97 ± 0.31	0.39 ± 0.01	0.15 ± 0.007	0.08 ± 0.003	4.59 ± 0.51
In ovo probiotics	*In ovo* probiotics	1.15 ± 0.02	0.18 ± 0.008	0.11 ± 0.03	9.94 ± 0.41	0.77 ± 0.05	0.18 ± 0.01	0.12 ± 0.02	7.85 ± 0.36	0.36 ± 0.01	0.15 ± 0.007	0.08 ± 0.003	4.56 ± 0.36
	HS	1.19 ± 0.04	0.15 ± 0.02	0.12 ± 0.005	9.55 ± 0.45	0.59 ± 0.01	0.14 ± 0.01	0.09 ± 0.005	6.81 ± 0.60	0.38 ± 0.01	0.16 ± 0.007	0.09 ± 0.003	4.42 ± 0.21
In ovo + in-water EO	*In ovo* + in-water EO	1.22 ± 0.02	0.18 ± 0.03	0.12 ± 0.01	10.3 ± 0.81	0.80 ± 0.05	0.19 ± 0.01	0.10 ± 0.01	8.02 ± 0.89	0.44 ± 0.01	0.15 ± 0.008	0.08 ± 0.003	5.53 ± 0.23
	HS	1.22 ± 0.03	0.15 ± 0.04	0.11 ± 0.004	11.4 ± 1.0	0.72 ± 0.02	0.17 ± 0.01	0.09 ± 0.004	7.61 ± 0.37	0.46 ± 0.01	0.16 ± 0.009	0.08 ± 0.003	5.74 ± 0.28
***p* value[3]**													
Treatments		0.232	<0.001	0.073	0.008	<0.001	<0.001	0.001	<0.001	<0.001	0.061	0.003	<0.001
Temperature		0.913	<0.001	0.222	0.296	<0.001	<0.001	<0.001	0.105	0.127	0.277	0.619	0.134
Treatments* Temperature		0.197	0.599	0.076	0.083	0.114	0.383	0.337	0.693	0.982	0.659	0.531	0.773

[1]Treatments groups include- Negative control treatment- chicks were offered a basal corn-soybean meal-wheat–based diet, Antibiotics treatment- chicks were offered NC + 0.05% bacitracin methylene disalicylate, *In ovo* folic acid (FA) treatment- eggs injected with 0.1 mL FA (FA; 95-102%; Thermo Scientific, USA) at 0.15 mg per egg, *In ovo* probiotics treatment- eggs injected with 0.2 mL of *Bacillus subtilis* fermentation extract, each egg received 10×10^6 CFU of the bacterium/0.2 mL saline diluent, and *In ovo* + in-water essential oil (EO) treatment- eggs injected with 0.2 mL of a saline + EO blend mixture at a dilution ratio of 2:1 and supplied the EO via the in-water route at the recommended dosage of 250 mL/1000 L of drinking water. [2]Temperature model include- Thermoneutral group (T)- birds were housed at 24ºC ± 0.2 from d 21 – 28, and Heat stress group (H)-birds were housed at 31ºC for 8hrs/day from d 21 – 28. [3]Means that do not share the same superscript are significantly different.

8.4.1.8 Gene expression analysis

8.4.1.8.1 Hepatic expression of cellular stress proteins (Heat Shock Proteins-HSP)

Of the two cellular stress proteins (HSP70 and HSP90) evaluated, significant differences were only recorded for the relative expression of HSP70 in the liver of birds in this study (Figure 8.3 a and b). HS increased ($P < 0.01$) the relative mRNA expression of HSP70. With regards to treatment effect, the *in ovo* FA and probiotics treatment both caused higher ($P < 0.01$) HSP70 expression only compared to the NC treatment; other treatments had statistically moderate expression of this stress protein (data not shown).

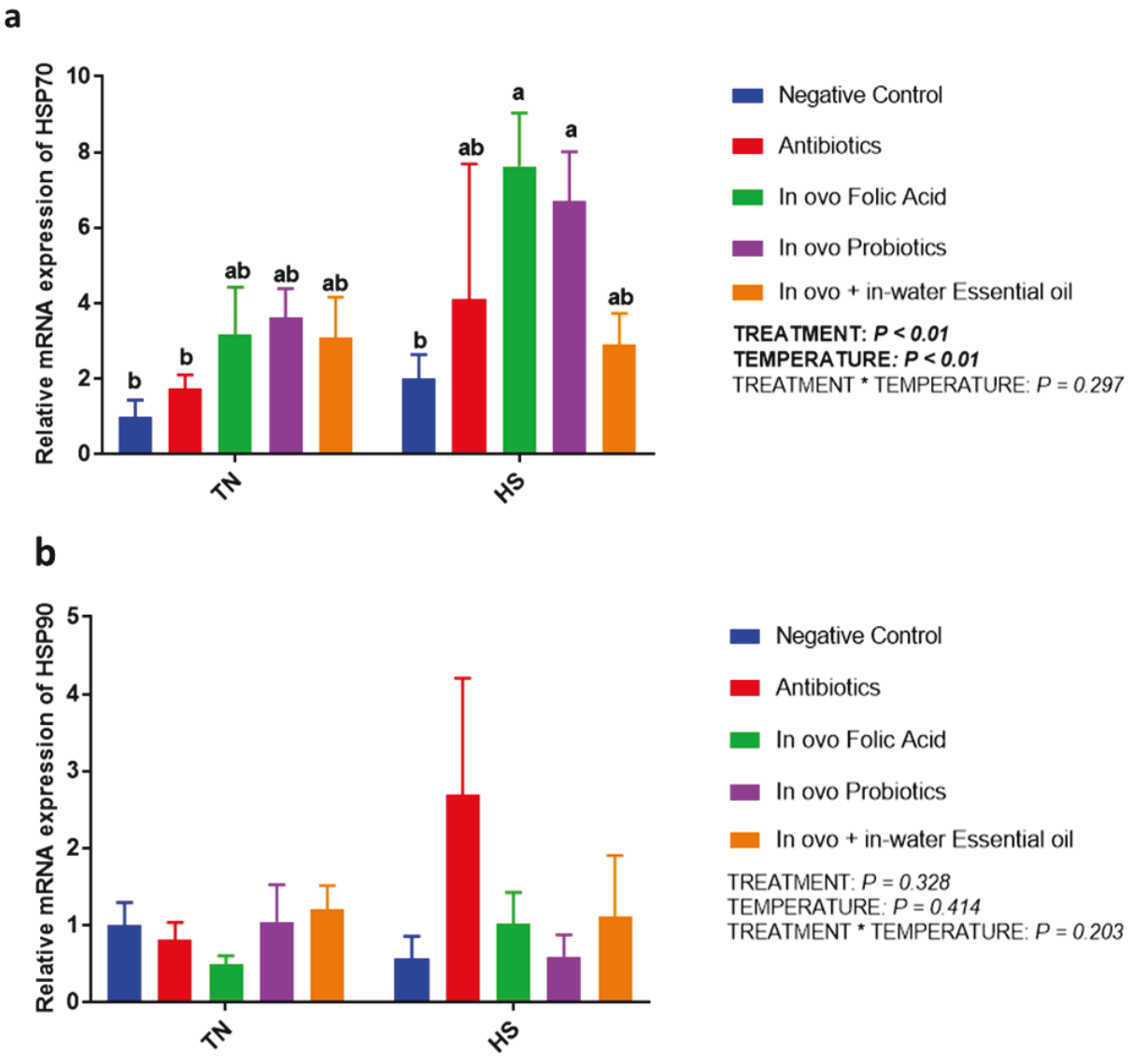


Figure 8.3 Hepatic heat shock proteins (HSP) expression.

Birds were allocated to treatments groups- Negative control treatment- chicks were offered

a basal corn-soybean meal-wheat–based diet, Antibiotics treatment- chicks were offered NC + 0.05% bacitracin methylene disalicylate, *In ovo* folic acid (FA) treatment- eggs injected with 0.1 mL FA (FA; 95-102%; Thermo Scientific, USA) at 0.15 mg per egg, *In ovo* probiotics treatment- eggs injected with 0.2 mL of *Bacillus subtilis* fermentation extract, each egg received 10 × 106 CFU of the bacterium/0.2 mL saline diluent, and *In ovo* + in-water essential oil (EO) treatment- eggs injected with 0.2 mL of a saline + EO blend mixture at a dilution ratio of 2:1 and supplied the EO via the in-water route at the recommended dosage of 250 mL/1000 L of drinking water. Birds were allotted to either a thermoneutral group (T)- birds were housed at 24ºC ± 0.2 from d 21 – 28, or a Heat stress group (H)-birds were housed at 31ºC for 8hrs/day from d 21 – 28.

8.4.1.8.2 Hepatic expression of lipogenic genes & their related transcription factors

Treatment, temperature model and their interaction had no effect on the relative mRNA expression of SCD (Stearoyl-CoA Desaturase), FAS (Fas Cell Surface Death Receptor), SBREP1, SBREP2 (Sterol-regulatory element binding proteins) and SCAP (SREBP cleavage activating protein) genes in the liver of broiler chickens in this study (Figure 8.4).

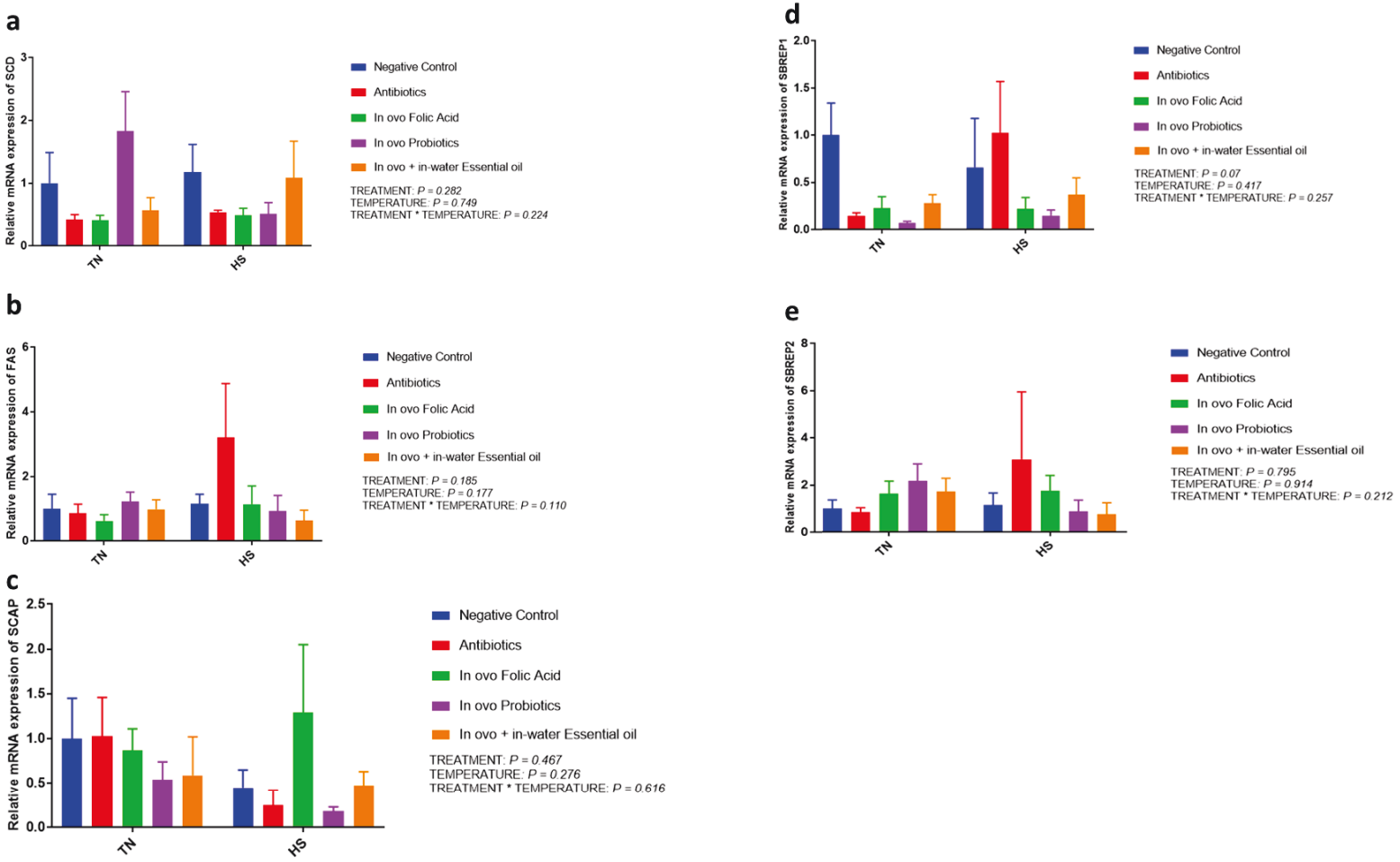


Figure 8.4 Expression of hepatic lipogenic genes and their related transcription factors.

Birds were allocated to treatments groups- Negative control treatment- chicks were offered a basal corn-soybean meal-wheat–based diet, Antibiotics treatment- chicks were offered NC + 0.05% bacitracin methylene disalicylate, *In ovo* folic acid (FA) treatment- eggs injected with 0.1 mL FA (FA; 95-102%; Thermo Scientific, USA) at 0.15 mg per egg, *In ovo* probiotics

treatment- eggs injected with 0.2 mL of *Bacillus subtilis* fermentation extract, each egg received 10×10^6 CFU of the bacterium/0.2 mL saline diluent, and *In ovo* + in-water essential oil (EO) treatment- eggs injected with 0.2 mL of a saline + EO blend mixture at a dilution ratio of 2:1 and supplied the EO via the in-water route at the recommended dosage of 250 mL/1000 L of drinking water. Birds were allotted to either a thermoneutral group (T)- birds were housed at 24ºC ± 0.2 from d 21 – 28, or a Heat stress group (H)-birds were housed at 31ºC for 8hrs/day from d 21 – 28.

8.4.1.8.3 Splenic expression of immune-related genes

Figure 8.5 shows the result obtained for all immune-related genes in the spleen. Only treatment differences were detected for IL-2 (interleukin 2) expression in the spleen. The *in ovo* FA treatment had higher ($P < 0.05$) expression of IL-2, only compared to the NC treatment, the expression levels of other treatments for this gene were statistically intermediate. An interaction of the main effects was observed for IL-4 (interleukin 4) expression. While HS increased ($P < 0.001$) the relative expression of this gene, the *in ovo* probiotics and *in ovo* + in-water EO treatment recorded higher ($P < 0.05$) expression of this gene under HS compared to TN conditions. Similarly, HS increased ($P < 0.01$) the relative expression of the IL-6 (interleukin 4) gene; however, the *in ovo* FA treatment recorded higher ($P < 0.01$) expression of this gene compared to other treatments (with the exception of the *in ovo* probiotic treatment), irrespective of the thermal challenge model. The expression levels for IFN-γ (Interferon-gamma) and IL-12B (interleukin 12B) genes were also increased by HS in this study. The relative expression of IL-1β (interleukin 1β) and IL-12A (interleukin 12A) were not affected by treatments, temperature, nor their interaction in this study.

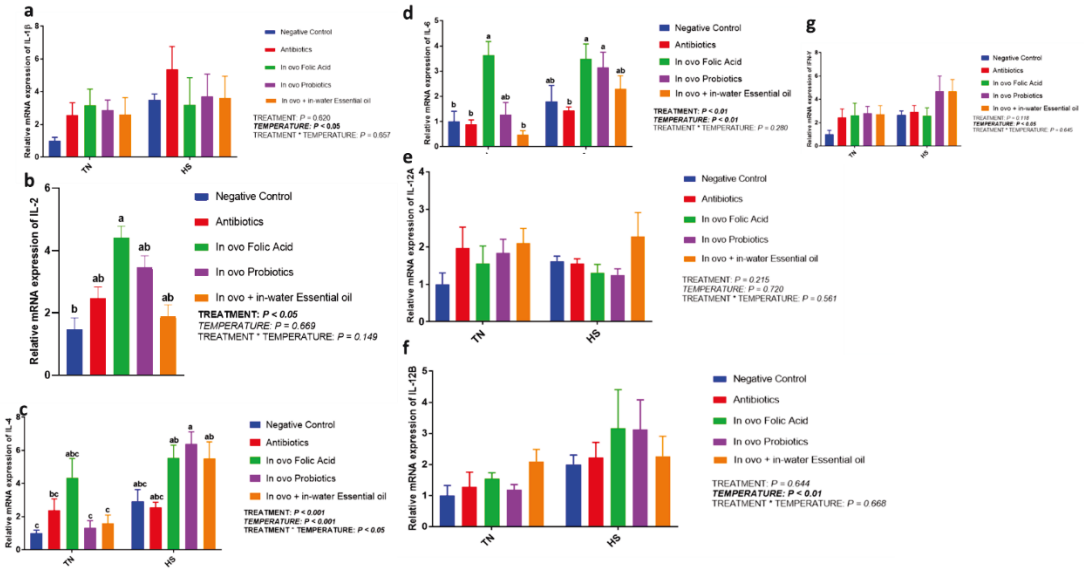


Figure 8.5 Expression of immune related genes in the spleen.

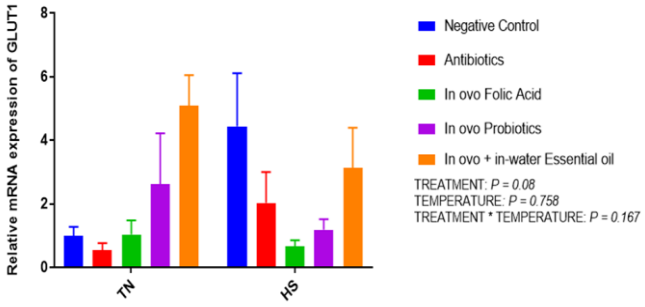
Birds were allocated to treatments groups- Negative control treatment- chicks were offered a basal corn-soybean meal-wheat–based diet, Antibiotics treatment- chicks were offered NC + 0.05% bacitracin methylene disalicylate, *In ovo* folic acid (FA) treatment- eggs injected with 0.1 mL FA (FA; 95-102%; Thermo Scientific, USA) at 0.15 mg per egg, *In ovo* probiotics treatment-

eggs injected with 0.2 mL of *Bacillus subtilis* fermentation extract, each egg received 10×10^6 CFU of the bacterium/0.2 mL saline diluent, and *In ovo* + in-water essential oil (EO) treatment-eggs injected with 0.2 mL of a saline + EO blend mixture at a dilution ratio of 2:1 and supplied the EO via the in-water route at the recommended dosage of 250 mL/1000 L of drinking water. Birds were allotted to either a thermoneutral group (T) - birds were housed at 24ºC ± 0.2 from d 21 – 28, or a Heat stress group (H)-birds were housed at 31ºC for 8hrs/day from d 21 – 28.

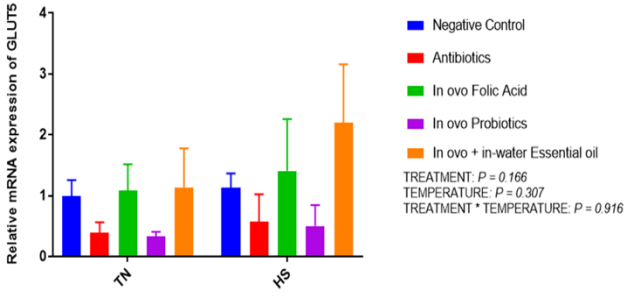
8.4.1.8.4 Expression of jejunal Glucose transporters genes

In this investigation, there were no differences in the relative mRNA expression of the evaluated glucose transporter genes (GLUT1- glucose transporter protein type 1; GLUT2- Glucose Transporter Type 2, and GLUT5- fructose transporter) in the jejunum (Figure 8.6).

a *p value*


Relative mRNA expression of GLUT1
0
2
4
6
8
TN
HS
Negative Control
Antibiotics
In ovo Folic Acid
In ovo Probiotics
In ovo + in-water Essential oil
TREATMENT: P = 0.08
TEMPERATURE: P = 0.758
TREATMENT * TEMPERATURE: P = 0.167

c


Relative mRNA expression of GLUT5
0
1
2
3
4
TN
HS
Negative Control
Antibiotics
In ovo Folic Acid
In ovo Probiotics
In ovo + in-water Essential oil
TREATMENT: P = 0.166
TEMPERATURE: P = 0.307
TREATMENT * TEMPERATURE: P = 0.916

b

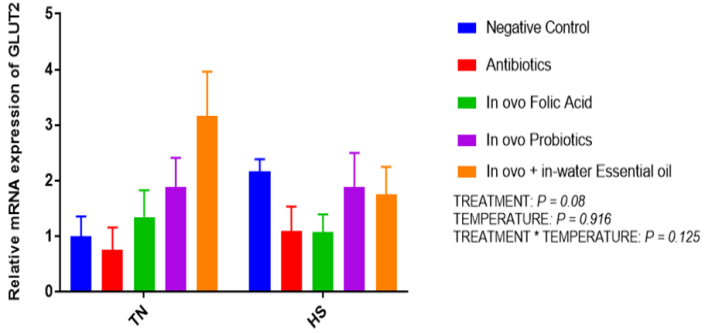

Relative mRNA expression of GLUT2
0
1
2
3
4
5
TN
HS
Negative Control
Antibiotics
In ovo Folic Acid
In ovo Probiotics
In ovo + in-water Essential oil
TREATMENT: P = 0.08
TEMPERATURE: P = 0.916
TREATMENT * TEMPERATURE: P = 0.125

Figure 8.6 Expression of glucose transporter genes in the jejunum.

Birds were allocated to treatments groups- Negative control treatment- chicks were offered a basal corn-soybean meal-wheat–based diet, Antibiotics treatment- chicks were offered NC + 0.05% bacitracin methylene disalicylate, *In ovo* folic acid (FA) treatment- eggs injected with 0.1 mL FA (FA; 95-102%; Thermo Scientific, USA) at 0.15 mg per egg, *In ovo* probiotics treatment- eggs injected with 0.2 mL of *Bacillus subtilis* fermentation extract, each egg received 10 × 106 CFU of the bacterium/0.2 mL saline diluent, and *In ovo* + in-water essential oil (EO) treatment- eggs injected with 0.2 mL of a saline + EO blend mixture at a dilution ratio of 2:1 and supplied the EO via the in-water route at the recommended dosage of 250 mL/1000 L of drinking water. Birds were allotted to either a thermoneutral group (T)- birds were housed at 24ºC ± 0.2 from d 21 – 28, or a Heat stress group (H)-birds were housed at 31ºC for 8hrs/day from d 21 – 28.

8.4.1.8.5 Expression of Jejunal amino acid transporters

The results of the relative mRNA expression of evaluated amino acid transporter genes (SLC1A1- Excitatory amino acid transporter 3 and SLC15A1- Peptide transporter 1) in the jejunum are detailed in Figure 8.7. HS increased ($P < 0.001$) the relative expression of the SLC1A1 gene. Interestingly, the *in ovo* + in-water EO treatment yielded the highest ($P > 0.05$) expression of this gene under HS condition; this was as much as a 3.5-fold increase ($P < 0.05$) when compared to the NC treatment. HS only showed a marginal tendency ($P = 0.06$) to increase the relative expression of the SLC15A1 gene.

