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DIETARY INCLUSION OF PROBIOTICS AND A PREBIOTIC IMPROVED THE HEALTH AND PERFORMANCE OF BROILERS CHALLENGED WITH *SALMONELLA TYPHIMURIUM*

By

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PREFACE AND DECLARATION

I, Natasha Du Toit declare that:

1. The research in this dissertation, except where otherwise indicated, is my own original research.

2. This dissertation has not previously been submitted for any degree purposes at any other university.

3. This dissertation does not contain other person's data, unless specifically acknowledged as being sourced from other persons. Where other written sources have been quoted, then: Their words have been rewritten but the information attributed to them has been referenced.

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LIST OF ABBREVIATIONS

% - Percentage
°C – Degrees Celsius
Abs - Antibodies
Ag - Antigens
AGP - Antibiotic Growth Promoter
AST - Aspartate aminotransferase
ATP - Adenosine triphosphate
CFU/ml - Colony Forming Units per millilitre
DNA - Deoxyribonucleic acid
FA - Fatty Acids
FCR - Feed Conversion Ratio
FOS - Fructooligosaccharides
g/kg – gram per kilogram
GIT - Gastrointestinal tract
GTF - Glucose Tolerance Factor
IgA - Immunoglobulin A
IgG - Immunoglobulin G
IgM - Immunoglobulin M
Kg – Kilogram
KGy – Kilograys
LPS - Lipopolysaccharide
m^3 - Cubic meter
MCFA - Medium Chain Fatty Acids
ME – Metabolizable Energy
MJ/kg – Mega joule per kilogram
mm^2 - millimetre square
MOS – Mannanoligosaccharides
P – Phosphate
PBS - Phosphate buffered saline
RNA - Ribonucleic acid
SCFA - Short Chain Fatty Acids
ST – *Salmonella Typhimurium*
TP - Total Protein
UI - Ulcer Index
VFA - Volatile Fatty Acids
µm - micro millimetre

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ABSTRACT

Salmonellosis is one of the most important foodborne zoonotic diseases throughout the world and poultry represents an important source of infection in humans. Chickens may become infected during incubation, in the brooding houses, through various vectors, such as feed and rodents or during slaughtering and processing.

The use of antibiotics have been reduced and even banned in some countries, due to the risk of bacterial populations developing resistance against the antibiotics. This lead to the exploration of alternative products for antibiotics as growth promoters, which include prebiotics, probiotics, organic acids, essential oils, plant extracts and many more. These products may improve animal health, productivity and microbial food safety in a natural way.

A feeding trial was conducted to investigate the effects of the dietary inclusion of probiotics and a prebiotic to improve the health and performance of broilers, which were challenged with *Salmonella typhimurium*. 1800 chicks (900 chicks exposed to Salmonella and 900 chicks not exposed) were randomly assigned to 6 dietary treatments for 5 weeks. The dietary treatments were: 1) No feed additives added, 2) A prebiotic (fructo-oligosaccharide) added to the feed, 3) Probiotic type 1 (Spore-forming bacteria) added to the feed, 4) Probiotic type 1 combined with the prebiotic added to the feed, 5) Probiotic type 2 (*Lactobacillus* spp.) added to the feed, 6) Probiotic type 2 combined with the prebiotic added to the feed.

The feed intake, average daily gain and body weight of the control (non-challenged) birds were significantly higher ($P<0.05$) than the Salmonella (challenged) birds. The Probiotic type 2 combined with the prebiotic improved the feed intake of the non-challenged birds compared to the non-challenged birds that received no supplementation or only a prebiotic. The challenged and non-challenged birds that did not receive any supplementation had lower body weights and average daily gains compared to the birds that received supplementation. The feed conversion ratio showed significant differences among the treatments ($P<0.003$) and between the control and Salmonella birds ($P<0.05$).

The non-challenged birds fed the Probiotic type 2 combined with a prebiotic and the challenged birds fed only the prebiotic, displayed a decrease in liver weight, compared to the other treatments. However, the duodenum, jejunum and caeca weights of the broilers were significantly ($P<0.05$) enlarged shortly after Salmonella exposure through the

inclusion of Probiotic type 1 and the two combination treatments in the diet. No significant differences were observed in the ileal weights after Salmonella exposure or after dietary supplementation.

The total serum protein and the aspartate amino transferase (AST) levels showed no significant differences between the groups and treatments. However, the albumin levels of the challenged birds were significantly lower ($P<0.05$) than the non-challenged birds. The globulin levels were higher for the challenged birds that did not receive any supplementation than those that received a prebiotic and probiotics. The albumin: globulin ratios were higher for the non-challenged birds than the challenged birds. In general, the challenged birds tended to have more lesions than the non-challenged birds on the gastrointestinal tract (GIT). There were no significant differences in the villous height, mucosal thickness and crypt depth of the duodenum, jejunum and ileum. However, the control birds supplemented with Probiotic type 2 combined with a prebiotic showed a thicker mucosa layer than the control that received no supplementation.

These findings indicate that the supplementation of a basal diet with probiotics and combination treatments of probiotics with a prebiotic can be used as growth promoters for broilers. These products, especially the Probiotic type 2 combined with the prebiotic, show promising effects as alternatives for antibiotics as pressure increases to eliminate the growth promotant antibiotics from being used in the livestock industry.

CHAPTER 1: INTRODUCTION

One of the most important foodborne zoonotic diseases throughout the world is Salmonellosis and poultry represents an important source of infection in humans (Landeras *et al*., 1998). Poultry is one of the most common vehicles in the transmission of salmonellosis. Chickens may become infected during incubation, in the brooding houses, through various vectors, such as feed and rodents or during slaughtering and processing (Hinton Junior. *et al*., 1990). The caeca in poultry are the primary reservoir for foodborne pathogenic bacteria such as paratyphoid salmonellae (Hudault *et al*., 1985). Severe gastroenteritis can occur when processed meat and meat by-products contaminated with human foodborne pathogens are ingested by humans (Fedorka-Cray *et al*., 1995).

Salmonella typhimurium (ST) is a facultative intracellular bacterial pathogen that can invade, survive, and multiply in different cells, including epithelial and phagocytic cells. It can survive intracellular in macrophages, which can be transported to regional lymph nodes, spleen and liver, where the bacteria can multiply (Turncock *et al*., 2002). Massive multiplication of bacteria occurs in the gut, following rapid invasion of tissue. Anorexia, adipsia, depression with ruffled feathers and increasing drowsiness are clinical signs of salmonellosis (Barrow, 2000).

Antibiotics are used world wide in the poultry industry to inhibit pathogens from poultry and prevent disease to improve meat and egg production. Antibiotics are used for a number of reasons, which include the reduction of faecal carriage of Salmonella, growth promotion stimulation, chemotherapy of Salmonella and other bacterial infections (Barrow, 2000). Common problems such as the development of drug-resistant bacteria, imbalance of normal microflora and the presence of drug residues in the body of the birds can occur after the use of dietary antibiotics (Awad *et al.,* 2009). The general use of antibiotics has been restricted by regulations. In Western countries, antibiotics can only be used under a veterinarian's supervision but in many other countries antibiotics are freely available (Barrow, 2000).

It has become important to develop alternatives for antibiotics such as the dietary inclusion of nondigestable ingredients that enhance microbial growth or beneficial microorganisms (Awad *et al*., 2009). Many pathogens are becoming increasingly resistant to antibiotics, but probiotics offer a significant treatment alternative to this problem (Horowitz, 2003). The term "probiotic" has been defined as "a live microbial feed supplement which beneficially affects the host animal by improving its intestinal balance".

A “prebiotic” is defined as a non-digestible food ingredient that beneficially affects the host by selectively stimulating the growth and/or activity of one or a limited number of bacteria in the colon. Synbiotics refers to the combination of probiotics and prebiotics (Patterson and Burkholder, 2003). Research has shown that by feeding combinations of probiotics and prebiotics tend to be more effective against Salmonella than when feeding them individually. The phenomenon of probiotics and prebiotics to prevent the growth of pathogens and to enhance the growth and performance of the host bird has lead to a widespread interest in these two biotics as alternatives for antibiotics. Research on other alternatives including organic acids, essential oils, bacteriocins, bacteriophages, enzymes, antimicrobial peptides, nucleotides and even plant extracts has also been done.

The aim of this experiment was to determine whether the dietary inclusion of two different types of probiotics and a prebiotic could ameliorate the effect of *Salmonella typhimurium* in broilers. A spore-forming and a non-spore forming probiotic were included in the diets and combinations of these probiotics with the prebiotic were also included in the experiment. The hypothesis was that:

- H_0: Inclusion of probiotics and a prebiotic will not ameliorate the effect of *Salmonella typhimurium* in broilers.
- H_a: Inclusion of probiotics and a prebiotic will ameliorate the effect of *Salmonella typhimurium* in broilers.

CHAPTER 2: LITERATURE REVIEW

2.1 ALTERNATIVES FOR ANTIBIOTICS

Antibiotics have been utilized in animal research for many years and are used for a number of reasons, which include reduction of faecal carriage of Salmonella, chemotherapy of Salmonella and other bacterial infections (including *E. coli* and *Mycoplasma*), and growth promotion. The use of antibiotics is being restricted by regulations without veterinary prescription and each country varies with the antibiotics they use. In many countries antibiotics are freely available, but in Western countries, antibiotics can only be used under a veterinarian's supervision (Barrow, 2000).

There is an ongoing issue regarding the use of antibiotics in animal feed to act as growth promoters. Large numbers of healthy animals are administered with low concentrations of antibiotics for long periods to increase the rate and efficiency of growth. These low levels of antibiotics are below the minimum inhibitory concentration of most pathogens. Antibiotic resistance in micro-organisms has been linked to the continuous use of antibiotics which could be transmitted from animals to humans. However, short-term application of antibiotics reduces this risk (Durrans, 2005).

A number of issues concerning the use of antibiotics were discussed by Barrow (2000): 1) Resistance can be monitored by using sentinel bacteria such as *E.coli* and gram positive micro-organisms. It would be more useful to study omnipresent bacteria than studying pathogen resistance which might not always be present. 2) Thought should be given to a restriction of antibiotics being used prophylactically, because of the evolutionary pressures being exerted by antibiotics, with only therapeutic use being allowed. 3) Antibiotics should perhaps not be used to reduce the intestinal carriage of food-borne pathogens. 4) The use of growth performing antibiotics which are banned in certain countries has lead to poultry and poultry meat posing as a potential public health risk. Therefore, to prevent the importation of such meat, trade controls might be introduced. 5) The use of disinfection and vaccination of dams should be emphasized more to reduce the problems of mortality and morbidity in young poultry which require medication.

In addition to animal health and economics, the use of antibiotics remains an important public health issue. The countries concerned as well as those countries which do not currently regard this issue as being important, should address the issues discussed above (Barrow, 2000).

The incidence of multiple antibiotic resistances in Salmonella from poultry has been very low, despite the extensive use of penicillin derivatives and more recently fluoroquindones. In the calf rearing industry over-use has led to the gradual evolutionary development of strains of *Salmonella typhimurium* resistant to several antibiotics. When serotypes such as *S. typhimurium* and *S. enteritidis* produce mortality then chemotherapy may be used in very young chickens. A variety of antibiotics is used for this purpose. Chemotherapy has also been used to reduce the frequency of egg contamination in broiler breeders or layers, where *S. enteritidis* causes problems. This commences prior to stock movement from rearing to laying accommodation, followed by oral administration of competitive exclusion preparation which restores the gut flora (Barrow, 2000).

The selection of resistant clones or the encouragement of resistance transfer in the intestines has always been a concern during the use of antibiotic therapy. Antibiotics are active against Salmonella as well as members of the gut flora, which are relatively inhibitory to Salmonella colonisation. For this reason, an oral administration of a gut flora preparation immediately after the withdrawal of chemotherapy is rational. Susceptibility to reinfection from the immediate environment of the birds increases as these organisms are eliminated and the multiplication of Salmonella increases until the gut is fully restored (Barrow, 2000)

In order to produce food cheaply and help animals realise their full genetic potential, a new performance enhancer is needed. Low feed conversion ratios (FCR), high daily weight gains, and shorter fattening times are traits that are necessary for good economic returns. The consumer has become accustomed to cheap foods which are produced under conditions ethically acceptable in terms of animal welfare and health. The antibiotics needed to fulfil these criteria will no longer be acceptable to consumers (Mellor, 2000). In North America, nearly half of all antibiotics used end up in livestock and poultry feeds. The amount of antibiotics being used for weight gain in animal food production has been reduced by legislation in parts of Europe (Reid and Friendship, 2002).

When antibiotic growth promoters (AGP's) are removed from diets the cost of poultry production is increased by approximately 3% as a result of poorer feed conversion. A higher risk of contamination and condemnation of carcasses in the processing plant, greater performance variability and increased mortality will all lead to additional financial losses which would also result in an increase in poultry production costs. The risk of carcass downgrades and microbial contamination with food poisoning bacteria are increased and are caused by the litter quality which may also be affected. To counteract

the performance and economic losses associated with AGP withdrawal, a range of nutritional and management measures are required. It is the poultry producer's ultimate goal to apply consumer-friendly and cost-effective strategies for suppressing the proliferation of pathogenic bacteria which was previously controlled by AGP's (Hruby, 2005).

In an attempt to smooth the transition from cheap food to "safe" food, the gap is already being populated by alternatives. The public is beginning to demand that this transition is achieved by a "natural" route. All such products must comply with certain standards and regulations. These alternative products must (i) not be toxic to the animal or their human handlers, (ii) not be mutagenic or carcinogenic, (iii) have little therapeutic use in human or veterinary medicine, (iv) not cause deleterious disturbances of the normal gut or be absorbed from the gut into edible tissue, (v) not promote Salmonella or give rise to environmental pollution, (vi) not cause cross-resistance to other antibiotics or be involved with transferable drug resistance and (vii) improve performance effectively and economically (Mellor, 2000). Strategies for improvement in animal health, productivity, and microbial food safety other than antibiotics, have been explored over the years (Joerger, 2003).

2.2 PREBIOTICS AND PROBIOTICS

2.2.1 Introduction

Several approaches, two of which are prebiotics and probiotics, have the potential to reduce contamination of poultry products and enteric disease in poultry (Patterson and Burkholder, 2003). The term "probiotic" has been defined as "a live microbial feed supplement which beneficially affects the host animal by improving its intestinal balance". A "prebiotic" is defined as a non-digestible food ingredient that beneficially affects the host by selectively stimulating the growth and/or activity of one or a limited number of bacteria in the colon. Synbiotics refers to the combination of probiotics and prebiotics (Patterson and Burkholder, 2003).

There are a few characteristics that describe the ideal probiotic and prebiotic. A probiotic must be of host origin, non-pathogenic, adhere to epithelium or mucus, modulate immune response and alter microbial activities, resistant to gastric acid and bile, persist in the intestinal tract, withstand processing and storage and produce inhibitory compounds. A prebiotic must not be hydrolyzed or absorbed by mammalian enzymes or tissues and must selectively enrich the environment for one or a limited number of beneficial bacteria.

Prebiotics beneficially alter the intestinal microbiota and the microbiota's activities as well as luminal or systemic aspects of the host defence system (Patterson and Burkholder, 2003).

The concept of prebiotics is relatively new, but effectively being used in the food industry, agriculture and human and veterinary medicine. It is known that non-digestible food ingredients are selectively fermented by bacteria which have positive effects on gut physiology and in response to this belief, prebiotics were developed. Beneficial probiotic effects, evidence of health-promoting effects and food protective activities are some of the criteria in the selection of certain strains (Walker and Duffy, 1998).

2.2.2 Prebiotics

Prebiotics are non-digestible carbohydrates, mostly short-chained monosaccharides called oligosaccharides. The growth of beneficial organisms in the gut is enhanced by some oligosaccharides, while others act as competitive attachment sites for pathogenic bacteria. Fructooligosaccharides (FOS), naturally found in onions and cereal crops, and mannanoligosaccharides (MOS) obtained from the cell walls of yeast (*Saccharomyces cerevisiae*), are the two prebiotics that have been studied the most (Griggs and Jacob, 2005). FOS derived from chicory are prebiotics that have bifidogenic effects, resist hydrolysis, reach the colon intact and are extensively fermented in the colon by the resident symbiotic anaerobic bacteria (Walker and Duffy, 1998).

Mannanoligosaccharides (MOS) act by binding and removing pathogens from the intestinal tract and stimulating the immune system (Patterson and Burkholder, 2003). Bacteria attach to the intestinal cells of the host with Type 1 fimbriae and this attachment enables the bacteria to cause disease in the host. Mannose, the main component of MOS, is a unique sugar which also contains receptors for Type 1 fimbriae. MOS functions as a competitive binding site to which the bacteria bind, after which they are carried out of the gut instead of binding to the intestine. *Salmonella typhimurium* colonisation of the intestine was minimised when the drinking water of broilers was supplemented with 2.5% mannose (Griggs and Jacob, 2005).

Inulin is another prebiotic produced and distributed by large companies in health foods and only gram amounts are needed to get a physiological effect. Prebiotics can modulate lipid metabolism, selectively modify the colonic microbiota and provide a substrate for beneficial GIT microbes such as *Bifidobacterium* and *Lactobacillus* species and others. The phenomenon of probiotics and prebiotics to prevent the growth of

pathogens and to enhance the growth and performance of the host bird has lead to a widespread interest in these two biotics as alternatives for antibiotics (Reid and Friendship, 2002).

2.2.3 Probiotics

Lactobacillus, Streptococcus, Bacillus, Bifidobacterium, Enterococcus, Aspergillus, Candida and Saccharomyces are important probiotic species in broiler nutrition (Lutful Kabir, 2009).

The most stable probiotic strains are by far the *Bacillus* spores as their spores are heat resistant and can stay viable during long-term storage. *Bacillus* species are classified as saprophytic gram-positive bacteria common in soil, water, dust and air. They enter the gut by being associated with food and are considered to be allochthonous. They are also involved in food spoilage (Cutting, 2006).

Spores can be found almost everywhere because of their ability to be dispersed in dust and water. In the gut of animals and insects the *Bacillus* spore-forming species are found which could have originated from ingestion of bacteria associated with soil. *Bacillus* species are able to survive temporarily and proliferate within the GIT showing that they exist in an endosymbiotic relationship with their host (Cutting, 2006).

Lactobacillus, Bifidobacterium and Streptococcus species are the most commonly used probiotics. The consumption of products like yogurt and milk are enriched with acid bacteria, such as Lactobacilli and Bifidobacteria, which aims at the consumer's well-being. These bacteria inhabit the intestinal tract of human infants and adults. Prebiotics selectively stimulates the growth of endogenous lactic acid bacteria and Bifidobacteria to improve the health of the host (Walker and Duffy, 1998).

Bifidobacterium bifidum can be described as anaerobic, rod-shaped Gram positive bacteria that are present in the intestinal flora of humans and animals (Estrada *et al*., 2001). This bacterium contributes to the fermentation process in the colon. They ferment carbohydrates to fructose-6-phosphate and their principal end products of fermentation are acetate and lactate. Bifidobacteria produce a wide range of antimicrobial agents such as antimicrobial peptides and lysozymes, which are effective against gram-positive and gram-negative organisms. These bacteria can also inhibit the growth of mammary and liver tumors, alter faecal bacterial enzyme activities and reduce antibiotic induced side-effects (Chichlowski *et al*., 2007).

The resistance of gastric acid, pancreatic enzymes and bile salts, colonisation of the intestinal tract and adherence to the intestinal mucosa are some of the beneficial effects shown by Lactobacillus and Bifidobacterium. They are relatively harmless and considered as important components of the gastrointestinal flora. Broad ranges of gastrointestinal disorders, in humans and animals, have been treated by lactic acid bacteria. The growth of many pathogens such as *Salmonella typhimurium*, *Staphylococcus aureus, Escherichia coli* and *Clostridium perfringens* has been inhibited by lactic acid bacteria (Rolfe, 2000).

2.2.4 Factors affecting probiotic response

The possibility exist that two different strains of the same bacterial species can yield different results. Two prebiotics containing different strains of *Lactobacillus acidophilus* may produce significantly different effects within the same host species. A *L. acidophilus* strain isolated from a chicken's gut will not adhere to the epithelium of the pig, because epithelial adhesion is host specific. Acid resistance and bile tolerance are also colonisation factors that may vary within species and their variability will be reflected in the effect obtained (Fuller, 1995).

Different preparation methods of a specific probiotic can cause variation in the results. For instance, the ability of Salmonella to adhere to the gut epithelium of chickens can be affected by the carbohydrate source in the growth medium. The adhesion capacity of these organisms also changes during the growth cycle (Fuller, 1995).

Probiotics can be administered to the host in various ways, such as a powder, tablet, liquid suspension, capsule, paste or spray. The amount and interval between doses may vary as probiotics may be given once or periodically at daily or weekly intervals. The probiotic effect obtained will therefore be affected by the amount and frequency of dosing. The preparation for probiotics cannot be relied upon to contain the number of viable organisms as stated on the label of the product and insufficient viable cells present in the probiotic can lead to negative results.

It is thought that the earlier the probiotic supplement is introduced, the more effective it will be. As previously mentioned, the gut microflora is in an unstable condition during the early stages of life and the organisms given orally will therefore find a niche which they can occupy. The animal receiving the probiotic must be subjected to adverse effects such as disease and stress for the probiotic to work effectively by reversing the effect. Probiotics work less effectively when used to stimulate the growth of a healthy

host. The conditions under which probiotics will have their maximum effects are clearly defined. Only if these conditions are met, will the results be positive and effective. Therefore, beneficial effects can be expected when the correct prebiotic is administered in the right way and at the right time (Fuller, 1995).

2.2.5 Colonisation and attachment of probiotics

The survival and stability of the probiotic strain, dose and frequency of administration, specificity of the probiotic strain relative to the host and health, age, nutritional status, stress and genetics of the host are all criteria affecting the efficiency of probiotics. Colonisation of bacterial probiotics are measured in colony forming units (CFU) and increase in number beginning at the beak then progressing distally to the colon (Chichlowski *et al*., 2007). Low anaerobic bacteria numbers are found in the crop, proventriculus and gizzard. This is due to the presence of oxygen consumed with the feed and the low luminal pH caused by hydrochloric acid in the proventriculus. Facultative anaerobes such as Lactobacilli, Streptococci and Enterobacteria occur in large bacterial numbers in the small intestine. *Bifidobacterium*, Bactericides and *Clostridia* species are other anaerobes which also occur in the small intestine at levels ranging from 10^4 – 10^8 CFU/mL. The colon and cecum, with colonisation of 10^{10} – 10^{13} CFU/mL is the most heavily colonised region of the GIT (Chichlowski *et al*., 2007).

There are three areas in the GIT which are colonised by autochthonous and allochthonous (probiotics) bacteria, namely the enterocyte surface, the caecal epithelia surface and colonic epithelia surface. These areas include three micro-environment components. The first is the digesta which is an ideal environment for many bacteria to flourish in and probiotics can be found attached to feed particles such as starch granules. The mucous blanket that covers the epithelial lining of the GIT, including the intestinal villi, caecal and colon surfaces, is the second micro-environment where microbes are found. The mucous serves as an environment where microbes can live and act as a nutrient source for the bacteria. Thirdly, bacteria can also exist on the surface of apical plasmalemma of epithelial cells lining these areas (Chichlowski *et al*., 2007).

The first step in the colonisation of the host enterocyte surface is the attachment to the enterocyte's plasmalemma and therefore permits probiotic organisms to resist peristalsis, mixing with digesta and mucus layer and removal from the gut. Probiotic bacteria which adhere to the enterocyte's plasmalemma are eliminated from the GIT a few days after supplementation ends, because they do not colonise the intestinal epithelium

permanently. One of the main selection criteria for a probiotic is the ability to adhere to mucus and epithelial cell surfaces. Studies on the adhesion and colonisation of probiotic bacteria are few, because of the intestinal enterocyte's complexity and the interaction amongst intestinal cell types within the intestinal tract (Chichlowski *et al*., 2007).

