Performance of Clostridium perfringens-challenged broilers inoculated with Effective Microorganisms

BY

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Declaration:

I, ………………………………………………….. declare that the thesis/dissertation, which I hereby submit for the degree …………………………………..……………… at the University of Pretoria, is my own work and has not previously been submitted by me for a degree at this or any other tertiary institution.

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TABLE OF CONTENTS   **PAGES**

ACKNOWLEDGMENTS

LIST OF ABREVIATIONS

AA- amino acids

ADG- average daily gain

AGP- antibiotic growth promoters

Al (OH3)- aluminium hydroxide

AME- apparent metabolisable energy

&- and

ANOVA- analysis of variance

ß- beta

BHI- Brain Heart Infusion

BW- body weight

BWG- body weight gain

CD- cadmium

CE- competitive exclusion

CFU- colony-forming units

CV- coefficient of variation

^{o}C- degrees Celsius

DoA- Department of Agriculture

E. coli-Escherichia coli

EE- exogenous enzymes

e.g.- for example

EM- effective microorganisms

et al.- et alia

EU- European Union

FCR- feed conversion ratio

FDA- food and drug administration

g- gram

>- greater than

GIT- gastro-intestinal tract

kg- kilogram

i.e.- that is

IgA- immunoglobulin A

IgM- immunoglobulin M

IBM- initial body weight

LAB- lactic acid bacteria

LSD- least significant difference

L- litre

Ltd- limited

ME- metabolisable energy

MJ- mega-joule

mm- millimetre

mL- millilitre

m^2- square metre

NRC- National Research Council

NSP- non-starch polysaccharides

%- percentage

P- probability

PCR- polymerase chain reactor

PEF- production efficiency factor

Pty- proprietary

SCFA- short chain fatty acids

SD- standard deviation

SEM- standard errors of means

<- smaller than

spp- several species

UK- United Kingdom

USA- United States of America

USDA-United States Department of Agriculture

VFA- volatile fatty acids

WHO- World Health Organisation

WVA- World Veterinary Association

ABSTRACT

The first study was conducted to evaluate the dietary inclusion of effective microorganisms (EM) on body weight (BW), feed intake, feed conversion ratio (FCR) and mortality of broilers, which had been either challenged or non-challenged with *Clostridium perfringens* (1mL of 1 x 10^8 CFU/mL orally). Six hundred and forty day-old Ross 788 broiler chicks were randomly allocated to thirty-two pens in groups of twenty birds per pen, giving a stocking density of ±18 birds per square meter from zero to forty days of age. The facility consisted of two rooms with sixteen pens per room. All the chicks were fed on a commercial maize-soya type diet, including a mash starter and a mash grower/finisher feed. At two weeks of age 320 chicks were inoculated with *Clostridium perfringens* type A through oral administration. The study had a randomised block design with four replicates and four treatments as: 1) Control-unmedicated; 2) antibiotic growth promoters (AGP) added to feed at 33g/kg; 3) EM added to feed and water at 50g/kg and 50mL/L respectively; and 4) AGP in feed at 33g/kg and EM (50g/kg) in feed and water (50mL/L). The inclusion of EM negatively affected water palatability that resulted in reduced water intake and increased FCR for the non-challenged broilers at 21 days of age. However, because feed intake was not affected, it was suggested that EM should rather be supplemented through the feed rather than through the water. The use of AGP alone or in combination with EM proved ($P<0.05$) broiler production performance. The cumulative feed intake, BW, FCR, average daily gain (ADG), cumulative water intake and production efficiency factor (PEF) of challenged broilers were not different ($P>0.05$) at 40 days of age. In this study the incidence of mortality was low (2.2%) and examination of livers and intestines showed only mild necrotic enteritis lesions. In conclusion, the findings of this study showed that EM under the current dosage failed to improve broiler production performance.

A second experiment was conducted as a follow-up study to evaluate the effect of EM on broiler performance when supplemented through the feed only. Cloacal swabs were taken from all day-old chicks and a day after inoculation with *Clostridium perfringens* for laboratory analysis of the microorganisms in the gut. All the chicks were fed on a

1

commercial two-phase maize-soya type diet consisting of a mash starter and a mash grower/finisher feed with additional fishmeal. The chicks from one room were inoculated orally with *Clostridium perfringens*, while the chicks from the other room remained unchallenged. The challenged group was inoculated orally with 1 mL (1 x 10^8 CFU/mL) of *Clostridium perfringens* as a single dose on day 14. The EM was supplemented to the broilers from day one through the feed. The supplementation of EM through the feed showed a poor performance for non-challenged whilst for the challenged showed an improved performance at 3 weeks. Both non-challenged and challenged broilers given EM had a poor performance at 6 weeks of age, and this showed inconsistent results throughout the experiment. However, it was found that the combination of both AGP and EM had a better performance than EM alone. It is noted when the results of broilers non-challenged versus challenged control groups were compared, the challenged ones showed a better performance. The broilers showed a low mortality of 1.3% and the causes were not related to the incidence of necrotic enteritis. The gross examination of the broilers inoculated orally with *Clostridium perfringens* showed mild intestinal lesions.

LIST OF TABLES

CHAPTER 1
INTRODUCTION

Healthy broilers are generally characterized as having a well functioning gastrointestinal tract (GIT). This is fundamental for the efficient conversion of feed for maintenance and for growth or production (Jin *et al*., 1997a). A most important characteristic of a well-functioning GIT is the balance of its bacterial population. Gut mucosal surfaces play a crucial role in the exclusion and elimination of potentially harmful dietary antigens and enteric microorganisms and with the aid of intestinal microflora, are essential in maintaining healthy flocks and minimizing losses associated with various diseases and stressors (Dalloul *et al*., 2003).

Broiler producers rely on the sub-therapeutic use of antibiotic growth promoters (AGP) in diets, which have proved to be an effective way of enhancing animal health status, uniformity and production efficiency and to prevent broilers from acquiring pathogenic bacterial infections. Healthy broilers with a stable intestinal microflora is thought to be maintained by using antibiotics in preventative dosages, but these low dosages have negative effects on *Lactobacilli* and other lactic acid producing bacteria. *Lactobacilli* constitute the largest part of aerobic microflora in the gastro-intestinal tract (GIT) and are very susceptible to antibiotics.

It has been reported that broilers are stressed by various factors such as transportation, overcrowding, vaccination, chilling and/or overheating which tend to create an imbalance in the intestinal microflora and a lowering of body defence mechanisms (Jin *et al*., 1997a). Such circumstance, AGP is often used to suppress or eliminate pathogenic organisms in the intestines. Montagne *et al*. (2003) found that the long term and extensive use of antibiotics for medical and veterinary purposes may eventually result in the selection for the survival of resistant bacterial species. Genes encoding for this resistance can be transferred to other formerly susceptible bacteria, thus posing a threat to animal and human health.

Due to the growing public concern over the transmission of and the proliferation of resistant bacteria through the food chain, the European Union decided in 1999 to ban four commonly used growth promoters namely: virginiamycin, spiramycin, tylosin and zinc bacitracin (Huyghebaert, 2003). Flavomycin, avilamycin and salinomycin were discontinued from animal feeds from the 1st January 2006. Various international bodies such as World Veterinary Association (WVA) and World Health Organisation (WHO) are also calling for the universal withdrawal of AGP in food-producing animals. South Africa has adopted a responsible approach towards the use of AGP and legal framework for the testing of antibiotic residue levels is controlled by the "Foodstuffs, Cosmetics and Disinfectants Act" (Maritz, 2005a).

Development of alternative products and improved management is therefore necessary to eliminate the dependence on AGP to maintain performance while maintaining the same productivity (Garrido *et al*., 2004). Santoma (2005) reported that after AGP was banned in Sweden 1986, animals have decreased their performance and mortality rates and medication costs have increased while carcass quality was somewhat impaired. In New Zealand zinc bacitracin is used as a script medication at levels that promote growth and performance, and they also banned some antibiotics such as avoparcin for use in animal feed that are particularly important in human medicine (Durrans, 2005).

The search for alternatives to antimicrobials has led to the use of effective microorganisms (EM), which are mixed cultures based on the blending of a multitude of microbes including organisms such as lactic acid bacteria, yeasts, actinomycetes and photosynthetic bacteria (Higa & Parr, 1994). EM acts similarly to probiotics as environmentally friendly microorganisms and are defined as living microorganisms which, when present at certain levels in the GIT, provide equilibrium of the intestinal microflora that will enhance the health of the host.

Addition of EM to diets or drinking water of broilers has shown improved body weight and quality of products (Hussain, 2000). Probiotic bacteria are selected from strains of microorganisms most beneficial to the host intestinal bacteria, and are classified as

colonizing species and they include *Lactobacillus spp., Enterococcus spp., Streptococcus spp.* and other species such as *Bacillus spp.* and the yeast *Saccharomyces cerivisiae* (Tomasik & Tomasik, 2003). These microorganisms inhibit the growth of potentially pathogenic microorganisms such as *Salmonella spp*, *Shingella spp*, *Campylobacter* and *E. coli* by lowering the pH through production of lactate, lactic acid and volatile fatty acids. Probiotics are often used as feed additive in poultry in intensive rearing systems. It was found that probiotics enhanced mineral absorption, synthesis and absorption of vitamins, especially of the B-vitamin group, which are important for the normal function of the nervous system and have positive effects on stress. The use of probiotics as a substitute was shown to promote growth and feed efficiency when introduced as an alternative to AGP (Jin *et al.*, 1997b). Several researchers have reported similar improvements on the growth performance on broilers given probiotics (Jin *et al.*, 1998; Jin *et al.*, 2000; Kralik *et al.*, 2004).

It has been reported that a well-established normal intestinal microflora competes with pathogens and hence decreases the risk of salmonellosis, *Clostridium perfringens*-associated lesions, campylobacteriosis and colibacillosis (Garrido *et al.*, 2004). The hypothesis of the study was that there is no difference between the efficacy of EM and AGP in preventing necrotic enteritis caused by *Clostridium perfringens*. This study was therefore undertaken to evaluate the use of effective microorganisms in water and feed on the performance of broilers challenged and non-challenged with *Clostridium perfringens* type A. In addition, intestinal lesions in broilers challenged with *Clostridium perfringens* type A were evaluated.

CHAPTER 2
Literature Review

2.1. Poultry management practices

Intensive livestock and poultry rearing practices have led to an increase in animal stress and incidences of diseases. Intestinal infection diseases such as coccidiosis, *Escherichia coli*, *Lactobacillus acidophilus*, *Campylobacter jejuni,* salmonellosis, necrotic enteritis and cryptosporidiosis are becoming increasingly prevalent in commercially-bred broilers and are inflicting severe economic losses on the poultry industry (Collett, 2004). The above microorganisms in nature always appear in communities. These microorganisms are dependent on each other and their environment and cannot easily be isolated from their habitats by routine culturing. Many bacteria have growth requirements which are fulfilled by their natural habitats and other synergistic bacterial species living in the same community. Examples of such habitats are the animal intestines, lake, river sediments and soil (Apajalahti *et al*., 2004).

There are a number of predisposing factors contributing to the high incidences of diseases with poor management and concurrent infection playing major roles. Management is one very important factor with direct implications on broiler health and welfare. The main production system used by South African farmers is litter floors in an environmentally controlled system. The main components of good management involve adequate housing, proper nutrition, and appropriate health care.

2.1.1 Housing

The first requirement for profitable broiler production is adequate housing, because broiler production is essentially a chick-brooding operation. The house should contain such equipment as is necessary to enable the regulation of factors such as temperature, moisture, ventilation (air quality) and light. It should also provide for efficient installation and operation of brooding, feeding, watering and other equipment.

According to Collett (2004), stress associated with poor management of the housing environment is closely related to factors such as:

a) Overstocking, resulting in rapid deterioration of litter quality and competition for access to feeders and drinkers.

b) Saturation of litter due to improper operation of ventilation systems.

c) Starvation associated with delays in feed delivery.

d) Disturbances associated with manual vaccination, weighing or partial depletion of flocks.

2.1.2 Nutrition

Proper nutrition in poultry production is needed with a balanced diet providing proteins, energy, vitamins, minerals and water in adequate proportions. Drinking water, although not considered a nutrient, deserves more attention in intensive poultry production. Good water helps in the digestion process, transportation of nutrients and the regulation of body temperature and elimination of waste. Water quality determines its fitness for consumption by broilers and in turn is essential for optimum productivity. Changes in feed composition that include the introduction of animal by-products or substitution of barley for maize result in an increase in the viscosity of ingesta, delayed passage rate, and alteration in the composition of intestinal flora.