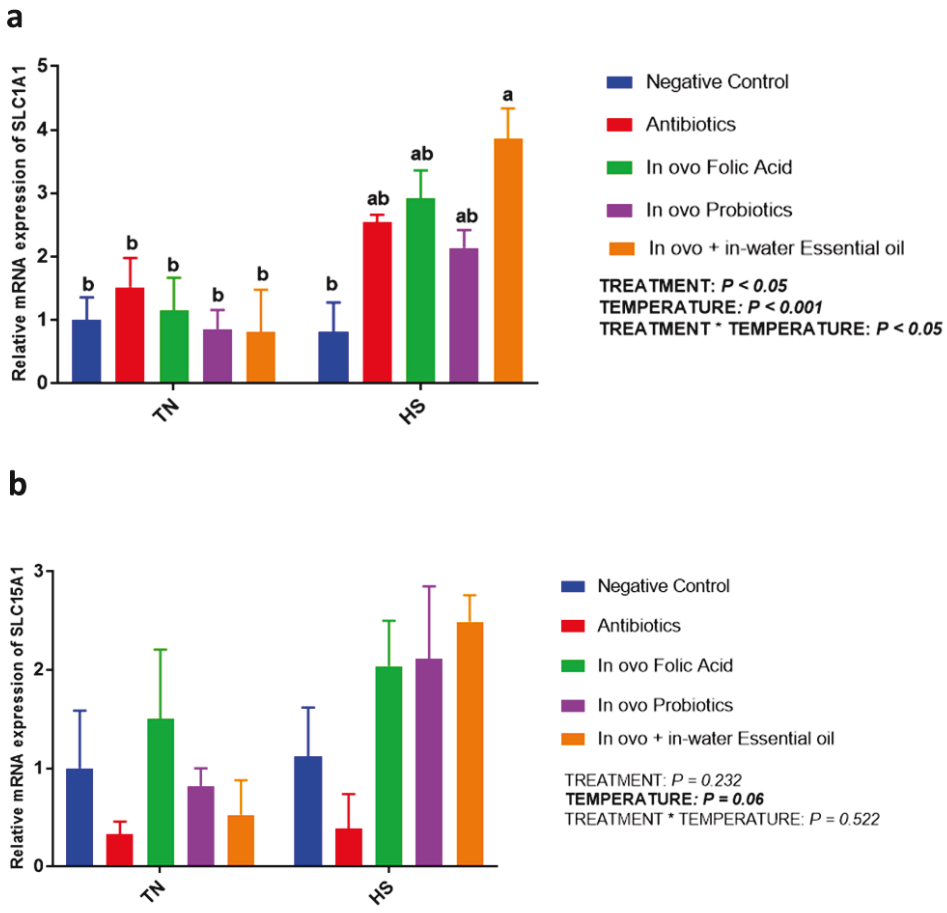


Figure 8.7 Expression of amino acid transporter genes in the jejunum.

Birds were allocated to treatments groups- Negative control treatment- chicks were offered a basal corn-soybean meal-wheat–based diet, Antibiotics treatment- chicks were offered NC + 0.05% bacitracin methylene disalicylate, *In ovo* folic acid (FA) treatment- eggs injected with 0.1 mL FA (FA; 95-102%; Thermo Scientific, USA) at 0.15 mg per egg, *In ovo* probiotics treatment- eggs injected with 0.2 mL of *Bacillus subtilis* fermentation extract, each egg received 10×10^6 CFU of the bacterium/0.2 mL saline diluent, and *In ovo* + in-water essential oil (EO) treatment- eggs injected with 0.2 mL of a saline + EO blend mixture at a dilution ratio of 2:1 and supplied the EO via the in-water route at the recommended dosage of 250

mL/1000 L of drinking water._Birds were allotted to either a thermoneutral group (T) - birds were housed at 24ºC ± 0.2 from d 21 – 28, or a Heat stress group (H)- birds were housed at 31ºC for 8hrs/day from d 21 – 28.

8.4.1.8.6 Jejunal intestinal barrier-related genes

Figure 8.8 shows the results of evaluated intestinal barrier-related genes in the jejunum. Compared to other treatments, the *in ovo* + in-water EO treatment consistently recorded higher ($P < 0.001$) expression of CLDN1 (Claudin 1) and CLDN3 (Claudin 3) genes in the jejunum. This increase was at least 4-fold in both cases. Interaction effect for both considered factors (*in ovo* treatments and temperature model) was also observed for CLDN4 (Claudin 4), OCLN (Occludin), ZO-2 (Zonula Occludens Protein 2) and MUC2 (Mucin 2) genes. As much as a 3-fold increase ($P < 0.001$) in the relative expression of CLDN4, ZO-2, and MUC2 genes in the ovo + in-water EO treatment is observed under TN condition compared to other treatments, however, the expression rate for these genes were unchanged under HS condition. Despite being non-significant, the relative expression of the MUC2 gene in the ovo + in-water EO treatment under HS condition was at least 17% higher ($P > 0.05$) than in other treatments. In a similar manner, the *in ovo* + in-water EO treatment also recorded a 2-fold increase ($P < 0.001$) in the relative expression of the OCLN gene under TN condition. Conversely, under HS condition, the antibiotic treatment recorded higher ($P < 0.001$) expression of the OCLN gene compared to the *in ovo* FA and NC treatment. The antibiotic, *in ovo* probiotics and the *in ovo* + in-water EO treatments all exhibited statistical similarity in the expression of the OCLN gene under HS condition. HS also reduced ($P \leq 0.05$) the relative expression of CLDN4, CLDN10 and MUC2 genes. The relative expression of ZO-1 (Zonula Occludens Protein 1) and ZO-3 (Zonula Occludens Protein 3) genes were not affected by treatments, temperature model, or their interaction in this study.

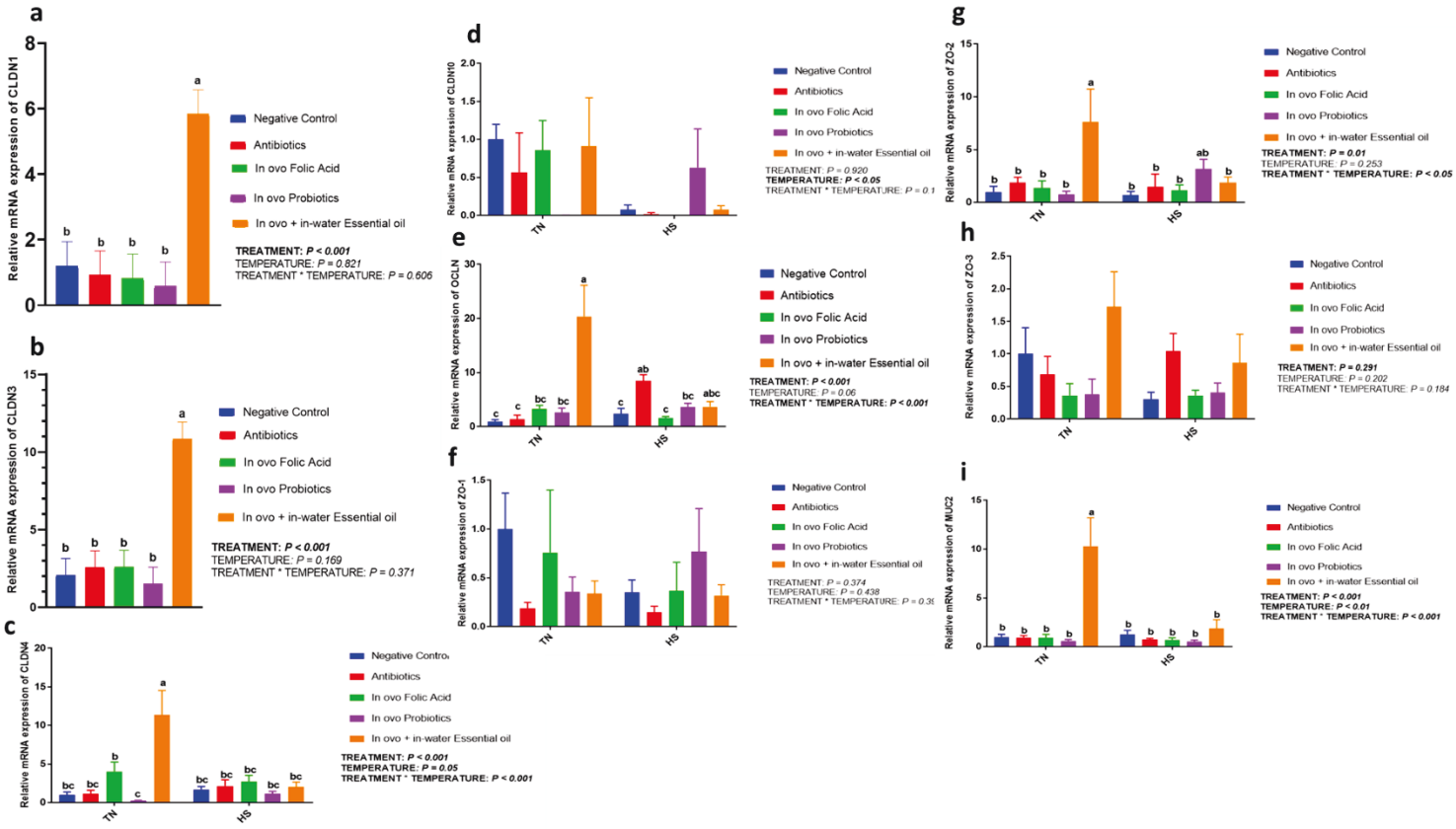


Figure 8.8 Expression of intestinal barrier-related genes in the jejunum.

Birds were allocated to treatments groups- Negative control treatment- chicks were offered a basal corn-soybean meal-wheat–based diet, Antibiotics treatment- chicks were offered NC + 0.05% bacitracin methylene disalicylate, *In ovo* folic acid (FA) treatment- eggs injected with 0.1 mL FA (FA; 95-102%; Thermo Scientific, USA) at 0.15 mg per egg, *In ovo* probiotics treatment- eggs injected with 0.2 mL of *Bacillus subtilis* fermentation extract, each egg received 10×10^6 CFU of the bacterium/0.2 mL saline diluent, and *In ovo* + in-water essential oil (EO) treatment- eggs injected with 0.2 mL of a saline + EO blend mixture at a dilution ratio of 2:1 and supplied the EO via the in-water route at the

recommended dosage of 250 mL/1000 L of drinking water. Birds were allotted to either a thermoneutral group (T) - birds were housed at 24ºC ± 0.2 from d 21 – 28, or a Heat stress group (H)-birds were housed at 31ºC for 8hrs/day from d 21 – 28.

8.5 Discussion

The challenges resulting from heat-induced hyperthermia in poultry which includes metabolic disorders, nutrient malabsorption, compromised gut health, immune dysregulation, oxidative stress, and poor growth performance, have made the need to combat HS a top priority for the global poultry industry. By utilizing a nutrigenomic approach, this study investigated the HS mitigating potential of three distinct *in ovo* delivered bioactive substances (probiotics, FA, and EO) optimized from previous independent studies (Oladokun et al., 2021a, 2021b; Oladokun and Adewole, 2022a). Data from this study provides preliminary evidence suggesting that successive delivery of EO blend (containing phytonutrients star anise, cinnamon, rosemary, and thyme) via *in ovo* and in-water routes might afford broiler chickens thermotolerance, especially via the regulation of amino acids transporter gene.

Of the three *in ovo* delivered bioactive substances in this study, only the probiotics recorded positive hatchability outcomes. Both *in ovo* delivered FA and EO recorded reduced hatchability. These hatchability outcomes are consistent with our previous report for the same *in ovo* delivered bioactive substances (Oladokun et al., 2021a, 2021b; Oladokun and Adewole, 2022a). Our laboratory has successfully validated the procedure for the *in ovo* delivery of probiotics (*Bacillus subtilis*), recording ~91 and ~ 96% hatchability rates in two different experiments (Oladokun et al., 2021a; Oladokun and Adewole, 2022a). Reported results on hatchability of *in ovo* delivered FA and EO, are quite variable in the few available literature. Increased (Gouda et al., 2021, 2022), decreased (Abdel-Halim et al., 2020; Abdel-Fattah and Shourrap, 2013) or unchanged hatchability values have been reported for *in ovo* delivered FA (Nouri et al., 2018; Ismail et al., 2019). Similarly, the few available results on *in ovo* delivered EO have also reported increased (Nadia et al., 2008; Sulaiman and Tayeb, 2020) or no effect of *in ovo* delivery of EO on hatchability outcomes (Saki and Salary, 2015; Toosi et al., 2016). Conceptually, the *in ovo* delivery of antioxidant-rich bioactive substances like FA and EO is expected to induce improved hatchability rates by abating free radical production (Nadia et al., 2008). Nonetheless, across the literature, it is apparent that asides the type of bioactive substances, other factors related to injected dose, volume, time and form of bioactive substances could also potentially influence hatchability outcomes (Oladokun and Adewole, 2020). We have previously reiterated that the forms of both *in ovo* delivered bioactive substances could be critical factors that influenced the observed hatchability outcomes (Oladokun et al., 2021b; Oladokun and Adewole, 2022a). While FA in its

powder form was utilized in this study, further studies are enjoined to consider evaluating liquid FA forms, if available, to ensure easy embryo absorption and utilization. The *in ovo* delivered EO blend was also formulated for in-water delivery; hence it contained emulsifiers. It would be necessary for other studies to utilize EO blends strictly formulated for *in ovo* delivery, if available. Furthermore, the *in ovo* probiotics and EO treatments recorded higher average chick navel score values (1.60 and 1.64) compared to the non-injected eggs (1.32), all modest average navel scores. Insignificant effects of this parameter were observed in previous studies involving these bioactive substances (Oladokun et al., 2021a, 2021b; Oladokun and Adewole, 2022a). The determinants of chick navel quality include the length of egg storage, age of breeder flock, health status of breeder flock, yolk absorption rate, incubation conditions (CO_2 concentration, temperature, and post-hatch conditions (access to feed and water) (Hamburger and Hamilton, 1951; Maatjens et al., 2014; Van Der Pol et al., 2014; Narinç and Aydemir, 2021). As all our injected eggs were obtained from the same source and subjected to similar conditions, none of these factors could possibly explain the observed results. Nonetheless, it is important to state that chick navel scoring is quite subjective and based on scorer's experience. To limit variability due to this subjectivity, only one experienced scorer was utilized in this experiment. Poor chick navel score has previously been correlated with poor chick post-hatch performance (Fasenko and O'Dea, 2008). However, recent studies have reported even improved post-hatch performance from chicks with poor navel scores (De Jong et al., 2019; de Jong et al., 2020; Heijmans et al., 2022), a trend consistent with this study. These reports suggest that chick navel scores should probably not be used as absolute indicators of chick quality but, at best, to complement other chick quality metrics.

In terms of growth performance, the antibiotics treatment is observed to improve ABWG at the starter phase (d1-14) and ABWG and FCR at d21-28 period, respectively, all in the absence of HS. This was not surprising, as the growth-promoting effects of antibiotics in poultry production are well substantiated across the literature (Engberg et al., 2000; Singh et al., 2000; Miles et al., 2006; Manafi et al., 2017). Consistent with the results presented here, other studies utilizing BMD, a common antibiotic used in poultry, have also reported improved ABWG and FCR in broiler chickens (Singh et al., 2000; Raza et al., 2019; Park et al., 2020; Li et al., 2022). Antibiotics are theorized to confer this growth-enhancing benefits by reducing growth-depressing microbial metabolites and increasing nutrient utilization efficiency (Reddy, 2011). Interestingly, the *in ovo* + in-water EO treatment afforded similar growth performance benefits (in terms of ABWG and

FCR) as the antibiotic treatment during the same period. In agreement with reported results, a few studies have also reported similar growth-enhancing capacity between antibiotics and EO blends under TN condition (Murugesan et al., 2015; Barbarestani et al., 2020). The antimicrobial, anti-inflammatory, antioxidant, and digestibility-enhancing properties inherent in the active components of most EO blends are deemed responsible for their growth-enhancing properties (Amad et al., 2011; Christaki et al., 2011; Feizi et al., 2014). Nonetheless, the growth-promoting properties of EO blends are reported to be variable depending on inclusion levels, bird age, and physiological and environmental conditions (Yang et al., 2009; Zeng et al., 2015).

Furthermore, an environmental (HS) challenge model was successfully validated in this study, as revealed by obtained results on rectal temperature measurements, specific blood biochemistry and antioxidant indices and molecular HS biomarkers. Considering that an increase in body temperature is a characteristic of homeotherms when exposed to temperature outside their thermoneutral zone, rectal temperature values are thus useful indicators of body temperature. The HS-challenged broiler chickens in this study consistently recorded significantly higher rectal temperatures across the four different time points evaluated. This observation is also true for other studies with effective HS regimes (Wan et al., 2017; He et al., 2019; Akinyemi and Adewole, 2022b). Under our experimental HS regime, ABWG and feed efficiency were reduced by HS. A plethora of studies has affirmed the negative effects of hyperthermia on growth performance indices, particularly ABWG and FCR in poultry (Farooqi et al., 2005; Azad et al., 2010; Quinteiro-Filho et al., 2010, 2012; Jahejo et al., 2016; Wen et al., 2019). A recent meta-analysis of 12 broiler chicken studies involving HS also concluded that HS significantly decreases ABWG and feed efficiency (Liu et al., 2020). A possible rationale for the hyperthermia-related growth depression observed includes increased energy devotion for physiological responses to HS (acclimation) at the expense of growth (Mujahid et al., 2007; Renaudeau et al., 2012). Intriguingly, under HS conditions, although insignificant, the *in ovo* + in-water EO treatment recorded the highest numerical improvement in ABWG, and feed efficiency compared to other treatments. The insignificant FCR values of the *in ovo* + in-water EO treatment were at least 19% lower than other treatments. The *in ovo* + in-water EO treatment FCR values were significantly lower than those of the *in ovo* probiotic treatment. As noted previously, the reported effects of EO blends in the literature are quite inconsistent, as EO bioactivities could be affected by bird age, host physiological status and administered dose (Zeng et al., 2015). While EO has been reported to

improve growth performance under HS condition (Ghazi et al., 2015; Saadat Shad et al., 2016), others have equally recorded no effect in poultry (Khosravinia, 2015; Büyükkılıç Beyzi et al., 2020). It is plausible that at higher dosages of the delivered EO blends, our observed beneficial result on growth performance could become more apparent.

Blood biochemical indices are typically valid predictors of general health, including responses to stress, as well as internal and external stimuli. Upon exposure to HS, the plasma levels of sodium, glucose, AST, albumin, globulin, and total protein were all reduced in broiler chickens in this study. Contrastingly, elevated levels of phosphorus and bile acids were detected in heat-challenged chickens. The reduction in plasma sodium and proteins were not unexpected and were in conformation with results reported in similar HS studies (Bonnet et al., 1997; Borges et al., 2004, 2007; Beckford et al., 2020; El-Gharib et al., 2021; Jimoh et al., 2022). While reduced sodium has been attributed to increased water consumption typical of heat-stressed birds (Borges et al., 2004), an increased rate of protein catabolism during HS triggers reduced protein deposition (Belhadj Slimen et al., 2016). Also higher blood levels of phosphorus reported by a few HS studies (Huang et al., 2018; Aslam et al., 2021) have been regarded to be an anti-stress defense mechanism triggered by glucocorticoid secretion (Weller et al., 2013). Notwithstanding, a few studies (Usayran et al., 2001; Rizk et al., 2017) have also reported decreased levels of blood phosphorus following HS, suggesting that the role of phosphorus in HS response is yet to be fully elucidated. Both AST and bile acid levels could be useful indicators of liver health. Presented values for both plasma metabolites were within published normal ranges for broiler chickens (Jaensch et al., 2000), suggesting that our HS regime did not cause hepatic damage. Our result on the effect of HS on blood glucose was more intriguing. Increased plasma glucose levels via hepatic gluconeogenesis triggered by corticosteroids secretion have been reported to be a neuroendocrine response to HS (Oladokun and Adewole, 2022b). This theory is well-buttressed across the literature (Qin et al., 2018; Chand et al., 2018; Ding et al., 2020a). However, McCormick et al. (1979) have previously reported that mild HS resulted in reduced blood glucose levels in non-fasted chickens compared to fasted ones. Wang et al. (2018a) have equally reported that Fayoumi chicken breeds respond uniquely to HS via blood glucose reduction compared to Leghorn breeds. The authors speculated that reducing blood glucose levels could be an adaptive physiological mechanism to "wait out" stress acquired by the Fayoumi breed. From the foregoing, it appears there could be a breed, HS intensity, and anorexic effect on blood glucose response to HS. More

studies are thus needed to provide clearer insight into these highlighted variables. Independent of HS, treatment effects on evaluated blood biochemical parameters were quite variable. The antibiotic treatment increased plasma levels of AST and A:G. This study provides another justification to encourage the discontinued use of antibiotics in poultry. While AST has been specified as a non-specific biomarker of liver health, reduced A:G has been correlated with an improved immune response (Dawson and Whittow, 2000). The use of several phytogenic bioactive substances, probiotics and the *in ovo* + in-water EO treatment have afforded reduced levels of both blood metabolites (Bityutskyy et al., 2019; Krauze et al., 2020; Oladokun et al., 2021b; Gazwi et al., 2022). The *in ovo* probiotics treatment recorded increased plasma levels of magnesium, phosphorus and reduced GGT levels. While magnesium plays a crucial role in ensuring immunocompetence (Sahin et al., 2005), phosphorus is involved in skeletal growth and energy production (Pond, 2005). Levels of both minerals have been reported to be increased following probiotics delivery (Capcarova et al., 2010). Blood GGT level is another useful index of liver function. Both probiotics and EO have been reported to reduce levels of this blood enzyme (Krauze et al., 2020). Plasma globulin levels were also increased by the *in ovo* FA and *in ovo* + in-water EO treatments. Globulins also play an important role in ensuring immunocompetence and maintaining liver function and has been reported to be enhanced by FA and EO supplementation (El-Gogary and El-Said, 2019; Rewatkar et al., 2019).

Furthermore, HS is known to undermine the redox status and induces oxidative damage in poultry (Zhang et al., 2018). Induced oxidative damage is often characterized by decreased levels of cellular antioxidant activities (SOD, TAC, etc.) and increased lipid peroxidation products (MDA). In the current study, HS decreased plasma levels of SOD and TAC alongside increased MDA levels. This result is consistent with reported results on HS in the literature (Ramnath et al., 2008; Xu et al., 2014; Safdari-Rostamabad et al., 2017; Xiong et al., 2020; Moustafa et al., 2021). Of all the treatments evaluated in this study, the *in ovo* probiotics treatments recorded the least TAC values, being significantly lower than those of the antibiotic treatment. Both enzymatic and non-enzymatic antioxidant defense systems are delineated by the TAC values. Considering the reported antioxidant properties of the other *in ovo* delivered treatments (FA and EO), this was not surprising (Büyükkılıç Beyzi et al., 2020; Gouda et al., 2020). Additionally, under HS conditions, the *in ovo* + in-water EO treatment recorded MDA levels that were at least 10% numerically lower than those of other *in ovo* treatments. Several studies have affirmed the antioxidants capacity of

EO either as a unit or blend (Montazeri et al., 2014; Ghazi et al., 2015; Yarmohammadi Barbarestani et al., 2020; Büyükkılıç Beyzi et al., 2020; Abdelnour et al., 2022)

The gastrointestinal tract (**GIT)** segments which are the main site of intestinal digestion and absorption, are considered highly vulnerable to HS, eliciting several adverse effects, including reduced villi height and increased crypt depth, resulting in reduced villi height:crypt depth (Liu et al., 2019; Song et al., 2019; Li et al., 2020). In this study, HS is seen to reduce duodenal villus width, as well as villus height and width in the jejunum. These morphometric and histopathological changes, possibly resulting from ischemia and hypoxia accompanied by epithelial shedding are consistent with other reports in heat-stressed birds (Burkholder et al., 2008; Silva et al., 2010; Deng et al., 2012; Ashraf et al., 2017; Li et al., 2020). Conversely, Quinteiro-Filho (2010) reported that HS (31 ± 1 and 36 ± 1°C/10 h per d) evoked no significant changes in villus and crypt morphology in broiler chickens. Fast intestinal mucosal re-epithelialization (usually occurring in less than 36 h after a stressful situation in chickens) might explain this insignificant effect (Burkholder et al., 2008). Although we recorded no significant interaction effects for our evaluated factors for this parameter, the *in ovo* + in-water EO treatment clearly increased villus height to crypt depth ratio across the three gut sections (duodenum, jejunum, and ileum), compared to the control treatment. This is to a great extent, consistent with our earlier report for the same treatment (Oladokun et al., 2021b). Several aromatic plants and their extracts are reported to enhance the intestinal morphology of broiler chickens owing to the antimicrobial, anti-inflammatory, and antioxidant properties of their bioactive components (Ding et al., 2020; Hong et al., 2012).

