Encapsulated butyric acid and *Bacillus subtilis* as antibiotic substitutes to mitigate heat stress and promote gut health and performance in broilers

by

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Declaration

I, Michaela Sharon Faulhaber, hereby declare that this dissertation, submitted for the MSc (Agric) Animal Science: Animal Nutrition degree at the University of Pretoria, is my own work and has not previously been submitted by me for a degree at any tertiary institution.

Signature: .....

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Abstract

The removal of in-feed antibiotics has created increased focus on the potential alternatives to replace antibiotic growth promotors. With rise in global warming and the inability of birds to efficiently dissipate heat, the need to find ways to reduce the incidence and risk associated with heat stress is important by modulating the gut to reduce the negative impacts caused by heat stress. Probiotics and encapsulated butyric acid have shown promise as alternatives to antibiotics as well as potential mitigators of the effect of heat stress on the gut health and performance in broilers. The study aimed to determine the response of broiler chickens supplemented with encapsulated butyric acid and *B. subtilis* combined and alone against zinc bacitracin with the intention of improving overall gut health, the microbiome composition and growth performance and to mitigate the effect of heat stress in male broilers. One thousand nine hundred and twenty (1920) day-old male Ross 308 chicks were distributed amongst two environmentally controlled houses which were either at heat stress conditions or thermoneutral conditions. Both houses were run simultaneously and consisted of six dietary treatments with eight replications per treatment and twenty broilers per pen. The dietary treatments were as follows: basal diet; basal diet with zinc bacitracin; basal diet with encapsulated butyric acid (EBA); basal diet with zinc bacitracin and encapsulated butyric acid; basal diet with a *B. subtilis*-based probiotic; and basal diet with *B. subtilis* and encapsulated butyric acid. Production parameters were observed on a weekly basis. On day 21 and 35, two birds per pen were euthanised and samples of the small intestine and digesta were collected for histomorphological and microbiome analysis, respectively. Overall gut health was also scored. Dietary inclusion of *B. subtilis* in combination with EBA revealed no significant improvement in growth performance although results were comparable to the antibiotic treatment. Thermotolerance of the birds were improved by the inclusion of a combination of *B. subtilis* and EBA. Protection of the gut integrity, villi-crypt structure and intestinal microbiota environment also ameliorated the adverse effect of heat stress on gut health, resulting in growth performance being comparable to AGP.

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List of Abbreviations

°C	Degrees Celsius
µm	Micrometres
µm:µm	Micrometres to micrometres
AGP	Antibiotic growth promoter
bp	Base pairs
BW	Live body weight
CFCR	Cumulative feed conversion ratio
CFI	Cumulative feed intake
cfu	Colony forming units
cm	Centimetres
CP	Crude protein
d	Days of age
D	Duodenum
DFM	Direct-fed microbials
DGGE	Denaturing gradient gel electrophoresis
DNA	Deoxyribonucleic acid
EBA	Encapsulated butyric acid
ESBL	Extended spectrum β-lactamase
EU	European Union
FCR	Feed conversion ratio
FI	Feed intake
g	Grams
GIT	Gastrointestinal tract
GLM	Generalised Linear Model
GT	Gauteng
HCl	Hydrochloric acid
HE	Haematoxylin and Eosin
HPA	Hypothalamo-pituitary-adrenal axis
HS	Heat-stressed
HT-NGS	High-throughput next-generation sequencing
I	Ileum
Inc	Incorporated
J	Jejunum
kg	Kilograms

Ltd	Limited
m	Meters
Max	Maximum
MB	Megabytes
MDR	Multi-drug resistant
mg	Milligrams
Min	Minimum
MJ	Megajoules
mm	Millimetres
MRSA	Methicillin-resistant *Staphylococcus aureus*
OTU	Operational taxonomic unit
PC1	Principal component one
PC2	Principal component 2
PCA	Principal component analysis
PCR	Polymerase chain reaction
PEF	Production efficiency factor
ppm	Parts per million
Pty	Proprietary
q-PCR	Quantitative polymerase chain reaction
RNA	Ribonucleic acid
rRNA	Ribosomal ribonucleic acid
RSA	Republic of South Africa
SAS	Statistical Analysis System
SCFA	Short-chain fatty acid
Spp	Species
SSCP	Single-strand conformation polymorphism
Temp	Temperature
TN	Thermoneutral
ton	Tonnes
T-RFLP	Terminal restriction fragment length polymorphism
v:v	Volume to volume
VFA	Volatile fatty acid
VH/CD	Villi height to crypt depth ratio
VRE	Vancomycin-resistant *Enterococcus*
α	Alpha
β	Beta

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Chapter 1: Introduction

1.1 Introduction

The ever-growing world population continues to accelerate the demand for protein, resulting in an increased pressure on the poultry industry to produce a bird reaching the required or higher slaughter weight but at a decreasing age (Koppenol *et al.*, 2015). Due to the increased consumption of poultry, the ever-growing human population and the need to intensify farming practices, producers have selected birds with a faster growth rate and higher carcass gain to meet the growing demand without much focus on the immunity and health of the bird. Intensification of the poultry industry has also increased the risk of any infectious disease being rapidly transferred from one individual to the next (Zhang *et al.*, 2011).

Gut health is a broad and complex term which encompasses a multitude of interconnecting factors including the microbiome, histomorphological structures of the mucosal layer, gut barrier function and permeability as well as the immune system. In poultry, feed intake and efficient nutrient absorption are determined by the health status of the gastrointestinal tract (Ducatelle *et al.*, 2018). As the growth period is shortened with an increase in feed efficiency, requirements for health care and nutrition of poultry are becoming more challenging. Attending to the miniscule changes occurring in the gut has become imperative as these changes are often ignored due to the subtle and microscopic damage created in the gut (Choct, 2009). The gastrointestinal tract plays many vital parts in nutrient digestion and absorption as well as a significant role as a barrier between the external and internal environment (Abdelqader & Al-Fataftah, 2016; Zhang *et al.*, 2017). The gut serves as a major location for potential exposure to environmental pathogens, thus a healthy gut performing at optimum is the foundation of optimal bird performance (Sugiharto, 2016). When gut health and function is hindered, digestion and absorption are impaired resulting in animal performance issues, decrease in feed efficiency, a higher prevalence of dysbacteriosis, and an increase in the prevalence of disease (Abdelqader & Al-Fataftah, 2016; Sugiharto, 2016). Poultry are particularly vulnerable to potential pathogenic microbes such as *Escherichia coli*, *Salmonella* spp., *Clostridium perfringens*, and C*ampylobacter sputorum* which compete in the small intestine with the host for nutrients and reduces the digestion of fat and fat-soluble vitamins (Gunal *et al.*, 2006). Therefore, to support the peak functioning of the intestinal mucosal barrier, the balance between epithelial cells, mucosal layer, microbiota and the immune system within the intestine is of importance (Choct, 2009; Sugiharto, 2016).

The risk of disease has partly been mitigated over decades by the use of antibiotics and has been exploited in both human medicine as well as the production of livestock to improve human and animal health (Novick, 1981; Dahiya *et al.*, 2006; Huyghebaert *et al.*, 2011;

Sugiharto, 2016). Recent data suggests that the usage of antibiotics in the livestock industry is greater than the entire human population (Van Boeckel *et al.*, 2015). Typically, there are three purposes for the use of antibiotics in livestock animals: therapeutic reasons (to cure a disease), prophylactic reasons (to prevent a disease) and to use as growth promotants (sub-therapeutic levels of antibiotics given to animals to increase growth rate and feed efficiency) (Huyghebaert *et al.*, 2011). The use of low-dose antibiotics has been a common practice for over 30 years; with the first recorded research trial taking place in 1946 (Buntyn *et al.*, 2016). However, the potential for microbial resistance to antibiotics has resulted in countries legislatively removing all antibiotics which are fed at subtherapeutic levels from animal feeds. Removal of antibiotics has resulted in decreased growth performance, decreased gut health and increased prevalence of diseases. Consequently, the need to research alternatives to these antibiotics has expanded as consumer pressure for “antibiotic-free meat” has intensified. These alternatives are needed to promote growth performance and gut health in broiler chickens similar or equivalent to that of AGPs.

Furthermore, heat stress has become a critical problem due to global warming. Heat stress negatively impacts the fast growing broiler birds, particularly during the summer seasons in temperate climates and in hot climates, because of their high metabolic rates and the poor ability to effectively dissipate heat (Alhenaky *et al.*, 2017). High ambient temperature has shown to influence bird behaviour (Wang *et al.*, 2018) and induce multiple physiological changes such as disruption of the systemic immune system and endocrine system which results in poor growth and increased mortality (Alhenaky *et al.*, 2017). Physiologically, blood and heat are shunted away from the digestive system in heat-stressed birds and relocated to the peripheral blood system to aid in the dissipation of heat (De Souza *et al.*, 2016). Without the necessary blood supply to the digestive system, the gastrointestinal tract begins to deteriorate, affecting the gut barrier function and gut integrity negatively through damage of the intestinal epithelium, histological injuries, alterations in the intestinal permeability and changing the conditions of the gut resulting in colonisation of pathogenic bacteria (Al-Fataftah & Abdelqader, 2014; Abdelqader & Al-Fataftah, 2016; Alhenaky *et al.*, 2017).

The past two decades have seen a significant increase in the marketing of probiotics and other alternatives to antibiotics to all sectors of livestock production (Buntyn *et al.*, 2016). The benefits of probiotics and butyric acid have been extensively studied and have shown promise in influencing gut health and production positively (Zhang *et al.*, 2011; Jayaraman *et al.*, 2017; Manafi *et al.*, 2018) as well as mitigating the negative effect of heat stress (Song *et al.*, 2014; Abdelqader & Al-Fataftah, 2016; Abdelqader *et al.*, 2017). Probiotics containing *Bacillus spp.* have gained interest as these are spore-forming bacteria that is resistant to heat and the effect of the pelleting process. This type of probiotic has shown to improve

performance, positively modulated the microbiota of the GIT, reduce pathogenic microbe colonisation and improve the nutrient digestibility of the poultry gut (Gadde *et al.*, 2017).

Encapsulated butyric acid is a protected source of butyric acid which prevents it from being absorbed in the upper digestive tract, delaying its release along the digestive tract and rendering it available more distally in the small intestine (Bortoluzzi *et al.*, 2017). Butyric acid is the direct energy source of epithelial cells of the intestine and assists in the proliferation and differentiation of these cells, to improve intestinal barrier function and integrity (Dehghani-Tafti & Jahanian, 2016), increase villi height and growth (Guilloteau *et al.*, 2010), and control intestinal pathogenic bacteria colonisation (Van Immerseel *et al.*, 2005; Hu & Guo, 2007).

With the removal of antibiotics, increase in global warming and increased focus on the gut health of poultry, it has become important to improve the efficacy of these potential antibiotic alternatives as cost endured due to disease and poor performance will ultimately affect profit. However, there is a paucity of literature available on the effects of encapsulated butyric acid and *Bacillus subtilis* used in combination and their effects on broiler production and overall gut health. Although significant research has been conducted with butyric acid and probiotics as single additives to poultry feed as potential antibiotic alternatives as well as potential compounds to combat the effect of heat stress, there is also very limited research on the combination of *Bacillus subtilis,* and encapsulated butyric acid provided to birds under heat stress. Therefore, further research is required to determine the effect of a combination of *Bacillus subtilis* and encapsulated butyric acid on the gut integrity, microbial colonisation in the intestine and growth performance as well as the effect on broilers which are exposed to heat stress.

1.2 Aim and objectives

The aim of this study was to determine the effect of encapsulated butyric acid and *Bacillus subtilis,* individually or in combination, against zinc bacitracin, on gut health, the microbiome, production and mitigating heat stress in male broilers.

In order to achieve the aim, the following four objectives were set:

1. To determine the effect of encapsulated butyric acid and *Bacillus subtilis* individually or in combination in comparison to zinc bacitracin on body weight gain, feed intake, feed conversion ratio and production efficiency factor of male broiler chickens.
2. To study the effect of encapsulated butyric acid and *Bacillus subtilis* both individually or in combination in comparison to zinc bacitracin on the gut

morphology in male broiler chickens by measuring villi height, crypt depth and villi height to crypt depth ratio.

3 To study the effect of encapsulated butyric acid and *Bacillus subtilis* individually or in combination in comparison to zinc bacitracin on the gut health and gut microbial diversity as well as macroscopic scoring of overall gut health in male broiler chickens .

4 To investigate the effect of encapsulated butyric acid and *Bacillus subtilis* individually or in combination in comparison to zinc bacitracin on mitigating the effect of heat stress in male broiler chickens.

1.3 Hypotheses

1. H_0: The supplementation of encapsulated butyric acid and *Bacillus subtilis* individually or in combination will have a beneficial effect on performance parameters, gut health, microbiome and gut morphology in male broilers.

   H_A: The supplementation of encapsulated butyric acid and *Bacillus subtilis* individually or in combination will not have a beneficial effect on performance parameters, gut health, microbiome and gut morphology in male broilers.

2. H_0: The supplementation of encapsulated butyric acid and *Bacillus subtilis* will have a beneficial effect on broiler performance and health similar to that of the AGP (zinc bacitracin).

   H_A: The supplementation of encapsulated butyric acid and *Bacillus subtilis* will not have a beneficial effect on broiler performance and health similar to that of the AGP (zinc bacitracin).

3. H_0: Encapsulated butyric acid and *Bacillus subtilis* will aid in mitigating the effects of heat stress in male broilers.

   H_A: Encapsulated butyric acid and *Bacillus subtilis* will not aid in mitigating the effects of heat stress in male broilers.

Chapter 2: Literature Review

2.1 Introduction

The modern freshly hatched chick increases its body weight by 25% in the first day and by approximately 5000% by 35 days, to a body weight of 2 kg (Choct, 2009). The high performance of the broiler bird is due to the intense selection for growth rate, strict attention to health as well as advances in nutrition and feed formulation. As the growth period is shortened with an increase in feed efficiency, the bird's requirements for health care and nutrition is becoming more demanding (Ducatelle *et al.*, 2018). Therefore, it has become more imperative to attend to miniscule changes occurring in the gut, which are frequently ignored because the damage is subtle and characterised by changes on a microscopic level in the gut (Choct, 2009). These small adjustments in the mucosal layer affect the nutrient utilisation efficiency because beneath the mucosal layer is a multitude of absorptive epithelial cells which are essential for nutrient transportation to the enterocytes (Choct, 2009; Rinttilä & Apajalahti, 2013; Sugiharto, 2016).

The aim for this literature review is to discuss the factors affecting gut health and performance by focussing on the morphology and histology of the gut, the microbiota of the gut as well as the disruption of homeostasis as a result of heat stress. Antibiotic use and removal thereof are briefly discussed. Thereafter, the use of substitutes for antibiotics with specific focus to the use of *Bacillus subtilis* (a probiotic) and encapsulated butyric acid (an organic acid) is deliberated.

2.2 Factors affecting gut health and performance

Gut health is a foremost subject for research in both humans and in animals. It is generally accepted the enhancement and maintenance of the health of the gut is far more multifaceted than just gut microbe modulation through probiotics or prebiotics (Choct, 2009). The semi-permeable single layered intestinal epithelium plays numerous important roles in nutrient digestion and absorption, however, it also plays a significant role as a barrier between external and internal environment (Abdelqader & Al-Fataftah, 2016; Zhang et al., 2017). The gut serves as a primary location for potential exposure to pathogens obtained from the environment, thus a healthy gut performing at optimum is the foundation of optimal bird performance (Sugiharto, 2016). The gastrointestinal tract is also the greatest immunological organ in the body (Choct, 2009). When gut health and function is hindered, digestion and absorption are impaired which leads to animal performance issues, increases in feed conversion, a higher prevalence of dysbacteriosis, and an increase in the prevalence of disease (for example necrotic enteritis) (Abdelqader & Al-Fataftah, 2016; Sugiharto, 2016).

The role of the intestinal mucosa as a site for absorption of nutrients and a barrier for the internal tissues against hostile luminal content makes the intestinal mucosa a significant factor affecting gut health and performance in poultry (Rinttilä & Apajalahti, 2013; Sugiharto, 2016). Therefore, to encourage the peak functioning of the intestinal mucosal barrier, the balance between epithelial cells, mucosal layer, microbiota and the immune system within the intestine is of importance (Choct, 2009; Sugiharto, 2016).

In addition to the above mentioned factors, the limited ability of chickens to dissipate heat along with their fast rates of metabolism render broiler chickens particularly susceptible to high environmental temperatures and heat stress (Zhang *et al.*, 2017). It has become gradually recognised that heat stress can affect the gut barrier function and integrity undesirably through damage of the intestinal epithelium, histological injuries, alterations in the intestinal permeability and changing the conditions of the gut resulting in colonisation of pathogenic bacteria (Al-Fataftah & Abdelqader, 2014; Abdelqader & Al-Fataftah, 2016; Alhenaky *et al.*, 2017). Therefore, anything that affects the gut health will most likely influence the animal in its entirety, altering its uptake of nutrients and requirements. The concept of "gut health" requires a multi-disciplinary approach and incorporates aspects such as nutrition, stress (such as presence of pathogenic microbes or heat stress), macro- and micro-structural integrity of the gut, the status of the immune system as well as the balance of the gut microbiota; which ultimately affects the overall performance of the bird.

2.2.1 Understanding the gut morphology and histology in chickens

The surface of the mucous membranes is lined with finger-like projections known as villi which, in the small intestine, increases the surface area to maximise absorption within the gut. Each villus surface is covered with simple columnar epithelium which lie upon a base of loose connective tissues called the lamina propria. Crypts are deep pits rich in stem cells and exist between the villi which extend to the muscularis mucosae. The single layered epithelial lining of the intestinal lumen is continuously renewed by stem cells in the crypts (Ducatelle *et al.*, 2018). Freshly formed cells travel up the villus and enter a distinct form of programmed death of cells known as anoikis, thereafter the cells exfoliate from the tip of the villus (Geyra *et al.*, 2001; Ducatelle *et al.*, 2018). During this migration, differentiation of the cells occur, making the cells near the tip of the villi the most important for the absorption of nutrients (Ducatelle *et al.*, 2018). The villi of the duodenum and jejunum are broader and tongue-shaped whereas in the ileum they become finger shaped (Choct, 2009). Generally, the surface area and length are the greatest in the proximal portion of the small intestine and gradually reduces to a minimum in the ileum just prior to the ileo-caecal junction (Choct, 2009). Shortly after hatching, the digestive organs and gastrointestinal tract (GIT) segments increase in size and weight

faster than that of body weight compared to all other organs and tissues (Yegani & Korver, 2008; Choct, 2009). Histomorphological measurements of the mucosa of the small intestine reveals that the villus doubles in height in the 48 hours post-hatch and attains a plateau at six to eight days of age in the duodenum and ten days of age in both the jejunum and ileum (Geyra *et al.*, 2001; Choct, 2009). Increase in surface area occurs in all segments until 3 days post-hatch, thereafter the jejunal area increases more rapidly than the ileum and duodenum (Geyra *et al.*, 2001; Yegani & Korver, 2008).

Not only is the increase in the weight of the GIT segments and digestive organs during gut development rapid and energy expensive, the energy and protein demand for the maintenance of the gut is higher compared to other organs (Xu *et al.*, 2003). Shortened villi lengths reduces the absorptive surface area for nutrients (Xu *et al.*, 2003), whereas deep crypt depths reveals rapid turnover of the intestinal tissues and an extraordinary demand for new tissue (Choct, 2009). Additional turnover of cells increases the nutrient requirements to maintain the digestive tract, with subsequent decrease in the efficiency of the bird. Resulting changes in gut morphology (decreased villus height and increased crypt depth) will negatively affect nutrient absorption, increase GIT secretions, increase presence of diarrhoea, decrease resistance to disease and hinder overall performance (Xu *et al.*, 2003; Choct, 2009). Therefore, the ideal intestinal morphology strives for longer villi and shallower crypts.

With the changes in the morphology of the gut and lowering of bird efficiency, measurements of crypt depth, villi height and the villus to crypt ratio has become the principle evaluation method of animal intestinal health status. The changes in morphology of the small intestine, which represents nutrient absorption ability, remains poorly understood due to considerable variability in experimental results of the morphometric measurements in the duodenum, jejunum and ileum (Xu *et al.*, 2003; De Verdal *et al.*, 2010). Histomorphological values of broilers at 23 days-of-age in the duodenum, jejunum and ileum are approximately 1400, 900 and 700 µm for villus height; 190, 170 and 160 µm for crypt depth; and 8, 6, and 5 for villi height to crypt depths ratio (De Verdal *et al.*, 2010). Not only are these morphometric measurements important in understanding the parameters of a healthy gut, the rapid growth of the GIT provides an ideal place for microbes to colonise. Moreover, studies of germ-free chickens reveal that they typically have smaller intestines and caeca which are lower in weight, which leads to the understanding that the microbiota also aid in the development of the digestive tract (Clavijo & Flórez, 2017).

2.2.2 The normal gut microbiota and diversity of the healthy chicken

The digestive system is a significant and vital pool of microorganisms. The microbiota of the gastrointestinal tract have one of the highest densities of cells documented for any

ecosystem, ranging from 10^7 to 10^{11} bacteria per gram of gut contents in poultry (Kogut, 2013; Rinttilä & Apajalahti, 2013; Zdunczyk *et al.*, 2015). The microbiota of the gut is defined as the entire community of microbes, including the commensal, synbiotic and pathogenic microorganisms, which typically colonise the gut of the chicken. The microbiome is defined as the collective biome of those symbionts (Clavijo & Flórez, 2017). Typical functions of the microbiota include modulation of the immune system, nutrient exchange, changes in the physiology of the digestive system as well as competitive exclusion of pathogens.

Understanding of the gut microbiota was previously constrained to those microorganisms that could be retrieved using culture-dependent techniques. These techniques, however, are unreliable as less than 20% of the microbes found within the GIT can be cultured due to unknown growth requirements and the fastidious nature of the intestinal bacteria (Clavijo & Flórez, 2017). Thus, culture-independent techniques have gained popularity of late to characterise the microbiota in the chicken GIT. In the early 2000s, molecular fingerprinting techniques such as single-strand conformation polymorphism (SSCP), denaturing gradient gel electrophoresis (DGGE), and terminal restriction fragment length polymorphism (T-RFLP) were used (Shaufi *et al.*, 2015). Although these technologies can be rapidly used in laboratories and are relatively inexpensive, they possess limitations such as low sensitivity, inaccuracy in abundance calculations, and low data reproducibility (Clavijo & Flórez, 2017). In recent years, molecular technologies are shifting to high-throughput next-generation sequencing (HT-NGS) with the use of 16S ribosomal RNA (rRNA) gene microarrays. HT-NGS offers a large scale in-depth investigation of the gut microbes allowing Omics studies and aiding in thorough and complex analysis of the environmental microbial communities (Shaufi *et al.*, 2015). Previous studies have utilised 16S rRNA to classify the gut microbiota within the chicken GIT (Shaufi *et al.*, 2015; Han *et al.*, 2016; Mancabelli *et al.*, 2016; Stanley *et al.*, 2016; Yan *et al.*, 2017; Pandit *et al.*, 2018). Bacterial 16S rRNA genes contains nine hypervariable flanked by extremely conserved regions, and are selected as polymerase chain reaction (PCR) sites (Choi *et al.*, 2015). The variation of sequences in the hypervariable regions allow accurate bacterial taxonomic estimation by comparing against 16S rRNA gene sequences that have been deposited into large databases. Stanley *et al.* (2016) utilised the V1-V3 regions for sequencing whereas Yan *et al.* (2017) used the V3 region only. Similarly, Shaufi *et al.* (2015) also used the V3 region for microbial sequencing but recognised that studies which integrated the V3-V4 region and longer MiSeq read chemistry may offer a improved resolution in the diversity of gut microbes and operational taxonomic unit (OTU) classification, therefore resolving discrepancies amongst culture-dependent and culture-independent taxations of the chicken microbiome.

In addition to microbiome analysis method, aspects such as environment, treatment, antibiotic use, age, feed additive, horizontal gene transfer, hygiene level, breed, diet, GIT

location, geography and temperature may influence the microbiota of the chicken GIT (Shaufi *et al.*, 2015). Therefore, profiles of the microbiome and composition vary greatly in previously reported studies. A study by Wei *et al.* (2013) used published and unpublished data on the GIT to analyse the microbiome of the intestine of broiler chickens. According to Clavijo & Flórez (2017), this research is the furthermost authoritative study available on the chicken microbiome diversity. Wei *et al.* (2013) found the occurrence of 915 OTUs (which is considered equivalent to species) classified in 13 phyla. In this study, phyla accounting for >90% of all sequences analysed were *Proteobacteria* (9.3%), *Bacteroidetes* (12.3%) and *Firmicutes* (70%). Shaufi *et al*. (2015) also found that the major phylum discovered in both the ileum and caeca were *Firmicutes* at all ages of the chicken counting 70% of all bacterial sequences. Wei *et al.* (2013) observed that the minority of bacteria included Cyanobacteria, Spirochactes, Synergisteles, Fusobacteria and Verrucomicrobiota. They also described 117 genera, among which *Clostridium*, *Lactobacillus, Bacteroides* and *Ruminococcus* predominated. Based on these studies, diversity of the microbes of the microbiota of the chicken is relatively low in comparison with other animals which may be due to the high rate of passage of food throughout the digestive system and low retention times; for example, the average retention time of a broiler at 29 days of age is between 4 and 5 hours whereas in humans it is on average 20 hours (Clavijo & Flórez, 2017).

Development of the intestinal microbiota in broiler chicks begins shortly after hatching by means of microbial exposure from the egg-shell surface (Rinttilä & Apajalahti, 2013). Therefore, the microbial inoculum at the early post-hatch stage is vital for microbial community establishment in the gut and the effects may persist over the lifetime of the broiler by guiding the development of the intestinal microbiota and immune system (Rinttilä & Apajalahti, 2013). There is a general consensus amongst research that the chicken population of gut microorganisms becomes more diverse and complex as the chicken ages (Lu *et al.*, 2003; Yegani & Korver, 2008; Shaufi *et al.*, 2015). Microbial studies have shown that the microbial community structure is fairly stable during 14 to 28 days of age (the period of rapid skeletal growth) and then changed significantly during the period of weight gain to 49 days of age (Lu *et al.*, 2003). Initially, the GIT of the day-old chick is colonised by facultative aerobes like *Lactobacillus, Enterobacteriaceae* and *Streptococcus*, as the environment of the intestine shows positive oxidation or reduction potential at hatching (Rinttilä & Apajalahti, 2013). Thereafter, consumption of oxygen by these bacteria changes the environment in the distal gut to further reducing conditions, facilitating subsequent growth and colonisation of oxygen-sensitive obligate anaerobes (Rinttilä & Apajalahti, 2013).

Although the digestive organs are strongly interconnected, microorganisms perform independent functions within each organ and therefore the taxonomic composition between these organs differ significantly (Clavijo & Flórez, 2017). In brief, varying species of

Lactobacillus dominate the crop and are responsible the fermentation of lactic acid and the breakdown of starch. The *Clostridiaceae* family is also present in the crop. Microbiota of the crop may be affected by the composition microbes in the feed before digestion (Han *et al.*, 2016). The gizzard is similar in that it is dominated by the same two genera as the crop (Clavijo & Flórez, 2017). The small intestine has been observed to have the highest abundance of bacteria, mainly *Enterococcus, Lactobacillus* and several *Clostridiaceae*; where *Lactobacillus* was the genus accounting for 70% of the total (Lu *et al.*, 2003; Shaufi *et al.*, 2015; Han *et al.*, 2016). Microbiota of the small intestine may be affected by the composition of nutrients of the ingested feed because the small intestine has the responsibility of nutritional absorption of digested feed (Han *et al.*, 2016). Moreover, the small intestinal bacteria utilise the same readily fermentable nutrients that is utilised by the host, hence the small intestine is a segment of the gut whereby competition for nutrients occurs between the host and its commensal bacteria (Yegani & Korver, 2008; Rinttilä & Apajalahti, 2013). Part of this energy loss can be recovered by the host by absorbing and metabolising volatile fatty acids (VFA) and lactic acid from microbial fermentation (Rinttilä & Apajalahti, 2013). As a result of the low pH and high rate of passage of contents within the intestine, the bacterial counts in the duodenum are low. Digesta travels from the duodenum past the jejunum and into the ileum, where there is a reduction in enzyme activities and deconjugation of bile acids occur. As a result, the number of bacteria increase through the segments up to 10^8 cells per millilitre of digesta in the distal ileum. Lastly, the digesta flows to the caeca which hosts the most diverse composition of microbiota. The microbiota in these organs are fundamental in digesting cellulose, starch and polysaccharide rich foodstuffs resistant to digestion in the small intestine. Principally, the caeca hosts Firmicutes, Bacteroides, Proteobacteria and Clostridiaceae (Clavijo & Flórez, 2017). These bacteria are also found in the distal ileum, entering during caecal emptying and reverse peristalsis but are inactive in the small intestine (Rinttilä & Apajalahti, 2013).

It remains unclear, however, how variations in the number of beneficial (*Bifidobacterium spp. and Lactobacillus spp.*) and the commensal bacteria (*Streptococcus, Clostridiaceae*, *Enterobacteriaceae* and *Enterococcus*) enhance the performance and health status of broiler chickens (Zdunczyk *et al.*, 2015). *Lactobacillus* is the genus of bacteria accounting for the greatest portion of the gut microbiota; it has been examined and used extensively in medicine as well as the food industry due to their principle function of lactate production and starch digestion (Yan *et al.*, 2017). *Lactobacilli,* are facultative anaerobes, subsequently producing enzymes and creating competitive exclusion of pathogenic bacteria (Jeong & Kim, 2014). Furthermore, *Lactobacilli* responsible for the production of lactic acid, which can act as a natural antimicrobial by disrupting the external membrane of gram-negative bacteria and reduce the pH of the intestine, inhibiting growth of pathogenic bacteria (Jeong & Kim, 2014). *Lactobacillus* has been highly linked with the feed efficiency of the host, however a gain in

body weight due to the presence of this genus has only been significant in infancy but not in adult birds (Yan *et al.*, 2017). Therefore, it could be inferred that *Lactobacillus* enrichment could generally improve the GIT and offer protection against pathogens as well as promote efficient energy and nutrient extraction in the host. In a study by Johnson *et al.* (2018), they found strong correlations amongst bacterial taxa at a genus level and bird weight as a measure of performance. They found a number of potentially pathogenic microorganisms that are negatively associated with performance, these included *Clostridium, Enterococcus* and unclassified *Enterobacteriaceae*, the latter typically representing *Escherichia coli*. *Streptococcus* in the ileum has shown significant negative correlation with body weight (Han *et al.*, 2016). Many species in this genus are known normal gut flora, however some have been associated with disease. For example, the *S. anginosus* group are connected to infections of many places in the body and abscess formation, whereas *S. mutans* and *S. mitis* groups are known pathogens of the buccal cavity (Han *et al.*, 2016).

2.2.3 Linking dysbiosis and the micro-architecture of the gut

The digestive system is a complex ecosystem containing three main interconnecting elements; the immune system, the intestinal epithelium and the commensal microbiota (Kogut, 2013). The digestive organs undergo anatomical and physiological changes during the post-hatch phase (Clavijo & Flórez, 2017). This rapid development of the gastrointestinal tract provides an perfect niche for microbes to colonise, and as mentioned previously, the microbiota play a vital role in GIT development by favouring the renewal and barrier function of the epithelium of the gastrointestinal tract (Kogut, 2013; Clavijo & Flórez, 2017).

The ability with which the epithelial lining of the intestine allows the passage of molecules via passive diffusion is known as intestinal permeability (Ducatelle *et al.*, 2018). Passive diffusion of potential destructive molecules from the lumen of the intestine into the cells of the epithelium is offset by efflux pumps which are plasma membrane-bound, called multi-drug resistant (MDR) pumps. A defect in these MDR pumps results in inflammation within the intestines. The permeability of the intestinal barrier also governed by the stability of the intercellular junctions (adherens junctions, desmosomes and tight junctions) that regulate the pathway of intercellular transport between neighbouring epithelial cells of the intestine (Ducatelle *et al.*, 2018). Alterations in molecular structure or reduction in expression of these intercellular junctions results in reduced absorption of nutrients, gut leakage as a result of increased passage of secretory water and ions, and increased macromolecule passage from the lumen which creates inflammation. Thus, there is a strong connection between dysbiosis, intestinal barrier dysfunction and inflammation (Ducatelle *et al.*, 2018).

