THE INFLUENCE OF EFFECTIVE MICROORGANISMS (EM) ON THE GROWTH, PRODUCTION AND EGG QUALITY OF THE COMMERCIAL LAYING HEN

By

AMANDA JACOBS

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Faculty of Natural and Agricultural Sciences

University of Pretoria

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Chapter 4 The Influence of the Method of EM (Effective Microorganisms) Supplementation on the Egg Quality of the Commercial Laying Hen

Abstract

In the first part of the study an experimental design was followed whereby 600 DeKalb Amber-link® commercial laying pullets were subjected to three levels of EM bokashi (0.5%, 1.0% and 1.5%) and a standard level of a coccidiostat in the starter (17% CP, 0.9% Ca and 0.4% available phosphate) and grower (15% CP, 0.8% Ca and 0.35% available phosphate) diets from day-old to 16 weeks of age. EM treatments did not significantly affect the average weekly body mass, average daily gain, average weekly feed intake, cumulative weekly feed intake, cumulative feed conversion ratio and the average bi-weekly shank length over the control. The coccidiostat treatment maintained significant lower body weights, average daily gains and worse feed conversion ratios than all the EM treatments and the control throughout the trial period of 16 weeks. Although not significant the EM1.5% level had the best feed conversion ratio, the highest body mass and the longest shank length at the end of the trial period at week 16. Mortalities were not treatment linked.

In the second part of the study it was determined whether EM and coccidiostat supplementation during the growing phase had any effect on performance in the laying phase and if EM and antibiotic supplementation during the laying phase has had any additional effect to EM and coccidiostat supplementation in the growing phase. For this, pullets that received three EM levels and a coccidiostat in the growing phase (Chapter 2) were halved for each treatment. One half from each treatment continued on the treatments received in the growing phase in the laying phase except the coccidiostat treatment which now received an antibiotic. The other halves received only a basal layer diet. EM and antibiotic supplementation during the laying phase did not influence egg production, egg weight, the percentage of uncollectable eggs, the total amount of eggs laid over the trial period and internal egg quality over supplementation during the growing phase alone. EM supplementation during the laying phase did not influence breaking strength, body weight and shank length over supplementation during the growing phase. Supplementing at 1.5% in the laying phase improved shell thickness over supplementation at this level in the growing phase. Supplementing 0.5% in the laying phase and 1.5% in both phases resulted in lighter yolk colours whilst 1.0%

supplementation in the laying phase increased the incidence of meat spots. Antibiotic treatment resulted in thinner shells and lower breaking strength but darker yolks.

In the third part of the study it was determined in two experiments whether method of EM supplementation (drinking water or the diet or both) exerted any significant effect on egg quality. For this, 50 DeKalb Amber-Link® day-old pullets were raised on basal starter and grower diets and subjected to two different dietary treatments in the subsequent laying phase - a basal layer diet or a diet containing 1.5% EM bokashi. In addition both these treatments received EM stock solution in the drinking water (1 : 1000). These treatments were compared to treatments receiving a basal layer diet or a diet containing 1.5% EM bokashi (both receiving no EM in the drinking water). In the first experiment EM supplementation method did not significantly influence the breaking strength of eggs, internal egg quality, the calcium and the phosphorus content and the ratio of these minerals in eggshell. Supplementation in the drinking water alone resulted in eggs with significantly thinner shells whilst supplementation in the diet alone resulted in eggs with lighter coloured yolks. Supplementation in the drinking water, regardless of diet, resulted in eggs with a higher incidence of meat spots. In the second experiment supplementation in the drinking water, regardless of diet, resulted in significantly less deterioration of egg quality over time. The best result was obtained with a combination of EM in the drinking water and the diet.

Samevatting

In die eerste gedeelte van die studie is 'n eksperimentele ontwerp gevolg waarvolgens 600 DeKalb Amber-Link® kommersiële lêhennetjies onderwerp is aan drie vlakke van EM bokashi (0.5%, 1.0% en 1.5%) en 'n standaard vlak van 'n koksidiostat in die aanvangs- (17% RP, 0.9% Ca en 0.4% beskikbare fosfaat) en die groei- (15% RP, 0.8% Ca en 0.35% beskikbare fosfaat) dieëte vanaf dagoud ouderdom tot 16 weke. Die EM behandelings het nie die gemiddelde weeklikse liggaamsmassas, die gemiddelde daaglikse toenames, die gemiddelde weeklikse voerinnames, die kumulatiewe weeklikse voerinnames, die kummulatiewe voeromset verhouding en die gemiddelde twee-weeklikse skenkellengte betekenisvol beïnvloed bo die kontrole nie.
Die koksidiostat behandeling het betekenisvol laer liggaamsmassas, gemiddelde daaglikse toenames en swakker voeromset verhoudings as al die EM behandelings en die kontrole gehandhaaf gedurende die hele proeftydperk van 16 weke. Alhoewel nie betekenisvol het die EM1.5% vlak die beste voeromset verhouding, die swaarste liggaamsmassa en die langste skenkellengtes gehad aan die einde van die eksperimentele periode op week 16. Mortaliteite was nie gekoppel and behandelings effekte nie.

In die tweede gedeelte van die studie is bepaal of EM en koksidiostat supplementasie gedurende die groeifase enige invloed gehad het op prestasie gedurende die lêfase en of EM en antibiotika supplementasie gedurende die lêfase enige addisionele effek gehad het tot EM en koksidiostat supplementasie in die groeifase. Hiervoor is die hennetjies wat drie EM vlakke en 'n koksidiostat in die groeifase ontvang het (Hoofstuk 2) gehalveer vir elke behandeling. Een helfte van elke behandeling het voortgegaan in die lêfase op die behandelings wat ontvang is in die groeifase, behalwe dat die koksidiostat behandeling nou 'n antibiotika in die lêdieët ontvang het. Die ander helftes het net 'n basale lêdieët ontvang. EM en antibiotika supplementasie gedurende die lêfase het nie eierproduksie, eiermassas, die persentasie abnormale eiers, die totale hoeveelheid eiers gelê oor die eksperimentele periode en interne eierkwaliteit beïnvloed oor supplementasie gedurende die groeifase alleen nie. EM supplementasie in die lêfase het nie die breeksterkte van eiers, liggaamsmassas en skenkellengtes beïnvloed oor supplementasie in die groeifase alleen nie. Supplementering teen 1.5% in die lêfase het dopdikte verbeter oor

supplementasie teen dié vlak in die groeifase. Voorsiening teen 0.5% in die lêfase en teen 1.5% in beide fases het ligter dooier kleure tot gevolg gehad, terwyl supplementasie teen 1.0% in die lêfase die insidensie van vleiskolle verhoog het. Antibiotika behandeling het gelei tot dunner doppe en laer breeksterktes maar donkerder dooier kleure.