The cellular stress protein; HSP70 have been documented as a biomarker of HS in poultry (Oladokun and Adewole, 2022b). In fact, HSP70 has been reviewed to be the most relevant to thermal tolerance of all HSP (Oladokun and Adewole, 2022b). As a confirmation of induced HS, a corresponding increase in hepatic expression of HSP70 was recorded for the birds in the HS group. In terms of *in ovo* delivered treatments, the *in ovo* FA and probiotics treatments recorded higher expression of this gene, compared to the control treatment, with the *in ovo* + in-water EO treatment recording intermediate expression of this gene. Oladokun and Adewole (2022b) provided insight into the relevance of this gene, suggesting that it is indeed a functional measure of cell restoration against stressors, and its expression level might be inversely proportional to a bird's thermal tolerance. Several studies involving probiotics and EO delivery have reported significant downregulation of this gene in heat-stressed poultry and have related this to improved

thermo-protective capacities (Hosseini et al., 2016; Wang et al., 2018b; Zhang et al., 2017). Interestingly, the reported treatment effect on HSP70 expression in this study followed a similar trend for accumulated MDA under HS among the *in ovo* delivered treatments, suggesting that the *in ovo* + in-water EO treatment shows a potential to afford better thermo-protection than the other *in ovo* treatments.

A growing body of evidence affirms the immunomodulatory role of HS in poultry (Niu et al., 2009; Quinteiro-Filho et al., 2010; Song et al., 2017). In consonance with previous reports (Helwig and Leon, 2011; Ohtsu et al., 2015; Adu-Asiamah et al., 2021; Patra and Kar, 2021), here we report that HS induces the release of pro-inflammatory cytokines (IL-4, IL-6, IL-12B, and IFN-γ) and mediators in the spleen of broiler chickens. With regards to treatments, the *in ovo* FA group induced higher splenic expression of IL-2 and IL-6, at least compared to the control group. An interaction effect was also observed for the IL-4 cytokines, with the antibiotic treatment recording the lowest numerical expression of this cytokine under HS. Notwithstanding, amongst all *in ovo* treatments, the *in ovo* + in-water EO treatment recorded the lowest numerical expression of this cytokine under HS. The Pro-inflammatory cytokine family plays an active role in the inflammatory response under HS condition. A possible hypothesis is that inflammatory activities are triggered at the expense of a humoral immune response following an HS event. Considering that our ABWG values under HS follow similar trend as IL-4 expression under HS condition. It is reasonable to speculate that heat-triggered inflammatory responses occur at an energy expense detrimental to growth performance. In any case, given the importance of cytokines in immunological development, response, and cell communication (Giansanti et al., 2006), the exact mechanism bordering their modulation under HS requires further elucidation.

Furthermore, nutrient transporters are known to play a major role in the absorption of digestion products in the gut (Goodman, 2010). While HS is observed to increase the mRNA expression of the SLC1A1 gene, the *in ovo* + in-water EO treatment yielded the highest expression of this gene under HS condition; this was as much as a 3.5-fold increase compared to the control group. The SLC1A1 gene belongs to the glutamate transporter family and is actively absorbed in the ileum and jejunum (Tauqir, 2016). Cellular circulation of the SLC1A1 gene is reportedly involved in providing energy to the intestinal cells (Iwanaga et al., 2006). While a few studies have reported the downregulation of this gene under HS conditions (Jahromi et al., 2016; Orhan et al., 2020), others have also reported its upregulation (Santos et al., 2019; Ma et al., 2021). Despite this

disparity in results, most studies (Chuang et al., 2020; Orhan et al., 2020; Wassie et al., 2022) have been unanimous in their report that external supplementation of phytogenics are capable of inducing increased SLC1A1 expression as a result of the integral role it plays in providing energy needed for the maintenance of optimum gut permeability and health. While a complete understanding of HS-induced regulation of the SLC1A1 gene is yet to be known, other factors such as genetic selection and dietary protein source and quality could potentially influence its regulation (Gilbert et al., 2010). More studies are thus needed in this regard to provide clearer insights. In any case, our results clearly show that under HS condition the *in ovo* + in-water EO treatment could improve poultry gut health via upregulation of the SLC1A1 gene.

Additionally, a critical part of optimum gut health is the maintenance of intestinal integrity or barrier function. The intestinal mucosa is regarded as the first line of defense against ingested pathogens, such as *Salmonella spp.* (Fagarasan, 2006). Several poultry studies have shown that HS could compromise the integrity of the intestinal barrier by disrupting the tight junction proteins, leading to increased intestinal permeability, of which gut inflammation is an innate response (Quinteiro-Filho et al., 2010; Alhenaky et al., 2017; Ruff et al., 2020). Claudins and Occludins are functional components of the tight junctions. Similarly, mucins (denoted as MUC2) secreted by the intestinal goblet cells are an important component of the chemical barrier critical to ensuring optimum intestinal integrity. In this study, HS downregulated the mRNA expression CLDN4, CLDN10 and MUC2 in the jejunum of broiler chickens. Our results are in conformation with several other studies in the literature that revealed that hyperthermia compromises the integrity of tight junctions in the gut (Schneeberger and Lynch, 2004; Varasteh et al., 2015; Mohyuddin et al., 2021; Liu et al., 2022). Independent of HS, our results showed that the *in ovo* + in-water EO treatment increased the expression levels of CLDN1, CLDN3, CLDN4, OCLN, ZO-2, and MUC_2. This suggests that the successive delivery of this EO blend is capable of maintaining intestinal barrier integrity and barrier function via the assembly of tight junction proteins. Several other studies involving various phytogenics have reported similar result and attributed this beneficial effect to the antioxidant properties of delivered bioactive substances (Humam et al., 2021; Lin and Lee, 2021; Liu et al., 2021; Ruan et al., 2021; Khalid et al., 2022; Mao et al., 2022). Besides under HS condition, the *in ovo* + in-water EO treatment also recorded about 17% higher expression of the MUC_2 gene in the jejunum compared to other treatments, although this did not reach statistical significance. Perhaps at a higher dosage of the *in ovo* + in-water EO treatment this benefit might

become statistically evident.

8.6 Conclusions

Summarily results from this study showed that induced HS impaired broiler chicken's growth performance (ABWG: -16% and feed conversion efficiency: -15%), antioxidant status (SOD: -13%, TAC: -46%, MDA: +39%), immune status, gut morphology, and intestinal barrier integrity. Independent of HS, the *in ovo* + in-water EO treatment improved broiler chicken gut morphology and intestinal barrier integrity. However, compared to the control treatment and other treatments in most cases the *in ovo* + in-water delivery of the EO treatment to heat-stressed birds induced a numerical improvement in feed conversion efficiency (+30%) and as much as a 3.5-fold significant upregulation of amino acid transporter gene (SLC1A1). Subject to further optimization of the reduced hatchability results obtained for the *in ovo* delivered EO through further studies, the successive delivery of this EO blend via the *in ovo* and in-water routes could potentially provide solutions to two critical challenges facing the poultry industry- finding effective alternatives to antibiotic growth promoters and HS mitigation. Indeed, more studies are needed to optimize EO dosages that would guarantee the utmost benefits.

9 CHAPTER 9 *IN OVO* DELIVERED BIOACTIVE SUBSTANCES AND GUT MICROBIOTA UNDER HEAT STRESS

MICROBIOCENOSIS OF THE CHICKEN CECA: IMPACT OF *IN OVO* DELIVERED BIOACTIVE SUBSTANCES, HEAT STRESS, AND ANTIBIOTIC GROWTH PROMOTERS

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9.1 Abstract

Although, the chicken gut microbiota plays important roles in host physiology and well-being, it can be impacted by several management and environmental factors such as the use of antibiotic growth promoters (AGP) and exposure to heat stress (HS). Probiotics, essential oils (EO), and vitamins (such as folic acid) are bioactive substances that could potentially modulate the gut microbiota and promote its resilience to stressful environmental factors, especially when supplied as early as during embryonic development. Consequently, this study evaluated the gut microbiota modulating potential of *in ovo* delivered probiotics, folic acid (FA), and *in ovo* + in-water delivered EO in broiler chickens, as compared to an in-feed AGP, under an HS challenge condition.

The results revealed alpha diversity was not affected by treatments, HS, or their interaction. AGP-treated birds had distinct beta diversity measure compared to all other treatments and HS combinations. Regarding taxonomic composition, HS reduced the proportion of members of the *Actinobacteria* phylum, while AGP supplementation, especially under thermoneutral condition, increased the proportion of members of this phylum compared to all other treatments. The cumulative proportions of bacteria in the genera *lachnoclostridium and Sellimonas* were increased by the *in ovo* + in-water EO and AGP treatments. The AGP treatment also increased the cumulative proportions of bacteria in the genera *Blautia*, *Lachnospiraceae, Anaerostipes, lachnoclostridium, Sellimonas,* all in the *Lachnospiraceae* family, while reducing the proportion of bacteria in the genera *Faecalibacterium, Negativibacillus, Oscillibacter, and Romboutsia* in the ceca. On the other hand, HS reduced the proportion of bacteria in the genera *Ruminococcus,*

Eubacterium, Blautia, and *Candidatus Soleaferrea*. Metagenomic prediction revealed that the AGP treatment enriched carbohydrate degradation, carbohydrate synthesis, and vitamin and amino acid biosynthesis-related pathways. Similarly, HS increased the pyruvate fermentation to acetate and lactate II, and polyamine biosynthesis I metabolic pathways.

Results from this study indicate that HS, AGP, and *in ovo* + in-water EO modified chicken cecal bacterial populations and suggest that the microbiota-mediated role of AGP in growth promotion is related to improved biosynthesis of essential nutrients (amino acids and vitamins especially) and utilization of carbon sources derived from host diet and microbiome.

9.2 Introduction

Poultry accounted for approximately one-third of global meat production in 2020 and is currently projected to constitute 47% of the protein consumed from all meat sources in 2031 (FAO, 2019; OECD/FAO, 2022). It is increasingly evident that much of the success of the global poultry industry can be traced to the use of antibiotic growth promoters (**AGP**) (Swaggerty et al., 2022). The beneficial effect of AGP on poultry production is thought to be exerted by AGP's capacity to modulate the birds' gut microbiota in favor of less growth-depressing metabolites like ammonia and bile degradation products (Niewold, 2007; Swaggerty et al., 2022). Despite this benefit accruing from AGP use in poultry production, it comes with a cost of drug competition with humans, as well as the dual burdens of antibiotic resistance in poultry products. The poultry industry is thus saddled with the challenge of finding other sustainable modulators of the gut microbiota.

The chicken gut microbiota constitutes a complex and dynamic microbial community comprising > 900 bacteria species (Zhu et al., 2002; Borda-Molina et al., 2018). In fact, it has been reported that about 80% of the bacteria species in the chicken cecum are yet to be cultured (Oakley et al., 2013; Torok et al., 2008). However, with advancement in 16S rRNA gene sequencing, metagenomics, metatranscriptomics, and metabolomics technologies in recent years, our understanding of this 'organ' previously referred to as a 'silent organ', has been enormous. It is now clear that the gut microbiota plays a critical role in gut development, nutrient digestion, metabolism, absorption, competitive exclusion of pathogens, and immunity (Rinttilä and Apajalahti, 2013; Clavijo and Flórez, 2018; Aggeletopoulou et al., 2019). Indeed, "dysbiosis"- a term used to describe an imbalance in the gut microbial structure in favor of pathogenic microbes has been linked to the pathologies of infectious bronchitis virus (Xu et al., 2022), influenza

(Yitbarek et al., 2018), Marek's disease (Perumbakkam et al., 2014), coccidiosis (Chen et al., 2020), and necrotic enteritis in poultry (Kiu et al., 2019). By reviewing the interrelationship between poultry gut microbiota and chicken performance, Carrasco et al. (2019) were able to infer that bird productivity might be correlated with microbial diversity. Notwithstanding, such productivity correlation might indeed be taxa and gut section specific rather than contingent on overall diversity.

As the gut microbiota is constantly interacting with the host, it is not surprising that a myriad of host-linked and environmental factors can modulate the composition and structure of the gut microbiota. These factors will include the host's age, breed, sex, sampled gut section, diet (including antibiotic use), housing conditions, management system, biosecurity, environmental stressors, particularly heat stress (HS) (Kers et al., 2018; Rostagno, 2019). Of all dietary modulators of the gut microbiota, perhaps AGP use has been the most successful. Several studies allege to AGP gut modulating potential (Schokker et al., 2017; Wisselink et al., 2017; Zhou et al., 2020). In spite of the success recorded with AGP, the potential of AGP inducing dysbiosis by having a more significant effect on the host's commensal microbiota rather than pathogenic microbiota has also been reported (Kogut, 2019). Croswell et al. (2009) have also reported that microbiota alterations after AGP withdrawal may persist even after several weeks. Additionally, bacterial-induced mucosa inflammation and susceptibility to pathogenic microbiota colonization may persist when animals are infected post-AGP withdrawal (Croswell et al., 2009; Kogut, 2019). Furthermore, amongst all environmental factors capable of modulating the gut microbiota, HS has been reported to possess the most significant effect (Cao et al., 2021). Oladokun and Adewole (2022b) have reported that heat-induced dysbiosis is indeed one of the physiological responses to HS in poultry. Lower levels of *Lactobacillus* and *Bifidobacterium* and higher levels of *Clostridium* and total coliforms are reported examples of HS-induced microbiota alterations (Shi et al., 2019; Chen et al., 2022). In terms of phyla, reported HS-induced gut microbiota signatures include increased *Firmicutes*, *Tenericutes*, *Proteobacteria* and reduced *Bacteroidetes* and *Cyanobacteria*. Several other genera with reported growth and feed efficiency correlation, including *Acinetobacter, Bacteroides, Coprococcus, Dorea, Faecalibacterium, Lactobacillus,* and *Streptococcus* have been reported to have unique responses to HS (He et al., 2021). With poultry known to have poor thermoregulatory capacity and projected global temperature increase (Masson-Delmotte et al., 2021; Oladokun and Adewole, 2022b), it is thus important that sustainable and timely solutions are proffered to the challenge of HS-induced gut microbiota

perturbations in poultry.

Research into several sustainable ways to modulate the poultry gut microbiota, especially via the use of several feed additives like prebiotics, probiotics, synbiotics, enzymes, organic acids, and essential oils, are on the rise (Aggeletopoulou et al., 2019; Kogut, 2019; Yadav and Jha 2019). The use of probiotics (defined as "live microorganisms which, when administered in adequate amounts, confer a health benefit on the host" (FAO/WHO, 2001) shows promising gut-modulating potential. For instance, the supplementation of *Lactobacillus johnsonii*, *Lactobacillus acidophilus* and *Bacillus* have been reported to increase the prevalence of favourable lactic acid bacteria population in the gut, whilst also showing potential to alleviate the incidence of necrotic enteritis in broiler chickens (Li et al., 2017; Qing et al., 2017; Hernandez-Patlan et al., 2019a). Similarly, although varying results have been reported, the capacity of essential oils (**EO**) to favorably modulate the gut microbiota has also been reported (Cross et al., 2007; Yin et al., 2017; Yang et al., 2020). Oregano EO has been reported to reduce gut coliform counts compared to AGP-supplemented birds (Skoufos et al., 2016). Similarly, thymol supplementation has been reported to reduce Escherichia coli counts in the gut of broiler chickens (Jang et al., 2007). Furthermore, vitamins are another category of feed additives that show the potential to modulate the gut microbiota (Biesalski, 2016), although they are scarcely researched. For instance, Riccio and Rossano (Riccio and Rossano, 2018) have reported the capacity of vitamin A to modulate gut microbiota resulting in increased intestinal barrier integrity and immune status. Vitamin E, an antioxidant-rich vitamin, has also been shown to reduce total anaerobes (pathobionts) count compared to control birds (Scocco et al., 2017). Folic acid (**FA**) is another B-complex vitamin rich in antioxidant activity whose effect on gut microbiota is largely unreported in the literature. Despite the foregoing, it is important to state that inconsistencies in the activities of these additives continue to be recorded in the literature due to several reasons, including differences in dose, form, and delivery routes (Oladokun et al., 2021a; Oladokun et al., 2021b). Under current poultry production systems, conventional in-feed or in-water routes constitute the major routes for bioactive substance delivery. However, the efficacy of bioactive substances delivered via these routes might be impaired due to several reasons, including potential negative interaction with other feed additives, thermal instability, and potential water quality limitations (Gadde et al., 2017; Oladokun and Adewole, 2020).

In ovo technology has thus been proposed as one means to ensure the efficacy of these gut-modulating bioactive substances (Oladokun and Adewole, 2020). Considering that the hen, which

used to be the status quo source of gut colonization, has been eliminated in the commercial poultry industry, the *in ovo* technology offers the opportunity to colonize the embryonic gut with beneficial microbiota very early on. *In ovo* delivery of several bioactive substances, including phytobiotics, prebiotics and probiotics showing promising results as a gut-optimizing strategy in poultry production (Oladokun and Adewole, 2020). Through several independent studies (Oladokun et al., 2021a; Oladokun et al., 2021b; Oladokun et al., 2022), our laboratory has reported that *in ovo* delivery of a probiotic product (*Bacillus subtilis* fermentation extract), as well as *in ovo* + in-water delivery of a commercial EO blend enhanced broiler chicken gut morphology, without compromising growth performance and gut homeostasis.

Additionally, divergence in reported results is observed in several HS-related poultry studies in the literature, possibly due to the complexity of the gut microbiota and all possible confounding factors highlighted above that could influence gut structure and diversity (Oladokun and Adewole, 2022b). Nevertheless, it is obvious that more studies are needed to better delineate the complete mechanism (s) underlying HS-induced gut microbiota responses in poultry. Accordingly, this study sought to evaluate the gut microbiota modulating potential of *in ovo* delivered probiotics, FA, and *in ovo* + in-water delivered EO in broiler chickens, compared to an AGP (Bacitracin), under an HS challenge condition.

9.3 Methods

9.3.1 *In ovo* procedure

Hatching eggs (n = 1,252; average weight of 65.07 ± 0.30 g (mean ± SE)) were sourced from 34-week-old Cobb 500 broiler breeders from Synergy hatchery, Nova Scotia, Canada, and incubated in a ChickMaster single-stage incubator (ChickMaster G09, Cresskill, NJ, USA) at the Hatchery facility of Dalhousie Faculty of Agriculture for 21 days. Incubation conditions were standard (37.5 °C and 55% relative humidity) from embryonic days (**ED**) 1 to 17 and then to an average of 32 °C and 68% from EDs 18 to 21. Eggs were candled on ED 12, and infertile eggs were disposed of. The remaining eggs (n = 1,216) were then allotted one of five treatment groups: (1) non-injected eggs (control; 98 eggs); (2) *in ovo* saline group (50 eggs; injected with 0.2 mL of physiological saline, i.e., 0.9% NaCl, Baxter Corporation, ON, Canada); (3) *in ovo* FA group (50 eggs, injected with 0.1 mL FA (FA; 95-102%; Thermo Scientific, USA) at 0.15 mg per egg; (4) *in ovo* probiotics group (50 eggs, injected with 0.2 mL of *Bacillus subtilis* fermentation extract, each egg received 10×10^6 CFU of the bacterium/0.2 mL saline diluent); and (5) *in ovo* essential

oil group (50 eggs; injected with 0.2 mL of a saline + essential oil blend mixture at a dilution ratio of 2:1). All treatments were replicated in four similar incubators operated under the same condition. FA were injected on ED 12, while the probiotics and essential oil group were injected on ED 18 following methods described by Oladokun et al. (2021a). Briefly, this procedure involved disinfecting the eggs with 70% alcohol swabs (BD alcohol swabs-catalog 326910, ON, Canada), followed by careful puncture of the air cell using an 18-gauge needle. A self-refilling injector (Socorex ultra-1810.2.05005, Ecublens, Switzerland) fitted with a 22-gauge needle (injection needle length—3 cm) at a 45-degree angle was then used to deliver the bioactive substances into the amnion and subsequent sealing of the punctured sites with sterile medical tapes (NexcareTM Flexible Clear Tape-7100187758, 3M, MN, USA). To reduce treatment variability, the non-injected eggs were also removed and put back in the incubator simultaneously as the other injected treatments. The injected *Bacillus subtilis* product (concentration of 10×10^{10} CFU/g) and the commercial essential oil blend (containing phytonutrients star anise, cinnamon, rosemary, and thyme oil) were obtained from Probiotech International Inc., St Hyacinthe, Quebec, Canada. The EO blend is registered by Health Canada as a Veterinary Health Product (**VHP**).

9.3.2 Experimental design, birds, diets, and management

Figure 9.1 gives an overview of the experimental layout utilized in this study. The experiment was conducted as a 5 x 2 factorial arrangement, 5 *in ovo* treatments and 2 temperature model as main factors. Hatched chicks (n = 480) were weighed and randomly allotted to five new treatment groups at the Atlantic Poultry Research Center, Dalhousie Faculty of Agriculture. Hatched chicks from the prior non-injected eggs treatment were assigned to treatment A) Negative control treatment (**NC**) - chicks were offered a basal corn-soybean meal-wheat–based diet, and (B) AGP diet - chicks were offered NC + 0.05% bacitracin methylene disalicylate (**BMD**). The eggs injected with FA, probiotics, and EO were also retained as treatments - (C) *In ovo* FA, (D) *In ovo* probiotics and (E) *In ovo* + in-water EO groups, respectively. The *in ovo* + in-water EO treatments included hatchlings from the *in ovo* EO group that also received EO at the recommended dosage of 250 mL/1000 L of drinking water via the water route. Chicks (mixed sex; 6 birds/cage) were randomly placed in either a thermoneutral room (**TN**) or environmentally controlled room (HS room) consisting of 40 battery cages each (dimension - 50cm × 60cm; stocking density - $0.076 m^2$/bird), with each treatment group having 8 replicate cages. Treatments were evenly represented at all cage tier levels. All birds had access to feed and water *ad libitum* throughout the study period (28 d). Diets (Table 9.1) were provided in mash form in both the starter (0-14d) and

grower (15-28d) phases. Lighting regime was set to generate 18 h light/ 6 h dark throughout the experiment and illumination was gradually decreased from 20 lux on day 0 to 5 lux on d 28. Rearing temperature in both experimental rooms were gradually decreased from 32 °C (d 0) to 24 °C (d 21). From d 21 to 28, birds in the TN room were raised under thermoneutral condition (24 °C ± 0.2) while birds in the environmentally controlled rooms were subjected to cyclic HS (8 h/d, 31 °C). Temperature and relative humidity in the rooms were continuously monitored and recorded using Extech Instruments (Nashua, USA) for both thermal groups.

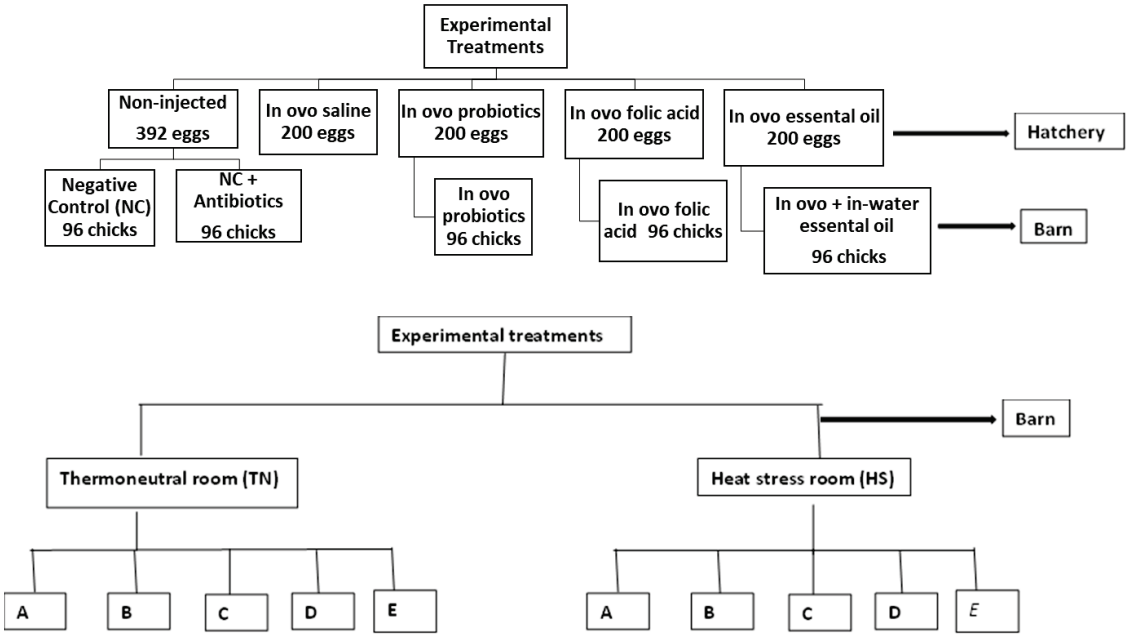


Figure 9.1 Schematic presentation of experimental layout in the hatchery and barn.