Biodiversity of the microbiota of the gut is important in indicating health, disease and stability of ecosystems (Choct, 2009; Yan *et al.*, 2017). An increase in the diversity of microbes in the gut has been linked to improved health in the elderly whilst a decreased diversity has been related to worsening of inflammatory bowel disease (Yan *et al.*, 2017). Thus, a stable and diverse gut microbiota is crucial for the bird to counterattack infections. When the normal gut microbiota composition is disrupted accompanied by inflammation of the intestine, a condition known as dysbiosis occurs (Ducatelle *et al.*, 2018). Typically, a gut microbiota shift favours atypical populations of microorganisms to predominate within the GIT (Kogut, 2013). Determining when an alteration in the composition of microbiota should be deemed dysbiosis is the principal challenge for scientists investigating the microbiome of the intestine of chickens (Ducatelle *et al.*, 2018). There is a potential two-fold effect on the host metabolism when dysbiosis occurs, 1) there is an alteration in the amount of beneficial gut bacteria versus pathogenic gut bacteria, therefore the ability of the host to obtain energy from food and to react to the intake of energy is affected, and 2) increasing the amounts of bacteria and bacterial products (for example lipopolysaccharides) originating from the microbes which circulate in the intestine and recognised by the innate immune system of the host, thus resulting in low-grade chronic inflammation in the gut (Kogut, 2013). Dysbiosis is typically seen at 20 – 30 days of age, which is relatively late in the life cycle of the commercial broiler chicken (Teirlynck *et al.*, 2011). Clinically, the foremost signs of dysbiosis in poultry are pale or orange droppings with food particles that are undigested, greasy wet droppings, foamy caecal droppings, reduced physical activity, increased intake of water, reduction in feed intake, reduced average daily gains as well as an increased feed conversion. At necropsy, thin, fragile walls of the intestine, watery or foamy intestinal contents and recurrent orange mucous and undigested feed in the intestines, gut ballooning and inflammation of the intestines can be observed in birds experiencing dysbiosis (Teirlynck *et al.*, 2011).

Stressors existing in the digesta can result in changes in the mucosa of the intestine as a result of the mucosal surface and intestinal content being in local proximity to one another (Xu *et al.*, 2003). Thus, exposure of the intestinal mucosa to multiple enteric pathogens can take place. Initially during the process of infection, pathogenic microbes attach to the brush border of epithelial cells of the intestine, which enables these pathogens to exploit the signalling pathways (Kogut, 2013). After the normal host-cell processes are weakened, pathogens are able to penetrate and cross the epithelial barrier. Pathogenic bacteria damage of the intestinal tract may reduce feed conversion efficiency as well as the rate of gain of body weight in broiler flocks (Yegani & Korver, 2008). Acute enteric damage will give rise to high mortality and disease. Necrotic enteritis causes lesions in the intestine and may be one of the most severe disease that occurs in the broiler chicken intestine (McDevitt *et al.*, 2006). The contributing organism for necrotic enteritis is *Clostridium perfringens*, which is an anaerobic

bacterium found in small numbers of less than 10^4 cfu in the GIT of birds. At these low numbers, this organism is not pathogenic. However, alterations in the GIT, due to various stressors, may provide conditions that favour clostridia causing the bacteria to proliferate resulting in necrotic enteritis (McDevitt *et al.*, 2006). When left untreated, mortality rates may be increased to 1% per day resulting in death of 10 to 40% of the birds in an affected flock. Furthermore, a far greater number of birds may be affected by sub-clinical necrotic enteritis, due to the fact that the disease in birds remain untreated because the disease often goes undetected, which ultimately affects the welfare and productivity of birds (McDevitt *et al.*, 2006). Birds that experience long-lasting intestinal enteritis are in a consistent mode of inflammation and recovery (Al-Baadani *et al.*, 2016). As a reaction to inflammation from toxins produced by pathogen or the pathogens themselves, changes in the villi and crypts are due to the renewal of villi can be observed. Thus, the effect of the inflammation and product ion of toxins from pathogens may contribute to suboptimal nutrient absorption and inferior performance of poultry (Xu *et al.*, 2003).

In research performed by Teirlynck *et al.* (2011), a method for macroscopically scoring of the gut health was developed. In their method, a total of ten parameters were assessed. Both sections cranial and caudal to the Meckel's diverticulum were scored individually for the following parameters: presence of ballooning; significant redness of the serosa or mucosa; reduction in the overall thickness of the gut wall, three seconds after dissecting the gut edges are flaccid; and abnormal gut content (mucous, water or gas) as well as undigested feed particles caudal to the ileocaecal junction. Based on the measurements obtained by Teirlynck *et al.* (2011), they found that variations in distention of the intestine between a gut which is health and one exposed to severe dysbacteriosis explained the tunica muscularis variations in thickness, concluding that ballooning and the flaccid aspects of the gut wall is connected to the decreased tone of the tunica muscularis. However, they acknowledged that the intestinal distention variations can only explain, in part, the observed decrease in villus height in the severe dysbacteriosis instances. Therefore, severe dysbacteriosis is linked to a decrease in the surface area available for absorption. Teirlynck *et al.* (2011) also found a change in the number and size of goblet cells which explained the presence of mucous in the gastrointestinal tract of affected birds.

This method proposed by Teirlynck *et al.* (2011), provides a potential simple way to test for possible dysbacteriosis without having to utilise HT-NGS (for microbe diversity analysis) or microscopic measurements (villi and crypt measurements). Although this may be true, a combination of all macroscopic and microscopic measurements as well as HT-NGS provides an all-encompassing view on describing a healthy gut and its outcome on the performance of the broiler chicken.

2.2.4 The effect of heat stress in poultry production

Heat stress occurs due to a negative balance among the net energy flowing from the broiler's body to its nearby environment and the amount of energy as heat created by the animal (Lara & Rostagno, 2013). Broilers have been intensively selected for high growth rates, thus the modern broiler genotype produces more body heat as a result of their higher metabolic activity (Lara & Rostagno, 2013; Abdelqader *et al.*, 2017). Along with their fast metabolisms, poultry have a limited ability to dissipate heat (due to the absence of sweat glands) which makes poultry particularly susceptible to high environmental temperatures and heat stress (Abdelqader *et al.*, 2017; Zhang *et al.*, 2017).

Body temperature exceeding the thermoneutral zone (approximately 18 to 22°C in broilers chickens) disrupts physiological homeostasis and reduces the functioning of both the immune and digestive systems, leading to inflammation of the gut and dysfunction, diminishing the health status of the bird and increasing mortality (Lin *et al.*, 2006; Wang *et al.*, 2018). As ambient temperature rises beyond the thermoneutral zone, birds will change their behaviour to prevent core temperature changes (Wang *et al.*, 2018). Modulation of temperature is transferred from sensible heat loss (through radiation via the comb, wattle, wing spreading and feet) to evaporative heat loss (like panting) (Wang *et al.*, 2018). These behavioural changes usually occur prior to the increase in core body temperature and the physiological alterations. Panting is accompanied with the loss of water which may result in dehydration in the broiler, thus heat-stressed chickens must drink more water to replenish their body water levels (Wang *et al.*, 2018). Wing spreading increase heat loss by radiation, however too frequent wing spreading has been linked to causing pale, dry, exudative meat in broilers. Heat conduction is promoted by squatting closely to the ground but is also linked to increased foot pad dermatitis frequency (Wang *et al.*, 2018). Birds also possess air sacs which add an extra system to facilitate the exchange of heat between their body and the outside environment (Lara & Rostagno, 2013). These air sacs are helpful during panting, providing air circulation on surfaces which contributes to increased gas exchange with the air and subsequently, evaporative heat loss.

Physiologically, blood and heat are shunted away from the digestive system in heat-stressed birds and relocated to the peripheral blood system, via peripheral vasodilation, to aid in the dissipation of heat (De Souza *et al.*, 2016). During this time, the gastrointestinal tract begins to deteriorate without the much needed blood supply. Therefore, it has become progressively recognised that heat stress has an negative effect on the gut barrier function and gut integrity through damage of the intestinal epithelium, histological injuries, changes in the intestinal permeability and changing the conditions of the gut resulting in colonisation of pathogenic bacteria (Al-Fataftah & Abdelqader, 2014; Abdelqader & Al-Fataftah, 2016; De Souza *et al.*, 2016; Alhenaky *et al.*, 2017). The damaging of intestinal epithelial cells and poor

outcomes as a result of heat stress will also enhance endotoxin infiltration into the blood circulation, which can result in additional responses systemic inflammation and multi-organ dysfunction (Abdelqader *et al.*, 2017). Furthermore, excessive panting decreases the partial pressure of carbon dioxide and the availability of calcium as well as increasing blood pH, which leads to an increase risk for respiratory alkalosis and lameness (Wang *et al.*, 2018).

The colonisation of food-borne pathogens in birds, such as *Campylobacter* and *Salmonella,* and their potential spread to the human food chain has been recognised as a public health and economic matter of interest in poultry production (Lara & Rostagno, 2013). Heat stress has been shown to lead to colonisation of pathogenic bacteria in farm animals, increased faecal shedding and horizontal transmission, with subsequent increase in risk of contamination of animal products. Furthermore, a stress response is associated primarily with the stimulation of the hypothalamo-pituitary-adrenal (HPA) axis and increased plasma concentrations of corticosterone which intensifies the detrimental effect of high body temperature (Lin *et al.*, 2006; Lara & Rostagno, 2013). Potentially pathogenic bacteria are able to exploit the neuroendocrine alterations in the host to encourage growth and pathogenicity (Lara & Rostagno, 2013). In addition, prolonged secretion of corticosterone when exposed to chronic stress may result in reduced immunity, depression, cardiovascular issues and muscle breakdown due to gluconeogenesis, as well as reduced cognition (Nawab *et al.*, 2018). Endocrinological changes as a result of broilers subjected to chronic heat stress conditions also stimulates lipid deposition through elevated *de novo* lipogenesis, decreased lipolysis and increased amino acid catabolism (Lara & Rostagno, 2013).

Therefore the effect of heat stress is multifaceted and negatively affects the physiological, immunological and gut health status of poultry resulting in huge economic losses in the poultry industry (Nawab *et al.*, 2018). As a result, alleviating heat stress is worthy not only in the traditional warmer regions of the world, but also those regions experiencing global climate change, due to global warming (Azad *et al.*, 2010).

2.3 Antibiotics in poultry

2.3.1 Antibiotics and their use

An antibiotic is defined as a microbial-produced compound which has the ability to eradicate or inhibit the growth of another microbe (Kumar & Singh, 2013). Antibiotics only treat infectious diseases which have bacteria as causative agents and are therefore ineffective against pathogens that are fungal or viral (Mehdi *et al.*, 2018). Typically, three main purposes for antibiotics are used in animal production: therapeutically, prophylactically and for growth promotion (Huyghebaert *et al.*, 2011; Kumar & Singh, 2013). Antibiotics used therapeutically are prescribed for the treatment of infections and is applied at therapeutic dosages that are higher than that utilised for preventative, control and productive purposes, over a short period

of time (Kumar & Singh, 2013). The prophylactical application of antibiotics are for the prevention or control of disease and is used in conditions where disease is most likely to occur as a result of a possible exposure to pathogenic organisms and environmental conditions (Kumar & Singh, 2013). Lastly, antibiotics are used at sub-therapeutic levels to increase the growth rate and/or to enhance feed efficiency in animals and is given over an extended period of time, generally known as antibiotic growth promotors (AGPs) (Huyghebaert *et al.*, 2011; Kumar & Singh, 2013).

The exact mechanism of action of numerous widely used antimicrobials have not yet been proven and remain unclear (Lillehoj & Lee, 2012; Brown *et al.*, 2017; Mehdi *et al.*, 2018). Demonstrating that the mode of action of antimicrobials is complex, and is contributed to the complexity of interactions amongst bacterial, environmental and host factors within the gastrointestinal tract of mammals (Brown *et al.*, 2017). Two primary hypotheses for the mode of actions of AGPs have been suggested: the bacteria-centric hypothesis (indirect effect) and the host-centric hypothesis (direct effect). Although the hypotheses are divided into these two groups, the mode of action of AGPs is still inherently complicated. The belief that the modulation of microbiota in the intestine is the main mode of action by AGPs has been suggested by observing the lack of enhanced growth response in germ-free mice (Brown *et al.*, 2017). Therefore, the bacteria-centric hypothesis proposes that changes in bacterial communities by AGPs results in enhanced growth, through modulation of the gut microbiota, to create a system that is more efficient (Brown *et al.*, 2017). The host-centric hypothesis focusses on the direct effect of AGPs on the host by acting as direct immunomodulatory agents that allow for resources to be shifted to metabolic functions (Brown *et al.*, 2017). Due to the symbiotic nature between bacterial and host cells in the gastrointestinal tract, it is difficult to separate components into separate units. It is speculated, however, that antibiotics work in several different ways, including interfering with cell wall synthesis; inhibiting protein synthesis by attaching to the ribosomal 50S subunit; inhibiting the protein synthesis by attaching to the ribosomal 30S subunit; inhibiting RNA synthesis; inhibiting DNA synthesis; hindering dihydrofolate reductase activity and lastly, by disruption os the bacterial membrane (Kumar & Singh, 2013).

Even in the absence of a distinct mode of action, antibiotics have still been extensively utilised over the past fifty years in combination with stringent biosecurity and hygienic procedures and has allowed the substantial growth of the poultry industry by preventing the impacts of the main avian diseases (Kumar & Singh, 2013; Mehdi *et al.*, 2018). Recent data suggests that the livestock industry uses twice as much of the globally produced antibiotics than that used by the human population as a whole (Xiong *et al.*, 2018). In perspective, the global average consumption is equivalent to 172, 148 and 45 mg per kilogram pig, chicken and cattle respectively (Xiong *et al.*, 2018). Furthermore, antimicrobial consumption is

projected to rise by 67% by 2030 with the majority (66%) being due to the growing number of animals raised for the production of food and the remaining portion (34%) due to the predicted change in farming practices to more intensified farming systems (Van Boeckel *et al.*, 2015).

2.3.2 Antibiotic resistance and the removal thereof

The euphoria of the beneficial effects of antibiotic use became short-lived, as bacteria began to establish different forms of resistance to prescribed antibiotics, with pathogenic bacteria being the most problematic. Microbes which have a high resistance to drugs and results in increased morbidity and mortality with associated non-response to therapeutic options are termed “superbugs” (Kumar & Singh, 2013). Bacterial resistance is contributed to a number of mechanisms, including i) activation of the efflux pump which actively pump to expel antibiotics from the bacterial cell thus decreasing intracellular concentration of antibiotics; ii) enzymatic modification or degradation of antibiotics (either within or outside the bacterial cell) rendering the antibiotic ineffective; iii) degradation of the antimicrobial compound; iv) utilisation of alternative metabolic pathways to those inhibited by the antimicrobial compound; v) overproduction of target enzyme; vi) modification of the antibiotic drug target within the bacterial cell; and vii) restriction in entry or access (cell permeability) which inhibits the antibiotic to reach the target site (Kumar & Singh, 2013; Van den Honert *et al.*, 2018).

Resistance is possible through horizontal gene transfer (genes carried by mobile genetic constituents, such as plasmids, phages, integrons and transposons) or through mutations (Mehdi *et al.*, 2018). The increase in the prevalence of antibiotic resistance in bacterial species is due to multifaceted combination of factors. Some factors are fundamental in nature, such as bacterial adaptation to changing environmental conditions as a result of their short generation interval and the intrinsic resistance of some bacteria (Van den Honert *et al.*, 2018). However, some of these factors are human made, such as extensive overuse and misuse of antibiotics as AGPs in the farming industry. Inappropriate use of antibiotics in the animal industry has resulted in intensive selection pressure, thus increasing and accelerated the probability for strains of bacteria to adapt and increase in number to produce a more resistant population (Van den Honert *et al.*, 2018). Typically, broilers are raised in confined housing under high stocking densities which is stressful and increases the density and efficacy of transmission of pathogens as well as the spread of antibiotic resistant bacteria (Brown *et al.*, 2017). Due to the impracticality of treating animals individually, mass medication of broad spectrum antibiotics in animal husbandry is a common practice. Thus the exploitation of low concentrations of antibiotics during an extended period of time also favours antibiotic resistant bacterial emergence (Van den Honert *et al.*, 2018).

As a result of the misuse of antibiotics, multidrug bacterial resistance in animal husbandry is a growing public health issue. There are many examples in literature of the presence of antimicrobial resistant bacteria in animal husbandry. Some of the most problematic multidrug resistant organisms in poultry include methicillin-resistant *Staphylococcus aureus* (MRSA), vancomycin-resistant *Enterococcus* (VRE), *Klebsiella pneumoniae* containing extended-spectrum β-lactamase (ESBL), *Escherichia coli*, *Salmonella*, and *Campylobacter jejuni* (Kumar & Singh, 2013; Van den Honert *et al.*, 2018; Xiong *et al.*, 2018).

Concerns about increasing resistance and the potential transferal to humans, due to sub-therapeutic use of AGPs in agriculture and the abusive use of antibiotics, has resulted in the ban of antibiotics as growth promotants in several countries. The initial country to legislatively ban the use of all antimicrobials for the purpose growth promotion was Sweden in 1986 (M'Sadeq *et al.*, 2015). Thereafter, Denmark banned the use of all AGPs in animal feeds in the year 2000 followed closely by the European Union (EU) in January 2006 (Xiong *et al.*, 2018). Agricultural industries in other countries, such as the United States of America (USA), were further urged by consumers to rear animals without AGPs (Dahiya *et al*., 2006; Huyghebaert *et al*., 2011). Although there is no current legislative ban of AGP use in livestock feeding in South Africa, large chicken food industries, such as the McDonald's Corporation and Kentucky Fried Chicken, have already publicised to endorse AGP-free poultry meats for consumers globally (Lillehoj & Lee, 2012). Similarly, there has been an increase in general consumer awareness and pressure to decrease the amount of antibiotics in the production of meat.

Although complete removal of antibiotics in the livestock industry is considered extreme and impossible, as this begins to ignore the welfare issues of infected animals, the current focus is on regulating the non-therapeutic application of human antimicrobial agents to livestock as well as the development of antimicrobial resistance in zoonotic pathogens (Brown *et al.*, 2017). The phasing-out of antibiotic use in broiler feed will unavoidably change the microbial ecology within the GIT of the broiler chicken (Dahiya *et al.*, 2006). The reduction of antibiotic use has forced the poultry industry to shift into a "post-antibiotic era" and substitutes for AGPs are needed to preserve the balance of the gut microbiota as well as improve production performance and efficiency.

2.4 Substitutes for antibiotics

Alternatives to antibiotics should ideally have the same beneficial effects to that of AGPs. Based on the proposed mechanisms of action of AGPs, potential substitutes for antibiotics are compounds which have microbe modulating and/or immunoregulatory effects in the gut (Huyghebaert *et al.*, 2011). Numerous alternatives to antibiotics, such as prebiotics, probiotics,

organic acids, phytonutrients and antimicrobial peptides, have been utilised by the animal industry for a variety of reasons. However, it is largely acknowledged that none of these alternatives are as effective as antibiotic growth promotants at a commercial level and can only partially compensate for the complete removal of in-feed antibiotics (Huyghebaert *et al.*, 2011). Although alternatives cannot replace the use of antibiotics, a combination of these feed additives have shown some capability to compensate for the loss in production (when AGPs are not utilised) with some beneficial economic returns (Lillehoj & Lee, 2012).

It is acknowledged that many different forms of antibiotic substitutes have been tested and used in the animal feed industry (specifically the poultry industry), however, a complete breakdown of these alternatives is beyond the scope of this literature review. Instead, focus in this section will be on probiotics (with emphasis on the *Bacillus subtilis* species) as well as organic acids (with specific attention to butyric acid).

2.4.1 Probiotics (direct-fed microbials)

Direct-fed microbials (DFM), often described as probiotics, are possible alternatives to antibiotics that have been extensively researched and used commercially (Teo & Tan, 2007; Sen *et al.*, 2012; Jeong & Kim, 2014; Abudabos *et al.*, 2015; Al-Baadani *et al.*, 2016; Manafi *et al.*, 2018; Wang *et al.*, 2018). A DFM is a single or mixed culture of live microorganisms (including bacteria, fungi and yeasts) which provide health benefits to the host when provided in sufficient amounts (Huyghebaert *et al.*, 2011; Alloui *et al.*, 2013). Multi-species probiotic preparations are thought to be more successful than probiotics of a single strain (Manafi *et al.*, 2018). In order for preparations to be considered as a probiotic, they must be a duplicate of the normal gut microbiota being resistant to processing, the effect of bile salts, acidity and digestive enzymes (Huyghebaert *et al.*, 2011; M'Sadeq *et al.*, 2015). Probiotics should also have reduced intestinal permeability, produce lactic acid and remain viable during transit through the GIT; thus it must be resistant to both the acidic environment of the stomach as well as the alkaline conditions in the duodenum (Alloui *et al.*, 2013; M'Sadeq *et al.*, 2015). In contrast to antibiotics, having either bactericidal or bacteriostatic effects, DFMs function through indirect mechanisms, such as modulation of the intestinal microbial populations, enhancements of the intestinal efficiency and innate immune system modulation of the host (Buntyn *et al.*, 2016).

Typical gut flora preparations (live obligate and facultative anaerobic bacteria) from normal and healthy adult avian individuals, free from pathogenic organisms, screened for antibiotic resistance and highly prolific, are given orally to newly hatched chicks to promote the immediate formation of the adult-type intestinal microbiota and provide an immediate resistance to the colonisation of pathogenic bacteria (Dahiya *et al.*, 2006). The inoculation of

newly hatched chicks begins the principle of competitive exclusion whereby the adhesion and colonisation of beneficial microbiota along the intestinal mucosa prevents the adhesion and invasion of pathogenic microorganisms resulting in competition for available nutrients and mucosal binding sites and the replacement of already adhered pathogens with beneficial microbiota (Alloui *et al.*, 2013; Ajuwon, 2016). Normal gut microbiota preparations have shown effectiveness against food-borne pathogens like *Clostridium perfringens*, *Salmonella spp., Clostridium botulinum*, *Campylobacter jejuni*, *Yersinia enterocolitica* and pathogenic *Escherichia coli* strains (Dahiya *et al.*, 2006). Most poultry producers and researchers agree that probiotic feed supplementation at an early age helps to maintain the integrity of the intestinal mucosa and promotes digestion and absorption, which ultimately improves the overall performance of the individual (Sikandar *et al.*, 2017).

Apart from competitive exclusion, other mechanisms of action of probiotics against pathogens include antimicrobial effect by secretion of bacteriocins, organic acids and hydrogen peroxide which inhibit development; lowering of gut pH due to VFA production during microbial fermentation; production of short-chain fatty acids (SCFA) in the intestine; immunomodulatory effects by stimulating gut associated lymphoid tissues and stabilisation of the gut microbiota (Alloui *et al.*, 2013). Probiotic inclusion may provide protection against pathogens and disease by decreasing the intestinal pH creating a hazardous environment to pathogens (Ajuwon, 2016). Probiotics may also increase the amount of VFA produced by increasing the availability of nutrients (Ajuwon, 2016). As mentioned previously, bacteria produce mostly butyrate, acetate and propionate during fermentation which are directly absorbed in the GIT and utilised as an energy source by the tissues. In poultry, butyrate may regulate growth through selective partitioning of nutrients away from the liver and adipose tissues to the muscle through selective up-regulation of muscle insulin receptor β-subunit expression (Ajuwon, 2016). Thus, increased production of VFA as well as the selective regulation of insulin signalling in tissues by probiotics may potentially stimulate growth. Moreover, increased VFA production may promote intestinal health and the integrity of the gut by stimulating the proliferation of the epithelial cells and increase villi height by stimulating cell mitosis (Park *et al.*, 2016). Manafi *et al.* (2018), found that multi-strain probiotics increased villi height and villi height to crypt depth ratio in the intestine of broilers. Thus, probiotic bacteria known to stimulate VFA production in poultry, such as *Lactobacillus spp.* and *Bacillus spp.*, may potentially regulate growth performance in poultry by up-regulation of VFA production and are valuable in the pursuit of low-dose antibiotic substitutes (Ajuwon, 2016).

Despite the popularity and promise of probiotics as antibiotic alternatives, a substantial limitation against the widespread adoption of probiotics is the apparent inconsistencies in their effects on the performance and gut health of poultry (Ajuwon, 2016). As with antibiotics, there exists a gap in the understanding of the precise mechanism of action of probiotics. This is

partly due to the complexity of the gut as a habitat for microbes and the variety of host-microbe and microbe-microbe interactions that arise within the gut (Ajuwon, 2016). A multitude of factors affect the efficacy of a probiotic namely; probiotic preparation method, probiotic species, the ability for probiotic microorganisms to persist in harsh environments, administration route, timing of application, farm sanitation, pathogen exposure, antibiotic use, bird age and immunological levels (Buntyn *et al.*, 2016; Junaid *et al.*, 2018).

Of all the probiotics available, *Lactobacillus*, *Bacillus*, *Streptococcus, Aspergillus, Bifidobacterium* and *Saccharomyces* species have been largely used in poultry to modulate the intestinal microbiota and inhibit colonisation of pathogens (Manafi *et al.*, 2018). *Lactobacillus* probiotics has been shown to increase the amount of lactic acid producing bacteria and decrease the gut lesion scores of broilers infected with *Salmonella* and coccidiosis (Alloui *et al.*, 2013). Probiotic yeasts, such as *Saccharomyces*, have been shown to stimulate the immune system of chicks without reducing the growth performance (Manafi *et al.*, 2018). Probiotics containing *Bacillus subtilis*, in particular, have been found to be the most beneficial in poultry (Alloui *et al.*, 2013). Apart from their ability to improve growth performance, *B. subtilis* is effective in inhibiting pathogen growth in the digestive tract of chickens, which can result in substantial economic losses.

2.4.2 *Bacillus subtilis* as a resilient probiotic

Direct-fed microbials consist of many varieties of bacterial species but *Bacillus spp.*, in particular, have shown to benefit growth performance and intestinal health (Gadde et al., 2017). Differing to most known probiotic species, being sensitive and incapable of surviving high temperatures, during feed processing, probiotics containing spore-forming bacteria are metabolically inactive and highly resilient to environmental conditions, such as high and low pH as well as extreme ow and high temperatures (Jeong & Kim, 2014). Cells of *Bacillus subtilis* can survive heating of up to 100°C for several minutes as well as survive in 0.5% of bile salts and are still able to germinate into vegetative cells (Teo & Tan, 2006). Thus, *Bacillus* bacteria have been considered as good contenders for feed additives due to their aerobic and endospore-forming nature which provides them the ability to survive environmental stresses, including transportation, storage and feed pelleting (Manafi *et al.*, 2018).

Bacillus subtilis spores main mode of action appears to be their capacity to produce an anaerobic environment in the GIT by rapid consumption of oxygen after germination (Jeong & Kim, 2014). It is assumed that this effect favours growth and proliferation of the natural microfloral *Lactobacilli*, which further leads to competitive exclusion of pathogenic microorganisms and lactic acid production to reduce pH and further control and regulate the colonisation of pathogenic bacteria in the intestine (Jeong & Kim, 2014). PB6 is a natural strain

of *Bacillus subtilis* that has been isolated from a chicken with a healthy gut and has been revealed *in vitro* to produce antimicrobial substances, with broad spectrum activity against numerous strains of *Campylobacter Spp.*, *E. coli* and *Clostridium Spp.* (Teo & Tan, 2005; Abudabos et al., 2013). It is also known that PB6 secretes surfactins which have anti-microbial activity (Teo & Tan, 2005; Jayaraman *et al.*, 2017).

The effect of *B. subtilis* on improvement of performance is well recorded (Teo & Tan, 2006, 2007; Jayaraman *et al.*, 2017). Under thermoneutral conditions, broilers supplemented with *Bacillus subtilis* PB6 had a higher body weight when compared with all other treatments in an experiment conducted by Jayaraman *et al.* (2017). Birds that received *B. subtilis* were found to have the lowest feed conversion ratio (FCR) than control groups, whether challenged by *Clostridium perfringens* or not (Abudabos *et al.*, 2013; Jayaraman *et al.*, 2017). Jayaraman *et al.* (2013) showed no statistically significant gain in body weight for unchallenged broilers, but when exposed to *C. perfringens* a numerical improvement in body weight gain for *B. subtilis* supplemented birds were noted. Under heat-stressed conditions, broiler birds supplemented with *B. subtilis* significantly improved body weight, average daily gain and feed efficiency as well as a lower mortality rate compared to those fed a basal diet without probiotics (Al-Fataftah & Abdelqader, 2014).

Apart from the effect of probiotic inclusion of *B. subtilis* on growth performance, its effect on the intestinal health has also been reviewed. Dietary inclusion of *Bacillus subtilis* was shown to increase the number of *Lactobacillus* and *Bifidobacterium* species and decrease *Clostridium* species in intestinal microbial counts under both thermoneutral and heat-stressed conditions (Al-Fataftah & Abdelqader, 2014). Similarly, Jeong & Kim (2014) also found a significant increase in *Lactobacillus* counts in the intestine and excreta as well as a significant reduction in *E. coli*, *Clostridium perfringens* and *Salmonella* counts. Broilers either infected with *E. coli* or not, showed *Lactobacilli* counts similar to broilers receiving an antibiotic when they were supplemented with *B. subtilis* PB6 (Teo & Tan, 2006). *B. subtilis* supplementation had a similar response in *C. perfringens* challenged broiler birds, with *B. subtilis* having a moderating activity in the proliferation of the pathogenic bacteria causing necrotic enteritis (Abudabos *et al.*, 2013; Jayaraman *et al.*, 2013). In contrast, Teo & Tan (2007) found no reduction in beneficial gut microbiota when broiler diets were supplemented with *B. subtilis* PB6, however a decrease in the quantity of *Clostridium* species and *E. coli* was seen.

Moreover, *B. subtilis* has been shown to have a positive effect on the gut and histomorphological integrity of villi against *C. perfringens*-induced necrotic enteritis in broiler chickens (Jayaraman *et al.*, 2013). Dietary inclusion of *B. subtilis* PB6 has shown to increase the duodenal and ileal villi height and villi surface area under both thermoneutral and heat-stressed conditions (Al-Fataftah & Abdelqader, 2014; Jayaraman *et al.*, 2017). These results are supported by Abudabos *et al.* (2013) and Jayaraman *et al.* (2013), who also found higher

intestinal weights and villi heights in the ileum and jejunum in *B. subtilis* supplemented birds compared to the other treatments.

Bacillus subtilis-based probiotics have shown to improve performance, modulate the intestinal microbiota positively, inhibit the colonisation of pathogenic bacteria, improve nutrient digestibility and enhance the immune system in the GIT of broilers (Sen *et al.*, 2012; Gadde *et al.*, 2017). Therefore, *B. subtilis* is gaining interest as a safe and resilient single-strain probiotic that may potentially be utilised as a substitute for antibiotics in poultry feed, however further research is needed to determine the exact mode of action as well as its exact effect on growth performance and gut health in broilers.

2.4.3 Organic acids

Organic acids such as acetic, propionic, lactic, citric, fumaric, butyric and tannic acids, have shown to affect intestinal health and bird performance positively (Sugiharto, 2016). Organic acids are widely used as both potent feed additives and as raw materials to inhibit pathogens such as *Salmonella spp.*, and *Enterobacteriaceae* (Dahiya *et al.*, 2006). This wide range of organic acids can vary physically and chemically, of which many are used as acidifiers in feed or as drinking water supplements (Huyghebaert *et al.*, 2011). Apart from their microbial modulatory effect, organic acids are chelating agents, increasing the availability of minerals in poultry and preventing mineral-phytate complexes from forming (Khodambashi Emami *et al.*, 2013). Researchers have revealed that organic acids may affect the microbiota of the GIT as well as intestinal morphology and digestibility of nutrients (Van Immerseel *et al.*, 2005; Hu & Guo, 2007).

Many organic acids are available in partially esterified forms or as salts of potassium, sodium or calcium which is advantageous over their acid forms due to salts being solid, less volatile and odourless (Huyghebaert *et al.*, 2011). Salts are also less corrosive, easier to handle and may be more water soluble than their acid counterparts (Huyghebaert *et al.*, 2011). Adaptation and subsequent drop in therapeutic activity of the acid may occur in birds exposed to acidifiers at an early age (Islam, 2012). Thus, it is suggested that acidifiers should be given to birds in the grower stage instead of in starter diets to prevent economic losses from heat stress (Islam, 2012).

Although extensively used in feeds, their mode of action has still not been sufficiently explained. The effect of organic acids on the gut microbiota may be due to cytoplasmic acidification causing uncoupling of energy regulation and production, and/or by the build-up of disassociated acid anions to reach toxic levels (Mani-López *et al.*, 2012). The capacity of organic acids to transform to its dissociated form is pH dependent and can enhance their antimicrobial effect (Huyghebaert *et al.*, 2011). The undissociated organic acid (non-ionised

and more lipophilic) can move freely through the microbial semi-permeable membrane into the cytoplasm of the cell, subsequently disrupting the normal physiology of certain types of bacteria (Mani-López *et al.*, 2012). Once within the cell, where pH is sustained near 7, the acid dissociates (releasing H^+ and anions), lowering the bacterial cell pH as well as decreasing the nutrient transport and bacterial cell enzymes such as catalases and decarboxylases are repressed (Dahiya *et al.*, 2006; Huyghebaert *et al.*, 2011; Mani-López *et al.*, 2012). Subsequently, the H^+-ATPase pump will consume energy to try to normalise the pH inside the bacterial cell which eventually stops the bacterial growth or even kill it (Dahiya *et al.*, 2006). The anionic portion of the acid is trapped within the bacteria, becoming toxic and leading to osmotic complications for the bacteria (Dahiya *et al.*, 2006). The ability for organic acids to inhibit microorganisms is reliant on on the pKa value (the dissociation constant or the pH at which the acid is half dissociated) and the higher the pKa value, the more effective the acid's antimicrobial effect (Huyghebaert *et al.*, 2011). Pathogenic bacteria of the gut also produce toxins which cause damage to the structure of the villi and crypts of the intestine. A reduction in the amount of pathogenic intestinal bacteria affects the histomorphology of the gut causing increased villi height, thus improving the structure of the gut in poultry (Sugiharto, 2016). Supplementation of organic acids in broiler chicken diets may improve nutrient absorption which may result in the improvement of growth performance (Sugiharto, 2016).