In die derde gedeelte van die studie is met twee eksperimente bepaal of metode van EM voorsiening (drinkwater of voer of beide) enige betekenisvolle effek uitgeoefen het op eierkwaliteit. Hiervoor is 50 DeKalb Amber-Link® dag-oud hennetjies grootgemaak op basale aanvangs en groei dieëte en onderwerp aan twee verskillende behandelings in die lêdieët in die daaropvolgende lêfase - 'n gewone basale lêdieët of 'n dieët wat 1.5% EM bokashi bevat het. Hierdie twee behandelings het addisioneel tot die dieëte 'n EM oplossing in die drinkwater ontvang teen 'n konsentrasie van 1: 1000. Hierdie twee behandelings is vergelyk met behandelings wat geen EM supplementasie in die drinkwater ontvang het nie en of 'n basale dieët of 'n dieët met 1.5% EM bokashi gevoer is. In die eerste eksperiment het die EM voorsienings metode nie die breeksterkte van eiers, interne eierkwaliteit, die kalsium en die fosfor inhoud van eierdoppe asook die verhouding van hierdie elemente tot mekaar betekenisvol beïnvloed nie. EM voorsiening in die drinkwater, ongeag van dieët, het gelei tot betekenisvol dunner doppe. EM voorsiening in die dieët alleen het gelei tot ligter dooier kleur. EM voorsiening in die drinkwater, ongeag van dieët, het 'n verhoogde insidensie van vleiskolle tot gevolg gehad. In die tweede eksperiment het supplmentasie in die drinkwater, ongeag van dieët, die bederfbaarheid van eiers oor tyd verbeter. Die beste resultaat is verkry met 'n kombinasie van EM in die drinkwater en die dieët.

Chapter 1

Probiotics – A Review with Special Emphasis on the Chicken

Introduction

Currently one third of the protein in the human diet comes from products of animal origin, which has been associated with improved physical and mental development of children (Briedenhann, 1999). For many consumers products from the domesticated fowl (*Gallus domesticus*) have become the cheapest source of animal protein (Abdulrahim *et al.*, 1999). The global demand for poultry products is continuously increasing significantly. Between 1989 and 1998 chicken meat consumption increased by 23% and egg consumption by 40% (Briedenhann, 1999).

As it is predicted that the production area will not increase sufficiently to meet the demand for food, improved technology is needed to increase productivity per unit area to meet the strong growing demand for animal products (Briedenhann, 1999). It is furthermore difficult to estimate on an industrial basis the total losses in production attributable to feeding poor quality feed ingredients to poultry and livestock. Because of the nutritional importance of protein and specific amino acids, decreased protein quality has the potential to cause significant losses in poultry production due to decreased efficiency (Grimes *et al.*, 1997).

The primary concerns of a poultry nutritionist therefore seem always to be productivity and efficiency in production or Return over Extra Outlay (REO) (Sefton, 1998). The use of antibiotics and growth promoters to increase productivity and efficiency is widespread and the general consensus appears to be that poultry production would be impossible without them (Sefton, 1998). Regulatory institutes and the general public are, however, becoming concerned with the use of antibiotics and growth promoting drugs. The poultry industry therefore faces many challenges to meet consumers' demands for safe and "natural" food in an efficient manner. Ways must be found to increase the efficiency of utilization of food and to increase productivity by keeping poultry healthy, both done in a

natural and non-invasive manner. Alternatives are available via the application of biotechnology – alternatives with an attractive Return over Extra Outlay (Sefton, 1998). One such an alternative, using biotechnology, is the addition of microbial cultures or "probiotics" to the gastrointestinal tract of both man and animals – a practice as old as time but only recently rediscovered. This review will attempt to inquire into the deeper purpose behind probiotic supplementation as a possible alternative to antibiotics and other pronutrients with the chicken as model.

"Probiotic" – Clearing of a Definition

The history of live microbial feed supplements dates back thousands of years. The first foods containing living microorganisms were fermented milks that are recorded in the Old Testament of the Bible. The emphasis being on the preservation of feed which would have inevitably had a beneficial effect on the health of the community (Fuller, 1992a).

The word "probiotic" is derived from the Greek meaning "for life" and has had several different meanings over the years such as "organisms and substances which contribute to intestinal microbial balance". The inclusion of the word "substances" gave the aforementioned definition a wide connotation, which would include antibiotics and therefore rendered this definition imprecise and unsuitable for use in animal husbandry. Today probiotics are defined as "a live microbial feed supplement which beneficially affects the host animal by improving its intestinal microbial balance" (Fuller, 1992a). The word "probiotic" and its definition has however been rejected by the EU Commission and the FDA, who specify "microorganism" and "direct-fed microbial" respectively. These organizations feel that the word "probiotic" is untransparent and that it has been used to oppose antibiotics in animal nutrition. The latter are actually probiotic in effect, though not in mode of action (Rosen, 1997). For the purpose of this review, however, the word "probiotic" will be used, as this is the most well known term to describe the addition of beneficial organisms to the animal gut.

Gut Microflora Metabolism – Friend or Foe?

Before supplementing an animal with microorganisms it is necessary to know the natural state of the microflora in the gut and its effect on the host. In the past it was generally believed that the microflora in the gut had adverse effects on the health of both man and animals. Indeed, the metabolic activities of the microflora in the alimentary tract can have wide-ranging implications on the health and performance of poultry, resulting in both detrimental and beneficial effects (Rowland, 1992).

Under normal circumstances the intestinal flora reduces the growth rate of the host. The degree of growth depression will vary between individual birds. It is unclear how and which organisms depress growth (Barrow, 1992). The microfloral populations of the small and large intestines in the chicken can, however, reach densities of almost 10^9 and 10^{11} per gram of ileal and caecal contents respectively. Such a concentration of metabolically active organisms evidently will influence not only the availability of substrate to the host but also the relative "well-being" of the host and hence its ability to grow rapidly (Apajalahti, 1999). Several hypotheses of microbial induced growth depression include reduced nutrient absorption (including reduced glucose and vitamin absorption) in the host due to competition with the gut flora and direct toxic effects of bacterial produced carcinogenic or mutagenic metabolites from substances derived from the diet or produced endogenously (Barrow, 1992).

One of the hypotheses seeking to explain microbial growth depression in the chicken due to malabsorption of nutrients connects the gut microflora with bile-acid metabolism. In the healthy animal bile acids are secreted in conjugated form and are deconjugated by bacteria in the ileum and large intestine. The conjugated acids, but only some of the deconjugated acids, are absorbed and recycled to the gut via the liver and bile. Certain species of the normal gut flora including *Streptococcus faecium*, *Clostridium perfringens*, streptococci and some of the bifidiobacteria and lactobacilli are able to deconjugate the various bile acids found in the chicken including cholic and taurocholic acid. The deconjugated acids are not readily absorbed and are therefore not available for recycling and subsequent release in the gut for the absorption of lipids and lipid-associated

vitamins. Ultimately, this would ultimately lead to a depression in growth (Cole & Fuller, 1984).

In the crop and caeca, microflora in the chicken may have nutritional significance when the hosts are in their natural environment where the diet may be of poor nutritional value. Fermentation in the crop produces a large number of organic acids that are available to the host. The extent of utilization of organic acids in the chicken is not known. Crop fermentation also influences the gut pH, which may exert an effect on enzyme activity. Nucleotides synthesized by lactobacilli are utilized by the host, but B vitamin synthesis contribute little to the chicken's requirements (Barrow, 1992). Vitamin A is also synthesized and may compensate to the chicken's requirements when reared on a deficient diet. None of the caecal flora degrades cellulose or xylan.