At the hatchery - *in ovo* saline eggs were injected with 0.2 mL of physiological saline, i.e., 0.9% NaCl, Baxter Corporation, ON, Canada); *in ovo* probiotics eggs were injected with 0.2 mL of *Bacillus subtilis* fermentation extract (each egg received 10 × 106 CFU of the bacterium/0.2 mL saline diluent); *in ovo* folic acid eggs were injected with 0.1 mL folic acid (95-102%; Thermo Scientific, USA; at 0.15 mg per egg); and *in ovo* essential oil eggs were injected with 0.2 mL of a saline + essential oil blend mixture at a dilution ratio of 2:1). At the barn- the non-injected eggs gave rise to treatment (A) Negative control treatment - chicks were offered a basal corn-soybean meal-wheat–based diet, and (B) Antibiotics diet - chicks were offered NC + 0.05% bacitracin methylene disalicylate (BMD). The eggs injected with folic acid, probiotics and essential oil were used to create treatments - (C) *In ovo* folic acid, (D) *In ovo* probiotics and (E) *In ovo* essential oil groups, respectively.

Table 9.1 Composition and nutritional contents of experimental diets1 (as-fed basis, percentage (%), unless otherwise stated).

Items	Phases			
	Starter (0-14 d)		Grower (15-28 d)	
	Control diet	Antibiotic diet	Control diet	Antibiotic diet
Ingredient Composition				
Corn (ground)	51.1	51.00	45.4	45.3
Soybean meal-46.5	41.4	41.5	36.3	36.3
Wheat	-	-	10	10
Animal/vegetable fat	2.93	2.97	4.22	4.26
Limestone	1.80	1.80	1.65	1.65
Dicalcium Phosphate	1.24	1.24	1.06	1.06
DL Methionine premix[2]	0.59	0.59	0.53	0.53
Vitamin/Mineral Premix [3, 4]	0.50	0.50	0.50	0.50
Salt	0.41	0.41	0.37	0.37
Lysine HCl	0.01	0.01	0.00	0.00
BMD 110G[5]	-	0.05	-	0.05
Total	100	100	100	100
Calculated composition				
Metabolizable energy (kcal/kg)	3,000	3,000	3,100	3,100
Crude protein	23	23	21.5	21.5
Calcium	0.96	0.96	0.87	0.87
Available phosphorus	0.48	0.48	0.44	0.44
Sodium	0.19	0.19	0.18	0.18
Digestible lysine	1.28	1.28	1.15	1.16
Digestible methionine + cysteine	0.95	0.95	0.87	0.87
Digestible Tryptophan	0.25	0.25	0.23	0.23
Digestible Threonine	0.89	0.89	0.82	0.82
Analyzed composition				
Dry Matter	90.7	90.8	93.2	93.5
Crude protein	24.8	25	22.5	23.8
Crude fat	5.50	5.79	6.84	6.85
Calcium	1.06	1.13	1.00	0.96

Potassium	1.14	1.16	0.99	1.04
Phosphorus	0.69	0.70	0.67	0.62
Sodium	0.19	0.21	0.21	0.16

[1]Basal diet (Control diet-NC) and antibiotic diet containing NC + 0.05% bacitracin methylene disalicylate; [2]DL-Methionine, 0.5 kg; wheat middling, 0.5 kg. [3]Starter vitamin-mineral premix contained the following per kg of diet: 9750 IU vitamin A; 2000 IU vitamin D3; 25 IU vitamin E; 2.97 mg vitamin K; 7.6 mg riboflavin; 13.5 mg Dl Ca-pantothenate; 0.012 mg vitamin B12; 29.7 mg niacin; 1.0 mg folic acid, 801 mg choline; 0.3 mg biotin; 4.9 mg pyridoxine; 2.9 mg thiamine; 70.2 mg manganese; 80.0 mg zinc; 25 mg copper; 0.15 mg selenium; 50 mg ethoxyquin; 1543 mg wheat middling's; 500 mg ground limestone. [4] Grower and Finisher vitamin-mineral premix contained the following per kg of diet: 9750 IU vitamin A; 2000 IU vitamin D3; 25 IU vitamin E; 2.97 mg vitamin K; 7.6 mg riboflavin; 13.5 mg Dl Ca-pantothenate; 0.012 mg vitamin B12; 29.7 mg niacin; 1.0 mg folic acid, 801 mg choline; 0.3 mg biotin; 4.9 mg pyridoxine; 2.9 mg thiamine; 70.2 mg manganese; 80.0 mg zinc; 25 mg copper; 0.15 mg selenium; 50 mg ethoxyquin; 1543 mg wheat middling's; 500 mg ground limestone. [5] Bacitracin methylene disalicylate (providing 55 mg/kg mixed feed); Alpharma, Inc., Fort Lee, NJ, USA.

9.3.3 16S rRNA gene sequencing and bioinformatics

On d28, 1 bird/cage (males) was randomly selected, weighed, and euthanized by electrical stunning and exsanguination and cecal digesta was collected. Microbial genomic DNA was subsequently isolated using the Qiagen DNeasy® PowerSoil Pro Kit (50) (Cat. No./ID: 47014) following the manufacturer's protocol. The concentration and purity of extracted DNA were then determined by spectrophotometry (Nanodrop ND1000, Thermo Scientific, Waltham, MA, USA). 16S rRNA gene amplicon libraries were then generated following the 16S Metagenomic Sequencing Library Preparation workflow from Illumina (Illumina, Inc., San Diego, CA) using PCR primers targeting the variable V3–V4 region of the 16S rRNA gene at Integrated Microbiome Resource, Dalhousie University in Halifax, Nova Scotia (Klindworth et al., 2013; Comeau et al., 2017). Bioinformatics analysis of microbiota data was as described by Oladokun et al. (2022), which followed protocols described in the Microbiome Helper pipeline (https://github.com/LangilleLab/microbiome_helper/wiki), based on the Quantitative Insight Into Microbial Ecology (**QIIME**) software package. Rarefaction curves were used to examine the individual alpha diversity for all samples (with the default observed OTUs as the metric). Alpha diversity comparisons were determined using the Shannon index and differences were tested by the Kruskal–Wallis statistical test set at $P < 0.05$. Beta diversity was visualized using Principal Coordinates Analysis (**PCoA**) based on weighted UniFrac distance matrix. The relative abundance at different taxonomic levels was visualized using stacked bar charts, while significant microbiota proportions were determined in the Statistical Analysis of Metagenomic Profiles (**STAMP**) software (Park et al., 2014) with an analysis of variance (**ANOVA**) test using the Benjamin–Hochberg false discovery rate as multiple test correction for treatments comparison and T-test for temperature model comparison. Corrected *p*-value was set at $P < 0.05$. To predict the function of cecal microbiota, data analysis was carried out using the Phylogenetic Investigation of Communities by Reconstruction of Unobserved States (**PICRUST2**) pipeline (Douglas et al., 2020). The PICRUST2 output was then analyzed using the ANOVA test and the Benjamin–Hochberg false discovery rate as multiple test correction for treatments comparison and T-test for temperature model comparison in the STAMP software.

9.4 Results

Sequencing yielded ~4.4 million raw reads from 80 broiler chicken cecal samples with an average of ~55 thousand reads per sample (Table 9.2). A total of ~3 million merged sequences were obtained, with an average of 38, 231 sequences per sample.

Table 9.2 Sequencing data quality control metrics for 80 ceca digesta samples.

S/N	Sample-id	Total raw reads	Total filtered reads	% Filtered	Total denoised	Total merged	% Merged	Total non-chimeric	% non-chimeric
1	Sample-1	34747	27610	79.46	26082	23315	67.1	19609	56.43
2	Sample-2	67154	53414	79.54	51446	45344	67.52	32155	47.88
3	Sample-3	56033	44920	80.17	42855	37426	66.79	27870	49.74
4	Sample-4	79522	64418	81.01	61999	54328	68.32	39374	49.51
5	Sample-5	124710	100577	80.65	98666	89071	71.42	47829	38.35
6	Sample-7	56679	45494	80.27	43806	38657	68.2	27928	49.27
7	Sample-8	53147	41435	77.96	39661	36227	68.16	31238	58.78
8	Sample-9	73398	59500	81.06	57279	51175	69.72	38527	52.49
9	Sample-10	32635	26280	80.53	25335	23271	71.31	20114	61.63
10	Sample-11	27060	21616	79.88	20599	18426	68.09	14675	54.23
11	Sample-12	65974	54200	82.15	52821	48206	73.07	36114	54.74
12	Sample-14	44626	36402	81.57	35083	32178	72.11	27787	62.27
13	Sample-16	62148	51237	82.44	49901	46282	74.47	39013	62.77
14	Sample-17	63777	51976	81.5	50374	46044	72.2	34777	54.53
15	Sample-18	50222	41056	81.75	39535	35033	69.76	26389	52.54
16	Sample-20	38760	31526	81.34	30291	27402	70.7	22668	58.48
17	Sample-21	6630	5394	81.36	5190	4867	73.41	4763	71.84
18	Sample-22	40273	32891	81.67	32028	29836	74.08	26851	66.67
19	Sample-23	42119	34717	82.43	34008	32138	76.3	25587	60.75
20	Sample-24	91348	74744	81.82	71699	63537	69.55	46208	50.58
21	Sample-25	64009	52725	82.37	50474	45153	70.54	34069	53.23
22	Sample-26	27976	22914	81.91	21911	19746	70.58	17546	62.72
23	Sample-27	40970	32972	80.48	31561	28749	70.17	24850	60.65
24	Sample-29	48839	40209	82.33	38782	35542	72.77	30551	62.55
25	Sample-30	33132	26775	80.81	25470	22860	69	18297	55.22
26	Sample-31	39846	31901	80.06	30656	26397	66.25	19407	48.71
27	Sample-32	57461	45123	78.53	42690	37135	64.63	27646	48.11

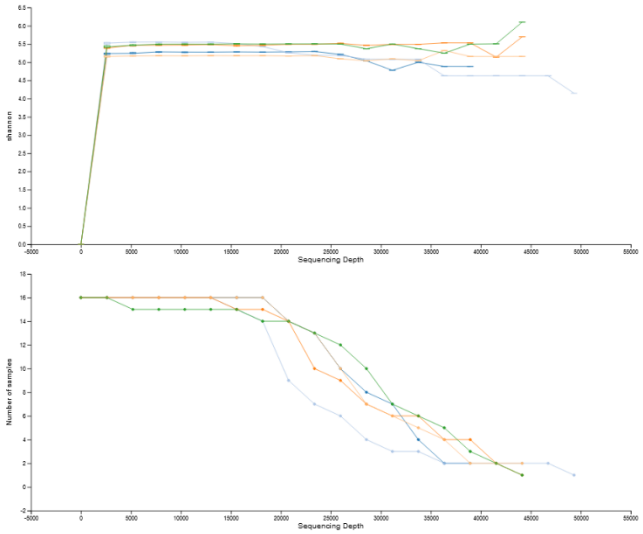
28	Sample-33	34739	28137	81	26972	24889	71.65	23078	66.43
29	Sample-34	39339	32365	82.27	31067	27389	69.62	21190	53.87
30	Sample-35	49942	40561	81.22	39451	36047	72.18	28173	56.41
31	Sample-36	37042	29903	80.73	28871	26055	70.34	18809	50.78
32	Sample-38	37950	30877	81.36	29742	26942	70.99	21840	57.55
33	Sample-40	39046	32161	82.37	30763	28114	72	23366	59.84
34	Sample-41	37062	30431	82.11	29220	26542	71.62	21372	57.67
35	Sample-42	57111	47249	82.73	45561	41028	71.84	29611	51.85
36	Sample-43	37509	30735	81.94	29570	26715	71.22	19530	52.07
37	Sample-45	75601	63441	83.92	61730	56921	75.29	41839	55.34
38	Sample-46	70904	58389	82.35	56560	51182	72.18	39473	55.67
39	Sample-47	41015	33645	82.03	32323	29374	71.62	25124	61.26
40	Sample-48	76672	63330	82.6	60903	55350	72.19	45426	59.25
41	Sample-49	41887	32882	78.5	31130	27170	64.86	22796	54.42
42	Sample-51	42926	33733	78.58	32261	29229	68.09	23696	55.2
43	Sample-52	62466	50091	80.19	47925	41599	66.59	30077	48.15
44	Sample-53	35178	28492	80.99	27065	24336	69.18	19920	56.63
45	Sample-54	66794	54846	82.11	53056	48207	72.17	38263	57.29
46	Sample-55	48873	39350	80.51	38246	35742	73.13	31706	64.87
47	Sample-56	25445	20390	80.13	19502	17920	70.43	16328	64.17
48	Sample-57	33729	27286	80.9	25949	24056	71.32	21533	63.84
49	Sample-59	55731	45164	81.04	43325	38593	69.25	30061	53.94
50	Sample-60	37149	29294	78.86	27939	24838	66.86	20408	54.94
51	Sample-62	58344	46308	79.37	44559	40446	69.32	31574	54.12
52	Sample-63	90129	73162	81.17	70390	62379	69.21	42598	47.26
53	Sample-64	93415	76046	81.41	73155	63338	67.8	44602	47.75
54	Sample-65	84669	67607	79.85	65266	59235	69.96	44278	52.3
55	Sample-66	70657	56641	80.16	54138	48420	68.53	36751	52.01
56	Sample-67	49158	39621	80.6	37684	33656	68.46	27681	56.31
57	Sample-68	56237	45466	80.85	43977	40123	71.35	30454	54.15
58	Sample-69	38144	30414	79.73	28673	25444	66.71	19903	52.18
59	Sample-70	80190	66116	82.45	64585	58731	73.24	40687	50.74
60	Sample-72	75556	60433	79.98	58339	52442	69.41	36349	48.11

61	Sample-73	77214	62462	80.89	59828	51962	67.3	38003	49.22
62	Sample-74	71556	56263	78.63	53679	47295	66.1	35857	50.11
63	Sample-75	52194	42061	80.59	39808	35552	68.12	27858	53.37
64	Sample-76	64021	51494	80.43	49403	44800	69.98	31965	49.93
65	Sample-77	45309	36755	81.12	35402	31085	68.61	22168	48.93
66	Sample-78	35506	28371	79.9	27024	24070	67.79	20253	57.04
67	Sample-80	61498	49807	80.99	46932	38809	63.11	26543	43.16
68	Sample-81	42312	31985	75.59	30694	27779	65.65	24297	57.42
69	Sample-82	85004	68544	80.64	66278	58667	69.02	39673	46.67
70	Sample-83	71708	56773	79.17	54752	48259	67.3	35412	49.38
71	Sample-84	55817	45386	81.31	43310	36966	66.23	25694	46.03
72	Sample-86	24618	19761	80.27	18400	15877	64.49	13804	56.07
73	Sample-87	55533	45197	81.39	43274	37433	67.41	25295	45.55
74	Sample-89	62657	50477	80.56	48366	42912	68.49	29711	47.42
75	Sample-90	44924	36763	81.83	34944	31178	69.4	25289	56.29
76	Sample-92	78177	62817	80.35	59565	51182	65.47	35455	45.35
77	Sample-93	89225	71565	80.21	69311	63464	71.13	49424	55.39
78	Sample-94	64364	50946	79.15	48134	38956	60.52	26537	41.23
79	Sample-95	55704	45223	81.18	42978	37305	66.97	25090	45.04
80	Sample-96	68656	55849	81.35	52920	44149	64.3	29259	42.62

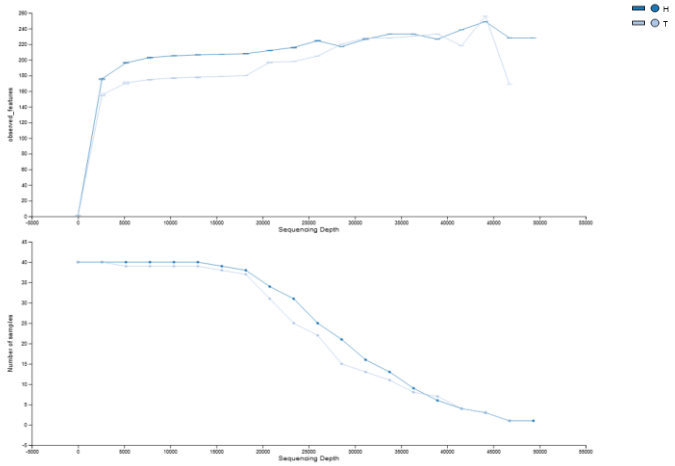
9.4.1 Alpha diversity—cecal microbiota richness

Rarefaction curves of observed features (richness) were used to estimate internal sample alpha diversity. The results revealed that sequencing depth was adequate to cover the bacterial diversity in the cecal samples (Figure 9.2).

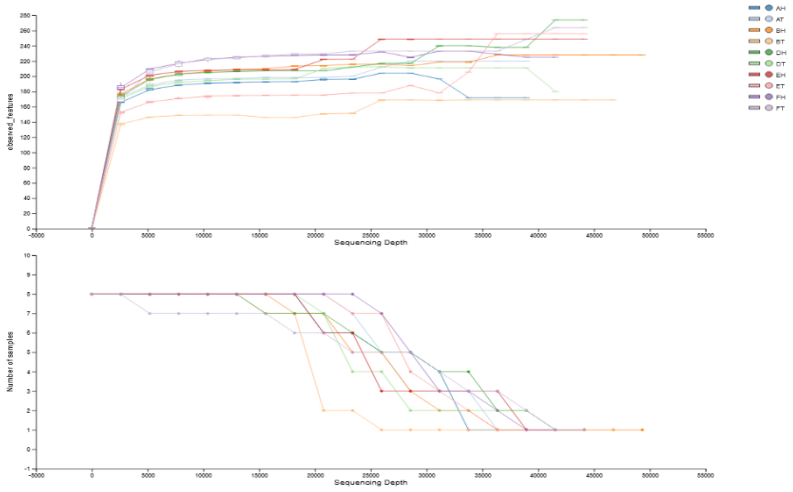
Alpha diversity index (Shannon index) as affected by treatments, temperature model or their interaction in this study is presented in Figure 9.3. Both main factors (treatments and temperature model) evaluated in this study recorded no effect on ($P > 0.05$) species richness in this study. Similarly, there was no significant interaction ($P = 0.822$) between evaluated treatments and temperature model on cecal microbial richness in this study. Notwithstanding, AGP treatment under the HS challenge recorded the highest numerical increase in alpha-Shannon index (Figure 9.3c).


shannon
Sequencing Depth
Number of samples
Sequencing Depth

(a)


H
T
observed_features
Sequencing Depth
Number of samples
Sequencing Depth

(b)


AH
AT
BH
BT
DH
DT
EH
ET
FH
FT
observed_features
Sequencing Depth
Number of samples
Sequencing Depth

(c)

Figure 9.2 Rarefaction curves of observed features and number of samples obtained from 16S rRNA gene V3–V4 sequences.

Based on a) treatments, b) temperature model, and c) treatment and temperature model interaction. Treatments groups include- A) Negative control treatment- chicks were offered a basal corn-soybean meal-wheat–based diet, B) Antibiotics treatment- chicks were offered NC + 0.05% bacitracin methylene disalicylate (BMD), D) *in ovo* folic acid (FA) treatment- eggs injected with 0.1 mL FA (FA; 95-102%; Thermo Scientific, USA) at 0.15 mg per egg, E) *In ovo* probiotics treatment- eggs injected with 0.2 mL of *Bacillus subtilis* fermentation extract, each egg received 10 × 106 CFU of the bacterium/0.2 mL saline diluent, and F) *in ovo* + in-water essential oil (EO) treatment- eggs injected with 0.2 mL of a saline + EO blend mixture at a dilution ratio of 2:1 and supplied the EO via the in-water route at the recommended dosage of 250 mL/1000 L of drinking water. Temperature models include- Thermoneutral group- birds were housed at 24ºC ± 0.2 from d 21 – 28, and Heat stress group (H)-birds were housed at 31ºC for 8hrs/day from d 21 – 28. Interaction effects include- AH) Negative control treatment under heat stress challenge, AT) Negative control treatment under thermoneutral condition, BH) Antibiotics treatment under heat stress challenge, BT) Antibiotics treatment under thermoneutral condition, DH) *in ovo* FA treatment under heat stress challenge, DT) *in ovo* FA treatment under thermoneutral condition, EH) *In ovo* probiotics treatment under heat stress challenge, ET) *In ovo* probiotics treatment under thermoneutral condition, FH) *in ovo* + in-water EO treatment under heat stress challenge, and FT)) *in ovo* + in-water EO treatment under thermoneutral condition. The box plots in the upper figure represent the distribution of the selected alpha diversity metric for each group of samples at each even sampling depth. The lower and upper whiskers of the box plot are the 9th and 91st percentiles of the distribution (respectively), while the lower and upper extents of the box are the 25th and 75th percentiles of the distribution (respectively). The horizontal bar through the middle of the box is the median of the distribution (i.e., the 50th percentile). Outlier points of these distributions are not shown. The line chart in the upper figure connects the median values of the alpha diversity metric distribution across the sampling depths. If a sampling depth is higher than the

number of sequences in a sample, that sample will not be included in the rarefaction plot at that sampling depth. The line chart in the lower figure illustrates the number of samples in each group (i.e., the sample size for each box plot) at each sampling depth.

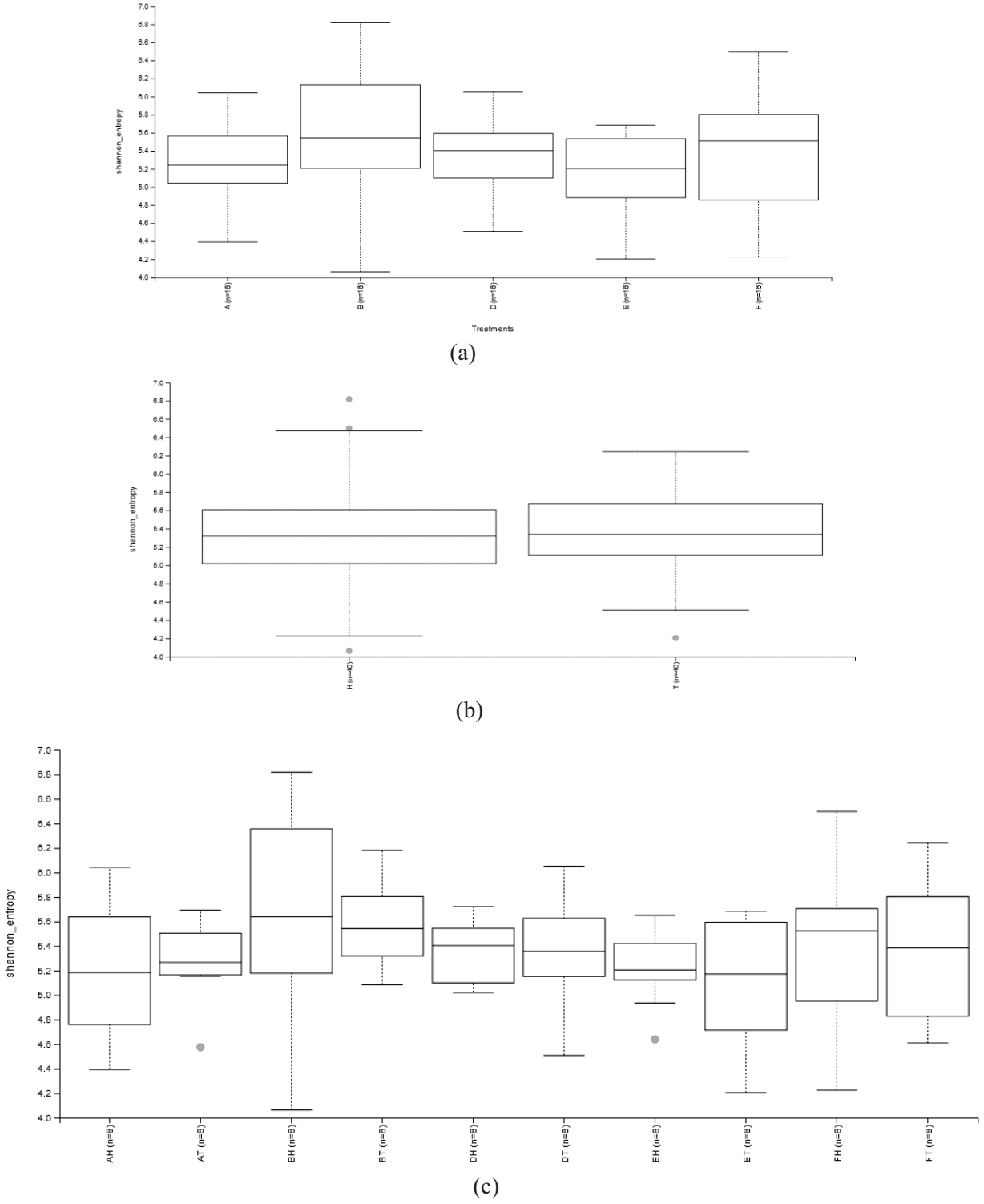


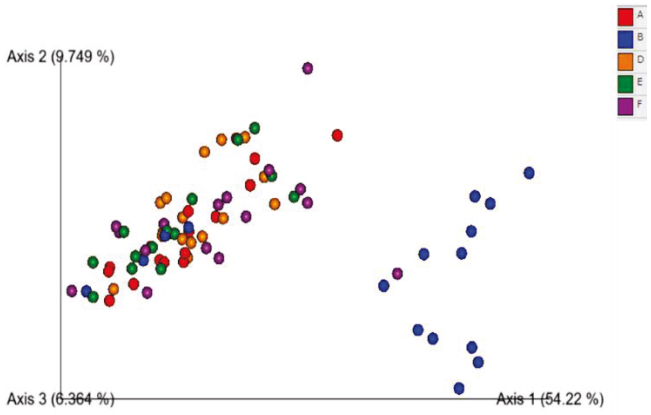
Figure 9.3 Alpha diversity plots.