Dose, type of organic acid product used and whether the acid is added in feeds or drinking water affects its efficacy in broiler diets (Dahiya *et al.*, 2006). Other factors which influence the antibacterial activity of an acid are chemical formula, pKa value of the acid, chemical form (acid, salt, coated or not), molecular weight, the nature of the microorganism, animal species and the buffering capacity of the feed (Huyghebaert *et al.*, 2011). In order for supplemented organic acids to improve performance and health of broilers, they must be administered in low dosages since excessive dosage may depress intestinal villi height, villi width, and crypt depth (Sugiharto, 2016). Blends of acids are preferred over supplements of only one acid as acid mixtures represent a range of pKa values and provide a broader spectrum of activity (Huyghebaert *et al.*, 2011; Sugiharto, 2016). This is since different types of organic acids diffuse into the cell cytoplasm through the bacterial cell wall and membrane at different rates (Sugiharto, 2016).

Antibacterial and host effects of some supplemented organic acids can play a role in being AGP alternatives but cannot fully replace antibiotics. Butyric acid, in particular, has shown to be significant source of energy for the cells of the gut epithelium and stimulates cell proliferation and differentiation as well as strengthen the mucosal barrier of the gut by encouraging the expression of tight junction proteins and by increasing the production of antimicrobial peptides in mucous (Huyghebaert *et al.*, 2011).

2.4.4 Encapsulated butyric acid as a potential short chain fatty acid

Fermentation by microbes primarily occurs in the caeca and colon, the by-products of which is short chain fatty acids (organic acids with one to six carbons) such as butyric acid. Although it is the least abundant of the primary SCFA produced, butyric acid is important as it is utilised by the intestinal epithelial cells as an immediate energy source to encourage their proliferation and differentiation as well as improve intestinal barrier function as well as aid in the development of the gut-associated lymphoid tissues (Abdelqader & Al-Fataftah, 2016; Dehghani-Tafti & Jahanian, 2016). Butyric acid has shown to increase the growth of villi and their heights (Guilloteau *et al.*, 2010; Levy *et al.*, 2015; Wu *et al.*, 2018), control intestinal pathogenic bacteria, such as *Salmonella* and *C. perfringens* as well as modulate the *Lactobacillus* populations (Van Immerseel *et al.*, 2005; Hu & Guo, 2007; Abdelqader *et al.*, 2017).

Because microbial fermentation is primarily in the hindgut, butyrate production in the small intestine is almost negligible (Smith *et al.*, 2012; Levy *et al.*, 2015). Free butyrate is quickly absorbed and metabolised by mucosa cells in the upper digestive tract of the chicken and continues to be metabolised and absorbed throughout the gastrointestinal tract starting from the crop (Levy *et al.*, 2015; Kaczmarek *et al.*, 2016). This limits the amount of butyrate reaching the small intestine (Kaczmarek et al., 2016). Along with its pungent smell, this makes butyrate less ideal as a feed additive (Levy *et al.*, 2015; Kaczmarek *et al.*, 2016). Because of these factors, butyric acid is often utilised in its butyrate form (a calcium or sodium salt) as it is more solid, stabile and has a reduced odour (Kaczmarek *et al.*, 2016). The efficacy of butyrate has, however, been found to increase when it is fed in a protected form such as encapsulation (Smith *et al.*, 2012). This microencapsulation prevents the rapid absorption in the upper digestive tract and thus rendering it available to be utilised further distal in the gastrointestinal tract, thus increasing the region that is exposed to the molecule (Van Immerseel *et al.*, 2005; Smith *et al.*, 2012; Kaczmarek *et al.*, 2016). Encapsulated or coated butyrate was shown to improve gut health by decreasing the pathogenic bacterial numbers in the gut (Van Immerseel *et al*., 2005). Therefore, it is suggested that the slow and varied release of protected sodium butyrate is more efficient in inhibiting the colonisation of pathogenic bacteria, as it is released and active along the length of the digestive tract (Bedford & Gong, 2018).

Abdelqader & Al-Fataftah (2016) found no significant change in final body weight and gain body weight in encapsulated butyric acid supplemented broilers under thermoneutral conditions. However, under heat-stressed conditions, final body weight and body weight gain were higher in birds that received encapsulated butyric acid than that of the control birds under the same heat stress conditions (Abdelqader & Al-Fataftah, 2016). Heat-stressed birds supplemented with encapsulated butyric acid also had a growth performance similar to that of

the control group under thermoneutral conditions (Abdelqader & Al-Fataftah, 2016). Supplementation of encapsulated butyric acid did not affect feed intake but was found to have similar growth performance results on body weight gain (Imran *et al.*, 2017). These findings are in conformity with Levy *et al.* (2015) and Kaczmarek *et al.* (2016), who reported that graded levels of coated butyric acid supplementation in broiler diets did improve performance without affecting feed intake. Researchers have shown that broilers fed a diet containing 2000 mg/kg sodium butyrate increased feed conversion compared with those fed a diet absent of sodium butyrate (Hu & Guo, 2007). However, in a study by Levy *et al.* (2015), they only fed up to 500g of an encapsulated butyric acid source and found an increase in feed conversion. The negative effect on feed conversion is likely due to too high levels of sodium or butyrate (Hu & Guo, 2007). Thus, it is worthy to note that variations in growth performance of broilers supplemented with butyric acid may be attributed to the inclusion level, environmental challenge and the type of microbial environment to which broilers are exposed (Wu *et al.*, 2018).

Furthermore, the regulation of growth by butyric acid or its sodium salt may be facilitated in part by its impact on the inflammatory response (Zhang *et al.*, 2011). Butyrate may have an anti-inflammatory effect facilitated by signalling pathways, such as the modulation of pro-inflammatory cytokines by inhibiting NF-kB activation (Guilloteau *et al.*, 2010). Thus, butyric acid is capable of reducing inflammation, subsequently restoring intestinal permeability (Guilloteau *et al.*, 2010).

Under thermoneutral conditions, encapsulated butyric acid improved the duodenal villi surface area and relative intestinal weight while villi height and absorptive epithelial cell area were unaffected (Abdelqader & Al-Fataftah, 2016; Imran *et al.*, 2017). Kaczmarek *et al.* (2016), however, found that birds fed diets supplemented with encapsulated butyrate had higher villi height than non-supplemented birds, whereas Levy *et al.* (2015) did not find any significant effect on the morphology of the gut with the addition of encapsulated butyric acid. Abdelqader & Al-Fataftah (2016) found that heat stress had a negative effect on the gut morphology of broilers, but when supplemented with butyric acid, the heat-stressed birds had similar villi heights, villi surface areas and relative intestinal weight than birds in thermoneutral conditions, thus confirming that butyric acid has a significant role in improving the recovery of villi damaged by heat stress. Similar results were found by Abdelqader *et al.* (2017) where intestinal morphology of heat-stressed cockerels supplemented with encapsulated butyric acid was similar to that of the thermoneutral control group. Dietary butyrate also alleviated damages to the villi thus decreasing the injury score in supplemented cockerels compared to the non-supplemented cockerels under heat stress, but no significant difference was seen in the thermoneutral control (Abdelqader *et al.*, 2017). Butyric acid is amongst the molecules known to promote intestinal epithelial cell restitution (an immediate response to damage) by providing energy to promote the proliferation of mucosal cells and support mechanisms

responsible for epithelial repair, thus improving the intestinal morphological structure (Abdelqader & Al-Fataftah, 2016; Wu *et al.*, 2018).

2.5 Conclusion

Broiler chickens are intensively selected and raised for high slaughter weight but at a decreasing age. Consequently, the poultry industry has become more intensive, increasing the risk of infectious diseases and pathogens being transferred from one individual to another. Antibiotics have been extensively used in the past and have partly aided livestock producers by decreasing disease and mortality as well as increase productive performance and growth. However, with the risk of antimicrobial resistance and the potential for transferal to humans, the livestock industry as a whole is under intense pressure to remove the use of low-dose supplementation of antibiotics.

Thus, research is focussed on finding potential substitutes to acclimate to the removal of antibiotics. Moreover, researchers have begun to review gut health as a way of improving the health and production in broilers. The microbial population as well as the gut histomorphology is integral in maintaining a healthy gastrointestinal tract. Dysbiosis, in particular, being of great concern as it results in decreased numbers of beneficial bacteria (such as *Lactobacillus*) and increased proportions of potentially pathogenic bacteria (such as *Salmonella spp.* and *Clostridium perfringens*). Changes in the microbial populations will subsequently cause damage to the gut mucosa, decreasing villi height and increasing crypt depths. These changes are further intensified by stressors such as heat stress and can result in great economical losses. Consequently, probiotics (such as *Bacillus subtilis*) and encapsulated organic acids (such as butyric acid) has gained interest as safe alternatives and may aid in mitigating the effects of environmental as well as pathogenic stressors by modulating the microbiota of the gastrointestinal tract, immune system modulation, increasing the integrity of the GIT and promote absorption in the small intestine.

Although many antibiotic alternatives show promise, none have shown to compensate fully in the absence of AGPs. The search for alternatives to antibiotic growth promoters is hindered by the lack of knowledge about the precise mechanisms of actions of the original antibiotics as well as the different alternatives. Further studies are needed to confirm the benefits as well as the exact mode of actions prior to any nutraceuticals being substantially used in the commercial poultry industry.

Chapter 3: Material and Methods

3.1 Introduction and ethics statement

This experiment aimed to test encapsulated butyric acid and *B. subtilis* PB6 both individually as well as in combination as potential AGP alternatives on the overall gut health and production performance with and without heat stress. Bird production performance were analysed on production parameters used within the poultry industry and gut health was analysed using microbial populations in the digesta, intestinal histomorphology and macroscopic gut health scores. All use of experimental animals was humane and in compliance with the guidelines from the Animal Ethics Committee of the University of Pretoria (ethical clearance number EC040-18). Chickens were humanely killed by cervical dislocation by trained individuals, which was in compliance with the method described by the South African Poultry Association Code of Practice June 2018.

3.2 Materials and methods

3.2.1 Animals and experimental design

One thousand nine hundred and twenty day-old Ross 308 chicks were obtained from Eagles Pride Hatchery (Roodeplaat, Gauteng, RSA). Day-old chicks were individually feather-sexed at the hatchery, and only male chicks were selected for the trial. The male chicks were obtained from a flock that was 60 weeks of age. The experiment was conducted at the Broiler Unit on the Hillcrest Experimental Farm (University of Pretoria, RSA) and separated into two environmentally controlled broiler houses (House 1 and House 2), each containing their own SKOV system. Both houses were run simultaneously and consisted of six dietary treatments with eight replications per treatment (48 pens in total) and twenty broilers per pen. At placement, twenty male broilers were randomly selected, weighed and allocated a pen number. Each chick received a neck tag with a unique number specifically designated to the pen. Broilers were housed in 1.2 m high meshed wire pens, each measuring 1.5 x 1.5 m (2.25 m^2) in size and reared under routine management practice. All pens contained fresh pine shavings for bedding covering a solid concrete floor, a tube feeder and five nipple drinkers. For the first seven days, extra pan feeders, paper squares and bell drinkers were provided. During the 35-day trial period, feed and water were provided *ad libitum*. The birds in House 2 were provided optimum rearing temperatures (thermoneutral conditions; TN) according to age and strain guidelines (Ross Broiler Management Handbook, 2018). House 1 was exposed to continual heat stress (HS) and remained at a temperature of approximately 3 - 5°C higher than that of the TN house. House temperature was captured 3 probes inside each house distributed along the centre of the house. Outside temperatures were measured using the same probes located on both sides of each house. Probe measured temperatures were

monitored using the SKOV environmental control system. Pen temperatures where captured using an infrared temperature gun three times a day (7:00, 16:00 and 20:00) during the first week, then twice a day (8:00 and 15:00) for the rest of the study period to ensure that the correct temperatures were met and birds in the HS house were showing signs of heat stress (panting, lethargy and spreading of wings and legs). The as-measured temperature profiles for the two houses are shown in Table A.1 Addendum A. The temperatures for both houses were controlled using fan and tunnel ventilation system. Negative pressure system was created, and a fan was used to extract air from the houses, creating a partial vacuum inside the house. Air entered the broiler house through air inlets which were evenly spaced around the sidewalls of the house. The houses received one hour darkness from 0-7d, two hour darkness from 8-14d, four hour darkness from 15-21d and eight hour darkness from 22-35d. The birds were vaccinated through drinking water for Gumboro disease at 10d and for commercial Newcastle disease at 16d and 23d.

3.2.2 Dietary treatments and feed analysis

The broilers were given a four-phase feeding program consisting of a pre-starter (0-7d; crumbles), starter (8-14d; crumbles), grower (15-28d; pellets) and finisher (29-35d; pellets). For each growth phase (pre-starter, starter, grower and finisher), a basal diet was fixed following recommendations set out by the breed specifications (Table 3.2.1) (Ross Broiler Management Handbook, 2018). A proximate analysis of the basal diets for all four growth phases was conducted, with results presented in Table 3.2.2. Analysed composition of the basal diets for the four phases (in Table 3.2.2) were marginally different to the formulated values for the same nutrients (in Table 3.2.1). The dietary treatments were as follows: 1) basal diet without any feed additives (Control); 2) basal diet with zinc bacitracin (AGP); 3) basal diet with encapsulated butyric acid (EBA); 4) basal diet with zinc bacitracin and encapsulated butyric acid (AGP + EBA); 5) basal diet with *Bacillus subtilis* source (*B. subtilis)*; and 6) basal diet with *B. subtilis* and encapsulated butyric acid (*B. subtilis* + EBA). Salinomycin was used as a coccidiostat and added to all diet phases at 0.05%. Zinc bacitracin was used as an AGP and included at 0.05% to the treatment diets where applicable. *B. subtilis* PB6 (CLOSTAT™; Kemin Industries, Inc, Sterkfontein, GT, RSA) was included at a rate of 500 g/ton and encapsulated butyric acid (ButiPEARL™; Kemin Industries, Inc, Sterkfontein, GT, RSA) was included at a rate of 300 g/ton. Presence of the *B. subtilis* PB6 in the final product was confirmed by agar plating of the feed samples (Kemin Industries, Inc, Sterkfontein, GT, RSA) and found to be more than the minimum 1 x 10^5 cfu/g required for sufficient inclusion (presented in Table 3.2.3). Samples of the treatment diets were tested for butyric acid recovery (Kemin Industries, Inc, Sterkfontein, GT, RSA) using the AOAC (20050, Method 986.13, and

found to be within the recommended recovery range of 80-120 mg/kg (presented in Table 3.2.3). The zinc bacitracin, *B. subtilis* and encapsulated butyric acid inclusions were added as required according to treatment diet on top of the basal mixture prior to mixing and pelleting.

Table 3.2.1 Ingredient and calculated nutrient composition of basal diet used during the study

	Pre-starter	Starter	Grower	Finisher
Ingredients (%)				
Yellow maize	53.523	60.456	64.848	68.288
Wheat bran	2.337	-	-	-
Soybean oilcake (CP 47%)	37.084	31.507	27.291	23.650
Sunflower oilcake (CP 36%)	3.000	4.000	3.000	3.000
Soybean oil (degummed)	-	-	1.605	2.037
Limestone flour	1.311	-	1.064	1.142
Course limestone	-	1.186	-	-
Monocalcium phosphate	1.152	1.249	0.704	0.598
Sodium chloride (salt)	0.250	0.239	0.247	0.263
Sodium bicarbonate	0.186	0.206	0.196	0.100
Broiler starter premix	0.300	0.300	-	-
Broiler grower premix	-	-	0.250	-
Broiler finisher premix	-	-	-	0.200
Methionine hydroxy analogue	0.376	0.338	0.312	0.272
Lysine HCl (78%)	0.266	0.301	0.280	0.229
Threonine (98%)	0.107	0.109	0.094	0.060
Salinomycin	0.050	0.050	0.050	0.050
Phytase (Atra® PHY)[1]	0.010	0.010	0.010	0.010
Pellet binder (Kembind™)[2]	0.200	0.200	0.200	0.200
Mould inhibitor[3]	0.150	0.150	0.150	0.150
Water	2.000	2.000	2.000	2.000
Calculated nutrients				
Dry matter (%)	87.877	87.894	87.935	87.949
Metabolisable energy (MJ/kg)	11.250	11.601	12.250	12.500
Crude protein (%)	24.000	22.000	20.000	18.500
Fat (%)	2.870	2.972	4.636	5.128
Fibre (%)	3.625	3.646	3.354	3.309
Ash (%)	5.807	5.523	4.667	4.487
Calcium (%)	1.000	0.900	0.800	0.800
Digestible phosphorus (%)	0.500	0.500	0.400	0.375
Sodium (%)	0.200	0.200	0.200	0.180
Chloride (%)	0.250	0.250	0.250	0.250
Potassium (%)	1.089	0.972	0.877	0.808
Digestible lysine (%)	1.300	1.200	1.075	0.950
Digestible methionine (%)	0.637	0.587	0.540	0.491
Digestible cysteine (%)	0.312	0.288	0.265	0.248
Digestible threonine (%)	0.845	0.780	0.699	0.618
Digestible tryptophan (%)	0.241	0.214	0.489	0.171
Digestible isoleucine (%)	0.880	0.796	0.713	0.653
Digestible arginine (%)	1.440	1.299	1.155	1.054
Digestible valine (%)	0.959	0.877	0.792	0.733

CP: crude protein; HCl: hydrochloric acid; MJ/kg : megajoules per kilogram

[1] Product of Danisco Animal Nutrition, distributed by Chemuniqué International (Pty) Ltd, Randburg, GT, RSA.

[2] Product of Kemin Industries, Inc, Sterkfontein, GT, RSA.

[3] Myco CURB® liquid a product of Kemin Industries, Inc, Sterkfontein, GT, RSA.

Table 3.2.2 Proximate composition of the basal diets used during the pre-starter, starter, grower and finisher phases

	Analysed composition (%)			
	Pre-starter (0-7d)	**Starter (7-14d)**	**Grower (14-28d)**	**Finisher (28-34d)**
Dry matter	88.393	88.658	87.716	88.700
Ash	6.226	5.402	5.111	4.575
Crude fibre	3.151	3.029	3.571	3.317
Crude protein	23.112	21.784	20.541	19.136
Ether extract	4.692	2.696	3.172	5.418
Calcium	1.129	0.927	0.840	0.625
Total phosphorus	0.691	0.726	0.573	0.546

Table 3.2.3 Recovery of *Bacillus subtilis* PB6 and EBA in the treatment diets during the pre-starter (0-7d), starter (7-14d), grower (14-28d) and finisher (28-34d) phases

	Treatment diets[1]				
	Control	**EBA**	**AGP + EBA**	***B. subtilis***	***B. subtilis* + EBA**
Pre-starter					
B. subtilis PB6[2] (cfu/g)	3.37×10^5	-	-	29.97×10^5	16.06×10^5
EBA[3] (mg/kg)	-	118.4	121.9	-	128.7
Starter					
B. subtilis PB6 (cfu/g)	3.51×10^5	-	-	32.01×10^5	20.83×10^5
EBA (mg/kg)	-	118.2	124.3	-	117.4
Grower					
B. subtilis PB6 (cfu/g)	0.70×10^5	-	-	31.93×10^5	19.72×10^5
EBA (mg/kg)	-	142.3	137.2	-	112.5
Finisher					
B. subtilis PB6 (cfu/g)	1.04×10^5	-	-	17.32×10^5	18.02×10^5
EBA (mg/kg)	-	110.0	136.1	-	149.9

cfu/g: colony forming unit per gram

[1] Control: basal diet; EBA: basal diet with encapsulated butyric acid; AGP + EBA: basal diet with zinc bacitracin and encapsulated butyric acid; *B. subtilis*: basal diet and *B. subtilis* source; *B. subtilis* + EBA: basal diet with *B. subtilis* and encapsulated butyric acid

[2] *B. subtilis* PB6 provided as CLOSTAT™ (Kemin Industries, Inc, Sterkfontein, GT, RSA)

[3] EBA: encapsulated butyric acid provided as ButiPEARL™ (Kemin Industries, Inc, Sterkfontein, GT, RSA)

3.2.3 Performance parameters

Birds and feed were weighed on day 0, 7, 14, 20, 28 and 34 on a pen basis. On day 20 and 34 (one day prior to slaughter and sampling), all birds were also weighed individually. All 48 pens in both houses were weighed separately. Birds were counted and placed into portable crates of known weight, weighed using a large scale and placed back into their original allocated pen. On days 20 and 34, each bird was identified by their tag number and placed individually on the scale to be weighed. For each phase, a fixed amount of feed per phase was calculated and distributed amongst the treatment pens. Residual feed was weighed on day 7, 14, 20, 28 and 34 and the data used to calculate the daily feed intake (FI) as well as the cumulative feed intake (CFI) per week. The feed conversion ratio (FCR) and cumulative FCR (CFCR) were corrected for bird mortalities and was calculated on the basis of gram of feed per gram of live BW gain. The production efficiency factor (PEF) was adapted to function in grams and calculated using the following equation:

Equation 1 $$PEF = \frac{\text{Liveability (\%)} \times \text{BW (g)}}{\text{Cumulative FCR} \times \text{Age (days)} \times 10}$$

3.2.4 Sampling and processing

On day 21 and 35, two birds per pen (192 birds per sampling day) were selected based on individual weights closest to the mean BW of birds in that pen. The selected broilers were euthanised via cervical dislocation prior to all sampling. Euthanised birds were dissected under aseptic conditions, the entire digestive system caudal to the proventriculus removed and relevant samples were taken. Gut health was first quantified by a scoring procedure based on the macroscopical appearance, prior to samples of different segments of the gut were collected for intestinal histology and digesta for analysis of the microbiome.

3.2.4.1 Macroscopic gut health scoring

Macroscopic scoring was performed at 21d and 35d by registered veterinarians belonging to the C4 Africa Group (Alphen Square North, Midrand, GT, RSA). Scoring was performed blinded, allowing no bias in the results. Each bird was scored immediately following the removal of the digestive system caudal to the proventriculus. The proposed method by Teirlynck *et al.* (2011) was followed and each bird received a score between 0 and 10 for intestinal dysbacteriosis parameters, where 0 represented a normal gastrointestinal tract (GIT) and 10 represented severe dysbacteriosis. A total of 10 parameters were assessed. Both sections cranial and caudal to the Meckel's diverticulum were scored individually for the following parameters: presence of ballooning; significant redness of the serosa or mucosa; reduction in the overall thickness of the gut wall, and abnormal gut content (mucous, water or gas) as well as undigested feed particles caudal to the ileocaecal junction as proposed by

Teirlynck *et al.*, 2011. Ballooning was assessed with the gut intact, thereafter 10cm incisions (situated approximately 10cm caudal and cranial from the Meckel's diverticulum) were made to expose the serosa for scoring. Thickness of the gut wall was evaluated by judging the flaccidity of the gut edges three seconds after opening of the intestinal segment by longitudinal incision. Caudal to the ileocaecal junction, a 10 cm incision was made to expose the digesta for scoring. An average score per pen was calculated from the scores of both birds and used as a single data point.

3.2.4.2 Intestinal histology and morphometric measurements

Samples approximately 3 cm in length were harvested from the centre of the duodenum (defined as extending from the end of the gizzard to the end of the duodenal loop); jejunum (defined as extending from the end of the duodenal loop to the Meckel's diverticulum); and ileum (defined as extending from the Meckel's diverticulum to the ileocaecal junction). Each section was immediately rinsed in phosphate buffered saline to remove digesta and fixed in 10% buffered formalin (v:v). Tissues were further cut into 4 mm thick transverse slices and processed at the Pathology Laboratory at the Faculty of Veterinary Sciences/Onderstepoort, University of Pretoria. Procedures included routine processing, paraffin wax embedding, sectioning, staining, and transfer onto microscope glass slides. For sectioning, ring-shaped transverse sections approximately 4 to 5 µm in thickness were cut and every tenth section was collected to ensure sufficient villi and crypts could be counted and measured. Sectioned segments were stained with haematoxylin and eosin (HE) and transferred to glass slides. Glass slides were viewed at a 5X magnification with a Zeiss AXIO Imager M2 microscope at the Laboratory for Microscopy and Microanalysis (Hatfield Campus, University of Pretoria, RSA). Images of nine randomly selected intact villi and crypts per intestinal section were captured to include the mucosal and submucosal layers. Villi height and crypt depth were measured using ImageJ software (a public domain Java image processing program). Villus height was measured from the tip of the villus to the villus-crypt junction, and crypt depth was defined as the depth between two adjacent villi. Each data point represented an average of the nine measurements taken for villi height and crypt depth for each intestinal sample respectively. Calculated data from the measurements included villus height to crypt depth ratio (µm:µm).

3.2.4.3 Microbiome analysis

Only birds housed in pens receiving certain treatments (Control, AGP, and *B. subtilis* + EBA) were selected for digesta sampling at 35d. Selected treatments were analysed due to the high costs involved for the analysis of the microbiome, thus treatments were selected that

were of most interest to the study. Samples were collected from both the heat-stressed and thermoneutral houses. Digesta samples, 10cm caudal and cranial to the Meckel's Diverticulum (20cm in total), were harvested from two birds of the same treatment, homogenised (pooled) in a labelled container and placed in the refrigerator. The populations of total bacteria were determined by q-PCR performed by Inqaba Biotechnical Industries (Pty) Ltd (Pretoria, RSA). Briefly, genomic DNA samples were PCR amplified using a universal primer pair (341F and 785R - targeting V3 and V4 of the 16S rRNA gene). Resulting amplicons were gel purified, end repaired and illumina specific adapter sequences were ligated to each amplicon. Following fluorometric quantification, the samples were individually indexed, and a further Ampure bead based purification step was performed. Amplicons were then sequenced on Illumina's MiSeq platform, using a MiSeq v3 (600 cycles) kit. Only reads of sufficient Q scores (>q20) and lengths were analysed. 20MB of data (2x300bp paired-end reads) were produced for each sample. For each sample, an individual breakdown of the different levels of classification (kingdom, phylum, class, order, family and genus and species) for each microbe was supplied and analysed.

3.3.5 Statistical analysis

The data for both the heat-stressed and thermoneutral houses (excluding the microbiome analysis) were analysed as a completely randomised design. The Statistical Analysis System (SAS, version 9.4) was used to conduct all statistical analysis. Repeated measures of analysis of variation using the generalised linear model (GLM) procedure of SAS were performed and included the effects of temperature (thermoneutral and heat stress), diets, replicates and the interactions of the main effects. Each replication served as an experimental unit. Significance of differences between means were evaluated using the Fischer's Test at a 95% confidence interval. Differences were accepted as significant when $P<0.05$.

The microbiome data at a phylum and a genus and species level was statistically analysed using the software program GenStat® (VSN International © 2017. GenStat for Windows 19th Edition. VSN International, Hemel Hempstead, UK.). Statistical analysis on the other classification levels was not performed. The Kruskal-Wallis one-way analysis of variance was used to test for differences in the percentage abundance between the Control, AGP and *B. subtilis* + EBA groups per microbe. If values were found to be significant ($P< 0.05$), a Mann-Whitney U test was used to compare each of the treatments against the other pairs-wise (Siegel, 1956). Thereafter, the two heat treatments were tested for differences in the percentage abundance of microbes using the Mann-Whitney U test. A Principal Component Analysis (PCA), a multivariate statistical technique, was utilised to the Phylum microbiome data, using the statistical program GenStat® (VSN International © 2017. GenStat for Windows

19th Edition. VSN International, Hemel Hempstead, UK.), in order to identify the main variates (microbes) that described the data and to simplify the interpretation of the data (Digby & Kempton, 1987; Krzanowski, 1988). PCA transformed the set of original correlated descriptors into a new set of principal components (linear combinations that explain the greatest amount of detected variability in the data). Descriptors were ranked so the variation in the dataset explained by the successive principal components decreases. The correlation structure of a group of multivariate observations was investigated and the axis along which maximum variability of the data occurs was identified and referred to as the first principal component (PC1;horizontal axis). The second principal component (PC2;vertical axis) was the axis along which the highest quantity of the outstanding variability lies subject to the constraint that the axes must be perpendicular. In this study the variates were the 16 microbes identified either exposed to heat stress conditions or thermoneutral conditions and three treatments (Control, AGP and *B. subtilis* + EBA diets), thus six treatment groups. The biplot reveals the similarity of points, where points closer together were similar and dissimilar points were further apart with respect to the variates that discriminate between them.

Chapter 4: Results

4.1 Performance parameters of treatment diets and house temperatures

The effect of the treatment diets and house temperatures on weekly body weights are presented in Table 4.1.1. Between 0 and 28 days of age, the body weights between treatments were not significantly different (P>0.05). However, at 34 days of age the AGP + EBA diet showed higher (P<0.05) body weights than the *B. subtilis* + EBA diet. The HS house had significantly higher (P<0.05) body weights at 0 days of age, whereas the TN house showed higher (P>0.05) body weights at 14 and 20 days of age. Although the bird's response to temperature treatments at 7, 28 and 34 days of age were not significantly different (P<0.05), a general trend exists whereby the TN house produced birds of higher body weight when compared with the HS house.

Table 4.1.1 The effects of different treatment diets and house temperatures on weekly body weights (g) of male broilers

	Days of Age					
	0	**7**	**14**	**20**	**28**	**34**
Treatment Diets[1]						
Control	43.63	179.34	437.01	811.91	1464.99	2047.82[ab]
AGP	43.56	180.94	440.95	823.10	1505.08	2059.29[ab]
EBA	43.44	180.76	436.53	804.57	1482.90	2042.42[ab]
AGP + EBA	43.69	181.58	441.11	818.01	1506.51	2102.98[a]
B. subtilis	43.84	179.86	435.23	807.65	1487.80	2045.21[ab]
B. subtilis + EBA	43.72	180.72	439.59	806.41	1503.18	2017.65[b]
SEM ±	0.235	1.299	3.760	7.858	27.236	22.894
House Temperature						
Heat-stressed	43.89[a]	179.78	433.53[b]	804.47[b]	1476.37	2046.81
Thermoneutral	43.41[b]	181.29	443.28[a]	819.41[a]	1507.11	2058.31
SEM ±	0.136	0.750	2.171	4.537	15.725	13.218

SEM: Standard error of the mean

[a,b] Column means within a treatment diet or house temperature without a common superscript differs significantly (P<0.05)

[1] Control: basal diet; AGP: basal diet with zinc bacitracin; EBA: basal diet with encapsulated butyric acid; AGP + EBA: basal diet with zinc bacitracin and encapsulated butyric acid; *B. subtilis*: basal diet and *B. subtilis* source; *B. subtilis* + EBA: basal diet with *B. subtilis* and encapsulated butyric acid.

The effect of the different treatment diets on weekly body weights with and without heat stress are presented in Table 4.1.2. Statistical analysis showed non-significant (P>0.05) body weight differences between broilers that received treatment diets that were in heat stress conditions. At 20 days of age, broilers exposed to thermoneutral conditions and received the AGP diet had significantly higher BW per bird than birds receiving the *B. subtilis* + EBA diet. Birds at 34 days of age that were exposed to TN conditions and received the Control; AGP; and AGP + EBA diets showed higher BW per bird (P<0.05) than the *B. subtilis* + EBA diet. In addition, the AGP + EBA diet had higher (P<0.05) body weights per bird than the *B. subtilis* diet.

Table 4.1.2 Weekly body weights (g) of male broilers subjected to heat stress and thermoneutral conditions fed different treatment diets

	Days of Age					
Treatment Diets[1]	***0***	***7***	***14***	***20***	***28***	***34***
Heat-stressed						
Control	43.50	177.00	429.81	800.03	1455.33	1998.06
AGP	43.69	180.63	435.69	812.31	1481.50	2035.38
EBA	43.94	178.81	431.31	798.75	1477.37	2042.34
AGP + EBA	43.81	181.13	436.81	807.88	1482.93	2078.40
B. subtilis	44.38	179.60	430.33	796.91	1472.65	2069.02
B. subtilis + EBA	44.00	181.50	437.25	810.94	1488.46	2057.65
SEM ±	0.332	1.838	5.317	11.113	38.518	32.377
Thermoneutral						
Control	43.75	181.69	444.21	823.78^{ab}	1474.65	2097.59^{ab}
AGP	43.44	181.26	446.22	833.88^{a}	1528.64	2083.20^{ab}
EBA	42.94	182.71	441.74	810.39^{ab}	1488.43	2042.50^{abc}
AGP + EBA	43.56	182.03	445.41	828.15^{ab}	1530.10	2127.55^{a}
B. subtilis	43.31	180.12	440.13	818.38^{ab}	1502.95	2021.40^{bc}
B. subtilis + EBA	43.44	179.93	441.93	801.89^{b}	1517.90	1977.65^{c}
SEM ±	0.332	1.838	5.317	11.113	38.518	32.377

SEM: Standard error of the mean

$^{a-c}$ Column means within a heat treatment without a common superscript differs significantly (P<0.05)

[1] Control: basal diet; AGP: basal diet with zinc bacitracin; EBA: basal diet with encapsulated butyric acid; AGP + EBA: basal diet with zinc bacitracin and encapsulated butyric acid; *B. subtilis*: basal diet and *B. subtilis* source; *B. subtilis* + EBA: basal diet with *B. subtilis* and encapsulated butyric acid.