The presence of the gut microflora affects protein and energy metabolism in the chicken in several ways. In protein metabolism little or no effect is induced by the gut microflora when adequate amounts of protein are fed. When birds are subjected to protein starvation the excretion of endogenous N are lower in the conventional state compared with the germ-free state (Muramatsu *et al.*, 1994). The presence of gut microflora modifies energy conversion and utilization in the chicken not only at a digestion step but also at an internal metabolism step. Association with the gut microflora increases the maintenance energy requirement of the chicken. However, the association with the gut microflora decreases fasting heat production. The presence of gut microflora therefore play either a buffering or counter-productive action in the energy utilization of birds, i. e. saving the heat loss when no energy is available, but lowering energy utilization when normal growth is maintained (Muramatsu *et al.*, 1994).
Other health implications of gut flora metabolism include the detoxification of dietary toxicants, an alteration in susceptibility of the host to tumour induction and involvement in the protection against disease (Fuller, 1992a).

Protection against disease may be the most valuable beneficial contribution of the gut microflora to the host. The intact intestinal flora resists implantation with non-

indigenous microorganisms because it consists of a community in its climax state. Its members have been selected for over a period of time and represent those microorganisms that can best cope with the biological and non-biological restrictions imposed by the ecosystem. Non-indigenous organisms, such as pathogens, that are less fit, cannot easily colonize this ecosystem (Hentges, 1992). Evidence for this protective effect of the gut flora stems form the observation that germ-free animals are more susceptible to disease than are the corresponding conventional animals with a complete intestinal flora. A stable intestinal flora population helps an animal resist infection by means of bacterial antagonism, bacterial interference, colonization resistance and competitive exclusion (Hentges, 1992; Fuller, 1989). Numerous studies have been done on the effect of gut microflora on the resistance to disease. For example, it has been shown that inoculating layers and broilers with normal caecal flora greatly reduced caecal colonization by salmonellae (Ziprin & Deloach, 1993). The mechanism by which native gut microflora prevents infection with *Salmonella* is not clear but the two most often cited mechanisms are occupation of the sites on the mucosa that *Salmonella* invade and the production of volatile fatty acids (Stavric, 1987). Conventional animals with a complete gut flora also have increased phagocytic activity and immunoglobulin levels as compared with germ-free (gnotobiotic) animals (Bealmear *et al.*, 1984 as cited by Fuller, 1989).

To summarize, in terms of nutritional benefit to the host the gut microflora would tend to be more beneficial when the chicken is in its natural environment, where it is subject to conditions of starvation and needs to conserve both protein and energy and synthesize essential components such as vitamin A. In such conditions the gut microflora would serve as a buffer. In terms of disease resistance in the host, however, the gut flora plays an intrinsic role that cannot be ignored in intensive poultry production systems. This is where probiotics may play an important role.

Why use Probiotics?

In a natural environment the establishment of a microbial population in the digestive tract of all warm-blooded animals, soon after birth, is inevitable (Jernigan & Miles, 1985). In

the wild state an animal obtains its gut flora from its immediate environment which is heavily contaminated with bacteria from its mother until it stabilizes as a complex collection of about 10^{14} microorganisms consisting of about 400 different types (Fuller, 1989). The microbial types that first establish, in most cases, are the forerunners of the final organisms that will colonize and persist in the digestive tract throughout the adult life of the animal. It is known that various types of colonizing bacteria are sensitive to changes that may occur in the digestive tract of the host. The host (and therefore also its environment) and microbial factors will determine the composition of this microflora population (Jernigan & Miles, 1985).

Under normal conditions there would be no need for the use of probiotics, as the population of microorganisms in the intestine of an animal would protect it against disease. In healthy chickens reared on a complete diet administration of probiotics is therefore unlikely to give any substantial direct nutritional benefit because the gut flora would be in a state of equilibrium (Barrow, 1992). Modern methods of perinatal care, however, limit the contact between mother and offspring and provide unnatural foods and environmental conditions (Barrow, 1992). This is especially true in the chicken. The egg is removed from the hen and hatched in a clean incubator. There is no direct contact with the hen and the chick acquires its flora from the incubator environment (Fuller, 1989).

The four most important factors that would influence an otherwise stable gut flora population are excessive hygiene, antibiotic therapy, diet and stress (Fuller, 1989). Examples of excessive hygiene in the chicken include the cleaning and disinfection of houses and housing layers on wire floors to prevent contact with the faeces. Whereas excessive hygiene prevents the acquisition of a protective flora, oral antibiotics suppress its activity even after it has been acquired (Fuller, 1989). Under these conditions the normal gut flora becomes deficient of the normal components that are responsible for disease resistance. Disturbance of the gut flora therefore leads to detrimental effects by allowing colonization by pathogens or growth-depressing bacteria (Barrow, 1992). One of the greatest stress factors in poultry production is the confinement of birds in dense populations. During stress conditions the general trend is for lactobacilli to decrease and

coliforms to increase (Fuller, 1989). Hormonal changes during stress can affect the production of mucus in the intestines which may in turn reduce the components of the gut flora which are usually associated with it (Fuller, 1989).

The use of probiotics seeks to rectify deficiencies in the gut flora induced by an unnatural environment and human management practices. Restoration of the gut flora will enable an animal to return to its normal, more "natural" resistant state (Fuller, 1989).

The Normal Intestinal Flora of Poultry

When supplying a probiotic to an animal it is necessary to know with what that specific probiotic will be dealing with in the gut. Fundamental to the understanding of the probiotic effect is the knowledge of how the specific microorganisms used can affect other microorganisms such as those that comprise the indigenous gut microflora or invading pathogens. If probiotic preparations are to survive and be active in the digestive tract, they have to be suitable for that environment and resist the host's protective mechanisms that are inhibitory to microbes (Jonsson & Conway, 1992). Before compiling a probiotic for chickens it is therefore necessary to quantify the conditions occurring in the gut, what the composition of the microbial population is with which a probiotic will interact and what happens when artificial conditions change an otherwise healthy and stable gut flora population.

The gut microflora of the healthy chicken is very complex and the interactions between different types of organisms are very complicated. Although the alimentary tract of the healthy newly hatched chick is sterile it rapidly becomes colonized by facultative anaerobes. Lactobacilli soon displace these types as the dominant organisms in the crop and small intestine. The caecal flora stabilizes 4-6 weeks after hatching (Barrow, 1992). Microflora from the crop that survive the low pH of the gizzard generally multiply in the small intestine. Organisms from the small intestine may be taken into the caeca. The microbial content of the cloaca and faeces depends on whether they contain material from the small intestine or from the caeca. Caecal droppings are discharged two to four times each day (Barrow, 1992).

Food is swallowed whole and is stored in the crop where a predominantly lactic acid semi-batch fermentation takes place. The E_h (oxidation/reduction potential) is fairly high so that ingested obligate anaerobes and other non-enteric organisms soon die. The predominant organisms in the crop are lactobacilli that adhere to the squamous epithelium of the crop. The lactobacilli produces mainly lactic and acetic acids such that the crop content pH of the healthy chicken is 4–5 with the result that less aciduric organisms do not normally grow to the same high numbers (Barrow, 1992). Lactobacilli that have been isolated from the crop include *Lactobacillus salivarius*, *L. fermentum* and a type resembling *L. acidophilus*. A carbohydrate-rich capsular layer mediates adhesion of lactobacilli to the crop. The number of lactobacilli in the crop will decrease with starvation but will remain sufficient to inoculate fresh food entering the crop. *E. coli* is present in the crop in low numbers possibly because of ingestion of faeces (Barrow, 1992). The pH of the proventriculus and gizzard is very low (pH 1-2) and microbial survival depends on acid tolerance.