2.1.3 Health

General health is a multifactorial facet. This requires regular monitoring of both the health of the flock (such as sections on different organs, determinations of lesion scores and identification of the intestinal flora) and the zootechnical performance (such as feed intake, weight gain, flock uniformity, litter score and climatic conditions) (Huyghebaert, 2003). Immunosuppression caused by systemic infections including infectious bursal disease, Marek's disease and chick anemia, predisposes flocks to clostridial infections. There is however several other causes of immuno-suppression including mycotoxin poisoning and stress, that pose significant risks in today's intensive production systems (Collett, 2004).

Innovations for improving animal health in animal production systems with special focus on other non-nutritional environmental factors in poultry farms have to be considered. Stocking density, excellent air quality, maintenance of litter quality (moisture content, bacterial activity, ammonia production) and hygiene are of great importance in maintaining a healthy productive flock (Collett, 2004).

2.2 **Biosecurity and hygiene**

Biosecurity plays a major role in controlling the pathogens in broiler operations. The broilers placed on a farm must be pathogen-free. Löhren (2004) reported that in Europe, even if the supplier guarantees pathogen-free day-old chicks, most companies test samples for diseases (chick box lines, dead on arrival, etc.) either on chicks before they are transported or on the farm before placement. Furthermore, when a previous flock had pathogens, efforts are being made to ensure that the infection cycle is interrupted. The critical points in the farm environment include: dust and dirt from previous flock, either in cracks in the floors or walls or in the close vicinity of the broiler house; living vectors such as rodents and dung beetles; and transportation of chicks (Löhren, 2004). It is important to note that with regard to fresh feed, salmonella often enters through certain raw materials and can persist in feed mills and re-infect heat-treated feed during cooling or later during storage and transportation.

Efforts must be made to render the farm rodent and wild-bird proof. Only authorized visitors such as veterinarians, flock supervisors and labourers should be allowed to enter the broiler farm or house. In the case of regular visitors, visits should be planned in a level and age-sensitive way, meaning from the youngest flocks first to the oldest flocks last. Farm-customised protective clothes and footwear must be worn and changing facilities and a footbath should be provided at the entrance of the broiler farm/house (Löhren, 2004).

2.3 Vaccination

The use of vaccines is a relatively new tool to control the spread of bacterial pathogens such as *Salmonella gallinarum pullorum*, *Salmonella enteritidis*, etc. However, Huyghebaert (2003) reported that there are no vaccines currently available against necrotic enteritis in the poultry industry and that vaccines *per se* may not be effective. Normally vaccines are intended to protect the broilers from clinical diseases and they have been used successfully in controlling viral diseases. There are generally two types of vaccines, inactivated vaccines and live vaccines.

2.3.1. Inactivated vaccines

Inactivated vaccines play a major role in the control of *Salmonella enteritidis*. They carry as an adjuvant either a mineral oil (oil-based vaccines) or aluminium hydroxide [Al (OH_3)]. Inactivated vaccines stimulate the immune system to produce antibodies, which neutralise the invading *Salmonellae* once they have penetrated the intestinal barrier. Oil-based *Salmonellae* vaccines provide higher protective levels than aluminium hydroxide based vaccines. The advantage of Al (OH_3) vaccines is that the birds better tolerate them than bacterial oil vaccines (Löhren, 2004).

2.3.2 Live vaccines

The first live vaccine was the *Salmonella gallinarum* vaccine for protection against *Salmonella gallinarum pullorum*. Live vaccines are applied orally against *Salmonella typhimurium* and against *Salmonella enteritidis*. Oral application stimulates the T cell system and the cellular immune system; thus they are active at the point of natural infection in the intestine by stimulating killer cells and phagocytes (Löhren, 2004). When mass vaccination is an option, spray administration of the vaccine is the preferred method. Spray vaccination is particularly true for vaccinating against Infectious Bronchitis and Newcastle disease. The main reason for this preference is that with live vaccines, strong, highly effective local immunity is reached on the mucous membrane. By spraying, one can reach the bird's mucous membranes in the eyes, nostrils, beak and deep into the respiratory track (Maree, 2007).

2.4 *Clostridium perfringens* **induced necrotic enteritis**

2.4.1 **Etiology/ cause**

Increasing interest in necrotic enteritis has been brought about by restrictions in the use of antimicrobial growth promoters (AGP). It is recognised that some components of the intestinal flora can contribute to the pathogens of severe clinical disease. High numbers of *Clostridium perfringens* type A bacteria are consistent with lesions from necrotic enteritis, a common disease caused by the alpha toxin (type A or C) of *Clostridium perfringens* (Kaldhusdal & Skjerve, 1996) in broilers. *Clostridium perfringens* is a gram-positive, spore forming anaerobic bacteria that causes serious disease in broilers through toxins they produce. *Clostridium perfringens* is divided into five types, A, B, C, D and E, based on the synthesis of four major lethal toxins, alpha, beta, epsilon and iota. Additionally, alpha toxin is produced by all types of *Clostridium perfringens* (Kalender, 2005).

Clostridium perfringens grow in the pH range 5-8 and at a substrate water activity of 0.93-0.97, and their endospores are the most resistant biological cell type. They can survive under extreme conditions, resisting heat, desiccation, acids and many chemical disinfectants (Johansson, 2006). A variety of mechanisms have been suggested to explain the growth suppressing effect of intestinal bacteria including *Clostridium perfringens*. One is through the production of toxic metabolites that irritate the gut mucosa, thereby inhibiting nutrient absorption (Wilson *et al*., 2005).

2.4.2 **Epidemiology**

Clostridium perfringens is frequently found in the intestinal tract of healthy broilers, usually at low levels ($<10^4$ CFU/g) and is spread in the poultry production and processing environment through faeces (Craven *et al*., 1999). Necrotic enteritis occurs as outbreaks characterised by depression, ruffled feathers, diarrhoea, huddling, anorexia and frequently high mortality (Wilson *et al*., 2005). Necrotic enteritis does not spread directly from bird to bird, but are ingested along with infected soil, faeces, or other infected materials (Wilson *et al*., 2005; Olkowski *et al*., 2006). Necrotic enteritis is reported to be commonly found in many bird species. Among chickens the disease is by far the most

common in broilers, but outbreaks in layer pullets and adult layer strain chickens have also been reported (Kaldhusdal & Løvland, 2002). Rapidly growing young birds, especially chickens and turkeys of three to twelve weeks of age are most susceptible to pathogens.

2.4.3 **Pathology**

A subclinical form of necrotic enteritis is characterised by the development of necrotic lesions in the gut wall and liver, as well as increased mortality (Craven, 2000). Mild lesions appear as small (in some cases barely visible to the naked eye) ulcers or light yellow spots on the surface of the mucosa, usually in the small intestines, in particular in the jejunum and ileum, and less commonly, the caeca. More severe lesions may be seen on membranes covering the entire mucosa of large segments of the large intestine, in some cases even the colo-rectum and the caecal tonsils (Kaldhusdal & Løvland, 2002).

Clostridium perfringens occurs as an acute clinical disease that eventually causes necrotic enteritis in broilers, but also as a subclinical disease with focal necrosis in the intestines. It may also manifests as *Clostridium perfringens*-associated hepatitic changes with cholangiohepatitis or fibrinoid necrosis in the liver, usually found during slaughtering or processing of broiler meat (Løvland & Kaldhusdal, 1999). An inflammation affecting the bile tree (cholangiohepatitis) is the common lesion type. Intrahepatic parts of the bile tree are most frequently affected, but gall bladder and extrahepatic bile duct changes may also be found. Another liver lesion type associated with *Clostridium perfringens* infection is multifocal hepatitis, histologically characterised by fibrinoid necrosis with or without an inflammatory response (Kaldhusdal & Løvland, 2002).

The environment in the gastrointestinal tract (GIT) is of vital importance for the growth of *Clostridium perfringens* and probably cause disturbance in the jejunum before *Clostridium pefringens* can start to proliferate in this part of the gut. Important factors to be considered are nutrition, pH, oxygen and the microflora in the jejunum, and different types of stress may also cause disturbances in the gut. It has been reported that the damage to the intestinal microflora caused by coccidial infection (e.g. *Eimeria, E.*

acervulina, *E. maxima* and to some extent *E. necatrix*) (Bedford, 2000). Changes in the normal intestinal microflora as a result of a change of ration or the use of fish meal, wheat, rye, or barley may predispose broilers to the rapid proliferation of *Clostridium perfringens* leading to necrotic enteritis which reduces growth performance, exacerbates intestinal diseases and even causes death (Choct, *et al*., 2005; Baba *et al*., 1996; Kaldhusdal & Skjerve, 1996).

However, consistence reproduction of necrotic enteritis by oral inoculation with *Clostridium perfringens* has produced varying results. Attempts to experimentally reproduce necrotic enteritis by oral inoculation in different labs has resulted in extremely variable results, including substantial mortality, severe clinical signs observed in the majority of treated birds and sub-clinical lesions of necrotic enteritis, seen as early as 5 hours after initial infusion of the broth cultures (Al-Sheikhly & Truscott, 1977). Kaldhusdal *et al*. (1999) found that the necrotic enteritis mortality and intestinal lesions of broilers were associated with substantially higher *Clostridium perfringens* counts.

3. Antimicrobial growth promoters (AGP)

3.1 AGP usage and benefits

Poultry possess a limited natural resistance and immunity against colonization or infection by pathogenic microorganisms such as *Salmonella, Shingella, Escherichia coli, Clostridium perfringens, Campylobacter* and other bacteria that are transmitted from animals to human beings through the food chain. Antibiotic growth promoters (AGP) were routinely administered to animal feeds in Europe and the rest of the world as a means to improve the performance of poultry whilst at the same time, reducing the incidence and severity of disease. Such effects are likely mediated through changes in the intestinal microflora which subsequently affect a multitude of factors including digestive enzyme output, gut motility, the quantity and quality of mucin secretion, rate of loss of enterocytes and alterations in the enteric and systemic immune system status (Bedford, 2005).

The general assumption is that AGP act as either a prophylaticum to endemic sub-clinical infection thus saving the poultry energy, or that they somehow affect the efficiency of digestion by modulating the composition of the intestinal microflora. These two mechanisms are not mutually exclusive. Most mechanisms have in common the intestinal microflora that provides both nutritional and defensive functions for the host.

3.2 Mode of action of AGP

The main site of AGP activity is within the GIT. A number of AGPs have the characteristic of growth promotants despite differences in their mechanism of action on microorganisms. It is apparent that performance enhancing is brought about by modifying mainly the intestinal gram-positive species, because of their much less complex cell wall structure (mainly consisting of peptidoglycan and both teichoic acid and teichuronic acid) compared to the gram-negative species whose cell walls consists of lipopolysaccharides (Huyghebaert, 2003).

The benefits of AGPs arise from their principal mode of action, which is directed at manipulation of the intestinal microflora. The interaction with the organisms of the gut results in improved digestion, metabolism and absorption of an array of essential nutrients including energy, protein, amino acids, minerals and vitamins. The benefits of AGPs in poultry can be broadly categorised into environmental, performance improvement and control of diseases (Page, 2005).

Besides nutritional and health effects, AGPs can enhance meat quality by reducing bacteria that produce metabolites with adverse effects on the carcass quality but with no actual effect on the health of the host itself (Apajalahti, 2005). The elimination of gut pathogens and the toxic metabolites produced, combined with selection of more efficient GIT microbial populations is key to the success of in-feed antimicrobials (Durrans, 2005). There are direct effects on the gut morphology (increased villus height and surface area and increased mucin secretion) that result in a lower feed requirement and excretion of less waste. The AGP usage may provide many additional benefits that are of value to

the consumer and animal producer, including reduced animal mortality, enhanced carcass yield, better pigmentation, drier litter and improvements in bedding quality, nutrient sparing effects, reduced manure production and decreased environmental contamination (Bafundo, 2004).

Bafundo (2004) recognized the following three concepts as reasonable explanations for improved growth with antibiotic usage and it is likely that it also depends on the environment:

3.2.1 *Control of sub-clinical disease*

Microbial populations in the GIT comprise of two distinct populations; one that exists in intimate association with the epithelium of the tract (e.g. *Lactobacilli spp.*) and one that occurs freely or attached to digesta particles within the lumen of the tract (Huyghebaert, 2003). The respective populations have different impacts on performance and the health status of the host animal. Therefore, the response of the natural bacterial populations to such products will vary and may, to a large extent, be dependent upon on several factors, such as the environment and the type of diet in as much as it affects viscosity-enhancing components.

Pathogenic organisms (e.g. *Clostridium perfringens*) inhibit the small intestines of broiler chickens as part of their microflora dynamics. Normally, populations of these organisms do not reach levels where overt disease is recognized. Some of these bacteria however produce toxic metabolites and microscopic lesions that have the ability to interfere with broiler growth. Even low dosages of antibiotics affect the levels of *Clostridium perfringens* by suppressing their growth (Huyghebaert, 2003).