The main and interactive effects of treatment and house temperature on weekly body weights for the trial period are presented in Table 4.1.3. No significant effects (P>0.05) on weekly body weights were found amongst different treatments. House temperature presented with significant (P<0.05) effects on BW of birds at 0, 14, and 20 days of age. Non-significant (P>0.05) interactive effects were observed between treatment and house temperature on the weekly body weights for the duration of the trial.

Table 4.1.3 The main and interaction effects of treatment diet and house temperature on weekly body weights (g) from 0 – 34d (P-values)

	Days of Age					
	0	**7**	**14**	**20**	**28**	**34**
Treatment[1]	0.8801	0.8608	0.8228	0.5137	0.8742	0.1931
Temperature[2]	0.0147	0.1578	0.0022	0.0224	0.1709	0.5401
Treatment x Temperature	0.3487	0.5461	0.9709	0.6769	0.9963	0.0688

[1] Control: basal diet; AGP: basal diet with zinc bacitracin; EBA: basal diet with encapsulated butyric acid; AGP + EBA: basal diet with zinc bacitracin and encapsulated butyric acid; *B. subtilis*: basal diet and *B. subtilis* source; *B. subtilis* + EBA: basal diet with *B. subtilis* and encapsulated butyric acid.

[2] Heat-stressed house was exposed to continual heat stress and remained at a temperature 3 - 5 ºC higher than that of the thermoneutral house.

Table 4.1.4 shows the effects of treatment diets and house temperatures on the weekly feed intakes of broilers from 0 – 34 days of age. Non-significant (P>0.05) differences in weekly feed intakes were found between 0 and 28 days of age. Between the ages of 28 and 34 days, significantly higher (P<0.05) feed intakes were observed in the AGP + EBA diet when compared with the AGP; EBA; *B. subtilis*; and *B. subtilis* + EBA treatment diets. Birds in TN conditions alone had higher body weights throughout the trial period, although significant differences were only observed between 0 and 20 days.

Table 4.1.4 The effects of different treatment diets (mean of all broilers within dietary treatment over temperature condition) and house temperatures (mean of all broiler within temperature conditions over all dietary treatments) on weekly feed intakes (g) of male broilers

	Days of Age				
	0 – 7	**7 – 14**	**14 – 20**	**20 – 28**	**28 – 34**
Treatment Diets[1]					
Control	163.22	325.59	583.18	1014.42	987.94[ab]
AGP	162.93	330.67	573.45	1004.73	976.34[b]
EBA	163.79	324.78	562.04	1013.92	971.79[b]
AGP + EBA	164.19	325.71	566.68	1028.68	1019.50[a]
B. subtilis	163.81	324.28	579.89	1023.49	979.99[b]
B. subtilis + EBA	163.58	325.79	562.27	1009.80	979.24[b]
SEM ±	1.237	3.257	11.851	13.479	13.410
House Temperature					
Heat-stressed	160.07[b]	322.73[b]	550.61[b]	1013.18	983.44
Thermoneutral	167.10[a]	329.54[a]	591.90[a]	1018.50	988.16
SEM ±	0.714	1.880	6.842	7.782	7.743

SEM: Standard error of the mean

[a,b] Column means within a treatment diet or house temperature without a common superscript differs significantly (P<0.05)

[1] Control: basal diet; AGP: basal diet with zinc bacitracin; EBA: basal diet with encapsulated butyric acid; AGP + EBA: basal diet with zinc bacitracin and encapsulated butyric acid; *B. subtilis*: basal diet and *B. subtilis* source; *B. subtilis* + EBA: basal diet with *B. subtilis* and encapsulated butyric acid.

Table 4.1.5 shows the effects of the different treatment diets on weekly feed intakes with and without heat stress. During 0 – 28 days of age, differences in weekly feed intakes were not significant (P>0.05) in birds exposed to both heat stress and thermoneutral conditions. Birds exposed to heat stress during the 28-34d period and received the AGP + EBA diet showed higher (P<0.05) weekly feed intakes in comparison with the Control diet, whereas the other treatment diets exhibited non-significant differences (P>0.05). Significantly higher (P<0.05) weekly feed intakes were observed in broilers subjected to thermoneutral temperatures who received the Control and AGP + EBA diets when compared with the EBA; *B. subtilis*; and *B. subtilis* + EBA diets.

Table 4.1.5 Weekly feed intakes (g) of male broilers exposed to heat stress or thermoneutral conditions fed different treatment diets

	Days of Age				
Treatment Diets[1]	**0 – 7**	**7 – 14**	**14 – 20**	**20 – 28**	**28 – 34**
Heat-stressed					
Control	158.63	319.19	551.81	998.47	951.61[b]
AGP	160.63	326.50	554.81	998.21	972.18[ab]
EBA	159.63	323.31	554.31	1006.59	981.52[ab]
AGP + EBA	162.50	322.06	533.38	1027.04	1012.28[a]
B. subtilis	158.00	321.52	559.92	1033.85	990.76[ab]
B. subtilis + EBA	161.06	323.81	549.41	1014.93	992.30[ab]
SEM ±	1.750	4.606	16.760	19.062	18.965
Thermoneutral					
Control	167.81	331.99	614.56	1030.36	1024.28[c]
AGP	165.23	334.84	592.09	1011.26	980.50[cd]
EBA	167.96	326.25	569.76	1021.24	962.06[d]
AGP + EBA	165.88	329.36	599.98	1030.32	1026.73[c]
B. subtilis	169.62	327.05	599.87	1013.13	969.22[d]
B. subtilis + EBA	166.09	327.76	575.12	1004.67	966.18[d]
SEM ±	1.750	4.606	16.760	19.062	18.965

SEM: Standard error of the mean

[a-d] Column means within a heat treatment without a common superscript differs significantly (P<0.05)

[1] Control: basal diet; AGP: basal diet with zinc bacitracin; EBA: basal diet with encapsulated butyric acid; AGP + EBA: basal diet with zinc bacitracin and encapsulated butyric acid; *B. subtilis*: basal diet and *B. subtilis* source; *B. subtilis* + EBA: basal diet with *B. subtilis* and encapsulated butyric acid.

The main and interactive effects between treatment diet and house temperature on the weekly feed intakes during the trial period are presented in Table 4.1.6. No significant (P>0.05) effects on weekly feed intakes amongst treatments were observed for the duration of the trial. Interactive effects of treatment diet and house temperature on weekly feed intakes was also non-significant (P>0.05) for the duration of the trial. The effect of house temperature on the weekly feed intakes of male broilers was significant (P<0.02) during 7 to 14 days of age and highly significant (P<0.0001) during 0 – 7 and 14 – 20 days of age. Thereafter, 20 – 34 days of age, the effect of house temperature on weekly feed intake was not significant (P>0.05).

Table 4.1.6 The main and interaction effects of treatment diet and house temperature on weekly feed intakes (g) from 0 – 34d (P-values)

	Days of Age				
	0 – 7	**7 – 14**	**14 – 20**	**20 – 28**	**28 – 34**
Treatment[1]	0.9838	0.7769	0.7140	0.8277	0.1514
Temperature[2]	<0.0001	0.0124	<0.0001	0.6306	0.6676
Treatment x Temperature	0.1558	0.9126	0.6085	0.7834	0.0984

[1] Control: basal diet; AGP: basal diet with zinc bacitracin; EBA: basal diet with encapsulated butyric acid; AGP + EBA: basal diet with zinc bacitracin and encapsulated butyric acid; *B. subtilis*: basal diet and *B. subtilis* source; *B. subtilis* + EBA: basal diet with *B. subtilis* and encapsulated butyric acid.

[2] Heat-stressed house was exposed to continual heat stress and remained at a temperature 3 - 5 ºC higher than that of the thermoneutral house.

The effects of different treatment diets and house temperatures on the cumulative feed intakes from 0 to 34 days of age are presented in Table 4.1.7. No significant (P>0.05) differences between treatment diets on cumulative feed intakes were found for the duration of the experimental trial. Significantly higher (P<0.05) cumulative feed intakes were observed in birds exposed to thermoneutral conditions than in heat-stressed conditions throughout the trial period (starting at 0 to 34 days). A general trend was observed whereby higher cumulative feed intakes were presented in birds exposed to thermoneutral conditions when compared with birds in heat stress conditions.

Table 4.1.7 The effects of different treatment diets (mean of all broilers within dietary treatment over temperature condition) and house temperatures (mean of all broiler within temperature conditions over all dietary treatments) on cumulative feed intakes (g) of male broilers

	Days of Age				
	0 – 7	**0 – 14**	**0 – 20**	**0 – 28**	**0 – 34**
Treatment Diets[1]					
Control	163.22	488.80	1071.99	2086.40	3074.35
AGP	162.93	493.59	1067.04	2071.78	3048.11
EBA	163.79	488.58	1050.62	2064.53	3036.33
AGP + EBA	164.19	489.90	1056.58	2085.26	3104.76
B. subtilis	163.81	488.09	1067.98	2091.48	3071.47
B. subtilis + EBA	163.58	489.36	1051.63	2061.43	3040.67
SEM ±	1.237	3.611	13.836	19.715	29.282
House Temperature					
Heat-stressed	160.07[b]	482.80[b]	1033.41[b]	2046.60[b]	3030.04[b]
Thermoneutral	167.10[a]	496.64[a]	1088.53[a]	2107.03[a]	3095.19[a]
SEM ±	0.714	2.085	7.988	11.382	16.906

SEM: Standard error of the mean

[a,b] Column means within a treatment diet or house temperature without a common superscript differs significantly (P<0.05)

[1] Control: basal diet; AGP: basal diet with zinc bacitracin; EBA: basal diet with encapsulated butyric acid; AGP + EBA: basal diet with zinc bacitracin and encapsulated butyric acid; *B. subtilis*: basal diet and *B. subtilis* source; *B. subtilis* + EBA: basal diet with *B. subtilis* and encapsulated butyric acid.

Table 4.1.8 shows the main and interaction effects of treatment diet and house temperature on the cumulative feed intakes of the male broilers. The effect of treatment diet alone on the cumulative feed intake of broilers was of no significant (P>0.05). The effect of house temperature on the cumulative feed intakes of the male broilers were highly significant showing a P-value less than 0.0001 between 0 – 20 days of age. For the duration of the trial starting at 0 – 28 days of age, temperature showed a significant influence (P<0.01) on the cumulative feed intakes of birds.

Table 4.1.8 The main and interaction effects of treatment diet and house temperature on cumulative feed intakes (g) from 0 – 34d (P-values)

	Days of Age				
	0 – 7	**0 – 14**	**0 – 20**	**0 – 28**	**0 – 34**
Treatment[1]	0.9838	0.9078	0.8193	0.8426	0.5620
Temperature[2]	<0.0001	<0.0001	<0.0001	0.0003	0.0079
Treatment x Temperature	0.1558	0.8095	0.6368	0.5907	0.2092

[1] Control: basal diet; AGP: basal diet with zinc bacitracin; EBA: basal diet with encapsulated butyric acid; AGP + EBA: basal diet with zinc bacitracin and encapsulated butyric acid; *B. subtilis*: basal diet and *B. subtilis* source; *B. subtilis* + EBA: basal diet with *B. subtilis* and encapsulated butyric acid.

[2] Heat-stressed house was exposed to continual heat stress and remained at a temperature 3 - 5 ºC higher than that of the thermoneutral house.

Table 4.1.9 shows the effect of different treatment diets on the cumulative feed intakes of birds which experienced heat stress and thermoneutral conditions during the experimental trial. Differences in cumulative feed intakes between treatment diets for heat-stressed birds were non-significant (P>0.05). In addition, broilers that experienced thermoneutral conditions had non-significant (P>0.05) differences in cumulative feed intakes amongst the treatment diets at 0 – 28 days of age. Starting at 0 to 34 days of age, thermoneutral birds who received the Control treatment had a higher (P<0.05) cumulative feed intake than the EBA and *B. subtilis* + EBA diets, however the latter two diets were not significantly different from each other.

Table 4.1.9 Cumulative feed intakes (g) of male broilers exposed to heat-stressed and thermoneutral conditions fed different treatment diets

	Days of Age				
Treatment Diets[1]	**0 – 7**	**0 – 14**	**0 – 20**	**0 – 28**	**0 – 34**
Heat-stressed					
Control	158.63	477.81	1029.62	2028.10	2979.70
AGP	160.63	487.13	1041.94	2040.14	3012.32
EBA	159.63	482.94	1037.25	2043.84	3025.37
AGP + EBA	162.50	484.56	1017.94	2044.98	3057.26
B. subtilis	158.00	479.52	1039.43	2073.29	3064.05
B. subtilis + EBA	161.06	484.88	1034.29	2049.22	3041.52
SEM ±	1.750	5.106	19.568	27.881	41.411
Thermoneutral					
Control	167.81	499.79	1114.35	2144.71	3168.99[a]
AGP	165.23	500.06	1092.15	2103.41	3083.91[ab]
EBA	167.96	494.22	1063.98	2085.22	3047.29[b]
AGP + EBA	165.88	495.24	1095.22	2125.54	3152.27[ab]
B. subtilis	169.62	496.67	1096.53	2109.66	3078.88[ab]
B. subtilis + EBA	166.09	493.85	1068.97	2073.65	3039.83[b]
SEM ±	1.750	5.106	19.568	27.881	41.411

SEM: Standard error of the mean

[a,b] Column means within a heat treatment without a common superscript differs significantly (P<0.05)

[1] Control: basal diet; AGP: basal diet with zinc bacitracin; EBA: basal diet with encapsulated butyric acid; AGP + EBA: basal diet with zinc bacitracin and encapsulated butyric acid; *B. subtilis*: basal diet and *B. subtilis* source; *B. subtilis* + EBA: basal diet with *B. subtilis* and encapsulated butyric acid.

The effects of different treatment diets and house temperatures on weekly feed conversion ratios of male broilers are presented in Table 4.1.10. For the duration of the trial, non-significant differences (P>0.05) on weekly feed conversion ratios amongst treatments were found. Significantly higher (P<0.05) weekly feed conversion ratios were found in broilers that were subjected to thermoneutral conditions than in birds that were heat-stressed during 0 – 7 and 14 – 20 days of age.

Table 4.1.10 The effects of different treatment diets (mean of all broilers within dietary treatment over temperature condition) and house temperatures (mean of all broiler within temperature conditions over all dietary treatments) on weekly feed conversion ratios of male broilers

	Days of Age				
	0 – 7	**7 – 14**	**14 – 20**	**20 – 28**	**28 – 34**
Treatment Diets[1]					
Control	1.20	1.27	1.56	1.50	2.22
AGP	1.18	1.28	1.51	1.47	2.22
EBA	1.19	1.27	1.53	1.50	2.16
AGP + EBA	1.19	1.26	1.50	1.50	2.13
B. subtilis	1.20	1.27	1.56	1.50	2.23
B. subtilis + EBA	1.20	1.26	1.54	1.50	2.23
SEM ±	0.011	0.010	0.037	0.023	0.049
House Temperature[2]					
Heat-stressed	1.18[b]	1.27	1.49[b]	1.51	2.18
Thermoneutral	1.21[a]	1.26	1.58[a]	1.48	2.22
SEM ±	0.007	0.006	0.021	0.014	0.028

SEM: Standard error of the mean

[a,b] Column means within a treatment diet or house temperature without a common superscript differs significantly (P<0.05)

[1] Control: basal diet; AGP: basal diet with zinc bacitracin; EBA: basal diet with encapsulated butyric acid; AGP + EBA: basal diet with zinc bacitracin and encapsulated butyric acid; *B. subtilis*: basal diet and *B. subtilis* source; *B. subtilis* + EBA: basal diet with *B. subtilis* and encapsulated butyric acid.

[2] Heat-stressed house was exposed to continual heat stress and remained at a temperature 3 - 5 ºC higher than that of the TN house.

The effect of the different treatment diets on the weekly feed conversion ratios of birds exposed to heat stress and thermoneutral conditions is shown in Table 4.1.11. Weekly feed conversion ratios in heat-stressed broilers between 0 – 28 days of age were not significantly different (P>0.05). Birds under thermoneutral conditions between 7 – 34 days of age also showed non-significant differences (P>0.05). Heat-stressed birds between 28 and 34 days of

age, had a higher (P<0.05) weekly feed conversion ratio for the Control treatment compared with the AGP + EBA treatment. In contrast, significant (P<0.05) differences in weekly feed conversion ratio at thermoneutral temperatures were only found during the first seven days where the AGP treatment exhibited a lower (P<0.05) weekly feed conversion ratio when compared with the *B. subtilis* diet.

Table 4.1.11 Weekly feed conversion ratios of male broilers exposed to heat stress and thermoneutral conditions fed different treatment diets

	Days of Age				
Treatment Diets[1]	**0 – 7**	**7 – 14**	**14 – 20**	**20 – 28**	**28 – 34**
Heat-stressed					
Control	1.19	1.26	1.49	1.51	2.27[a]
AGP	1.17	1.28	1.47	1.50	2.20[ab]
EBA	1.18	1.28	1.52	1.51	2.13[ab]
AGP + EBA	1.18	1.26	1.44	1.52	2.07[b]
B. subtilis	1.17	1.29	1.53	1.53	2.17[ab]
B. subtilis + EBA	1.17	1.27	1.46	1.50	2.21[ab]
SEM ±	0.016	0.015	0.052	0.033	0.069
Thermoneutral					
Control	1.22[ab]	1.27	1.63	1.49	2.18
AGP	1.19[b]	1.27	1.55	1.45	2.23
EBA	1.20[ab]	1.27	1.55	1.49	2.19
AGP + EBA	1.20[ab]	1.25	1.57	1.49	2.18
B. subtilis	1.24[a]	1.26	1.60	1.47	2.29
B. subtilis + EBA	1.22[ab]	1.25	1.61	1.50	2.24
SEM ±	0.016	0.015	0.052	0.033	0.069

SEM: Standard error of the mean

[a,b] Column means within a heat treatment without a common superscript differs significantly (P<0.05)

[1] Control: basal diet; AGP: basal diet with zinc bacitracin; EBA: basal diet with encapsulated butyric acid; AGP + EBA: basal diet with zinc bacitracin and encapsulated butyric acid; *B. subtilis*: basal diet and *B. subtilis* source; *B. subtilis* + EBA: basal diet with *B. subtilis* and encapsulated butyric acid.

The effect of treatment on the weekly feed conversion ratios presented with non-significant (P>0.05) results for the duration of the trial (Table 4.1.12). The effect of house temperature on the weekly feed conversion ratios showed a high level of significance (P<0.01) at 0 – 7 and 14 – 20 days of age. No significant interactive effects were observed between treatment diet and house temperature for the duration of the trial.

Table 4.1.12 The main and interaction effects of treatment diet and house temperature on weekly feed conversion ratios from 0 – 34d (P-values)

	Days of Age				
	0 – 7	**7 – 14**	**14 – 20**	**20 – 28**	**28 – 34**
Treatment[1]	0.8061	0.6917	0.8286	0.9510	0.5530
Temperature[2]	0.0005	0.1794	0.0019	0.1319	0.2748
Treatment x Temperature	0.3816	0.9297	0.8577	0.9766	0.6953

[1] Control: basal diet; AGP: basal diet with zinc bacitracin; EBA: basal diet with encapsulated butyric acid; AGP + EBA: basal diet with zinc bacitracin and encapsulated butyric acid; *B. subtilis*: basal diet and *B. subtilis* source; *B. subtilis* + EBA: basal diet with *B. subtilis* and encapsulated butyric acid.

[2] Heat-stressed house was exposed to continual heat stress and remained at a temperature 3 - 5 ºC higher than that of the TN house.

Table 4.1.13 shows the effect of the treatment diets and house temperatures on the cumulative feed conversion ratios of male broilers during the trial period. No significant differences in the cumulative feed conversion ratios amongst all the treatment diets were found during the course of the experimental trial. Significantly higher cumulative feed conversion ratios were found at 0 – 7 and 0 – 20 days of age when birds exposed to thermoneutral conditions were compared with birds that underwent heat stress.

Table 4.1.13 The effects of different treatment diets (mean of all broilers within dietary treatment over temperature condition) and house temperatures (mean of all broiler within temperature conditions over all dietary treatments) on the cumulative feed conversion ratios of male broilers

	Days of Age				
	0 – 7	**0 – 14**	**0 – 20**	**0 – 28**	**0 – 34**
Treatment Diets[1]					
Control	1.20	1.24	1.39	1.44	1.61
AGP	1.18	1.24	1.37	1.42	1.59
EBA	1.19	1.25	1.38	1.43	1.59
AGP + EBA	1.19	1.23	1.36	1.42	1.58
B. subtilis	1.20	1.25	1.40	1.44	1.61
B. subtilis + EBA	1.20	1.24	1.38	1.43	1.60
SEM ±	0.011	0.007	0.017	0.013	0.014
House Temperature[2]					
Heat-stressed	1.18[b]	1.24	1.36[b]	1.42	1.59
Thermoneutral	1.21[a]	1.24	1.41[a]	1.44	1.61
SEM ±	0.007	0.004	0.010	0.008	0.008

[a,b] Column means within a treatment diet or house temperature without a common superscript differs significantly ($P<0.05$)

[1] Control: basal diet; AGP: basal diet with zinc bacitracin; EBA: basal diet with encapsulated butyric acid; AGP + EBA: basal diet with zinc bacitracin and encapsulated butyric acid; *B. subtilis*: basal diet and *B. subtilis* source; *B. subtilis* + EBA: basal diet with *B. subtilis* and encapsulated butyric acid.

The influence of treatment diet on the cumulative feed conversion ratios of male broilers during the trial presented as non-significant (P>0.05) as presented in Table 4.1.14. House temperature revealed a high significance (P<0.01) on the cumulative feed conversion ratios of the broilers during 0 – 7 and 0 – 20 days of age. The interactive effects of treatment diet and house temperature on the cumulative feed conversion ratios presented as non-significant (P>0.05).

Table 4.1.14 The main and interaction effects of treatment diet and house temperature on cumulative feed conversion ratios from 0 – 34d (P-values)

	Days of Age				
	0 – 7	**0 – 14**	**0 – 20**	**0 – 28**	**0 – 34**
Treatment[1]	0.8061	0.7107	0.7038	0.6598	0.5914
Temperature[2]	0.0005	0.4886	0.0017	0.1907	0.1493
Treatment x Temperature	0.3816	0.9407	0.8304	0.8325	0.9729

[1] Control: basal diet; AGP: basal diet with zinc bacitracin; EBA: basal diet with encapsulated butyric acid; AGP + EBA: basal diet with zinc bacitracin and encapsulated butyric acid; *B. subtilis*: basal diet and *B. subtilis* source; *B. subtilis* + EBA: basal diet with *B. subtilis* and encapsulated butyric acid.

[2] Heat-stressed house was exposed to continual heat stress and remained at a temperature 3 - 5 ºC higher than that of the TN house.

The effect of the different treatment diets on the cumulative feed conversion ratios of broilers exposed to heat stress and TN conditions is presented in Table 4.1.15. For all treatment diets, no significant differences (P>0.05) were found in the cumulative feed conversion ratios of birds when exposed to heat stress conditions. Similarly, thermoneutral conditions observed non-significant differences (P>0.05) in cumulative feed conversion ratios between treatment diets for the duration of the trial, starting from 0 – 14 days of age. Cumulative FCR at 0 – 7 days of age in thermoneutral conditions presented a significantly (P<0.05) lower difference between AGP diet when compared with the *B. subtilis* diet only.

Table 4.1.15 Cumulative feed conversion ratios of male broilers exposed to heat-stressed and thermoneutral conditions fed different treatment diets

	Days of Age				
Treatment Diets[1]	**0 – 7**	**0 – 14**	**0 – 20**	**0 – 28**	**0 – 34**
Heat-stressed					
Control	1.19	1.24	1.36	1.43	1.60
AGP	1.17	1.24	1.36	1.42	1.59
EBA	1.18	1.25	1.38	1.43	1.59
AGP + EBA	1.18	1.23	1.33	1.41	1.57
B. subtilis	1.17	1.24	1.38	1.45	1.60
B. subtilis + EBA	1.17	1.23	1.34	1.41	1.59
SEM ±	0.016	0.010	0.025	0.019	0.020
Thermoneutral					
Control	1.22^{ab}	1.25	1.43	1.46	1.62
AGP	1.19^{b}	1.24	1.39	1.42	1.59
EBA	1.20^{ab}	1.24	1.39	1.43	1.60
AGP + EBA	1.20^{ab}	1.23	1.40	1.43	1.60
B. subtilis	1.24^{a}	1.25	1.42	1.44	1.62
B. subtilis + EBA	1.22^{ab}	1.24	1.41	1.45	1.62
SEM ±	0.016	0.010	0.025	0.019	0.020

SEM: Standard error of the mean

^{a,b} Column means within a heat treatment without a common superscript differs significantly (P<0.05)

[1] Control: basal diet; AGP: basal diet with zinc bacitracin; EBA: basal diet with encapsulated butyric acid; AGP + EBA: basal diet with zinc bacitracin and encapsulated butyric acid; *B. subtilis*: basal diet and *B. subtilis* source; *B. subtilis* + EBA: basal diet with *B. subtilis* and encapsulated butyric acid.

The effects of different treatment diets and house temperatures on the production efficiency factors of male broilers is presented in Table 4.1.16. Non-significant differences in the production efficiency factors of birds were observed in the different treatment diets up until 0 – 28 days of age. At 0 – 34 days of age, significantly lower (P<0.05) production efficiency factors were found in birds who received the *B. subtilis* + EBA diet when compared with the AGP + EBA diet, excluding the interactive effect of house temperature. House temperature showed differences in the production efficiency factor that were not significant (P>0.05) for the duration of the trial.

Table 4.1.16 The effects of different treatment diets (mean of all broilers within dietary treatment over temperature condition) and house temperatures (mean of all broiler within temperature conditions over all dietary treatments) on the production efficiency factors of male broilers

	Days of Age				
	0 – 7	**0 – 14**	**0 – 20**	**0 – 28**	**0 – 34**
Treatment Diets[1]					
Control	212.24	249.66	287.55	355.63	365.64[ab]
AGP	218.08	252.21	296.49	371.69	373.33[ab]
EBA	215.90	249.34	287.89	362.55	369.28[ab]
AGP + EBA	217.09	254.11	296.12	370.25	382.37[a]
B. subtilis	213.19	247.69	285.10	360.35	365.52[ab]
B. subtilis + EBA	215.42	252.56	289.89	367.08	362.48[b]
SEM ±	3.293	3.219	5.213	8.197	6.118
House Temperature					
Heat-stressed	217.25	248.53	292.55	362.57	370.68
Thermoneutral	213.39	253.33	288.47	366.62	368.86
SEM ±	1.901	1.858	3.010	4.733	3.532

SEM: Standard error of the mean

[a,b] Column means within a treatment diet or house temperature without a common superscript differs significantly (P<0.05)

[1] Control: basal diet; AGP: basal diet with zinc bacitracin; EBA: basal diet with encapsulated butyric acid; AGP + EBA: basal diet with zinc bacitracin and encapsulated butyric acid; *B. subtilis*: basal diet and *B. subtilis* source; *B. subtilis* + EBA: basal diet with *B. subtilis* and encapsulated butyric acid.

The effect of the different treatment diets on the production efficiency factor for the heat-stressed and thermoneutral houses at 7, 14, 20, 28 and 34 days of age is presented in Table 4.1.17. PEF data was calculated using liveability percentage, individual body weights, CFCR and age in days. Analysis of PEF values for birds under heat stress for the duration of the study as well as birds up to 28 days of age in thermoneutral conditions had non-significant differences (P>0.05). At 34 days of age raised in thermoneutral temperatures, broilers receiving the AGP + EBA diet had significantly (P<0.05) higher PEF values than both the *B. subtilis* and *B. subtilis* + EBA diets. The *B. subtilis* + EBA diet was also significantly (P<0.05)

lower in PEF values than the AGP diet at 34 days of age when not raised heat-stressed. In the same conditions, both the AGP and EBA diets had non-significant differences in PEF values compared with each other as well as all other treatment diets.

Table 4.1.17 Production efficiency factor of male broilers exposed to heat stress and thermoneutral conditions fed different treatment diets

	Days of Age				
Treatment Diets[1]	**0 – 7**	**0 – 14**	**0 – 20**	**0 – 28**	**0 – 34**
Heat-stressed					
Control	211.94	246.85	289.89	356.49	358.20
AGP	219.20	249.07	295.82	365.84	369.34
EBA	214.93	245.82	287.52	362.23	370.63
AGP + EBA	217.71	251.65	299.28	366.93	380.78
B. subtilis	219.34	245.85	284.94	356.24	372.02
B. subtilis + EBA	220.36	251.93	297.83	367.67	373.09
SEM ±	4.657	4.552	7.373	11.593	8.652
Thermoneutral					
Control	212.54	252.46	285.21	354.77	373.07^{abc}
AGP	216.96	255.36	297.15	377.54	377.33^{ab}
EBA	216.87	252.85	288.26	362.87	367.93^{abc}
AGP + EBA	216.46	256.56	292.97	373.58	383.96^{a}
B. subtilis	207.03	249.53	285.25	364.46	359.02^{bc}
B. subtilis + EBA	210.49	253.20	281.96	366.49	351.87^{c}
SEM ±	4.657	4.552	7.373	11.593	8.652

SEM: Standard error of the mean

$^{a-c}$ Column means within a heat treatment without a common superscript differs significantly (P<0.05)

[1] Control: basal diet; AGP: basal diet with zinc bacitracin; EBA: basal diet with encapsulated butyric acid; AGP + EBA: basal diet with zinc bacitracin and encapsulated butyric acid; *B. subtilis*: basal diet and *B. subtilis* source; *B. subtilis* + EBA: basal diet with *B. subtilis* and encapsulated butyric acid.

The main and interactive effects of treatment and house temperature on the production efficiency factors of male broilers from day 0 to 34 is shown in Table 4.1.18. From 0 to 34 days of age, non-significant (P>0.05) influences of the main effects (treatment diet and house temperature) on the production efficiency factors of the male broilers were found. Similarly, the interactive effects of treatment diet and house temperature on the production efficiency factors of the male broilers were also found to be non-significant.

Table 4.1.18 The main and interaction effects of treatment diet and house temperature on the production efficiency factors from 0 – 34d (P-values)

	Days of Age				
	0 – 7	**0 – 14**	**0 – 20**	**0 – 28**	**0 – 34**
Treatment[1]	0.8029	0.7298	0.5337	0.7238	0.2374
Temperature[2]	0.1554	0.0718	0.3408	0.5467	0.7174
Treatment x Temperature	0.5631	0.9910	0.8488	0.9886	0.3149

[1] Control: basal diet; AGP: basal diet with zinc bacitracin; EBA: basal diet with encapsulated butyric acid; AGP + EBA: basal diet with zinc bacitracin and encapsulated butyric acid; *B. subtilis*: basal diet and *B. subtilis* source; *B. subtilis* + EBA: basal diet with *B. subtilis* and encapsulated butyric acid.

[2] Heat-stressed house was exposed to continual heat stress and remained at a temperature 3 - 5 ºC higher than that of the TN house.

4.2 Macroscopic gut health scoring at 21 and 35 days of age

Table 4.2.1 shows the effects of different treatment diets and house temperatures on the macroscopic gut health scores of male broilers at 21 and 35 days of age. At 21 days of age, significantly higher ($P<0.05$) gut health scores were in birds who received the AGP + EBA diet than birds who received both the Control and *B. subtilis* + EBA diets. Broilers who received the *B. subtilis* diet had significantly higher 35 day gut health scores when compared with the EBA and *B. subtilis* + EBA diets. At both 21 and 35 days of age, the *B. subtilis* + EBA diet present with the lowest and second lowest scores respectively. House temperature showed non-significant ($P>0.05$) differences in gut health scores at both 21 and 35 days of age.

Table 4.2.1 The effects of different treatment diets (mean of all broilers within dietary treatment over temperature condition) and house temperatures (mean of all broiler within temperature conditions over all dietary treatments) on gut health scores[1] of male broilers at 21 and 35 days of age

	Days of age	
	21	**35**
Treatment Diets[2]		
Control	1.03[b]	1.84[ab]
AGP	1.44[ab]	1.91[ab]
EBA	1.22[ab]	1.50[b]
AGP + EBA	1.72[a]	2.00[ab]
B. subtilis	1.00[ab]	2.31[a]
B. subtilis + EBA	0.94[b]	1.59[b]
SEM ±	0.181	0.202
House Temperature		
Heat-stressed	1.33	1.70
Thermoneutral	1.11	2.02
SEM ±	0.104	0.117

SEM: Standard error of the mean

[a,b] Column means within a treatment diet or house temperature without a common superscript differs significantly (P<0.05)

[1] Score of 0 represents a normal gut and 10 severe dysbacteriosis

[2] Control: basal diet; AGP: basal diet with zinc bacitracin; EBA: basal diet with encapsulated butyric acid; AGP + EBA: basal diet with zinc bacitracin and encapsulated butyric acid; *B. subtilis*: basal diet and *B. subtilis* source; *B. subtilis* + EBA: basal diet with *B. subtilis* and encapsulated butyric acid.