Alpha diversity (Shannon index) box plots show (a) no significant difference (P = 0.285, Kruskal-Wallis test) between evaluated treatments, (b) no significant difference (*P* = 0.908, Kruskal-Wallis test) between Heat stress (H) and

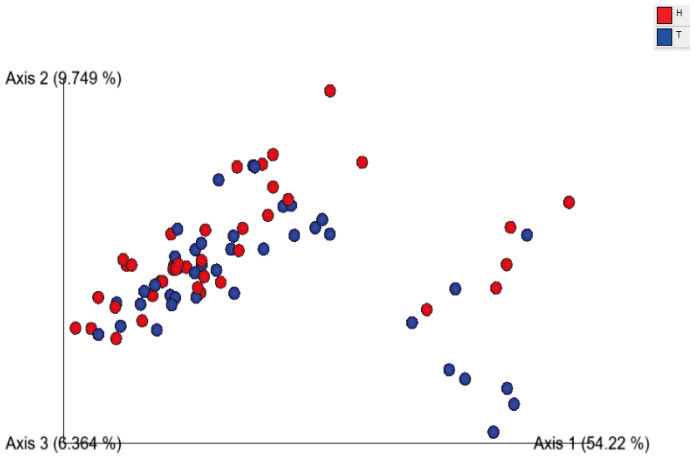
Thermoneutral (T) groups, and (c) no significant interaction effect (P = 0.822, Kruskal-Wallis test) between treatment and temperature groups. Treatments groups include- A) Negative control treatment - chicks were offered a basal corn-soybean meal-wheat–based diet, B) Antibiotics treatment - chicks were offered NC + 0.05% bacitracin methylene disalicylate (BMD), D) *in ovo* folic acid (FA) treatment - eggs injected with 0.1 mL FA (FA; 95-102%; Thermo Scientific, USA) at 0.15 mg per egg, E) *In ovo* probiotics treatment- eggs injected with 0.2 mL of *Bacillus subtilis* fermentation extract, each egg received 10×10^6 CFU of the bacterium/0.2 mL saline diluent, and F) *in ovo* + in-water essential oil (EO) treatment - eggs injected with 0.2 mL of a saline + EO blend mixture at a dilution ratio of 2:1 and supplied the EO via the in-water route at the recommended dosage of 250 mL/1000 L of drinking water. Temperature groups include - Thermoneutral group- birds were housed at 24ºC ± 0.2 from d 21 – 28, and Heat stress group (H)- birds were housed at 31ºC for 8hrs/day from d 21 – 28. Interaction effects include- AH) Negative control treatment under heat stress challenge, AT) Negative control treatment under thermoneutral condition, BH) Antibiotics treatment under heat stress challenge, BT) Antibiotics treatment under thermoneutral condition, DH) *in ovo* FA treatment under heat stress challenge, DT) *in ovo* FA treatment under thermoneutral condition, EH) *In ovo* probiotics treatment under heat stress challenge, ET) *In ovo* probiotics treatment under thermoneutral condition, FH) *in ovo* + in-water EO treatment under heat stress challenge, and FT)) *in ovo* + in-water EO treatment under thermoneutral condition. Boxes in the boxplots denote interquartile range, solid middle line in the boxes denote the median, all other symbols outside the boxes represent outliers.

9.4.2 Beta diversity— shaping the cecal microbiota

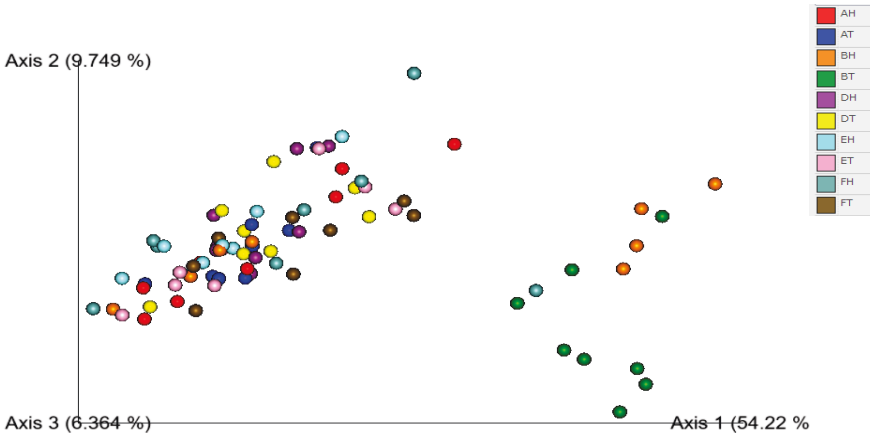
Multidimensional scaling (PCoA) based on weighted UniFrac distances was done to graphically explore the bacterial community heterogeneity (Figure 9.4). On a treatment basis, the AGP treatment showed an almost clear cluster separation (except for n = 4 outliers), compared to other treatments (Figure 9.4a). On the other hand, no unique cluster separation was observed between the cecal microbiota profile of HS and TN birds. When the interaction effect between evaluated treatments and thermal challenge was considered, the ceca microbiota profile of birds offered AGP under TN conditions showed clear cluster separation when compared to all other treatment*temperature combinations (Figure 9.4c).



(a)



(b)



(c)

Figure 9.4 PCoA plots based on weighted UniFrac metric.

a) treatment comparison, b) temperature comparison, and c) treatment and temperature group interaction comparison. Treatments groups include - A) Negative control treatment - chicks were offered a basal corn-soybean meal-wheat–based diet, B) Antibiotics treatment- chicks were offered NC + 0.05%

bacitracin methylene disalicylate (BMD), D) *in ovo* folic acid (FA) treatment- eggs injected with 0.1 mL FA (FA; 95-102%; Thermo Scientific, USA) at 0.15 mg per egg, E) *In ovo* probiotics treatment- eggs injected with 0.2 mL of *Bacillus subtilis* fermentation extract, each egg received 10×10^6 CFU of the bacterium/0.2 mL saline diluent, and F) *in ovo* + in-water essential oil (EO) treatment- eggs injected with 0.2 mL of a saline + EO blend mixture at a dilution ratio of 2:1 and supplied the EO via the in-water route at the recommended dosage of 250 mL/1000 L of drinking water. Temperature groups include- Thermoneutral group (T) - birds were housed at 24ºC ± 0.2 from d 21 – 28, and Heat stress group (H)-birds were housed at 31ºC for 8hrs/day from d 21 – 28. Interaction effects include- AH) Negative control treatment under heat stress challenge, AT) Negative control treatment under thermoneutral condition, BH) Antibiotics treatment under heat stress challenge, BT) Antibiotics treatment under thermoneutral condition, DH) *in ovo* FA treatment under heat stress challenge, DT) *in ovo* FA treatment under thermoneutral condition, EH) *In ovo* probiotics treatment under heat stress challenge, ET) *In ovo* probiotics treatment under thermoneutral condition, FH) *in ovo* + in-water EO treatment under heat stress challenge, and FT)) *in ovo* + in-water EO treatment under thermoneutral condition.

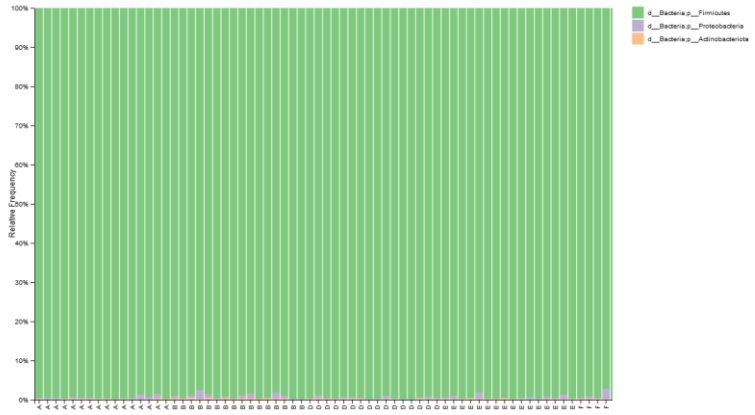
9.4.3 Taxonomic composition

Irrespective of treatment, temperature, and treatment*temperature effects, phyla *Firmicutes*, *Proteobacteria* and *Actinobacteria* were the most abundant (> 95%) phyla in this study (Figure 9.5). The relative abundance of the most dominant bacteria at the class, order and family taxa are presented in Figure 9.6. The relative abundance of the top ten most abundant bacteria genera, as influenced by treatments, temperature model or their interaction, is shown in Figure 9.7.

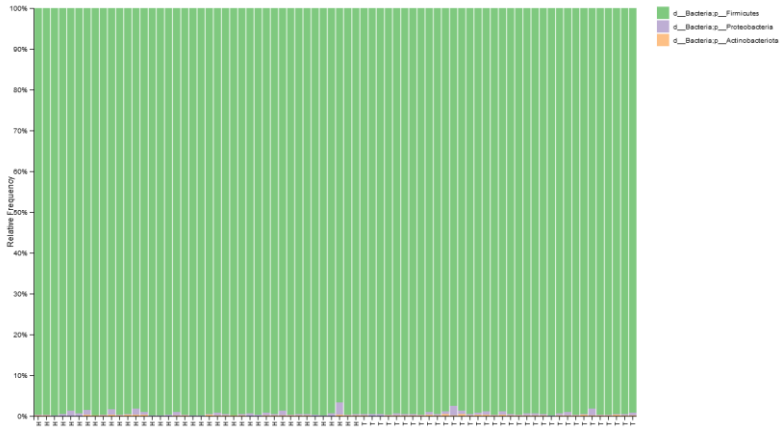
To confirm differences in ceca microbiota taxonomic profile, significant differences in cumulative proportion of bacteria across all taxa was also evaluated. Taxa with significantly different microbiota proportions are thus presented in Figures 9.8.1 and 9.8.2. At the phylum level, an interaction effect was observed for both treatment and temperature challenge. While HS alone reduced the proportion of members of the *Actinobacteria* phylum, AGP supplementation, especially under TN condition, increased the proportion of members of this phylum compared to all other treatment and temperature conditions. The only exception to this effect was observed in the *in ovo* + in-water EO treatment under TN condition, which had a similar proportion of the *Actinobacteria* phylum as the AGP treatment under the same condition. Both AGP treatment and HS challenge independently increased the proportion of bacteria in the genus *Ruminococcus*. However, the cecal microbiota of birds fed the AGP treatment under TN condition recorded a higher ($P < 0.05$) proportion of bacteria in this genus compared to other treatments, irrespective of temperature challenge model. Also, the *in ovo* + in-water treatment recorded a similar increase ($P < 0.05$) in proportion of bacteria in the genus *Ruminococcus_torques* group compared to other treatments. Furthermore, while AGP treatment increased ($P < 0.05$) the proportion of bacteria in the genus *Eubacterium*, HS independently reduced ($P < 0.001$) it. However, none of the *in ovo* delivered treatments could reverse this decrease ($P < 0.02$) under HS condition, compared to the AGP-treated birds under TN condition. A similar trend of AGP-induced increase ($P < 0.001$) in genus *Blautia* and HS-induced reduction ($P < 0.01$) of the same genus was also observed. Notwithstanding, the AGP-induced bacteria increase in the genus *Blautia* was higher ($P < 0.001$) than every other treatment and temperature combination. The proportion of bacteria in the genus *Lachnospiraceae* was also increased ($P < 0.01$) by both AGP and HS treatment independently. However, under HS challenge, the AGP treatment only recorded a higher ($P < 0.05$) proportion of this genus than the probiotic treatment, every other treatment exhibited statistical similarity. No effect of HS on the proportion of bacteria in the genus *Gordonibacter* was recorded in this study. However, the AGP treatment increased ($P < 0.01$) the proportion of this genus compared to other

treatments. While the AGP treatment was observed to reduce the proportion of bacteria in the genera F*aecalibacterium*, N*egativibacillus*, *Oscillibacter*, and *Romboutsia*, no effect of thermal challenge on the proportion of bacteria in these genera was observed. However, AGP treated birds reduced bacteria in these genera, compared to all other treatments.

Only treatment effect was recorded for the cumulative proportions of bacteria in the genera *Anaerostipes*, *Butyricicoccus, lachnoclostridium, Monoglobus, Sellimonas, Turicibacter, and Incertae_Sedis* (undefined). The AGP treatment increased ($P < 0.05$) the proportion of bacteria in these genera compared to other treatment groups. The only exception was for the *lachnoclostridium and Sellimonas genera* where the *in ovo* + in-water treatment had a similar increase of this genera as the AGP treatment. When thermal challenge was considered independently, HS was observed to increase ($P < 0.01$) the proportion of bacteria in the genera *Anaerotruncus*, *Candidatus Soleaferrea*, *Flavonifractor*, and *Lactobacillus* compared to TN condition.



(a)



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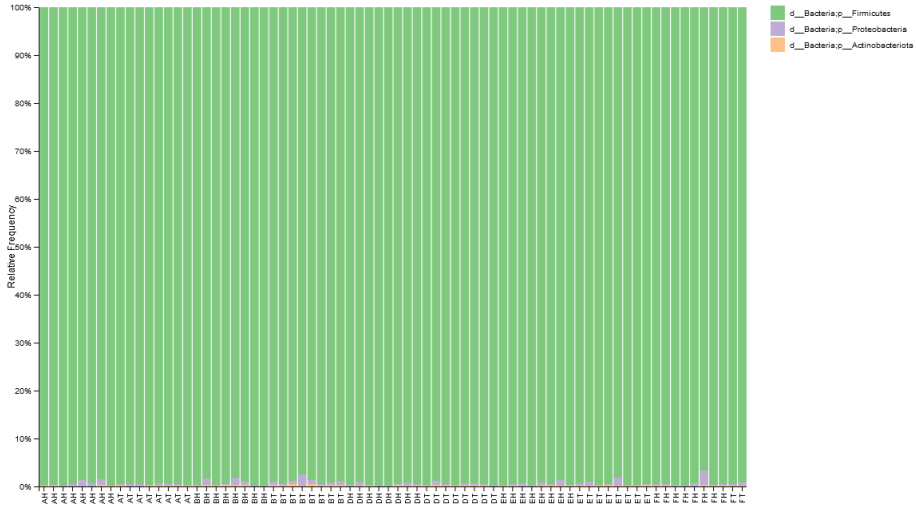
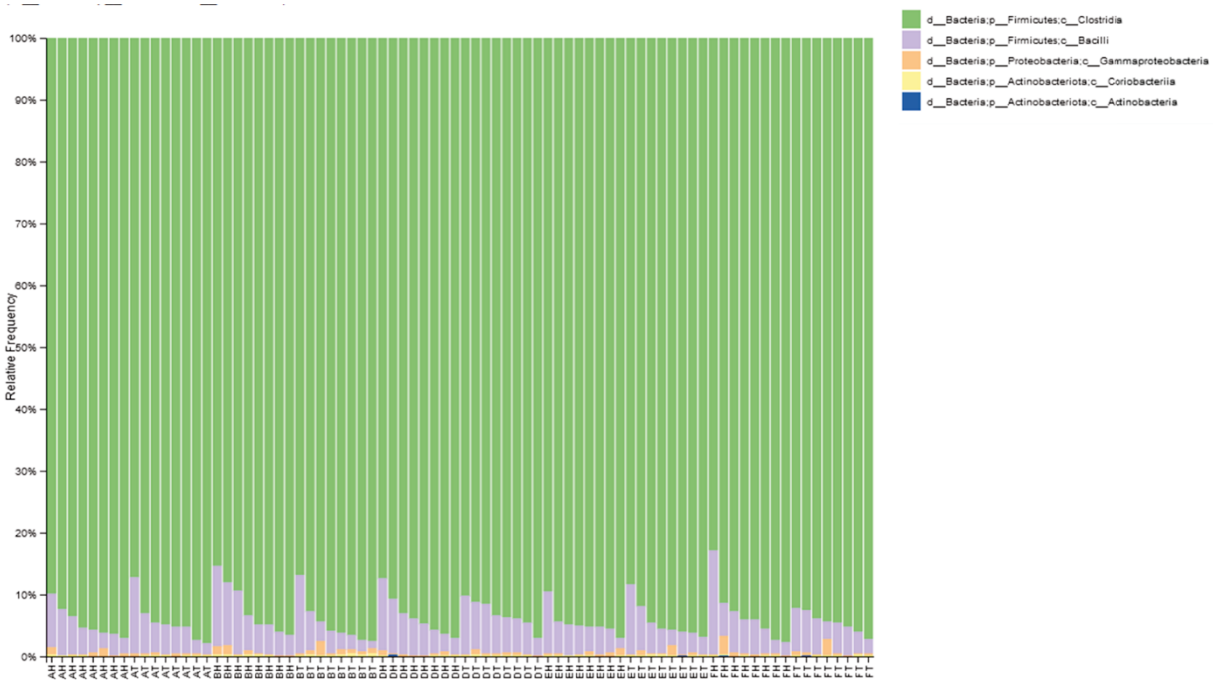


(c)

Figure 9.5 Ceca microbiota taxonomic composition of the dominant phyla.

Based on based on a) treatments, b) temperature model, and c) treatment and

temperature model interaction. Treatments groups include- A) Negative control treatment- chicks were offered a basal corn-soybean meal-wheat–based diet, B) Antibiotics treatment- chicks were offered NC + 0.05% bacitracin methylene disalicylate (BMD), D) *in ovo* folic acid (FA) treatment- eggs injected with 0.1 mL FA (FA; 95-102%; Thermo Scientific, USA) at 0.15 mg per egg, E) *In ovo* probiotics treatment- eggs injected with 0.2 mL of *Bacillus subtilis* fermentation extract, each egg received 10 × 106 CFU of the bacterium/0.2 mL saline diluent, and F) *in ovo* + in-water essential oil (EO) treatment- eggs injected with 0.2 mL of a saline + EO blend mixture at a dilution ratio of 2:1 and supplied the EO via the in-water route at the recommended dosage of 250 mL/1000 L of drinking water. Temperature models include- Thermoneutral group(T)- birds were housed at 24ºC ± 0.2 from d 21 – 28, and Heat stress group (H)-birds were housed at 31ºC for 8hrs/day from d 21 – 28. Interaction effects include- AH) Negative control treatment under heat stress challenge, AT) Negative control treatment under thermoneutral condition, BH) Antibiotics treatment under heat stress challenge, BT) Antibiotics treatment under thermoneutral condition, DH) *in ovo* FA treatment under heat stress challenge, DT) *in ovo* FA treatment under thermoneutral condition, EH) *In ovo* probiotics treatment under heat stress challenge, ET) *In ovo* probiotics treatment under thermoneutral condition, FH) *in ovo* + in-water EO treatment under heat stress challenge, and FT)) *in ovo* + in-water EO treatment under thermoneutral condition.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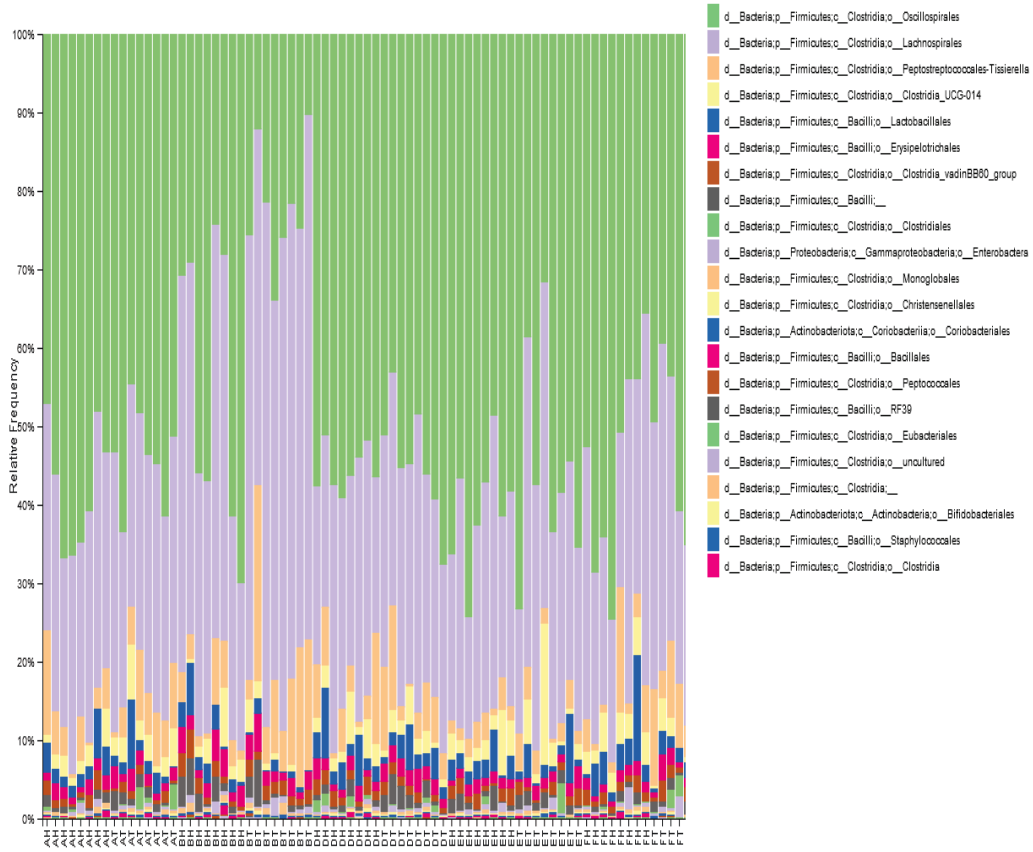
d__Bacteria;p__Firmicutes;c__Bacilli
d__Bacteria;p__Proteobacteria;c__Gammaproteobacteria
d__Bacteria;p__Actinobacteriota;c__Coriobacteriia
d__Bacteria;p__Actinobacteriota;c__Actinobacteria
Relative Frequency
100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%