3.2.2 *Enhanced nutrient availability*

When intestinal populations of bacteria rise, competitive exclusion occurs in the gut. Diminishing the populations of pathogenic bacteria makes available measurable levels of critical nutrients such as energy, amino acids, vitamins and minerals that can then be used by the host animal for growth. Other mechanisms that have been suggested for the mode of action of AGPs include; reducing the production of microbial metabolites such as ammonia and bile degradation products, reduction of microbial use of nutrients and reduction of the metabolic costs of the immune system by inhibiting pathogens (Niewold, 2005).

3.2.3 *Minimising activation of the immune response*

In situations where the intestinal immune response is activated, a number of immune stimulators are released by activated macrophages (IL-1, IL-6, etc.) (Huyghebaert, 2003). These substances bring about a cascade of effects that fuel immune stimulation by shunting available nutrients and energy away from the growth process. By minimising bacterial (antigenic) exposure, antibiotics permit nutrients to be used for normal metabolic processes and growth; similarly the catabolic effects of immune stimulation are avoided.

3.3 **Antibiotic resistance**

According to Maritz (2005a) antimicrobial resistance refers to an antimicrobial that previously was effective in killing or inhibiting the growth of a particular microorganism lost its efficacy so that bacterial infection would be more difficult or expensive to treat. Resistance develops when a bacterium survives exposure to an antibiotic that normally kills the bacterial population, usually after a mutation occurred. That mutation could promote antibiotic resistance via increased resistance to the absorption of the antibiotic through its cell wall, increased metabolism of the antibiotic to a non-inhibitory form, or induction of alternative metabolic products that permit circumvention of the inhibitory action of the antibiotics (Edens, 2003).

Antibiotic use and antibiotic resistance are clearly connected (Huyghebaert, 2003). Antibiotics have been used to treat human and animal diseases as well as to improve productivity of farm animals for a long time. However, the continuous use of antibiotics promotes a survival advantage to drug-resistant strains of bacteria that have been implicated in foodborne illnesses and other infections (Montagne *et al.*, 2003). On the other hand any benefit realised in moderating antibiotic resistance in animals by the removal of AGP may in part be offset by increasing disease problems and consequent use of therapeutic AGP, as has been shown in Europe (Edens, 2003).

The development of antimicrobial resistance has become a global problem. The resistance to antibiotics initially emerged shortly after the introduction of the penicillins and has since been observed for all antimicrobial drug classes. Although decreased bacterial susceptibility could adversely affect clinical outcome, it has been observed that susceptibility to antimicrobials is not shared equally among bacterial species, or even between different strains of the same bacterial species (Edens, 2003). Although the development of resistance varies between bacterial species, the rate and extent of the development of resistance among bacteria common to the animal production environment is unknown. However, resistance will develop among some bacterial species and strains, even at low drug concentrations (Fedorka-Cray & Robens, 2005). A major concern that resulted in the EU ban of AGP in animal production was that many of the multiple antibiotic resistant strains of bacteria are capable of passing resistance factors to unrelated bacteria. This fuelled from a growing concern that the use of antimicrobial drugs in veterinary medicine and animal husbandry may compromise animal health if resistant bacteria develop in animals and are transferred to humans via the food chain or the environment (van Vuuren, 2005).

3.4 **International position regarding AGP**

It appears that there is a link between antibiotic resistance in humans and antibiotic use in animals, with the majority of the resistant bacterial strains that threaten public health originating from the overuse and misuse of antibiotics in animal therapy (Maritz, 2005a).

In 1999, the EU prohibited the use of antibiotics as animal growth-promoters (Montagne *et al*, 2003), while in the USA, although it is routinely given to boost the nutritional benefits of animal feed, most broiler companies are now encouraging reduction in the use of AGP to some extent. The US Centers for Disease Control and Prevention, Food and Drug Administration (FDA), United States Department of Agriculture (USDA) and many government agencies involved in promotion and regulation of health activities around the world are vigorously involved in developing programmes intended to monitor the emergence of antimicrobial resistance and to decrease the use of antimicrobial drugs where possible (Fedorka-Cray & Robens, 2005).

In Australia, a range of AGPs such as arsenical compounds, flavophospholipol, bacitracin and virginiamycin are employed. In South Africa, there is increased consumer pressure to ban AGP. Maritz (2005a) reported that South Africa had adopted a more responsible approach towards the use of AGP and a legal framework for the testing of the antibiotic residue is controlled by the "Foodstuffs, Cosmetics and Disinfectants Act", of the Department of Agriculture (DoA), which is responsible for the national monitoring of levels for exported meat and putting controls in place.

According to Durrans (2005), South Africa can use existing legal loopholes to export meat products to Europe that have been produced using in-feed antimicrobials by ensuring there are no antibiotic residues in the final product. However, there is increasing pressure from European producers to close this loophole, which will mean that the ability to compete in the market place will be severely affected by the ban on AGP use. As early as 1986, Sweden introduced a general ban on the use of in-feed antimicrobials. The prohibition increased the incidence of sub-clinical diseases and as a consequence the use of therapeutical doses of antibiotics to treat animals increased considerably (Edens, 2003).

3.5 **Cost implications of AGP removal**

The major advantage of the antibiotic ban will be an improvement in consumer perception on the wholesomeness of products of animal origin. The disadvantages relate to the increment in cost of production due to increased feed prices, lower stocking densities, poorer production and lack of control of sub-clinical diseases, especially under poor management conditions. According to the study by Maritz (2005b) feed cost will increase due to the incorporation of higher digestible feed ingredients and expensive alternatives to AGP. Furthermore, broiler producers need to take into account the cost implications of poorer production results such as higher mortalities, lower growth rates and poorer feed efficiencies. Broilers raised in the absence of antibiotics have been shown to present higher risk to consumers from disease-causing pathogens and bacterial contamination, such as air sacculitis and necrotic enteritis (Bafundo, 2004).

The in-feed antimicrobial ban has challenged the farmers not to use poorly digestible ingredients that risk not only poor performance, but an additional danger of bacterial overgrowth and subsequent disease intestinal disorders (Bedford, 2000). Wegener (2005) reported that the construction and environment of houses, hygiene, management and feed composition all contribute to the occurrence of necrotic enteritis. However, the use of coccidiostats of the ionophore type prevented its occurrence, and broiler productivity was increased due to genetic improvement. Interestingly, it was reported that the effects of termination of AGP in Denmark on poultry production was small and decreased feed efficiency by minus 2.3% that was largely offset by savings in the cost of AGP. No changes in weight gain or mortality and overall net cost associated with productivity losses were experienced (Wierup, 2005). Among the most gut-specific pathogens, *Clostridium perfringens* is assumed to represent the main health problem associated with the ban on the use of in-feed antibiotics as growth promoters.

Choct (2001) reported that when the antibiotic ban was first introduced in Europe, it affected the intensive farming industry with a huge increase in mortality and morbidity rates of animals as well as a drop in production. The report also revealed that the poultry

industry had a sporadic outbreak of necrotic enteritis that, in sub-clinical cases, decreased growth and feed efficiency and, in severe cases, caused large numbers of deaths.

4. **The alternatives to AGP**

The phasing out or banning of AGP will cause difficulties for commercial poultry producers through poorer growing efficiency and increased stress and disease levels. It is important that alternatives to growth promoters are properly evaluated and due consideration is given to the quality, safety and efficacy of each product. The choice of development of alternatives to antibiotics that work through similar mechanisms, promoting growth whilst enhancing the efficiency of feed conversion and disease control should be applied. An effective replacement for antibiotics should have a significant and safe for both animal and human beings (Collett, 2004).

There are different strategies, numerous feed additives, and combination of nutrition and management approaches that have been implemented to cope with the negative side effects of AGP withdrawal. Commercial and scientific evidences of beneficial effects on performance and control of subclinical diseases have been shown for plant extracts (essential oils, herbs, spices and botanicals), exogenous enzymes (non-starch polysaccharides such as ß-glucans and xylans), organic acids (formic, lactic, propionic, etc.), prebiotics (oligosaccharides of galactose, fructose or mannose, etc.), probiotics (yeasts such as saccharomyces and other live organisms such as effective microorganisms), immune enhancers such as certain fatty acids, carotenoids, vitamins, chelated minerals and peptides, vaccines (inactivated and active vaccines) and other management practices (Kaldhusdal, 2003).

4.1 **Plant extracts (essential oils, herbs, spices and botanicals)**

Plant extracts are standardised products that have characteristic physiological effects on the host including stimulation of appetite, secretion of endogenous enzymes, immuno-stimulation and promoting increased antimicrobial activities. The basic ingredients of

21

plant extract preparations are secondary plant metabolites like allylisothiocyanates, thymol, carvacrol, cinnamaldehyde, capsaicin, piperin and numerous other active agents (Veldman *et al.* 2005b).

The addition of oils, herbs, spices and botanicals in feed as feed additives increases the secretion of digestive fluids and improves the immune system of broilers, and besides the improved health, a better nutrient digestibility, reduced frequency of digestive disorders and also increased performance of broilers are ensured (Wenk, 2005). The phytochemicals such as essential oils or dietary fibre contribute to a balanced intestinal microflora (eubiosis), an optimal precondition for an effective protection against pathogenic organisms and intact immune system (Maritz, 2005b).

Sarica *et al.* (2005) reported that flavomycin and two herbal natural feed additives (thyme and garlic) with and without xylanase-based enzyme complex had no significant effect on the growth performance. Furthermore, total plasma cholesterol concentration, dry matter content of excreta, the weights of heart, pancreas, liver, gizzard and spleen, except for small intestine, hot and cold carcass yielded the concentrations of the total aerobic bacteria and *E. coli* in the small intestine when incorporated into wheat-based broiler diets. Denli *et al.*, (2004) reported significant effects on body weight gain and feed efficiency of quails supplemented thyme and black seed essential oils and the decrease in intestinal pH and liver weight was experienced. The results obtained from Alçiçek *et al.* (2004) further confirms that the supplementation of a mixture of herbal essential oils to the diet significantly improved the body weight gain, feed conversion ratio and carcass yield of broilers.

4.2 Exogenous enzymes

Most nutritionists prefer to use maize in the diets of poultry. This is primarily due to the perception that maize has a relatively high and consistent nutritional value for poultry. However, it has been demonstrated that maize is variable in its chemical composition and

22

nutritional value for poultry and that the use of exogenous feed enzymes can reduce the variability in the feeding value of maize and improve the performance of growing broiler chickens (Cowieson *et al.*, 2005). The mechanisms by which exogenous enzymes mediate their effects include improvements in nitrogen and energy retention, reduction in endogenous losses, improvements in feed intake and the beneficial effects on the microbial community in the distal GIT (Cowieson *et al*. 2005).

The introduction or change of diet disturbs the establishment of intestinal microflora and in the case of introducing a high non-starch polysaccharide (NSP) diet, such as wheat or barley-based diets, the change favours the proliferation of anaerobic organisms (Choct, 2001). The NSP in animal feedstuffs are a complex group of components differing widely in chemical composition, physical properties and physiological activity. The NSP includes hemicelluloses, pectins, oligosaccharides, arabinoxylans and ß-glucans (consisting of both the soluble and non-soluble fraction). The viscous nature of NSP is the primary cause for its anti-nutritive effect in poultry, because of the increased bulk and viscosity of the intestinal contents that decrease the rate of diffusion of substrate and digestive enzymes and hinder their effective interaction at the mucosal surface (Huyghebaert, 2003).

The NSP present in viscous cereals such as barley, wheat, rye, etc. pass through the gut of broilers largely untouched affecting digesta viscosity and gut microflora growth. Exogenous enzymes (EE) improve nutrient digestibility and decrease the incidence of wet litter in broilers. Wheat is an important feed ingredient in poultry diets, accounting for up to 70% of the metabolisable energy and 40% of protein requirement of broilers compared to other cereal grains. Hew *et al.* (1998) reported that the apparent metabolisable energy (AME) and nitrogen digestibility of wheat due to exogenous xylanases, and the improvement in apparent digestibility of amino acids (AA) with EE reflected a reduced endogenous AA loss, resulting from the amelioration of the anti-nutritive effects of wheat NSP.