2.2.6 Nutrient metabolism

Probiotic organisms can be divided into anaerobes and facultative anaerobes, based on their tolerance to oxygen. Bifidobacteria and Lactobacillus are from the facultative anaerobe genera and used in probiotic bacterial consortia which reduce the redox potential in the gut and render the environment suitable for obligate anaerobes. Obligate anaerobic bacteria are only capable of anaerobic fermentation. Carbohydrates and proteins undergo anaerobic fermentation and through substrate level phosphorilation yield metabolic intermediates that act as electron acceptors. Metabolic end-products such as lactate, succinate and volatile fatty acids, acetate, propionate and butyrate, and hydrogen, carbon dioxide, methane and bacterial biomass are the results of anaerobic fermentation. The volatile fatty acids formed by intestinal bacteria are absorbed and metabolised by the bird and contributes to the energy requirements of the host. Bacterial metabolites from the breakdown and fermentation of proteins, such as ammonia, phenols and amines, are toxic (Chichlowski *et al*., 2007).

2.2.7 Mechanisms of action

It is not clear what the exact mechanism of action of probiotics is, but it has been proposed that they produce toxic compounds inhibitory to pathogens, compete for substrates, and competitively exclude potentially pathogenic bacteria (Patterson and Burkholder, 2003). There are four major mechanisms known to be involved in the development of a micro-environment favouring beneficial micro-organisms. The competition for essential nutrients, creation of a micro-ecology that is hostile to other bacterial species, production and secretion of antimicrobial metabolites and the elimination of available receptor sites are some of the mechanism expressed by certain favourable characteristic possessed by beneficial micro-organisms. The prevention of pathogen colonisation in the GIT is executed through these mechanisms (Edens, 2003).

Probiotics also have beneficial effects on broiler performance, certain haematobiochemical parameters, modulation of intestinal microflora, improving sensory characteristics of dressed broiler meat, intestinal histological changes, pathogen inhibition,

promoting microbiological meat quality of broilers and immunomodulation (Lutful Kabir, 2009).

Work done in mammals, specifically humans is what the perception of probiotics function in poultry is based on. The necessary protection is provided by a delicate balance among microbes in the GIT of chickens that prevents potential bacteria and pathogens to invade and cause disruption of poultry's normal bodily functions (Edens, 2003).

A symbiotic relationship has evolved between animals/humans (being the hosts) and micro-organisms, whereby the host have developed a defence strategy and micro-organisms protect and provide certain benefits to the host, such as modification of its immune system. A complex physiological and host defence mechanism must be established for this symbiotic relationship to work. Then can colonisation of bacteria be prevented by microbes from the GIT (Edens, 2003).

The bioavailability of vitamins and proteins in the GIT are increased with probiotics. This is caused by bacterial strains that produce lactic acid which leads to an increase in acidification of the gut pH. Calcium bioavailability is also improved by probiotics (Horowitz, 2003). Antimicrobials are known to be produced by some *Bacillus* species contained in commercial products (Cutting, 2006).

Implantation on cell membranes is blocked when probiotics act by binding lectin receptor sites on the pathogenic bacteria. Other mechanisms used to inhibit pathogens in the gut are the decrease in the production of toxic amines and ammonia, the formation of hydrogen peroxide (a bactericide), the initiation of a non-specific immunostimulation and the production of anti-enterotoxins. It is not surprising that the microflora in the GIT have a major influence on overall health and disease, because nearly seventy percent of the body's immune system is based in the GIT (Horowitz, 2003).

Anticancer properties have also been demonstrated by probiotics. There are a few mechanisms with which Lactobacilli play an anticancer role: They suppress the metabolic action of bacteria, they directly suppress the induction and growth of tumors and they neutralise procarcinogenic substances before they are converted to active carcinogens in the intestinal tract. Zacconi *et al*. (1992) stated that probiotics can influence the blood cholesterol level by the inhibition of cholesterol synthesis or by decreasing its level directly by assimilation.

2.2.7.1 Prevention of pathogen colonisation

The function of probiotic administration to poultry is to enhance their health and to reduce enteric and systemic diseases. Probiotic bacteria physically block opportunistic pathogen colonisation through their ability to colonise areas, which are also favourable to Salmonella, within the intestinal tract such as intestinal villus and colonic crypts. This mechanism is known as "competitive exclusion" (Chichlowski *et al*., 2007). This approach can be used by inoculating 1-day old chicks with an adult microflora which indicates that the intestinal microbiota has an impact on intestinal function and resistance to disease. Instead of adding one or a few bacterial species to an established microbial population, the competitive exclusion approach provides the chick instantaneously with an adult intestinal microbiota (Patterson and Burkholder, 2003). The competitive exclusion bacteria may be composed of a single specific strain, several strains or even several bacterial species. These bacteria are found in the GIT and are classified as non-pathogenic (Doyle and Erickson, 2006).

The efficacy of competitive exclusion products have been proposed and can be explained by the modulation of the immune system occurring in the animal, the production of volatile fatty acids or bacteriocins which limits the growth of pathogens, competition for essential nutrients by the competitive exclusion bacteria limiting the ability of the pathogen to grow and the native and competitive exclusion flora which lines the intestine, physically obstructing attachment sites for the pathogens. The use of antibiotics, infected breeders stress, contaminated hatchery areas, molting, disease and feed withdrawal are factors that can reduce the efficacy of competitive exclusion treatment (Doyle and Erickson, 2006).

The experimental inoculation of *Salmonella* organisms into newly hatched chicks causes the excretion of bacteria in large numbers for a long period of time. Inoculation of adult birds causes bacteria to be excreted in smaller numbers for shorter periods. Adult birds have a complex intestinal microflora that gives the bird a high degree of protection and any disruption in this flora can result in increased excretion of Salmonella. The high level of hygiene in the hatchery is the cause of newly hatched chicks to have poor resistance to pathogens and therefore the chicks need a few weeks to obtain this flora naturally (Barrow, 2000).

Another way that probiotics can exclude the colonisation of pathogens is to prevent their adhesion to the gastrointestinal epithelium. It varies from organism to organism how they prevent pathogens from attaching and colonizing. The physical micro-environment of the intestinal tract can be changed in such ways that pathogens cannot

survive. Probiotics compete with pathogens for nutrients and therefore preventing them from growing and functioning in the gut. As already mentioned, probiotics also produce end-products such as volatile fatty acids that lower the pH and make it impossible for pathogens to survive (Chichlowski *et al*., 2007).

2.2.7.2 Maintenance of epithelial barrier integrity

The epithelial barrier maintains functional integrity by two mechanisms. The "mucous blanket", is the first mechanism and is a thick layer of mucus secreted by the goblet cells which is dispersed throughout the luminal epithelium in the small intestine. Mucin, glycolipids, small proteins, lipids, glycoproteins and soluble receptors that recognise adhesion proteins which facilitate bacterial attachment, are the constituents of this mucus. The administration of probiotics can alter the effects of intestinal bacteria when these bacteria trigger enterocyte inflammation.

Tight junctions are the second mechanism that ensures epithelial barrier integrity. These tight junctions form an unbroken biological barrier by letting the intestinal epithelial cells attach to one another, preventing bacteria and large molecules from the digesta to enter. The apical junction complex is the collective term for the tight junction and zonula adherens (Chichlowski *et al*., 2007).

2.2.7.3 Enhancement of intestinal immune function

The immune system is challenged by the intestinal enterocytes, because digestion and nutrient uptake must occur without causing responses to food or organisms living in the gut. The enterocytes also monitor the epithelial cell's surface for the presence of pathogens, therefore, a constant interaction amongst different cell types, innate and adaptive immune system and bacteria. The organization of the intestine and inter-digitations of immune cells throughout the epithelial tissue achieves a balance between a hyper-response and no response. Horowitz (2003) stated that the gut is responsible for nearly seventy percent of the immune system. More lymphocytes exist in the gut than in any other tissue, and the gut itself is therefore often referred to as the largest immune organ in the body and indicate the size of the gut and amount of surface area in contact with the external environment (Chichlowski *et al*., 2007).

The loss of nutrients, access of pathogens as well as the ability of the immune system to detect pathogens in the lumen can be prevented by the intestinal epithelium's enterocytes that provide a barrier. This barrier is escaped by pathogens and they enter

the gut via M cells, which comprise approximately 1% of total intestinal epithelia. These M cells sample antigens and transport them to nearby immune cells, revealing their phagocytic properties (Chichlowski *et al*., 2007).

The action of probiotics to down regulate specific signalling pathways can influence the inflammatory response created by pathogens. Probiotics can also modulate the expression of pro- and anti-inflammatory cytokines. Yurong *et al*. (2005) stated that probiotics can increase the IgA in the lumen and IgA, IgM, IgG producing ß cells and the T cells in the caecal tonsils which leads to the increase in density of the microvilli and length of the caecal tonsils. Haghighi *et al*. (2005) reported that the formation of natural antibodies against antigens in the gut and serum are increased by the oral administration of probiotics.

Koenen *et al*. (2004) found that different effects on the GIT and immune system of birds were caused by the same bacterial strain, but depends on their genetics and age, which suggests that different doses of probiotics at different intervals may be required by different types of birds.

2.2.8 Potentiating the effectiveness of probiotics

The selection of more efficient strains, gene manipulation, the combination of strains, the combination of probiotics and the synergistically acting components are a few methods that may be used to potentiate the effectiveness of probiotics. The best way, from a practical point of view, to enhance the efficacy of probiotics is through the combination of probiotics and synergistically acting components. Either intensifying one of the mechanisms or extending the range of probiotics can be a starting point (Bomba *et al*., 2002).

Synbiotics is a mixture of pre- and probiotics that affects the host beneficially and their protective, stimulative effects are restricted to the colon. This mixture improves the survival and implantation of live microbial dietary supplements in the GIT and selectively stimulates the growth of health-promoting bacteria by improving the host's welfare and activates their metabolism (Gibson and Roberfraid, 1995).

Bomba *et al*. (2002) defined potentiated probiotics as: "Bio preparations containing synergistically acting components of natural origin and production strains of microorganisms that potentiate their probiotic effect on the small intestine and colon and their beneficial effect on the host by intensifying a mechanism or by extending the range of their probiotic action". There are two criteria that potentiated probiotics must comply to (i)

their protective, stimulative effect must be expressed in all parts of the digestive tract and (ii) they must be more effective than their components separately.

Lactobacillus bacteria that produce lactic acid may prevent coliform bacteria diarrhoea by interacting with enterotoxins in an indirect way, by influencing *E.coli* populations or metabolism or by neutralising the enterotoxins directly. Phytocomponents such as phytins and phytic acid can stimulate lactobacilli to produce lactic acid.

Probiotics and non-specific substrates, for example Lactobacillus and peptides, work better in combination rather than when they are separate. Together, they form a combined action by reducing mortalities after diarrhoea, halving the amount of digestive disorders and greatly improving animal health (Bomba *et al*., 2002).

The supplementation of whey can enhance the effects of certain *Lactobacillus* strains by increasing body weight gain and resistance to Salmonellae. The cell numbers and lactic acid production by Lactobacilli and Streptococci are increased when whey is supplemented (Bury *et al*, 1998). It is also found that whey proteins such as ά-lactalbumin and β-lactoglobulin are excellent growth promoters of Bifidobacteria. It has long been known that milk and milk products in chicken's diets affects changes in the intestinal microflora, leading to harmful microorganisms being reduced. Dried whey contains about 61% lactose and the caecae pH of chickens can be reduced by lactose (DeLoach *et al*., 1990). It is believed that lactose acts by promoting the growth of lactose-fermenting bacteria that compete with the Salmonella for colonisation or produce substances toxic to Salmonella.

Alternatives for the use of antibiotic growth promoters, such as organic acids together with probiotics and specific carbohydrates such as yeast-cell walls were mentioned by Jensen (1998). Fermented feed can be used as an alternative to organic acids in combination with probiotics. Probiotics and antibiotics is another combination that exists and can obtain additive advantages. Probiotics are referred to as the natural substitutes for feed antibiotics. Nousiainen and Setälä (1993) mention that by using an antibacterial feed additive, the natural flora is weakened which makes it easier for probiotic bacteria to establish in the animal's digestive tract. An interesting aspect of this combination is that Lactobacilli preparations may protect the animal against the side effects of antibiotics.

These effects may cause bacterial translocation which can lead to septicaemia, caused by an increase in resistant species and a decrease in more sensitive species. The combination of *L. casei*, *L. acidophilus* and *L. bulgaricus* as the probiotic mix, together with

antibiotics, will prevent ampicillin-resistant bacteria to increase as well as their translocation to the liver.

Some microbes are able to accumulate metal ions in the cell or bind them in the external environment at the cell surface. Yeast and Lactobacilli can condense selenium in high concentrations from their growth media into their cells and produce an organic form of selenium from inorganic Se (Bomba *et al*., 2002). Yeast can produce a glucose tolerance factor (GTF), which has trivalent chromium as the active constituent and is also a cofactor for potentiating insulin. Therefore, yeast serves as a feed supplement of the non-toxic, bioactive chromium form. An essential nutrient for microbes is iron, but not for Lactobacillus. Instead, Lactobacillus uses manganese and cobalt as nutrients. Probiotics which are used extensively, such as Bifidobacteria and Lactobacilli, can bind iron and therefore reduce the availability of iron to pathogens.

Probiotic preparations require a complex solution to ensure high effectiveness aimed at the product and how it is applied (Bomba *et al*., 2002).

2.3 OTHER ALTERNATIVES FOR ANTIBIOTIC GROWTH PROMOTERS

2.3.1 Organic acids

Organic acids are well known to extend the shelf life of perishable food ingredients, preventing food deterioration as well as being utilised as preservatives and food additives. They are formed through fermentation and widely distributed in nature as normal constituents of plant and animal tissues. In monogastrics, organic acids aid in the proliferation of the intestinal wall and are important sources of energy for the cells of the intestinal wall (Perdok *et al*., 2003).

This group includes the saturated straight-chain monocarboxyclic acids and their derivatives. Organic acids are generically referred to as fatty acids (FA), volatile fatty acids (VFA) or weak, carboxyclic acids (Cherrington *et al*., 1991). These acids are incorporated into human foods and used as direct additives or indigenous and starter cultures, which are added to dairy, vegetable and meat products and accumulate these acids over time through their fermentation activity. The production of short chain fatty acids (SCFA), acetate, propionate and butyrate in the GIT of humans and animals are highly concentrated in areas where anaerobic microfloras are predominant (Ricke, 2003).

Formic, acetic, propionic, butyric, lactic, sorbic, fumaric, malic, tartaric and citric acid are organic acids commonly used in the feed industry. By improving the palatability of

the diet through lactic and citric acid, voluntary feed intake is enhanced and feed intake is depressed when high levels of organic acids are included in the diet (Perdok *et al*., 2003).

To reduce or eliminate pathogenic bacteria and fungal contamination, a feed additive such as propionic acid has been used. Mature digestive tract microflora are absent in young chicks and the microflora are quite susceptible to the colonisation by Salmonellae. Therefore, protection of young chicks against colonisation by pathogens is important. High initial lactic acid concentrations, high levels of undissociated propionic acid and high total propionic acid concentrations are conditions that produce a pH of 5.0 in the caeca which reduces the numbers of Salmonellae (Hume *et al*., 1993).

Normally, mixtures of organic acids or their salts are used because of their additive or synergistic effects (Perdok *et al*., 2003). Blends of organic acids such as formic and propionic acids are more effective against Salmonella, in their different stages of dissociation and non-dissociation, than single acids alone. The right combination of dissociated and undissociated organic acids is needed for a well balanced acidifier. Choosing organic acids with different pKa values (the pH at which the acid is 50% dissociated), such as formic and propionic acid, will cause dissociated and undissociated acids to be present at the same pH. The advantage of this is that dissociated organic acids that can reduce the pH and undissociated organic acids that have direct antimicrobial effects are simultaneously present (Lücstädt, 2005).

Organic acid's mode of action is to improve protein digestion and increase energy production. The decrease in gastric pH and bacterial growth, an increase in nutrient digestibility and improvement in pepsin activity are some of the mechanisms for the growth promoting effects of organic acids. The gastrointestinal tract's microbiota can be influenced by acids through changes in the physical conditions, which are less appropriate for the growth of pathogenic species and can be lethal to some pathogens (Perdok *et al*., 2003).

Organic acid's antibacterial activity is related to the acids ability to dissociate, which is determined by the pKa-value of the respective acid, to reduce the pH and the pH of the surrounding milieu (Canibe *et al*., 2002). The optimum pH range for Salmonella to grow is between 6.5 and 7.5, although they can live in a pH range from 4-9. Therefore, bacterial growth will be inhibited if there are low pH values in the environment (Lücstädt, 2005). A decrease in pH causes the antibacterial activity of organic acids to increase. By increasing the concentrations of organic acids and increasing the length of the carbon chain, the antibacterial effects of organic acids are also increased (Canibe *et al.,* 2002).

The inherent resistance of the target microorganisms, the physiological status of the pathogen in the feed over long periods of time and extrinsic factors of the environment which the acids are added to, are all factors that can influence the effectiveness of organic acids (Davidson, 2001).

During a lifecycle of Salmonella, it can grow aerobically and in a short period of time, change environments to an anaerobic metabolism to survive and colonise the gastrointestinal tract, which is a highly fermentative environment. High concentrations of SCFA are produced by other organisms in the GIT and Salmonella would be required to survive these high concentrations, but it would also be generating and exporting fermentation organic acids of its own.

The ability of SCFA to survive multiple environmental stresses has implications for the gastrointestinal ecology competitiveness of Salmonellae and foodborne pathogens, but also raises issues regarding the use of organic acids in food processing.

2.3.2 Bacteriocins

Other than the producing strain, bacteriocins are proteinaceous compounds lethal to bacteria. They are classified based on their molecular weight differences and, as a group, they are heterogeneous. Some bacteriocins can consist of smaller peptides containing 19-37 amino acids or large peptides with molecular weights of up to 90 000. Bacteriocins can have a narrow activity spectrum, where closely related species are inhabited or they can have a broad spectrum, which includes many different bacterial species. They are thought to have been present in many foods eaten since ancient times, and are often therefore considered as natural (Joerger, 2003).

The bacteriocin nisin has GRAS (generally recognized as safe) status and attention has been given to nisin and other bacteriocins produced by lactic acid bacteria, which are beneficial to human health and food production. The notion that bacteriocins might be useful for survival in the intestinal tract is supported by the fact that intestinal bacteria isolated from chicken caeca are able to synthesise bacteriocins *in vitro*. The ecology of the intestinal microbiota can be influenced by bacteriocins, as suggested from experiments with bacteriocins-producing bacteria. For example, intestinal *Salmonella typhimurium* counts in chickens were lowered when an avian *Escherichia coli* strain genetically engineered to produce the bacteriocin microcin 24, was continuously administered in the water supply. A more cost effective approach would be to administer bacteriocins-producing bacteria rather than the bacteriocins themselves.

The issue of resistance, as with antimicrobial compounds, also has to be considered for bacteriocins. Most of the low molecular weight bacteriocins appear to interact with the bacterial membrane, although the mechanism of action for all bacteriocins is not known. Changes in the bacterial membrane which are targeted by a bacteriocin usually causes resistance (Joerger, 2003).

2.3.3 Bacteriophages

Bacteriophages are defined as viruses that infect and multiply in bacteria and after replication many are released into the environment by lysis of the host bacterium. It will be necessary to do significant research before phage therapy can be implemented and not all phages would be suitable for phage therapy. Some phages can temporarily integrate their genome into the bacterium's genome where it is replicated as well as the bacterial genome where new traits are introduced or the expression of host traits are modified and other phages produce progeny without destroying their bacterial host (Joerger, 2003).

Specificity, effectiveness in killing their target bacteria, natural residence in the environment and self-replication and self-limitation are characteristics of bacteriophages which make them attractive as therapeutic agents for proliferating enteric pathogens in animals and for carriage control (Doyle and Erickson, 2006).

Their highly discriminatory nature is another characteristic that makes bacteriophages so attractive. Known specialists bacteriophages can interact with specific bacteria which express specific binding sites and if these receptors are absent, then the bacteria remain unaffected. A significant challenge for phage therapy is this narrow host range; for example, there is not a known phage that is lytic for all *Salmonella* serovars. Only a small part of the Salmonella serovars spectrum will be lysed by a particular Salmonella phage and won't even be lytic for all members of one particular serovar. There are a few reasons indicating the difficulty for therapy or prophylaxis against bacteria. The immune system will give little or no support to intestinal phage therapy. The establishment of population levels comparable to those prior phage administration and bacterial multiplication can only be performed when any target bacteria harbour resistance mechanisms or escape phage attacks (Joerger, 2003).

The chance of bacteria-phage collisions appears to be reduced by the viscosity of intestinal content and higher phage concentrations relative to the bacteria might be required for effective treatment. Bacteria which are found in certain part of the intestinal

tract can, in large numbers, exhibit non-specific phage binding and prevent phage diffusion by acting as a mechanical barrier.

Some of the failures of phage therapy attempts could also occur because of difference in bacteria's physiology grown under laboratory conditions and bacteria inhabiting host environments. It could be that most of the bacteria inside the host do no longer express the phage receptors prevalent in laboratory cultures. The physiology of bacteria could be of such a kind that phage replication is slower or completely inhabited and that selective binding to and replication of phages in bacteria of different physiological states have been recognized (Joerger, 2003).

Phages are exposed to a number of factors during phage treatment that might limit their activity. Phages are subject to attack by antibodies when circulating in the blood stream. The reticuloendothelial system can also remove phages from circulation. It is therefore feasible to select phage variants not as susceptible to removal from circulation in the body. The use of bacteriophages in agriculture or for food safety applications is still unknown at this stage, but they are the most promising agents that could complement and sometimes replace current antibiotics (Joerger, 2003).

2.3.4 Antimicrobial peptides

It appears that the production of small antimicrobial peptides is not confined to bacteria, but occurs in all eukaryotic organisms studied so far. Antimicrobial peptides are small molecules with a molecular mass of 1 to 5 kDa. Their mode of action involves the cell membranes of targeted organisms and the interaction with negatively charged membranes are facilitated by certain elements contained in their structure. The development of resistance to the eukaryotic peptides might require changes to the membrane due to the fact that these peptides resemble some of the small bacteriocins such as nisin (Joerger, 2003).

The proteinaceous nature of antimicrobial peptides makes them vulnerable to proteolytic enzymes as was the case with bacteriocins. Defensins and bactenecin are some of the peptides that exhibit toxic effects and by reducing the range and concentration of the peptides might limit this effect through proteolysis. Biological production of antimicrobial peptides by micro-organisms, tissue cultures, and transgenic animals need to be attempted, as chemical synthesis appears too costly for large-scale production thereof. Transgenic plants could also be used for peptide production and peptide-containing plant material could be added to animal feed, but it will be required, through extensive research,

to identify peptides that influence intestinal microbiota in the same way as currently used antibiotics (Joerger, 2003).

2.3.5 Nucleotides

Nucleotides are made up of three components: A nitrogenous heterocyclic base derivative of a pyrimidine or purine, a pentose (ribose or deoxyribose) and one or more phosphate groups. These three components form a low-molecular-weight intracellular compound by which nucleotides are recognised. Nucleotides are the basic building blocks of the nucleic acids DNA and RNA and are now used commercially as feed additives for the improvement of animal growth and disease resistance (Mishra and Hertrompf, 2006). Nucleotides are important to cellular metabolism by playing roles in transferring chemical energy, biosynthetic pathways, and act as co-enzyme components as well as biological regulators. By increasing the availability of precursors of RNA synthesis, nucleotides can facilitate protein synthesis (Chiofalo *et al.*, 2006).