Little multiplication of organisms occurs in the duodenum because of the relatively high flow rate of the contents. At this flow it would be difficult for bacteria to multiply sufficiently fast to avoid being washed out. Attachment to the epithelial cells is therefore virtually a prerequisite for microorganisms to colonize this region. Because the epithelium is continuously regenerating and sloughing off cells and overlying mucus, bacteria can only colonize this region if their generation time is faster than the sloughing rate (Jonsson & Conway, 1992). Colonization of the duodenal villi by *Ent. hirae* may result in growth depression. The caeca are filled with a thick viscous fluid containing no food particles. In these organs the highest viable counts and most complex microflora exists. Here the kinetics of bacterial growth resembles those of a batch culture. More than 200 strains reside here and most are obligate anaerobes. A layer of bacteria, hundreds of cells thick, is present in the caeca, embedded in the mucus lining the epithelium. This layer allows for rapid colonization of fresh contents entering the caeca and play no significant role in the protection against pathogens (Barrow, 1992).

As stated in the previous section, a number of naturally occurring and artificial factors such as disease, the immune response, age, diet and orally administered antibiotics can affect the composition of the normal, stable gut flora. These changes are important in an assessment of the need for probiotic use but are poorly understood and have not been fully characterized (Barrow, 1992).

The most obvious change in an adult established flora induced by dietary changes occurs at the anterior end of the gastrointestinal tract. Little change occurs in the caeca. Increased carbohydrate ingestion stimulates the lactobacilli whereas diets rich in protein suppress the lactobacilli, while coliforms, clostridia and streptococci increase in numbers in the crop. Appropriate dietary changes may promote the colonization by probiotics (Barrow, 1992). The most common practice to promote colonization and render manipulation of the gut flora is the use of antibiotics over the short term simultaneously with probiotic organisms resistant to that antibiotic (Barrow, 1992). Without such a drastic selective pressure the adult intestinal flora is difficult to change simply by oral administration of microorganisms. It would be easier to establish a beneficial organism soon after hatching before other organisms are able to colonize (Barrow, 1992).

Composition of Probiotics

Probiotics may contain one or several (up to eight or nine) strains of microorganisms, multiple-strain preparations being active against a wider range of conditions and in a wider range of animals (Fuller, 1989). They may contain organisms of the indigenous flora, especially where adhesion is a prerequisite, or not.

The lactobacilli have remained the most commonly used probiotic organisms. Lactic acid bacteria (LAB) comprise a wide range of genera including a considerable number of species, all of which are able to ferment carbohydrates to lactic acid as major end product. They are typically Gram-positive, usually catalase negative and strictly anaerobic non-sporeforming bacteria. However, lactic-acid producing, sporeforming bacteria are found in the genera *Bacillus* (aerobic) and *Sporolactobacillus* although these are not considered LAB because of their physiological and biochemical properties (Klein,

1997). The most important genera of the LAB used as probiotics are *Lactobacillus*, *Enterococcus* and *Bifidobacterium* (Klein, 1997). The use of lactobacilli stems from studies which have shown that when gut flora develops after birth, as the lactobacilli increase, other components of the flora decrease (Fuller, 1989). The use of *L. acidophilus* and other lactobacilli was also stimulated by the desire to ensure that the organism would survive in the gut. Survival in the gastrointestinal tract are expressed by their resistance to low pH and / or to bile and temperature growth ranges (Klein, 1997). Lactobacilli commonly used in probiotic preparations include *L. delbreuckii* subsp. b*ulgaricus*, *L. acidophilus*, *L. casei*, *L. fermentum*, *L. plantarum*, *L. brevis*, *L. cellobiosus*, *L. lactis* and *L. reuteri* (Fuller, 1992a). The bifidiobacteria currently being used as probiotics are *Bifidobacterium adolescentis*, *Bif. animalis*, *Bif. bifidum*, *Bif. infantis*, *Bif. longum* and *Bif. thermophilum* (Fuller, 1992a).

Probiotics also contain bacteria belonging to the genera *Leuconostoc*, *Pediococcus*, *Propionibacterium* and *Bacillus*. Two common strains of *Enterococcus faecium* (M74 & SF68) are human isolates commonly used in animal probiotic preparations. Yeasts (*Saccharomyces cerevisiae* and *Candida pintolopesii*) and moulds (*Apergillus niger* and *A. oryzae*) are commonly used in animal products (Fuller, 1992a).

A number of empirical observations have suggested that some preparations containing dead bacteria are effective probiotics, but the use of live organisms is emphasized for many products with the implication that intestinal colonization is essential for efficacy (Barrow, 1992).

Probiotics for chickens are designed to either replace beneficial organisms that are not present in the alimentary tract or to provide the chicken with the effects of beneficial bacteria. There are two major groups of probiotic preparations: those that are primarily intended to be effective in the crop and the anterior regions of the alimentary tract and those whose effect is directed mainly in the caeca. It is likely that both types are, to some extent, effective throughout the gut (Barrow, 1992). Criteria used to compose a probiotic for chickens include adhesion to the crop epithelium, the ability to grow in the nutritional

environment of the gut, the ability to tolerate a low pH to survive the gizzard environment and the ability to resist innate or microbially produced inhibitory mechanisms. The lactobacilli are the strains that adhere to the crop epithelium and are the most widely used in the preparation for probiotics for chickens. Avian strains of *L. acidophilus*, *L. salivarius* and *L. fermentum* will adhere to the crop. It was found that human strains of *L. acidophilus, L. helveticus* and *L. brevis* wash rapidly out of the gastrointestinal tract of the chicken indicating the importance of use the of specie specific strains (Barrow, 1992).

Intestinal microflora from adult chickens have been reported to protect chicks from Salmonella colonization. Attempts have been made to identify components of the natural protective flora in chickens with the aim of developing probiotic treatments with a known bacterial composition. Cecal material that successfully protect chicks contain, on a per gram basis, approximately 10^8 coliforms, 10^4 fecal streptococci and more than 10^9 anaerobes including *Bacteroides*, *Bifidobacteria*, *Clostridia*, *Eubacteria*, *Fusobacterium*, *Propionibacteria* and several Gram positive cocci (Oyarzabal & Conner, 1995). Bacterial isolates which have protected chicks against salmonellae infection included *Bifidobacterium bifidum*, *E. faecium*, *Pediococcus* spp., *Streptococcus faecalis*, *S. faecium*, lactobacilli, coli-aerogenes bacteria, clostridia and nonsporing anaerobes (Stavric, 1987; Oyarzabal & Conner, 1995). These types of probiotics which aim to protect against salmonellae infection can contain up to 50 species isolated from the caecal contents of a donor hen. In addition to disease resistance these probiotic cultures will also improve growth (Stavric, 1987). It has been shown that isolates from the natural gut flora can also protect the chicken against *E. coli* (Weinack *et al.*, 1981 as cited by Fuller, 1989), *Campylobacter fetus* subsp. *jejuni* (Soerjadi *et al.*, 1982 as cited by Fuller, 1989). *Clostridium perfringens*, *Cl. botulinum* (Snoeyenbos *et al.*, 1983 as cited by Fuller, 1989) and *Yersinia enterocolitica* (Soerjadi Liem *et al.*, 1984b as cited by Fuller, 1989). The specific bacteria responsible for the latter effects are not known although it is speculated that lactobacilli, streptococci, coliforms and strictly anaerobic bacteria my play a role in the protection (Impey *et al.*, 1982 as cited by Fuller, 1989).