Table 4.2.2 shows the results of birds fed different treatment diets raised with and without heat stress and the effect on the average macroscopic gut health scores at 21 and 35 days of age. Heat-stressed birds at 21 days of age showed significantly (P<0.05) higher gut health scores in the AGP + EBA diet when compared with the other treatment diets (excluding the AGP diet).Birds at 21 days of age in thermoneutral conditions showed no significant (P>0.05) differences in scores between any of the treatment diets. Numerically, birds exposed to both heat stress and thermoneutral conditions who received the Control and *B. subtilis* +

EBA diets had the lowest macroscopic gut health scores. At 35 days of age, heat-stressed birds showed higher (P<0.05) gut health scores in the AGP + EBA and *B. subtilis* diets in comparison to the EBA diet alone. At the same age, birds exposed to thermoneutral conditions only showed significantly higher gut health scores in the *B. subtilis* diet when compared with the *B. subtilis* + EBA diet. Thermoneutral birds at 35 days of age showed no statistically significant differences in gut health scores in the first four treatment diets when compared with either the *B. subtilis* or *B. subtilis* + EBA diets, however numerical differences exist where the *B. subtilis* + EBA diets had the lowest gut scores in comparison to the other treatment diets.

Table 4.2.2 Macroscopic gut scoring[1] of male broilers expose to heat stress and thermoneutral conditions fed different treatment diets at 21 and 35 days of age

	Heat-stressed		**Thermoneutral**	
Treatment Diets[2]	**21 d**	**35 d**	**21 d**	**35 d**
Control	1.25^{b}	1.56^{ab}	0.81	2.13^{ab}
AGP	1.56^{ab}	1.69^{ab}	1.31	2.13^{ab}
EBA	1.00^{b}	1.25^{b}	1.44	1.75^{ab}
AGP + EBA	2.13^{a}	2.06^{a}	1.31	1.94^{ab}
B. subtilis	1.06^{b}	2.13^{a}	0.94	2.50^{a}
B. subtilis + EBA	1.00^{b}	1.50^{ab}	0.88	1.69^{b}
SEM ±	0.255	0.286	0.255	0.286

SEM: Standard error of the mean

^{a,b} Column means with different superscripts differs significantly at P<0.05

[1] Score of 0 represents a normal gut and 10 severe dysbacteriosis

[2] Control: basal diet; AGP: basal diet with zinc bacitracin; EBA: basal diet with encapsulated butyric acid; AGP + EBA: basal diet with zinc bacitracin and encapsulated butyric acid; *B. subtilis*: basal diet and *B. subtilis* source; *B. subtilis* + EBA: basal diet with *B. subtilis* and encapsulated butyric acid.

The main and interaction effects of treatment and house temperature on gut health scores at 21 and 35 days of age is presented in Table 4.2.3. The influence of treatment diet on the gut health scores of birds was significant (P<0.05) at 21 days of age and non-significant (P>0.05) at 35 days of age. House temperature presented a non-significant influence on the gut health scores at 21 days of age and an influence that tended to be significant (P=0.0541) at 35 days of age. The interactive effects of treatment diet and house temperature presented as not significant (P>0.05) at both 21 and 35 days of age.

Table 4.2.3 The main and interaction effects of treatment diet and house temperature on gut health scores[1] at 21 and 35 days of age (P-values)

	Days of age	
	21	**35**
Treatment[2]	0.0215	0.0756
Temperature[3]	0.1419	0.0541
Treatment x Temperature	0.2713	0.8506

[1] Score of 0 represents a normal gut and 10 severe dysbacteriosis

[2] Control: basal diet; AGP: basal diet with zinc bacitracin; EBA: basal diet with encapsulated butyric acid; AGP + EBA: basal diet with zinc bacitracin and encapsulated butyric acid; *B. subtilis*: basal diet and *B. subtilis* source; *B. subtilis* + EBA: basal diet with *B. subtilis* and encapsulated butyric acid.

[3] Heat-stressed house was exposed to continual heat stress and remained at a temperature 3 - 5 ºC higher than that of the TN house.

4.3 Intestinal morphology of the duodenum, ileum and jejunum at 21 and 35 days of age

Inspection under the light microscope revealed normal histological structures of the intestinal mucosa (villi and crypts), tunica muscularis and submucosa of the duodenum, jejunum and ileum in birds within the heat-stressed and thermoneutral houses at 21 days of age (Figures A.1, A.2, A.3, A.4, A.5 and A.6 in Addendum A).

Table 4.3.1 shows the effect of the treatment diets and house temperature on the microscopic histomorphological measurements (villi heights, crypt depths and villi height to crypt depth ratios) of the small intestine of 21 day old male broilers. Non-significant differences in the villi heights of the duodenum and jejunum were observed amongst the treatment diets. Significantly higher ($P<0.05$) ileal villi heights were observed in birds who received the Control and the AGP diets compared with the AGP + EBA diet. Duodenal crypt depths of birds who received the *B. subtilis* + EBA diet were significantly deeper than those birds who received the Control and the *B. subtilis* diets. Jejunal crypt depths amongst all treatment diets were not significantly ($P>0.05$) different. Ileal crypt depths presented as significantly larger in birds that received the EBA diet than the *B. subtilis* diet. The *B. subtilis* + EBA diet a showed significantly ($P<0.05$) lower villus height to crypt depth ratio (VH/CD) than both the Control and the *B. subtilis* diets. Jejunal VH/CD showed differences amongst treatment diets that were not significant ($P>0.05$). Numerically, birds raised in thermoneutral conditions had higher villi heights in all three sections of the small intestine, however only villi heights in the jejunum and ileum were significant ($P<0.05$). 21 day old birds exposed to heat stress conditions had lower

Table 4.3.1 The effects of different treatment diets (mean of all broilers within dietary treatment over temperature condition) and house temperatures (mean of all broiler within temperature conditions over all dietary treatments) s on histomorphological measurements of the small intestine at 21 days of age

	Villus height (µm)			Crypt depth (µm)			Villus height to crypt depth (µm:µm)		
	Duodenum	**Jejunum**	**Ileum**	**Duodenum**	**Jejunum**	**Ileum**	**Duodenum**	**Jejunum**	**Ileum**
Treatment Diets[1]									
Control	1779.86	1072.94	613.12^{a}	209.41^{b}	174.77	182.67^{ab}	8.81^{a}	6.32	3.42^{a}
AGP	1727.51	1086.60	609.53^{a}	215.91^{ab}	167.11	194.31^{ab}	8.27^{ab}	6.66	3.25^{abc}
EBA	1727.81	1094.07	595.67^{ab}	217.85^{ab}	170.29	202.83^{a}	8.09^{ab}	6.51	2.99^{bc}
AGP + EBA	1693.97	1065.57	565.33^{b}	218.83^{ab}	165.82	200.10^{ab}	8.02^{ab}	6.65	2.91^{c}
B. subtilis	1711.26	1123.35	596.50^{ab}	209.56^{b}	168.07	180.76^{b}	8.69^{a}	6.76	3.33^{ab}
B. subtilis + EBA	1728.73	1122.70	574.71^{ab}	234.76^{a}	171.47	193.99^{ab}	7.51^{b}	6.74	2.99^{bc}
SEM ±	35.800	33.199	14.495	8.223	6.275	7.461	0.416	0.336	0.137
House Temperature									
Heat-stressed	1718.76	1049.68^{b}	559.24^{b}	209.64^{b}	161.08^{b}	187.87	8.53	6.66	3.04
Thermoneutral	1737.63	1138.74^{a}	625.71^{a}	255.80^{a}	178.09^{a}	197.01	7.94	6.55	3.25
SEM ±	20.669	19.168	8.369	4.748	3.623	4.307	0.240	0.194	0.079

SEM: Standard error of the mean

$^{a-c}$ Column means within a treatment diet or house temperature without a common superscript differs significantly ($P<0.05$)

[1] Control: basal diet; AGP: basal diet with zinc bacitracin; EBA: basal diet with encapsulated butyric acid; AGP + EBA: basal diet with zinc bacitracin and encapsulated butyric acid; *B. subtilis*: basal diet and *B. subtilis* source; *B. subtilis* + EBA: basal diet with *B. subtilis* and encapsulated butyric acid.

crypt depths in all three sections of the small intestine, however only duodenal and jejunal crypt depths were significantly different. Duodenal, jejunal and ileal VH/CD were not significantly different amongst birds exposed to heat stress and thermoneutral conditions.

The effect of the different treatment diets at 21 days of age on the villus height, crypt depth and villus height to crypt depth ratio in the small intestine for the two houses is presented in Table 4.3.2. Villi height measurements at 21 days of age in the duodenum, jejunum and ileum of heat-stressed birds revealed non-significant differences between treatments. Crypt depth measurements in the same circumstances showed non-significant ($P>0.05$) differences in jejunal and ileal measurements. However, duodenal measurements in heat-stressed broilers showed significantly ($P<0.05$) lower crypt depths in the *B. subtilis* treatment diet when compared with the AGP + EBA; and *B. subtilis* + EBA diets. Ratios of villus height to crypt depth in heat-stressed birds revealed non-significant ($P>0.05$) differences between the treatment diets in the jejunum and ileum. Calculated villus height to crypt depth ratio showed that the duodenal measurements of heat-stressed birds were not significantly ($P>0.05$) different when the AGP; EBA; AGP + EBA; and *B. subtilis* + EBA diets were compared, although the former four diets had lower ($P<0.05$) villus height to crypt depth ratios in relation to the *B. subtilis* diets. At 21 days of age, birds kept in thermoneutral conditions showed significantly higher villi heights in the Control diets when compared with the AGP + EBA and *B. subtilis* diets. Similarly, ileal villi measurements also showed significantly higher villi heights in the Control diets when compared with the AGP + EBA and *B. subtilis* diets but the *B. subtilis* + EBA diet also had a shorter ($P<0.05$) height in comparison with the Control. Villi measurements from the jejunum in non-stressed birds revealed non-significant ($P>0.05$) differences between all treatments. Duodenal crypt depths at 21 days of age in birds raised without heat stress revealed that the *B. subtilis* + EBA crypt depths were greater ($P<0.05$) in relation to the Control, AGP and AGP + EBA treatment diets. Jejunal crypt depths showed significantly ($P<0.05$) deeper crypts only in the Control diets when compared to the AGP diet in birds that were exposed to thermoneutral conditions for 21 days. In the same environmental conditions, ileal samples had only significantly ($P<0.05$) greater crypt depths in the EBA treatment when compared with the *B. subtilis* treatment. Ratios of villus height to crypt depth in the duodenum revealed the Control diets had a higher ($P<0.05$) ratio when compared with the *B. subtilis* and *B. subtilis* + EBA diets in the birds which did not experience heat stress. Villus height to crypt depth ratio of the broilers raised in thermoneutral conditions produced differences that where not statistically significant ($P>0.05$) in the jejunum. In the ileum, however, villi height to crypt depth ratios were lower ($P<0.05$) in the AGP + EBA treatment when compared with the Control and *B. subtilis* diets. The EBA diet had significantly ($P<0.05$) lower villi height to crypt depth ratios in comparison to the Control treatment diet.

Table 4.3.2 Histomorphological measurements of the small intestine of male broilers exposed to heat-stressed and thermoneutral conditions at 21 days of age

	Villus height (µm)			Crypt depth (µm)			Villus height to crypt depth (µm:µm)		
Treatment Diets[1]	**Duodenum**	**Jejunum**	**Ileum**	**Duodenum**	**Jejunum**	**Ileum**	**Duodenum**	**Jejunum**	**Ileum**
Heat-stressed									
Control	1707.88	992.80	543.83	205.14^{ab}	158.12	169.77	8.52^{ab}	6.47	3.30
AGP	1723.44	1026.48	579.88	214.57^{ab}	169.92	194.13	8.27^{b}	6.19	3.05
EBA	1693.86	1078.44	555.23	213.17^{ab}	165.81	188.54	8.20^{b}	6.55	2.99
AGP + EBA	1702.08	1054.67	545.26	222.98^{a}	158.68	197.28	8.04^{b}	6.82	2.87
B. subtilis	1755.45	1048.64	580.58	184.15^{b}	157.10	185.41	10.01^{a}	6.73	3.15
B. subtilis + EBA	1729.83	1097.03	550.69	217.84^{a}	156.87	192.08	8.13^{b}	7.20	2.90
SEM ±	50.629	46.951	20.499	11.629	8.874	10.551	0.589	0.477	0.194
Thermoneutral									
Control	1851.85^{a}	1153.09	682.41^{a}	213.67^{d}	191.42^{a}	195.57^{ab}	9.10^{c}	6.17	3.55^{a}
AGP	1731.59^{ab}	1146.73	639.19^{ab}	217.26^{d}	164.30^{b}	194.49^{ab}	8.27^{cd}	7.14	3.45^{abc}
EBA	1761.76^{ab}	1109.71	636.12^{ab}	222.53^{cd}	174.76^{ab}	217.12^{a}	7.98^{cd}	6.47	2.98^{bc}
AGP + EBA	1685.86^{b}	1076.46	585.39^{b}	214.68^{d}	172.96^{ab}	202.92^{ab}	8.00^{cd}	6.48	2.95^{c}
B. subtilis	1667.07^{b}	1198.07	612.42^{b}	234.97^{cd}	179.05^{ab}	176.10^{b}	7.38^{d}	6.79	3.51^{ab}
B. subtilis + EBA	1727.64^{ab}	1148.37	598.72^{b}	251.67^{c}	186.08^{ab}	195.91^{ab}	6.89^{d}	6.27	3.07^{abc}
SEM ±	50.629	46.951	20.499	11.629	8.874	10.551	0.589	0.477	0.194

SEM: Standard error of the mean

[a-d] Column means within a heat treatment without a common superscript differs significantly (P<0.05)

[1] Control: basal diet; AGP: basal diet with zinc bacitracin; EBA: basal diet with encapsulated butyric acid; AGP + EBA: basal diet with zinc bacitracin and encapsulated butyric acid; *B. subtilis*: basal diet and *B. subtilis* source; *B. subtilis* + EBA: basal diet with *B. subtilis* and encapsulated butyric acid.

The main and interaction effects of treatment and house temperature on villi height, crypt depth and villus height to crypt depth ratio of the small intestine of 21 day old male broilers is presented in Table 4.3.3. The interactive effects of treatment diet and house temperature on villi height in the duodenum, jejunum and ileum were non-significant (P>0.05), as was treatment diet as a main effect. House temperature alone, however, had a highly significant (P<0.01) influence on the villi height in the jejunum and ileum but not in the duodenum. The interactive effects of treatment diet and house temperature on crypt depth in the three sections of the small intestine were non-significant (P>0.05), as was treatment diet as a main effect. House temperature alone, however, had a significant influence on the crypt depth in the duodenum and jejunum but not in the ileum. The influence of treatment diet on the VH/CD of birds was non-significant in the duodenum and jejunum but was significant (P<0.05) in the ileum. Both the house temperature as a main effect and the interactive effects of treatment diet and house temperature on the VH/CD presented as non-significant.

Table 4.3.3 The main and interaction effects of treatment diet and house temperature on histomorphological measurements of the small intestine at 21d (P-values)

	Villus height			**Crypt depth**			**Villus height to crypt depth**		
	D	**J**	**I**	**D**	**J**	**I**	**D**	**J**	**I**
Treatment[1]	0.667	0.743	0.142	0.284	0.927	0.215	0.267	0.943	0.044
Temperature[2]	0.520	0.002	<0.0001	0.019	0.001	0.137	0.086	0.705	0.066
Treatment x Temp	0.306	0.511	0.117	0.128	0.273	0.423	0.096	0.529	0.890

D: Duodenum, J: Jejunum, I: Ileum, Temp: Temperature

[1] Control: basal diet; AGP: basal diet with zinc bacitracin; EBA: basal diet with encapsulated butyric acid; AGP + EBA: basal diet with zinc bacitracin and encapsulated butyric acid; *B. subtilis*: basal diet and *B. subtilis* source; *B. subtilis* + EBA: basal diet with *B. subtilis* and encapsulated butyric acid.

[2] Heat-stressed house was exposed to continual heat stress and remained at a temperature 3 - 5 ºC higher than that of the TN house.

Inspection under the light microscope revealed normal histological structures of the intestinal mucosa (villi and crypts), tunica muscularis and submucosa of the duodenum, jejunum and ileum in HS and TN houses at 35d (Figures A.7, A.8, A.9, A.10, A.11 and A.12 in Addendum A).

The effects of different treatment diets and house temperatures on the histomorphological measurements of the small intestine of broilers at 35 days of age is presented in Table 4.3.4. For all segments of the small intestine, the differences in villi height, crypt depth and VH/CD were not significant amongst all treatment diets. Birds exposed to thermoneutral conditions had significantly higher ($P<0.05$) villi heights in the jejunum and ileum but not in the duodenum. At 35 days of age, duodenal, jejunal and ileal crypt depths were not significantly different ($P>0.05$) between birds exposed to heat stress and thermoneutral conditions, although numerically deeper crypt depths were found in the duodenal and jejunal samples. Birds exposed to thermoneutral conditions showed higher VH/CD ratios overall, however only jejunal and ileal samples showed significantly higher ($P<0.05$) results.

Table 4.3.5 reveals the effect of the different treatment diets on the histomorphological measurements in the duodenum, ileum and jejunum for broilers at 35 days of age with and without heat stress. Birds which experienced heat stress for 35 days showed no significant ($P>0.05$) differences in the heights of villi between treatments. Likewise, crypt depths between treatment diets of heat-stressed birds were also not significantly ($P>0.05$) different in duodenal and ileal samples. Jejunal samples, however, showed deeper ($P<0.05$) crypts in the Control diets when compared with the AGP and EBA diets separately, although the differences between the latter two diets where observed to be non-significant. When the AGP + EBA; *B. subtilis*; and *B. subtilis* + EBA diets were compared, differences in jejunal crypt depths were not significant ($P>0.05$). Differences in the villi height to crypt depth ratios in the duodenum and jejunum of heat-stressed birds were not significantly ($P>0.05$) different between all treatment diets. Ileal samples, however, showed lower ($P<0.05$) ratios in the Control and EBA treatments when compared with the AGP treatment diet. Broilers which experienced thermoneutral temperatures to 35 days of age showed villi heights that were non-significant ($P>0.05$) throughout the small intestine. The effect of the treatment diets on duodenal and ileal crypt depths revealed non-significant ($P>0.05$) differences in broilers kept in thermoneutral conditions. Jejunal crypt depths showed only deeper ($P<0.05$) crypts in the AGP diet in relation to the *B. subtilis* diet. Significantly ($P<0.05$) lower villus height to crypt depth ratios in the duodenum of birds without heat stress were only observed in *B. subtilis* diet when compared with the EBA diet. Jejunal villi height to crypt depth data in broilers without heat stress showed that both the Control and *B. subtilis* + EBA diets had higher ($P<0.05$) ratios when compared with the AGP diet, while ratios in the ileum showed non-significant differences amongst all treatment diets.

Table 4.3.4 The effects of different treatment diets (mean of all broilers within dietary treatment over temperature condition) and house temperatures (mean of all broiler within temperature conditions over all dietary treatments) on histomorphological measurements of the small intestine at 35 days of age

	Villus height (µm)			Crypt depth (µm)			Villus height to crypt depth (µm:µm)		
	Duodenum	**Jejunum**	**Ileum**	**Duodenum**	**Jejunum**	**Ileum**	**Duodenum**	**Jejunum**	**Ileum**
Treatment Diets[1]									
Control	1826.46	1370.15	753.93	204.54	166.79	173.69	9.17	8.33	4.33
AGP	1804.44	1302.78	775.88	194.85	163.81	167.47	9.37	8.05	4.74
EBA	1879.55	1320.97	753.01	189.11	160.50	171.57	10.07	8.26	4.45
AGP + EBA	1832.65	1276.55	746.10	192.83	162.55	170.73	9.66	8.02	4.48
B. subtilis	1842.73	1339.41	748.79	204.30	161.05	172.24	9.12	8.51	4.47
B. subtilis + EBA	1898.08	1328.39	756.95	204.95	158.05	172.20	9.43	8.42	4.50
SEM ±	47.507	34.924	20.651	6.570	5.374	6.910	0.362	0.287	0.202
House Temperature									
Heat-stressed	1872.06	1279.20[b]	711.48[b]	201.73	162.78	170.72	9.41	7.95[b]	4.27[b]
Thermoneutral	1822.58	1366.89[a]	800.07[a]	195.13	161.73	171.91	9.53	8.58[a]	4.72[a]
SEM ±	27.428	20.163	11.923	3.793	3.103	3.989	0.209	0.166	0.117

SEM: Standard error of the mean

[a,b] Column means within a treatment diet or house temperature without a common superscript differs significantly ($P<0.05$)

[1] Control: basal diet; AGP: basal diet with zinc bacitracin; EBA: basal diet with encapsulated butyric acid; AGP + EBA: basal diet with zinc bacitracin and encapsulated butyric acid; *B. subtilis*: basal diet and *B. subtilis* source; *B. subtilis* + EBA: basal diet with *B. subtilis* and encapsulated butyric acid.

Table 4.3.5 Histomorphological measurements of the small intestine of male broilers exposed to heat-stressed and thermoneutral conditions at 35 days of age

	Villus height (µm)			Crypt depth (µm)			Villus height to crypt depth (µm:µm)		
Treatment diets[1]	**Duodenum**	**Jejunum**	**Ileum**	**Duodenum**	**Jejunum**	**Ileum**	**Duodenum**	**Jejunum**	**Ileum**
Heat-stressed									
Control	1852.27	1341.41	710.64	215.78	177.12^{a}	175.62	8.78	7.69	3.95^{b}
AGP	1805.18	1266.91	726.84	200.64	152.20^{b}	158.01	9.17	8.38	4.77^{a}
EBA	1859.62	1218.64	691.25	191.38	154.29^{b}	176.66	9.83	7.95	3.95^{b}
AGP + EBA	1843.90	1215.84	716.40	198.36	161.86^{ab}	171.40	9.42	7.58	4.31^{ab}
B. subtilis	1955.74	1354.41	713.76	206.96	169.08^{ab}	172.76	9.50	8.22	4.34^{ab}
B. subtilis + EBA	1915.64	1277.96	709.97	197.28	162.11^{ab}	169.89	9.74	7.90	4.29^{ab}
SEM ±	67.185	49.390	29.204	9.292	7.600	9.772	0.512	0.406	0.286
Thermoneutral									
Control	1800.66	1398.89	797.23	193.31	156.47^{cd}	171.77	9.55^{ab}	8.96^{a}	4.71
AGP	1803.70	1338.65	824.88	189.06	175.42^{c}	176.93	9.58^{ab}	7.73^{b}	4.71
EBA	1899.49	1423.30	814.77	186.84	166.72^{cd}	166.47	10.30^{a}	8.57^{ab}	4.94
AGP + EBA	1821.40	1337.27	775.79	187.30	163.23^{cd}	170.06	9.91^{ab}	8.47^{ab}	4.64
B. subtilis	1729.72	1324.40	783.83	206.64	153.02^{d}	171.72	8.73^{b}	8.80^{ab}	4.60
B. subtilis + EBA	1880.52	1378.81	803.94	212.62	155.52^{cd}	174.52	9.13^{ab}	8.95^{a}	4.71
SEM ±	67.185	49.390	29.204	9.292	7.600	9.772	0.512	0.406	0.286

SEM: Standard error of the mean

$^{a-b}$ Column means with different superscripts differs significantly at P<0.05

[1] Control: basal diet; AGP: basal diet with zinc bacitracin; EBA: basal diet with encapsulated butyric acid; AGP + EBA: basal diet with zinc bacitracin and encapsulated butyric acid; *B. subtilis*: basal diet and *B. subtilis* source; *B. subtilis* + EBA: basal diet with *B. subtilis* and encapsulated butyric acid

The main and interaction effects of treatment diet and house temperature on histomorphological measurements of the duodenum, jejunum and ileum of 35 day old male broilers is shown in Table 4.3.6. The influence of treatment diet on the villus height, crypt depth and villus height to crypt depth ratio presented as non-significant (P>0.05). The influence of house temperature as a main effect on the villi height was highly significant (P<0.005) in the jejunum and ileum but non-significant in the duodenum. The influence of house temperature as a main effect on the crypt depth of all three sections of the small intestine were not significant (P>0.05). The influence of house temperature on the VH/CD ratios of the duodenum were not significant but presented as significant in the jejunum and the ileum. The interactive effect of treatment diet and house temperature presented as non-significant amongst all histomorphological measures, except jejunal crypt depth which presented as significant (P<0.05).

Table 4.3.6 The main and interaction effects of treatment diet and house temperature on histomorphological measurements of the small intestine at 35d (P-values)

	Villus height			**Crypt depth**			**Villus height to crypt depth**		
	D	**J**	**I**	**D**	**J**	**I**	**D**	**J**	**I**
Treatment[1]	0.743	0.529	0.933	0.348	0.927	0.993	0.457	0.804	0.819
Temperature[2]	0.206	0.003	<0.0001	0.222	0.812	0.834	0.671	0.009	0.008
Treatment x Temp	0.461	0.303	0.913	0.481	0.041	0.764	0.551	0.239	0.516

D: Duodenum, J: Jejunum, I: Ileum, Temp: Temperature

[1] Control: basal diet; AGP: basal diet with zinc bacitracin; EBA: basal diet with encapsulated butyric acid; AGP + EBA: basal diet with zinc bacitracin and encapsulated butyric acid; *B. subtilis*: basal diet and *B. subtilis* source; *B. subtilis* + EBA: basal diet with *B. subtilis* and encapsulated butyric acid.

[2] Heat-stressed house was exposed to continual heat stress and remained at a temperature 3 - 5 ºC higher than that of the TN house.

4.4 Microbiome and diversity

Results from the 16s rRNA HT-NGS analysis showed that bacteria were the dominating microorganisms of the ilea at a kingdom level in chickens at 35 days of age in both the heat stress and thermoneutral treatments (Figure 4.4.1). In both the heat-stressed and thermoneutral houses, birds receiving the Control diet had the greatest abundance of microbes of unknown organisms with thermoneutral birds having the highest percentage of unknown organisms.

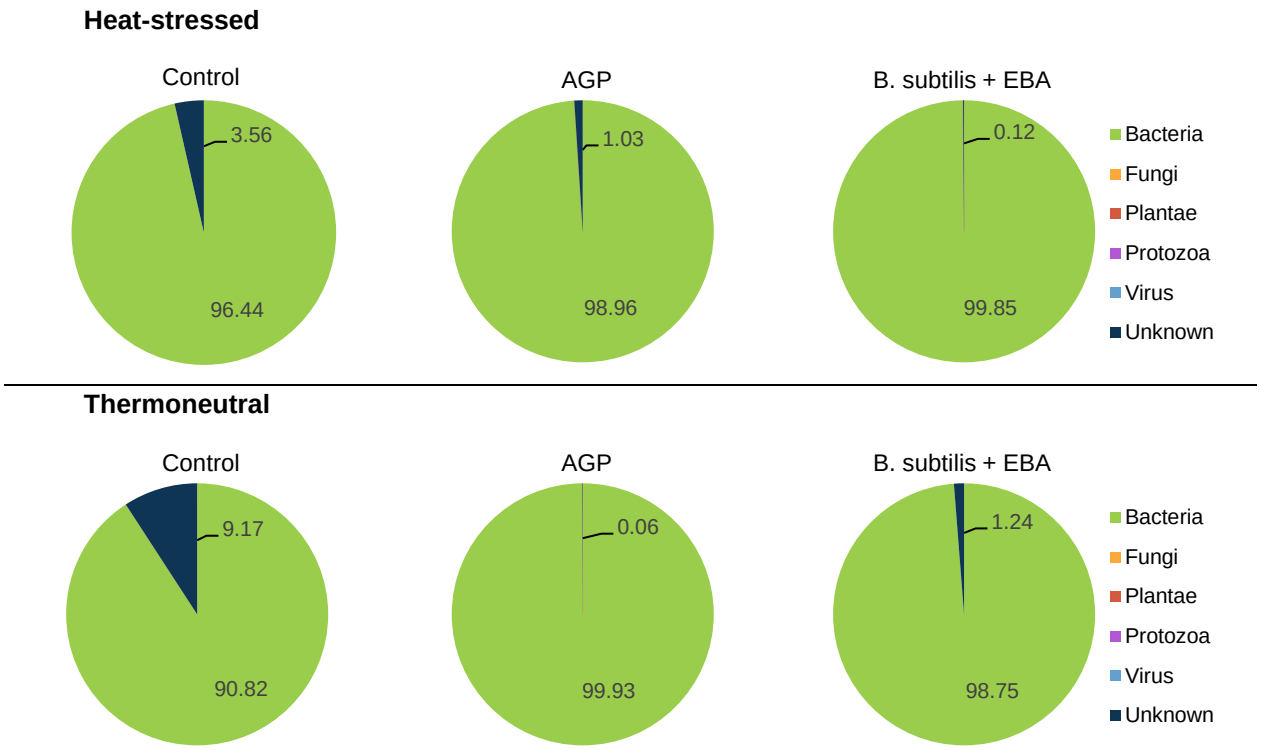

Figure 4.4.1 Composition (%) of the ileal microbiome at kingdom level of broilers at 35d exposed to heat-stressed and thermoneutral conditions receiving Control, AGP and *Bacillus subtilis* + EBA diets
(Control: basal diet; AGP: basal diet with zinc bacitracin; *B. subtilis* + EBA: basal diet with *B. subtilis* and encapsulated butyric acid)

At a phylum level, percentage abundance of *Firmicutes* in the ilea amongst treatments were similar in birds which experienced heat stress, however in thermoneutral conditions, the Control group had the highest abundance of *Firmicutes* followed by the *B. subtilis* + EBA, with the AGP treatment group having the lowest ($P<0.05$) abundance when compared with the Control (Table 4.4.1 and Figure 4.4.2). Similarly, the *Proteobacteria* percentage remain similar amongst treatments under heat stress conditions whereas in thermoneutral conditions, the AGP diet showed the highest abundance which is significantly higher ($P<0.05$) than that of the *B. subtilis* + EBA group.

Table 4.4.1 The effect of feed additive and house temperature on the percentage abundance of the ileal microbiome at a phylum level of broilers exposed to heat-stressed and thermoneutral conditions at 35 days of age

	Heat-stressed			Thermoneutral		
	Control[1]	**AGP[1]**	***B. subtilis* + EBA[1]**	**Control[1]**	**AGP[1]**	***B. subtilis* + EBA[1]**
Actinobacteria	0.9475	1.5297	0.7811	0.0287	0.1195	0.2761
Ascomycota	0.0022	0.0011	0.0014	0.0018	0.0022	0.0067
Bacteriodetes	0.0000	0.0004	0.0004	0.0005	0.0001	0.0002
Basidiomycota	0.0001	0.0001	0.0000	0.0007	0.0000	0.0001
Bryophyta	0.0001	0.0005	0.0000	0.0007	0.0000	0.0001
Chlamydiae	0.0000	0.0001	0.0000	0.0000	0.0000	0.0000
Ciliophora	0.0035	0.0054	0.0231	**0.0076^{a}**	**0.0007^{b}**	**0.0040^{a}**
Cyanobacteria	0.0000	0.0000	0.0000	0.0000	0.0001	0.0000
Firmicutes	24.59	25.92	29.77	**36.21^{a}**	**10.78^{b}**	**29.05^{ab}**
Fusobacteria	0.0000	0.0000	0.0000	0.0002	0.0001	0.0000
Glomeromycota	0.0000	0.0001	0.0000	0.0000	0.0000	0.0000
Proteobacteria	41.95	55.35	40.02	**45.07^{ab}**	**56.76^{a}**	**26.12^{b}**
Tracheophyta	0.0000	0.0001	0.0002	0.0006	0.0002	0.0000
Virrucomicrobia	0.0000	0.0009	0.0000	0.0008	0.0000	0.0002
Not assigned	0.0001	0.0000	0.0000	0.0006	0.0001	0.0002
Unknown	**32.51^{a}**	**17.19^{b}**	**29.41^{ab}**	**22.06^{b}**	**32.34^{ab}**	**41.88^{a}**

^{a,b} Row means within a house temperature without a common superscript differs significantly (P<0.05)

[1]Control: basal diet; AGP: basal diet with zinc bacitracin; *B. subtilis* + EBA: basal diet with *B. subtilis* and encapsulated butyric acid

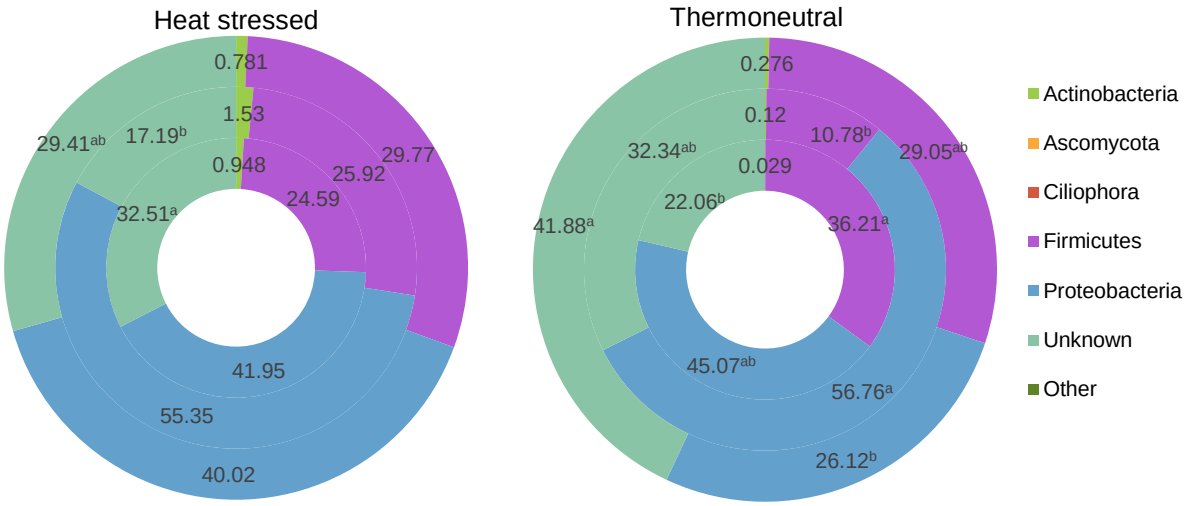

Figure 4.4.2 Composition of the ileal microbiome at a phylum level of broilers at 35d exposed to heat-stressed and thermoneutral conditions. The inner, middle and outer rings indicate the Control, AGP and *Bacillus subtilis* + EBA diets respectively. ^{ab} Values without a common superscript differ significantly (P<0.05)

Control: basal diet; AGP: basal diet with zinc bacitracin; *B. subtilis* + EBA: basal diet with *B. subtilis* and encapsulated butyric acid. Microorganisms in "Other" constituted those phyla which had very low average abundance (<0.001%).