Nucleotides assist with the normal development, maturation and repair of the gut and immune cells of humans and rats, therefore, animals lacking nucleotides in their diet will show lower immune responses. The growth and maturation of enterocytes are expedited when nucleotides are administered and the intestinal recovery after diarrhoea or food deprivation is accelerated (Yu *et al*, 2002).

Animals which are confronted with pathogens and other forms of stress can not reflect their genetic potential. The development of young animals are supported when RNA/nucleotides are incorporated, which leads to stabilised health as their natural immune response is actively fostered. Numerous trials have been done to investigate the effects of nucleotides on various animals and found that performance improves as well as feed conversion ratios. Nucleotides have also shown to reduce stress and mortality in poultry, increase egg production and hatchability. Furthermore, the development of the intestinal tract accelerated after feeding nucleotides from day-one, which resulted in a better feed conversion rate in later growth stages (Hoffmann, 2007).

Ascogen (Chemoforma Ltd, Switzerland), a supplementary product which contains RNA extracted from yeast, nucleotides, precursors of nucleotides, organic acids and thermolysed yeast, has proven to enhance IgG concentration, intestinal villous height and food intake (Chiofalo *et al.*, 2006).

To control performance, health and stress in livestock, balanced formulations of nucleotides must be regarded as a management tool (Hoffmann, 2007).

2.3.6 Enzymes

One of the most commonly included additives in animal feed is exogenous enzymes. Biochemical reactions are initiated or accelerated through enzymes acting as organic catalysts. Proteases, amylases, lipases and phospholipases are examples of some feed enzymes that act as supplements to endogenous enzymes (Durrans, 2005).

The major beneficial effect of adding enzymes to monogastric diets are related to the increased amounts of nutrients which can be released from the diet and absorbed from the GIT. Nutrient availability is therefore the focus point of enzyme effects. Bedford and Schulze (1998) mentioned that by retaining nutrients from feeds containing enzymes, the GIT will have to invest less energy. This could cause a reduction in anti-nutritive activity and therefore lead to reduced endogenous nitrogen losses in the GIT.

The viscosity in the GIT can be reduced by enzymes, such as hemicellulase, pentosanase, β-glucanase, pectinase and ά-galactosidase, which act on the fibrous components of poultry feed. A large response to enzyme supplementation is found in feeds with high viscosity in the GIT. High molecular weight viscous aggregates are formed in the GIT by cell wall components which are high in xylose and β-glucans. This leads to a reduction in the passage rate, promotion in endogenous enzyme losses, a reduction in the diffusion of digestive enzymes and the stimulation of bacterial proliferation. The time available for bacteria in the digesta to multiply before passage occurs will increase and is a result of the slower passage rate (Verstegen and Williams, 2002).

Enzyme mixtures have been developed, because enzymes are substrate specific and a cocktail can act on a number of substrates present in animal feed. Investigations on enzyme preparations which will reduce undesirable bacterial populations as well as benefit the desired gut micro flora are underway (Durrans, 2005).

Two phases, the ileal and caecal phase, have been classified to explain the effects of enzymes on gut microflora. By increasing the rate of digestion and limiting the amounts of substrates available to the microflora, the enzymes reduce the number of bacteria in the ileal phase. In the caecal phase, beneficial bacteria are fed soluble, poorly absorbed sugars produced by enzymes. These bacteria then produce volatile fatty acids which may be of benefit by providing energy for the bird as well as controlling Salmonella populations (Yang *et al*., 2009).

2.3.7 Essential oils

Steam distillation methods are used to extract essential oils, known as volatile, natural vegetable products from herbs and spices. Synthetically, quite a few essential oils are produced. Inhibition observed against bacteria, yeast and fungi, the inactivation of enzyme systems and causing increased permeability of the microbial cell wall are some of the broad spectrum antimicrobial activities observed (Perdok *et al*., 2003).

They are used in embalment, preservation of foods and as antimicrobial, analgesic, sedative, anti-inflammatory treatment, spasmolytic and local anesthesic remedies, due to their antiseptic, i.e. bactericidal, virucidal, fungicidal and medical properties as well as fragrance for which they are known for. The pharmaceutical and food uses of essential oils are more widespread as alternatives to synthetic chemical products, due to their fungicidal and bacterial properties (Bakkali *et al*., 2007).

Some essential oils show anti-oxidant activity *in vitro*. Their range of claimed effects *in vivo* are stimulating appetite, increasing secretion of digestive pancreatic enzymes and enhancing volatile fatty acid production due to gut flora modification. Identical forms of essential oil's active compounds can be produced synthetically with identical chemical structure to the naturally occurring raw materials and extracts. These products must be at least 99,5% identical to the natural materials (Williams and Losa, 2002).

Derived from their specific bioactive components, many plants have beneficial multifunctional aspects (Perdok *et al*., 2003). Through synergistically effects essential oils can function individually and in combination with other feed additives and therefore essential oils can enhance their effects (Williams and Losa, 2002). Essential oils exhibit greater effects combined in carefully studied ratios and levels than when each is alone. However, when wrong combinations are made essential oils can also act antagonistically (Perdok *et al*., 2003). Plants are protected through the important role of essential oils as antifungal, antibacterial, insecticides, antiviral and herbivores, by reducing their appetite for such plants (Bakkali *et al*., 2007).

Essential oils are thought to improve animal performance by reducing sub-clinical infections, improving palatability, regulating intestinal microbial flora and stimulating endogenous enzymes and digestion (Perdok *et al*., 2003).

The stimulation of endogenous enzymes by spice extracts and regulation of gut microbial flora are two specific areas to indicate the potential for these products in animal nutrition. Both of these could therefore help maintain the health and performance of the

host. The characteristics of food substrates in the gut can be altered by increasing the concentration of amylase and other endogenous enzymes. Reduced digesta viscosity is one effect, and this was demonstrated in a broiler trial using wheat and barley based diets. An improved feed efficiency of 5% from 1-40 days of age and significant effects on the percentage birds with sticky droppings and reduction in digesta viscosity were seen when a blend of essential oils was included (Williams and Losa, 2002).

The effects of essential oils in different diets vary. Broilers were fed diets based on wheat or maize and the viscosity was reduced in the wheat-based diets with little effect in the less viscous substrate produced on the maize-based diet. Improved feed absorption and utilisation can be the result of these changes in the intestinal substrate. Essential oils in specific blends can produce benefits on performance of the birds and maintaining health. Benefits of 2-6% can be expected when compared with controls without any growth promoters (Williams and Losa, 2002).

2.3.8 Plants, their extracts, oils and herbs

Plants have evolved with effective antimicrobial and antifungal defence systems. Combining their strengths with an effective promoter of beneficial gut bacteria gives a stronger defence mechanism against bacteria causing production losses. The common characteristic of all herbs and spices reveals a variety of biological effects and a variety of different chemical substances (Jones, 2002). Stimulating appetite would be the initial effect of adding plant extracts to animal feeds. The olfactory nerves and gustatory papillae can be stimulated by the aroma of added plant extracts (Perdok *et al*., 2003).

The applications of plants in poultry diets are multiple. High levels of metabolically active vitamin D_3 have been found in a number of plants and these plants can be useful in reducing problems concerning egg quality associated with age, under adverse conditions.

In many plants known to have antimicrobial properties, elevated levels of chemicals occur. It has been found that these chemicals, which are naturally produced by plants to combat their natural pathogens, are effective against pathogenic bacterial species commonly found in animals and humans. SPE1 is a powerful, safe new plant extract which has been identified and patented by the Belgian company Vitamex. *In vitro* tests have shown the active ingredient to be used as an alternative for antibiotic growth promoters and it contains medium chain fatty acids (MCFA) of chain lengths C6, C8, C10. SPE1 is well defined in relation to its composition, has very predictable and reproducible zootechnical effects and so far no mechanism by which microbial resistance can develop,

has been described. Villous morphology of the animal's GIT is improved and better performances are also obtained (Bruggeman *et al*., 2002).

The potential benefits of using phytogenics in livestock nutrition are increased feed intake, growth performance and performance parameters, stimulation of digestion, reduced incidence of diarrhoea, and improved reproductive parameters and feed efficiency, leading to higher profitability. The reduction in performance related to inappetance is prevented by phytogenic feed additives, due to their mode of action. Phytogenic feed additives supports digestion by having a positive influence on saliva and gastric acid production. This increases appetite as well as feed intake. Acidification of the stomach through gastric acid causes a low pH which is important for pepsin, the protein digestive enzyme, and the inhibition of pathogen growth which prefers a higher pH (Jones, 2002). There are a few phytogenic products available on the market, but, due to a large variation in chemical composition they vary greatly in their efficiency and dosage rate.

Digestive secretion, stimulation of the immune system, promotion of feed intake and acting as an antibacterial, antiviral, coccidiostat, anti-inflammatory, anthelmintic and antioxidant agents are reported beneficial effects of essential oils, herbs and botanicals used in animal feed. These additives may contain unacceptable levels of heavy metals, pesticides and other contaminants which make the approval of these additives very difficult to obtain and the activity of relevant compounds in these additives is not always possible to measure. Differences in geographical growing areas, storage conditions, season, extraction method and harvesting times are reasons why there is variation in the activity of there additives (Durrans, 2005).

Herbal medicines are also being investigated as an alternative to antibiotics for the optimisation of GIT health as some of their effects may be anti-fungal and anti-oxidant (Verstegen and Williams, 2002).

2.3.9 Other alternatives

Important natural growth promoters such as yeast products can also be used as alternatives to antibiotics. It was first reported by Eckles and Williams (1925) that *Saccharomyces cerevisiae* could be used as a growth promoter for ruminants. World wide commercial yeast products are used in animal production specifically for animal feeding. Gao *et al.* (2008) found that the growth performance of broilers were improved by yeast cultures and that the intestinal mucosal morphology of broilers, their immune functions, calcium and phosphate digestibility are affected by yeast cultures

The use of conjugated linoleic acid is another component being investigated. Differences in GIT metabolism, particularly in relation to the effect of linoleic acids on the immune response of animals, may be due to the natural occurring positional and geometric isomers of linoleic acid. This may have important implications for GIT health improvement, if shown to be valid (Verstegen and Williams, 2002).

2.2 SALMONELLA

2.2.1 Description

Salmonella is classified as a gram-negative facultative rod-shaped bacterium in the same proteobacterial family as *Escherichia coli*, the family Enterobacteriaceae, trivially known as “enteric” bacteria (Todar, 2005).

2.2.2 Antigenic structure

The genus Salmonella has three kinds of major antigens with diagnostic, identifying applications: Somatic (O) or cell wall antigens, surface antigens and flagellar (H) antigens (Todar, 2005). The O antigens are determined by specific sugar sequences on the cell surface and occur on the surface of the outer membrane. Overlying the O antigen is the superficial Vi antigen, which is only present in a few serovars with *Salmonella typhimurium* being the most important. A useful epidemiological tool as the H antigen can be used to determine the spread of infection and its source (Giannella, 1996).

The epitopes found on repeated hydrophilic linear or branched subunits of carbohydrates, are the O antigens and can be recognised by agglutination with absorbed antiserum. The epitopes are shared between different serotypes and single or more than one epitopes may be present. Through transduction (phage-mediated) or conjunction (plasmid) the extraneous genes from other *Salmonella* or *Citrobacter* strains may be obtained by the epitopes which might change them. This explains why epitopes are not constant for any serotype. Through mutation in the lipopolysaccharide (LPS) genes and loss of the O antigen, *Salmonella* strains can become resistant to phage activity, because O antigens also act as receptors for various bacteriophages (Barrow, 2000).

A complex LPS structure is found in the cell envelope of Salmonellae and is liberated during culture and on cell lysis. The lipopolysaccharide may be important in determining virulence of the organism or function as an endotoxin. There are three components that form this macromolecular endotoxin complex, and they are the outer O-polysaccharide coat, the middle portion (R core) and the inner lipid A coat.

There are a few reasons why lipopolysaccharide structure is so important. Firstly, the O antigen specificity is caused by the repeating sugar unit’s nature in the outer O-polysaccharide chains. The virulence of the organism may also be determined by the O antigen specificity. Secondly, infection by a variety of Gram-negative bacteria sharing a core structure can be prevented by protective antibodies directed against the R core and

may also moderate the lethal effects of these bacteria. Thirdly, in the pathogenesis of many clinical manifestations of Gram-negative infections, an important role may be played by the endotoxin component of the cell wall. Endotoxins have a few functions; they alter lymphatic function, depress myocardial function cause fever and activate the serum complement, kinin, and clotting systems. Many of the manifestations of septic shock that can occur in systemic infections can also be caused by circulating endotoxin (Giannella, 1996).

Many serotypes posess flagellar (H) antigens, with two flagella antigen genes are present and only one of which is expressed at any one time (phase 1 or phase 2). The organisms tend to change from one phase to another and are regulated by the spontaneous inversion of a DNA segment that controls expression. The expression of either gene may be associated with several epitopes (Barrow, 2000). Flagellar antigens are heat-labile proteins. A characteristic pattern of agglutination is formed when Salmonella cells are mixed with flagella-specific antisera which leads to bacteria being loosely attached to each other by their flagella and can be dissociated by shaking. Bacteria with corresponding H antigens can also be immobilized through antiflagellar antibodies (Todar, 2005).

The flagellar (designated H antigens) and lipopolysaccharide (LPS) (designated O antigens) are the major components of the bacterial surface used in epidemiological investigations. Genera of the Enterobacteriaceae are shared with antigenic epitopes and cross-reactions with closely related taxa such as Citrobacter may occur (Barrow, 2000).

2.2.3 Habitats

The intestinal tract of humans and animals is the principal habitat of the Salmonella. *Salmonella* serovars can be ubiquitous, have unknown habitats or can be found predominantly in one particular host. Grave diseases associated with bloodstream invasion are caused by Typhi and Paratyphi A which are strictly human serovars. Salmonellosis is transmitted through faecal contamination of water or food. Diverse clinical symptoms, ranging from asymptomatic infection to serious typhoid-like syndromes in infants or highly susceptible animals are caused by ubiquitous (non-host-adapted) *Salmonella* serovars such as *Salmonella* typhimurium. Foodborne toxic infections in human adults are mostly caused by ubiquitous *Salmonella* organisms (Todar, 2005).

Human or animal excretion disseminates Salmonellae into soil, water, soil and sometimes plants used as food, all of which forms the natural environment. Salmonella

can be excreted when humans and animals, wild or domesticated, are clinically diseased or after having had salmonellosis and remain carriers. If the environmental conditions of pH, temperature and humidity are favourable, Salmonella can survive in water for several weeks and in soil for several years. It does not seem as if *Salmonella* organisms can multiply significantly in their natural environment out of the digestive tract (Todar, 2005).

2.2.4 EPIDEMIOLOGY

The complexities of the 'paratyphoid' serotype's epidemiology is caused by the serotypes being shed in large numbers from the alimentary tract without even showing signs of disease as well as not being host specific. The major sources of infection can be limited to poultry, feed and the environment.

2.2.4.1 Poultry as a source of infection

Very high numbers of Salmonella are orally ingested by the chicks into the gut immediately after hatching and extensive shedding in the faeces follows. It is unknown what the exact course of events is during the incubation of infected eggs. Infection in the incubator may be caused by contaminated eggshell and ingestion of contaminated fluff or other dust by the hen, which may result in extensive contamination of the hatchery which could continue for a long time. Extensive cross-contamination of other birds in the same house can occur with infections lasting for the lifetime of a broiler. New infections can also be introduced by replacing birds and can be transfer between houses on the same site.

External temperature, use of antibiotics and growth promoters, and accompanying infections by other agents such as *Eimeria* and infectious bursal disease, both which aggravate Salmonella infection, are external factors that can affect the duration of faecal excretion (Barrow, 2000). The "all in – all out" system which has been the general adoption of chicken rearing in batches has considerably reduced the transfer of infection from crop to crop. Stress in birds can be reduced by improving the design of lairages on farms and transport in uncrowded, correctly ventilated vehicles, which are important in reducing the spread of infection (British medical journal, 1977).

2.2.4.2 Feed as a source of infection

The animal protein component is one of the major sources of infection for poultry feed. The faecal material from wild birds and reptiles can contaminate feed sources which are air dried through the sun in countries of production, especially dried fish meal which has traditionally been an important protein component. Carbohydrates, mineral components and even proteins from other sources may also be contaminated. Feed can also get contaminated at the time of milling or by inappropriate storage and rodents (Barrow, 2000).

The viability of *Salmonella typhimurium* in feed and litter contamination was studied by Nashed (1986). He found that at 37ºC the organism remained viable in the feed for up to 6 weeks and in litter for up to 2 weeks. At room temperature the organism remained viable for up to 71 weeks in the feed and 78 weeks in the litter and at 7°C the organism remained viable for up to 79 weeks in the feed and litter.

The most efficient methods of reducing contamination in feedstuffs are heat pelleting and/or steam pelleting, however the number of Salmonella in the feeds are reduced but not eliminated. The manufacturing of the feed has to be controlled from its animal and marine protein constituents. Special attention should be paid to feed for breeding flocks, as infection may be introduced into poultry by birds hatched from lightly contaminated eggs (British medical journal, 1977).

2.2.4.3 Environment as a source of infection

Salmonella are everywhere in the environment and the organism seems to have adapted to changes in its environment. Salmonella has survived antibacterial drugs, chemical treatments and improvements in sanitation practices (Shackelford, 1988).

The environment contains many sources of infection for poultry including rodents, housing, wild birds and cats which may have access to the water sources, animal attendants and housing. The fact that poultry rearing occurs in different climates must be considered when discussing housing. In temperate climates it may be easier to achieve restricted vermin access when birds are totally enclosed. It is a common way in hotter climates to enhance ventilation through open-sided housing, making vermin control difficult to achieve. Rodents can cause infection either by becoming infected from stock in a house and passing that infection to the next batch of birds in the house or by introducing new strains (Barrow, 2000).

Other potential sources of infection are mammals, like domestic cats which are kept close to a poultry house or on site to reduce the number of rodents. Clothing, especially footwear, worn by personnel is a way in which the *Salmonella* organism can be transferred between sites or houses. When infection is introduced, it can spread rapidly within a flock and cause heavy contamination in the house. Depending on the construction type and age, it may be difficult to clean the contaminated house after depopulation, which may lead to contamination of the next batch of birds (Barrow, 2000).

2.2.4.4 Transmission to humans

The major mode of transmission for non-typhoidal salmonellosis is contaminated food, because salmonellosis has an enormous animal reservoir. These organisms can also be harboured by cows, chickens, pigs, turkeys, wildlife and other domestic animals. Animal products are the main vehicle of transmission, because of Salmonellae's ability to survive in meat and animal products that are not thoroughly cooked (Giannella, 1996).

These organisms lack a specific animal reservoir, which is why the epidemiology of typhoid fever and other enteric fevers involves person to person spread. The major mode of spread is through contaminated human faeces, with contaminated water the usual vehicle. In some cases contaminated food might also be a vehicle. Powerful epidemiologic tools such as bacterial phage lysotyping of Salmonella isolates and plasmid DNA fingerprinting can be used to study salmonellosis outbreaks and tracing the spread of the organism in the environment (Giannella, 1996).

The contamination of poultry skin and feathers may be the cause of faecal shedding. Extensive cross-contamination can occur when birds are kept in close proximity of each other in crates and stress can lead to increased shedding during depopulation and transport to slaughter premises. Birds can also be contaminated through the crates during transportation. Carcass contamination levels of 50% to 100% in retail outlets can be caused by infection levels of less than 5% in a flock. Infection can spread through plucking machines, faecal contamination of scalding tanks and ruptured intestines during evisceration. Infection can also be reintroduced to previously uninfected carcasses through pooling of giblets (Barrow, 2000).

The economics of slaughter dictates that only limited measures can be taken, although considerable effort is made to reduce spread by chemical treatment and water additives. The best approach is to ensure that Salmonella-free birds are slaughtered before the infected batches. Greater frequencies of infection can be caused by pooling

material such as liquid egg and minced meat. Another possible source of infection are table eggs, although the levels of infection are low and below the incidence of contaminated carcasses. A contamination rate ranging from 5-7% is attributed to the shell and up to 0.3% of the egg contents (Barrow, 2000).

2.2.5 SALMONELLOSIS IN POULTRY

Salmonellosis in animals could result in serious economic losses to farmers and food producers. *Salmonella* serotypes that have been introduced in animal feed can be excreted by fowls and pigs without becoming ill. Healthy animals which contain salmonellas in their gut may contaminate meat during slaughter and dressing. Animals which excrete *Salmonella* serotypes without showing any ill symptoms would have to be eliminated in order to control salmonellosis in humans (British medical journal, 1977).

The existence of human and animal carrier states, its large and varied animal reservoir and the lack of a concerted nationwide program to control Salmonella, causes salmonellosis to be a major public health problem (Giannella, 1996). A common vehicle in the transmission of salmonellosis is poultry. Methods through which chickens can be colonised with Salmonella are through cross-contamination in the brooding houses, when chickens are fed contaminated feed, and during slaughtering and processing. Young chickens are more susceptible to Salmonella colonisation than older birds (Hinton *et al*., 1990).

The serotype is related to the capacity of *Salmonella enterica* to produce disease in poultry. Clinical salmonellosis in poultry is well known to be produced by serotypes such as typhimurium and enteritidis.

It is true for chicks and poults that mortality rates can vary from less than 10% to over 80% in severe outbreaks. Mortality and morbidity is also strain dependant under experimental conditions and strain virulence can vary widely from 0% to almost 100%, even within serotypes. However, extreme levels of clinical manifestation will seldom be experienced in the field because of a rapid rise in resistance during the first two days of life and the fact that the infection levels are never 100% within the first day (Barrow, 2000).

It is generally considered that the transmission of infection occurs orally and rapid tissue invasion occurring after massive bacterial multiplication in the gut. The accumulation of faecal material around the vent is an indication of the presence of enteritis and death following 4-10 days after infection. Different degrees of stunting will be shown by convalescent birds for a few weeks afterwards (Barrow, 2000).

Once colonised in one broiler, the organism can be shared with other broilers internally and externally. The organism can be transferred from one broiler's excrement to at least the feathers of other broilers being transported in the same container. Poultry that have paratyphoid (a disease of young and old chickens), caused by *Salmonella enteritidis* and *Salmonella typhimurium*, would show signs of profuse watery diarrhoea and dehydration in acute cases, pasting of vent, laboured breathing and huddling together (Anjum, 1997).

Organisms multiply after invasion in the liver and spleen and spread to other organs which cause a systemic infection. General malaise follows and develops into a combination of anorexia and dehydration which ends up being the cause of death (Barrow *et al*, 1987). Severe enteritis, focal necrotic lesions in the mucosa of the small intestine, spleen and liver conjestion, kidneys enlargement and conjestion, cheesy cecal cores, peri-hepatitis, peri-corditis, co-agulation of yolk material in the yolk sac, air-saculitis, purulent arthritis and cell-death may occur as pathological changes and lesions (Saif, 2003). In the incidence of clinical disease, the host's genetic background may be important, as with field outbreaks of fowl typhoid. Between different inbred lines under experimental conditions, the mortality produced by a virulent strain may vary from 25-100% (Barrow, 2000).

It is very difficult to obtain the estimates of cost that paratyphoid salmonellosis cause to the poultry industry. Direct damage may be because of clinical infection causing losses to newly hatched chicks. However, these events are not economically significant and are relatively rare. The public health impact of poultry-derived human infection may cause indirect effects, which can be divided into costs associated with the losses of earnings and productivity, and the treatment of human infection. National costs, such as compensation, the cost of insurance and replacement, associated with eradication have occurred in several governments, including those of Sweden, the UK and the Netherlands (Barrow, 2000).