A probiotic culture can be presented to an animal in several ways. The type of preparation will depend on the sort of use intended. Probiotics may be presented in the form of powders (loose or in capsules), tablets, granules or pastes. They may be administered by direct insertion into the mouth or by inclusion in the food e. g. in pelleted feeds (Fuller, 1989) or the drinking water (Watkins & Kratzer, 1984). Experiments have also been done with the administration to newly hatched chicks by spraying a probiotic into the surrounding atmosphere (Fuller, 1992a). In spite of careful selection of strains, it seems unlikely that it would be possible to establish permanently the probiotic organism in the intestinal tract and multiple dosing is essential if the full probiotic effect is to be obtained (Fuller, 1992a).

Characteristics of a Good Probiotic

An effective probiotic is required to operate under a variety of different environmental conditions and to survive in many different forms. Therefore, it should show the following characteristics:

- It should be capable of being prepared as a viable product on an industrial scale (Fuller, 1992a).
- A probiotic should be stable and capable of remaining viable for long periods under storage and field conditions (Fuller, 1989). It is especially important that strains used as probiotics must not lose viability in the period between addition to the feed and consumption by chickens (Haddadin *et al.*, 1996).
- It should have the ability to survive (not necessarily grow) and metabolize in the gut environment e. g. be resistant to low pH, organic acids (Fuller, 1992a) and bile salts (Haddadin *et al.*, 1996). In the chicken tolerance of a low pH is important in allowing extensive colonization of the small intestine by survival of the gizzard environment (Barrow, 1992).
- It should consist of a strain or strains that is capable of exerting a beneficial effect on the host animal, e. g. increased growth, egg production or resistance to disease (Fuller, 1989).
- A probiotic culture should include host-specific strains that adhere to the gut epithelial surface (Fuller, 1989). In the chicken this includes adhesion to the crop.

The ability of strains to adhere to the crop is particularly important for organisms that have a slower rate of multiplication in the feed slurry present in the crop (Barrow, 1992).

- It should be non-pathogenic and non-toxic to the host animal (Fuller, 1989).
- Although it has not been possible to determine the minimum effective dose required in chickens, a probiotic should preferably be presented in large numbers of organisms and as viable cells.
- The strains used in the probiotic culture must be capable of growth in a bulk medium that is easy to prepare and give rise to a high cell count prior to feeding (Haddadin *et al.*, 1996).
- Additional factors such as optimal temperature for growth and resistance to unsaturated fatty acids may also play a significant role in determining colonization by a probiotic and should therefore be taken into account when compiling a probiotic for a certain animal species (Barrow, 1992).

The Mode of Action

The underlying basis for most of the effects claimed for probiotics is an effect on the gut microflora – either its composition or its metabolic activity. The most important ways in which a probiotic organism may exert a beneficial effect on its host is to modify these metabolic processes in the gut. Such beneficial effect could be achieved in theory by a variety of mechanisms (Rowland, 1992):

- By suppressing reactions which result in the generation of toxic or carcinogenic metabolites. This may be done by the provision of enzymes that detoxify ingested substances or their active metabolites. An example in the chicken is the reduction of urease activity of gut microflora when *Lactobacillus casei* is supplemented in the diet. This leads to a reduction in the production of toxic ammonia in the gut (Yeo & Kim, 1997). Another example is the suppression of nitroreductase that is involved in the synthesis of carcinogenic nitrosamines by lactobacilli (Goldin & Gorbach, 1984 as cited by Fuller, 1989).
- By stimulating enzymatic reactions involved in the detoxification of potentially toxic substances either ingested or formed endogenously. An example in humans is the

ingestion of fermented milks containing lactobacilli possessing β-galactosidase activity which raises the intestinal lactase activity which assists lactose digestion and therefore counteracting symptoms associated with lactose intolerance.

- By stimulating mammalian enzymes involved in the digestion of complex nutrients, or where such enzymes are absent (due to genetics or disease) providing a bacterial source of these enzymes.
- By promoting the digestion of previously indigestible substances for instance cellulose and phytate phosphorus.
- By synthesizing vitamins and other essential nutrients not provided in sufficient quantities in the diet.
- By influencing the function and morphology of the digestive tract (Jonsson & Conway, 1992). For instance, lactic acid secreted by lactobacilli may encourage better absorption of calcium and phosphorus from the digestive tract (Haddadin *et al.*, 1996).
- Probiotics displace or dilute normal gut flora organisms that activate ingested substances to toxic or carcinogenic derivatives.
- Probiotics generate conditions in the gut, which alter the rate of bacterial activation of ingested chemicals, e. g. lowering of pH affects ammonia production and bile acid metabolism.

The beneficial effects of probiotics may be mediated by a direct antagonistic effect against a specific group of organisms, resulting in a decrease in their numbers or by any effect on their metabolism or by stimulation of the immune system (Fuller, 1989). These include:

- The suppression of viable bacterial numbers by production of antibacterial compounds (e. g. organic acids and hydrogen peroxide), competition for nutrients and for adhesion sites. Lactic acid bacteria are known to be associated with the gut wall of chickens and may prevent colonization by pathogens due to competition for adhesion sites on the gut epithelial surface. *Lactobacillus* spp. are also capable of producing large amounts of lactate from simple carbohydrates and concomitantly can withstand a high degree of acidity which is usually fatal to other bacteria for example *E. coli* (Jernigan & Miles, 1985). Another example is the inoculating of chicks with

the normal gut flora of adult hens, making them resistant to salmonellae infection. This concept is known as competitive exclusion (Stavric, 1987).

- The alteration of gut microbial metabolism by means of increased or decreased enzyme activity.
- The stimulation of immunity by means of increased antibody levels and increased macrophage activity. In order for bacteria to have these kind of systemic effects it may be necessary for them to migrate from the gut to the systemic circulation. It has been shown that lactobacilli can translocate (Berg, 1983 as cited by Fuller, 1989) and can survive in the spleen, liver and lungs for quite a time (Bloksma, 1981 as cited by Fuller, 1989). These findings of a systemic effect on immunity indicate that probiotics have the potential, not only to affect the balance of the gut flora, but to influence the pathogenesis of diseases which occur in tissues remote from the intestinal tract (Fuller, 1989).
- Probiotics may have anticarcinogenic activities. The anticarcinogenic properties of lactobacilli can be divided into three categories: a) the inhibition of tumour cells (Reddy *et al.*, 1973 as cited by Fuller, 1989) b) the suppression of bacteria which produces enzymes such as β- glucosidase, β-glucuronidase and azoreductase which are responsible for the release of carcinogens from innocuous complexes.

What can be Expected with Probiotic Supplementation in Chickens?