(a)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d__Bacteria;p__Firmicutes;c__Clostridia;o__Lachnospirales
d__Bacteria;p__Firmicutes;c__Clostridia;o__Peptostreptococcales-Tissierella
d__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridia_UCG-014
d__Bacteria;p__Firmicutes;c__Bacilli;o__Lactobacillales
d__Bacteria;p__Firmicutes;c__Bacilli;o__Erysipelotrichales
d__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridia_vadinBB60_group
d__Bacteria;p__Firmicutes;c__Bacilli;__
d__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales
d__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__Enterobacteria
d__Bacteria;p__Firmicutes;c__Clostridia;o__Monoglobales
d__Bacteria;p__Firmicutes;c__Clostridia;o__Christensenellales
d__Bacteria;p__Actinobacteriota;c__Coriobacteriia;o__Coriobacteriales
d__Bacteria;p__Firmicutes;c__Bacilli;o__Bacillales
d__Bacteria;p__Firmicutes;c__Clostridia;o__Peptococcales
d__Bacteria;p__Firmicutes;c__Bacilli;o__RF39
d__Bacteria;p__Firmicutes;c__Clostridia;o__Eubacteriales
d__Bacteria;p__Firmicutes;c__Clostridia;o__uncultured
d__Bacteria;p__Firmicutes;c__Clostridia;__
d__Bacteria;p__Actinobacteriota;c__Actinobacteria;o__Bifidobacteriales
d__Bacteria;p__Firmicutes;c__Bacilli;o__Staphylococcales
d__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridia
Relative Frequency
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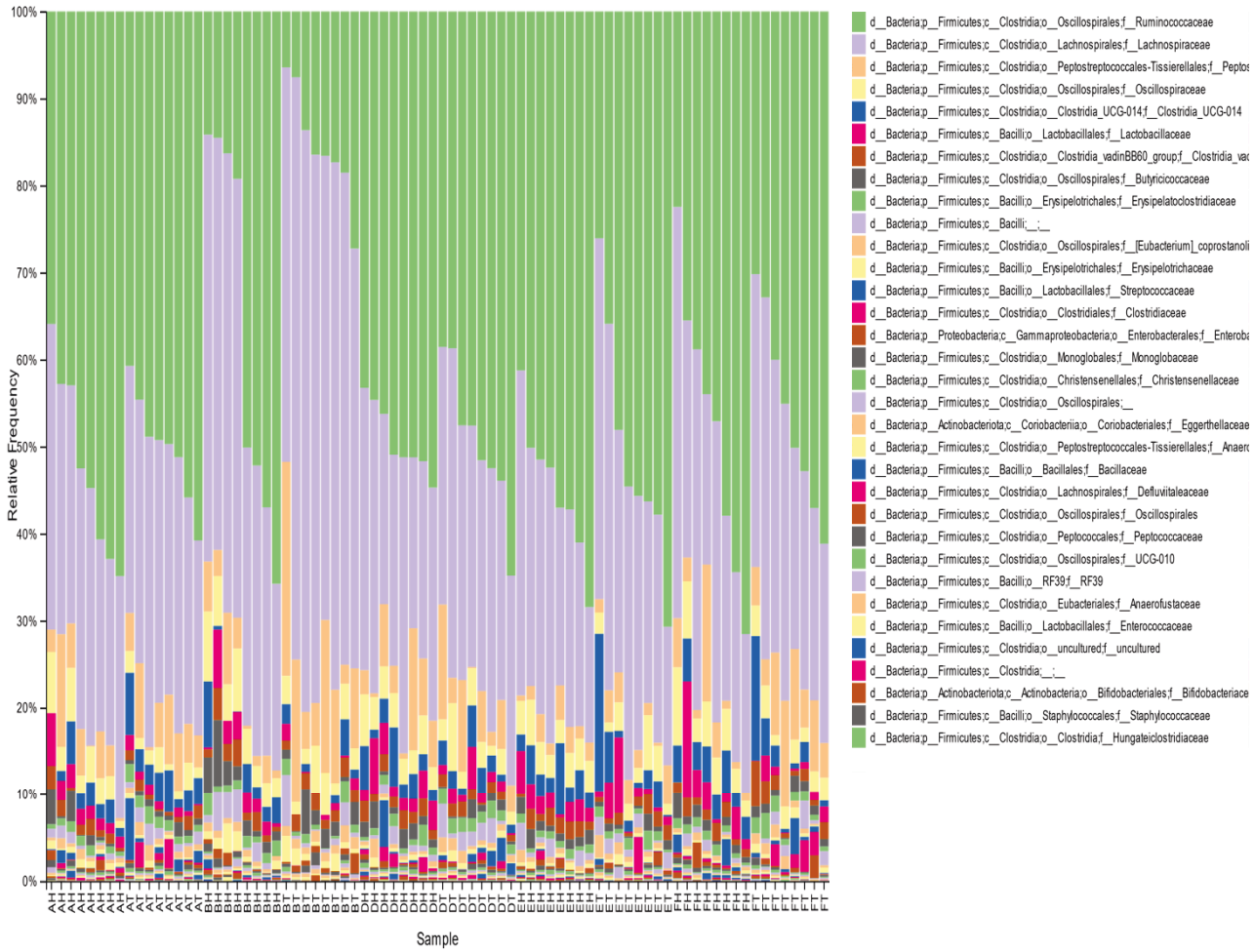
70%
60%
50%
40%
30%
20%
10%
0%

(b)

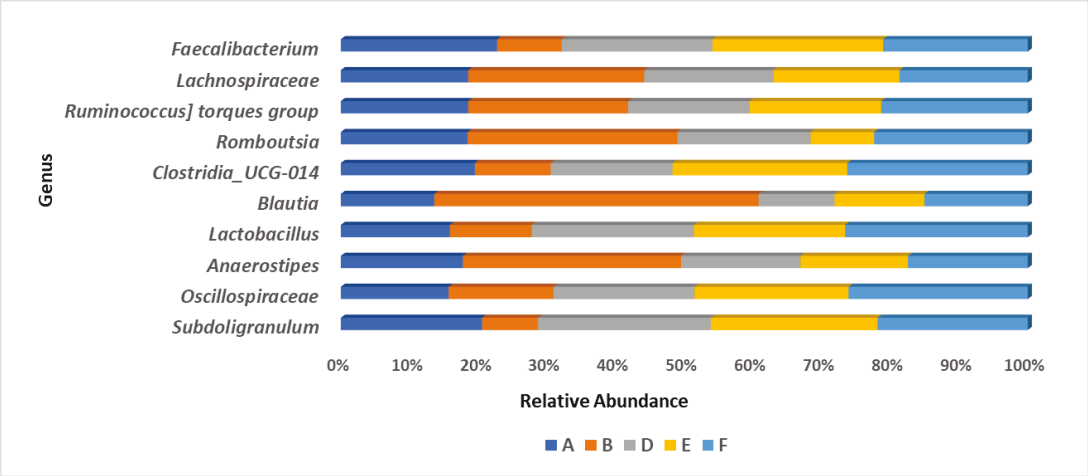


(c)

Figure 9.6 Ceca microbiota taxonomic profile at the (a) class, (b) order and (c) family level.

For Interaction effects AH) Negative control treatment (chicks were offered a basal corn-soybean meal-wheat–based diet) under heat stress challenge (birds were housed at 31ºC for 8hrs/day from d 21 – 28), AT) Negative control treatment under thermoneutral condition (birds were housed at 24ºC ± 0.2 from d 21 – 28), BH) Antibiotics treatment (chicks were offered NC + 0.05% bacitracin methylene disalicylate) under heat stress challenge, BT) Antibiotics treatment under thermoneutral condition, DH) *in ovo* folic acid (eggs injected with 0.1 mL folic acid (FA; 95-102%; Thermo Scientific, USA) at 0.15 mg per egg) treatment under heat stress challenge, DT) *in ovo* FA treatment under thermoneutral condition, EH) *In ovo* probiotics (eggs injected with 0.2 mL of *Bacillus subtilis* fermentation extract, each egg received 10×10^6 CFU of the bacterium/0.2 mL saline diluent) treatment under heat stress challenge, ET) *In ovo* probiotics treatment under

thermoneutral condition, FH) *in ovo* + in-water essential oil treatment (eggs injected with 0.2 mL of a saline + essential oil blend mixture at a dilution ratio of 2:1 and supplied the essential oil via the in-water route at the recommended dosage of 250 mL/1000 L of drinking water) under heat stress challenge, and FT)) *in ovo* + in-water essential oil treatment under thermoneutral condition.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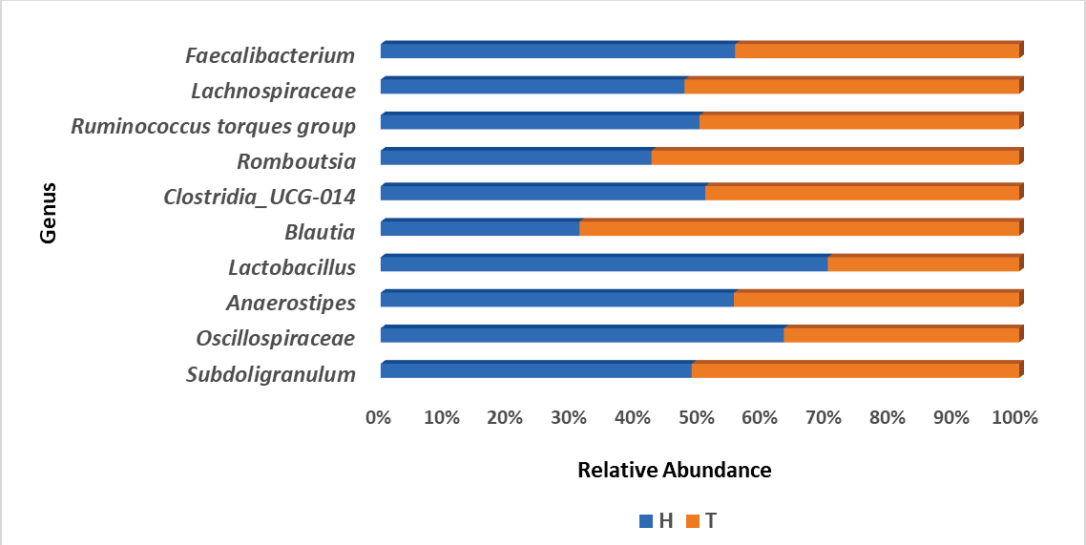
Lachnospiraceae
Ruminococcus] torques group
Romboutsia
Clostridia_UCG-014
Blautia
Lactobacillus
Anaerostipes
Oscillospiraceae
Subdoligranulum
Genus
0%
10%
20%
30%
40%
50%
60%
70%
80%
90%
100%
Relative Abundance
A
B
D
E
F

(a)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Clostridia_UCG-014
Blautia
Lactobacillus
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Oscillospiraceae
Subdoligranulum
Genus
0%
10%
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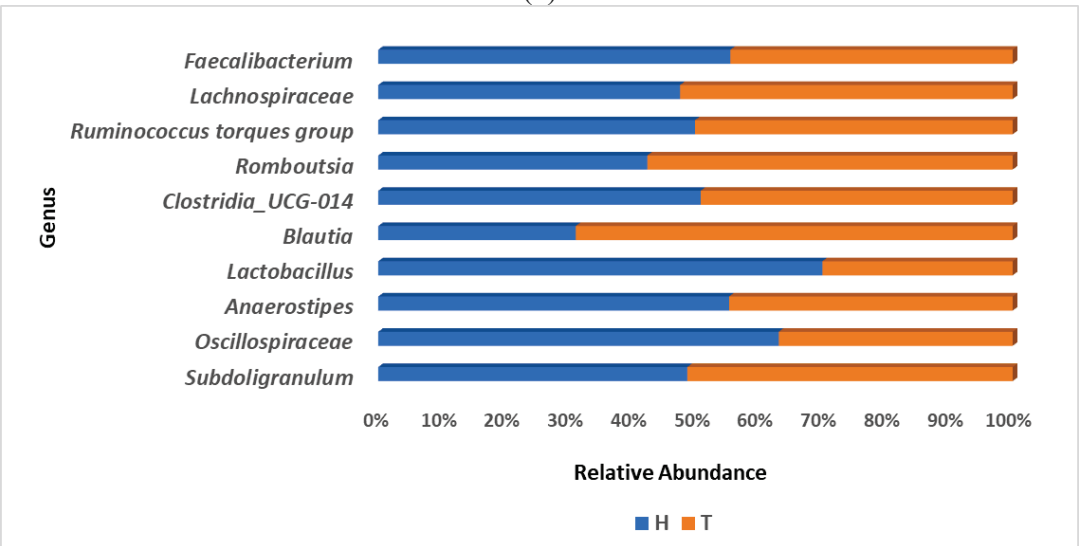
30%
40%
50%
60%
70%
80%
90%
100%
Relative Abundance
H
T

(b)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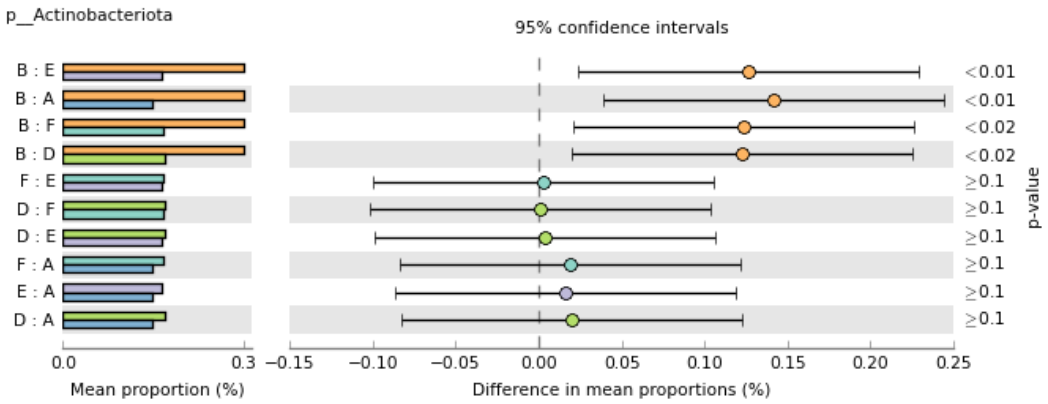
Lachnospiraceae
Ruminococcus torques group
Romboutsia
Clostridia_UCG-014
Blautia
Lactobacillus
Anaerostipes
Oscillospiraceae
Subdoligranulum
Genus
0%
10%
20%
30%
40%
50%
60%
70%
80%
90%
100%
Relative Abundance
H
T

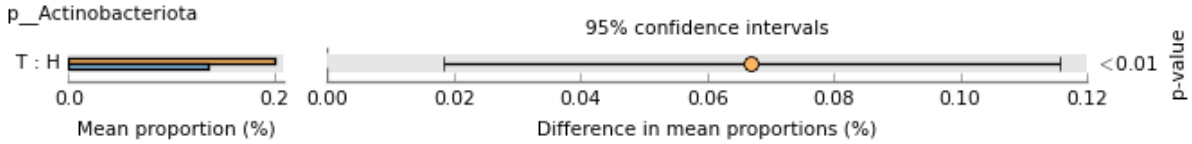
(c)

Figure 9.7 Microbiota profile of the most dominant genera in broiler chicken ceca.

Based on a) treatments, b) temperature model, and c) treatment and temperature model interaction. Treatments groups include- A) Negative control treatment - chicks were offered a basal corn-soybean meal-wheat–based diet, B) Antibiotics treatment - chicks were offered NC + 0.05% bacitracin methylene disalicylate (BMD), D) *in ovo* folic acid (FA) treatment- eggs injected with 0.1 mL FA (FA; 95-102%; Thermo Scientific, USA) at 0.15 mg per egg, E) *In ovo* probiotics treatment- eggs injected with 0.2 mL of *Bacillus subtilis* fermentation extract, each egg received 10 × 106 CFU of the bacterium/0.2 mL saline diluent, and F) *in ovo* + in-water essential oil (EO) treatment- eggs injected with 0.2 mL of a saline + EO blend mixture at a dilution ratio of 2:1 and supplied the EO via the in-water route at the recommended dosage of 250 mL/1000 L of drinking water. Temperature models include- Thermoneutral group (T)- birds were housed at 24ºC ± 0.2 from d 21 – 28, and Heat stress group (H)-birds were housed at 31ºC for 8hrs/day from d 21 – 28. Interaction effects include- AH) Negative control treatment under heat stress challenge, AT) Negative control treatment under thermoneutral condition, BH) Antibiotics treatment under heat stress challenge, BT) Antibiotics treatment under thermoneutral condition, DH) *in ovo* FA treatment under heat stress challenge, DT) *in ovo* FA treatment under thermoneutral condition, EH) *In ovo* probiotics treatment under heat stress challenge, ET) *In ovo* probiotics treatment under thermoneutral condition, FH) *in ovo* + in-water EO treatment under heat stress challenge, and FT)) *in ovo* + in-water EO treatment under thermoneutral condition



(a)



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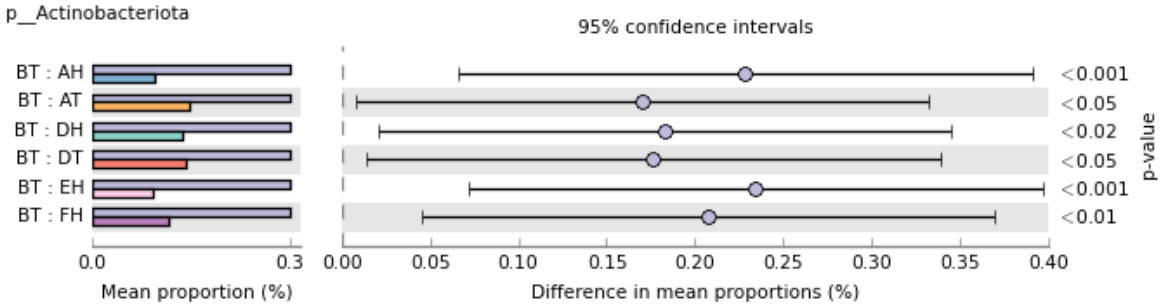
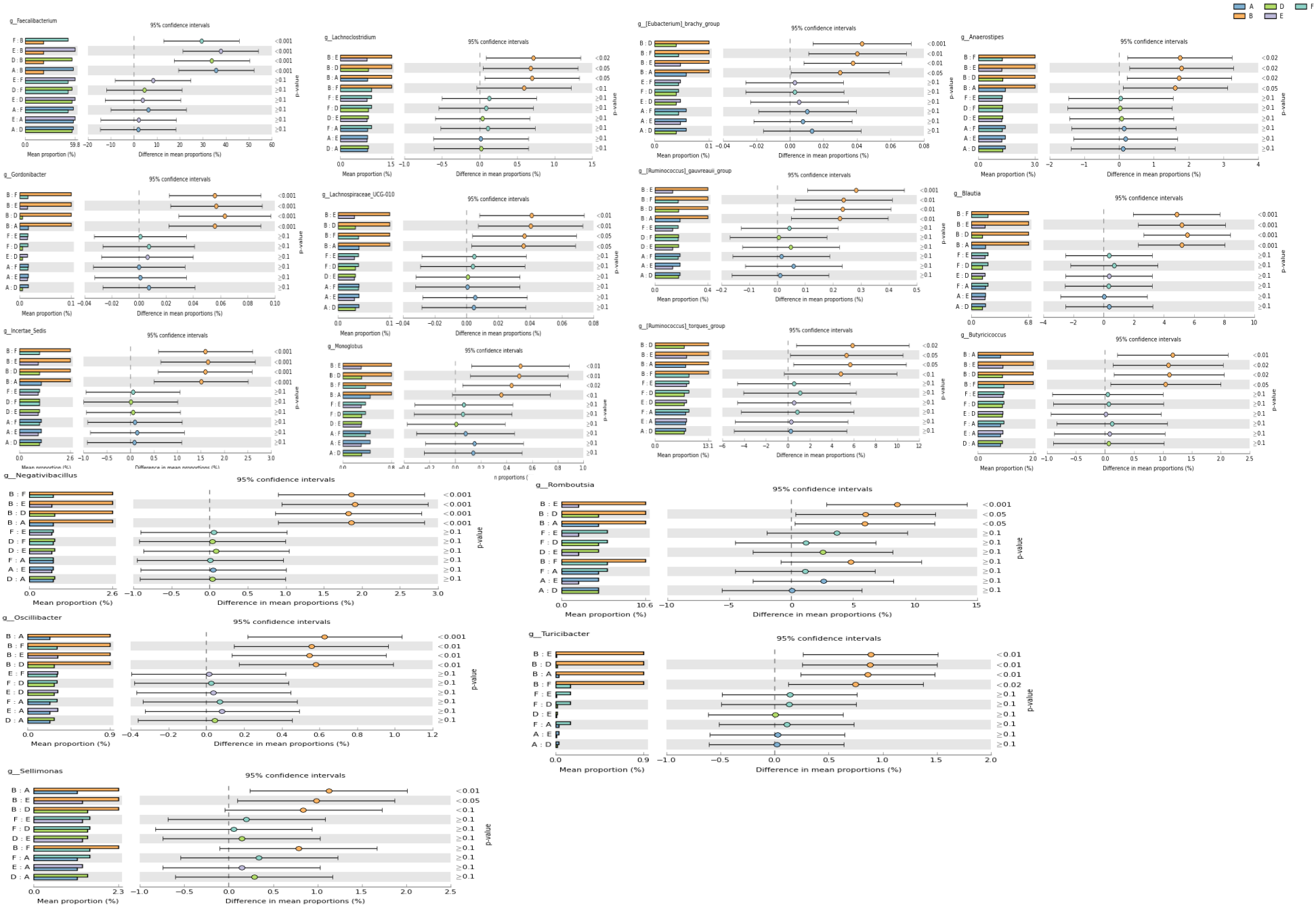


(c)

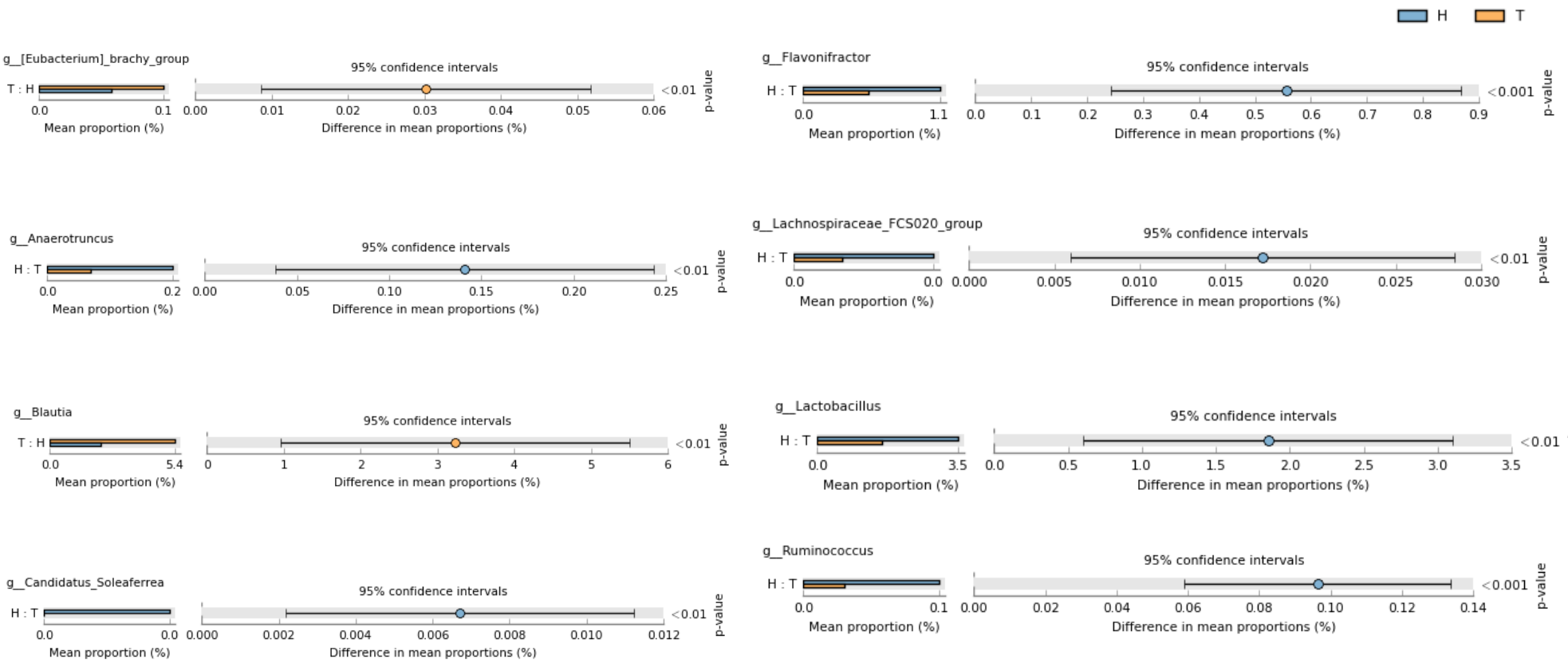
Figure 9.8 Significant differences (ANOVA and T-test, B–H FDR corrected p value, $P < 0.05$) in cumulative proportions of bacteria in the phylum Actinobacteria.