2.2.6 PATHOGENICITY IN POULTRY

A wide variety of infections in poultry are produced by strains of paratyphoid Salmonella, but depends on portal of entry, bacterial serotype and strain, genetic background of the host and age of the bird. In physiologically and immunologically healthy adult chickens, the paratyphoid serotypes are unable to produce severe systemic disease, unlike the 'typhoid" serotype such as *S. typhi* in humans and *S. gallinarum* in chickens. Clinical diseases are normally only produced in chickens or turkey poults that are infected

within a few hours of hatching. The capacity to produce systemic disease in newly hatched chicks varies among strains of *Salmonella typhimurium*, with mortalities ranging from 0% to 100% (Barrow, 2000).

Salmonella must undergo an infectious disease cycle to invade the host, which consists of pathogen entry, establishment and multiplication, avoidance of host defences, damage and exit. Salmonella can easily gain access to the intestinal system of the host and colonise it, due to the prevalence of Salmonella contamination of different food products (Ricke, 2005). Massive multiplication of bacteria in the gut follows quickly after infection of newly hatched chicks by the oral route.

The initial contact of Salmonella to the epithelium of the host's (humans/chickens) gastrointestinal system is the clinical beginning of a Salmonella infection. Very high numbers of bacteria reaches the gut and extensive shedding can occur in the faeces. Adverse conditions such as low pH in the GIT and antimicrobial or physical barriers had to be overcome by the Salmonella by the time it reaches the intestines. Colonisation of the intestine through attachment is the next challenge that the invading bacteria must face. The Peyer's patches in the distal ileum are the preferred entry point for Salmonella (Ricke *et al*, 2005).

Salmonella relies on other mechanisms for survival once they have been taken up by the cell, and has evolved ways to avoid targeting by the phagosome-lysosome fusion pathways. An acidic pH is required for *Salmonella typhimurium* to induce replication and survival within the cells. Salmonella then resides in the vacuole of phagocytic and non-phagocytic cells. The presence of lysosomal glycoprotein and removal of the surface marker assists with changes to the vacuole as soon as Salmonella enters. Subsequent neutrophil accretion (heterophils in birds) is then caused by the Type III secretion system as well as fluid accretion in the ileum. Necrosis of the surrounding tissue and diarrhoea are caused by the neutrophil addition, which brings about the symptoms of disease (Ricke *et al*, 2005).

Accumulation of food and fluid around the vent indicating gut dysfunction characterises the disruption of intestinal function and is not observed after parenteral administration of the bacteria (Barrow, 2000).

It is not required to use the virulence plasmid of *S. enteritidis* or *S. typhimurium* for virulence in the young chick. The genetic lineage of the bird can cause variation in the severity of the disease produced. Whether the bacteria were inoculated orally or parenterally, the oral inoculation of a virulent strain of *Salmonella typhimurium* produced

extreme variation in mortality. The trait is inherited in a mendelian fashion, and is the result of a single gene *Sal*1, which is neither associated with the major histocompatibility complex nor sex-linked (Barrow, 2000).

Unlike serotypes such as *S. typhi* and *S. gallinarum*, paratyphoid serotypes of Salmonella are not host-adapted or host-specific. *Salmonella typhimurium* and *S. enteritidis* generally do not produce typical typhoid infections in adult poultry, but do in adult mice. In the absence of disease, the paratyphoid Salmonella serotypes nevertheless colonise the alimentary tract, although adult birds are relatively resistant to systemic multiplication by serotypes. Faecal excretion of large numbers of *Salmonella* bacteria for many weeks is the result of day-old chicks being orally infected.

Adult birds have a complex gut flora which inhibits pathogen colonisation and is the reason why infected adult birds tend to show a reduction of *Salmonella* bacteria (Barrow, 2000).

2.2.7 SALMONELLOSIS IN HUMANS

Salmonellosis is an infection caused by *Salmonella* bacteria and three forms of salmonellosis have been recognised: 1) Enteric fever, 2) Gastroentiritis, 3) Septicemia. When the patient is not experiencing intestinal symptoms and the bacteria can not be isolated from the faecal specimens, then Salmonella infection is in the septicemic form and can be an intermediate stage of infection. The resistance of the patient and the Salmonella isolate virulence determines the severity of the infection. It also determines whether the infection remains localised in the intestine or disseminates to the bloodstream (Giannella, 1996).

The dose of bacteria will determine the incubation period for Salmonella gastroenteritis (food poisoning). About 6 to 48 hours after ingestion of contaminated food or water, symptoms usually begin to show in the form of abdominal pain, nausea and vomiting. Diarrhoea is the cardinal manifestation with headaches and myalgia being common symptoms. Fever (38°C to 39°C) and chills are also common and usually takes 2 to 7 days for the fever to reside (Giannella, 1996).

A severe form of Salmonella is Enteric fever. Any species of Salmonella may cause this type of disease, but typhoid fever is the best studied Enteric fever, which is caused by *S. typhi*. An incubation period of 10 to 14 days is needed for the symptoms to start showing. Gastroenteritis may precede enteric fever, but resolves before the onset of systemic disease. Constipation, fever, myalgia, anorexia and headaches are the non-

specific symptoms of enteric fever. If antibiotics are not promptly administered, severe infections such as Enteric fevers can occur which may be fatal (Giannella, 1996).

The feeding of animal excrement to livestock is a contributing factor to this foodborne illness. Heat processing kills bacterial pathogens, but it is an expensive process. Farmers use deep-stacking and ensiling to process animal waste, but 43°C to 60°C are the maximal temperatures achieved in stacked poultry litter. These temperatures are below the inactivation of pathogenic Salmonella and *E. coli* spp. and far below the United States Department of Agriculture's (USDA) recommended cooking temperature of 71°C to 77°C for potentially manure tainted meat products (Haapapuro *et al*, 1997).

The incidence of food poisoning will diminish if clinical Salmonella and Salmonella causing no symptoms of infection in poultry and pigs can be reduced. Salmonellosis accounts for about 80% of all food poisoning in Britain in which the cause is determined. Salmonellosis in humans can be prevented by thorough cooking, correct storage and, if unavoidable, adequate reheating of food, together with good hygiene in the kitchen (Britain medical journal, 1977).

2.2.8 PATHOGENESIS IN HUMANS

Enteric fevers, gastroenteritis, septicaemia, focal infections and an asymptomatic carrier state are some of the symptoms which Salmonellosis consists of, with particular serovars showing a strong propensity to produce a particular disease. With the ingestion of contaminated food, most non-typhoidal Salmonellae enter the body. The ability to replicate intracellular, the ability to invade cells, the elaboration of toxins and a complete lipopolysaccharide coat are a variation of attributes known as virulence factors needed by Salmonella to be fully pathogenic (Giannella, 1996).

The organisms colonise the ileum and colon after ingestion; they then invade the intestinal epithelium and reproduce within the epithelium and lymphoid follicles. The mechanism by which Salmonellae invade the epithelium is by binding to specific receptors on the epithelial cell surface (See Figure 2.1 below). Pinocytosis of the organism is stimulated through the organism's invasion which induces the enterocyte membrane to undergo "ruffling". Invasion involves increases in cellular inositol phosphate and calcium and is dependant on the cell cytoskeleton to rearrange multiple genes in chromosomes and plasmids which are involved in attachment and invasion which are under genetic control. The organisms multiply intracellular after invasion of the epithelium, they then

spread to mesenteric lymph nodes and via the systemic circulation, spread through out the body, where they are taken up by the reticuloendothelial cells (Giannella, 1996).

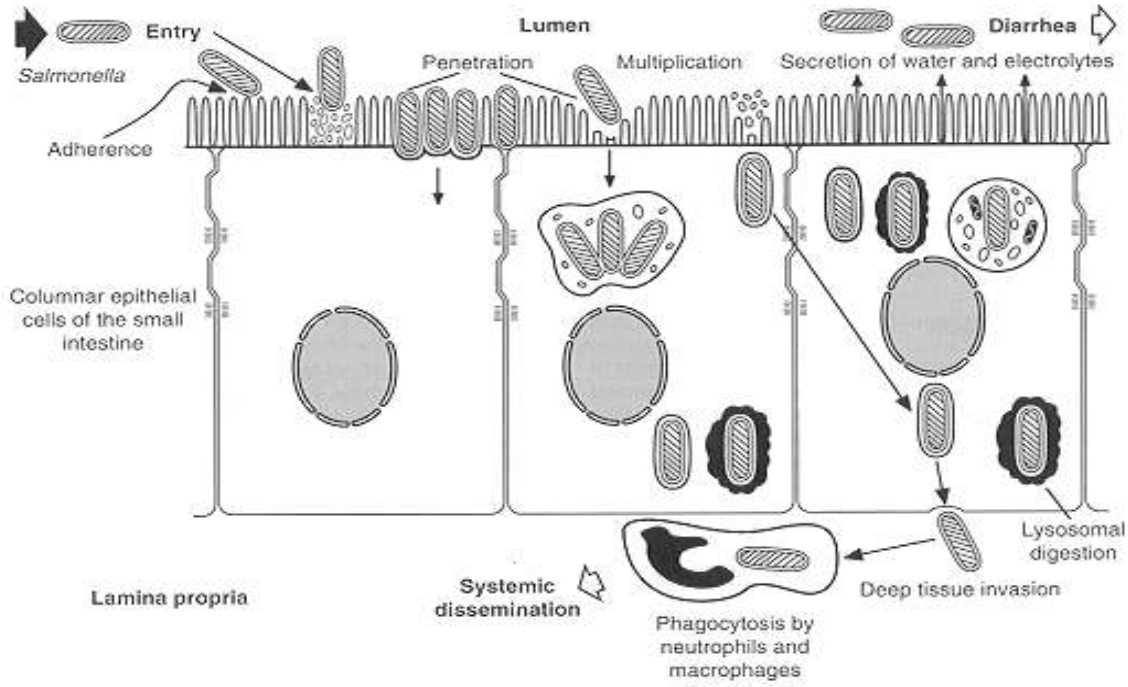

Figure 2.1: Invasion of intestinal mucosa by Salmonella (Giannella, 1996)

The organism is confined and controlled by the reticuloendothelial system. Some organisms may infect the gallbladder, liver, meninges, spleen, bones and other organs, depending on the serotype and effectiveness of the host defences against the serotype. Gastroenteritis is the most common human Salmonella infection and remains confined to the intestines with most of the serovars being killed in extraintestinal sites. An acute inflammatory response is induced by most Salmonellae after invasion of the intestines, which can cause ulceration. Protein synthesis can be inhibited through the elaboration of cytotoxins by Salmonella. Various proinflammatory cytokines are synthesized and released by epithelial cells when the mucosa is invaded. These cytokines can also be responsible for damaging the intestines as well as evoke an acute inflammatory response (Giannella, 1996).

Common symptoms of inflammation such as diarrhoea, fever, leukocytosis, abdominal pain and chills occur as a result of the intestinal inflammatory reaction. Mucus, polymorphonuclear leukocytes and blood may also occur in the stools. The appearance of an acute inflammatory reaction is associated with strains of Salmonella that penetrate the intestinal mucosa. The small and large intestines secretes fluids and electrolytes, which is caused by diarrhoea. Salmonella do not escape the phagosome, but do penetrate the

intestinal epithelial cells. Therefore, minimal intracellular spread and ulceration of the epithelium will occur. From the basal side of epithelial cells, the Salmonella escape into the lamina propria. An increase in cyclic AMP induced secretion is follows, which is caused by the invasion of the intestinal mucosa followed by activation of mucosal adenylate cyclase. Intestinal secretion may be stimulated by one or more enterotoxins-like substances which are elaborated by *Salmonella* strains (Giannella, 1996).

2.2.9 HOST DEFENCES

The resistance of intestinal colonisation and invasion of Salmonella is an important role executed by host defences. Salmonellae can be killed by normal gastric acid which has a pH of less than 3.5. In the stomachs of healthy individuals, there is a reduction of Salmonella ingested which results in fewer or no organisms entering the intestines. By sweeping ingested Salmonella through quickly, the bowel is protected by normal small intestine motility. Through anaerobes, normal intestinal microflora protects against Salmonella and SCFA are liberated which are thought to be toxic to Salmonellae. The host is therefore more susceptible to salmonellosis when antibiotics alter the anaerobic intestinal flora.

The intestine is also protected against Salmonellae by secretary or mucosal antibodies without which the host becomes more susceptible to salmonellosis. There are certain factors that render the host more susceptible to salmonellosis and they include: i) impaired systemic immunity (eg. Carcinomatosis, leukemias, lymphomas, diabetes mellitus and acquired immunodeficiency syndrome (AIDS)), ii) Intestine (eg. antibiotic administration, gastrointestinal surgery), iii) Hemolytic anemias (eg. sickle cell anemia and other hemoglobinopathies) and iv) the stomach and examples of specific conditions are Achlorhydria and gastric surgery (Giannella, 1996).

2.2.10 BIOLOGICAL PREVENTION AND CONTROL METHODS

2.2.10.1 Competitive exclusion

Heavy excretion of large numbers of the inoculated bacteria for a long period of time is caused by experimental inoculation of newly hatched chickens with *Salmonella* organisms. The excretion of smaller numbers of bacteria for shorter periods occurs when adult birds are inoculated. The adult bird has a high degree of protection conferred by the presence in the alimentary tract of a complex intestinal microflora and is the reason for this difference. Increased excretion of Salmonella results when this flora is disrupted. Within

24 hours, young birds will have the same resistance to colonisation as adult birds, if the newly hatched chicks are treated with the gut flora of adult birds. The high level of hygiene in the hatchery is largely the cause of the newly hatched chick and poult having poor resistance. Several weeks are required for the young bird to gain this flora naturally. The resistance of young poultry to Salmonella colonisation has been increased by the application of competitive exclusion. Flora in broth cultures, caecal contents or faeces suspensions are equally effective (Barrow, 2000).

2.2.10.2 Vaccination

The development of vaccines against non-host specific serotypes for the use in poultry has been exclusively empirical. A reason for this is that information regarding Salmonella serotypes associated with human food poisoning, especially their colonisation and immunity is missing. The age of the bird at the time of vaccination and challenge, the strain of Salmonella used for challenge, the route and bacterial dose used for inoculation can cause the outcome of experimental infection to vary greatly. Variable protection is found with different types of non-living vaccines which have been used in the field and experimentally generating an immune response (Barrow, 2000).

In very young chicks, live weaker vaccines have the ability to colonise the alimentary tract against Salmonella. The organisms multiply extensively when vaccines are administered to newly hatched chicks, because of the absence of the normal complex microbial flora found in adult birds are absent. The colonisation by other *Salmonella* strains inoculated within the following few hours is therefore prevented (Barrow, 2000).

Oral administration via drinking water, food or spray would be the ideal route. Oral vaccination may include the use of an invasive strain to stimulate maximum immunity, although the ideal vaccine should be avirulent for chickens, because immunogenicity may be correlated with invasiveness. The vaccine should not affect productivity or produce disease in the progeny, while protection should last as long as possible. An integral part of Europe's control programme is the control in breeders and layers where protection is required for many months. Several days are required after vaccination for a protective immunity to develop and vaccinations should therefore be compatible with the use of competitive exclusion. By using a live vaccine strain that shows the colonisation-blocking effect (a form of competitive exclusion that occurs between closely related enteric bacteria) in newly hatched birds could overcome this delay of immunity development. In the early

life of the young chick, a degree of protection can be produced against salmonellosis by means of vaccination with competitive exclusion products (Barrow, 2000).

2.2.10.3 Probiotics

Detail of this section was already discussed in Section 2.2. Probiotics are known as non-viable and viable products which produces a beneficial effect in the animal when the existing gut flora is altered after oral administration. Probiotics are also known as bacteria which have originated in the gut or derived from elsewhere. When the composition of the intestinal flora is altered by displacing the existing micro-organisms which are harmful, with beneficial micro organisms in terms of nutrition or resistance to pathogens, confers to this beneficial effect. The idea of probiotics has become intensified in countries where the use of antibiotics has been limited (Barrow, 2000).

2.2.10.4 Hygiene and Management

The importance of hygiene and management are in poultry rearing to avoid Salmonella and other pathogenic bacteria contamination can not be emphasised enough. It has been demonstrated by large breeding companies and research institutes that it is possible to rear birds totally free of zoonotic pathogens. A considerable degree of success can be obtained by following a number of rules. Several codes of practice exist in the UK, which are appropriate for different areas of production, including layers, broilers, hatcheries and breeders (Barrow, 2000).

Another limiting factor is the quality of the house in terms of ambient temperature and age since older housing is more difficult to clean and obvious additional potential problems with environmental contamination implies with open-sided housing. Entry restriction, appropriate clothing, washing and cleaning of staff clothing are important management schemes for staff and entry of personnel. It is also important to monitor Salmonella carriage by staff (Barrow, 2000).

Cross contamination in the abattoir has been reduced by abolishing wiping cloths, reducing floor dressing and by improving the design for washing and cleaning. Areas in large abattoirs and staff associated with gut contents can be separated from other sections, but this is difficult in small abattoirs. Cross-contamination has caused widespread outbreaks of salmonellosis, therefore methods of handling and chilling the poultry in the processing plant must be designed to minimise cross-contamination (British medical journal, 1977).

A recognised principal factor is the guidelines for housing and management that are available and contribute to reducing the spread of infections (Barrow, 2000). Possibilities for the reduction in microbial loads on carcasses are spray scalding, provision of hand wash nozzles for manual work stations, automation of the eviscerating process, immersion chilling, and cooling of carcasses packed in plastic bags. Normal hygiene precautions will protect the meat workers from infection and also protect the meat from contamination from the workers (Corry and Hinton, 1997).

Swabs should be taken on a regular basis to monitor the environment and poultry for Salmonella. Disinfection and sanitation of housing is important and normally the houses are disinfected by a wash, dry, disinfect, dry and fog process. Recommended commercial products are available which have been tested with the standard assays for activity in the presence of organic material. The most effective of the commercial products are phenolics, especially in the presence of organic material such as feed and faeces. Products are also inactivated with old wooden material, found as structural components of older housing. Formaldehyde and gluteraldehyde are the most effective chemicals, although the toxicity of these chemicals presents problems (Barrow, 2000).

CHAPTER 3: MATERIALS AND METHODS

3.1 EXPERIMENTAL DESIGN AND TREATMENTS

Ethical approval was obtained for the trial by the AUCC (Animal Use and Care Committee), University of Pretoria, Pretoria. Day-old commercial Ross 788 broiler chicks were obtained from Eagle's Pride Hatchery (Pretoria). The chicks were sexed and vaccinated at the hatchery against New Castle Disease and Infection Bronchitis virus. The growth trial was conducted in a broiler facility on the experimental farm of the University of Pretoria, Hatfield, Pretoria. This facility is divided into two separate sections, each with its own ventilation and drinking systems and temperature control.

Twelve treatment groups (6 Salmonella-exposed groups and 6 non-exposed groups) were studied during the trial. The Salmonella-exposed groups were housed in one section and the non-exposed groups in the other section of the broiler house. Initially, a total number of 2400 chicks were placed in the two sections of the house (1200 chicks per section). The chicks were randomly assigned to 60 pens (30 pens per section), each treatment having 5 replicates with 40 chicks per replicate. The 40 chicks per pen were reduced to 30 chicks per pen on the day of Salmonella inoculation (at day 4 of age) by removing all outliers and poor quality chicks, without taking sex into consideration, to ensure minimum variation among the starting weights of the chicks. The following six treatments were repeated for both of the main groups, the one exposed to and the other not exposed to *Salmonella typhimurium* (ST) (2x3x2 design; twelve treatments in total):

- No feed additives (neither probiotics nor prebiotics added to the feed)
- Only prebiotics added to the feed (no probiotics)
- Probiotic Type 1 added to the feed (no prebiotics)
- Probiotic Type 1 and prebiotics added to the feed
- Probiotic Type 2 added to the feed (no prebiotics)
- Probiotic Type 2 and prebiotics added to the feed

Figure 3.1 illustrates a layout of the broiler house with the treatments being randomly divided between the pens. The dietary treatments were provided to the birds from day-old until termination of the trial at day 35. The same prebiotic was used (fructo-oligosaccharides; Larrem (Pty) Ltd, South Africa) for all treatment groups which received prebiotics. The Probiotic Type 1 contained spore-forming bacteria (*Brevibacillus laterosporus*; Bioworx (Edms) Bpk, South Africa) and the Probiotic Type 2 consisted of

Lactobacillus spp (Larrem (Pty) Ltd). The prebiotics and both the probiotic products were included in the diets according to manufacturer's specifications (as given in Table 3.1). The chicks from the exposed groups were orally inoculated on day 4 of age with 0.5 mL of phosphate buffered saline (PBS) containing $1x10^{10}$ colony forming units (CFU)/mL of *Salmonella typhimurium*.

FIGURE 3.1: Layout of the broiler house to demonstrate how the treatments were randomly divided between pens

Pen 1 Treatment 1	Pen 2 Treatment 4	Pen 3 Treatment 6	Pen 4 Treatment 2	Pen 5 Treatment 5	Pen 6 Treatment 1	Pen 7 Treatment 3	Pen 8 Treatment 4
	Pen 9 Treatment 2	Pen 10 Treatment 5	Pen 11 Treatment 3	Pen 12 Treatment 6	Pen 13 Treatment 2	Pen 14 Treatment 4	Pen 15 Treatment 1
	Pen 16 Treatment 6	Pen 17 Treatment 3	Pen 18 Treatment 1	Pen 19 Treatment 4	Pen 20 Treatment 5	Pen 21 Treatment 3	Pen 22 Treatment 6
Pen 23 Treatment 5	Pen 24 Treatment 3	Pen 25 Treatment 1	Pen 26 Treatment 4	Pen 27 Treatment 2	Pen 28 Treatment 6	Pen 29 Treatment 5	Pen 30 Treatment 2

TREATMENTS:
1- Non-additive
2- Prebiotic
3- Probiotic Type 1
4- Probiotic Type 1 with prebiotic
5- Probiotic Type 2
6- Probiotic Type 2 with prebiotic

3.2 HOUSING

An environmentally controlled broiler house on the experimental farm, University of Pretoria, Hatfield, was used. The house consisted of two separate sections. One section was used for the non-exposed chickens and the other section of the house for the chickens exposed to ST. Each facility had its own ventilation system and was divided into

30 pens each, equipped with its own tube feeder, bell drinker and infra-red heating lights. The pens had an area of 1.5m² and there was an open space of 50cm between pens. The house had concrete floors and shavings were used as litter material. The temperature and ventilation of the facilities were closely monitored and regulated through the use of infrared heating lamps and electrical fans. This ensured a similar environment for both sections of the house. A lighting programme of 23-hours light and 1-hour darkness was employed. All environmental and management factors were the same for the two sections of the house and standard broiler management procedures were followed. The broiler facilities were thoroughly cleaned and disinfected before commencement of each trial.

The broiler houses and all the equipment inside were cleaned with Vet One Plus and disinfected with Vet GL 20 (Immunovet Services, Johannesburg). Foot dips, filled with Vet Fluid-O (Immunovet services, Johannesburg), were placed at all entrances of the broiler houses. Formaldehyde in combination with potassium permanganate was used to fumigate bedding once placed inside the broiler houses. For fumigation 120mL formalin was mixed with 60g of potassium permanganate per 2.83 m³.

3.3 FEED

The birds received a broiler starter diet from day 1 to 7, a broiler grower diet from day 8 to 28 and a broiler finisher diet from day 29 to 35. The composition of the diets is shown in Table 3.2, Table 3.3 and Table 3.4, respectively. All the raw materials for the rations were obtained from Meadow Feeds, Delmas. Feed formulation software (Format International, UK) was used to formulate the diets according to the nutrient specifications prescribed by Ross Poultry Breeders. After the raw materials had been weighed, the bags were irradiated with 5 kGy (Isotron South Africa, Kempton Park) to prevent Salmonella exposure of chicks via the feed. After irradiation, the feed was thoroughly mixed with a disinfected feed mixer at the Poultry Section of the Agriculture Research Council (ARC), Irene and stored in a cool, dry and disinfected area until it was used. All feed were fed as mash.