The beneficial claims made for probiotic supplementation in chickens are numerous and can be summarized in the following:

- An improved growth rate in broilers (Yeo & Kim, 1997; Abdulrahim *et al.*, 1999) and body weight gain in laying hens during the laying phase (Nahashon *et al.*, 1994b). This is generally regarded as being due to the suppression of growth depressing microorganisms (Fuller, 1992a).
- An improved utilization of feed in broilers (Abdulrahim *et al.*, 1999) and laying hens (Grimes *et al.*, 1997; Haddadin *et al.*, 1996). This may be achieved by increased efficiency of the existing digestive processes or by promoting the digestion of previously indigestible substances. It has been shown that supplementation of *Ent. Faecium* in chickens may allow them to digest cellulose (Fuller, 1992a).

- An increased feed intake in laying hens (Nahashon *et al.*, 1994b) and broilers (Yeo & Kim, 1997).
- Increased egg production, egg weights and egg size but these are seldom significant (Fuller, 1992a; Miles *et al.*, 1981). Several studies have however found significant increases in the level of egg production (Haddadin *et al.*, 1996; Nahashon *et al.*, 1994b).
- An increase in the total number of large eggs laid was found in several studies (Cerniglia *et al.*, 1983; Nahashon *et al.*, 1994b; Grimes *et al.*, 1997).
- Improved health. This includes resistance to infectious diseases either by direct antagonism or by the stimulation of immunity (Fuller, 1992a).
- Improved nitrogen and energy utilization in broilers (Mohan *et al.*, 1996) and higher protein digestibility in laying hens (Grimes *et al.*, 1997).
- Improved shell quality in layers when marginal levels of calcium are fed (Bolden & Jensen, 1985). Improvement in shell thickness due to an increase in calcium assimilation (Mohan *et al.*, 1996).
- Improved internal egg quality (Haddadin *et al.*, 1996) and improved retention of calcium, fat, phosphorus, copper and manganese in laying hens (Nahashon *et al.*, 1994a; Nahashon *et al.*, 1994b).
- A reduction in the total cholesterol content of egg yolk and blood serum (Mohan *et al.*, 1996; Abdulrahim *et al.*, 1996; Haddadin *et al.*, 1996).
- Decreased ammonia production in the gut. Ammonia (including the ammonium ion) produced from amino acid degradation in the body is converted to uric acid in the chicken. A significant amount of uric acid is excreted in the gastrointestinal tract and hydrolyzed into ammonia by microbial urease. Ammonia, one of the microbial products that is known to be harmful to all animals, may enter the bloodstream and exert toxic effects. Dietary probiotic supplementation may suppress the growth of the bacteria that produce urease. This may indirectly lead to an increase in feed intake and weight gain in chickens (Yeo & Kim, 1997).
- A reduction in mortality within 24 hours after hatching by spraying eggs with microencapsulated lactic acid bacteria (Sefton, 1998).

- A reduction of caecal colonization by nonlactose-fermenting and lactose-fermenting salmonellae by inoculating chicks with normal caecal flora. This phenomenon is known as competitive exclusion (Ziprin & Deloach, 1993).
- *Lactobacillus acidophilus* supplementation in laying hens increased the cellularity of Peyer's patches in the ileum indicating a stimulation of the mucosal immune system that responds to antigenic stimuli by secreting immunoglobulin A (IgA) (Nahashon *et al.*, 1994b).
- A possible improved availability of phytate phosphorus by mold and yeast produced phytase enzymes. This will inevitability lower the cost of dietary phosphorus provision for broilers and laying hens (Day *et al.*, 1987).
- Equal growth rates in chicks fed diets with essential amino acids at suboptimal levels but with probiotic supplementation and chicks fed adequate levels of amino acids but with no probiotic supplementation (Dilworth & Day, 1978 as cited by Jernigan & Miles, 1985).
- Greater pigmentation and fat deposition in broilers (Burkettt *et al.*, 1977 as cited by Jernigan & Miles, 1985).
- A reduction in the occurrence of pasted vents associated with a reduction in mortality (DaMassa, 1980 as cited by Jernigan & Miles, 1985).

Not all the claims made for using probiotics in chickens are beneficial, however. For instance, it was found that supplementation of the diet of broilers with lactobacilli led to a lowering of the liver biotin content when a diet marginal in biotin is fed. This may be due to direct competition of the microorganisms with the host for dietary biotin (Watkins & Kratzer, 1983; Buenrostro & Kratzer, 1983). This phenomenon causes growth depression and may indirectly increase the incidence of Sudden Death Syndrome in broilers (Buenrostro & Kratzer, 1983). Furthermore, no effect was noticed in the fertility and hatchability of the eggs of turkeys and Bobwhite quail. It may therefore be assumed that the fertility and hatchability of chicken eggs will also not be influenced by probiotic addition (Miles *et al.*, 1981c as cited by Jernigan & Miles, 1985). Just as the normal gut microflora compete with the host, probiotics will also compete with the host for essential nutrients and this may prove to be detrimental.

Problems with Probiotics

One constant feature of the probiotic effect is its variability. Probiotics are not a single entity and many factors can influence their performance. The main difficulty with probiotics is that their targeting is somewhat imprecise. It is not always entirely clear if the effects of orally administered probiotic strains are directed to the crop, small intestine or large intestine. For instance, it was found that lactobacilli given in milk and targeted for the stomach, was found to be effective in reducing *E. coli* counts in the stomach and duodenum (Pusztai *et al.*, 1990). The viability of a probiotic product is a second but certainly the most important aspect but this cannot always be assured. Strains within the same species can show huge variance in livability because of differences in metabolism (Fuller, 1992b).

The composition of the host animal gut flora may influence the performance of a probioticum in the host. Probiotics act by affecting the composition of the host's intestinal flora. A prerequisite for a positive response is the presence of microorganisms, which has an adverse effect on the host e. g. growth depression. If the growth-depressing organism is absent then no growth promotion will be obtained. Similarly, no probiotic response will be obtained if the probiotic organism has already been acquired naturally (Fuller, 1992b).

Whether a single dosing or continuous dosing regime is followed may also influence the probiotic effect. Although the minimum effective dose that elicits a response is not known, it has been found that the probiotic effect disappears after cessation of dosing. In chickens it has been found that the probiotic organisms cannot be recovered from the gut 7 days after dosing. Permanent colonization of the gut by ingested organisms therefore does not seem to be a likely outcome (Fuller, 1992b).

The age and type of animal may influence the performance of a probiotic. The gut microflora, physiology, and immune status of an animal change as it gets older. Because the flora is still in a state of flux during the neonatal period, it may be easier to influence

the flora during this period rather than later in life when a relatively stable flora has been established (Fuller, 1992b).

The type of preparation will influence the performance of a probiotic. Probiotics come in a variety of different forms and one type of preparation may be more suitable than another for a particular animal. Host specificity therefore plays a very important role. If colonization of the gut is necessary, a good probiotic for a pig may not be suitable for use in a chicken (Pusztai *et al.*, 1990; Fuller, 1992b).

The production methods of microorganisms included in a probioticum may affect its performance in the gut. The way a probiotic organism will behave in the intestinal tract can be affected by the way it was grown and harvested. For example, the type of carbohydrate energy source provided for growth may affect epithelial adhesion and suspension of probiotic organisms in milk may enhance their ability to adhere and therefore improve colonization in the gut (Fuller, 1992b).