Based on a) treatments, b) temperature model, and c) treatment and temperature model interaction. Treatments groups include - A) Negative control treatment - chicks were offered a basal corn-soybean meal-wheat–based diet, B) Antibiotics treatment - chicks were offered NC + 0.05% bacitracin methylene disalicylate (BMD), D) *in ovo* folic acid (FA) treatment - eggs injected with 0.1 mL FA (FA; 95-102%; Thermo Scientific, USA) at 0.15 mg per egg, E) *In ovo* probiotics treatment - eggs injected with 0.2 mL of *Bacillus subtilis* fermentation extract, each egg received 10 × 106 CFU of the bacterium/0.2 mL saline diluent, and F) *in*

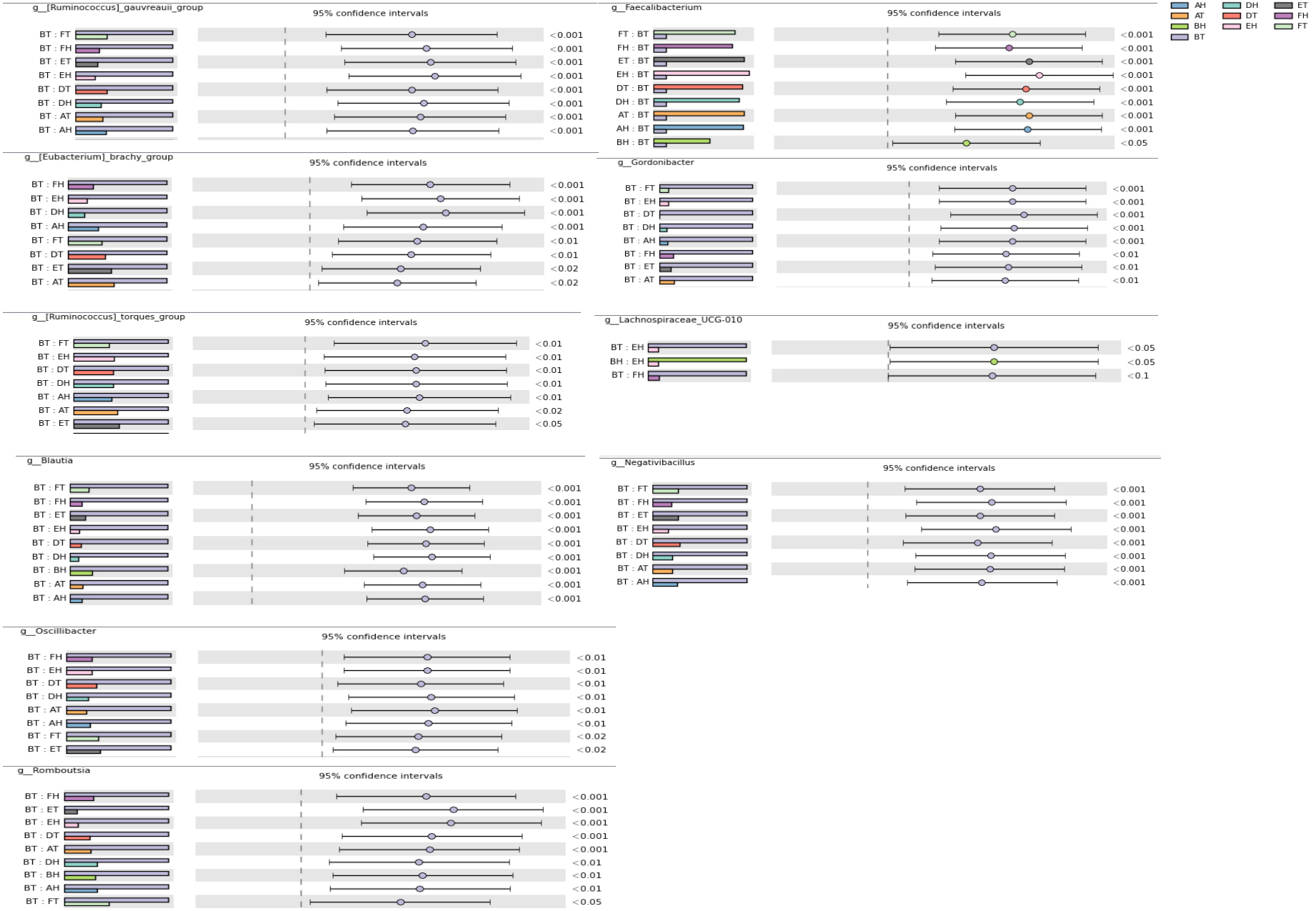
ovo + in-water essential oil (EO) treatment- eggs injected with 0.2 mL of a saline + EO blend mixture at a dilution ratio of 2:1 and supplied the EO via the in-water route at the recommended dosage of 250 mL/1000 L of drinking water. Temperature models include- Thermoneutral group (T) - birds were housed at 24ºC ± 0.2 from d 21 – 28, and Heat stress group (H) -birds were housed at 31ºC for 8hrs/day from d 21 – 28. Interaction effects include - AH) Negative control treatment under heat stress challenge, AT) Negative control treatment under thermoneutral condition, BH) Antibiotics treatment under heat stress challenge, BT) Antibiotics treatment under thermoneutral condition, DH) *in ovo* FA treatment under heat stress challenge, DT) *in ovo* FA treatment under thermoneutral condition, EH) *In ovo* probiotics treatment under heat stress challenge, ET) *In ovo* probiotics treatment under thermoneutral condition, FH) *in ovo* + in-water EO treatment under heat stress challenge, and FT)) *in ovo* + in-water EO treatment under thermoneutral condition.


A
B
D
E
F
g__Faecalibacterium
g__Lachnoclostridium
g__[Eubacterium]_brachy_group
g__Anaerostipes
g__Gordonibacter
g__Lachnospiraceae_UCG-010
g__[Ruminococcus]_gauvreauii_group
g__Blautia
g__Incertae_Sedis
g__Monoglobus
g__[Ruminococcus]_torques_group
g__Butyricicoccus
95% confidence intervals
Mean proportion (%)
Difference in mean proportions (%)
p-value
g__Negativibacillus
g__Romboutsia
g__Oscillibacter
g__Turicibacter
g__Sellimonas

(a)


H
T
g__[Eubacterium]_brachy_group
T : H
95% confidence intervals
<0.01
p-value
0.0
0.1
Mean proportion (%)
0.00
0.01
0.02
0.03
0.04
0.05
0.06
Difference in mean proportions (%)
g__Flavonifractor
H : T
95% confidence intervals
<0.001
p-value
0.0
1.1
Mean proportion (%)
0.0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0.9
Difference in mean proportions (%)
g__Anaerotruncus
H : T
95% confidence intervals
<0.01
p-value
0.0
0.2
Mean proportion (%)
0.00
0.05
0.10
0.15
0.20
0.25
Difference in mean proportions (%)
g__Lachnospiraceae_FCS020_group
H : T
95% confidence intervals
<0.01
p-value
0.0
0.0
Mean proportion (%)
0.000
0.005
0.010
0.015
0.020
0.025
0.030
Difference in mean proportions (%)
g__Blautia
T : H
95% confidence intervals
<0.01
p-value
0.0
5.4
Mean proportion (%)
0
1
2
3
4
5
6
Difference in mean proportions (%)
g__Lactobacillus
H : T
95% confidence intervals
<0.01
p-value
0.0
3.5
Mean proportion (%)
0.0
0.5
1.0
1.5
2.0
2.5
3.0
3.5
Difference in mean proportions (%)
g__Candidatus_Soleaferrea
H : T
95% confidence intervals
<0.01
p-value
0.0
0.0
Mean proportion (%)
0.000
0.002
0.004
0.006
0.008
0.010
0.012
Difference in mean proportions (%)
g__Ruminococcus
H : T
95% confidence intervals
<0.001
p-value
0.0
0.1
Mean proportion (%)
0.00
0.02
0.04
0.06
0.08
0.10
0.12
0.14
Difference in mean proportions (%)

(b)


AH
AT
BH
BT
DH
DT
EH
ET
FH
FT
g__[Ruminococcus]_gauvreauii_group
95% confidence intervals
BT : FT <0.001
BT : FH <0.001
BT : ET <0.001
BT : EH <0.001
BT : DT <0.001
BT : DH <0.001
BT : AT <0.001
BT : AH <0.001
g__Faecalibacterium
95% confidence intervals
FT : BT <0.001
FH : BT <0.001
ET : BT <0.001
EH : BT <0.001
DT : BT <0.001
DH : BT <0.001
AT : BT <0.001
AH : BT <0.001
BH : BT <0.05
g__[Eubacterium]_brachy_group
95% confidence intervals
BT : FH <0.001
BT : EH <0.001
BT : DH <0.001
BT : AH <0.001
BT : FT <0.01
BT : DT <0.01
BT : ET <0.02
BT : AT <0.02
g__Gordonibacter
95% confidence intervals
BT : FT <0.001
BT : EH <0.001
BT : DT <0.001
BT : DH <0.001
BT : AH <0.001
BT : FH <0.01
BT : ET <0.01
BT : AT <0.01
g__[Ruminococcus]_torques_group
95% confidence intervals
BT : FT <0.01
BT : EH <0.01
BT : DT <0.01
BT : DH <0.01
BT : AH <0.01
BT : AT <0.02
BT : ET <0.05
g__Lachnospiraceae_UCG-010
95% confidence intervals
BT : EH <0.05
BH : EH <0.05
BT : FH <0.1
g__Blautia
95% confidence intervals
BT : FT <0.001
BT : FH <0.001
BT : ET <0.001
BT : EH <0.001
BT : DT <0.001
BT : DH <0.001
BT : BH <0.001
BT : AT <0.001
BT : AH <0.001
g__Negativibacillus
95% confidence intervals
BT : FT <0.001
BT : FH <0.001
BT : ET <0.001
BT : EH <0.001
BT : DT <0.001
BT : DH <0.001
BT : AT <0.001
BT : AH <0.001
g__Oscillibacter
95% confidence intervals
BT : FH <0.01
BT : EH <0.01
BT : DT <0.01
BT : DH <0.01
BT : AT <0.01
BT : AH <0.01
BT : FT <0.02
BT : ET <0.02
g__Romboutsia
95% confidence intervals
BT : FH <0.001
BT : ET <0.001
BT : EH <0.001
BT : DT <0.001
BT : AT <0.001
BT : DH <0.01
BT : BH <0.01
BT : AH <0.01
BT : FT <0.05

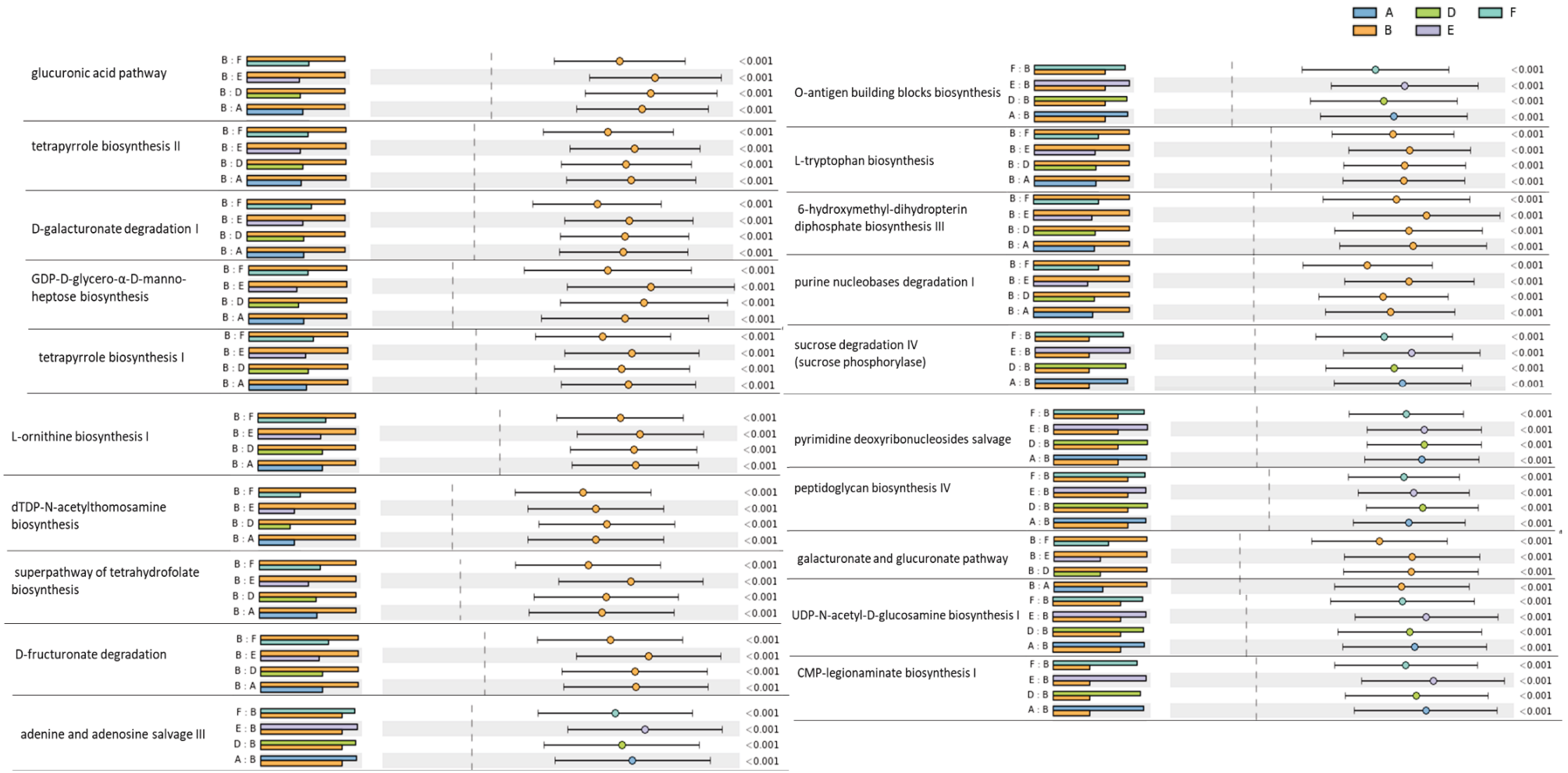
(c)

Figure 9.9 Significant differences (ANOVA and T-test, B–H FDR corrected p value, $P < 0.05$) in cumulative proportions of bacteria at the Genus taxa.

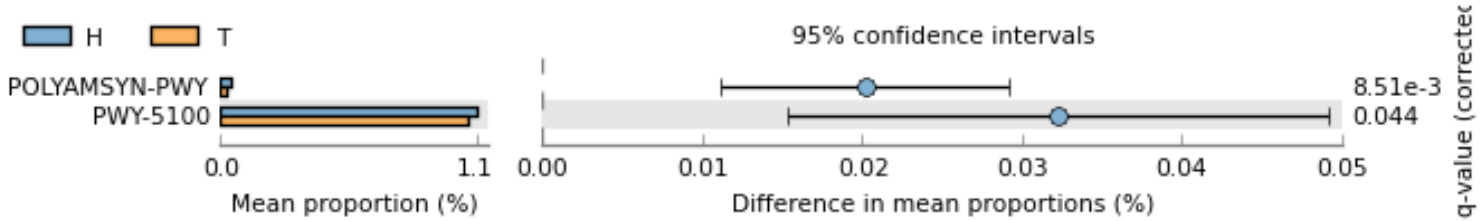
Based on a) treatments, b) temperature model, and c) treatment and temperature model interaction. Treatments groups include - A) Negative control treatment - chicks were offered a basal corn-soybean meal-wheat–based diet, B) Antibiotics treatment - chicks were offered NC + 0.05% bacitracin methylene disalicylate (BMD), D) *in ovo* folic acid (FA) treatment- eggs injected with 0.1 mL FA (FA; 95-102%; Thermo Scientific, USA) at 0.15 mg per egg, E) *In ovo* probiotics treatment - eggs injected with 0.2 mL of *Bacillus subtilis* fermentation extract, each egg received 10 × 106 CFU of the bacterium/0.2 mL saline diluent, and F) *in ovo* + in-water essential oil (EO) treatment- eggs injected with 0.2 mL of a saline + EO blend mixture at a dilution ratio of 2:1 and supplied the EO via the in -water route at the recommended dosage of 250 mL/1000 L of drinking water. Temperature models include - Thermoneutral group (T) - birds were housed at 24ºC ± 0.2 from d 21 – 28, and Heat stress group (H)-birds were housed at 31ºC for 8hrs/day from d 21 – 28. Interaction effects include- AH) Negative control treatment under heat stress challenge, AT) Negative control treatment under thermoneutral condition, BH) Antibiotics treatment under heat stress challenge, BT) Antibiotics treatment under thermoneutral condition, DH) *in ovo* FA treatment under heat stress challenge, DT) *in ovo* FA treatment under thermoneutral condition, EH) *In ovo* probiotics treatment under heat stress challenge, ET) *In ovo* probiotics treatment under thermoneutral condition, FH) *in ovo* + in-water EO treatment under heat stress challenge, and FT)) *in ovo* + in-water EO treatment under thermoneutral condition.

9.4.4 Predicted function of ceca microbiota

The predicted microbiota function is presented in Figures 9.9.1 and 9.9.2. The AGP treatment under TN conditions increased ($P < 0.01$) the number of functional genes involved in superpathways of tetrahydrofolate biosynthesis, superpathway of β-D-glucuronosides degradation, methanogenesis from acetate, L-fucose degradation 1, dTDP-N-acetylthomosamine biosynthesis, 6-hydroxymethyl-dihydropterin diphosphate biosynthesis, tetrapyrrole biosynthesis, purine nucleobases degradation 1, D-galacturonate degradation, L-tryptophan biosynthesis, thiazole component of thiamine diphosphate biosynthesis 1, and galacturonate and glucuronate pathways compared to other treatments, irrespective of thermal challenge conditions. Conversely, the same AGP treatment under TN conditions decreased ($P < 0.05$) the number of functional genes involved in the pyrimidine deoxyribonucleosides salvage, UDP-N-acetyl-D-glucosamine biosynthesis 1, peptidoglycan biosynthesis IV, O-antigen building blocks biosynthesis, and CMP-legionaminate biosynthesis 1 pathway. Only treatments effect was observed for genes involved in Glucuronic acid pathways, GDP-D-glycero-α-D-manno-heptose-biosynthesis, D-fructuronate degradation, L-ornithine biosynthesis 1, adenine and adenosine salvage III, and sucrose degradation IV pathways. In this case, the AGP treatment recorded higher ($P < 0.001$) number of genes involved in GDP-D-glycero-α-D-manno-heptose-biosynthesis, D-fructuronate degradation, L-ornithine biosynthesis 1 and Glucuronic acid pathways. On the contrary, the same AGP treatment recorded a lower ($P < 0.001$) number of functional genes involved in adenine and adenosine salvage III, and sucrose degradation IV pathways. Furthermore, genes involved in pyruvate fermentation to acetate and lactate II and superpathway of polyamine biosynthesis I were increased ($P = 0.04$) by HS in this study.


A
B
D
E
F
glucuronic acid pathway
B : F
B : E
B : D
B : A
<0.001
tetrapyrrole biosynthesis II
D-galacturonate degradation I
GDP-D-glycero-α-D-manno-heptose biosynthesis
tetrapyrrole biosynthesis I
L-ornithine biosynthesis I
dTDP-N-acetylthomosamine biosynthesis
superpathway of tetrahydrofolate biosynthesis
D-fructuronate degradation
adenine and adenosine salvage III
F : B
E : B
D : B
A : B
O-antigen building blocks biosynthesis
L-tryptophan biosynthesis
6-hydroxymethyl-dihydropterin diphosphate biosynthesis III
purine nucleobases degradation I
sucrose degradation IV (sucrose phosphorylase)
pyrimidine deoxyribonucleosides salvage
peptidoglycan biosynthesis IV
galacturonate and glucuronate pathway
UDP-N-acetyl-D-glucosamine biosynthesis I
CMP-legionaminate biosynthesis I

(a)



(b)

Figure 9.10 Predicted functions of ceca microbiota.

Based on a) treatments, and b) temperature model. Treatments groups include - A) Negative control treatment - chicks were offered a basal corn-soybean meal-wheat–based diet, B) Antibiotics treatment - chicks were offered NC + 0.05% bacitracin methylene disalicylate (BMD), D) *in ovo* folic acid (FA) treatment - eggs injected with 0.1 mL FA (FA; 95-102%; Thermo Scientific, USA) at 0.15 mg per egg, E) *In ovo* probiotics treatment- eggs injected with 0.2 mL of *Bacillus subtilis* fermentation extract, each egg received 10 × 106 CFU of the bacterium/0.2 mL saline diluent, and F) *in ovo* + in-water essential oil (EO) treatment - eggs injected with 0.2 mL of a saline + EO blend mixture at a dilution ratio of 2:1 and supplied the EO via the in-water route at the recommended dosage of 250 mL/1000 L of drinking water. Temperature models include- Thermoneutral group (T) - birds were housed at 24ºC ± 0.2 from d 21 – 28, and Heat stress group (H)-birds were housed at 31ºC for 8hrs/day from d 21 – 28.

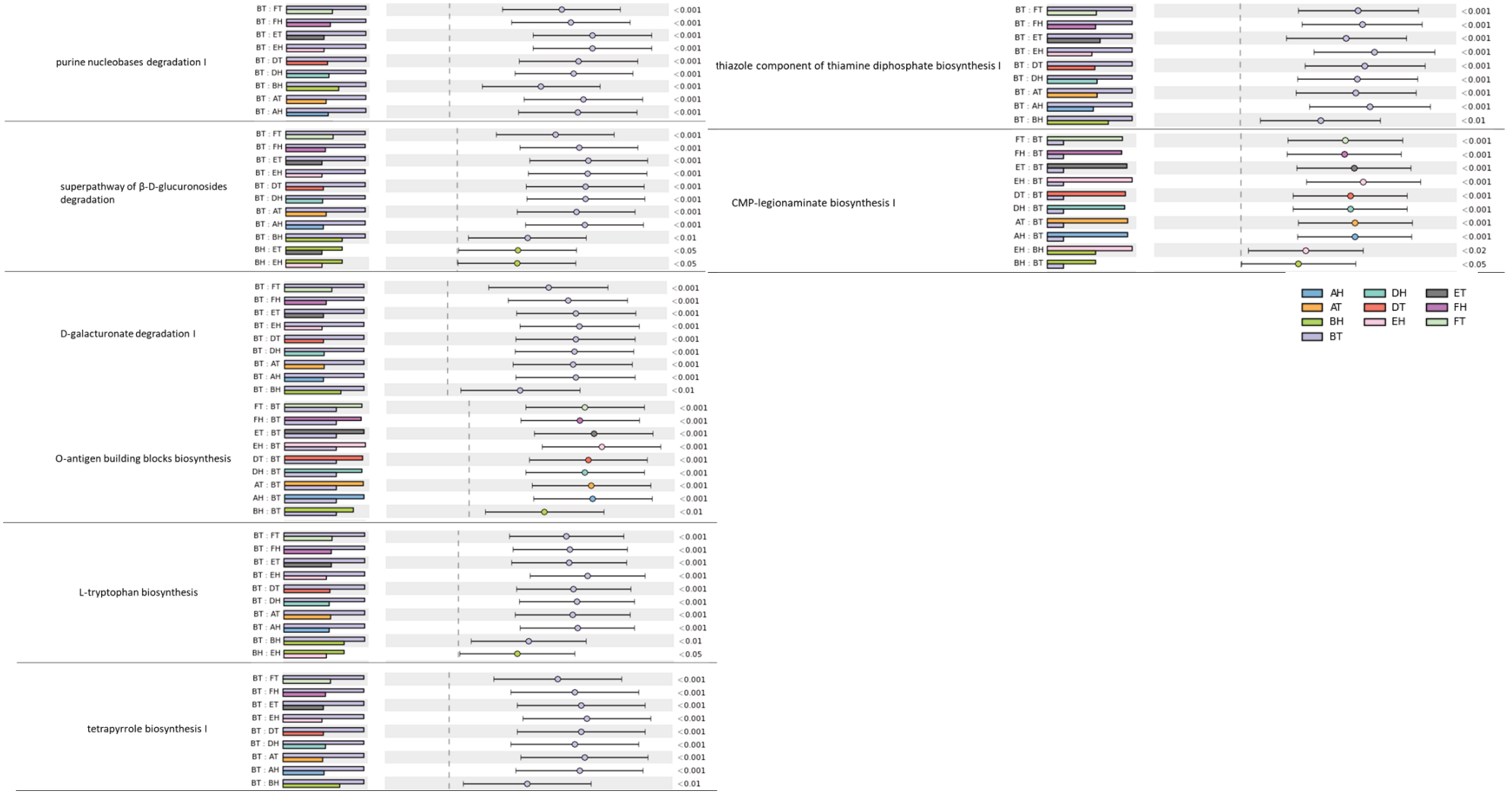


Figure 9.11 Predicted functions of ceca microbiota based on treatment and temperature model interaction.