The basal feed (kg) was divided into 6 parts, each representing a treatment. The probiotics and prebiotic treatments were added to the feed according to recommended dosages (see Table 3.1)

Table 3.1 Inclusion rates of pro- and prebiotics according to manufacturer's specifications

	Treatments			
Diets	**Prebiotic (g/kg)**	**Probiotic type 1 (g/kg)**	**Probiotic type 2 (g/kg)**	**Probiotic type 2 combined with a prebiotic (g/kg)**
Starter	1.50	0.86	3.00	2.50
Grower	1.00	0.26	2.50	2.00
Finisher	1.00	0.14	2.50	2.00

Table 3.2 Raw material composition and nutrient levels of the starter diet

Ingredient	**% Inclusion**
Yellow maize	59.6
Soya oil cake 47%	26.4
Local fish meal 65%	11.0
Monocalcium phosphate	1.29
Limestone 36%	1.07
Premix	0.50
Salt	0.15
Calculated nutrient levels	**g/kg**
ME (MJ/kg)	12.7
Crude protein	243
Lysine	14.7
Methionine	4.80
Calcium	11.0
Available phosphorous	5.10
Sodium	1.66
Fat	38.8
Fibre	26.9

Table 3.3 Raw material composition and nutrient levels of the grower diet

Ingredient	**% Inclusion**
Yellow maize	64.83
Soya oil cake 47%	15.72
Local fish meal 65%	10.0
Extruded full fat soya	6.74
Monocalcium phosphate	1.19
Limestone 36%	0.84
Premix	0.50
Salt	0.19
Calculated nutrient levels	**g/kg**
ME (MJ/kg)	13.22
Crude protein	215
Lysine	12.7
Methionine	4.37
Calcium	9.50
Available phosphorous	4.70
Sodium	1.70
Fat	50.0
Fibre	27.31

Table 3.4 Raw material composition and nutrient levels of the finisher diet

Ingredient	**% Inclusion**
Yellow maize	71.0
Soya oil cake 47%	13.55
Local fish meal 65%	8.2
Extruded full fat soya	4.5
Monocalcium phosphate	1.0
Limestone 36%	1.0
Premix	0.50
Salt	0.25
Calculated nutrient levels	**g/kg**
ME (MJ/kg)	13.33

Crude protein	190
Lysine	10.8
Methionine	3.88
Calcium	9.0
Available phosphorous	4.03
Sodium	1.78
Fat	46.43
Fibre	26.78

3.4 GENERAL HUSBANDRY AND VETERINARY CARE

All birds received fresh water and feed *ad libitum*. The bell drinkers were cleaned daily. Standard broiler management practices were followed and environmental conditions were controlled and adjusted throughout the duration of the trial. All chicks were vaccinated on day 13 of age against Gumboro disease with TAD Gumborovac (Lion Bridge Pretoria). All the chickens were closely monitored twice a day for any abnormal symptoms and behaviour. A veterinarian would have been notified of any abnormalities among the chickens during the trial.

To prevent cross-contamination between birds or contamination of the environment or staff, strict biosecurity measures were applied. The only persons that were allowed into the broiler facilities were the responsible veterinarian, principal researcher and participating student. When entering the building, researchers wore protective clothing which included over-alls, latex gloves, gumboots and facemasks. After the trial, the used gloves and facemasks were placed in a biohazard waste box for incineration. The over-alls and gumboots were kept in a room that was part of the house, but outside the area where the chicks were held. Vet Hand (Immunovet services, Johannesburg) disinfecting soap was used by the researchers to shower immediately after leaving the house. An antibiogram for the specific ST that was used during the experiment was prepared by the Department of Microbiology, Faculty of Health Sciences, University of Pretoria before the project started. This ensured that accidental infections could be treated immediately and effectively. The farm manager of the Experimental Farm, Hatfield, Mr Roelf Coertze was aware of the trial and all other farm animals were closely monitored for any signs of salmonellosis.

Bird performance in terms of growth, feed intake and feed conversion ratio was measured weekly up to 5 weeks of age, where after the trial was terminated. The

condition of the chickens were monitored twice daily by observation of abnormal behaviour or movement, breathing or ability to obtain food and water. All mortalities were recorded. On days 7, 14, 21, 28 and 35 of age, three birds per pen were sacrificed by cervical dislocation. The small intestine was divided into its different sections, i.e. the duodenum, jejunum and ileum. Each separate section of the small intestine, and also the caeca were weighed and expressed as percentage of body weight. The small intestine was also examined for intestinal lesions and stored for villous morphological measurements. The GIT and caeca were stored in Millonig's buffered formalin solution, which prevented the tissues from shrinking (Appendix A). Blood samples were taken and the aspartate amino transferase (AST), total serum protein (TSP), as well as globulin and albumin levels in the serum were determined by Clinical Pathology, Onderstepoort, and University of Pretoria.

3.5 SAMPLE ANALYSIS

3.5.1 Salmonella contamination of birds and environment

Cloacal swabs from 10 chicks per pen were tested weekly for the presence of Salmonella. Environmental swabs were also taken. The swabs were incubated in Rappaport-Vassiliadis medium (Merck, Germany) and plated onto Rambach medium (Merck, Germany) at the Department of Microbiology and Plant Pathology, University of Pretoria.

The Rambach agar was prepared as follows: 1 vial of liquid mix was added to 1000mL of distilled water and mixed by swirling until the mixture was completely dissolved. 1 vial of nutrient powder was added and mixed by swirling until it was completely suspended. The composition of the mixture consisted of peptones, sodium chloride, sodium deoxycholate, chromogenic mix, propylene glycol and agar-agar. The mixture was heated on a magnetic stirrer and was totally suspended when no visual particles stuck to the glass wall. The medium was then cooled and gently shaken from time to time, which prevented the medium from clotting. The medium was poured into petri dishes. The ready plates were opaque and pink in colour and were stored to dry and solidify before they was used for plating of the broth (Merck Microbiology Manual, 2007).

Enterobacteriaceae are able to multiply readily, due to the nutritive substrates in the Rambach agar. Rambach agar enables species of Salmonella to be differentiated unambiguously from other bacteria by means of adding propylene glycol to the culture medium. Acid is formed when Salmonella gets into contact with propylene glycol and in combination with a pH indicator; the colonies have a characteristic red colour. The

medium contains a chromogene which indicates the presence of β-galactosidase splitting, a characteristic for coliforms and will help differentiate coliforms from Salmonellae. Coliform microorganisms grow as blue-green or blue-violet colonies. Other *Enterobacteriaceae* and gram negative bacteria, such as *Proteus, Pseudomonas, Shigella, Salmonella typhimurium and Salmonella paratyphi A* grow as colourless to yellow colonies (Rambach, 1990; Gruenewald *et. al*, 1991).

The Rappaport-Vassiliadis medium, which consisted of peptone from soymeal, magnesium chloride hexahydrate, sodium chloride, diphotassium hydrogen phosphate, potassium di-hydrogen phosphate and malachite-green, was prepared as follows: 43g/L of the medium was suspended in distilled water and then dispensed into test tubes. The tubes were gently autoclaved. The broth showed a clear dark-blue colour. The cotton buds of the swabs were cut off, inserted into the broth test tubes, and then incubated for 24 hours at 43°C. The material from the resulting cultures were then streaked out onto the Rambach agar and incubated for 24 hours at 36°C (Merck Microbiology Manual, 2007). Below are some examples of what the cultures looked like after they have been incubated.

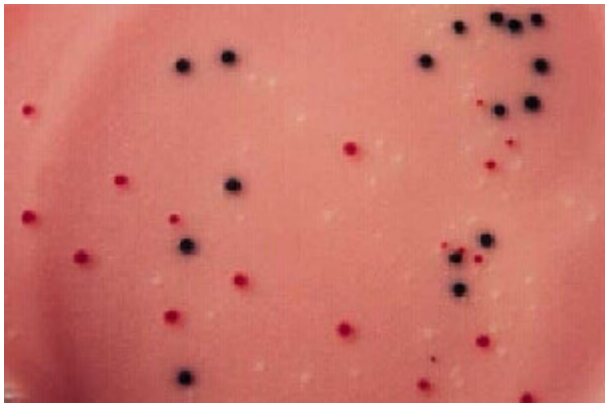

Figure 3.2*:* Colonies of *Salmonella enteritidis, E.coli* and *Proteus* plated on Merck, Rambach® Agar (Merck Microbiology Manual, 2007).

Figure 3.3: Agar plates showing the growth of *Escherichia coli (left) and Salmonella enteritidis (right)* (Merck Microbiology Manual, 2007).

The Rappaport-Vassiliadis medium is used for the selective enrichment of Salmonella with the exception of *S. typhi* and *S. paratyphi* A from foodstuffs and other material. The malachite green and magnesium chloride concentrations of this culture medium are less than those of the Salmonella Enrichment Broth, to improve the growth of Salmonella at 43°C (Vassiliadis *et al*., 1983). Peptone from soymeal is also used for the same reason (Van Schothorst *et al*., 1983; Fricker *et al*., 1985). Lowering pH to 5.2 also increases selectivity.

3.5.2 Intestinal damage

The duodenum, jejunum and ileum were spread out on filter paper immediately after sacrificing the chickens. These intestines were opened by a longitudinal incision along the antimesenteric side and examined for lesions after the removal of the contents. The extent of the haemorrhage was measured according to a scale: 0-absence, 1-slight haemorrhage, 2-moderate haemorrhage and 3-severe haemorrhage (Villegas *et al*., 2001).

3.5.3 Preparation for villous morphological measurements

The duodenum, jejunum and ileum had to undergo specific procedures, as described below, before villous morphological measurements could be taken, and were conducted at the Department of Soil Science and Plant Production, University of Pretoria.

3.5.3.1 Dehydration process

Cross sections of intestinal samples, preserved in formaldehyde and glutaraldehyde mixing fluid, went through a dehydration process first, before embedding

could commenced. Chamberlain (1932) stated: “The process of hardening and dehydration must be gradual; if the material should be transferred directly from water to absolute alcohol, hardening and dehydrating would be brought about in a very short time, but the violent osmosis would cause a ruinous contraction of the most delicate parts”. The tissue samples passed through a 30, 50, 70 volume percent of distilled water and ethanol. The tissue samples then passed through a 100 volume percent of ethanol which was repeated twice. Thereafter, a 30, 50 and 70 volume percent of paraffin solvent (xylene) in ethanol followed. The tissue samples then passed through a 100 volume percent of paraffin solvent (xylene) which was repeated twice. Paraffin wax was used for tissues in ethanol and transfer to a paraffin solvent (xylene). Shavings of paraffin wax was added to the tissue sample in solvent and stood at room temperature for one day. More shavings of paraffin wax was added the next day and continued adding new wax each day, until the shavings of wax did not dissolve anymore. The samples were then moved to a 59°C oven for 24 hours and more wax shavings were added. After melting, the mixture was poured out the next day and new wax shavings were added and put back in the oven. This step was repeated twice. The samples were then taken out of the oven and it was noted that no odour of the solvent was detectable at that point. The samples were then embedded (O’Brien and Mc Cully, 1981).

3.5.3.2 Embedding of tissue samples in paraffin wax

The wax, containing the tissue sample, had to be melted to start the embedding process. A metal mold was used and smeared with a thin layer of glycerol. The glycerol promotes the separation of the mold from the wax when it has solidified. The mold was filled three-quarters with hot wax from the oven. A thin congealed layer formed at the bottom of the mold. The tissue sample was then transferred from their container and arranged in the congealing wax. A warm spatula was used to transfer the tissue and a warm needle was used to manoeuvre the tissue into place. A plastic cassette was placed into the wax over the tissue sample. The cassette has holes through which the wax pressed to the surface. It was important to make sure that there were no air bubbles in the wax surrounding the sample and a warm needle was used to remove them. A label was placed onto the wax surface to identify the sample. The mold was then placed onto a cold plate that was -11°C and left there to solidify completely. The wax contraction freed the block easily from the metal mold (O’Brien and Mc Cully, 1981).

3.5.3.3 Preparing slides from the embedded tissue samples

The wax surrounding each sample was cut off parallel to the sample to form a square. The sample was placed in position on the microtome and the thickness adjusted to about 7-8µm. By rolling a lever on the side, the sample moved up and down over a sharp blade and started sectioning the sample. Lint of wax containing repetitions of the sample was formed with the sectioning process. A piece of the lint was cut off and placed into a 40°C water bath. This stretched out the sample and eliminated any crinkles in the lint. The slide was then prepared by adding a drop of Haupt solution onto the slide and rubbing it over the length of the slide, leaving it to dry. This solution helped fixate the sample to the slide. The slide was used to scoop the lint out of the water bath onto the length of the slide and was then put on a medium hot plate to dry. The slides could then be stored. A few drops of formalin between the slides helped fixate the samples to the slides (O'Brien and Mc Cully, 1981).

3.5.3.4 Staining the slides

Mayer's Hematoxylin and Eosin staining protocol was used. Appendix B shows the method for staining. The following procedure was used: The slides were stained in Mayer's hematoxylin solution for 15 minutes and then rinsed under running tap water. Dehydration followed, where the slides passed through 30 and 70 volume percent of ethanol, followed by two changes of absolute (100%) ethanol. The sections were then counterstained in Eosin Y solution for about 30 seconds to 1 minute. Sections were then deparafinized by going through 30 and 70 volume percent of xylene in ethanol, followed by two changes of absolute xylene. A xylene based mounting medium was used to mount the "cover glasses" onto each slide. The slides were then left to dry, before they could be stored.

Hematoxylin and Eosin stains have been used for at least a century. It works well with a variety of fixatives and displays a broad range of cytoplasmic, nuclear and extracellular matrix features. Hematoxylin can be identified as a deep blue-purple colour and stains nucleic acids by a complex, incompletely understood reaction. Eosin is pink in colour and stains proteins non-specifically. The cytoplasm and extracellular matrix have varying degrees of pink staining and nuclei are stained blue, in typical tissue (Fischer *et al*, 2008). Before hematoxylin can be used as a nuclear stain, it must be oxidized to hematein and combined with a metallic ion. That is why aluminium ammonium sulphate is one of the reagents used to make up the stain. Hematoxylin solutions are classified as

progressive or regressive based on dye concentration. Mayer's hematoxylin, which was used, is a progressive stain and has a lower dye concentration which selectively stains nuclear chromatin without staining cytoplasmic structures (Sigma-Aldrich, 2009).

3.5.3.5 Measuring the villus height and crypt depth

For the morphometric study of intestinal mucosa, Nikon ACT-1 system software was used in conjunction with a light microscope fitted with a camera, the Nikon DXM1200F, at the Department of Microscopy, University of Pretoria. The camera transferred the image from the microscope to the computer screen.

A computer mouse was used to manually delimit the height of each villus from the top point to the transition into the crypt zone (Figure 3.4). The studied intestinal mucosa area comprised of the entire mucosal thickness from the top point of the villus to the *muscularis mucosae* (Figure 3.5). The measurements were expressed in microns. The analysis was made under a 4x magnification using specimens (duodenum, jejunum and ileum) in which the villi and the crypts were perpendicular to the *muscularis mucosae*. Three replicates per treatment were used and measurements were taken from two chickens per pen for which an average villus height and crypt depth were calculated.

The following measurements were obtained:

Villus height – from the top of the villus to the villus-crypt junction (Figure 3.4)

Total mucosal thickness – from the top of the villus to the inner border of the *muscularis mucosae* (Figure 3.5)

Crypt depth – represented by the difference between the total mucosal thickness and the villus height

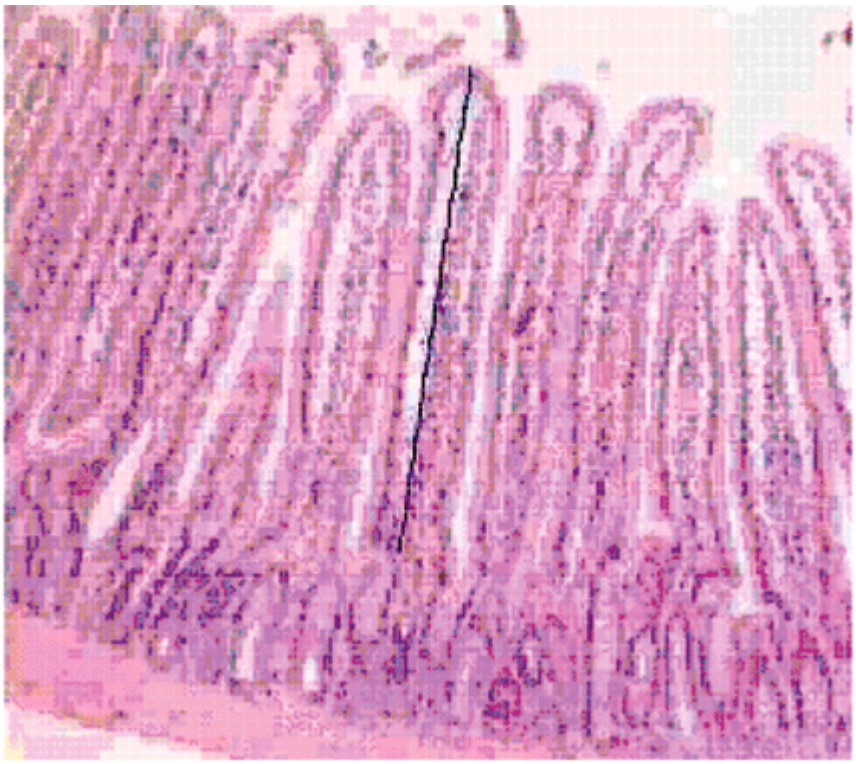

Figure 3.4 Histology of a remnant jejunum illustrating the villus height measurement (Ribeiro *et al*, 2004)

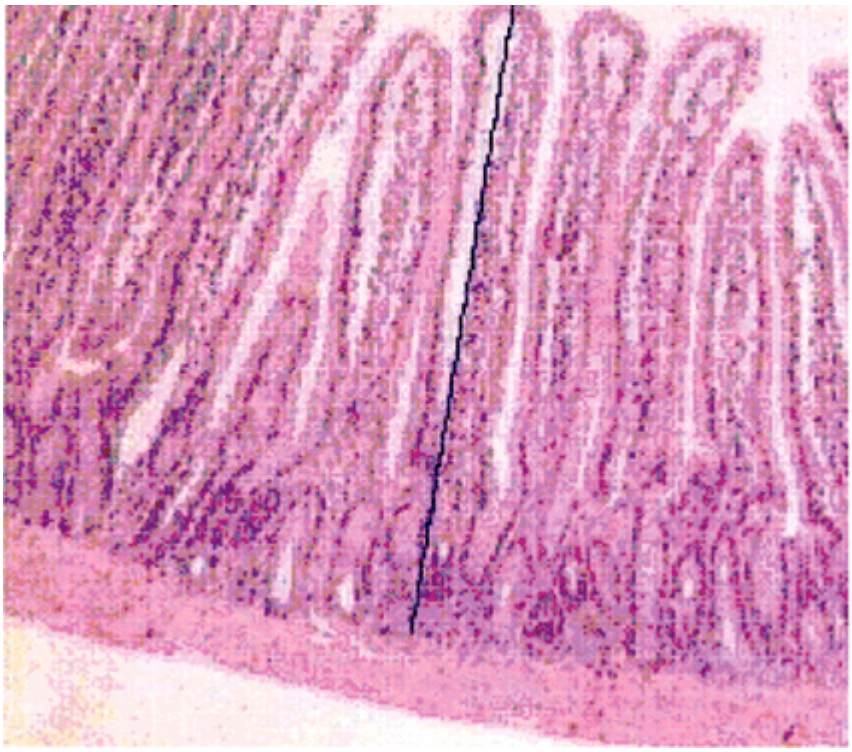

Figure 3.5 Histology of a remnant jejunum illustrating the total mucosal thickness measurement (Ribeiro *et al*, 2004)

3.5.4 Immunoglobin measurements and biochemical variables

Before the birds were sacrificed during each slaughter, blood samples were taken from the heart. Serum was collected after centrifuging. The serum was analysed for: Total Serum Protein (TSP), aspartate transaminase activity (AST) and albumin and globulin levels by the Department of Clinical Pathology Onderstepoort, University of Pretoria.

Serum albumin

Serum samples were collected for albumin analyses. Albumin concentration was measured on a TECHNICON RA-1000® system (Miles Inc., Diagnostics Division, Tarrytown, New York, USA) according to standard procedures, as explained in the Technicon RA Systems Manual (Method No. SM4-0131E94, May 1994). This albumin method is based on the work of Doumas *et al*. (1971) who automated the original manual method of Rodkey (1965).

Total Serum Protein (TSP) - Serum samples were collected for TSP analyses. TSP concentrations were measured on a TECHNICON RA-1000® system (Miles Inc., Diagnostics Division, Tarrytown, New York, USA) according to standard procedures, as explained in the Technicon RA Systems Manual (Method No. SM4-0147E94, May 1994). This method is based on the work of Skeggs & Hochstrasser (1964) who automated the manual method of Weichselbaum (1946).

Globulin – Serum globulin values were calculated as the difference between TSP and albumin.

Aspartate Aminotransferase (AST) - Serum samples were collected for AST analyses. AST concentrations were measured on a TECHNICON RA-1000® system (Miles Inc., Diagnostics Division, Tarrytown, New York, USA) according to standard procedures, as explained in the Technicon RA® Systems Manual (Method No. SM4-0137E94, May 1994). The Technicon RA® system AST method is based on work by Karmen (1955) who originated a procedure that coupled malate dehydrogenase and NADH to the aminotransferase reaction. Bergmeyer *et al*. (1978) modified this procedure to eliminate side reactions and to optimize substrate conditions.

3.6 STATISTICAL ANALYSIS

An analysis of variance with the GLM model (Statistical Analysis Systems, 2009) was used to determine the significance between control and Salmonella groups, different treatments and slaughters for the balanced data. Means and standard deviations (SD) were calculated. A significance level of 5% (P=0.05) between means was determined by using the Bonferoni test (Samuel, 1989). If $P<0.05$, significant differences occurred between the two groups, i.e. Control (non-challenged) and Salmonella (challenged) groups. If $P<0.003$, significant differences occurred within the two groups, meaning differences among the treatments.

CHAPTER 4: RESULTS

4.1 FEED INTAKE

In general, the non-challenged birds had higher feed intakes from day 14 to day 35 than the Salmonella-challenged birds, although these differences were only significant on day 28 (Table 4.1) for all treatments. Within the non-challenged group, supplementation of Probiotic 2 in combination with the prebiotic improved feed intake on day 35, when compared with the non-challenged birds that received no supplementation or only prebiotics.

4.2 AVERAGE DAILY GAIN

The non-challenged birds showed higher average daily gains on day 21 and day 28 than the challenged birds with significant differences between the groups on day 28 (Table 4.2). In the non-challenged group, the birds that received the prebiotic treatment showed higher average daily gain values on day 28 when compared with the non-challenged birds that received Probiotic 2 in combination with the prebiotic treatment. On day 35, within both the non-challenged and challenged groups, birds that did not receive any additives had lower average daily weight gain than those that received additives, although only significant for the Probiotic 2 in combination with the prebiotic treatment within the non-challenged group and Probiotic 1 in combination with the prebiotic treatment within the challenged group.

4.3 BODY WEIGHT

The non-challenged birds showed higher body weights from day 21 onwards than the challenged birds, with significant differences between the two groups on day 35 (Table 4.3). Within both the non-challenged and challenged groups, birds that did not receive any additives had lower body weights than those that received pre- and/or probiotics, although only significant for the Probiotic 1 combined with prebiotic treatment within the non-challenged group and the prebiotic treatment within the challenged group.

4.4 FEED CONVERSION RATIO

Very little significant differences between treatment means were noted for feed conversion ratio (Table 4.4). On day 7, the feed conversion ratio of birds that received

additives seemed to be higher within the challenged group, but this effect disappeared from day 14 onwards.