Conclusion

Probiotic supplementation has considerable advantages over antibacterial supplements currently in use. They do not induce resistance to antibiotics that will compromise therapy. They are not toxic and therefore will not produce undesirable side-effects when being fed and, in the case of broilers, will not produce toxic residues in the carcass. Probiotics may stimulate immunity, whereas the immune status remains unaffected by antibiotic treatment. Probiotics may be cheaper than other pronutrients. At present the minimum effective dose is not known, but if it can be established that the probiotic effect are established after minimal dosing then the cost will fall far below that which is at the moment when probiotics tend to consist of preparations containing large numbers of bacteria which are fed continuously (Fuller, 1989).

The use of probiotics in animal husbandry is now an acceptable practice and is on the increase. Future research and development will focus on the development of non-viable probiotics and the genetic manipulation of strains that will make this an even more

comfortable method to improve productivity, efficiency and the general health of densely housed poultry. It may still be early days for probiotics but the general public's increased awareness of food safety and animal welfare will ensure that the probiotic concept won't just fade away into the past.

Chapter 2

The Influence of EM (Effective Microorganisms) on the Growth Performance of Commercial Laying Pullets

2.1. Introduction

The performance and economic return of a commercial layer flock is virtually determined by the quality of the pullet produced in the growing programme, particularly during the first 6 to 10 weeks of age. The quality of the pullet at the time her production cycle begins will determine how profitable she will be during her period of lay. Therefore, the aim of the growing program is to produce a pullet with a properly developed skeleton and an optimal body mass at the start of the egg production cycle. Therefore, the importance of top quality pullet management and nutrition during this phase can not be overstated. It determines the success of the entire egg production operation.

Confinement of pullets can be a predisposing factor to increased susceptibility to disease because of dense bird populations. Instead of antibiotics, probiotics may be used to reduce disease susceptibility by improving the health of the gastrointestinal tract and subsequent performance of the pullets (Nahashon *et al.*, 1994a). With the banning of various antibiotics and other pronutrients (including anticoccidials) that were traditionally used to maintain high production and performance in the poultry industry, in numerous countries, probiotic preparations are also being increasingly used in poultry diets to enhance growth rate and improve food utilization (Mohan *et al.*, 1995).

There are numerous forms of probiotic products that are used in poultry feeds at various concentrations and their effect can vary. Most of these products contain only a single strain of viable organisms and this is mostly from the genus *Lactobacillus*. There are few reports concerned with the aspect of probiotic supplementation in the growing chick and when simulating growth in the domestic fowl most of the studies use the broiler chick as a model. Watkins & Kratzer (1983) found a depression in the growth of broilers but no effect on feed conversion ratio when *Lactobacillus* was orally dosed. Yeo & Kim (1997)

found an increase in the average daily gain but not in feed intake in broilers receiving a diet containing *Lactobacillus casei*. Watkins & Kratzer (1984) found no difference in body weight and feed conversion ratio in broilers when *Lactobacillus* was administered in the drinking water. Results that have been obtained with single strain *Lactobacillus* supplementation in the growing chick therefore vary greatly. The precise reason for conflicting results are not always clear but Abulrahim *et al.* (1999) suggests that the response of different breeds might modify results. No studies have been done on the influence of probiotics on the growing phase of the commercial laying pullet.

It has been proposed that multiple-strain probiotic cultures are active against a wider range of conditions and therefore may be more successful in improving performance (Fuller, 1989). Mohan *et al.* (1996) found an improved weight gain and feed conversion ratio over the control at the end of 8 weeks in broiler chicks when the starter and finisher diets were supplemented with a multiple strain probiotic containing *Lactobacillus acidophilus*, *Lactobacillus casei*, *Bifidobacterium bifidum*, *Aspergillus oryzae* and *Torulopsis*.

In view of these results and the problems facing the laying hen industry regarding the adverse effects of bird density and an inevitable ban on the use of antibiotics, a study was undertaken to investigate the effect of a multiple-strain probiotic on the growth performance of the commercial laying pullet. The multiple-strain probiotic named EM (Effective Microorganisms) used in this study consists of primarily lactic acid bacteria (*Lactobacillus planarium* ATCC 8014, *Lactobacillus casei* ATCC 7469 and *Streptococcus lactis* IFO 12007), yeast (*Saccharomyces cerevisiae* IFO 0203 and *Candida utilis* IFO 0619), actinomycetes (*Streptomyces albus* ATCC 3004 and *Streptomyces griseus* IFO 3358) and fermenting fungi (*Aspergillus oryzae* IFO 5770 and *Mucor hiemalis* IFO 8567) (Phillips and Phillips, 1996).

For the purpose of this part of the study the effect of different dietary inclusion levels of EM in comparison to a commercial coccidiostat was investigated on the growth

performance of a uniform population of commercial laying pullets. The hypothesis tested was if EM has a potential positive influence on the growth of commercial laying pullets.

2.2. Trial design

At the onset of this study a total of 669 DeKalb Single Comb White Leghorn, Amber-link®, day-old pullet chicks were obtained from a commercial hatchery. Thirty replicate groups, each consisting of 20 pullets, were assigned randomly to a control or diets containing a coccidiostat, 0.5% EM bokashi, 1.0% EM bokashi or 1.5% EM bokashi. Therefore, each treatment was represented by six replicates.

2.3. Diets

From week 0 to week 6 chicks received the various treatments in a starter diet and from week 6 to 16 in a grower diet (Table 2.3.1). Food and water were provided *ad libitum* throughout the experimental period of 0 to 16 weeks of age. In addition to the different dietary treatments all the chicks had access to water containing a vitamin and electrolyte supplement (Vitelar®) during the first three days to reduce stress associated with adaptation. This supplement contained no feed additives.

The EM treatment diets were compiled as follow. EM bokashi was prepared according to the APNAN user's guide (Phillips & Phillips, 1986) whereby 1 liter of EM stock solution was mixed with 5 liters of molasses and 100 liters of water to form an extended EM mixture. The extended EM mixture is then added to an organic mixture and allowed to ferment anaerobically for 3 weeks to form EM bokashi (Table 2.3.2). This EM bokashi was then included in the experimental diets at the various experimental inclusion levels (0.5%, 1.0% and 1.5%). Diets were formulated to account for nutrients derived from the bokashi (Table 2.3.2). Nutritional contributions, which may have originated from the EM *per se*, could not be accounted for. The coccidiostat treatment diet contained different prescribed levels of a commercial coccidiostat premix taking into account which diet was fed, the starter or the grower diet (Table 2.3.1). All feeds and bokashi were formulated and supplied by Rainbow Feeds (Pty) Ltd.

Representative samples of each diet were collected and chemically analyzed (Table 2.3.3 and Table 2.3.4) according to the methods of the AOAC (1990). Feed nitrogen levels were determined by Kjeldahl analysis. Crude fat was extracted using the Soxhlet apparatus. Crude fibre levels were determined using the Wijkström method (Application note of tecator AN02/78A).

2.4. Housing

Chicks were housed in 30 separate floor pens (area 2 × 3m) in an environmentally controlled broiler house on the premises of the experimental farm of the University of Pretoria. Before the onset of the study the broiler house and all accompanying equipment were cleaned and disinfected by professional, industrial cleaners.