4.3 **Organic acids**

Durrans (2005) reported that organic acids were originally used in animal feeds as mold inhibitors that were found to have an effect as potential bactericides and were able to produce bacteriostatic and bactericidal properties under specific conditions. Water and feed acidification have an important role to play in gut flora management. The pro-nutrient potential of commercial acid preparations is thought to rise from the antibacterial effects of their ionizing properties. Acid ionizing varies according to type, concentration and mix of the acid. It can be modified by the pH, buffering capacity and the water activity of the feed, water and the gut content. The effects of acids are higher than salt, because the acid has acidification in combination with the anion effects. Formic acid is an effective acidulant that acts by inhibiting microbial enzymes. Generally, the antimicrobial activity of formic acid primarily controls yeasts and some bacteria such as *E. coli*, coliforms, *enterococcus*, *Staphylococcus, Salmonella* and *Clostridium perfringens* (Huyghebaert, 2003).

Commercially available organic acids such as acetic, butyric, formic, fumaric, lactic, propionic and sorbic acids in the calcium, potassium or sodium salt forms are reported to have both growth promoting and feed conversion effects by decreasing intestinal pH, creating a healthier microbial population and increasing nutrient absorption (Durrans, 2005; van Kol, 2005). However, van Campenhout *et al*. (2001) reported that the mixture of organic acids (Acid Lac® Dry) did not reduce *Clostridium perfringens* counts to the same extent as the antibiotic, but improved feed conversion ratio. Additionally, organic acids in their undissociated forms are able to pass through the cell membrane of bacteria, thus lowering the pH of the cell causing the organism to use some of the energy in its effect trying to restore the normal balance. The lower pH conditions thus protect the animal from pathogenic infections especially at young ages. There has been interest in the effect of organic acids on feed efficiency and broiler performance. The effectiveness of organic acids in poultry may also depend on the composition of the diet and its buffering capacity (Choct, 2001).

4.4 Immune enhancers

A phenotypic inbreeding for production parameters of poultry might result in genotypic linkages with an altered immune responsiveness (Huyghebaert, 2003). The immune system of broilers plays an important role as defence mechanism against pathogenic infections. These qualities can only be obtained if the health status of the bird is high. Therefore, a lot of energy is invested in prophylactic measures such as vaccination and chemophrophylaxis against infectious diseases. However, health and immune responsiveness are not only maintained and improved by vaccinations and hygiene, but also through an adequate supply of nutritional components to the broilers. The immune reactivity can be modulated by nutritional concentration such as alterations in minerals, vitamins, essential fatty acids and other substances such as oligosaccharides (Huyghebaert, 2003). There are significant health benefits that can be derived from using immune stimulants such as ß-glucans as feed ingredients. Other ß-glucans are reported to have the ability to protect broilers against gram-positive organisms such as viruses, bacteria, fungi and parasites (Maritz, 2005b). The immunomodulators such as CD_3, CD_4, IgM-marked B-cells, and neutrophils are examples of ß-glucans.

Most of the immune cells in the body are located within the intestine as part of the gut associated lymphoid tissue (for example IgA antibodies). The lymphoid tissues provide protection by preventing adhering to intestinal epithelial cells and by inhibiting the absorption of toxins. In addition, lymphocytes can kill bacteria directly through antibody-dependent cell-mediated cytotoxicity (Huyghebaert, 2003). In order to reduce or remove AGP and other prophylactic drugs in feed, there is a major search for immunomodulators. Furthermore, the mucosa-associated lymphoid tissue with its phagocytic M-cells is reported to play an important role especially against particles and macromolecules.

The structural elements of bacteria such as lipoposaccharides, also called endotoxins, are among the most important immunostimulants, which are also very toxic, causing inflammation, resulting in fever, reduced appetite and impaired performance of broilers

(Huyghebaert, 2003). The use of immunostimulants is reported to overcome stress-induced immunosuppression and to re-adjust the immune function to a normal level.

4.5 **Prebiotics**

A way of manipulating the gut microflora is by supplementation of the diet with small fragments of a special group of carbohydrates. These carbohydrates can selectively stimulate some or all of the beneficial microorganisms in the gut, thus bringing about changes in the intestinal microflora that in turn affects the host in a beneficial way. These carbohydrates are known as prebiotics in the feed industries. The overall effect of prebiotics on host health and well-being is similar to that of probiotics (Choct, 2001). There are potentially hundreds of different prebiotics that are naturally available or can be produced from polysaccharides. The commercially available prebiotic products are mainly oligosaccharides of galactose, fructose or mannose.

Prebiotics are non-digestible feed ingredients with selective effects on the intestinal microflora (Huyghebaert, 2003). The commonly used prebiotics such as fructo-oligosaccharides, inulin and non-digestible oligosaccharides have been reported to enhance the communities of intestinal microflora with the GIT (Durrans, 2005). Insulin is a blend of fructan chains that are widely distributed in nature as plant storage carbohydrates, and is extracted from chicory roots (Veldman *et al*. 2005a). The ß-bonds of the inulin structure prevent it from being hydrolytically digested in the upper tract of broilers, making them available for fermentation to short chain fatty acids (SCFA) by the intestinal microflora. Normal intestinal microflora such as *Lactobacillus* or *Bifidobacterium* utilize inulin for fermentation more efficiently that other groups of bacteria and produce SCFA and lactate.

Mannan-oligosaccharides have also been used as prebiotics, but they do not selectively enrich the beneficial bacterial populations (Patterson & Burkholder, 2003). Fritts & Waldroup (2003) conducted a study to evaluate the use of Bio-Mos®, a mannan-

oligosaccharide derived from the cell wall of yeast for growing turkeys. Bio-Mos®
supplementation improved feed conversion efficiency.

4.6 **Probiotics**

Prior to termination of the AGP, the poultry industry anticipated problems with necrotic
enteritis. However, necrotic enteritis is still a minor health problem largely because
producers continue to use ionophores for the prophylactic treatment of necrotic enteritis
and coccidiosis. Ionophores are antimicrobials approved in many countries to prevent
coccidiosis, a parasitic disease that predisposes broilers to necrotic enteritis. Ionophores
may also directly suppress *Clostridium perfringens* and therefore necrotic enteritis. The
use of vaccines against coccidia instead of ionophores resulted in outbreaks of necrotic
enteritis (Wierup, 2005).

Probiotics are defined as mono or mixed cultures of live microorganisms that indirectly
benefit the host by improving the properties of the indigenous microflora (Fuller, 1992).
Probiotics are given orally to poultry to help birds fight illnesses and diseases. Although
probiotics have been introduced as alternative to antibiotics; their effects on poultry
production are not consistent, resulting in uncertainty and scepticism for the development
of the product (Jin *et al*., 1998).

The normal intestinal microflora in farm animals is important due to its effect on the
production of livestock on the quality and safety of animal products. Caecal microflora
protects broilers against bacterial infection, a healthy intestinal microflora contributes to
small intestine function including digestion and nutrient absorption that is the limiting
factor determining growth rate (Gong *et al*., 2002). It is clear that the microflora provide
real benefits to the animal. For example, the microflora provides both nutrition and
protection to broilers, in the form of fermentation of products and prevention of
colonization by pathogens.

4.6.1 **The efficacy of probiotics on broiler production**

Edens (2003) reported that the use of probiotics has the potential for reducing the risk of infection from pathogens and eliminating the possibility of inducing of antibiotic resistance among pathogenic organisms. Furthermore, the potential for carcass contamination from gut-associated pathogens and public concern appear to be reduced. The positive effects of probiotics on broilers can either be from a direct nutritional effect of the probiotics, or a health effect, with probiotics acting as bioregulators of the intestinal microflora and reinforcing the host's natural defences (Karaoglu & Durdag, 2005).

Kralik *et al*. (2004) found that supplementation of lactic bacteria *Enterococcus faecium* M-74 probiotics resulted in increased body weight, better feed conversion and resulted also in decreased *E. coli*. Furthermore, probiotics insured the intestinal microflora balance and stimulated other bacterial species to produce nutrients that positively influenced fattening traits of broilers. They also found sporogenic bacteria from genuses *Bacillus spp*. and *Clostridium spp*. that showed a strong resistance in the digestive system.

Feeding broilers probiotics helps maintain beneficial intestinal microflora and may modulate the mucosal immune system and enhancing the host's resistance to enteric pathogens. Probiotics for poultry have been generally divided according to their site of colonization and proposed mode of action.

4.6.2 **Mode of action of probiotics**

Some probiotic strains can be selected for their beneficial properties which include their activity against pathogenic microorganisms, enzyme activities that favour absorption of essential nutrients and/or ions, and presence of substances with capacity for adherence to epithelium (Gusils *et al*., 1999). Mixed bacterial cultures reduce the quantity of toxins produced. *Lactobacillus* species are one of the most important probiotic strains. These bacteria produce large amounts of lactic acid from monosaccharide and disaccharide substrates that result in a lower pH, which is fatal to many bacteria (Maritz, 2005a).

Bacillus subtilis increases the anaerobic condition of the digesta leading to increased numbers of bacterial lactic acid forming bacteria.

Lactobacilli colonize the crop and affects both nutritional and performance parameters. However, under conditions of stress or major bacterial challenge, the *Lactobacilli* population will decline. *Bifidobacterium* act in the caecum and enhance immuno-stimulation, competition with the pathogenic bacteria and the production of volatile fatty acids (VFA) that provide metabolic energy to the host. *Bifidobacterium* produces substances such as lactic acid and acetate that inhibit a wide range of gram-positive and gram-negative bacteria *in vitro* (Barrow, 1992).

Huyghebaert (2003) reported that some probiotics (*Lactobacillus spp* and *Bacillus spp*) have beneficial effects on performance in young birds especially when they have been stressed by adverse environmental conditions (e.g. resulting in elimination of wet droppings). However, supplementation with *Streptococcus spp* has been reported to give adverse effects on performance of growing birds. Variation in the effects of probiotics on broilers obtained from various studies may be attributed to the differences in the strains and forms of bacteria used and in the concentrations of dietary supplements (Jin *et al*., 1997b).

The target of probiotics is to improve gut health by selecting for beneficial microflora and suppressing intestinal and foodborne pathogens, therefore, they must possess certain favourable characteristics such as 1) creation of a micro-ecology that is hostile to other bacterial species, 2) elimination of available receptor sites, 3) production and secretion of antimicrobial metabolites, and 4) competition for essential nutrients (Edens, 2003; Sarica *et al*., 2005). Since growth requirements of the bacteria differ, it should be possible to shift the microbial community from pathogenic to non-pathogenic direction by changing the gut dynamics through dietary modulations (Apajalahti, 2004). A research by Owings *et al*. (1990) showed that the supplementation of *Streptococcus faecium* had a better broiler performance in water and feed when compared with antibiotics.

4.6.2.1 **Volatile fatty acid (VFA) production and lowering of pH**

Disruption of the normal intestinal microflora populations with antibiotics will abolish the protective mechanism, because the concentrations of VFA produced by the intestinal bacteria will decrease and gut pH will increase towards a more alkaline range. In newly hatched chicks in commercial hatcheries, the VFA concentration and pH are not sufficient to chemically suppress pathogens (Edens, 2003), therefore, supplementation of probiotic microorganisms will be very beneficial. In the case of commercial broiler chickens, overgrowth of *Clostridium perfringens* is the most serious alteration of intestinal microflora.

In the ceaca, an anaerobic environment develops and favours the growth of organisms such as *Bifidobacterium spp.* and *Bacteriodes spp.* In an anaerobic environment, *Bifidobacterium spp.* and *Bacteriodes spp.,* along with other lactic acid bacteria (LAB), create a micro-ecology that can be characterized by an acid pH resulting from the production of VFA and lactic acid and antimicrobial substances that effectively exclude or kill many different pathogens (Edens, 2003). When conditions favour the growth of bacterial species such as *Clostridium perfringens*, there is the potential for the digestion and absorption of essential nutrients and vitamins to be impaired. Dietary fat is a major source of energy in broiler feeds. *Clostridium perfringens* has the ability to inhibit the action of bile, preventing the breakdown of fat. This can result in reduced absorption of fat-soluble vitamins, impacting on growth and efficiency.

4.6.2.2 **Activity against pathogenic microorganisms**

The GIT of broilers contains a species-diverse group of microflora such as gram-negative and gram-positive microorganisms. These microorganisms reach a typical equilibrium state, which depends on many factors such as location in the GIT, integrity of the intestinal mucosa, and transit time of chymus Huyghebaert (2003). This intestinal microflora has a specific barrier impact, such as induction of anatomical and physiological changes in the intestinal cell wall structure, immunological modifications in the GIT, and enhancement of broiler resistance to enteropathogenic bacteria. Any imbalance in the microflora could lead to the colonisation of pathogens or to a microflora

that could impair growth (Huyghebaert, 2003). Jin *et al*. (1997b) reported that the addition of probiotic *L. acidophilus* or a mixture of *Lactobacillus* depressed the pathogens such as coliforms in the intestinal contents.