4.5 LIVER WEIGHT

Within the non-challenged group, supplementation of Probiotic 2 in combination with the prebiotic showed lower liver weights on day 14, when compared with the non-challenged birds that received no supplementation or probiotic 1 (Table 4.5). Within the challenged group, the prebiotic supplementation showed lower liver weights on day 14, when compared to the challenged birds that received no supplementation or Probiotic 1 combined with prebiotic treatment. These effects disappeared from day 14 onwards.

4.6 DUODENUM WEIGHT

As shown in Table 4.6, broilers had significantly enlarged duodenums shortly after exposure to *Salmonella typhimurium* (day 7). This effect became less evident from day 14, with only significant differences for the birds that did not receive any additives and those that received Probiotic 1 among the non- challenged birds and Probiotic 2 combined with a prebiotic treatment among the challenged birds.

4.7 JEJUNUM WEIGHT

The jejunum weights showed a similar pattern as the duodenum weights (Table 4.7). Both groups, challenged and non-challenged birds, showed enlarged jejunums shortly after exposure to *Salmonella typhimurium* (day 7), but became less evident from day 14 onwards. The only significant differences occurred among the challenged birds that received no supplementation or Probiotic 1 treatment.

4.8 ILEUM WEIGHT

Neither Salmonella exposure of the birds nor feed treatments showed any clear effects on ileum weight (Table 4.8).

4.9 CAECA WEIGHT

As shown in Table 4.9, broilers had significantly enlarged caecae shortly after exposure to *Salmonella typhimurium* (day 7). This effect had disappeared by day 14 with only significant differences within the non-challenged group that received Probiotic 1 and the prebiotic combination treatment.

4.10 BLOOD/SERUM VARIABLES

Salmonella-exposure and addition of two types of probiotics and a prebiotic had no definite effect on total serum protein levels (Table 4.10). Shortly after exposure to Salmonella, albumin levels of the challenged birds were significantly lower than the albumin levels of non-challenged birds for most treatments. However, this effect had disappeared by day 14 (Table 4.11). As shown in Table 4.12, the challenged birds that did not receive any additives on day 7 had higher globulin levels than those that received pre- and/or probiotics, although only significant for the Probiotic 2 treatment. However, this effect had disappeared by day 14. The non-challenged birds showed higher albumin: globulin ratios than the challenged birds shortly after exposure to Salmonella, as shown in Table 4.13. This effect became less evident from day 14 onwards. Within the non-challenged group, supplementation of the prebiotic showed higher aspartate amino transferase levels on day 14, when compared to the non-challenged birds that received no supplementation or other additives (Table 4.14). This effect became less evident from day 14 onwards.

4.11 LESIONS

In general, the challenged (Salmonella) birds had more lesions than the non-challenged (non-challenged) birds from day 14 onwards. As shown in Table 4.15, within the Salmonella group, supplementation of the prebiotic and Probiotic 2 treatment showed higher lesion counts on day 28, when compared to the Salmonella birds that received no supplementation or other additives. This effect became less evident from day 28 onwards.

4.12 VILLOUS MORPHOLOGICAL MEASUREMENTS

There were no significant differences in duodenal villus height, total mucosal length and crypt depth among the challenged and non-challenged birds on days 21 (Table 4.16.1) and 35 (Table 4.16.2). However, the duodenal villi height, mucosal thickness and crypt dept showed higher values on day 35 than day 21. The non-challenged birds supplemented with the Probiotic 2 and prebiotic combination showed a thicker mucosa layer than the non-challenged birds that received no supplementation, on day 21. There were no significant differences in the jejenal (Table 4.17.1, Table 4.17.2) and ileal (Table 4.18.1, Table 4.18.2) villi height, mucosal length and crypt depth among the treatment groups or between the challenged and non-challenged birds on days 21 and 35.

Table 4.1: Weekly feed intake of broilers that were challenged with *Salmonella typhimurium* receiving probiotics and/or a prebiotic

Treatments	Day 7		Day 14		Day 21		Day 28		Day 35	
	Non-challenged	Challenged	Non-challenged	Challenged	Non-challenged	Challenged	Non-challenged	Challenged	Non-challenged	Challenged
No additives	122.4 (±6.459)	128.1 (±20.90)	372.9 (±9.417)	349.0 (±27.48)	553.1 (±24.75)	528.3 (±23.26)	839.4 [1ab] (±64.67)	727.2 [2] (±96.05)	1060 [a] (±122.2)	1028 (±99.62)
Prebiotic ^	124.1 (±8.785)	141.2 (±12.07)	372.3 (±8.847)	354.8 (±17.95)	555.9 (±12.62)	529.1 (±21.94)	834.8 [1ab] (±64.61)	754.6 [2] (±50.62)	1059 [a] (±121.9)	1097 (±153.5)
Probiotic 1*	133.2 (±14.71)	140.2 (±15.43)	375.4 (±13.76)	362.8 (±15.87)	562.9 (±19.49)	536.9 (±23.88)	820.2 [1ab] (±79.02)	729.4 [2] (±68.95)	1089 [ab] (±152.7)	1028 (±125.3)
Probiotic 1 with prebiotic	126.0 (±6.311)	137.4 (±29.17)	368.1 (±12.78)	343.9 (±23.82)	554.7 (±5.880)	525.3 (±28.00)	893.3 [1a] (±27.72)	656.7 [2] (±79.56)	1142 [1ab] (±28.27)	1011 [2] (±132.0)
Probiotic 2[#]	131.7 (±3.216)	140.9 (±16.20)	373.5 (±10.32)	361.2 (±10.96)	554.2 (±15.07)	523.8 (±35.71)	834.0 [1ab] (±50.61)	758.1 [2] (±68.83)	1086 [ab] (±84.24)	1066 (±49.603)
Probiotic 2 with prebiotic	130.1 (±14.63)	140.0 (±7.640)	365.6 (±13.15)	369.0 (±8.220)	557.6 (±16.81)	518.5 (±26.23)	776.2 [1b] (±7.364)	696.3 [2] (±57.42)	1185 [1b] (±32.34)	1037 [2] (±75.47)

[12] Row means with the same superscript do not differ significantly (P>0.05)
[ab] Column means with the same superscript do not differ significantly (P>0.003)
^ Prebiotic (Fructo-oligosaccharide)
*** Probiotic 1: Consisted of the spore-forming bacteria *Brevibacillus laterosporus***
[#] Probiotic 2: Consisted of *Lactobacillus spp.*

Table 4.2: Weekly average daily gain of broilers that were challenged with *Salmonella typhimurium* receiving probiotics and/or a prebiotic

Treatments	Day 7		Day 14		Day 21		Day 28		Day 35	
	Non-challenged	Challenged	Non-challenged	Challenged	Non-challenged	Challenged	Non-challenged	Challenged	Non-challenged	Challenged
No additives	15.43 (±1.066)	13.56 (±1.950)	28.14 (±1.769)	25.68 (±2.678)	41.41 (±2.686)	40.96 (±4.559)	75.00 ^{1ab} (±4.786)	60.00 ^{2} (±10.09)	79.76 ^{a} (±8.607)	83.07 ^{a} (±5.888)
Prebiotic ^	15.42 (±1.045)	13.37 (±1.289)	27.56 (±1.055)	26.76 (±2.326)	43.13 (±0.553)	40.42 (±4.066)	82.17 ^{1a} (±19.26)	71.17 ^{2} (±9.169)	85.11 ^{ab} (±14.97)	90.97 ^{ab} (±4.188)
Probiotic 1*	15.29 (±0.294)	14.92 (±1.746)	27.77 (±1.254)	28.27 (±2.312)	42.09 (±1.597)	41.71 (±3.994)	74.25 ^{1ab} (±10.30)	64.30 ^{2} (±7.066)	90.92 ^{ab} (±7.815)	85.17 ^{ab} (±13.52)
Probiotic 1 with prebiotic	15.56 (±0.506)	13.82 (±0.213)	27.19 (±1.337)	25.11 (±2.349)	41.95 (±3.081)	39.54 (±2.466)	80.76 ^{1ab} (±3.427)	60.90 ^{2} (±8.671)	90.85 ^{ab} (±3.332)	94.85 ^{b} (±14.06)
Probiotic 2$^{\#}$	15.34 (±0.755)	14.58 (±0.797)	27.60 (±1.057)	26.74 (±0.476)	40.69 (±1.340)	38.82 (±3.416)	73.74 ^{ab} (±9.292)	69.33 (±7.469)	88.61 ^{ab} (±11.46)	87.86 ^{ab} (±5.394)
Probiotic 2 with prebiotic	15.19 (±1.150)	15.22 (±2.103)	26.82 (±1.662)	27.27 (±1.510)	44.24 (±1.920)	38.00 (±2.175)	67.42 ^{b} (±2.724)	66.81 (±6.001)	95.89 ^{1b} (±7.686)	84.61 ^{2ab} (±8.768)

^{12} Row means with the same superscript do not differ significantly (P>0.05)
^{ab} Column means with the same superscript do not differ significantly (P>0.003)
^ Prebiotic (Fructo-oligosaccharide)
*** Probiotic 1: Consisted of the spore-forming bacteria *Brevibacillus laterosporus***
$^{\#}$ Probiotic 2: Consisted of *Lactobacillus spp*.

Table 4.3: Weekly body weight of broilers that were challenged with *Salmonella typhimurium* receiving probiotics and/or a prebiotic

Treatments	Day 7		Day 14		Day 21		Day 28		Day 35	
	Non-challenged	Challenged	Non-challenged	Challenged	Non-challenged	Challenged	Non-challenged	Challenged	Non-challenged	Challenged
No additives	150.6 (±7.467)	138.2 (±12.29)	347.6 (±19.29)	318.0 (±24.01)	639.5 (±37.28)	604.7 (±37.24)	1163 [1] (±31.96)	1025 [2] (±88.42)	1721 [1a] (±81.77)	1606 [2a] (±126.4)
Prebiotic ^	150.5 (±7.320)	136.2 (±9.024)	343.5 (±14.69)	323.5 (±22.79)	645.4 (±13.05)	606.5 (±46.58)	1221 [1] (±141.7)	1105 [2] (±47.19)	1816 [1ab] (±67.02)	1741 [2b] (±21.93)
Probiotic 1*	149.6 (±2.062)	147.1 (±12.22)	344.0 (±8.849)	345.0 (±28.13)	638.7 (±15.00)	636.9 (±52.99)	1158 (±78.50)	1087 (±86.07)	1795 [1ab] (±130.4)	1683 [2ab] (±159.3)
Probiotic 1 with prebiotic	151.5 (±3.542)	139.3 (±1.491)	341.9 (±9.028)	315.1 (±16.01)	635.5 (±14.84)	591.9 (±21.38)	1201 [1] (±27.27)	1018 [2] (±81.00)	1837 [1b] (±41.55)	1682 [2ab] (±107.5)
Probiotic 2[#]	150.0 (±5.285)	144.7 (±5.578)	343.2 (±8.644)	331.9 (±7.680)	628.1 (±10.60)	603.6 (±29.07)	1144 (±57.41)	1089 (±67.93)	1765 [ab] (±134.0)	1704 [ab] (±84.70)
Probiotic 2 with prebiotic	148.9 (±8.050)	149.2 (±14.72)	336.7 (±18.97)	340.1 (±12.64)	646.1 (±25.91)	606.1 (±21.43)	1118 (±13.46)	1074 (±59.74)	1790 [1ab] (±54.66)	1666 [2ab] (±69.35)

[12] Row means with the same superscript do not differ significantly (P>0.05)
[ab] Column means with the same superscript do not differ significantly (P>0.003)
^ Prebiotic (Fructo-oligosaccharide)
*** Probiotic 1: Consisted of the spore-forming bacteria *Brevibacillus laterosporus***
[#] Probiotic 2: Consisted of *Lactobacillus spp*.

Table 4.4: Weekly feed conversion ratio of broilers that were challenged with *Salmonella typhimurium* receiving probiotics and/or a prebiotic

Treatments	Day 7		Day 14		Day 21		Day 28		Day 35	
	Non-challenged	Challenged	Non-challenged	Challenged	Non-challenged	Challenged	Non-challenged	Challenged	Non-challenged	Challenged
No additives	1.16 (±0.144)	1.10 [a] (±0.521)	1.90 (±0.091)	1.95 (±0.080)	1.91 (±0.083)	1.86 (±0.180)	1.60 (±0.046)	1.74 (±0.138)	1.90 (±0.035)	1.77 (±0.151)
Prebiotic ^	1.17 [1] (±0.059)	1.55 [2b] (±0.196)	1.93 (±0.060)	1.90 (±0.103)	1.84 (±0.046)	1.88 (±0.112)	1.49 (±0.242)	1.53 (±0.129)	1.80 (±0.149)	1.72 (±0.186)
Probiotic 1*	1.26 (±0.124)	1.38 [b] (±0.202)	1.93 (±0.060)	1.84 (±0.092)	1.91 (±0.055)	1.85 (±0.132)	1.59 (±0.121)	1.62 (±0.085)	1.71 (±0.116)	1.74 (±0.148)
Probiotic 1 with prebiotic	1.17 [1] (±0.064)	1.45 [2b] (±0.322)	1.94 (±0.038)	1.96 (±0.068)	1.90 (±0.125)	1.90 (±0.090)	1.58 (±0.037)	1.55 (±0.128)	1.80 [1] (±0.056)	1.55 [2] (±0.272)
Probiotic 2#	1.25 (±0.089)	1.40 [b] (±0.172)	1.93 (±0.059)	1.93 (±0.073)	1.95 (±0.035)	1.93 (±0.059)	1.63 (±0.152)	1.56 (±0.049)	1.76 (±0.142)	1.74 (±0.112)
Probiotic 2 with prebiotic	1.25 (±0.160)	1.28 [ab] (±0.208)	1.95 (±0.059)	1.94 (±0.089)	1.86 (±0.118)	1.95 (±0.032)	1.65 (±0.066)	1.49 (±0.080)	1.77 (±0.127)	1.76 (±0.152)

[12] Row means with the same superscript do not differ significantly (P>0.05)
[ab] Column means with the same superscript do not differ significantly (P>0.003)
^ Prebiotic (Fructo-oligosaccharide)
*** Probiotic 1: Consisted of the spore-forming bacteria *Brevibacillus laterosporus***
 # Probiotic 2: Consisted of *Lactobacillus spp*.

Table 4.5: Weekly liver weight of broilers that were challenged with *Salmonella typhimurium* receiving probiotics and/or a prebiotic

Treatments	Day 7		Day 14		Day 21		Day 28		Day 35	
	Non-challenged	Challenged	Non-challenged	Challenged	Non-challenged	Challenged	Non-challenged	Challenged	Non-challenged	Challenged
No additives	4.27 (±0.423)	4.43 (±0.840)	5.09 [a] (±0.597)	5.47 [a] (±0.520)	3.79 (±0.215)	3.67 (±0643)	3.55 (±0.397)	3.31 (±0.557)	2.98 [1] (±0.511)	3.62 [2] (±0.375)
Prebiotic ^	4.64 (±0.308)	4.43 (±0.316)	4.88 [ab] (±0.487)	4.41 [b] (±0.538)	3.98 (±0.202)	3.87 (±0.953)	3.27 (±0.581)	3.25 (±0.314)	3.73 (±0.568)	3.54 (±0.342)
Probiotic 1*	4.60 (±0.419)	4.60 (±0.513)	5.28 [a] (±0.675)	4.88 [ab] (±0.671)	3.71 (±0.366)	3.64 (±0.227)	3.48 (±0.309)	3.32 (±0.448)	3.09 [1] (±0.334)	3.72 [2] (±0.520)
Probiotic 1 with prebiotic	4.62 (±0.508)	4.47 (±0.408)	5.04 [1a] (±0.429)	5.73 [2a] (±0.754)	3.77 (±0.303)	3.57 (±0.341)	3.28 (±0.623)	3.02 (±0.359)	3.70 (±0.410)	3.77 (±0.730)
Probiotic 2[#]	4.70 (±0.504)	4.45 (±0.163)	4.55 [ab] (±0.969)	4.89 [ab] (±0.453)	3.87 (±0.296)	3.49 (±0.212)	3.40 (±0.413)	3.29 (±0.382)	3.64 (±0.519)	3.42 (±0.705)
Probiotic 2 with prebiotic	4.20 (±0.449)	4.25 (±0.294)	3.97 [1b] (±0.401)	5.01 [2ab] (±0.625)	3.86 [1] (±0.555)	3.05 [2] (±0.045)	2.84 [1] (±0.539)	3.51 [2] (±0.215)	3.46 (±0.404)	3.41 (±0.285)

[12] Row means with the same superscript do not differ significantly (P>0.05)
[ab] Column means with the same superscript do not differ significantly (P>0.003)
^ Prebiotic (Fructo-oligosaccharide)
*** Probiotic 1: Consisted of the spore-forming bacteria *Brevibacillus laterosporus***
[#] Probiotic 2: Consisted of *Lactobacillus spp*.

Table 4.6: Weekly duodenum weights of broilers that were challenged with *Salmonella typhimurium* receiving probiotics and/or a prebiotic

Treatments	Day 7		Day 14		Day 21		Day 28		Day 35	
	Non-challenged	Challenged	Non-challenged	Challenged	Non-challenged	Challenged	Non-challenged	Challenged	Non-challenged	Challenged
No additives	2.42 [1a] (±0.222)	3.24 [2a] (±0.282)	2.02 [1] (±0.114)	2.40 [2] (±0.164)	1.46 (±0.077)	1.70 (±0.156)	1.44 (±0.150)	1.39 (±0.096)	1.03 (±0.094)	1.14 (±0.097)
Prebiotic ^	2.57 [1a] (±0.409)	2.97 [2ab] (±0.340)	2.20 (±0.228)	2.48 (±0.207)	1.48 (±0.131)	1.63 (±0.116)	1.31 (±0.056)	1.32 (±0.104)	0.97 (±0.057)	1.03 (±0.069)
Probiotic 1*	2.97 [b] (±0.135)	2.98 [ab] (±0.506)	2.07 (±0.102)	2.28 (±0.124)	1.59 (±0.137)	1.54 (±0.148)	1.35 (±0.046)	1.39 (±0.287)	0.93 (±0.020)	1.02 (±0.091)
Probiotic 1 with prebiotic	2.71 [1ab] (±0.294)	3.14 [2ab] (±0.285)	2.08 [1] (±0.156)	2.45 [2] (±0.105)	1.59 (±0.050)	1.69 (±0.147)	1.30 (±0.028)	1.33 (±0.077)	0.98 (±0.085)	1.05 (±0.040)
Probiotic 2#	2.35 [1a] (±0.455)	2.88 [2ab] (±0.260)	2.29 (±0.140)	2.27 (±0.152)	1.54 (±0.161)	1.55 (±0.206)	1.30 (±0.081)	1.27 (±0.181)	0.97 (±0.040)	0.94 (±0.060)
Probiotic 2 with prebiotic	2.69 [ab] (±0.454)	2.83 [b] (±0.401)	2.14 (±0.236)	2.34 (±0.185)	1.52 (±0.127)	1.59 (±0.242)	1.25 (±0.059)	1.36 (±0.127)	1.03 (±0.075)	1.04 (±0.098)

[12] Row means with the same superscript do not differ significantly (P>0.05)
[ab] Column means with the same superscript do not differ significantly (P>0.003)
^ Prebiotic (Fructo-oligosaccharide)
*** Probiotic 1: Consisted of the spore-forming bacteria *Brevibacillus laterosporus***
 # Probiotic 2: Consisted of *Lactobacillus spp*.

Table 4.7: Weekly jejunum weights of broilers that were challenged with *Salmonella typhimurium* receiving probiotics and/or a prebiotic

Treatments	Day 7		Day 14		Day 21		Day 28		Day 35	
	Non-challenged	Challenged	Non-challenged	Challenged	Non-challenged	Challenged	Non-challenged	Challenged	Non-challenged	Challenged
No additives	3.00 [1] (±0.567)	3.55 [2a] (±0.171)	2.57 (±0.135)	2.71 (±0.218)	1.88 (±0.090)	2.04 (±0.124)	1.59 (±0.220)	1.67 (±0.222)	1.15 (±0.073)	1.31 (±0.117)
Prebiotic ^	3.21 (±0.340)	3.30 [ab] (±0.236)	2.67 (±0.266)	2.87 (±0.313)	1.77 [1] (±0.123)	2.07 [2] (±0.181)	1.52 (±0.209)	1.61 (±0.139)	1.22 (±0.135)	1.25 (±0.086)
Probiotic 1*	3.07 (±0.320)	3.11 [b] (±0.172)	2.46 (±0.317)	2.64 (±0.228)	1.77 (±0.093)	1.92 (±0.191)	1.51 (±0.147)	1.61 (±0.189)	1.13 (±0.103)	1.25 (±0.124)
Probiotic 1 with prebiotic	3.07 [1] (±0.352)	3.54 [2a] (±0.292)	2.43 [1] (±0.170)	2.76 [2] (±0.215)	1.71 (±0.203)	1.93 (±0.320)	1.43 (±0.056)	1.47 (±0.152)	1.12 (±0.051)	1.30 (±0.130)
Probiotic 2[#]	2.82 (±0.338) [1]	3.20 [2ab] (±0.299)	2.65 (±0.126)	2.75 (±0.281)	1.64 (±0.341)	1.80 (±0.054)	1.53 (±0.097)	1.62 (±0.187)	1.23 (±0.112)	1.19 (±0.053)
Probiotic 2 with prebiotic	3.21 (±0.303)	3.24 [ab] (±0.264)	2.65 (±0.338)	2.68 (±0.135)	1.78 (±0.287)	1.85 (±0.256)	1.44 (±0.146)	1.60 (±0.151)	1.24 (±0.042)	1.25 (±0.162)

[12] Row means with the same superscript do not differ significantly (P>0.05)
[ab] Column means with the same superscript do not differ significantly (P>0.003)
^ Prebiotic (Fructo-oligosaccharide)
*** Probiotic 1: Consisted of the spore-forming bacteria *Brevibacillus laterosporus***
[#] Probiotic 2: Consisted of *Lactobacillus spp*.

Table 4.8: Weekly ileum weights of broilers that were challenged with *Salmonella typhimurium* receiving probiotics and/or a prebiotic

Treatments	Day 7		Day 14		Day 21		Day 28		Day 35	
	Non-challenged	Challenged	Non-challenged	Challenged	Non-challenged	Challenged	Non-challenged	Challenged	Non-challenged	Challenged
No additives	2.53 [1] (±0.547)	2.94 [2ab] (±0.237)	2.02 (±0.449) [1]	2.37 (±0.231) [2]	1.65 (±0.239)	1.75 (±0.112)	1.52 (±0.136)	1.55 (±0.147)	1.03 (±0.108)	1.12 (±0.100)
Prebiotic ^	2.51 (±0.286)	2.70 [ab] (±0.333)	2.24 (±0.277)	2.47 (±0.253)	1.65 (±0.162)	1.82 (±0.101)	1.32 (±0.109)	1.37 (±0.113)	1.07 (±0.018)	1.02 (±0.108)
Probiotic 1*	2.64 (±0.366)	2.57 [a] (±0.381)	2.19 (±0.266)	2.25 (±0.247)	1.64 (±0.280)	1.65 (±0.249)	1.36 (±0.072)	1.44 (±0.190)	1.04 (±0.112)	1.08 (±0.053)
Probiotic 1 with prebiotic	2.42 [1] (±0.323)	3.03 [2b] (±0.159)	2.12 (±0.182)	2.33 (±0.183)	1.72 (±0.183)	1.68 (±0.265)	1.25 (±0.084)	1.34 (±0.058)	1.05 (±0.120)	1.10 (±0.090)
Probiotic 2#	2.53 (±0.467)	2.54 [a] (±0.284)	2.39 (±0.146)	2.38 (±0.275)	1.59 (±0.117)	1.53 (±0.060)	1.39 (±0.082)	1.35 (±0.093)	1.05 (±0.063)	0.99 (±0.055)
Probiotic 2 with prebiotic	2.70 (±0.286)	2.70 [ab] (±0.212)	2.20 (±0.339)	2.21 (±0.182)	1.67 (±0.218)	1.69 (±0.228)	1.29 (±0.134)	1.46 (±0.060)	1.04 (±0.142)	1.04 (±0.088)

[12] Row means with the same superscript do not differ significantly (P>0.05)
[ab] Column means with the same superscript do not differ significantly (P>0.003)
^ Prebiotic (Fructo-oligosaccharide)
*** Probiotic 1: Consisted of the spore-forming bacteria *Brevibacillus laterosporus***
[#] Probiotic 2: Consisted of *Lactobacillus spp*.