Table 4.4.2 The main effects of dietary treatment and house temperature on the percentage abundance of the ileal microbiome at a phylum level of broilers at 35 days age (P-values)

	Dietary treatment[1]		House temperature[2]
	Heat-stressed	Thermoneutral	
Actinobacteria	0.671	0.119	**0.037**[4]
Ascomycota	0.724	0.952	0.251
Bacteriodetes	0.200	0.305	0.611
Basidiomycota	0.592	**0.054**[3]	0.158
Bryophyta	0.127	0.336	0.816
Chlamydiae	0.368	-	1.000
Ciliophora	0.878	**0.013**[4]	0.708
Cyanobacteria	-	-	1.000
Firmicutes	0.954	**0.035**[4]	0.560
Fusobacteria	-	0.592	0.489
Glomeromycota	0.368	-	1.000
Proteobacteria	0.483	**0.076**[3]	0.617
Tracheophyta	0.592	0.366	0.609
Virrucomicrobia	0.124	0.109	0.602
Not assigned	0.368	0.992	0.359
Unknown	**0.096**[3]	0.264	0.519

[1]Control: basal diet; AGP: basal diet with zinc bacitracin; *B. subtilis* + EBA: basal diet with *B. subtilis* and encapsulated butyric acid.

[2]Heat-stressed house was exposed to continual heat stress and remained at a temperature 3 - 5 ºC higher than that of the TN house.

[3] Significant effect at P<0.10 (10% confidence level) only

[4] Significant effect at P<0.05 (5% confidence level)

Table 4.4.2 shows the main effects of treatment diet and house temperature on the ileal microbiome at a phylum level of male broilers at 35 days of age. In the heat-stressed house, the treatment diets only had a tendency towards significant effect (P<0.10) on the percentage abundance of the ileal microbiome in the Unknown phylum category. Birds fed the different treatment diets which were exposed to thermoneutral conditions were significantly different at a 5% confidence level in the percentage abundance in the *Ciliophora* and *Firmicutes* phyla. Dietary treatment has a tendency towards a significant effect (P<0.1) on the percentage abundance of *Basidiomycota* and *Proteobacteria* phyla in birds exposed to thermoneutral environment. House temperature had a significant effect (P<0.05) on the percentage abundance for the *Actinobacteria* phylum only, whereas all other phyla was not affected by house temperature.

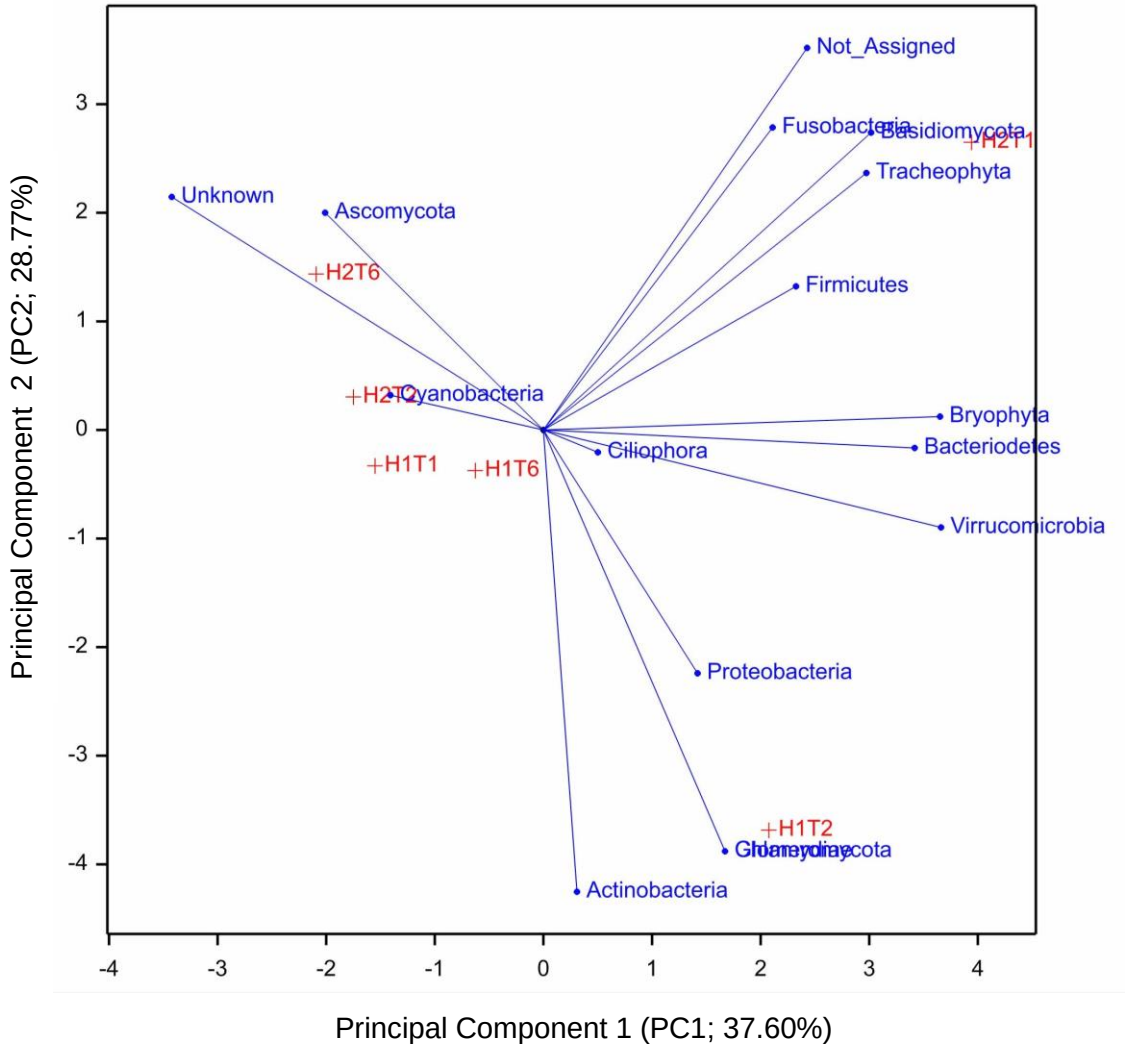

Figure 4.4.3 Principal component biplot of the percentage abundance of various phyla of ileal microbes of 35-day old birds exposed to heat stress and thermoneutral conditions

(H1: Heat stress conditions, H2: Thermoneutral conditions; T1: Control diet, T2: AGP diet, T6: *B. subtilis* and EBA diet)

A two-dimensional plot of the scores for the first two principal components are presented and accounts for 66.37% (PC1+PC2 = 37.60+28.77) of the total variation in the 16 phyla identified (Figure 4.4.3). A contrast on the PC2 (vertical axis) was observed between the heat-stressed and thermoneutral houses, which were mostly associated with microbes identified as "Unknown" and *Ascomycota* for the *B. subtilis* + EBA diet; “Not_Assigned", *Fusobacteria, Basidiomycota* and *Tracheophyta* with the Control diet and *Cyanobacteria* for AGP diet for birds exposed to thermoneutral conditions. In contrast, birds subjected to heat stress conditions had mostly *Proteobacteria, Glamydia, Glomeromycota* and *Actinobacteria* as the phyla associated with the AGP

diet, while the Control and *B. subtilis* + EBA diets in heat-stressed birds were more similar to the AGP diets in birds subjected to thermoneutral conditions. PC1 (horizontal axis) contrasts heat-stressed birds fed the AGP diet with thermoneutral birds fed the Control diet, which were also mostly dissimilar. Furthermore, the *B. subtilis* + EBA diet contrasted the least between the two heat treatments.

As with the 16s rRNA analysis at a phylum level (Figure 4.4.2), analysis showed the same trends in abundance of unknown microorganisms at a class level amongst the treatment diets and heat treatments (Figure 4.4.4). Percentage abundance of *Bacilli* was lowest (10.78%) in the AGP-fed birds and the greatest (40.71%) in the Control group in thermoneutral birds. It was observed that *Gammaproteobacteria* was the dominant bacteria in the heat-stressed birds fed the AGP group only, whereas in thermoneutral birds, the AGP diet showed the highest abundance followed by the Control and *B. subtilis* + EBA, respectively. *Actinobacteria* remained higher in heat-stressed birds than in thermoneutral birds, with highest observed abundances in the AGP group.

HT-NGS at an order level reveals that the composition of the microbiota is dominated by *Enterobacteriales* in heat-stressed birds, with the AGP diet having the highest abundance followed by the Control and the *B. subtilis* + EBA treatment groups, respectively (Figure 4.4.5). A similar trend was observed in the abundance of *Enterobacteriales* in the ilea of thermoneutral birds. The

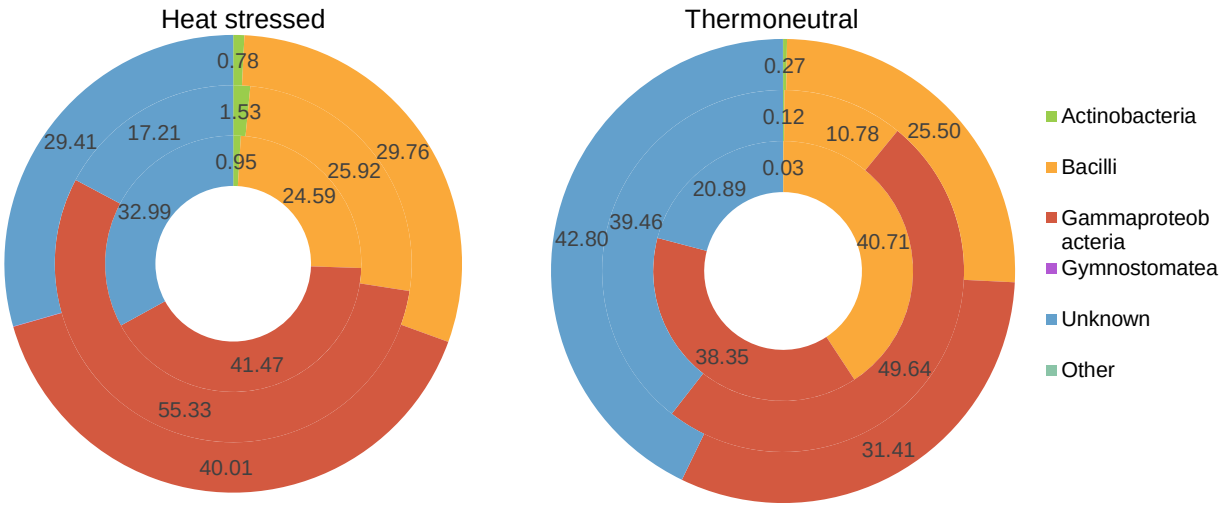

Figure 4.4.4 Composition of the ileal microbiome at a class level of broilers at 35d exposed to heat-stressed and thermoneutral conditions. The inner, middle and outer rings indicate the Control, AGP and *Bacillus subtilis* + EBA diets respectively.

Control: basal diet; AGP: basal diet with zinc bacitracin; *B. subtilis* + EBA: basal diet with *B. subtilis* and encapsulated butyric acid. Microorganisms in the "other" category constituted those classes which had very low abundance (<0.001%).

percentage abundance of *Lactobacillales* in thermoneutral birds was the lowest in the AGP group (10.51%) followed by a two-fold increase in proportion in the *B. subtilis* + EBA group (25.34%) and a further two-fold increase in the Control group (40.38%). However, in heat-stressed birds, these values remained similar amongst treatment diets, however the *B. subtilis* + EBA group had the highest percentage abundance of *Lactobacillales*. A significant increase in the abundance of *Pseudomonadales* in heat-stressed birds were observed compared with thermoneutral birds, with the *B. subtilis* + EBA group having the greatest increase followed by the AGP group.

Similar trends were seen in analysis of 16S rRNA at a family level (Figure 4.4.6) when compared with 16S rRNA analysis at an order level (Figure 4.4.5). Notably, abundance of *Corynebacteriaceae* was highest in heat-stressed birds compared with thermoneutral birds, although small differences were observed amongst treatment diets within heat-stressed birds. The percentage abundance of *Enterococcaceae* present in the ilea of heat-stressed birds were highest in the AGP and Control diets, whereas thermoneutral birds showed almost negligible quantities amongst the treatment diets.

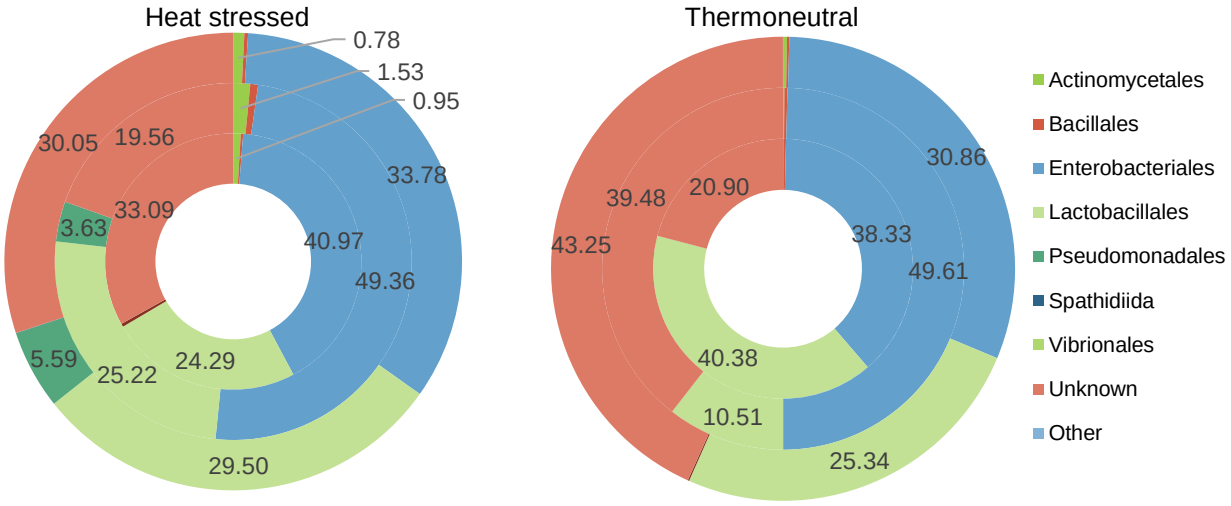

Figure 4.4.5 Composition of the ileal microbiome at an order level of broilers at 35d exposed to heat-stressed and thermoneutral conditions. The inner, middle and outer rings indicate the Control, AGP and *Bacillus subtilis* + EBA diets respectively.

Control: basal diet; AGP: basal diet with zinc bacitracin; *B. subtilis* + EBA: basal diet with *B. subtilis* and encapsulated butyric acid. Microbes in the "other" category constituted those orders which had very low abundance (<0.001%).

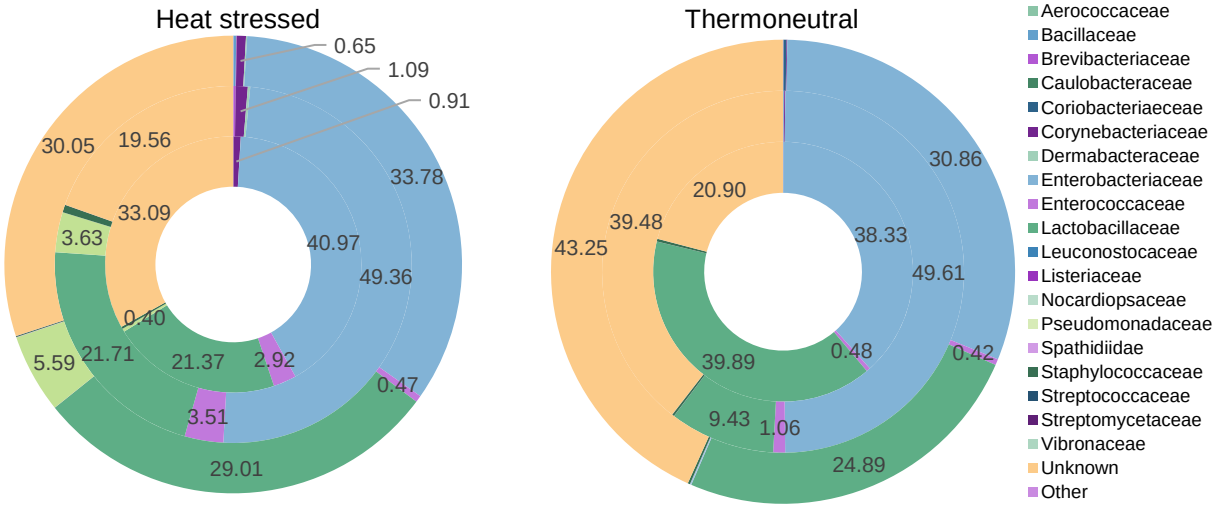

Figure 4.4.6 Composition of the ileal microbiome at a family level of broilers at 35d exposed to heat-stressed and thermoneutral conditions. The inner, middle and outer rings indicate the Control, AGP and *Bacillus subtilis* + EBA diets respectively.

Control: basal diet; AGP: basal diet with zinc bacitracin; *B. subtilis* + EBA: basal diet with *B. subtilis* and encapsulated butyric acid. Microbes in the "other" category constituted those families which had very low abundance (<0.001%).

Table 4.4.3 shows the percentage abundance of the ileal microbiota at a genus and species level in male broilers at 35 days of age which were exposed to thermoneutral and heat-stressed conditions. In 35-day old birds subjected to heat stress conditions, it was observed that the AGP and *B. subtilis* + EBA treatments had a higher percentage abundance (P<0.05) of *Firmicutes* bacteria when compared with the Control birds.

The main effects of treatment diet and house temperature on the percentage abundance of the ileal microbiota at a genus and species level of broilers at 35 days age is shown in Table 4.4.4. In thermoneutral birds, the percentage abundances of *Symbiodinium spp.* and the Uncultured bacterium was significantly (P<0.05) different amongst treatment diets whereas the uncluttered organism was only significant at a 10% confidence level. In heat-stressed birds, the percentage abundance tended to be significantly different (P<0.10) amongst *Enterobacter spp.* and the Bacterium group, whereas the abundance of *Firmicutes bacteria* was significantly different (P<0.05). The percentage abundance of *Corynebacterineae bacterium* and *Pseudomonas spp.* were different at a high level of significance (P<0.01) amongst the two heat treatments. Similarly, when comparing between the two heat treatments, birds had significantly different (P<0.10) abundances in *Actinomycetales bacterium*, *Brachybacterium spp., Corynebacterium spp.,* as well as *Gamma proteobacterium*.

Figure 4.4.7 shows the composition of ileal microbiota at a genus and species level of broilers at 35 days of age which were exposed to heat-stressed and thermoneutral conditions. Within birds

which were exposed to heat stress conditions, the percentage abundance of *Actinomycetales bacterium, Bacillus spp., Corynebacterineae bacterium, Eggerthella spp., Lactobacillus aviarius,* and *Lactobacillus crispatus* were the greatest in the Control diet when compared with all other treatment diets, whereas the *Firmicutes bacteria, Lactobacillus spp.,* and *Lactococcus spp.* were the genera most abundant in the thermoneutral conditioned house. Similarly, the highest percentage abundance in the AGP diet with respect to the other treatment diets was observed in *Actinomycete, Brachybacterium spp., Brevibacterium spp., Cronobacter spp., Dickeya spp., Gamma proteobacterium, Nocardiopsis spp., Paenibacillus spp.,* and *Proteus spp.,* whereas in the thermoneutral conditions, the AGP diet only had the greatest abundance in the *Blautia spp.* and *Proteobacterium.* Within the heat-stressed house, birds who were fed the *B. subtilis* + EBA diet had the highest abundance of *Caulobacter daechungenesis, Enterobacteriaceae bacterium, Klebsiella spp., Lactobacillus johnsonii, Lactobacillus gasseri, Lactobacillaceae bacterium, Streptococcus spp., Symbiodinium spp.,* and *Tisochrysis lutea,* however in thermoneutral conditions, the highest abundance were in *Enterobacter spp., Lactobacillus fermentum, Lactobacillus reuteri,* and *Weissella spp.*

Table 4.4.3 Percentage abundance of the ileal microbiome at a genus and species level of 35-day old broilers exposed to heat-stressed and thermoneutral conditions and different feed additives

	Heat-stressed			Thermoneutral		
	Control[1]	AGP[1]	*B. subtilis* + EBA[1]	Control[1]	AGP[1]	*B. subtilis* + EBA[1]
Actinomycetales bacterium	0.2234	0.0196	0.0911	0.0001	0.0010	0.0023
Actinomycete	0	0.0010	0	0.0002	0.0001	0
Aerococcus spp.	0.0004	0	0.0206	0	0.0181	0.0215
Bacillus spp.	0.6120	0.0148	0.3051	0.0219	0.0682	0.0039
Bacterium	0.3115	0.0026	0.0936	0.0432	0.1269	0.0147
Blautia spp.	0	0	0	0	0.0037	0
Brachybacterium spp.	0.0183	0.2201	0.0820	0.0006	0.0034	0.0009
Brevibacterium spp.	0.0062	0.2076	0.0333	0.0044	0.0208	0.0091
Caulobacter daechungensis	0	0	0.0037	0	0	0
Corynebacterineae bacterium	0.0295	0.0045	0.0032	0	0	0.0002
Corynebacterium spp.	0.9147	1.0855	0.6478	0.0221	0.0944	0.2639
Cronobacter spp.	0.0002	0.0170	0.0010	0.0003	0.0006	0.0006
Dickeya spp.	0	0.0034	0	0	0.0003	0.0002
Eggerthella spp.	0.0021	0	0	0.0001	0	0
Enterobacter spp.	0.0130	0.3320	0.5910	0.0640	0.2080	5.7360
Enterobacteriaceae bacterium	0.0180	0.1250	8.9390	0.0210	0.5340	0.0060
Enterobacteriales bacterium	0.0019	0.0603	0.0018	0.0040	0.0105	0.0023
Enterococcus spp.	2.9180	3.5070	0.4690	0.4840	1.0630	0.4170
Erwinia spp.	0.0004	0.0008	0.0017	0.0006	0.0009	0.0053
Escherichia coli	32.90	31.59	19.37	33.72	46.80	23.68
Escherichia spp.	7.656	13.852	1.191	3.614	1.575	1.318
Firmicutes bacteria	**0.0004[b]**	**0.0125[a]**	**0.0217[a]**	0.5246	0.0145	0.0011
Gamma proteobacterium	0.0961	2.4374	0.6348	0.0064	0.0267	0.4475
Klebsiella spp.	0.0022	1.5946	3.5948	0.0819	0.3535	0.0637
Lactobacillaceae bacterium	0.0001	0.0070	0.0939	0.0001	0.0006	0.0020
Lactobacillus acidophilus	1.1310	6.1690	0.7730	0.0210	1.7370	0.5470
Lactobacillus aviarius	0.00006	0	0	0	0	0
Lactobacillus crispatus	4.2970	0.0100	0.2890	0.1010	0.3000	1.1860
Lactobacillus fermentum	0.2009	0.0127	0.0265	0.3111	0.0021	0.9141
Lactobacillus gasseri	0.0006	0.0003	0.2498	0.1463	0.0006	0.0015
Lactobacillus johnsonii	12.95	8.21	22.46	15.45	0.65	2.94
Lactobacillus reuteri	1.3070	2.4570	0.3580	0.1430	0.4730	11.6250
Lactobacillus salivarius	1.1371	0.0371	0.1692	1.2902	0.1383	1.5972
Lactobacillus spp.	0.3420	4.8080	4.5840	22.4310	6.1250	6.0790
Lactococcus spp.	0.0002	0.0001	0	0.0057	0.0001	0
Listeria spp.	0.0023	0.0009	0.0049	0.0002	0.0027	0.0047
Nocardiopsis spp.	0.0012	0.0046	0	0.0002	0.0004	0.0002
Paenibacillus spp.	0.0001	0.0015	0.0005	0	0	0
Proteobacterium	0.4790	0.0200	0.0010	0.0020	7.1180	0
Proteus spp.	0.0013	0.7048	0.0109	0.1647	0.0007	0.0001
Pseudomonas spp.	0.3960	3.6270	5.5940	0.0030	0.0050	0.0930
Salmonella spp.	0.0001	0.0008	0.0001	0.0061	0.0006	0.0024
Shigella spp.	0.3769	1.1533	0.0844	0.6541	0.1315	0.0425
Staphylococcus spp.	0.2782	0.6855	0.0672	0.3281	0.1941	0.1520
Streptococcus spp.	0.0001	0.0015	0.0062	0.0003	0.0042	0.0016
Streptomyces spp.	0	0.0091	0.0089	0.0004	0.0002	0.0089
Symbiodinium spp.	0.0016	0.0052	0.0229	**0.0032[b]**	**0.0007[a]**	**0.0033[b]**
Tisochrysis lutea	0	0	0.0069	0	0	0
Uncultured bacteria	27.78	16.04	28.81	**11.16[b]**	**32.10[ab]**	**41.45[a]**
Uncultured organism	3.5570	1.0280	0.1190	9.219	0.0630	1.3020
Weissella spp.	0.0008	0.0001	0.0008	0.0009	0.0020	0.0052
Other	0.0205	0.0154	0.1586	0.0407	0.0110	0.04642

[a,b] Row means within a house temperature without a common superscript differs significantly (P<0.05)

[1]Control: basal diet; AGP: basal diet with zinc bacitracin; *B. subtilis* + EBA: basal diet with *B. subtilis* and encapsulated butyric acid.

Table 4.4.4 The main effects of dietary treatment and house temperature on the percentage abundance of the ileal microbiome at a genus and species level of broilers at 35 days age (P-values)

	Dietary treatment		House temperature
	Heat-stressed	Thermoneutral	
Actinomycetales bacterium	0.928	0.503	**0.075**[1]
Actinomycete	0.368	0.592	1.000
Aerococcus spp.	0.305	0.592	1.000
Bacillus spp.	0.477	0.125	0.927
Bacterium	**0.096**[1]	0.159	0.939
Blautia spp.	-	-	-
Brachybacterium spp.	0.605	0.947	**0.078**[1]
Brevibacterium spp.	0.550	0.366	0.325
Caulobacter daechungensis	0.368	-	-
Corynebacterineae bacterium	0.682	0.368	**0.011**[2]
Corynebacterium spp.	0.693	0.204	**0.092**[1]
Cronobacter spp.	0.266	0.802	0.573
Dickeya spp.	0.124	0.592	0.870
Eggerthella spp.	0.124	0.368	0.745
Enterobacter spp.	**0.099**[1]	0.225	0.167
Enterobacteriaceae bacterium	3.987	0.390	0.818
Enterobacteriales bacterium	0.598	0.267	0.280
Enterococcus spp.	0.925	0.852	0.690
Erwinia spp.	0.391	0.947	0.867
Escherichia coli	0.523	0.193	0.480
Escherichia spp.	0.193	0.270	0.862
Firmicutes bacteria	**0.039**[2]	0.151	0.678
Gamma proteobacterium	0.646	0.990	**0.058**[1]
Klebsiella spp.	0.340	0.878	0.791
Lactobacillaceae bacterium	0.338	0.153	0.938
Lactobacillus acidophilus	0.217	0.594	0.871
Lactobacillus aviarius	0.368	-	-
Lactobacillus crispatus	0.608	0.261	0.878
Lactobacillus fermentum	0.172	0.763	0.837
Lactobacillus gasseri	0.230	0.832	0.605
Lactobacillus johnsonii	0.216	0.169	0.659
Lactobacillus reuteri	0.520	0.144	0.372
Lactobacillus salivarius	0.253	0.387	1.000
Lactobacillus spp.	0.416	0.983	0.519
Lactococcus spp.	0.592	0.368	1.000
Listeria spp.	0.158	0.107	0.979
Nocardiopsis spp.	0.592	0.992	1.000
Paenibacillus spp.	0.592	-	-
Proteobacterium	0.370	0.111	0.570
Proteus spp.	0.546	0.377	0.207
Pseudomonas spp.	0.162	0.324	**0.007**[2]
Salmonella spp.	0.208	0.632	0.215
Shigella spp.	0.216	0.432	0.660
Staphylococcus spp.	0.879	0.561	0.797
Streptococcus spp.	0.111	0.717	0.764
Streptomyces spp.	0.155	0.427	0.685
Symbiodinium spp.	0.944	**0.016**[2]	0.959
Tisochrysis lutea	0.124	-	-
Uncultured bacteria	0.216	**0.039**[2]	0.894
Uncultured organism	0.310	**0.055**[1]	0.178
Weissella spp.	0.690	0.535	0.203
Other	0.442	0.652	0.419

[1] Significant effect at P<0.1 (10% confidence level) only

[2] Significant effect at P<0.05 (5% confidence level)

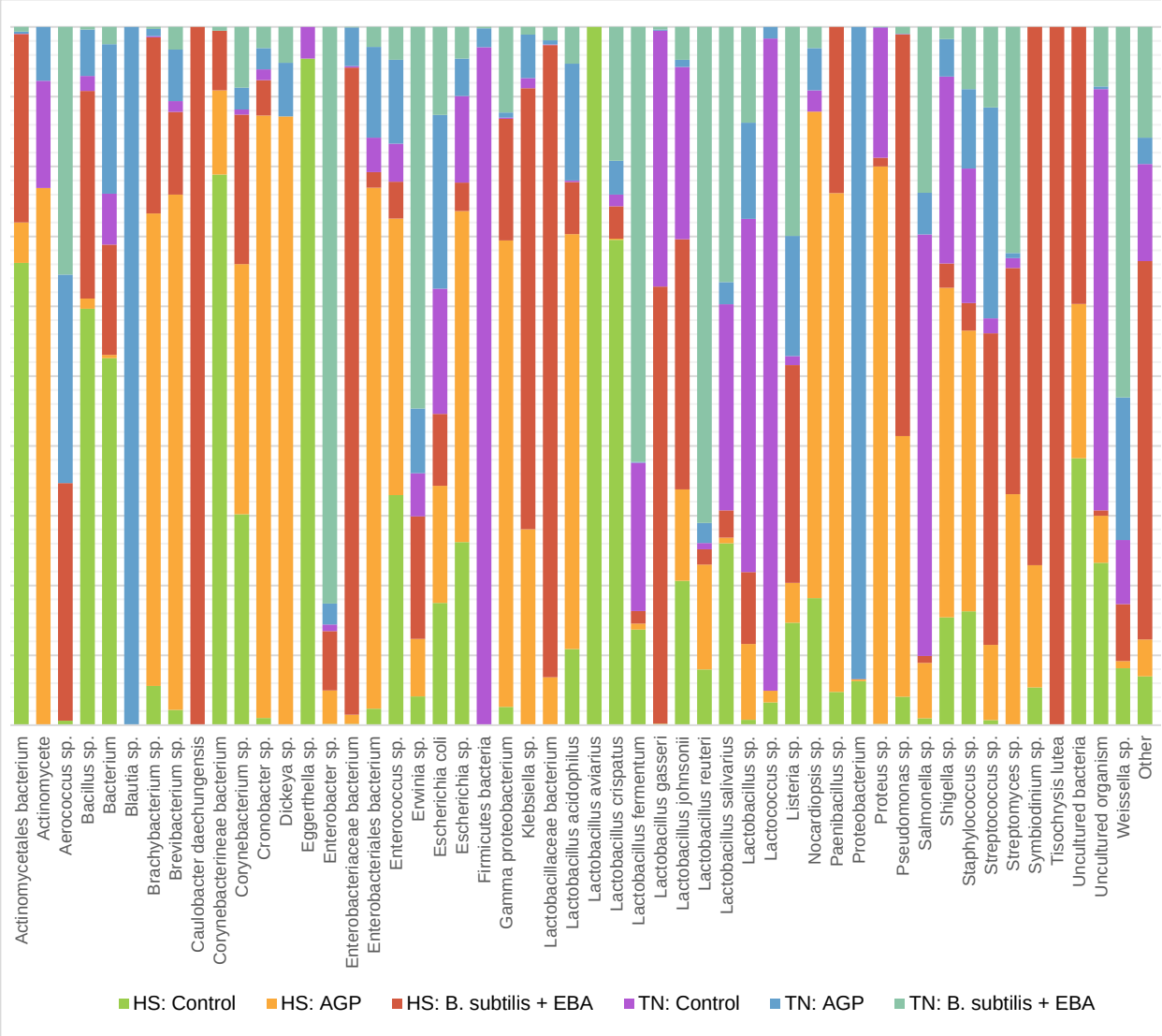

Figure 4.4.7 Composition of the ileal microbiome at a genus and species level of broilers at 35d exposed to heat-stressed and thermoneutral conditions. The inner, middle and outer rings indicate the Control, AGP and *Bacillus subtilis* + EBA diets respectively.