Treatments groups include- A) Negative control treatment- chicks were offered a basal corn-soybean meal-wheat–based diet, B) Antibiotics treatment- chicks were offered NC + 0.05% bacitracin methylene disalicylate (BMD), D) *in ovo* folic acid (FA)

treatment- eggs injected with 0.1 mL FA (FA; 95-102%; Thermo Scientific, USA) at 0.15 mg per egg, E) *In ovo* probiotics treatment- eggs injected with 0.2 mL of *Bacillus subtilis* fermentation extract, each egg received 10 × 106 CFU of the bacterium/0.2 mL saline diluent, and F) *in ovo* + in-water essential oil (EO) treatment- eggs injected with 0.2 mL of a saline + EO blend mixture at a dilution ratio of 2:1 and supplied the EO via the in-water route at the recommended dosage of 250 mL/1000 L of drinking water. Temperature models include- Thermoneutral group (T)- birds were housed at 24ºC ± 0.2 from d 21 – 28, and Heat stress group (H)-birds were housed at 31ºC for 8hrs/day from d 21 – 28. Interaction effects include- AH) Negative control treatment under heat stress challenge, AT) Negative control treatment under thermoneutral condition, BH) Antibiotics treatment under heat stress challenge, BT) Antibiotics treatment under thermoneutral condition, DH) *in ovo* FA treatment under heat stress challenge, DT) *in ovo* FA treatment under thermoneutral condition, EH) *In ovo* probiotics treatment under heat stress challenge, ET) *In ovo* probiotics treatment under thermoneutral condition, FH) *in ovo* + in-water EO treatment under heat stress challenge, and FT)) *in ovo* + in-water EO treatment under thermoneutral condition.

9.5 Discussion

Given the importance of ceca microbiota in poultry performance and health (Stanley et al., 2013; Proszkowiec-Weglarz et al., 2020), an exposition on the interactions between the host and its resident microbiota, especially under varying management condition, would be insightful. AGP has been theorized to improve growth promotion in poultry by the modulation of cecal microbiota dynamics (Zhou et al., 2020). Here, we provide evidence on microbiota-mediated mode of action of AGP and *in ovo* delivered bioactive substances under varying thermal conditions.

Alpha diversity (Shannon index) was not affected by *in ovo* delivered treatments, AGP, and HS in this study, suggesting similarity in the number of resident species. Similar to this outcome, several other studies have also reported no effect of nutritional supplements (Bortoluzzi et al., 2017; Chang et al., 2020; Proszkowiec-Weglarz et al., 2020; Oladokun et al., 2021a; Kouzounis et al., 2022; Oladokun et al., 2022), AGP (Danzeisen et al., 2011; Pourabedin et al., 2015; Kumar et al., 2018; Proctor et al., 2019) and HS (Xing et al., 2019; Liu et al., 2020; Nelson et al., 2020; Emami et al., 2022) on alpha diversity metrics in poultry. Notwithstanding, extreme events that trigger gut dysbiosis have been reported as capable of altering bacteria counts and ecological niches (Thibodeau et al., 2015). Considering that intestinal dysbiosis has been reported to be a physiological response to HS (Oladokun and Adewole, 2022b) it is only rational to expect HS-induced changes in alpha diversity. However, the effect of HS on alpha diversity might depend on the intensity and duration of thermal challenge. For instance, HS-induced changes in specie richness after 2 wk heat exposure have been reported in broiler chickens and laying hens (Hsieh et al., 2017; Wang et al., 2018c). Furthermore, unlike the results reported for alpha diversity, AGP, especially under TN condition, caused a shift in beta diversity compared to other evaluated treatments in this study. This indicates that AGP triggered a spatial turnover in taxonomic profile abundances in the ceca. Unsurprisingly, the modification of gut microbial diversity is purportedly AGP's growth-promoting mode of action (Dibner and Richards, 2005). In conformation with the result presented here, several other studies have reported the capacity of several AGPs (bacitracin inclusive) to induce a shift in beta diversity in poultry (Czerwiński et al., 2012; Costa et al., 2017; Banerjee et al., 2018; Kumar et al., 2018; Le Roy et al., 2019; Johnson et al., 2019; Adewole, 2020; Elokil et al., 2020; Lemos et al., 2020). Nonetheless, more studies are needed to provide holistic insights into the cause and effect of such AGP-induced shift in microbial diversity. Moreover, AGP-driven shifts in microbial structure have been associated with decreased adaptive immune system stimulation in chickens (Czerwiński et al., 2012; Costa et al., 2017; Banerjee et

al., 2018; Le Roy et al., 2019; Johnson et al., 2019; Yitbarek et al., 2019; Elokil et al., 2020; Lemos et al., 2020) and is reported to be dose-dependent (Johnson et al., 2019).

Furthermore, independent of treatment combinations from this study, the ceca microbial composition at the phylum taxa was majorly dominated by *Firmicutes*, *Proteobacteria* and *Actinobacteria*. This is consistent with reported ceca microbiota census (Corrigan et al., 2015; Rodrigues et al., 2020; Oladokun et al., 2021a; Erinle et al., 2022a; Oladokun et al., 2022). Nevertheless, both the *in ovo* + in-water EO and the AGP treatments increased the proportion of bacteria in the phylum *Actinobacteria* in this study. Phylum *Actinobacteria* consists of non-motile, anaerobic Gram-positive bacteria with varying metagenomic functions reported in the literature. While *Actinobacteria* have been negatively correlated with vitamin B6 metabolism, glycolysis/gluconeogenesis, and increased susceptibility to *S. Enteritidis* infections (Mon et al., 2015; Hernandez-Patlan et al., 2019b), other studies have equally reported beneficial role of this phylum related to primary bile acid biosynthesis and drug metabolism, anti-infectives, gut homeostasis and improved growth performance (Ogawara, 2014; Landwehr et al., 2016; Binda et al., 2018; Das et al., 2020). A recent study has also highlighted *Actinobacteria*'s role in short-chain fatty acid (**SCFA**) production (Mazalli et al., 2022). Given the healthy state of the flock in this study, it is probable that the beneficial effect of this phylum was in effect.

At the genus taxa, AGP increased the proportion of bacteria in the genera *Blautia*, *Lachnospiraceae, Anaerostipes, lachnoclostridium, Sellimonas* in this study. The proportions of bacteria in the genera *lachnoclostridium and Sellimonas* were also increased by the *in ovo* + in-water EO treatment. Intriguingly, all of the highlighted bacteria genera belong to the family *Lachnospiraceae*. The *Lachnospiraceae* family comprises anaerobic, fermentative, and chemoorganotrophic bacteria with strong hydrolyzing activities. They are early gut colonizers known for their ability to utilize diet-derived polysaccharides in the production of SCFA, especially butyrate, important energy source for the host (Vacca et al., 2020; Hankel et al., 2021). In agreement with the results presented here, an increased proportion of members of this bacteria family following AGP supplementation are well reported in the literature (Zhu et al., 2019; Trela et al., 2020; Erinle et al., 2022b). Our laboratory has also reported increased proportion of members of this bacteria family in AGP supplemented birds, alongside improved growth performance in the same birds (Oladokun et al., 2022), suggesting that the *in ovo* + in water EO treatment might also be able to exploit similar microbiota-mediated approach to improve growth performance in poultry. In a similar manner, AGP was found to increase the proportion of other

genera associated with increased SCFA production, including *Ruminococcus* groups (alongside the *in ovo* + in water EO treatment), *Eubacterium*, *Butyricicoccus*, and *Monoglobus.* Members of these genera are able to positively modulate improved gut homeostasis via fiber degradation to yield SCFA as energy metabolites (Fernández-Rubio et al., 2009; Torok et al., 2013; Geirnaert et al., 2014; Adewole, 2020; Adewole et al., 2021; Hao et al., 2022). These metabolites positively influence gut health by acting as energy sources for the enteric cells, maintaining intestinal barrier integrity, inhibiting proinflammatory pathways, and countering colonic oxidative stress (Geirnaert et al., 2014). Additionally, AGP was found to differentially increase the proportions of members of genera *Gordonibacter, Turicibacter,* while reducing the proportions of *Faecalibacterium, Negativibacillus, Oscillibacter, and Romboutsia* in the ceca. The *Faecalibacterium, Turicibacter,* and *Romboutsia* genera are commensal SCFA-producing species capable of inducing anti-inflammatory actions in the gut (Wang et al., 2016; Clavijo and Flórez, 2018; Adewole et al., 2021; Yang et al., 2022). The *Negativibacillus* genus has also been positively correlated with improved body weights in rats (Yan et al., 2017; Wang et al., 2019). Conversely, t*he Gordonibacter* genus has been implicated in immunological stress conditions (Kong et al., 2022), as well as in the etiology of inflammatory bowel disease conditions in humans (Würdemann et al., 2009). Our team has previously buttressed the cost-benefit microbiota properties of AGP use in broiler production (Oladokun et al., 2022). These incidental properties give another ground to question the sustainability of AGP use in broiler production. Is there a possibility that at an undefined timepoint, the genera associated with negative effects outweigh those with reported positive effects either via competitive exclusion, dominance, or succession? Additionally, in view of HS dysbiotic role in ceca microbiota homeostasis (Oladokun and Adewole, 2022b) its reduction of SCFA-producing genera (*Ruminococcus, Eubacterium, and Blautia)* and *Candidatus Soleaferrea)* in this study was not surprising. The genus *Candidatus Soleaferrea* have been negatively correlated with body weight in chicken (Xiang et al., 201), as well as increased susceptibility to subclinical necrotic enteritis infection (Zhao et al., 2022). What was more surprising, was the increased proportion of other potentially beneficial genera (*Lachnospiraceae*, *Anaerotruncus, Flavonifractor, and Lactobacillus)* with reported SCFA-producing capacity (Gong et al., 2002b; Polansky et al., 2016; Clavijo and Flórez, 2018; Hankel et al., 2021) under HS condition. *Lactobacillus,* for instance, are popular probiotic candidate in poultry production as a result of their bacteriostatic and SCFA-producing abilities (Gong et al., 2002b) and are generally reported to be reduced by HS in poultry (Al-Fataftah et al., 2014; Song et al., 2014;

Zhang et al., 2017). Despite the paucity of studies in this respect, one study reported an increased proportion of the *Lactobacillus* genus following a heat stress challenge (34–38 °C for 28 d) with no rationale offered. It is possible that the reported beneficial effects of the *Lactobacillus* genus are indeed species-specific, with HS potentially increasing the proportions of species with putatively non-beneficial effects. In any case, more studies are needed to provide comprehensive insight in this regard.

Finally, using PICRUSt2 (Douglas et al., 2020), the metagenomes of broiler chicken ceca microbiome were predicted. The prediction revealed AGP treatment significantly enriched metabolic pathways related to carbohydrate/sugar degradation (β-D-glucuronosides degradation, L-fucose degradation 1, D-galacturonate degradation, D-fructuronate degradation, glucuronic acid, and galacturonate and glucuronate pathways), energy generation and carbohydrate biosynthesis (methanogenesis from acetate, purine nucleobases degradation 1, dTDP-N-acetylthomosamine biosynthesis, and GDP-D-glycero-α-D-manno-heptose-biosynthesis), vitamin biosynthesis (tetrahydrofolate biosynthesis, 6-hydroxymethyl-dihydropterin diphosphate biosynthesis, tetrapyrrole biosynthesis, thiazole component of thiamine diphosphate biosynthesis 1) and amino acid biosynthesis (L-tryptophan biosynthesis and L-ornithine biosynthesis 1). In contrast, metabolic pathways related to nucleotide and nucleoside biosynthesis (pyrimidine deoxyribonucleosides salvage and UDP-N-acetyl-D-glucosamine biosynthesis 1), cell structure biosynthesis (peptidoglycan biosynthesis IV) and specific carbohydrate synthesis (O-antigen building blocks biosynthesis, CMP-legionaminate biosynthesis 1) were depleted with AGP supplementation. The highlighted metabolic pathways induced by our treatments combinations in this study are, to a great extent, consistent with the presented results on taxonomic composition and growth performance (data not shown). Consistent with our report, previous studies have strongly correlated the *Ruminococcus* genus with increased enrichment of the tetrahydrofolate biosynthesis and methanogenesis from acetate pathways (Hu et al., 2021; Van Heck et al., 2022). Similarly, mice microbiota treated with AGP has also presented an increase in the carbohydrate biosynthesis (dTDP-N-acetylthomosamine biosynthesis) pathway (Eslabão et al., 2022). D-glucuronic acid— an end product of microbial glucuronide deconjugation catalyzed by β-glucuronidase has also been associated with AGP (vancomycin) treatment of human microbiota (Sunwoo et al., 2020). Higher abundance of microbial genes involved in the L-tryptophan biosynthesis pathway has also been reported in AGP (vancomycin) treated humans (Madany et al., 2022). O-antigens from the AGP-induced reduction of O-antigen building blocks biosynthesis

metabolic pathway are a critical part of lipopolysaccharide membrane, an immunotolerance response of microbial community in the gut (Delcour et al., 2009). However, it is unclear if a reduced microbial O-antigen building blocks biosynthesis directly correlates with reduced lipopolysaccharide biosynthesis. Downregulated O-antigen building blocks biosynthesis has been associated with microbial inhibitory effect of a live bacterial product in humans (Dizman et al., 2022). Furthermore, it is rational that HS increases the pyruvate fermentation to acetate and lactate II pathway, as presented in this study: considering that this pathway has been reported to be utilized by *Thermotoga maritima,* a hyperthermophilic anaerobic eubacterium which produces fermentation products (like acetate and acetate) using glucose as a substrate (Schröder et al., 1994). In summary, the differentially enriched metabolic pathways suggest that the AGP treatment afforded high potency for biosynthesis of essential nutrients (amino acids and vitamins especially) and utilization of carbon sources derived from both diet and the host in order to ensure improved growth performance, particularly under TN condition.

9.6 Conclusions

Under the reported experimental conditions, AGP supplementation caused a significant shift in microbial diversity than HS did. Both *in ovo* delivered treatments and thermal challenge differentially influenced ceca microbiota taxonomic composition. While HS reduced the proportion of members of the *Actinobacteria* phylum, the *in ovo* + in-water EO and AGP treatments increased the proportion of bacteria in this phylum. The cumulative proportions of bacteria in the genera *lachnoclostridium and Sellimonas* were both increased by the *in ovo* + in-water EO and AGP treatment. The AGP treatment also increased the cumulative proportions of bacteria in the genera *Blautia*, *Lachnospiraceae, Anaerostipes, lachnoclostridium, Sellimonas,* all in the *Lachnospiraceae* family, while reducing the proportion of bacteria in the genera *Faecalibacterium, Negativibacillus, Oscillibacter, and Romboutsia* in the ceca. On the other hand, HS reduced the proportion of bacteria in the genera *Ruminococcus, Eubacterium, Blautia,* and *Candidatus Soleaferrea* in this study. Metagenomic prediction revealed that the AGP treatment enriched carbohydrate degradation, carbohydrate synthesis, vitamin biosynthesis, and amino acid biosynthesis-related pathways. Similarly, HS increased the pyruvate fermentation to acetate and lactate II and polyamine biosynthesis I metabolic pathways. This study provides critical insight into microbiota-mediated role of AGP in growth promotion. Results from this study suggests that the microbiota-mediated role of AGP in growth promotion is related to improved biosynthesis of

essential nutrients (amino acids and vitamins especially) and utilization of carbon sources derived from the host diet and microbiome. Also, considering the close similarities in taxonomic profile between the *in ovo* + in-water EO and AGP treatment in this study, the *in ovo* + in water EO treatment might be a good candidate for further optimization study as a potential alternative to AGP. Additionally, all differentially regulated bacteria genera highlighted from this study could be potential candidates for precision biotics development, especially when supplied as synbiotics as a result of the fiber degrading ability to yield SCFA reported for most of these bacteria.

CHAPTER 10 CONCLUSIONS AND FUTURE DIRECTIONS

Global restrictions on the use of AGP have necessitated new approaches to maintaining poultry gut health. In the post-AGP era, if birds are to reach their maximum genetic potential, maximum attention needs to be placed on the early establishment of immunocompetence and optimum gut health. Consequently, this thesis has extensively evaluated the *in ovo* delivery of bioactive substances and its effect on growth performance and different aspects of gut health (morphology, microbiota, and immune system). As a strategic means of optimizing poultry gut health and performance, results from this thesis reveal that the *in ovo* delivery of probiotics, EO, and FA shows promising benefits as alternatives to AGP in the post-AGP era.

It is apparent that the successful development of effective alternatives to AGP is contingent upon a good understanding of AGP's gut modifying mechanisms. This thesis provided mechanistic insights into the gut microbiota-mediated mode of action of AGP. From four different experiments, this thesis suggests that AGP is able to improve growth performance in broiler chickens by increasing the abundance of bacteria in the family Lachnospiraceae. The increased abundance of bacteria in this family in the AGP treatments was parallel with growth performance data for the same treatment in this thesis. Increased abundance of bacteria in the family Lachnospiraceae has been associated with increased butyrate production (Vacca et al., 2020; Hankel et al., 2021), a supplementary source of energy for the gut enteric cells, which leads to improved weight gain. Members of the Lachnospiraceae are thus potential candidates for precision post-biotics development. Despite the growth-enhancing benefits obtainable from AGP use in poultry, this thesis shows that such benefits come at a metabolic cost. For the first time in the literature, this thesis reports that AGP might be capable of increasing the concentrations of blood electrolytes- sodium and chloride (Chapters 4 and 7). High concentrations of these electrolytes are implicated in the incidences of dehydration, edema, acidosis, poor bone development, and decreased humoral immunity (Oviedo-Rondón et al., 2001; Pohl et al., 2013). The antibiotic treatment was also found to increase plasma levels of AST (Chapter 5), a non-specific biomarker of liver health. At the molecular level, the AGP treatment downregulated the hepatic expression of the CLCA1 (chloride channel accessory 1) gene in male broiler chicken transcripts (Chapter 6). The downregulation of this gene is reported to enhance the pro-inflammatory cytokines associated with increased energy demands (Dietert et al., 2014; Mamber et al., 2020). These findings further

justify the unsustainability of the continuous use of AGP in the poultry industry. This thesis also provided information on the effect of AGP on the broiler chicken ceca metagenome. The growth-promoting effect of AGP is shown to be exerted by differential enrichment of metabolic pathways related to the biosynthesis of essential nutrients (amino acids and vitamins especially) and utilization of carbon sources derived from both diet and the host (Chapter 9).

Furthermore, this thesis showed that the *in ovo* delivery of probiotics (*Bacillus subtilis* fermentation extract) was mostly comparable with the AGP treatments' ability to ensure gut microbiota homeostasis, enhanced gut morphology, and feed conversion efficiency (Chapters 3 and 4). The procedure for amniotic delivery of *Bacillus subtilis* fermentation extract was also successfully validated in two separate experiments, recording > 90% hatchability. An optimum dosage (10×10^6 CFU) of this probiotics product was also validated (Chapter 4). Additionally, *in ovo* delivery of FA (0.15 mg FA/egg) was also comparable to AGP in performance effect in this thesis (Chapter 7). *In ovo* delivery of FA (0.15 mg FA/egg) enhanced broiler chicken gut morphology and FCR in a similar capacity as the in-feed antibiotic treatment, with birds consuming less quantity of feed. However, the *in ovo* delivery of FA recorded a negative hatchability outcome. Furthermore, this thesis showed that maximum benefits are derivable from successive delivery of EO via *in ovo* and in-water route rather than either of these routes independently (Chapter 5). Successive delivery of a commercial EO blend (containing star anise, cinnamon, rosemary, and thyme) via *in ovo* and in-water route improved broiler chicken's antioxidant status (TAC: +400%) and blood biochemical profile (creatine kinase: -69.5% and AST: -23%). Also, successive delivery of EO via in *in ovo* and in-water route improves broiler chicken gut integrity under TN condition and show potential thermotolerance effect (a 3.5-fold significant upregulation of SLC1A1) under HS condition.

Based on the findings from this thesis, a few future research leads are recommended. To guarantee positive hatchability outcomes from the *in ovo* delivery of FA and EO, the use of liquid FA forms and EO formulated for *in ovo* delivery is advised. Subsequent in-feed supplementation of probiotics following *in ovo* delivery to stimulate significant post-hatch performance is another area that warrants further investigation. Also, considering the observed result on probiotics' role on immunoglobulin concentration, it would be worthwhile to investigate the effect of *in ovo* delivered probiotics under a disease challenge model. This thesis also provides preliminary evidence suggesting unique sex-controlled hepatic differential gene expression in broiler chickens offered *in ovo* EO treatments. *In vivo* studies that take sex into consideration are needed to validate

the results presented here fully. Finally, as most of the gut-promoting benefits derived from the successive delivery of EO via *in ovo* + in-water was under TN condition, further optimization of EO dosages via these routes to guarantee maximum benefits under HS condition is needed.

Finally, considering that questions and not answers are the basis of scientific inquiry, further hypotheses thus arise from this thesis. Based on the results of *in ovo* delivery of probiotics in this study and previously reported speculation that a single time point delivery of *in ovo* probiotics might not guarantee maximum growth performance gains, it is rational to inquire; are the benefits accruing from *in ovo* probiotics use transient? Additionally, it was reported that AGP upregulated the hepatic expression of SLC5A10 (solute carrier family 5-member 10) in male broiler chicken transcripts in this thesis. Slc5a10 (encoding SGLT5) is a mannose, fructose, and to a less degree, a glucose and galactose transporter (Wright, 2013; Chittka et al., 2018). Although glucose transporter 2 (GLUT2) is considered the main sugar transporter relevant to liver function (Leturque et al., 2005). Fukuzawa et al. (2013) have reported exacerbated hepatic steatosis induced by diminished sodium-dependent fructose uptake in SGLT5-deficient mice. Based on this, it is necessary to ask, is the SLC5A10 gene a valid biomarker of liver health?

Conclusively, all *in ovo*-delivered bioactive substances were mostly comparable to the AGP treatment in their ability to optimize broiler chicken gut health and performance. However, the successive delivery of EO via *in ovo* and in-water routes seemed to afford the most gut-optimizing benefits. Nonetheless, the hatchability outcome of this delivery route would need to be optimized through further studies. While this thesis may not have provided answers to all our inquiry, it is hopeful that it has indeed provided sufficient background to justify that someday in the near future we might only need to “feed the egg and not the chick” our bioactive substances.

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