Table 4.9: Weekly caeca weights of broilers that were challenged with *Salmonella typhimurium* receiving probiotics and/or a prebiotic

Treatments	Day 7		Day 14		Day 21		Day 28		Day 35	
	Non-challenged	Challenged	Non-challenged	Challenged	Non-challenged	Challenged	Non-challenged	Challenged	Non-challenged	Challenged
No additives	1.19 [ab] (±0.167)	1.26 (±0.141)	0.70 [1] (±0.105)	0.89 [2] (±0.108)	0.72 (±0.074)	0.64 (±0.027)	0.56 (±0.090)	0.57 (±0.037)	0.36 (±0.083)	0.43 (±0.041)
Prebiotic ^	1.16 [ab] (±0.107)	1.11 (±0.229)	0.87 (±0.127)	0.91 (±0.146)	0.67 (±0.073)	0.66 (±0.042)	0.50 (±0.069)	0.58 (±0.050)	0.39 (±0.062)	0.39 (±0.053)
Probiotic 1*	1.34 [1a] (±0.230)	1.14 (±0.357) [2]	0.77 (±0.145)	0.79 (±0.098)	0.68 (±0.086)	0.63 (±0.071)	0.56 (±0.078)	0.51 (±0.059)	0.35 (±0.054)	0.39 (±0.052)
Probiotic 1 with prebiotic	1.08 [1b] (±0.333)	1.26 [2] (±0.107)	0.75 [1] (±0.125)	0.93 [2] (±0.115)	0.67 (±0.081)	0.65 (±0.089)	0.49 (±0.048)	0.51 (±0.047)	0.40 (±0.058)	0.41 (±0.052)
Probiotic 2#	1.19 [ab] (±0.267)	1.27 (±0.144)	0.83 (±0.065)	0.85 (±0.135)	0.62 (±0.141)	0.61 (±0.054)	0.51 (±0.082)	0.53 (±0.080)	0.40 (±0.069)	0.34 (±0.046)
Probiotic 2 with prebiotic	1.24 [ab] (±0.152)	1.22 (±0.112)	0.92 (±0.182)	0.93 (±0.119)	0.62 (±0.119)	0.65 (±0.045)	0.50 (±0.056)	0.57 (±0.092)	0.39 (±0.068)	0.35 (±0.057)

[12] Row means with the same superscript do not differ significantly (P>0.05)
[ab] Column means with the same superscript do not differ significantly (P>0.003)
^ Prebiotic (Fructo-oligosaccharide)
*** Probiotic 1: Consisted of the spore-forming bacteria *Brevibacillus laterosporus***
[#] Probiotic 2: Consisted of *Lactobacillus spp*.

Table 4.10: Weekly total serum protein levels of broilers that were challenged with *Salmonella typhimurium* receiving probiotics and/or a prebiotic

Treatments	Day 7		Day 14		Day 21		Day 28		Day 35	
	Non-challenged	Challenged	Non-challenged	Challenged	Non-challenged	Challenged	Non-challenged	Challenged	Non-challenged	Challenged
No additives	25.18 (±2.692)	26.48 (±2.275)	28.04 (±4.081)	26.78 (±3.420)	25.66 [1] (±2.323)	29.98 [2] (±1.908)	29.76 (±3.256)	27.10 (±1.844)	30.82 (±2.079)	29.24 (±2.586)
Prebiotic ^	23.10 (±2.861)	25.22 (±2.083)	26.04 (±3.205)	26.02 (±4.225)	26.44 (±2.486)	26.70 (±1.678)	26.54 (±3.128)	27.26 (±0.871)	30.02 (±4.305)	30.08 (±2.993)
Probiotic 1*	25.74 (±2.224)	25.90 (±2.038)	23.94 (±2.889)	23.56 (±1.901)	26.36 (±2.292)	26.80 (±2.608)	28.08 (±3.273)	29.42 (±3.168)	30.18 (±3.090)	30.12 (±2.823)
Probiotic 1 with prebiotic	24.86 (±0.976)	23.32 (±2.686)	25.22 (±1.857)	26.72 (±1.502)	26.48 (±0.887)	26.10 (±2.164)	26.18 (±1.666)	28.24 (±3.085)	31.68 (±1.968)	29.90 (±5.109)
Probiotic 2[#]	23.36 (±0.416)	21.62 (±2.144)	26.08 (±1.724)	25.76 (±3.198)	28.06 (±1.385)	28.78 (±2.983)	26.94 [1] (±3.129)	30.54 [2] (±2.781)	28.86 (±2.754)	31.40 (±2.416)
Probiotic 2 with prebiotic	23.68 (±1.281)	25.60 (±2.119)	25.88 (±3.235)	26.30 (±4.337)	23.88 (±2.674)	25.06 (±1.791)	27.18 (±0.991)	27.26 (±2.605)	32.60 (±3.386)	29.74 (±2.406)

[12] Row means with the same superscript do not differ significantly (P>0.05)
[ab] Column means with the same superscript do not differ significantly (P>0.003)
^ Prebiotic (Fructo-oligosaccharide)
*** Probiotic 1: Consisted of the spore-forming bacteria *Brevibacillus laterosporus***
[#] Probiotic 2: Consisted of *Lactobacillus spp*.

Table 4.11: Weekly albumin levels of broilers that were challenged with *Salmonella typhimurium* receiving probiotics and/or a prebiotic

Treatments	Day 7		Day 14		Day 21		Day 28		Day 35	
	Non-challenged	Challenged	Non-challenged	Challenged	Non-challenged	Challenged	Non-challenged	Challenged	Non-challenged	Challenged
No additives	15.40 [1] (±1.658)	13.78 [2] (±1.314)	14.74 (±1.101)	15.98 (±1.597)	15.34 (±0.896)	16.34 (±0.996)	17.22 (±0.753)	17.00 (±1.471)	17.42 (±0.789)	16.64 (±0.623)
Prebiotic ^	14.30 (±1.340)	14.26 (±0.956)	15.06 (±2.277)	14.52 (±1.504)	16.02 (±1.402)	15.58 (±1.383)	15.24 (±1.071)	15.92 (±0.926)	16.94 (±1.071)	16.44 (±1.159)
Probiotic 1*	16.20 [1] (±0.957)	14.50 [2] (±1.102)	13.86 (±1.299)	14.26 (±0.891)	15.84 (±1.539)	15.48 (±0.847)	16.22 (±2.434)	17.52 (±1.627)	16.64 (±1.078)	16.82 (±1.169)
Probiotic 1 with prebiotic	16.06 [1] (±1.242)	13.52 [2] (±1.460)	14.96 (±1.394)	14.74 (±0.832)	15.72 (±0.593)	15.68 (±1.013)	15.84 [1] (±0.963)	17.54 [2] (±1.137)	17.32 (±0.540)	16.44 (±1.566)
Probiotic 2[#]	14.66 [1] (±1.230)	13.04 [2] (±0.713)	14.40 (±0.930)	15.10 (±2.254)	17.08 (±0.691)	15.92 (±1.486)	16.38 (±0.444)	17.60 (±0.992)	16.26 (±1.508)	17.42 (±1.605)
Probiotic 2 with prebiotic	14.52 (±0.801)	14.18 (±1.043)	14.68 (±0.890)	15.18 (±1.851)	15.24 (±1.522)	14.78 (±0.998)	16.38 (±1.047)	17.02 (±1.134)	17.96 [1] (±1.218)	16.06 [2] (±0.740)

[12] Row means with the same superscript do not differ significantly (P>0.05)
[ab] Column means with the same superscript do not differ significantly (P>0.003)
^ Prebiotic (Fructo-oligosaccharide)
*** Probiotic 1: Consisted of the spore-forming bacteria *Brevibacillus laterosporus***
[#] Probiotic 2: Consisted of *Lactobacillus spp*.

Table 4.12: Weekly globulin levels of broilers that were challenged with *Salmonella typhimurium* receiving probiotics and/or a prebiotic

Treatments	Day 7		Day 14		Day 21		Day 28		Day 35	
	Non-challenged	Challenged	Non-challenged	Challenged	Non-challenged	Challenged	Non-challenged	Challenged	Non-challenged	Challenged
No additives	9.78 [1] (±1.424)	12.70 [2a] (±2.992)	13.30 (±3.177)	10.80 (±1.944)	10.32 [1] (±1.605)	13.64 [2] (±1.057)	12.54 (±2.972)	10.10 (±0.464)	13.40 (±2.111)	12.60 (±2.433)
Prebiotic ^	8.80 (±2.602)	10.96 [ab] (±1.422)	10.98 (±1.182)	11.50 (±2.741)	10.42 (±1.819)	11.12 (±1.365)	11.30 (±2.349)	11.34 (±1.301)	13.08 (±3.860)	13.64 (±2.422)
Probiotic 1*	9.54 (±1.820)	11.40 [ab] (±1.039)	10.08 (±1840)	9.30 (±1.187)	10.52 (±1.137)	11.32 (±2.153)	11.86 (±2.434)	11.90 (±2.847)	13.54 (±2.539)	13.30 (±1.728)
Probiotic 1 with prebiotic	8.80 (±1.482)	9.80 [ab] (±1.614)	10.26 (±2.011)	11.98 (±2.039)	10.76 (±0.832)	10.42 (±1.420)	10.34 (±1.197)	10.70 (±3.250)	14.38 (±1.724)	13.46 (±4.038)
Probiotic 2[#]	8.70 (±1.294)	8.58 [b] (±1.574)	11.68 (±1.593)	10.66 (±1.519)	10.98 (±1.018)	12.86 (±2.652)	10.56 (±2.704)	12.94 (±2.246)	12.60 (±1.764)	13.98 (±0.912)
Probiotic 2 with prebiotic	9.16 (±1.438)	11.42 [ab] (±1.221)	11.20 (±2.526)	11.12 (±2.563)	8.64 (±1.318)	10.28 (±1.612)	10.80 (±1.461)	10.24 (±2.521)	14.64 (±2.314)	13.68 (±1.831)

[12] Row means with the same superscript do not differ significantly (P>0.05)
[ab] Column means with the same superscript do not differ significantly (P>0.003)
^ Prebiotic (Fructo-oligosaccharide)
*** Probiotic 1: Consisted of the spore-forming bacteria *Brevibacillus laterosporus***
[#] Probiotic 2: Consisted of *Lactobacillus spp*.

Table 4.13: Weekly albumin: globulin ratios of broilers that were challenged with *Salmonella typhimurium* receiving probiotics and/or a prebiotic

Treatments	Day 7		Day 14		Day 21		Day 28		Day 35	
	Non-challenged	Challenged	Non-challenged	Challenged	Non-challenged	Challenged	Non-challenged	Challenged	Non-challenged	Challenged
No additives	1.59 [1] (±0.221)	1.14 [2] (±0.285)	1.15 [1] (±0.225)	1.49 [2] (±0.133)	1.51 (±0.183)	1.20 (±0.060)	1.43 (±0.282)	1.53 (±0.251)	1.33 (±0.226)	1.36 (±0.272)
Prebiotic ^	1.74 [1] (±0.530)	1.31 [2] (±0.155)	1.37 (±0.153)	1.29 (±0.171)	1.57 (±0.270)	1.42 (±0.232)	1.38 (±0.218)	1.42 (±0.219)	1.39 (±0.456)	1.23 (±0.193)
Probiotic 1*	1.75 [1] (±0.338)	1.27 [2] (±0.070)	1.40 (±0.207)	1.55 (±0.167)	1.51 (±0.158)	1.41 (±0.279)	1.41 (±0.324)	1.53 (±0.346)	1.26 (±0.209)	1.28 (±0.108)
Probiotic 1 with prebiotic	1.88 [1] (±0.423)	1.40 [2] (±0.211)	1.52 (±0.416)	1.27 (±0.255)	1.47 (±0.139)	1.52 (±0.193)	1.55 (±0.192)	1.77 (±0.578)	1.22 (±0.156)	1.27 (±0.244)
Probiotic 2[#]	1.73 (±0.390)	1.55 (±0.222)	1.25 (±0.188)	1.43 (±0.204)	1.57 (±0.136)	1.28 (±0.270)	1.64 (±0.443)	1.39 (±0.239)	1.30 (±0.161)	1.25 (±0.068)
Probiotic 2 with prebiotic	1.63 [1] (±0.349)	1.25 [2] (±0.096)	1.35 (±0.253)	1.40 (±0.215)	1.78 (±0.192)	1.47 (±0.256)	1.54 (±0.278)	1.75 (±0.464)	1.24 (±0.138)	1.18 (±0.129)

[12] Row means with the same superscript do not differ significantly (P>0.05)
[ab] Column means with the same superscript do not differ significantly (P>0.003)
^ Prebiotic (Fructo-oligosaccharide)
*** Probiotic 1: Consisted of the spore-forming bacteria *Brevibacillus laterosporus***
[#] Probiotic 2: Consisted of *Lactobacillus spp.*

Table 4.14: Weekly aspartate amino transferase levels of broilers that were challenged with *Salmonella typhimurium* receiving probiotics and/or a prebiotic

Treatments	Day 7		Day 14		Day 21		Day 28		Day 35	
	Non-challenged	Challenged	Non-challenged	Challenged	Non-challenged	Challenged	Non-challenged	Challenged	Non-challenged	Challenged
No additives	425.6 (±266.0)	294.4 (±69.37)	296.2 [a] (±169.9)	197.6 (±17.73)	160.0 (±20.31)	168.0 (±12.73)	172.6 (±4.16)	364.2 (±439.6)	179.6 (±40.46)	213.8 (±90.00)
Prebiotic ^	458.8 (±439.6)	175.4 (±47.68)	798.6 [1b] (±1163)	175.4 [2] (±25.88)	161.8 (±10.76)	154.8 (±11.35)	430.8 (±578.5)	171.6 (±12.58)	344.6 (±422.0)	193.6 (±7.701)
Probiotic 1*	431.6 (±289.1)	385.0 (±269.2)	190.4 [a] (±47.61)	205.6 (±32.58)	148.4 (±22.24)	159.4 (±3.36)	187.6 (±38.88)	166.8 (±18.70)	203.6 (±60.19)	183.2 (±12.68)
Probiotic 1 with prebiotic	405.6 (±276.3)	287.4 (±132.5)	330.4 [a] (±125.6)	192.4 (±26.43)	156.0 (±13.21)	182.4 (±56.45)	172.6 (±10.76)	162.4 (±14.12)	251.8 (±77.08)	235.8 (±74.03)
Probiotic 2[#]	499.2 (±174.4)	255.8 (±92.42)	249.6 [a] (±108.0)	187.6 (±24.17)	173.2 (±34.99)	168.0 (±16.45)	164.2 (±18.28)	385.6 (±447.6)	267.0 (±208.2)	182.6 (±27.23)
Probiotic 2 with prebiotic	406.0 (±254.3)	278.6 (±101.6)	246.8 [a] (±80.08)	206.2 (±49.03)	156.2 (±15.77)	162.6 (±11.15)	178.6 (±12.34)	179.2 (±17.66)	290.0 (±190.8)	202.6 (±65.16)

[12] Row means with the same superscript do not differ significantly (P>0.05)
[ab] Column means with the same superscript do not differ significantly (P>0.003)
^ Prebiotic (Fructo-oligosaccharide)
*** Probiotic 1: Consisted of the spore-forming bacteria *Brevibacillus laterosporus***
[#] Probiotic 2: Consisted of *Lactobacillus spp*.

Table 4.15: Weekly lesion counts of broilers that were challenged with *Salmonella typhimurium* receiving probiotics and/or a prebiotic

Treatments	Day 7		Day 14		Day 21		Day 28		Day 35	
	Non-challenged	Challenged	Non-challenged	Challenged	Non-challenged	Challenged	Non-challenged	Challenged	Non-challenged	Challenged
No additives	0	0	0	0.267 (±0.435)	0.067 (±0.149)	0.467 (±0.183)	0	0.067 [a] (±0.149)	0.560 [1] (±0.683)	1.667 [2] (±0.408)
Prebiotic ^	0	0	0.333 (±0.746)	0.333 (±0.408)	0.267 (±0.279)	0.733 (±0.723)	0.200 [1] (±0.182)	1.666 [2b] (±1.225)	1.333 (±1.000)	1.000 (±0.667)
Probiotic 1*	0.267 (±0.596)	0	0.067 (±0.149)	0.200 (±0.447)	0.800 (±0.606)	0.933 (±1.011)	0.067 (±0.149)	0.267 [a] (±0.279)	1.200 (±0.931)	1.400 (±1.211)
Probiotic 1 with prebiotic	0	0	0	0.533 (±0.837)	0.400 (±0.279)	1.067 (±0.925)	0.267 (±0.365)	0.133 [a] (±0.298)	0.800 (±0.606)	1.200 (±0.691)
Probiotic 2[#]	0	0	0.133 (±0.298)	0.333 (±0.471)	0.667 (±0.527)	0.333 (±0.408)	0.267 [1] (±0.435)	1.800 [2b] (±1.660)	0.867 (±1.145)	1.200 (±0.506)
Probiotic 2 with prebiotic	0	0	0.067 (±0.149)	0.133 (±0.182)	0.600 (±0.548)	0.467 (±0.506)	0.067 (±0.149)	0.533 [a] (±0.691)	0.466 (±0.558)	0.933 (±1.011)

[12] Row means with the same superscript do not differ significantly (P>0.05)
[ab] Column means with the same superscript do not differ significantly (P>0.003)
^ Prebiotic (Fructo-oligosaccharide)
*** Probiotic 1: Consisted of the spore-forming bacteria *Brevibacillus laterosporus***
[#] Probiotic 2: Consisted of *Lactobacillus spp*.

Table 4.16.1: Duodenal measurements of broilers that were challenged with *Salmonella typhimurium* receiving probiotics and/or a prebiotic on day 21

Treatment	Day 21					
	Villi height		Mucosal thickness		Crypt depth	
	Non-challenged	Challenged	Non-challenged	Challenged	Non-challenged	Challenged
No additives	350.1 (±22.69)	404.0 (±48.71)	423.0 [a] (±27.05)	477.4 (±26.55)	72.89 (±17.96)	73.39 (±30.81)
Prebiotic^	520.3 (±167.2)	773.5 (±349.5)	641.5 (±162.3)	854.2 (±365.0)	121.2 (±6.274)	80.78 (±15.65)
Probiotic 1*	537.6 (±112.4)	617.2 (±164.5)	624.2 (±132.0)	708.1 (±157.8)	86.53 (±23.32)	90.91 (±24.27)
Probiotic 1 with prebiotic	593.6 (±148.0)	823.1 (±312.0)	705.4 (±171.4)	985.1 (±470.7)	111.7 (±25.53)	161.9 (±166.6)
Probiotic 2#	424.7 (±71.75)	527.3 (±41.65)	523.7 (±90.75)	613.1 (±36.08)	98.99 (±19.00)	85.78 (±8.003)
Probiotic 2 with prebiotic	780.8 (±268.1)	777.7 (±198.4)	960.2 [b] (±369.0)	957.3 (±277.2)	179.4 (±101.8)	179.6 (±81.78)

[12] **Row means with the same superscript do not differ significantly (P>0.05)**
[ab] **Column means with the same superscript do not differ significantly (P>0.003)**
^ Prebiotic (Fructo-oligosaccharide)
*** Probiotic 1: Consisted of the spore-forming bacteria *Brevibacillus laterosporus***
 # Probiotic 2: Consisted of *Lactobacillus spp.*

Table 4.16.2: Duodenal measurements of broilers that were challenged with *Salmonella typhimurium* receiving probiotics and/or a prebiotic on day 35

Treatment	Day 35					
	Villi height		Mucosal thickness		Crypt depth	
	Non-challenged	Challenged	Non-challenged	Challenged	Non-challenged	Challenged
No additives	1079 (±106.5)	1061 (±214.4)	1351 (±99.84)	1278 (± 297.2)	272.1 (±74.27)	217.3 (±86.58)
Prebiotic^	1031 (±32.26)	1003 (±197.4)	1256 (±64.39)	1334 (±323.4)	225.0 (±34.86)	330.76 (±229.3)
Probiotic 1*	919.0 (±140.4)	996.3 (±152.6)	1145 (±82.17)	1231 (±142.9)	225.7 (±76.44)	234.8 (±86.70)
Probiotic 1 with prebiotic	1212 (±200.9)	1023 (±86.06)	1373 (±208.3)	1178 (±52.97)	161.4 (±33.47)	156.0 (±57.96)
Probiotic 2#	1227 [1] (±77.01)	788.6 [2] (±114.8)	1443 [1] (±52.973)	931.1 [2] (±116.9)	216.2 (±26.47)	142.6 (±63.84)
Probiotic 2 with prebiotic	1193 (±222.1)	1080 (±116.0)	1373 (±240.0)	1284 (±138.7)	180.2 (±38.53)	204.0 (±22.75)

[12] Row means with the same superscript do not differ significantly (P>0.05)
[ab] Column means with the same superscript do not differ significantly (P>0.003)
^ Prebiotic (Fructo-oligosaccharide)
*** Probiotic 1: Consisted of the spore-forming bacteria *Brevibacillus laterosporus***
 # Probiotic 2: Consisted of *Lactobacillus spp*.

Table 4.17.1: Jejenal measurements of broilers that were challenged with *Salmonella typhimurium* receiving probiotics and/or a prebiotic on day 21

Treatment	Day 21					
	Villi height		Mucosal thickness		Crypt depth	
	Non-challenged	Challenged	Non-challenged	Challenged	Non-challenged	Challenged
No additives	852.9 (±235.5)	591.2 (±203.8)	1081 (±359.4)	695.0 (±217.0)	227.9 [1] (±128.3)	103.9 [2] (±42.85)
Prebiotic^	737.7 (±149.8)	906.7 (±179.0)	927.2 (±204.5)	1090 (±163.5)	189.5 (±61.55)	182.8 (±31.21)
Probiotic 1*	847.0 (±139.1)	855.7 (±349.8)	1061 (±197.6)	1021 (±418.7)	213.7 (±59.23)	165.6 (±84.67)
Probiotic 1 with prebiotic	773.6 (±174.9)	617.1 (±125.2)	962.5 (±152.7)	736.2 (±149.9)	188.9 (±23.48)	119.2 (±31.91)
Probiotic 2#	655.8 (±189.6)	544.0 (±138.1)	807.8 (±232.0)	658.9 (±193.0)	152.0 (±57.70)	115.0 (±61.06)
Probiotic 2 with prebiotic	653.1 (±152.4)	778.8 (±210.0)	791.6 (±200.7)	916.7 (±224.7)	138.5 (±48.48)	137.9 (±17.42)

[12] Row means with the same superscript do not differ significantly (P>0.05)
[ab] Column means with the same superscript do not differ significantly (P>0.003)
^ Prebiotic (Fructo-oligosaccharide)
*** Probiotic 1: Consisted of the spore-forming bacteria *Brevibacillus laterosporus***
 # Probiotic 2: Consisted of *Lactobacillus spp.*

Table 4.17.2: Jejenal measurements of broilers that were challenged with *Salmonella typhimurium* receiving probiotics and/or a prebiotic on day 35

Treatment	Day 35					
	Villi height		Mucosal thickness		Crypt depth	
	Non-challenged	Challenged	Non-challenged	Challenged	Non-challenged	Challenged
No additives	848.2 (±241.7)	865.5 (±148.5)	1013 (±324.2)	1028 (±177.5)	164.8 (±83.23)	162.4 (±29.63)
Prebiotic^	840.7 (±159.4)	933.9 (±380.9)	987.8 (±163.4)	1092 (±478.1)	147.1 (±98.24)	158.2 (±102.2)
Probiotic 1*	932.0 (±190.1)	983.6 (±281.5)	1142 (±210.7)	1180 (±355.2)	210.0 (±30.58)	196.7 (±78.82)
Probiotic 1 with prebiotic	808.0 (±111.8)	925.4 (±281.8)	954.4 (±153.8)	1107 (±323.9)	146.4 (±46.59)	181.4 (±45.21)
Probiotic 2#	959.6 (±404.0)	1099 (±300.0)	1183 (±514.7)	1318 (±452.9)	223.8 (±120.8)	219.3 (±157.5)
Probiotic 2 with prebiotic	952.1 (±195.3)	1113 (±361.0)	1151 (±205.2)	1312 (±416.2)	199.2 (±18.74)	198.8 (±59.68)

[12] Row means with the same superscript do not differ significantly (P>0.05)
[ab] Column means with the same superscript do not differ significantly (P>0.003)
^ Prebiotic (Fructo-oligosaccharide)
*** Probiotic 1: Consisted of the spore-forming bacteria *Brevibacillus laterosporus***
 # Probiotic 2: Consisted of *Lactobacillus spp*.