Control: basal diet; AGP: basal diet with zinc bacitracin; *B. subtilis* + EBA: basal diet with *B. subtilis* and encapsulated butyric acid. Microorganisms in the "other" category constituted those phyla which had very low abundance (<0.001%). HS = Heat-stressed; TN: Thermoneutral

Chapter 5: Discussion

Previous studies have shown that probiotics and butyric acids may be used as growth promotors and may affect the GIT microbiota, bird performance, carcass yield, and the histomorphology of the small intestine (Al-Fataftah & Abdelqader, 2014; Imran *et al.*, 2017; Manafi *et al.*, 2018). This study investigated the response of broiler chickens supplemented with encapsulated butyric acid and *Bacillus subtilis* probiotic alone and in combination against an antibiotic growth promoter (zinc bacitracin) with the intention of improving overall gut health, the microbiome and production. For the duration of the study, the most significant differences amongst treatment groups for all performance parameters were during the finisher phase (28 – 34 days of age) which may be due to a stimulation in the growth efficiency of broilers which usually begins to stagnate during this phase of production. These results are similar to previous studies in which few to no effects were seen with addition of butyric acid (Levy *et al.*, 2015) nor probiotics (Junaid *et al.*, 2018) throughout the production cycle. Most notably, the combination of AGP and EBA consistently had the highest feed intake and body weight and the best feed conversion of all treatment groups irrespective if exposed to thermoneutral or heat-stressed conditions. Improvement in BW and FCR may be as a result of microencapsulation of butyric acid with palm oil which allows for targeted release of butyrate at an ileum level, thus improving gut health and improving protein digestibility (Imran *et al.*, 2017). In contrast, a combination of EBA and *B. subtilis* within the diet consistently resulted in the poorest feed intake, body weight, feed conversion and production efficiency results when compared with either feed additive provided in singularity in the diet. These results are in corroboration with the observations made by Agboola *et al.* (2015), where broilers fed a diet containing organic acids (formic acid, ammonium formate, propionic acid and ammonium propionate) in combination with a probiotic of *Lactobacillus sporogenes* and *Saccharomyces cerevisiae* also produced numerically the lowest body weight gain and feed intake. The dietary additions of organic acids and probiotics had no significant effects on performance traits in studies by Agboola *et al.* (2015) which is consistent with the findings in this study, and indicates a lack of additive effect of both dietary supplements. Although the exact reasoning remains unclear, these results suggests that an undesirable interaction effect may exist between EBA and *B. subtilis*, resulting in poorer production performance in broiler birds. Probiotics typically positively modulate the gut microbiota and butyric acid is known to improve the integrity of the GIT; these feed additives complement each other by improving gut health and ultimately growth performance. Therefore, the poorer growth performance observed when EBA and *B. subtilis* was fed in combination is most peculiar and requires further investigation. Studies by Teo & Tan (2007) have demonstrated that when broilers are treated with *B. subtilis* PB6, feed intake and FCR was comparable to the antibiotic

control. Similarly, growth performance in diets containing a combination of EBA and *B. subtilis* during this study was comparable to that of the antibiotic inclusive diet.

Previous studies have highlighted that broilers are more susceptible to heat stress and their metabolic demands for rapid rate of growth presents unique challenges encouraging adaptive responses in absorptive function (Al-Fataftah & Abdelqader, 2014), changes in behavioural characteristics (Wang *et al.*, 2018), and increased mortality which results in significant economic losses within the poultry industry (Abdelqader *et al.*, 2017). In this study, house temperature was observed to have an effect on the growth production of birds. During this trial, birds exposed to thermoneutral conditions, irrespective of diet, outperformed those birds who were heat-stressed. Typically, birds in the thermoneutral house showed higher weekly body weight gain and feed intake during the entire trial period, which subsequently increased the feed conversion ratio throughout the trial with significant differences observed at 0-7d, 14-20d and 28-34d periods. Similar results were obtained by Al-Fataftah & Abdelqader (2014), who found that heat-stressed Hubbard male broilers fed a basal diet as well as a *B. subtilis* supplemented diet both had lower final body weights and average daily feed intakes than broilers that were exposed to thermoneutral conditions throughout the trial period. The negative impacts of heat stress on feed intake and growth performance observed in this study were also in agreement with other previous reports (Lin *et al.*, 2006; Song *et al.*, 2014) and may be attributed to the reduction in feed intake as a defence mechanism initiated by birds to reduce their metabolic heat production and acclimate to the increased environmental temperature (Al-Fataftah & Abdelqader, 2014).

The digestive system is a complex ecosystem consisting of three main interconnecting elements: the intestinal epithelium, the immune system and the commensal microbiota (Kogut, 2013). A stable and diverse gut microbiota is essential for the broilers to resist infections. When the composition of normal gut microbiota is disrupted in combination with intestinal inflammation, dysbiosis occurs which induces a cascade of reactions in the GIT which includes reduced digestibility of nutrients and impaired intestinal barrier function (Teirlynck *et al.*, 2011; Ducatelle *et al.*, 2018). This, in turn, increases the risk of inflammation and the translocation of bacteria. Dysbiosis is identified by undigested food particles; thin, fragile, translucent intestinal walls; watery and foamy intestinal contents; presence of orange mucous; ballooning of the gut and intestinal inflammation (Teirlynck *et al.*, 2011). Teirlynck *et al.* (2011) developed a method for macroscopically scoring gut health to provide an indication of the severity of dysbiosis in the poultry gut. They found that higher scores were indicative of decreased villi length, thinning of the *tunica muscularis* and an increase in the T-lymphocyte infiltration. In the present study, 21 day-old broilers which received a combination of *B. subtilis* and EBA showed the lowest macroscopic gut health scores, indicating that this group had the best gut health amongst the treatment groups. This trend followed through to birds at 35 days of age whereby birds which received a combination of the probiotic and butyric acid obtained

one of the lowest overall gut health scores, second to that of EBA supplemented alone. These results suggest that the combination of *B. subtilis* and EBA increases the integrity of the gut and improved the microbial composition of the gut thus influencing the overall gut health. As seen in the growth parameter results in this study, the combination of zinc bacitracin and EBA produced some of the worst gut health scores, decreased villi height and increased crypt depth at both 21 and 35 days of age. Shortened villi decreases surface area for the absorption of nutrients (Xu *et al.*, 2003), whilst deeper crypts reveal rapid cell turnover of the intestinal tissues and a high demand for new tissue (Choct, 2009). Additional turnover of cells increases the nutrient requirements to maintain the digestive tract, resulting in a decrease in bird efficiency (Choct, 2009). This further solidifies the idea that a negative response is produced when an AGP and EBA is fed in combination which ultimately affects gut health. Microbes increase the integrity of the gut (Kohl, 2012). Very high gut integrity created by the inclusion of EBA may result in a decrease absorption of nutrients. In contrast, inclusion of an AGP decrease the number of microbes in the gut, decrease gut integrity and increase absorption of nutrients.

During heat stress, blood and heat are shunted away from the digestive system to facilitate heat dissipation and the digestive system begins to deteriorate as a result of the decreased blood supply to the tissues (De Souza *et al.*, 2016). Interestingly, thermoneutral birds in this experiment showed the highest scores (irrespective of diet), indicating a higher risk of dysbiosis. An exact reason for this observation is unknown and could not be found in other studies. Heat stress in 21 day-old birds impaired the intestinal villi-crypt system and decreased villi height and crypt depth and increased the ratio between villi height to crypt depth, while inclusion of *B. subtills* and EBA in combination partially reversed the decrease in villi height in the duodenum and jejunum. Divergence of the systemic blood flow away from the digestive system can cause ischemia and hypoxia in the intestinal epithelial cell which can generate reactive oxygen species that damage epithelial cells quickly, inducing intestinal lesions, decrease the integrity of the mucosa and impair cellular homeostasis (Al-Fataftah & Abdelqader, 2014). The mitigation of the effects of heat stress on the gut by the combination of *B. subtilis* and EBA is further concreted by the improved gut health observed in the macroscopic gut health scores.

Encapsulated butyric acid is a protected source of butyric acid which prevents it from being absorbed in the upper digestive tract, delaying its release along the digestive tract and rendering it available more distally in the small intestine (Bortoluzzi *et al.*, 2017). Butyric acid is the direct energy source of epithelial cells of the intestine and assists in the proliferation and differentiation of these cells, to improve intestinal barrier function and integrity (Dehghani-Tafti & Jahanian, 2016), increase villi height and growth (Guilloteau *et al.*, 2010), and control intestinal pathogenic bacteria colonisation (Van Immerseel *et al.*, 2005; Hu & Guo, 2007). In this study, encapsulated butyric acid at 21 days in the absence of AGP had a targeted response by bypassing the duodenum, rendering the compound

to be more available further down the digestive tract and increasing villi height in the jejunum but not in the ileum.

The gastrointestinal tract is sensitive to stressors, which results in a multitude of changes, including alteration of the normal microbiota (Al-Fataftah & Abdelqader, 2014). A stable and diverse gut microbiota is essential for a bird to resist infections. When the normal microbiota composition is disrupted and accompanied by inflammation of the intestine, dysbiosis occurs (Ducatelle *et al.*, 2018). A shift in the composition of the gut microbiota often favours abnormal populations of microorganisms to predominate in the gut (Kogut, 2013). It is beneficial to note that taxonomic profiles described for the different sections of the gastrointestinal tract differ considerably between studies and are influences by various factors such as sex, presence of stressors, individual genetics, diet, the use of antimicrobials and the technique used (Clavijo & Flórez, 2017). This makes comparative studies difficult as a refined comprehensive analysis of the microbiome of a healthy gut is scarce. It remains unclear how variations in the amount of beneficial (*Lactobacillus spp., Bifidobacterium spp.*) and commensal bacteria (*Clostridiaceae*, *Enterobacteriaceae*, *Streptococcus* and *Enterococcus*) improve the health status and performance of broiler chickens (Zdunczyk *et al.*, 2015).

In the present study, a combination of *B. subtilis* and EBA showed the highest abundance of *Caulobacter daechungenesis, Enterobacteriaceae bacterium, Klebsiella spp., Lactobacillus johnsonii, Lactobacillus gasseri, Lactobacillaceae bacterium, Streptococcus spp., Symbiodinium spp.,* and *Tisochrysis lutea.* Birds which received this diet typically had the most abundant levels of beneficial microbes (*Lactobacillus spp.*) and commensal bacteria (*Streptococcus spp.* and *Enterobacteriaceae*). *Lactobacillus* is the genus of bacteria accounting for the greatest portion of the gut microbiota (>90%) and has the principle function of lactate production and starch digestion (Yan *et al.*, 2017; Clavijo & Flórez, 2017). *Lactobacilli* are facultative anaerobes, subsequently producing enzymes and creating competitive exclusion of pathogenic bacteria (Jeong & Kim, 2014). Furthermore, lactic acid produced by this group of microbes can act as a natural antimicrobial by disrupting the outer membrane of gram-negative bacteria and reduce the pH of the intestine, inhibiting growth of pathogenic bacteria (Jeong & Kim, 2014). In a study by Abdelqader *et al.* (2017), they showed that dietary butyrate supplied to heat-stressed cockerels improved intestinal microbiota by promoting the growth of *Lactobacillus* and *Bifidobacterium* and inhibiting the growth of *Clostridium* and coliforms. They suggested that the protective effect may be due to butyrate having the ability to inhibit growth of pathogenic enteric bacteria, reducing the production of toxic compounds which damage the intestinal epithelium (Abdelqader *et al.*, 2017).

Diets containing antibiotics as a constituent had the highest percentage abundance of *Gammaproteobacteria* and *Escherichia spp.* This result suggests that broad spectrum antibiotics decrease the abundance of beneficial healthy microbes and allows potentially pathogenic bacteria

to compete for attachment sites, colonise, and increase the risk of disease. *E.coli*, in particular, belongs to this group of bacteria and is a major concern in the poultry industry. *E. coli* is considered to be a zoonotic bacterium that is potentially pathogenic to human and has been identified as a potential reservoir for the dissemination of resistance to antibiotics in other pathogenic bacteria, including *Salmonella* (Clavijo & Flórez, 2017).

In the present study, birds exposed to heat stress had the highest levels of *Actinomycetales bacterium, Brachybacterium spp., Corynebacterineae bacterium, Corynebacterium spp., Gamma proteobacterium* and *Pseudomonas spp.* when compared to birds exposed to thermoneutral conditions. Heat stress has been shown to lead to colonisation of pathogenic bacteria in farm animals, increased faecal shedding and horizontal transmission, with subsequent increase in risk of contamination of animal products (Lara & Rostagno, 2013). Potentially pathogenic bacteria are able to exploit the neuroendocrine alterations in the host response to heat stress to promote growth and pathogenicity.

Chapter 6: Conclusions

Growth performance was not significantly improved with a supplementation of encapsulated butyric acid and *Bacillus subtilis* fed either in combination or alone throughout the trial period, with exception of the finisher phase (between 28 and 35 days of age). The effect of heat stress negatively affected growth performance, however *B. subtilis* and EBA fed in combination did not mitigate the effect in these conditions. Supplementation of a combination of *B. subtilis* and EBA showed a reduced feed intake, reduced weekly body weights, poorer feed conversion and production efficiency in the finisher phase. Observations of the combination of these feed additives did, however, show a growth performance comparable to that of the antibiotic growth promotor. Most noteworthy was the improved growth performance in birds supplemented with a combination of antibiotic growth promotors and EBA fed broilers during the trial period. In contrast, gut health observations revealed that birds receiving this diet had reduced villi height and highest crypt depth. Thus, a possible interaction effect occurs when antibiotics and butyric acid is fed in combination and this requires further research to determine the mechanism of action when fed in combination.

B. subtilis and EBA improved the overall health of the gut indicative by the lower gut health scores with birds fed this diet under heat stress conditions. Improvement in histomorphology and gut integrity was observed by an increase in villi height, decrease in crypt depth and an increase in the ratio between the two parameters. The negative effect on growth performance due to heat stress was partly ameliorated by the improvement in the health of the gastrointestinal tract.

Modulation of the gut microbiota towards commensal and beneficial colonies of bacteria was observed when *B. subtilis* and EBA were fed in blend and subjected to heat stress conditions. Principally, this blend increased the percentage abundance of *Lactobacillus spp., Streptococcus spp.* and *Enterobacteriaceae spp.* whilst competitively excluding the colonisation of pathogenic microbes such as *Escherichia coli.* The exact composition and functioning of the intestinal microbiota in poultry are poorly understood. Although many studies have focussed on the microbiota of the poultry gut, these studies lack consistency with variation in the results obtained due to a multitude of factors including, sex, sample technique, presence of stressors, breed and age. Future endeavours should apply focus on understanding and sequencing the complete microbiome of the chicken intestine in order to get a better understanding of the interactions and functioning within the GIT.

In order to improve the current experimental design, it is recommended that the temperature level at which birds exposed to heat stress conditions be increased. Birds in this study were continually heat stressed which may have resulted in the birds becoming more adapted to the environment of increase temperature above the thermoneutral zone of broilers. Thus, it is recommended that future studies on the effect of heat stress in broilers be on cyclical heat stress which will remove the possibility of adaptation to the condition of increased temperature.

Furthermore, inoculation of broilers with pathogenic bacteria such as *Clostridium perfringens* may provide a further understanding of the capability of *B. subtilis* to competitively exclude pathogen colonisation, the extent to which EBA can increase the integrity of the gut as well as the effect of these feed additives on the overall gut health in broilers exposed to heat stress conditions.

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Addendum A

Table A.1 Temperature profile of the heat-stressed and thermoneutral houses (day 0 to 34)

	Heat-stressed			Thermoneutral			
Days of age	**Max Temp**	**Average**	**Min Temp**	**Max Temp**	**Average**	**Min Temp**	**HS – TN[1]**
0	39.7	36.3	31.8	34.1	31.0	26.6	5.2
1	39.9	35.7	31.5	34.9	32.7	27.8	3.0
2	38.9	36.2	30.8	36.5	32.8	26.5	3.4
3	39.6	36.0	29.8	35.0	31.5	28.6	4.6
4	39.6	36.8	31.2	36.3	32.8	29.6	4.0
5	39.9	36.9	30.8	36.0	32.2	27.3	4.7
6	40.7	36.4	30.6	37.3	33.1	28.0	3.3
7	39.6	36.4	31.8	36.5	32.5	29.1	3.9
8	38.4	35.2	33.0	34.1	32.2	28.9	3.0
9	38.4	34.8	30.1	34.4	29.9	25.7	4.9
10	38.1	34.3	29.6	34.7	30.9	26.7	3.4
11	37.1	33.4	29.3	33.8	30.0	25.1	3.4
12	35.9	33.1	29.0	31.4	29.1	25.7	4.0
13	35.7	32.0	28.4	31.6	27.4	23.7	4.6
14	35.4	32.2	28.8	30.7	26.8	23.4	5.4
15	35.7	30.7	21.2	30.4	26.4	22.0	4.3
16	34.0	29.9	26.7	30.7	26.2	21.1	3.7
17	32.4	29.6	25.8	28.8	25.7	21.8	3.9
18	31.6	30.3	26.2	27.4	23.6	20.9	6.7
19	32.6	28.6	24.1	29.4	24.2	20.3	4.4
20	32.5	28.1	23.3	27.7	23.7	19.2	4.3
21	32.1	29.2	24.0	30.4	23.8	18.7	5.4
22	31.0	27.7	24.4	28.9	24.6	20.0	3.1
23	32.1	26.9	22.2	29.9	23.1	18.8	3.8
24	32.0	26.8	25.3	26.4	23.8	20.3	3.0
25	30.2	27.4	24.1	27.6	24.0	17.7	3.3
26	30.8	27.4	23.5	27.7	24.1	20.8	3.3
27	29.9	25.1	21.3	24.7	22.1	18.1	3.0
28	26.0	23.4	21.3	23.9	21.2	18.2	2.2
29	28.7	25.1	20.0	25.5	20.6	16.8	4.5
30	27.7	23.9	19.3	24.0	20.8	17.0	3.1
31	27.5	23.8	21.1	23.7	20.4	17.3	3.4
32	26.0	22.8	21.5	21.4	19.8	17.5	3.1
33	26.7	23.3	21.0	22.2	20.4	17.5	3.0
34	26.5	23.1	21.4	22.0	19.9	17.5	3.2

[1] HS – TN = the difference between average temperatures of the heat-stressed house and the thermoneutral house

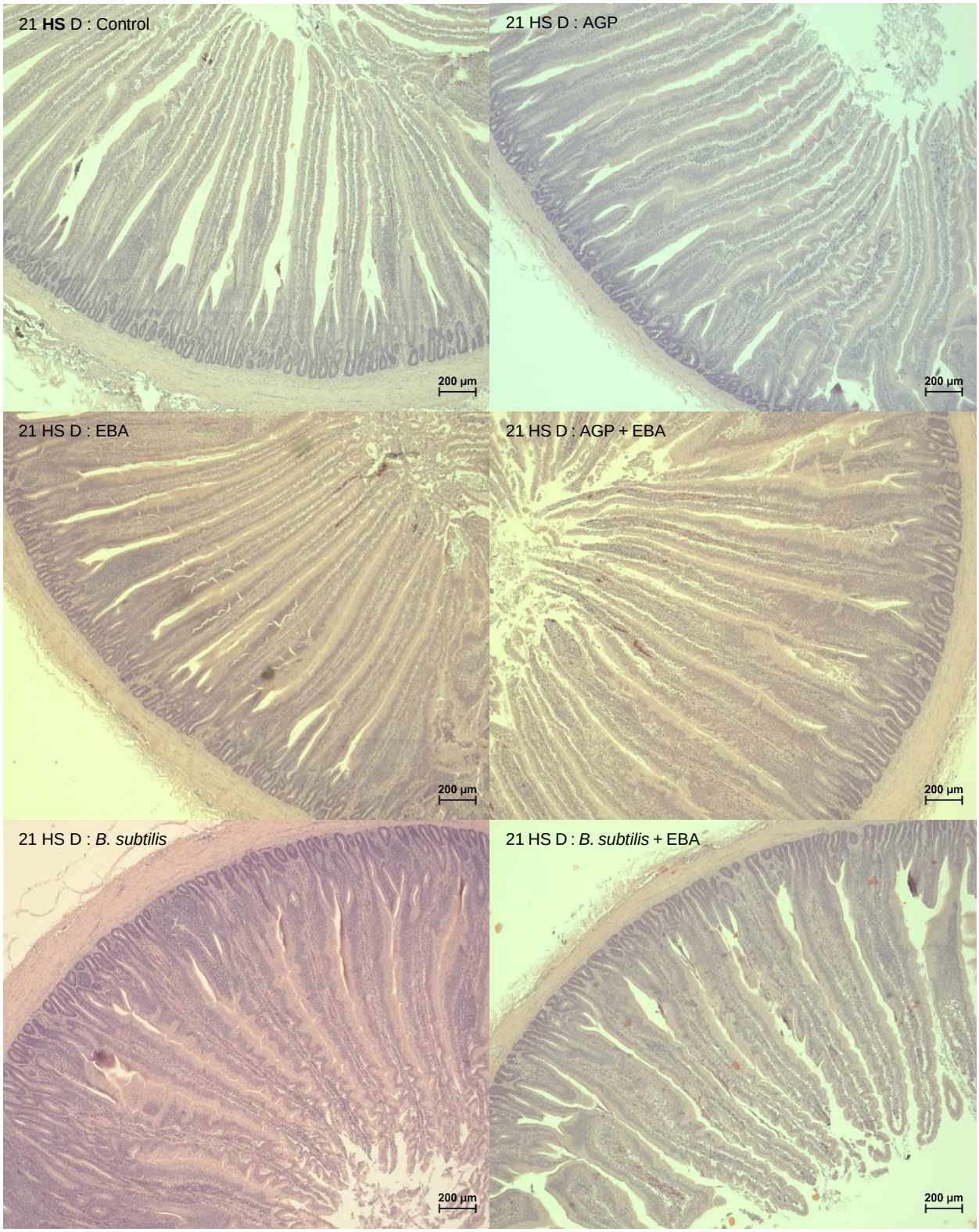

Figure A.1 Representative photomicrographs of 21d duodenal mucosa and submucosa of the treatment diets of heat-stressed broilers, stained with HE, Bar: 200 µm at 5x magnification.

21 HS D: 21d heat-stressed duodenum

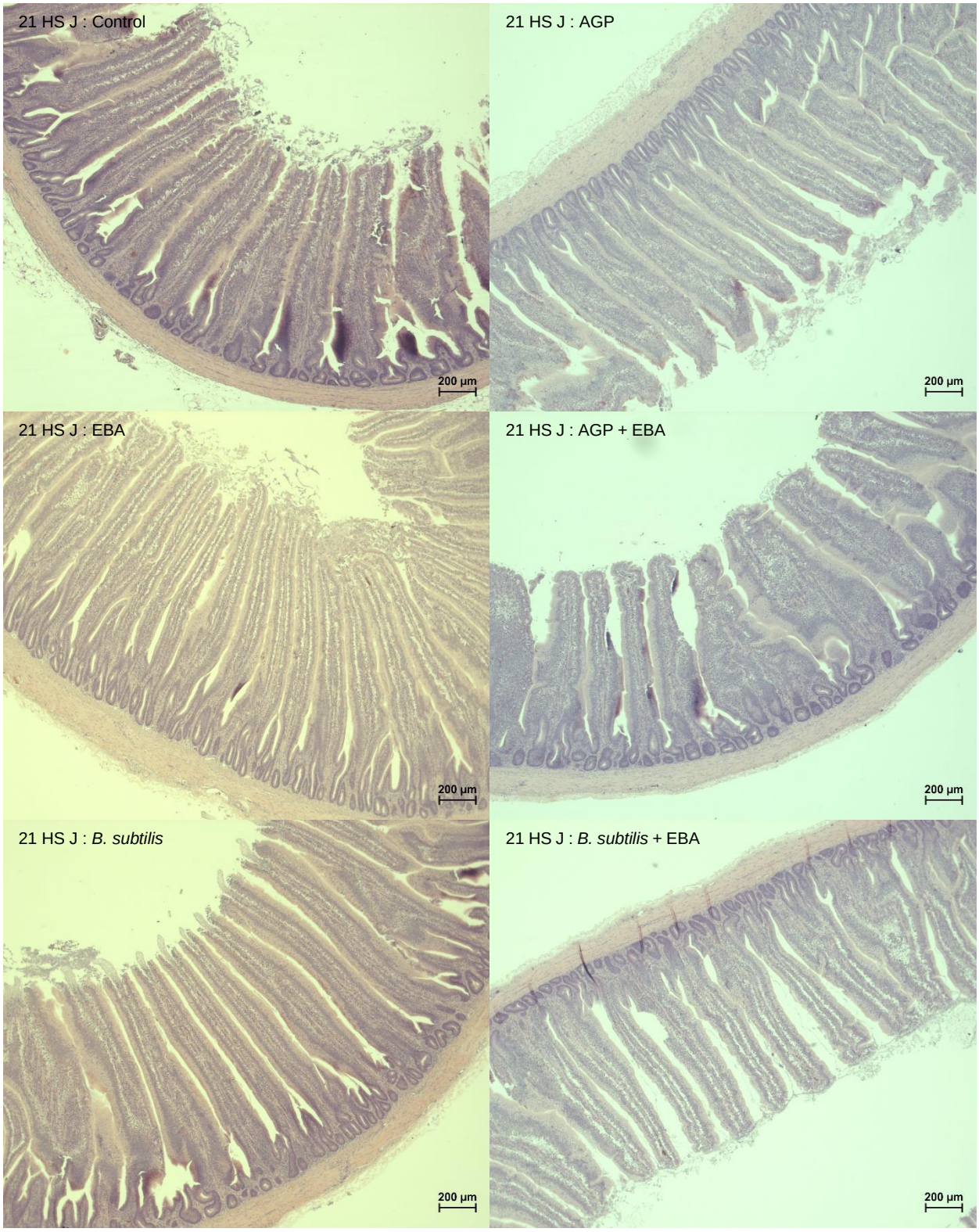

Figure A.2 Representative photomicrographs of 21d jejunal mucosa and submucosa of the treatment diets of heat-stressed broilers, stained with HE, Bar: 200 µm at 5x magnification.

21 HS J: 21d heat-stressed jejunum

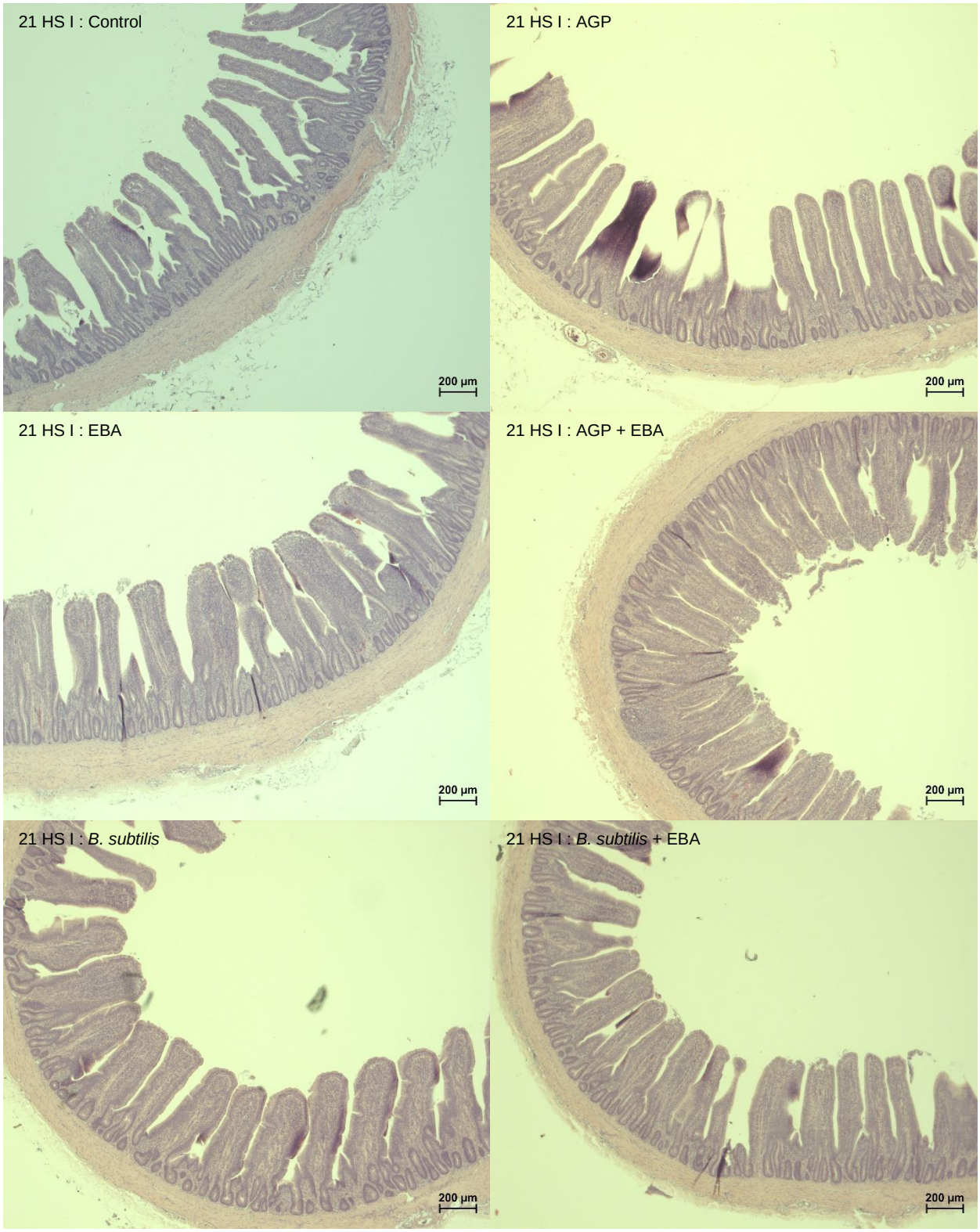

Figure A.3 Representative photomicrographs of 21d ileal mucosa and submucosa of the treatment diets of heat-stressed broilers, stained with HE, Bar: 200 µm at 5x magnification.