4.6.2.3 **Competitive exclusion**

Other prophylactic measures to control necrotic enteritis have been proposed. Oral administration of a mix culture of bacteria (competitive exclusion) from healthy birds to newly hatched chicks reduced the incidence of necrotic enteritis and a decreased colonisation was observed (Craven *et al*., 1999). Probiotics, i.e. live microbial feed supplement that beneficially affects the host by improving the intestinal balance, have been shown to reduce gross intestinal lesions of necrotic enteritis (Hofacre *et al*., 1998). Dalloul *et al*. (2003); Lee *et al*. (2007) reported that *Lactobacillus*-based probiotic administration induced protective immunity against *Eimeria acervulina* infection.

A popular mechanism of action concept of probiotics, which have been used in poultry, is "competitive exclusion" (CE) of bacterial pathogens. The CE is the mixture of bacteria derived from the gut of the healthy chickens or beneficial bacteria (e.g. lactic acid bacteria) administered orally to day-old chicks to establish beneficial gut microflora (Sun *et al*., 2005). The CE concept is applied when day old chicks are treated with microflora resulting in a colonizing resistance towards potentially pathogenic microorganisms and the treatment is administered as a spray directly onto chicks, applied in drinking water or as a top dressing on feed to prevent *Salmonella* and *Campylobacter* colonization. In contrast to the continuous administration of probiotics, the CE treatment is given only once, although now a second or third dosage at a later stage is considered to be beneficial. A probiotic product is composed of pure cultures of one or more microorganisms, but the exact composition of the CE microflora is not completely known.

Maintenance of antibiotic resistance is an energetically expensive process for a bacterium. Removal of antibiotics that promote resistance and seeding the bird with antibiotic sensitive probiotic organisms gradually leads to development of a bacterial population that is sensitive to antibiotics. The bacteria that are used as probiotic

organisms have an ecological advantage in the GIT, because they can multiply more efficiently than the antibiotic resistant forms that spend extra energy for maintenance of the resistance factors rather that for reproduction (Edens, 2003). Use of probiotic bacteria has a competitive advantage that constitutes the basis of the competitive exclusion concept. However, it is important to understand that bacteria with plasmid(s) bearing antibiotic resistance factors do not simply disappear. Those bacteria can survive for long periods before they lose resistance-bearing plasmid(s) and continue to pose a potential problem to poultry production as well to human health. As long as there are populations of bacteria in the GIT that express competitive advantages, the potential pathogens can be kept in check.

4.6.3 **Conclusion**

The hypothesis of the study was that there is no difference between the efficacy of EM and AGP in preventing necrotic enteritis caused by *Clostridium perfringens*. The study was undertaken to evaluate the use of EM in water and feed on the performance of broilers challenged and non-challenged with *Clostridium perfringens* type A. With respect to poultry production, an important goal is to determine the optimal microflora for the broiler (maximum benefits with minimum costs) and then be able to manipulate the microflora through diet, supplements, etc, to obtain the desired microflora. Probiotics are used with the goal of altering the microflora for the benefit of animal health and production. Probiotic bacteria are meant to improve the gut health, but these are likely to be effective only if the requirements for their growth are fulfilled or if their physiological effect is independent for the survival.

CHAPTER 3

Investigating the effect of Effective Microorganisms on growth performance and gut health of broiler chickens challenged with *Clostridium perfringens*

3.1 **Introduction**

The sub-therapeutic use of antibiotic growth promoters (AGP) in broiler diets for the prevention of pathogenic bacterial infections has been effective in enhancing bird health, uniformity and production efficiency for more than fifty years. However, increased public concern on the development and spread of antibiotic resistance in bacteria and the possible presence of antibiotic residuals in poultry products has led to a search for alternatives to antibiotics in broiler diets (Gong *et al*., 2002). The preventative dosages of antibiotics was originally thought to promote a stable intestinal microflora and Bedford (2000) and Dibner & Richards (2005) has been found that it negatively affect *Lactobacilli* and other lactic acid producing bacteria. These bacteria constitute the largest part of anaerobic intestinal microflora, and play a crucial role in maintaining a healthy and efficient digestive system. Research has established that normal intestinal microflora competes with pathogens and hence decreases the risk of salmonellosis, *Clostridium perfringens*-associated lesions, campylobacteriosis, and colibacillosis (Garrido *et al*., 2004). High numbers of *Clostridium perfringens* type A bacteria in the small intestines of broilers are consistent with lesions from necrotic enteritis, a very common disease caused by the alpha toxin (type A or C) of *Clostridium perfringens* (Kaldhusdal & Skjerve, 1996).

The search for acceptable alternatives to antimicrobials that do not compromise productivity has led to the use of EM which are a mixed culture of microorganisms comprising lactic acid bacteria, yeasts, actinomyces and photosynthetic bacteria (Higa & Parr, 1994). EM like probiotics are environmentally friendly microorganisms, but unlike commercial probiotic products they are generally composed of a larger number of different microorganisms. Probiotics, when supplied at ideal levels in the GIT, provide equilibrium to the intestinal flora that can impute a beneficial effect on the health of the host. The use of probiotics was shown to

promote growth and feed efficiency when introduced as an alternative to AGP (Jin *et al*., 1997a), but little work has been done to demonstrate the efficacy of EM.

This study was undertaken to evaluate the effect of EM on the performance of broilers which had been either challenged with *Clostridium perfringens* type A or not. The null-hypothesis of the study was that EM is not effective in preventing necrotic enteritis caused by *Clostridium perfringens*.

3.2 Materials and Methods

3.2.1 Housing and Management

The study was conducted at the poultry unit of the Agricultural Research Council, Nutrition and Food Science, Irene, on the Gauteng Highveld. Six hundred and forty 'as hatched' Ross 788 broiler chicks were obtained from a commercial hatchery. Upon arrival at the research site, the chicks were examined and any that were obviously sick or dehydrated were culled. The chicks were randomly allocated to two separate rooms containing 16 pens each with 20 chicks per pen at a stocking density of 18 chicks/m^2.

Standard management procedures were followed for all treatments as recommended by the suppliers of the chicks. All the chicks were kept on wood-shaving litter floors. Environmental control was facilitated by means of gas heaters and extractor fans. Each pen was equipped with a tube feeder and chicken fountain drinker. For the first seven days, an additional 4L-chick founts were placed in each pen. The house temperature was kept close to 32 ºC during the first day, thereafter it was decreased periodically to 21 ºC over a period of three weeks. Continuous light was provided for the first 48 hours, followed by a lighting pattern of 23-hours light, 1-hour darkness for the remainder of the growing period.

The chicks were vaccinated against Infectious Bronchitis at the hatchery, while vaccination for Newcastle and Gumboro Diseases were done on days 14 and 18

respectively, at the research unit. All chicks were fed a commercial two-phase maize-soya type diet consisting of a mash starter and grower feed, formulated to meet or exceed the nutritional requirements of broilers as recommended by the National Research Council (NRC) (1994). The composition and nutrient content of these diets are shown in Table 3.1.

3.2.2 Experimental design and dietary treatments

Three hundred and twenty chicks from one room were challenged with *Clostridium perfringens* at two weeks of age, while the same number of birds in the other room remained unchallenged. Each group was then randomly allocated to four treatments resulting in a randomised block design (challenged x treatment) replicated four times.

The treatments consisted of the following:

Treatment 1: Unmedicated broiler rations (Control).

Treatment 2: Broiler rations containing Zinc bacitracin as antibiotic growth promoters (AGP) at 33g/kg only.

Treatment 3: Broiler rations containing 0.1% effective microorganisms (EM) at 50mL/L in drinking water plus EM Bokashi at 50g/kg in the feed.

Treatment 4: Broiler rations containing AGP (Zinc bacitracin) with EM in drinking water at 50mL/L plus EM Bokashi in feed at 50g/kg (AGP + EM).

Table 3.1: Composition and nutrient content of basal diets fed to broilers

Ingredients and composition (g/kg as fed)	Starter	Grower/Finisher
Yellow maize (8.6%)	67.00	74.73
Maize Gluten (60%)	17.92	4.77
Fullfat Soya beans	5.20	5.23
Bokashi	4.00	8.0
Limestone	0.55	1.20
Salt	1.20	0.90
L-Lysine HCL	0.89	0.76
DL-Methionine	0.35	0.25
Vitamin/ Mineral Premix	1.33	1.33
Coccidiostat ©	0.04	0.08
Zinc Bacitracin (where applicable)	0.03	0.05
Monocalcium Phosphate	1.49	2.70
ME (MJ/ kg)	125	134
Dry matter (g/ kg)	850	906
Oil ether extract (g/ kg)	37.5	55
Crude protein (g/ kg)	230	196
Crude fibre (g/ kg)	24.4	26.7
Calcium (g/ kg)	10	9.0
Phosphorus (g/ kg)	3.4	3.9
Available phosphorus (g/ kg)	4.2	1.5
Salt (g/ kg)	3.2	3.4
Sodium (g/ kg)	1.8	1.8
L Lysine (g/ kg)	7.6	7.2
Methionine (g/ kg)	6.4	5.9
Methionine & cystine (g/ kg)	4.1	3.9

3.2.2.1 Preparation of EM

The liquid culture of the EM product used in this study was supplied by EMROSA (Pty) Ltd. (Centurion, South Africa) and contained a mixture of lactic acid bacteria with 8.3×10^6 CFU/mL (*Lactobacillus plantarium* species), yeasts with 1.8×10^5 CFU/mL (*Candida valida* species), actinomycetes with 3×10^3 CFU/mL (*Streptomyces albus* species) and fermenting fungi with 1.1×10^5 CFU/mL (*Aspergillus oryzae* species). Original EM solution is a yellowish liquid with a pleasant odour and sweet-sour taste and should be kept at a pH of 3 to enhance preservation. For the water supplementation, the EM was added daily to the chickens at two weeks of age to the drinking water in the chicken

fountains at a rate of 50mL/L of water. For feed supplementation, EM Bokashi (the fermented form of EM) was prepared separately, and mixed into the feed at 50g/kg feed. The EM Bokashi consisted of LAB with 2.7 x 10^3 CFU/g (*Lactobacillus rhamnosus* species), yeasts (*Candida valida* species), actinomycetes with 1.7 x 10^6 CFU/g (*Streptomyces albus* species) and fermenting fungi with 2.5 x 10^6 CFU/g (*Aspergillus oryzae* species).

EM Bokashi was prepared as follows: 150mL molasses in 15 litres of water was prepared to give a ratio of 1:1000 according to the manufacture's guidelines. The mixture was then mixed with microbial EM inoculant and the mixture was poured over 100g of wheaten bran. A handful of the mixture was squeezed into a ball to estimate the required moisture level. If squeezed, it was supposed to remain compact and spring back without crumbling, and when touched, it was supposed to crumble. The material was then placed in an airtight plastic bag, away from direct sunlight to create anaerobic conditions for fermentation to occur. After four weeks, the EM Bokashi was spread out on a plastic sheet in the shade to dry before storage. The final EM Bokashi had an estimated moisture level of 30%.

Each day EM Bokashi was weighed and thoroughly mixed by hand with the feed in a small drum at a rate of 50g per kg feed before being given to broilers as instructed by the supplier. The EM liquid and EM Bokashi were supplemented from day 14 until 40 after the chicks were challenged with *Clostridium perfringens*. Zinc Bacitracin was used as the AGP at 0.33g/ 100kg and Salinomycin© as *Coccidiostat* was also added at a rate of 0.5g/100kg.

3.2.2.2 Induction of necrotic enteritis

Clostridium perfringens was isolated from the intestinal mucosa of a chicken with necrotic enteritis according to the method of Onderdonk & Allen (1994), and cultured under anaerobic conditions for 24 hours at 37°C on Blood Columbia Agar Base containing 7% horse blood (BTA) (Oxoid Limited, Basingstone, Hampshire, UK). The

bacteria formed glistening grey colonies of 3-5mm diameter with a double zone of beta-haemolysis.

The bacteria were positively identified microscopically and through specific tests. *C. perfringens* are gram-positive rods, catalase negative and non-motile and, when placed onto lactose egg-yolk agar, give lipase negative, lactose positive and lecithinase positive reactions. They were also typed using a polymerase chain reaction (PCR) and gel electrophoresis. The bacteria were then stored at -85°C in Brain Heart Infusion (BHI) broth with 0.5% cysteine added. The inoculum was prepared by thawing the broth at 37°C and plating it on BTA overnight under the same conditions as previously described. The bacteria were then checked for purity, placed in 100mL of BHI broth and incubated at 37°C overnight. The following day the bacterial suspension was adjusted to an optical density of 1 (1 x 10^8 CFU/mL) using 450nm wavelength (Onderdonk & Allen, 1994). At two weeks of age, all chicks in the challenged groups were inoculated individually *per os* with 1mL of this suspension.