Table 4.18.1: Ileal measurements of broilers that were challenged with *Salmonella typhimurium* receiving probiotics and/or a prebiotic on day 21

Treatment	Day 21					
	Villi height		Mucosal thickness		Crypt depth	
	Non-challenged	Challenged	Non-challenged	Challenged	Non-challenged	Challenged
No additives	490.1 (±228.8)	463.3 (±202.3)	601.8 (±243.3)	557.9 (±229.3)	111.7 (±26.42)	94.69 (±30.73)
Prebiotic^	364.9 (±50.33)	581.0 (±173.6)	496.3 (±64.38)	714.4 (±220.9)	131.4 (±24.77)	133.4 (±47.52)
Probiotic 1*	460.8 (±80.43)	664.8 (±49.91)	582.4 (±113.9)	769.4 (±50.56)	121.6 (±47.52)	104.7 (±11.04)
Probiotic 1 with prebiotic	544.2 (±113.7)	733.8 (±102.6)	665.1 (±129.9)	880.6 (±102.9)	120.9 (±65.09)	146.8 (±27.01)
Probiotic 2#	495.8 (±181.8)	481.0 (±68.42)	597.7 (±213.5)	602.3 (±104.2)	101.9 (±31.90)	121.3 (±38.14)
Probiotic 2 with prebiotic	652.2 (±214.7)	640.8 (±278.7)	781.6 (±245.5)	753.2 (±340.0)	129.4 (±35.57)	112.4 (±65.14)

[12] Row means with the same superscript do not differ significantly (P>0.05)
[ab] Column means with the same superscript do not differ significantly (P>0.003)
^ Prebiotic (Fructo-oligosaccharide)
*** Probiotic 1: Consisted of the spore-forming bacteria *Brevibacillus laterosporus***
 # Probiotic 2: Consisted of *Lactobacillus spp.*

Table 4.18.2: Ileal measurements of broilers that were challenged with *Salmonella typhimurium* receiving probiotics and/or a prebiotic on day 35

Treatment	Day 35					
	Villi height		Mucosal thickness		Crypt depth	
	Non-challenged	Challenged	Non-challenged	Challenged	Non-challenged	Challenged
No additives	852.3 [1] (±159.3)	599.8 [2] (±122.9)	949.1 (±166.3)	699.3 (±156.9)	96.82 (±7.151)	99.56 (±36.58)
Prebiotic^	683.0 (±181.5)	613.5 (±97.29)	789.4 (±208.7)	713.8 (±99.27)	106.3 (±35.52)	100.3 (±18.70)
Probiotic 1*	725.1 (±183.1)	686.7 (±98.50)	824.6 (±159.9)	798.0 (±120.0)	99.44 (±26.53)	111.3 (±30.31)
Probiotic 1 with prebiotic	746.0 (±65.66)	750.4 (±152.6)	915.1 (±177.8)	833.7 (±156.1)	169.1 [1] (±112.9)	83.285 [2] (±4.772)
Probiotic 2#	701.0 (±159.2)	497.3 (±58.68)	801.3 (±123.0)	603.7 (±60.72)	100.3 (±36.34)	106.4 (±8.151)
Probiotic 2 with prebiotic	574.5 (±181.6)	550.3 (±129.0)	682.4 (±212.7)	691.1 (±119.2)	107.8 (±32.68)	140.8 (±17.80)

[12] Row means with the same superscript do not differ significantly (P>0.05)
[ab] Column means with the same superscript do not differ significantly (P>0.003)
^ Prebiotic (Fructo-oligosaccharide)
*** Probiotic 1: Consisted of the spore-forming bacteria *Brevibacillus laterosporus***
 # Probiotic 2: Consisted of *Lactobacillus spp.*

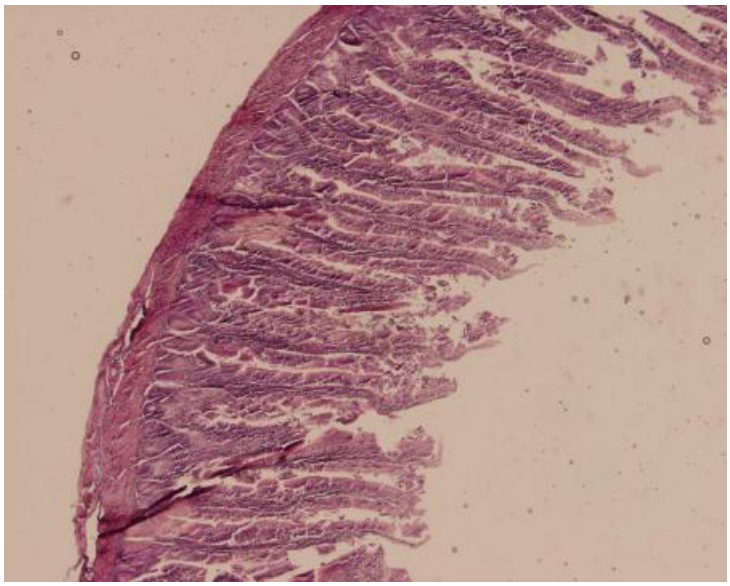

Figure 4.1: Histology of the ileum of the challenged birds treated with the Probiotic type 1 treatment on day 21.

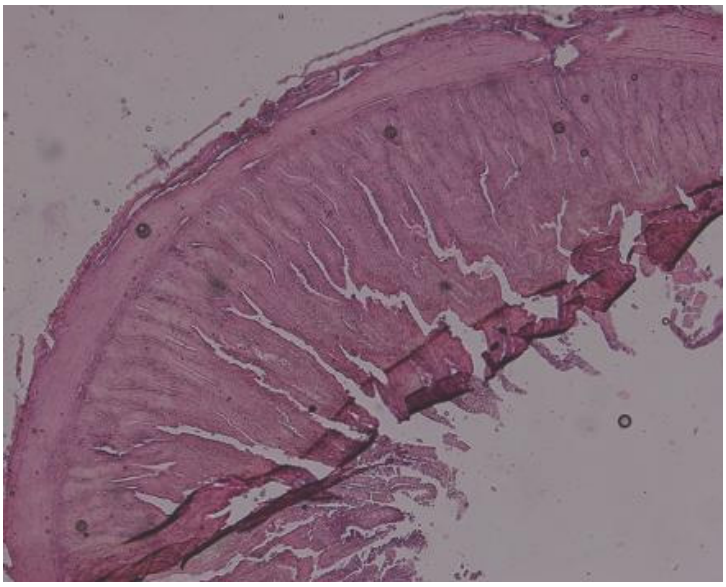

Figure 4.2: Histology of the duodenum of the challenged birds treated with the Probiotic type 1 combined with a prebiotic treatment on day 35.

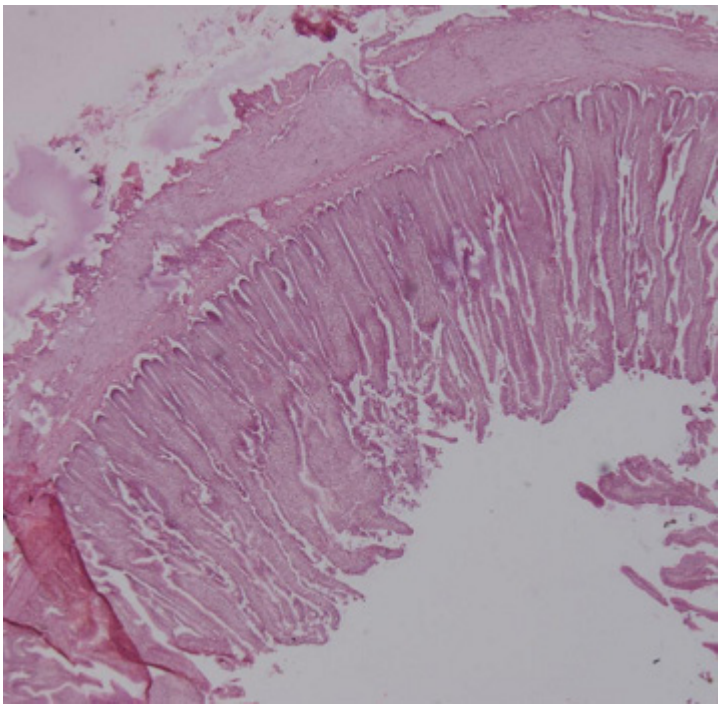

Figure 4.3: Histology of the jejunum of the non-challenged birds treated with a Prebiotic on day 21.

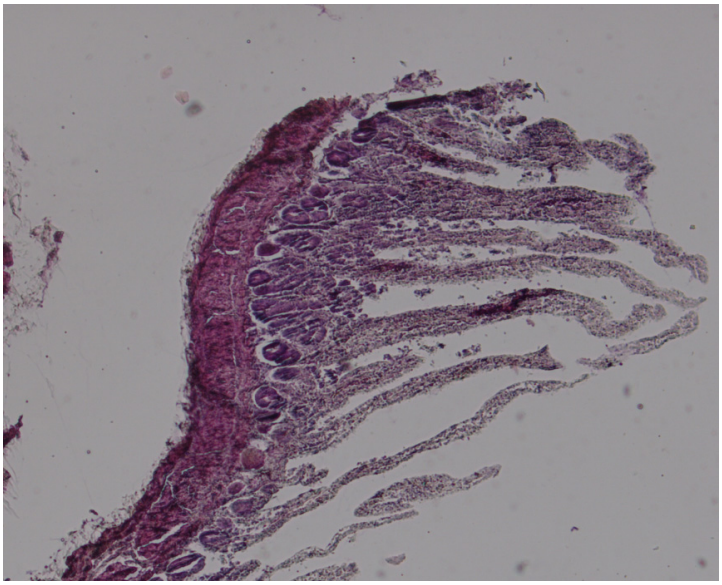

Figure 4.4: Histology of the jejunum of the non-challenged birds treated with the Probiotic type 2 combined with a prebiotic treatment on day 35.

CHAPTER 5: DISCUSSION

Antibiotics are used world wide in the poultry industry to improve meat and egg production and preventing poultry pathogens from causing diseases. However, common problems such as the development of drug-resistant bacteria, imbalance of normal microflora and drug residues in the body of the birds, have arisen due to the use of dietary antibiotics (Awad *et al*., 2009). Therefore, alternative approaches to antibiotics to non-challenged bacterial diseases in humans and veterinary medicine has been developed. The idea of these alternatives being "natural" is one of the reasons why consumers are so interested (Reid and Friendship, 2002).

The role of a diet is to modulate various functions of the body and also provide enough nutrients to fulfill metabolic requirements of the body. Beneficial micro-organisms, such as probiotics, prebiotics and synbiotics, hold considerable promise for the healthcare industry which can be harnessed by the food manufacturers (Awad *et al.*, 2009).

The dietary inclusion of probiotics and a prebiotic to improve the health and performance of broilers challenged with *Salmonella typhimurium* was investigated in this feeding trial. Six dietary treatments were randomly assigned to 1800 chicks (900 chicks exposed to Salmonella and 900 chicks not exposed) for 5 weeks. The dietary treatments were: 1) No feed additives added, 2) A prebiotic (fructo-oligosaccharide) added to the feed, 3) Probiotic type 1 (Spore-forming bacteria) added to the feed, 4) Probiotic type 1 combined with the prebiotic added to the feed, 5) Probiotic type 2 (*Lactobacillus* spp.) added to the feed, 6) Probiotic type 2 combined with the prebiotic added to the feed.

Overall, the non-challenged birds performed better than the Salmonella challenged birds. The non-challenged birds achieved higher feed intakes, average daily gains and bodyweight than the challenged birds, which indicates that Salmonella affected the performance of the challenged birds.

The integrity of the intestinal barrier is positively affected by the gut microbiota with its metabolic, tropic and protective function. A progressive increase of intestinal permeability is caused by intestinal barrier dysfunction which induces a switch from physiological to pathological inflammation that is characteristic of disease. This has a direct impact on gut microbial composition and susceptibility to enteric pathogens. Poor growth rate and productivity in livestock and poultry is generally caused by stress situations, such as disease, which explains why the challenged birds performed worse than the non-challenged birds regarding feed intake, average daily gain and body weight

and overall growth performance. Intestinal inflammation is promoted and maintained by an increase of pathogenic bacteria and a decrease in health-promoting bacteria. Toxins and other substances are produced by intestinal pathogens which interfere with epithelial metabolism. Therefore, uncontrolled pathological inflammation can be triggered by the pathogenic phenotype (Gaggìa *et al*, 2010). Probiotics and prebiotics neutralises these toxins produced by pathogens, they also stimulate immunity and produces a systemic effect on feed utilisation (Choudhari *et al*, 2008). Younus (2006) also found that birds infected with *Salmonella typhimurium* had lower weight gains compared to non-challenged birds. In this study, the treatment of broilers, both challenged and non-challenged, with probiotics in combination with a prebiotic improved the performance parameters of the birds and proved more effective than the supplementing Probiotics or Prebiotic alone.

When Probiotic type 2 was combined with the prebiotic, feed intake of the non-challenged birds improved, which correlates with the results of Ignatova *et al*. (2009), who found that the total feed intake was higher for chickens fed a probiotic-supplemented diet than those fed a basal diet without additives. The Probiotic type 2 in combination with the prebiotic treatment given to the non-challenged birds and the Probiotic type 1 combined with the prebiotic treatment given to the challenged birds increased the average daily gain of the birds, compared to the other treatments. Yang *et al*. (2009) found that the average daily gain of birds increased when combination treatments were given and proved to be more effective in reducing Salmonella than treatments alone. These results are also in agreement with the findings of Awad *et al*. (2009) which proved that birds supplemented with a synbiotic showed an increase in average daily gain compared to birds receiving no supplementation or only probiotics.

Probiotics deliver many lactic acid bacteria into the GIT upon consumption. Enzymes and other beneficial substances are delivered into the intestines by these micro-organisms which modifies the intestinal milieu (Lutful Kabir *et al*, 2009). Probiotic microbes and pathogenic bacteria start competing for nutrients. The growth of pathogenic micro-organisms in the intestines is supressed on the one hand and on the other the bioavailability to dietary minerals, growth rate and feed efficiency is increased. Lactobacilli bacteria ferment lactose to lactic acid which reduces the pH to a level that harmful bacterial cannot tolerate which favours increased activity for intestinal enzymes and digestibility of nutrients (Choudhari *et al*, 2008).

The non-challenged birds that received the Probiotic type 1 and prebiotic combination treatment gained higher bodyweights than the challenged birds that received

only the prebiotic treatment. These results relates to the findings of Takahashi *et al*. (2005) where birds fed diets supplemented with probiotics and prebiotics showed an increase in body weight. Vincente *et al*. (2007) also found that the body weight of birds was increased by treating them with probiotics. It could be that the additives given to the challenged birds reduced the Salmonella counts and therefore enabled the birds to achieve better body weights and average daily gains as proven in the results. Selected probiotic cultures controls food-borne pathogens in the birds gut and potentially increase performance parameters such as growth rate and body weight gain. The main application of probiotics is the prevention of gastrointestinal infection and disease more than a curative approach. This is because probiotics action is to modulate the gastrointestinal environment reducing the risk of disease synergistically with the immune system of the host, unlike antibiotics which aim at killing pathogen bacteria (Gaggìa *et al*, 2010).

The treatments did not have any significant effects on the feed conversion ratio of the broilers, although the challenged birds that received additives showed a slight improvement in the feed conversion ratio on day 7. The findings of Pelícia *et al*. (2004) and Ahmad (2004) relate to these results. However, Ahmad (2006) found a discrepancy regarding feed conversion ratio results, as some studies showed that the supplementation of probiotics in feed of birds improved the feed conversion ratio while others suggested no such effect. The results of Awad *et al*. (2009) revealed that birds supplemented with synbiotics had lower feed conversion ratios than the birds receiving no supplementation or probiotics.

The non-challenged birds and the challenged birds fed the Probiotic type 2 combined with the prebiotic treatment and the prebiotic treatment alone, respectively, displayed a decrease in liver weight, compared to no supplementation, Probiotic type 1 combined with the prebiotic treatment and Probiotic type 1 alone. These results are in agreement with the findings of Awad *et al*. (2009). They proved that birds supplemented with synbiotics showed a decrease in liver weight compared to birds supplemented with probiotics and no supplementation. However, Pelícia *et al.* (2004), Islam *et al*. (2004) and Tarun (2008) found that probiotics in the diet had no effect on liver weight.

The GIT of the birds did reveal changes after the supplementation of additives in the feed. The duodenum and jejunum of the non-challenged and challenged birds were enlarged on day 7, which was shortly after Salmonella exposure. However, the challenged birds had enlarged duodenum and jejunums with no supplementation in the diet; whereas the non-challenged birds showed enlarged duodenums after Probiotic type 1 treatment

was supplemented. Takahashi *et al*. (2005) proved similar results and found that the small intestine of birds fed additives in the diet had higher weights. However, Pelícia *et al*. (2004) proved otherwise and found that the addition of probiotics and prebiotics had no effect on the digestive tract of birds, probably because the intestinal flora is balanced and the additives showed no response in such situations. There were no significant changes in the ileal weight after Salmonella-exposure or by adding additives to the diet. However, the non-challenged and challenged birds displayed enlarged caecae on day 7. The only significant difference was among the non-challenged birds where the Probiotic type 1 treatment caused higher cecal weights than the Probiotic type 1 combined with the prebiotic treatment.

By supplementing feed additives to the feed did not significantly affect the blood serum variables. The total serum protein showed no effect. The challenged birds revealed lower albumin levels than the non-challenged birds, for most of the treatments with no significant differences among the treatments. During the first week of the trial, the challenged birds that received no supplementation revealed significantly higher globulin levels compared to the birds that received the Probiotic type 2 treatment with no other significant differences among the treatments or between the groups. Fernández *et al*. (2001) proved similar results and found that the globulin levels increased in the presence of infection. This could probably mean that the Probiotic type 2 treatment reduced the Salmonella infection, therefore revealing lower globulin levels in the challenged birds. The non-challenged birds had higher albumin: globulin ratio than the challenged birds, but was only significant during the first week of the trial. Overall, the aspartate amino transferase (AST) values did not show any significant effects. In the non-challenged group the prebiotic treatment had extremely high levels of AST on day 7 compared to the other treatments showing no such effect. This result can not be explained.

Treating birds with probiotics and a prebiotic did not have significant effects on the villus height, mucosal thickness or crypt depth of the duodenum. Although, the non-challenged birds revealed a thicker mucosa on day 21 after being treated with the Probiotic type 2 combined with the prebiotic. Pelícia *et al*, 2004 proved that birds fed probiotics and prebiotics in the diet and challenged with Salmonella had no differences in the length of the duodenum and jejunum. However, Edens (2003) and Awad *et al*. (2009) suggested otherwise and found that the villus height increased and the crypt depth decreased when chickens were fed probiotics. The jejunum and ileum revealed no significant differences or effects on villous height, mucosal thickness or crypt depth.

In this study, the Probiotic type 2 combined with the prebiotic treatment proved to be the most effective for improving broiler performance. According to Sharif (2003) a combination of non-defined probiotics and prebiotics leads to more effective non-challenged of Salmonella than the treatments individually.

CHAPTER 6: CONCLUSION

This study showed that birds infected with *Salmonella typhimurium* had poorer performance in terms of body weight, average daily gain and feed intake which caused the non-challenged birds to perform better than the challenged birds. The broilers had enlarged duodenums, jejunums and caecae on day 7. Lower albumin levels were shown by the challenged birds compared to the non-challenged birds. The Probiotic type 2 treatment proved to decrease the Salmonella infection in the challenged birds by having lower globulin levels. No effects were shown by the total serum protein and AST levels.

Neither Salmonella-exposure nor supplementation with probiotics and a prebiotic affected the villous height, crypt depth and mucosal thickness of the duodenum, jejunum and ileum of broilers, with the only exception being on day 21 when the non-challenged birds had a thicker mucosa in the duodenum after being treated with the Probiotic type 2 combined with a prebiotic.

This study demonstrated that the supplementation of a basal diet with probiotics to have promising effects on the performance of the broilers in terms of feed intake, average daily gain and body weight. The feed conversion ratio did not show any significant changes. By treating broilers with only a prebiotic did not show any significant effects on their performance.

The combination of a probiotic and a prebiotic, especially the non-spore forming Probiotic type 2 combined with a prebiotic showed the most promising results. The probiotics alone did show growth-promoting effects, but not as effective as the synbiotics. This study also demonstrated that non-spore forming probiotics showed better results than spore-forming probiotics. It is therefore recommended to supplement a basal diet with feed additives, especially synbiotics, one of which is non-spore forming probiotics, to improve the performance and feed efficiency of broilers.

As pressure increases to eliminate antibiotic growth promoters in animal feed these products might be promising alternatives to improve poultry production without relying on antibiotics.

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APPENDIX A

Millonig's buffered formalin solution

The following reagents are needed to prepare 50L of a 10%-buffered formalin solution:

- 857.2g Sodium dihydrogen phosphate (NaH_2PO_4)
- 173.9g Sodium hydroxide (NaOH)
- 242.8g Glucose ($C_6H_{12}O_6$)
- 5L 40%-formalin (H_2CO)

Method:

Dissolve each of the dry chemicals separately in 1800ml water on a magnetic stirrer. Add the 3 solutions to the 5L formalin in a 50L container. Fill the container with TAP water up to the 50L mark.

APPENDIX B

Mayer's Hematoxylin and Eosin staining protocol

The following reagents were used to make up the hematoxylin solution:

- Aluminium ammonium sulphate 50g
- Hematoxylin 1g
- Sodium iodate 0.2g
- Citric acid 1g
- Distilled water1000ml

The chemicals were dissolved in the order listed above. For example, the aluminium ammonium sulphate was dissolved in 1000ml distilled warm water (±40°C) first. When the aluminium was completely dissolved, the hematoxylin was added, and when the hematoxylin was dissolved, the sodium iodate was added, etc.

The Eosin Y solution was made up as follows:

Firstly, the Eosin Y Stock solution (1%) was made up by mixing the following reagents until they dissolved and was then stored at room temperature:

- Eosin Y 10g
- Distilled water 200ml
- 95% Ethanol 800ml

Secondly, the Eosin Y working solution (0.25%) was made up by mixing the following together, and stored at room temperature:

- Eosin Y stock solution 250ml
- 80% Ethanol 750ml
- Glacial acetic acid (concentrated) 5ml