21 HS I: 21d heat-stressed ileum

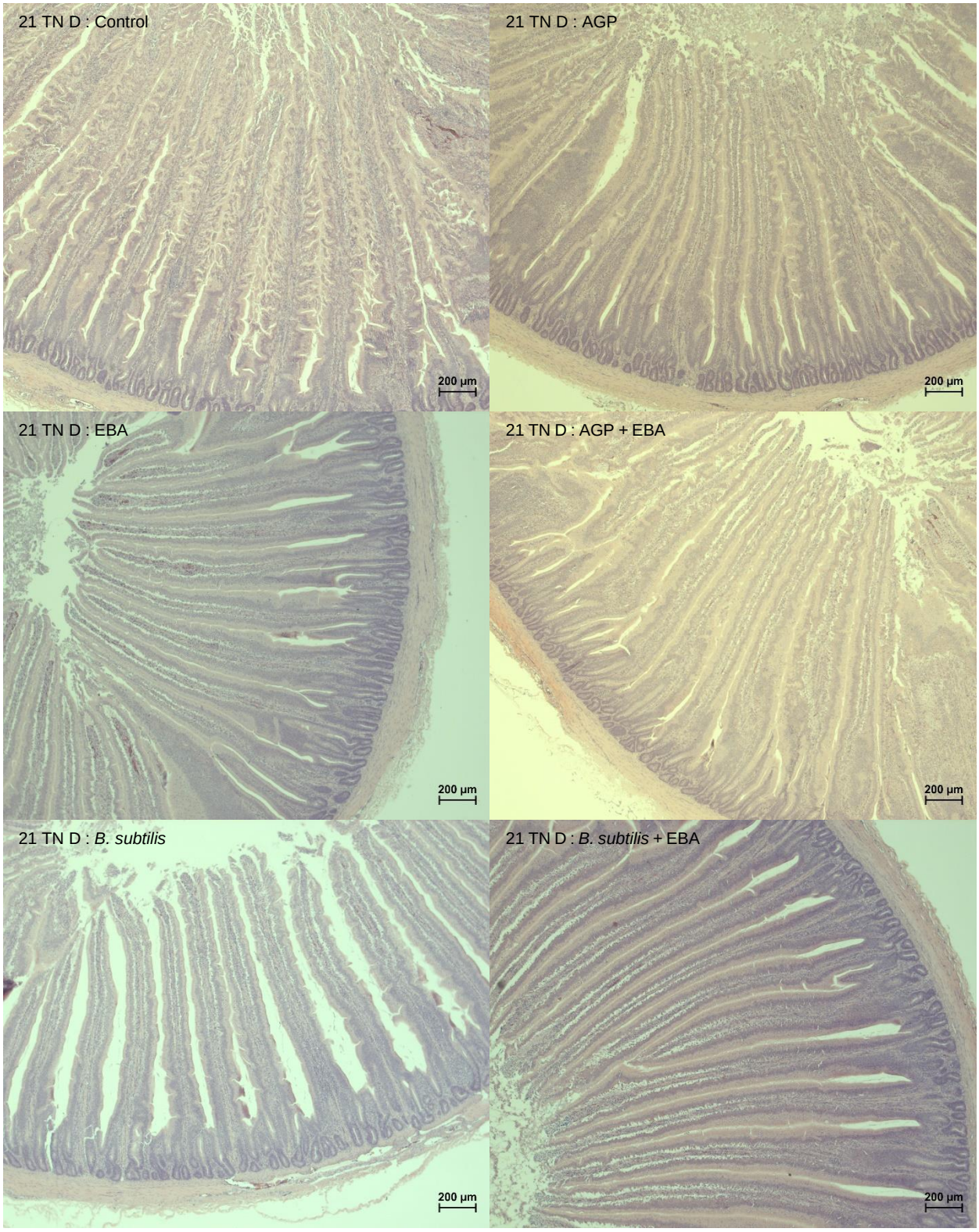

Figure A.4 Representative photomicrographs of the duodenal mucosa and submucosa of the treatment diets of thermoneutral broilers at 21d, stained with HE, Bar: 200 µm at 5x magnification.
21 TN D: 21d thermoneutral duodenum

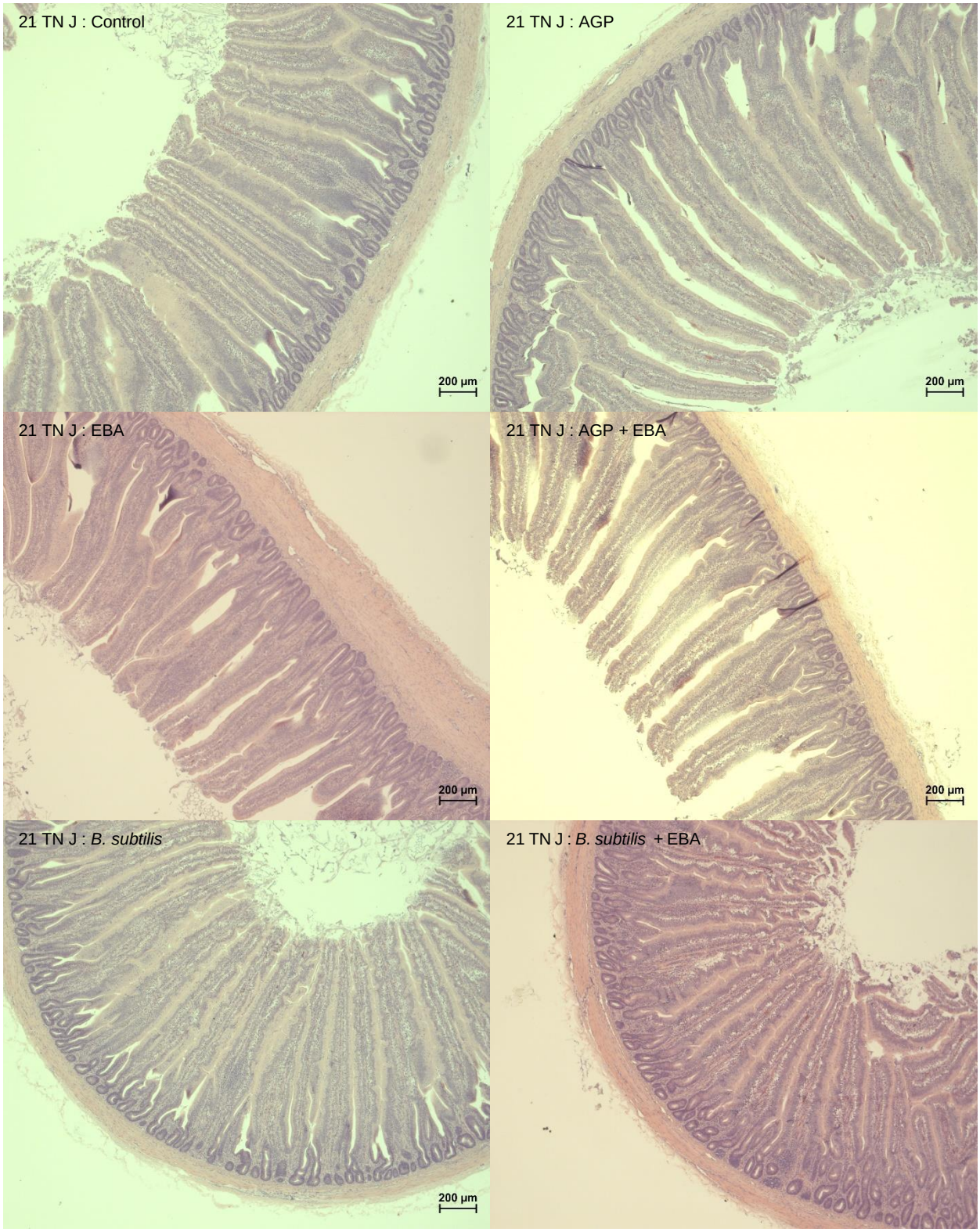

Figure A.5 Representative photomicrographs of the jejunal mucosa and submucosa of the treatment diets of thermoneutral broilers at 21d, stained with HE, Bar: 200 µm at 5x magnification.

21 TN J : 21d thermoneutral jejunum

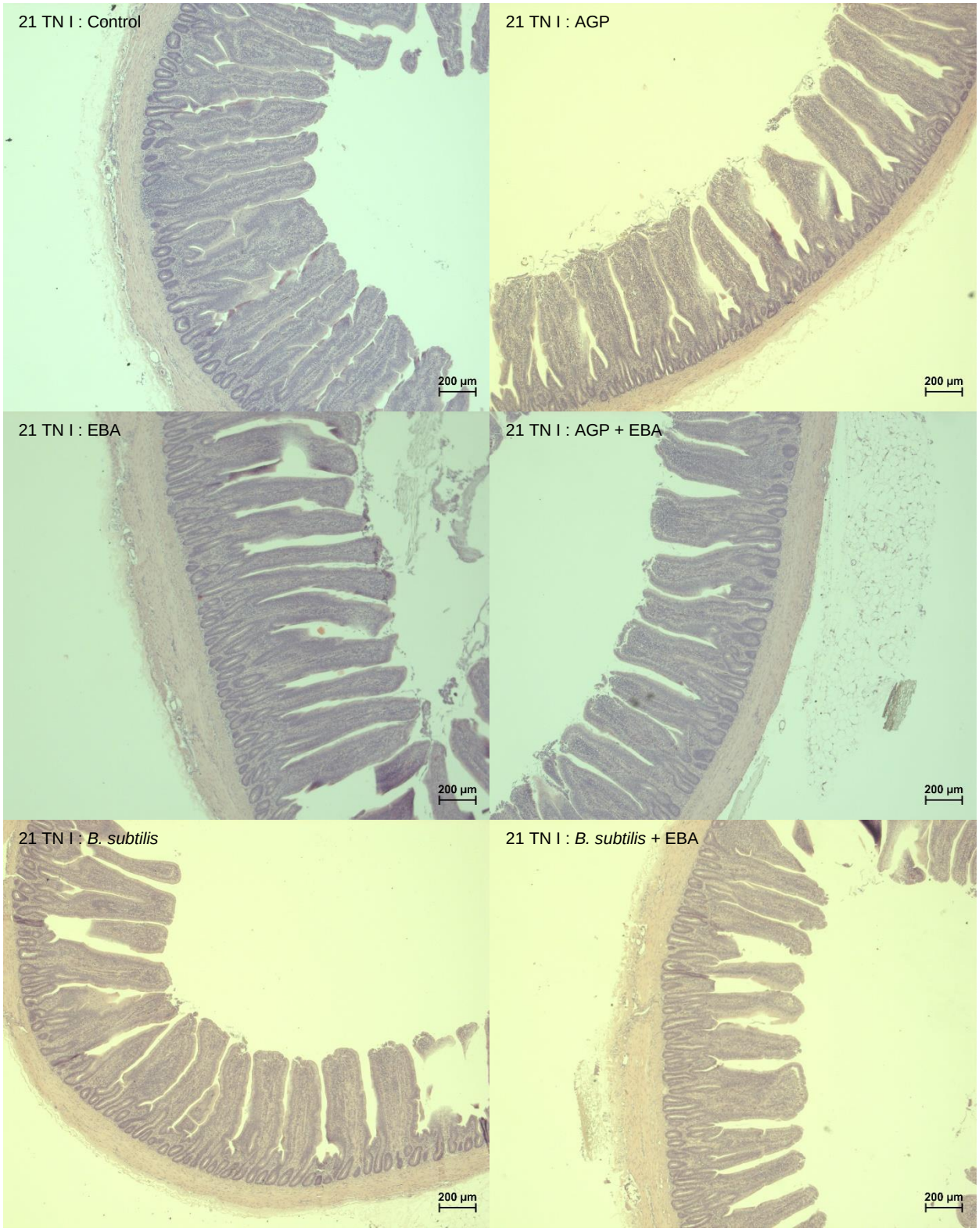

Figure A.6 Representative photomicrographs of the ileal mucosa and submucosa of the treatment diets of thermoneutral broilers at 21d, stained with HE, Bar: 200 µm at 5x magnification.

21 TN I: 21d thermoneutral ileum

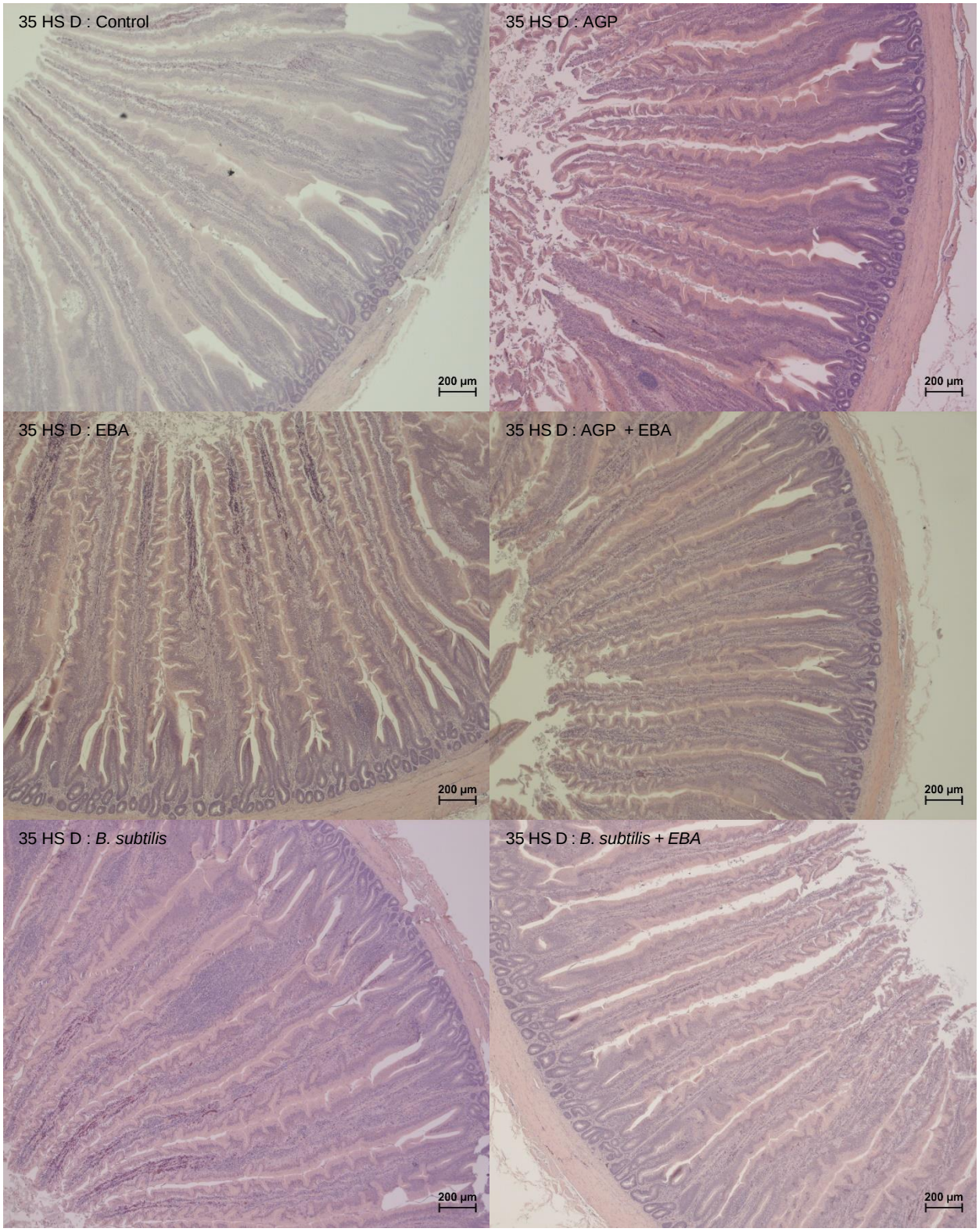

Figure A.7 35d representative photomicrographs of the duodenal mucosa and submucosa of the treatment diets of heat-stressed broilers, stained with HE, Bar: 200 µm at 5x magnification.

35 HS D: 35d heat-stressed duodenum

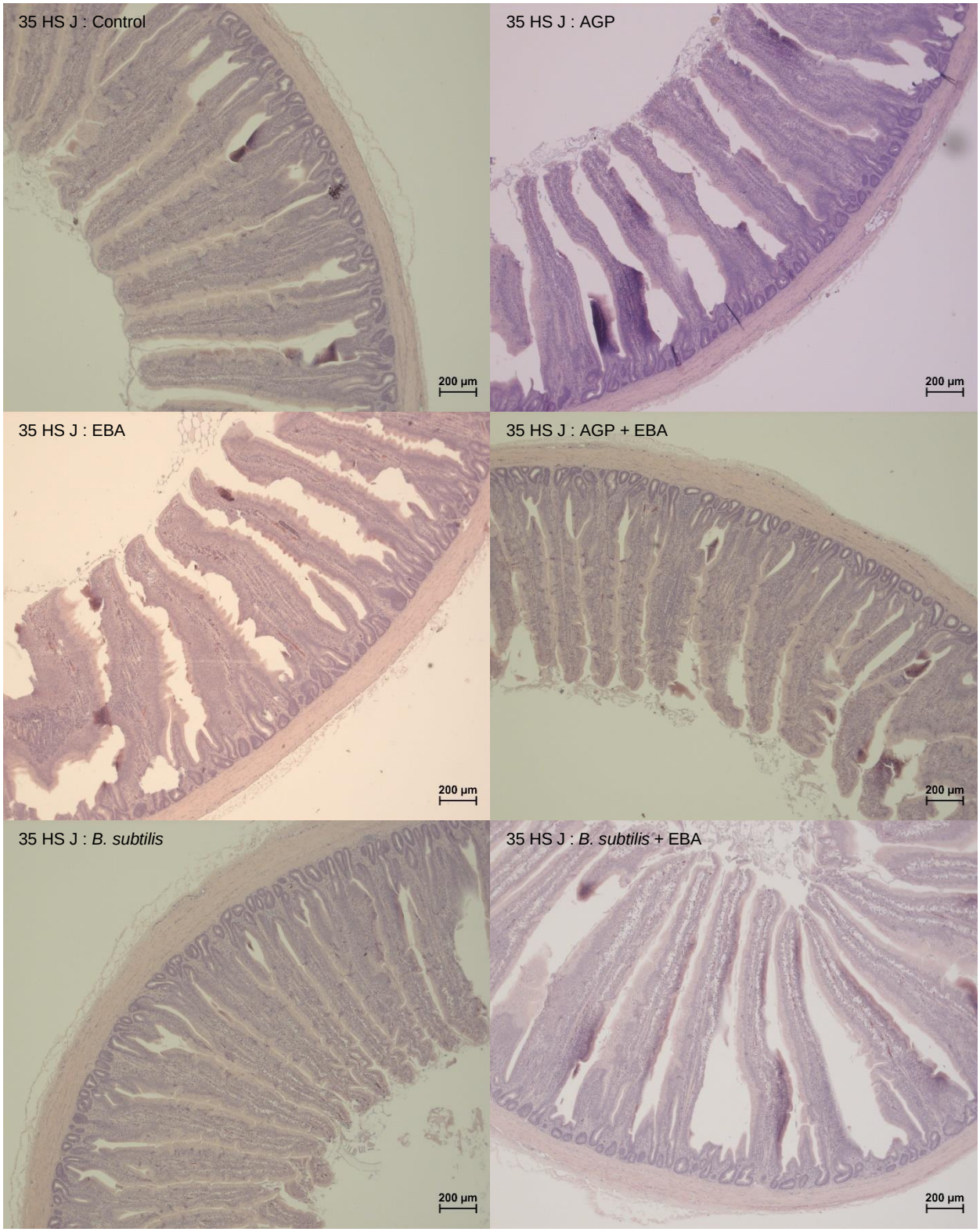

Figure A.8 35d representative photomicrographs of the jejunal mucosa and submucosa of the treatment diets of heat-stressed broilers, stained with HE, Bar: 200 µm at 5x magnification.

35 HS J: 35d heat-stressed jejunum

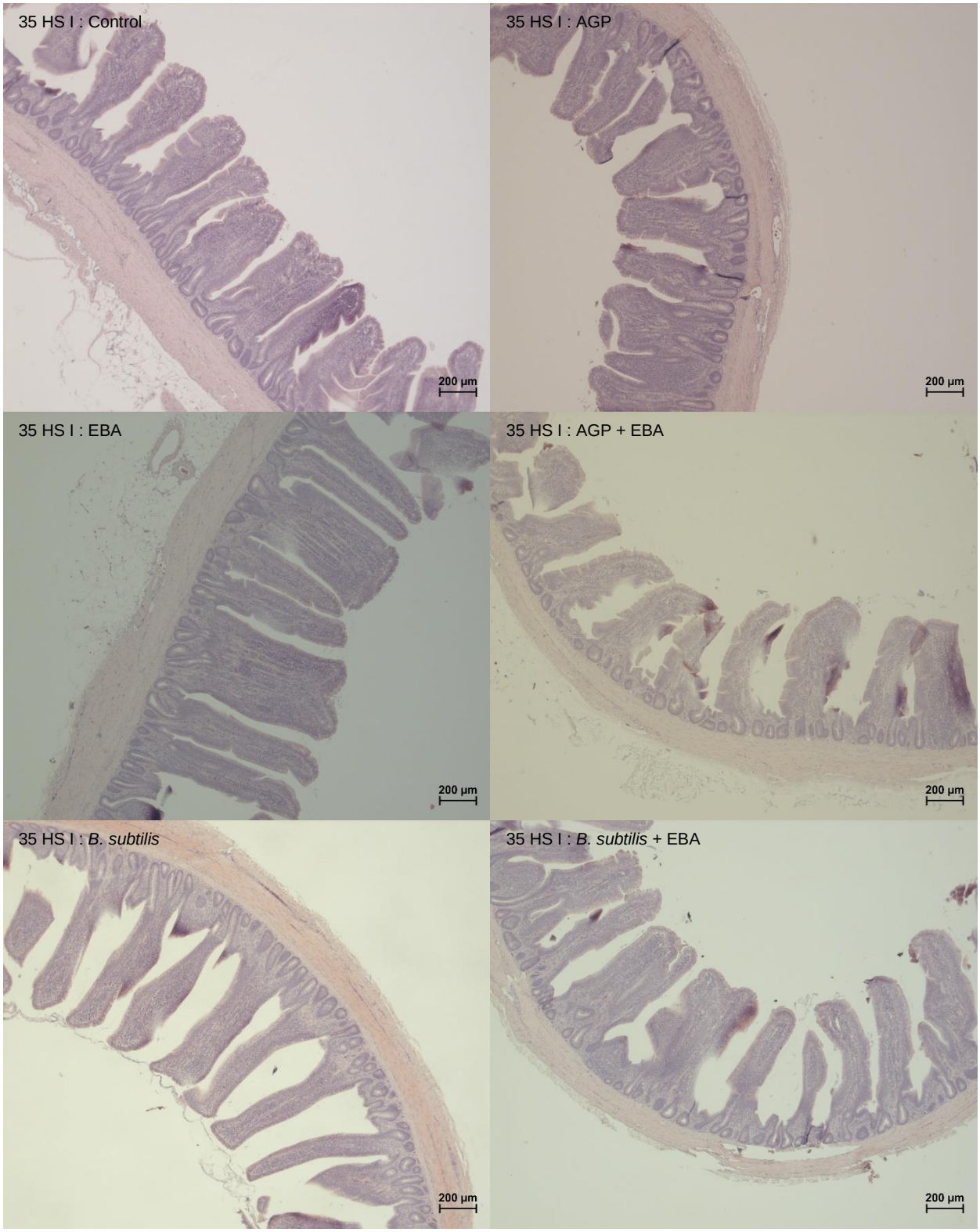

Figure A.9 35d representative photomicrographs of the ileal mucosa and submucosa of the treatment diets of heat-stressed broilers, stained with HE, Bar: 200 µm at 5x magnification.

35 HS I: 35d heat-stressed ileum

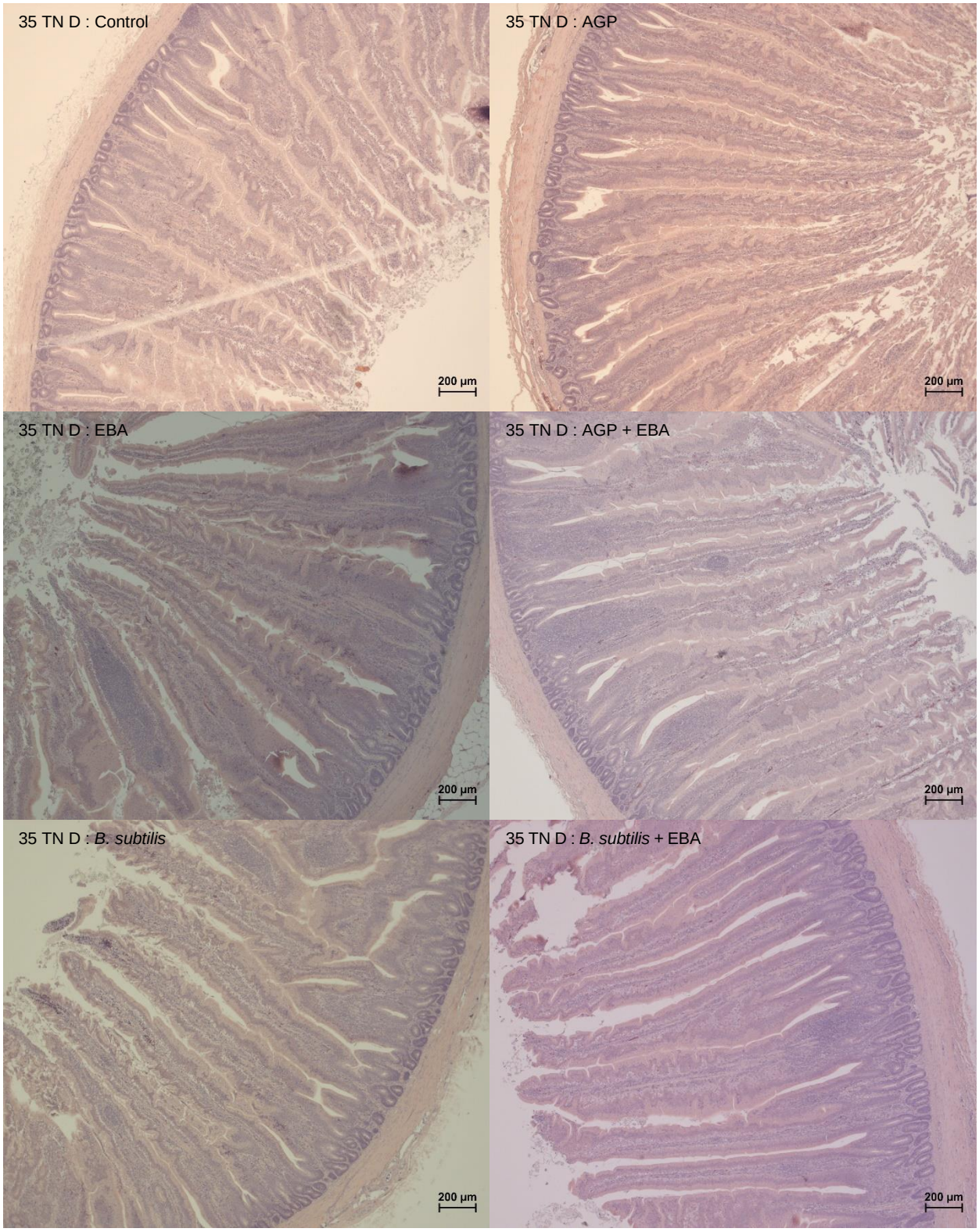

Figure A.10 Representative photomicrographs of 35d duodenal mucosa and submucosa of the treatment diets of thermoneutral broilers, stained with HE, Bar: 200 µm at 5x magnification.

35 TN D: 35d thermoneutral duodenum

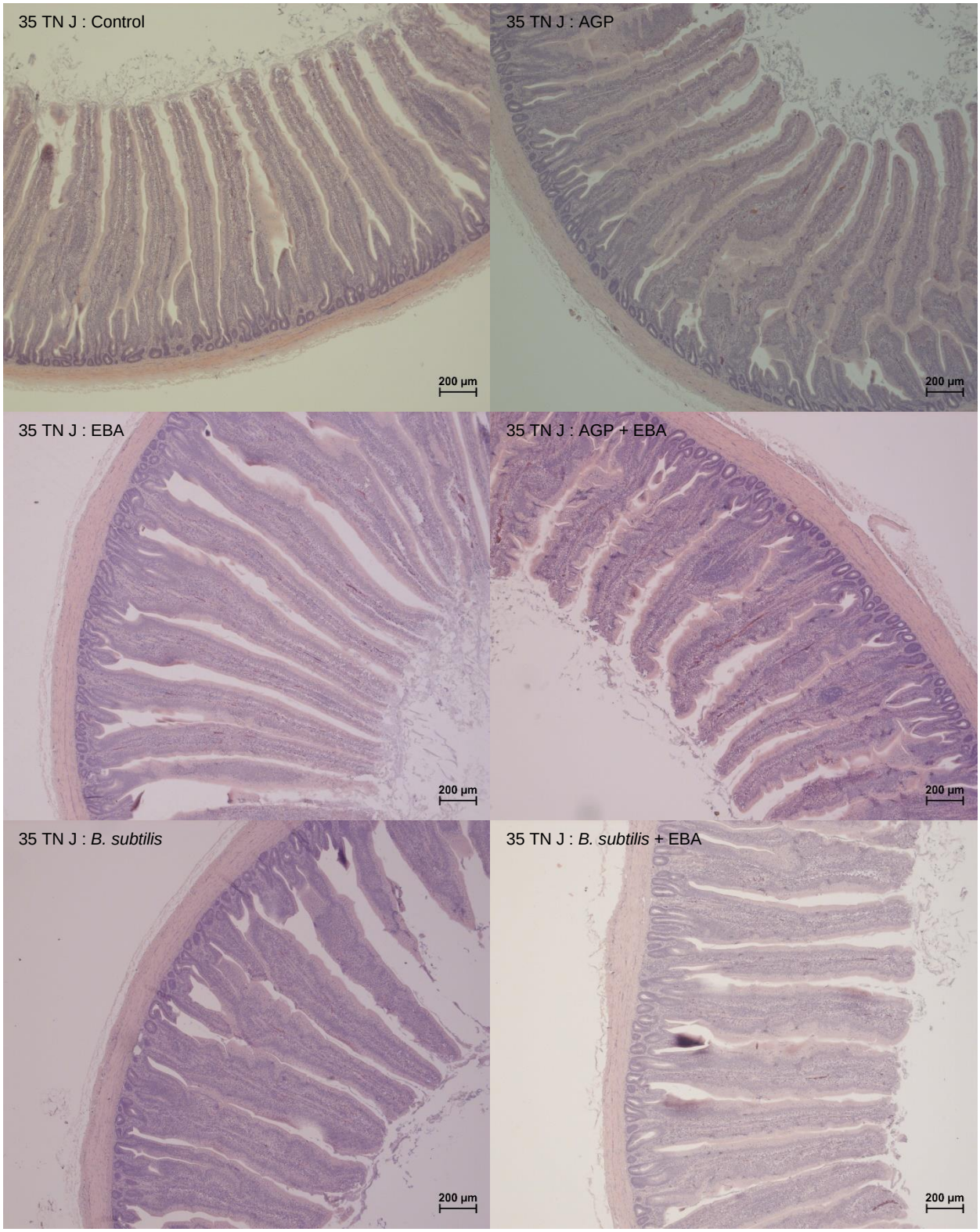

Figure A.11 Representative photomicrographs of 35d jejunal mucosa and submucosa of the treatment diets of thermoneutral broilers, stained with HE, Bar: 200 µm at 5x magnification.

35 TN J: 35d thermoneutral jejunum

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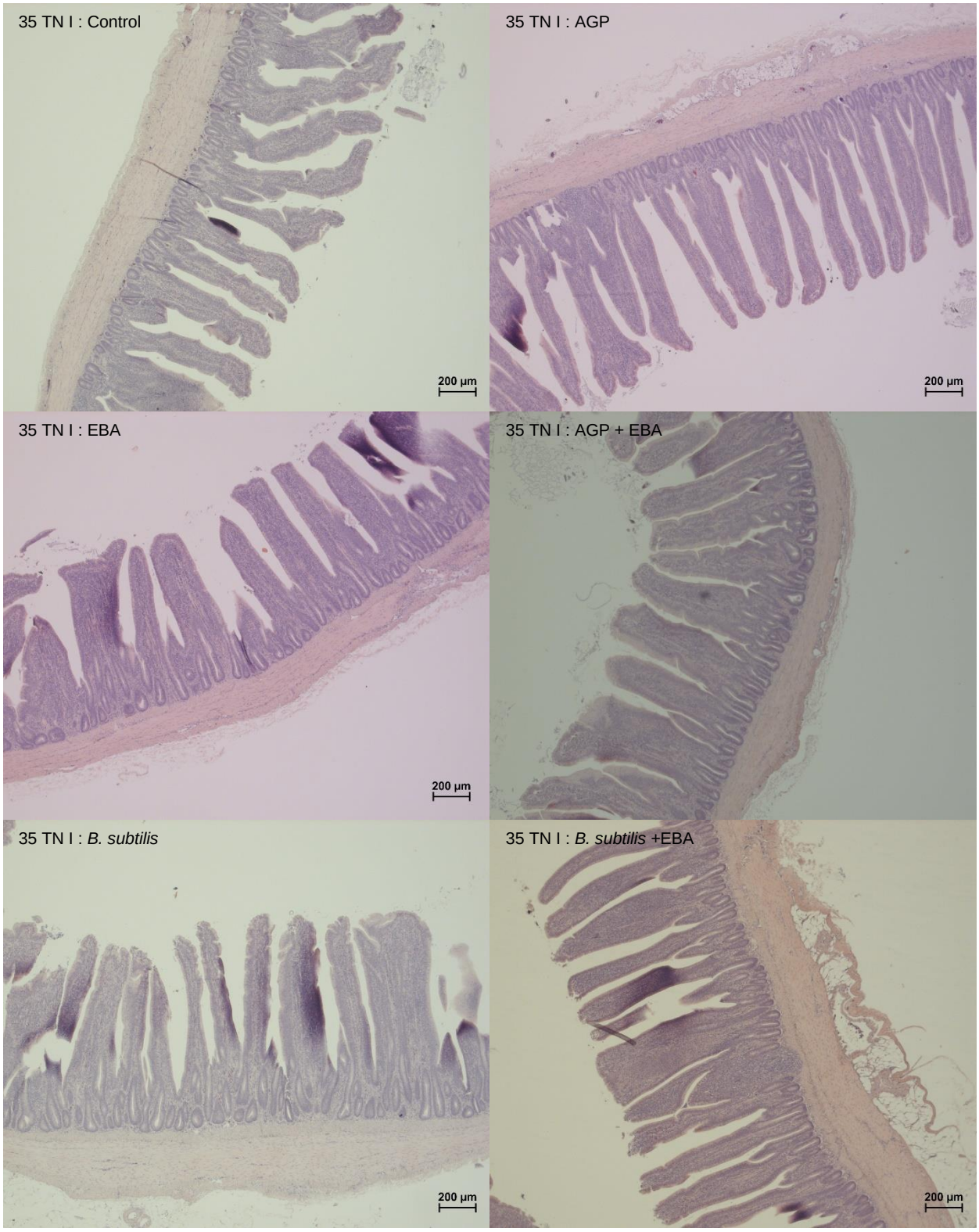

Figure A.12 Representative photomicrographs of 35d ileal mucosa and submucosa of the treatment diets of thermoneutral broilers, stained with HE, Bar: 200 µm at 5x magnification.

35 TN I: 35d thermoneutral ileum