Just before inoculation, pre-challenged chickens were subjected to cold stress by reducing environmental temperature to 23°C for twenty hours to induce immunosuppression and to effectively allow the colonisation of pathogens (Edens, 2003). During this period, feed was also withdrawn to minimise the occurrence of regurgitation (Collier *et al*., 2003). The room temperature was kept at 32 ºC during the first day, thereafter it was decreased in stages to 21 ºC over a period of three weeks.

3.2.3 Measurements and observations

Performance parameters of the broilers were measured in terms of weekly feed and water intake, body weight and feed conversion ratio (FCR) for the periods, 0-21 and 0-40 days of age. Total feed intake per pen was done by weighing feed orts weekly to determine cumulative feed intake per chick. Where mortalities occurred, average feed intake per bird was determined by weighing feed orts for the particular pen on the day. Water was weighed back daily to determine cumulative water intake per chick. Cumulative feed intake was divided by the body weight gain for determination of FCR. The data was

corrected for mortality by weighing the body weights of the dead birds and percentage mortality was determined at the end of the study. Pens were checked twice daily for mortality and all dead birds were weighed and submitted for *post mortem* examination by the consulting veterinarian. Records were kept in terms of daily minimum-maximum temperatures and daily procedures. The production efficiency factor (PEF) for all pens were determined by the formula, PEF = ([Live weight (kg) x Livability %]/ [FCR x age (days)] x 100, where Livability = (100 – Mortality rate) and Mortality rate = (number of dead birds/ total number of birds) x 100.

On day 21, two birds per pen were sacrificed and on day 35 one bird per pen was sacrificed. The abdomens were opened and the gastro-intestinal tracts exposed and segmented. Small intestines and livers were observed macroscopically for lesions. The necrotic lesions were scored according to the method of Collier *et al.* (2003). The necrotic lesions were scored on a scale of 0 to 3 as shown in Table 3.2.

Table 3.2: Criteria for scoring of necrotic enteritis in the intestines and liver of chickens challenged and non-challenged with *C. perfringens*

Score	0	1	2	3
Description	No dot lesions	One to five small dot lesions (<1mm diameter)	More than five small dot lesions (<5mm), but fewer than five larger dot lesions (>5mm diameter)	More than five larger lesions and erosive ones (>5mm diameter)

3.2.4 **Statistical analysis**

Data was analysed using the statistical programme, GenStat (2000). The experiment was designed as a randomised block designed with 4 replicates and 4 treatments. Analysis of variance (ANOVA) was used to test for differences between treatments. The data was tested as normal with homogeneous treatment variances. Treatment means were

separated using Fishers protected t-test least significant difference (LSD) at the 5% level of significance (Snedecor & Cochran, 1980). If the F-probability from the ANOVA was significant at 5%, treatment groups were compared in terms of differences in live weight, feed intake, feed conversion ratio and mortality at twenty-one and forty days of age and the production efficiency factor (PEF) at forty days of age. At day 40 the challenged group was compared with the control treatments of the non-challenged group using the t-test.

3.3 **Results and Discussion**

Performance of the broilers challenged and non-challenged were evaluated at 21 days of age as presented in Tables 3.3 and 3.4 and at 40 days of age as presented in Tables 3.5 and 3.6. At 21 days of age, in the non-challenged group, no differences ($P>0.05$) were observed between treatments for initial body weight (IBW), feed intake and the cumulative feed intake. Therefore, AGP and EM did not affect feed intake singly or in combination. The broilers given EM, however, had a lower 21-day body weight (BW) ($P<0.05$) compared to the control, AGP and combination of AGP and EM. Given that the water intake was significantly lower ($P<0.05$) in the EM group than in the other three groups, it could be that the inclusion of EM in water may have negatively affected water intake, probably because it reduced palatability, resulting in a lower ($P<0.05$) final BW and higher ($P<0.05$) feed conversion ratio (FCR). This contradicts the reports by Kralik *et al.* (2004) and Timmerman (2006) that supplementation of probiotics in water improved broiler performance. Water is critical for digestion and metabolism processes and directly affects gain and conversion efficiency (McDonald *et al*., 1995). Since feed intake does not seem to have been affected by the inclusion of EM in feed, this could probably be the preferred route of supplementation of EM rather than through water. Alternatively the dose of EM in water could have been too high. More work would need to be done to determine whether it affects palatability in water and whether it is dose dependent.

At 21 days of age, the challenged broilers on the other hand did not differ significantly ($P>0.05$) in terms of cumulative feed intake, IBW, feed intake, cumulative feed intake, average daily gain (ADG), BW and FCR in all the treatments. Water intake was again lower ($P<0.05$) where EM was added to the water (Table 3.4).

At 40 days, in the non-challenged group, there was no significant difference ($P>0.05$) in the feed intake, cumulative feed intake and water intake amongst the treatments, whilst broilers on AGP and the combination of AGP and EM treatments had higher ($P<0.05$) BW, ADG, and production efficiency factor (PEF) index values than those on EM alone (Table 3.5). This seems to indicate that the use of AGP alone or in combination with EM

resulted in better broiler performance than the other treatments, agreeing with the findings of Jin *et al*. (1998). There does not appear to be any advantage in using the combination of AGP and EM over the use of AGP alone and no advantage at all in using EM alone. The control and EM treatments showed significantly higher (P<0.05) FCR values than the AGP and combination of AGP and EM treatments, which further demonstrated poor broiler performance. Of note was the fact that the EM group had a pronounced lower cumulative water intake (P<0.05) compared to the other treatments.

For the challenged birds at 40 days of age, it was interesting to note that no significant (P>0.05) difference was observed between any of the treatments (Table 3.6). It was clear that challenging the birds with *Clostridium perfringens* was not effective in reducing broiler performance. Furthermore, supplementation with AGP or EM did not improve broiler performance in the face of *Clostridium perfringens* challenge. The results of this study agree with the findings from Karaoglu & Durdag (2005), who found probiotic supplementation had no effect on the growth performance of broilers, while contradicting Jin *et al*. (2000) who promoted the use of probiotics to improve broiler performance. There appeared to be no advantage in using AGP or EM in the face of a *Clostridium perfringens* challenge. One would have expected a difference in performance between the control and the groups having AGP. It could be that the challenge may not have produced the desired result of reducing performance.

In a bid to shed more light on the apparent lack of effect of a *Clostridium perfringens* challenge, Table 3.7 shows the results of the t-test where the control treatment of the challenged trial were compared with the control of the non-challenged trial without main treatment effects at 40 days of age. Broiler chickens performed better (P<0.05) in terms of cumulative water intake and PEF for non-challenged than challenged control groups, and it could be that low mortality rates might have played a vital role in the performance. No differences (P>0.05) were found in terms of cumulative feed intake, ADG, BW, body weight gain (BWG) and FCR. The PEF show a tendency towards reduced production efficiency in the challenged group which may significantly affect the profit.

A mortality rate of 2.2% obtained from this study was low and the causes of death for both challenged and non-challenged birds were reportedly from *E. coli* infection, Septicemia, Pneumonia, Autolysis and Yolksac infection. Intestinal and liver lesions of the challenged and non-challenged birds in all treatments at 21 and 28 days of age produced mild necrotic enteritis and the livers had fewer lesions compared to the intestines.

Cowen *et al*. (1987) reported that broilers challenged with *Clostridium perfringens* alone failed to induce mortality or other symptoms of necrotic enteritis while Olkowski *et al*. (2006) reported that none of the challenged broilers produced overt clinical signs of necrotic enteritis and there were no mortalities associated with oral exposure to high doses of *Clostridium perfringens*. Since the causes of death in the study were not directly related to *Clostridium perfringens* infection it could be that the challenge only lowered the immunity of the broilers as shown by the causes of death. It has been shown that simultaneous or concurrent infections of *E. necatrix* and *Clostridium perfringens* resulted in the increased clostridial populations in the intestine and high mortality (Baba *et al*., 1996). Since in this study, the birds were challenged with *Clostridium perfringens* alone it may explain why there was a failure to induce overt necrotic enteritis. It may also be that the use of cold stress to induce immunosuppression was not effective.

3.4 **Conclusions**

The findings of the present study showed that EM under the current dosage failed to improve broiler performance especially in the non-challenged group. The results in the challenged group were not conclusive because of a failure to induce overt necrotic enteritis. This could be attributed to inability of cold stress to induce immunosuppression. It is recommended that EM, when given to broilers, should be supplemented in feed rather than in water since it resulted in a reduced water intake. There is need for more work to evaluate the effect of EM when given in feed alone. Dose response studies should also be conducted in future to determine the effect of different dosages in water and feed on performance.

Table 3.3: Mean values of production performance parameters of broiler chickens up to 21 days of age, receiving either antibiotic growth promoters (AGP), Effective Microorganisms (EM) or both

Treatments	Initial body weight (g)	Daily feed intake (g)	Cumulative feed intake (g)	Daily water intake (g)	Cumulative water intake (g)	Average daily gain (g/d)	Body weight (g)	Feed conversion ratio
Control	40.81	526.2	882.9	847.0[a]	1576[a]	527.9[a]	568.7[a]	1.565[bc]
AGP	41.44	495.8	859.1	880.0[a]	1698[a]	562.9[a]	604.4[a]	1.422[a]
EM	40.75	497.1	837.4	740.0[b]	1368[b]	461.3[b]	502.0[b]	1.668[c]
AGP + EM	40.69	496.9	870.9	872.0[a]	1679[a]	559.4[a]	600.1[a]	1.452[ab]
P-value	0.208	0.364	0.22	0.009	0.008	0.002	0.002	0.013
SEM[1]	0.255	13.51	14.52	24.1	54.6	13.99	14.08	0.045
LSD[2]	0.817	43.24	46.45	77.0	174.8	44.75	45.04	0.143
CV%[3]	1.2	5.4	3.4	5.8	6.9	5.3	4.9	5.8

[a,b,c]Means in the same column without common superscript differ significantly
[1]**SEM** = pooled standard errors of the means
[2]**LSD** = least significant difference
[3]**CV%** = coefficient of variation

Table 3.4: Mean values of production performance parameters of broiler chickens challenged with *Clostridium perfringens* type A up to 21 days of age, receiving either antibiotic growth promoters (AGP), Effective Microorganisms (EM) or both

Treatments	Initial body weight (g)	Daily feed intake (g)	Cumulative feed intake (g)	Daily water intake (g)	Cumulative water intake (g)	Average daily gain (g/d)	Body weight (g)	Feed conversion ratio
Control	41.56	561.1	876.7	795.7[a]	1438[a]	469.3	510.9	1.723
AGP	40.56	506.6	811.0	800.0[a]	1494[a]	466.9	507.5	1.597
EM	41.38	500.3	815.5	749.5[b]	1358[b]	462.6	504.0	1.619
AGP + EM	41.56	503.3	828.9	804.0[a]	1466[a]	490.0	531.5	1.560
P-value	0.113	0.109	0.140	0.007	0.018	0.284	0.273	0.183
SEM[1]	0.293	17.65	19.61	9.11	24.6	9.97	9.99	0.049
LSD[2]	0.938	56.47	62.72	29.14	78.6	31.90	31.95	0.158
CV%[3]	1.4	6.8	4.7	2.3	3.4	4.2	3.9	6.1

[a,b]Means in the same column without common superscript differ significantly
[1]**SEM** = pooled standard errors of the means
[2]**LSD** = least significant difference
[3]**CV%** = coefficient of variation

Table 3.5: Mean values of production performance parameters of broiler chickens up to 40 days of age, receiving either antibiotic growth promoters (AGP), Effective Microorganisms (EM) or both

Treatments	Daily feed intake (g)	Cumulative feed intake (g)	Daily water intake (g)	Cumulative water intake (g)	Average daily gain (g/d)	Body weight (g)	Feed conversion ratio (g)	Production efficiency factor (index)
Control	807	3380	1340	5800^a	45.05^{ab}	1786^{ab}	1.895^b	236.3^{ab}
AGP	757	3306	1362	5936^a	46.19^a	1835^a	1.802^a	248.6^a
EM	764	3208	1246	5196^b	41.67^b	1665^b	1.926^b	210.5^b
AGP + EM	792	3427	1319	5815^a	47.92^b	1895^a	1.809^a	249.5^a
P-value	0.320	0.151	0.133	0.002	0.031	0.030	0.007	0.025
SEM[1]	20.2	63.6	32.5	97.2	1.221	45.0	0.022	8.09
LSD[2]	64.7	203.3	103.8	311.1	3.906	143.9	0.071	25.89
CV%[3]	5.2	3.8	4.9	3.4	5.4	5.0	2.4	6.9

[a,b]Means in the same column without common superscript differ significantly
[1]**SEM** = pooled standard errors of the means
[2]**LSD** = least significant difference
[3]**CV%** = coefficient of variation

Table 3.6: Mean values of production performance parameters of broiler chickens challenged with *Clostridium perfringens* type A up to 40 days of age, receiving either antibiotic growth promoters (AGP), Effective Microorganisms (EM) or both

Treatments	Daily feed intake (g)	Cumulative feed intake (g)	Daily water intake (g)	Cumulative water intake (g)	Average daily gain (g/d)	Body weight (g)	Feed conversion ratio (g)	Production efficiency factor (index)
Control	774	3549	1180	5063	44.79	1802	1.970	182.9
AGP	862	3537	1201	5123	45.45	1825	1.938	200.2
EM	799	3476	1095	4849	45.41	1814	1.917	198.1
AGP + EM	778	3465	1088	4940	45.30	1804	1.921	196.8
P-value	0.610	0.739	0.166	0.207	0.859	0.920	0.233	0.097
SEM[1]	50.7	64.9	39.5	90.1	0.610	26.6	0.018	4.65
LSD[2]	162.3	207.6	126.4	288.3	1.952	85.2	0.059	14.88
CV%[3]	12.6	3.7	6.9	3.4	2.7	2.9	1.9	4.8

[a,b]Means in the same column without common superscript differ significantly
[1]**SEM** = pooled standard errors of the means
[2]**LSD** = least significant difference
[3]**CV%** = coefficient of variation

Table 3.7: Mean values of production performance parameters of broilers challenged with *Clostridium perfringens* type A versus broilers that were not challenged up to 40 days of age

Treatments	Cumulative feed intake (g)	Cumulative water intake (g)	Average daily gain (g/d)	Body weight (g)	Body weight gain (g)	Feed conversion ratio	Production efficiency factor (index)
Non-challenged Means (SD)	3380 ±81.9	5800[a] ±236.0	45.05 ±2.53	1786 ±98.58	1745 ±97.95	1.895 ±0.073	236.3[a] ±21.57
Challenged Mean (SD)	3549 ±125.0	5063[b] ±210.2	44.79 ±1.33	1802 ±56.94	1760 ±56.57	1.970 ±0.027	182.9[b] ±8.94
P-value	0.064	0.003	0.866	0.796	0.805	0.103	0.004

[a,b]Means in the same column without common superscript differ significantly
SD = Standard deviation

CHAPTER 4

Evaluating the effect of Effective Microorganisms on broilers inoculated with *Clostridium perfringens*

4.1 Introduction

The concern that the continuous use of antibiotics gives a survival advantage to drug-resistant strains of the bacteria have been reinforced by the occurrence of foodborne illnesses and other infections (Montagne *et al*., 2003). Currently, scientists are searching for alternative feed additives that may be used to alleviate the problems associated with the withdrawal of the antibiotic growth promoters (AGP) from feed. A popular alternative to the use of the antibiotics has been the use of effective microorganisms (EM) which are environmentally friendly microorganisms. Effective Microorganisms are a mixed culture of microorganisms comprising lactic acid bacteria, yeasts, actinomycetes and photosynthetic bacteria (Higa & Parr, 1994) which are supposed to suppress the growth of pathogenic bacteria and to aid in digestion.

An earlier study indicated that EM failed to improve broiler performance in broilers either challenged with *Clostridium perfringens* or not challenged. This was attributed to the fact that the EM supplementation resulted in reduced water intake, thereby negatively affecting the feed conversion ratio of the broilers. Furthermore, the results in the challenged group were not conclusive because of the failure to impair broiler performance and to induce overt necrotic enteritis. The aim of this study was to evaluate the effect of increasing the dose of the EM on the performance of broilers after orally inoculating the broilers with high dosages of *Clostridium perfringens*. The EM was also only added in the feed and not in the drinking water of the broilers.

4.2 Materials and Methods

4.2.1 Housing and Management

The same housing and management as described in Experiment 1 was continued in this experiment. All chicks were fed a commercial two-phase maize-soya type diet consisting of a mash starter and grower/ finisher formulated to meet or exceed the nutritional requirements of broilers as recommended by NRC (1994) as in Experiment 1, but unlike in Experiment 1, fishmeal was included in the rations for inducing *Clostridium perfringens*.

As in the previous experiment, the chicks from one room were inoculated orally with *Clostridium perfringens*, while the chicks from the other room remained unchallenged. The challenged group of birds was inoculated orally with 1 mL (1 x 10^8 CFU/mL) of *Clostridium perfringens* as a single dose on day 14. Cloacal swabs were taken from all chicks on the day of arrival and a day after inoculation with *Clostridium perfringens* to be analysed for the presence of *Clostridium perfringens*.

4.2.2 Experimental design and dietary treatments

Three hundred and twenty chicks from one room were challenged with *Clostridium perfringens* at two weeks of age, while the birds in the other room remained unchallenged. Each group was then randomly allocated to four treatments.

Treatment 1: Unmedicated broiler rations (Control).

Treatment 2: Broiler rations containing Zinc bacitracin as antibiotic growth promoters (AGP) at 33g/kg.

Treatment 3: Broiler rations containing EM Bokashi at 50g/kg in the feed.

Treatment 4: Broiler rations containing AGP (Zinc bacitracin) at 33g/kg and EM Bokashi in feed at 50g/kg (AGP + EM).

Table 4.1: Composition and nutrient content of basal diets fed to broilers in the study

Ingredients and composition (g/kg as fed)	Starter	Grower/Finisher
Yellow maize (8.6%)	63.08	73.99
Maize Gluten (60%)	14.13	4.77
Fullfat Soya beans	8.41	9.00
Fishmeal	4.50	2.00
Bokashi	4.00	3.20
Limestone	0.55	1.20
Salt	1.20	0.90
L-Lysine HCL	0.89	0.66
DL-Methionine	0.35	0.26
Vitamin/ Mineral Premix	1.33	1.20
Coccidiostat ©	0.04	0.08
Zinc Bacitracin (where applicable)	0.03	0.05
Monocalcium Phosphate	1.49	2.70
ME (MJ/ kg)	125	134
Dry matter (g/ kg)	850	906
Oil ether extract (g/ kg)	37.5	55
Crude protein (g/ kg)	230	196
Crude fibre (g/ kg)	24.4	26.7
Calcium (g/ kg)	10	9.0
Phosphorus (g/ kg)	3.4	3.9
Available phosphorus (g/ kg)	4.2	1.5
Salt (g/ kg)	3.2	3.4
Sodium (g/ kg)	1.8	1.8
L Lysine (g/ kg)	7.6	7.2
Methionine (g/ kg)	6.4	5.9
Methionine & cystine (g/ kg)	4.1	3.9

4.2.2.1 **Preparation of EM**

Preparation of EM was done as in Experiment 1. EM Bokashi was supplemented to the chicks from day-old until the termination of the experiment on day 40 through the feed. EM Bokashi was added in the feed during feed mixing. No EM was added in water in this experiment.

4.2.2.2 **Induction of necrotic enteritis**

The same procedure of *Clostridium perfringens* culture was followed as in Experiment 1. The bacterial suspension was adjusted to an optical density of 1 x 10^8 CFU/mL using 450nm wavelength (Onderdonk & Allen, 1994). At two weeks of age, challenged groups were inoculated individually *per os* with 1mL of this suspension.

4.2.3 **Measurements and observations**

Performance parameters of the broilers were measured in terms of weekly feed and water intake, body weight and feed conversion ratio (FCR) for the periods, 0-21 and 0-40 days of age and the same procedure was followed as in Experiment 1. The laboratory analysis of cloacal swabs of the all the challenged chicks showed a positive *Clostridium perfringens*.

4.2.4 **Statistical analysis**

Data was analysed using the statistical programme, GenStat (2000). The experiment was designed as a randomised block designed with 4 replicates and 4 treatments. Analysis of variance (ANOVA) was used to test for differences between treatments. The data was tested as normal with homogeneous treatment variances. Treatment means were separated using Fishers protected t-test least significant difference (LSD) at the 5% level of significance (Snedecor & Cochran, 1980). If the F-probability from the ANOVA was significant at 5% treatment groups were compared in terms of differences in live weight, feed intake, feed conversion ratio and mortality at twenty-one and forty days of age and the production efficiency factor (PEF) at forty days of age. At day 40 the challenged

group was compared with the control treatments of the non-challenged group using the t-test.

4.3 **Results and Discussions**

The performance of the non-challenged broilers and those challenged with *Clostridium perfringens* were evaluated at 21 days of age as presented in Table 4.2 and 4.3 and at 40 days of age presented in Table 4.3 and 4.5. The initial body weight, water intake, average daily gain (ADG), body weight (BW) and feed conversion ratio (FCR) for the non-challenged broilers were not significantly different (P>0.05) among the control, AGP, EM and the combination of both AGP and EM. It shows that none of these treatments had any nutritional advantage to the birds. However, feed intake and cumulative feed intake of broilers from the control, AGP and combination of both AGP and EM treatments were higher (P<0.05) than that broilers from EM treatment. This agrees with the previous results (Botlhoko *et al*., 2006 unpublished data) though in that study, it was reported that the inclusion of EM in water resulted in less water intake. For this part of this study, cumulative water intake for the control and AGP group were also higher (P<0.05) than EM and the combination of both AGP and EM. The supplementation of EM might have negatively affected the feed intake, and this agrees with the study by Buenrostro & Kratzer (1983) as cited by Jacobs (2000) that feed intake was decreased when chicks were inoculated with *Lactobacillus acidophilus*.

The initial body weight, feed intake, and cumulative feed intake for challenged broilers of the control, AGP, EM and combination of both AGP and EM treatments were not significant (P>0.05) as shown in Table 4.2. Water intake and ADG in the EM and the combination of both AGP and EM treatments were significantly (P<0.05) higher than control and AGP. The combination of both AGP and EM showed a higher (P<0.05) cumulative water intake than EM, AGP and control, whilst EM was higher (P<0.05) than AGP and control respectively. The broilers given EM had a higher (P<0.05) BW and FCR compared to control, AGP and combination of both AGP and EM. The supplementation of EM in feed yielded positive results where it was clearly improving the water intake and therefore BW and FCR. This result confirms findings of Jin *et al.*

(1988) and Kralik *et al.* (2004) that probiotics improved broiler performance. The administration of EM in feed instead of water might be one of the factors affecting its effectiveness, when comparing that with the previous results (Botlhoko *et al.*, 2006 unpublished data) where inclusion of EM in water negatively affected the performance of broilers when compared with the present results. This agrees with the study by Watkins & Kratzer (1984) as cited by Jacobs (2000). Another factor might be that the early administration of EM shows to give the broilers immunity. Generally, it is noted that EM showed an improved broiler performance when compared with non-challenged at 3 weeks. This might be that the challenge with *Clostridium perfringens* did not suppress their performance under the current dosage or the competitive exclusion has played the vital role in the performance of the broilers.

The feed intake, cumulative feed intake, water intake, cumulative water intake, ADG, BW, and PEF showed significant ($P<0.05$) differences for the control, AGP, EM and the combination of both AGP and EM treatments at 40 days of age (Table 4.3). In this results only FCR for non-challenged broilers did not show significance ($P>0.05$) difference among treatments. Control and the combination of both AGP and EM had a higher ($P<0.05$) feed intake than the combination of both AGP and EM. Control, AGP, and the combination of both AGP and EM had a higher ($P<0.05$) cumulative feed intake than EM. The trend in the earlier results (non-challenged, Table 4.2) showed to be continued where the lack of EM to improve feed intake which is crucial for the general performance of ADG, BW and PEF. Control had a higher ($P<0.05$) water intake than AGP, EM and the combination of both AGP and EM respectively. Control and AGP showed a better ($P<0.05$) cumulative water intake than EM and the combination of both AGP and EM. Control, AGP and combination of both AGP and EM showed a higher ($P<0.05$) ADG, BW, and FCR than EM. This result shows the inconsistency reported earlier in this study in the challenged broilers at 3 weeks where EM improved broiler performance in terms of water intake, ADG, BW and FCR. This confirms the investigations related to the use of probiotics being inconsistent (Jin *et al.*, 1998).

No significant (P>0.05) differences were observed for challenged broilers in terms feed intake and cumulative feed intake among treatments (Table 4.4). This shows that neither AGP nor EM or their combination had any effect in terms of feed intake. The combination of both AGP and EM had a high (P<0.05) cumulative water intake than EM, AGP and control respectively. The combination of both AGP and EM showed significantly (P<0.05) higher ADG than AGP, EM and control respectively. The trend in high performance by the combination of both AGP and EM continued and this might be that broilers respond well than when is given alone and contributes to better ADG and BW. The AGP, EM and combination of both AGP and EM had a significantly (P<0.05) higher BW than control. The AGP had a better (P<0.05) FCR compared to the combination of both AGP and EM, EM and control respectively. The AGP and the combination of both AGP and EM had a significantly (P<0.05) high PEF than EM and control respectively. At this stage AGP shows to have a better performance in terms of BW, FCR and PEF and this confirms the results by Wilson *et al*. (2005) where Zinc Bacitracin reduced intestinal levels of *Clostridium perfringens* in broilers while improving growth rate. However, EM showed a significant (P<0.05) performance when compared to control in terms of water intake, cumulative water intake, BW, and FCR. Jacobs (2000) reported that the supplementation of EM in either water or feed or both in water and feed did not improve phosphorus and calcium content in terms of eggshells. Furthermore in that study was found that EM did not show significant (P>0.05) improvement of eggshell strength and finally it had a higher incidence of bloodspot and light yolk colours.

There were no significant (P>0.05) differences in terms of cumulative feed intake, cumulative water intake and PEF for the control treatments of broilers non-challenged versus challenged at day 40 (Table 4.5). However, challenged broilers showed a higher (P<0.05) ADG, BWG and FCR than non-challenged broilers. The result of the present study contradicts our previous results (experiment 1) were challenged broilers showed a poor performance than control treatments, and the challenge with *Clostridium perfringens* did not suppress the performance of the broilers or cause any mortality related to necrotic enteritis. The high performance of control groups of challenged broilers at this stage is

not known and need further research for investigation. Non-challenged broilers had a significantly higher (P<0.05) BW than challenged.

The result of this study shows a very low mortality rate of 1.3% for both non-challenged and challenged broilers and the causes of mortalities were from Pneumonia, Yolk-sac infection and Autolysis. The causes of mortalities were not related to necrotic enteritis and this confirms our previous study (experiment 1). The challenged broilers had a mild necrotic enteritis lesions and confirms reports by Løvland & Kaldhusdal, (1999); Choct (2001) and further reports by Olkowski *et al*. (2006) where non of the challenged broilers produced overt clinical signs and no mortality associated with necrotic enteritis with oral administration of high *Clostridium perfringens*.

The gross examination of broilers challenged orally *Clostridium perfringens* at day 21 had fewer intestinal lesions while day 28 had larger amount of lesions. Day 28 showed promising results in terms of causing the overt necrotic enteritis and the inclusion of fishmeal might have also contributed slightly (Al-Sheikhly & Truscott, 1977; Olkowski *et al*., 2006). The results show that the response to challenge may differ from experiment to experiment. In a pilot trial carried out earlier, the broilers were inoculated with *Clostridium perfringens* on day one where a high mortality rate was experienced and was related to necrotic enteritis. For the non-challenged broilers, a few intestinal lesions were noted on both day 21 and 28, even though strict biosecurity measures were ensured. This seems to be in agreement with the observation that *Clostridium perfringens* is a common inhabitant of the chicken intestinal tract, with no apparent impact in the host as indicated by Ficken & Wages (1997) cited by Wilson *et al*. (2005).

4.4 **Conclusions**

EM did not improve the broiler performance as reported in the previous study (experiment 1), however, it is noted that the early supplementation of EM showed desired results by a slightly improved performance and a reduced mortality. It shows that the effectiveness of EM on the performance of broilers at 6 weeks of age showed to be weakened compared at 3 weeks of age. This might be that the number of necrotic lesions

in the intestines has increased with the increasing growth of the broilers and therefore suppressed the performance. The cold stress, withdrawal of the feed and the inoculation with *Clostridium perfringens* might have also negatively influenced the performance of the broilers. Further research is required to determine the most effective source of probiotics and the inoculation of *Clostridium perfringens* be coupled with the pre-challenge with coccidiosis to produce the overt intestinal necrotic lesions.

Table 4.2: Mean values of production performance parameters of broiler chickens up to 21 days of age, receiving either antibiotic growth promoters (AGP), Effective Microorganisms (EM) or both

Treatments	Initial body weight (g)	Daily feed intake (g)	Cumulative feed intake (g)	Daily water intake (g)	Cumulative water intake (g)	Average daily gain (g/d)	Body weight (g)	Feed conversion ratio
Control	68.88	535.6^a	1039^a	2.733	36.21^a	26.0	615	1.91
AGP	69.00	526.6^a	1053^a	2.470	37.43^a	17.1	430	2.92
EM	69.00	456.2^b	917^b	2.420	30.45^b	16.4	415	2.65
AGP + EM	67.88	507.7^a	1013^a	2.110	22.68^c	22.4	540	2.15
P-value	0.464	0.005	0.024	0.189	0.001	0.061	0.063	0.086
SEM^1	0.564	12.18	27.1	0.182	1.618	2.41	0.051	0.309
LSD^2	1.806	38.95	86.6	0.582	5.176	7.70	0.162	0.989
CV%^{3}	1.6	4.8	5.4	15.0	10.2	23.5	20.3	24.8

[a,b,c]Means in the same column without common superscript differ significantly
[1]**SEM** = pooled standard errors of the means
[2]**LSD** = least significant difference
[3]**CV%** = coefficient of variation

Table 4.3: Mean values of production performance parameters of broiler chickens challenged with *Clostridium perfringens* type A up to 21 days of age, receiving either antibiotic growth promoters (AGP), Effective Microorganisms (EM) or both

Treatments	Initial body weight (g)	Daily feed intake (g)	Cumulative feed intake (g)	Daily water intake (g)	Cumulative water intake (g)	Average daily gain (g/d)	Body weight (g)	Feed conversion ratio
Control	70.81	49.50	976	1.93[c]	25.76[d]	18.84[c]	0.4725[d]	2.487[c]
AGP	69.50	54.60	1105	2.52[bc]	38.42[c]	27.66[c]	0.6525[c]	1.902[bc]
EM	70.72	53.40	1115	3.74[a]	46.43[b]	33.19[a]	0.7703[a]	1.623[a]
AGP + EM	69.50	57.60	1123	3.31[ab]	50.94[a]	29.32[a]	0.6825[b]	1.825[b]
P-value	0.477	0.495	0.128	0.023	0.001	0.001	0.001	0.001
SEM[1]	0.765	35.8	42.5	0.353	1.404	0.380	0.008	0.072
LSD[2]	2.494	119.6	142.0	1.129	4.491	1.269	0.027	0.239
CV%[3]	2.2	13.3	7.9	24.6	7.0	2.8	2.5	7.3

[a,b,c,d]Means in the same column without common superscript differ significantly
[1]**SEM** = pooled standard errors of the means
[2]**LSD** = least significant difference
[3]**CV%** = coefficient of variation

Table 4.4: Mean values of production performance parameters of broiler chickens up to 40 days of age, receiving either antibiotic growth promoters (AGP), Effective Microorganisms (EM) or both

Treatments	Daily feed intake (g)	Cumulative feed intake (g)	Daily water intake (g)	Cumulative water intake (g)	Average daily gain (g/d)	Body weight (g)	Feed conversion ratio	Production efficiency factor (index)
Control	980^a	2899^a	5.22^a	97.5^a	33.10^a	1.230^a	2.50	140.5^a
AGP	839^b	2762^a	4.14^b	88.9^a	25.95^{ab}	0.980^{ab}	3.22	94.6^{ab}
EM	696^c	2368^b	2.21^c	57.0^c	20.57^b	0.787^b	3.32	67.2^b
AGP + EM	885^{ab}	2792^a	4.20^b	70.8^b	31.74^a	1.180^a	2.52	134.4^a
P-value	0.005	0.001	0.001	0.001	0.013	0.012	0.131	0.017
SEM^1	40.1	51.0	0.246	3.36	2.28	0.079	0.282	14.37
LSD^2	128.2	163.0	0.786	10.75	7.28	0.253	0.902	45.99
CV%^{3}	9.4	3.8	12.5	8.9	16.3	15.2	19.5	26.3

^{a,b,c}Means in the same column without common superscript differ significantly
^{1}**SEM** = pooled standard errors of the means
^{2}**LSD** = least significant difference
^{3}**CV%** = coefficient of variation

Table 4.5: Mean values of production performance parameters of broiler chickens challenged with *Clostridium perfringens* type A up to 40 days of age, receiving either antibiotic growth promoters (AGP), Effective Microorganisms (EM) or both

Treatments	Daily feed intake (g)	Cumulative feed intake (g)	Daily water intake (g)	Cumulative water intake (g)	Average daily gain (g/d)	Body weight (g)	Feed conversion ratio (g)	Production efficiency factor (index)
Control	981	3080	2.50[b]	71.6[c]	39.15[c]	0.859[b]	2.249[c]	135.2[c]
AGP	997	2950	3.85[ab]	100.3[b]	47.24[b]	1.273[a]	1.785[a]	226.7[ab]
EM	855	2923	2.86[b]	99.9[b]	41.44[c]	1.143[a]	2.007[bc]	194.2[b]
AGP + EM	1144	3293	4.62[a]	126.0[a]	51.35[a]	1.198[a]	1.835[b]	254.0[a]
P-value	0.370	0.233	0.040	0.002	0.001	0.024	0.005	0.002
SEM[1]	106.6	125.7	0.470	6.35	0.984	0.074	0.064	12.76
LSD[2]	353.4	420.2	1.503	20.31	3.289	0.247	0.214	42.68
CV%[3]	21.4	8.2	27.2	12.8	4.4	13.2	6.5	12.6

[a,b,c]Means in the same column without common superscript differ significantly
[1]**SEM** = pooled standard errors of the means
[2]**LSD** = least significant difference
[3]**CV%** = coefficient of variation

Table 4.6: Mean values of production performance parameters of broiler chickens challenged with *Clostridium perfringens* type A versus non-challenged up to 40 days of age

Treatments	Cumulative feed intake	Cumulative water intake	Average daily gain	Body weight	Body weight gain	Feed conversion ratio	Production efficiency factor
	(g)	(g)	(g/d)	(g)	(g)		(index)
Non-challenged Means (SD)	2899 ±152.3	97.5 ±18.3	33.10 ±1.5	1.230 ±0.1	1159 ±52.8	2.505 ±0.1	140.5 ±19.1
Challenged Mean (SD)	3078 ±252.9	71.6 ±36.7	38.90 ±8.2	0.867 ±0.5	1361 ±286.7	2.260 ±0.3	132.6 ±11.1
P-value	0.158	0.092	0.003	0.017	0.003	0.026	0.584

[a,b]Means in the same column without common superscript differ significantly
SD = Standard deviation

CHAPTER 5

5.1 **Summary and recommendations**

The European Union banned the use of antimicrobial growth promoters (AGP) in 1999 and the remained ones were discontinued in 2006. There was a general fear that the AGP had a transmission and the proliferation of resistant bacteria through the food chain and that could compromise the health of human beings. Therefore, a search for the alternative feed additives had to be used to alleviate the problems associated with the withdrawal of AGP from the feed. A popular alternative to the use of the antibiotics has been the use of EM, which are probiotics used as environmentally friendly microorganisms that has been proved to enhance broiler feed efficiency, general performance and protection of broilers against pathogens. However, the use of probiotics is associated with inconsistency in producing the contradicting results. This study was undertaken to evaluate the effect of effective microorganisms (EM) on the performance of broilers which had been either challenged or not-challenged with *Clostridium perfringens* type A.

In the first study the EM was administered through the feed and water, however, the administration of EM through the water showed to have negatively influenced the performance of broilers. The challenged broilers did not show any overt clinical necrotic enteritis and the mortality was not associated with necrotic enteritis. Generally, the use of EM failed to improve broiler performance.

In the second study EM was administered through the feed only and the broilers were inoculated with *Clostridium perfringens*. The broilers were offered EM early on day one. The results showed that EM improved the broiler performance at three weeks of age and at day forty continued to perform poorly. It showed the early administration of EM has added an advantage in the better performance. It is noted that the combination of both AGP and EM showed a better performance of broilers compared when EM is given alone. The broilers had mild necrotic intestinal lesions, and did not suppress the performance or cause any mortality.

In the first study it was recommended that EM when given to broilers should be supplemented in feed rather than in water since it resulted in a reduced water intake. There is need for more work to evaluate the effect of EM when given in feed alone. Dose response studies should also be conducted in future to determine the effect of different doses in water and feed on performance. Finally, in the second study, it was recommended that further research is required to determine the most effective source of probiotics and the inoculation of *Clostridium perfringens* be coupled with the pre-challenge with coccidiosis to produce the overt intestinal necrotic lesions.

CHAPTER 6
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