The temperature of the house was maintained at 32 °C for the first week and then lowered to 29 °C (Week 2), then to 26 °C (Week 3) and then maintained as close as possible to 23 °C for the remainder of the study. All supplementary heat was removed on day 52. Ventilation took place through fans in the ceiling of the broiler house. Conventional vaccination and lighting programmes were followed. The light intensity was continuously lowered to reduce flightiness. Chicks were not de-beaked, but were wingbanded for identification purposes. All sexing errors were removed from the study on day 64 and treated like mortalities.

2.5. Parameters recorded

Chicks were weighed weekly per replicate to determine the average weekly body mass per chick and to determine the average daily gain as a derived parameter. Feeders were weighed weekly to determine the average weekly feed intake per chick, the cumulative weekly feed intake per chick and the cumulative weekly feed conversion ratio. Average feed intake was corrected for mortalities. Shank length was measured bi-weekly. Three chicks were caught randomly per replicate and the right shank measured with a special ruler calibrated in millimeters obtained from Amber-link Chick Sales (Pty) Ltd. Average

shank length was then determined. Mortalities were noted daily but were so few that it was of no significant importance and therefore were not treatment linked.

2.6. Statistical analysis

Data were analyzed using the SAS® system. This system is an integrated system of software providing complete control over data management, analysis, and presentation and is marketed by SAS Institute South Africa (Pty) Ltd, 1st Floor North Wing, President Place, 1 Hood Avenue, Rosebank, P. O. Box 3469, Parklands 2121, South Africa. Data were subjected to analysis of variance using the General Linear Models (GLM) procedure. Tukey's Studentized Range (HSD) Test was used to determine the significance of differences between treatment means at the $P<0.05$ level. Body mass curves were fitted using the REG procedure of SAS®.

Table 2.3.1. Basal experimental diets for growing pullets (0 – 16 weeks)[1]

Ingredients	Starter Diet (0 – 6 Weeks) (% Dry)	Grower Diet (6 – 16 Weeks) (% Dry)
Yellow Maize	59.00	66.30
Wheat Bran	10.00	9.70
Sunflower Oilcake	15.00	15.70
Full Fat Soya	12.70	5.40
Monocalcium Phosphate	1.20	1.03
Limestone	1.30	1.15
Salt	0.33	0.32
Lysine HCl	0.13	-
SCX-P1*	0.50	-
SCX-P2**	-	0.50
Calculated Composition	**(Dry Basis)**	**(Dry Basis)**
ME (MJ / kg)	12.30	12.31
Crude Protein (g / kg)	170.16	149.97
Lysine (g / kg)	8.03	5.60
Methionine (g / kg)	3.24	3.03
Fat (g / kg)	51.38	41.16
Crude Fibre (g / kg)	51.11	50.00
Calcium (g / kg)	9.05	7.99
Available Phosphorus (g / kg)	3.98	3.51
Sodium (g / kg)	1.52	1.50

1 For the different EM treatment diets, 0.5%, 1.0% and 1.5% EM bokashi were added to the diet. Coccidiostat treatment diets contained 500 g / t (starter) and 250 g / t (grower) of a commercial coccidiostat premix respectively. Diets were formulated to account

for nutritional contributions from the bokhasi *per se*. Possible nutritional contributions from the EM *per se* could not be taken into account.

*Vitamin and Mineral Premix for Chick Starter diets. Supplies the following per ton of diet: vitamin A, 12 000 000 IU; vitamin D3, 2 500 000 IU; vitamin E, 30 000 IU; vitamin B1, 2000 mg; vitamin B2 , 6000 mg; vitamin B6, 4000 mg; vitamin B12 , 30 mg; vitamin K3, 6000 mg; niacin, 40 000 mg; pantothenic acid, 10 000 mg; folacin, 1000 mg; biotin, 50 mg; choline, 500 mg; g anti oxidant, 125g; Fe, 15g; Mn, 60 g; Cu, 6g; $ZnSO_4$, 40 g; Co, 0.5g; I, 1g; Se, 100 mg; carrier, 3.68kg.

**Vitamin and Mineral Premix for Poultry Grower diets. Supplies the following per ton of diet: vitamin A, 10 000 000 IU; vitamin D3, 2 000 000 IU; vitamin E, 20 000 IU; vitamin B1, 2000 mg; vitamin B2 , 5000 mg; vitamin B6, 3000 mg; vitamin B12 , 15 mg; vitamin K3, 2000 mg; niacin, 30 000 mg; pantothenic acid, 4 000 mg; folacin, 500 mg; biotin, 60 mg; choline, 400 mg; g anti oxidant, 125g; Fe, 20g; Mn, 70 g; Cu, 6g; $ZnSO_4$, 40 g; Co, 0.5g; I, 1g; Se, 100 mg; carrier, 3.86kg.

Table 2.3.2. Calculated composition and nutritional contribution of EM bokashi[1]

Ingredients	**(% Dry)**
Hominy Chop	15.00
Wheat Bran	70.00
Full Fat Soya	5.00
Bone Meal	5.00
Fish Meal	5.00
Calculated Composition	**(Dry Basis)**
ME (MJ / kg)	8.32
Crude Protein (g / kg)	176.50
Lysine (g / kg)	8.49
Methionine (g / kg)	2.97
Fat (g / kg)	53.00
Crude Fibre (g / kg)	88.50
Calcium (g / kg)	13.55
Total Phosphorus (g / kg)	12.93
Sodium (g / kg)	0.92

[1]Nutritional contribution of EM *per se* not taken into account

Table 2.3.3. Analyzed composition of the starter experimental diets (0 – 6 weeks)

Analyzed Composition (Dry Basis)	EM0.5%	EM1.0%	EM1.5%	Coccidiostat	Control
% Dry	90.954	90.846	90.784	90.345	90.446
Crude Protein (%)	18.288	17.774	18.078	17.774	18.684
Ether Extract (%)	6.036	6.033	6.172	6.136	6.505
Crude Fibre (%)	6.660	6.572	6.977	6.592	7.268
Ash (%)	6.185	6.182	5.483	6.234	6.248

Table 2.3.4. Analyzed composition of the grower experimental diets (6 – 16 weeks)

Analyzed Composition (Dry Basis)	EM0.5%	EM1.0%	EM1.5%	Coccidiostat	Control
% Dry	89.041	88.916	89.307	89.664	89.588
Crude Protein (%)	17.041	17.861	16.941	15.492	16.690
Ether Extract (%)	4.729	6.593	5.788	5.038	5.706
Crude Fibre (%)	10.621	9.194	9.545	9.508	11.168
Ash (%)	4.194	5.217	5.100	5.059	4.615

2.7. Results

The addition of different levels of EM bokashi to the starter and grower diets of commercial laying pullets did not influence the average bi-weekly shank length significantly except in an isolated case (Table 2.7.1). In the eleventh week a significant difference existed between the 1.0% EM level and the coccidiostat, the 1.0% EM level having an average longer shank length than the coccidostat treatment (84.833mm vs. 81.000mm). Although not statistically significant, a numerical difference existed throughout Weeks 5 to 15 between the shank lengths of the chicks fed the diets containing the coccidiostat and the rest of the EM treatments and the control. At the end of the trial period at Week 15 the EM 1.5% level had the longest shank length (96.167mm) followed by the 0.5% level (95.556mm), the 1.0% level (95.278mm) and the control (94.389mm). The coccidiostat treatment had the shortest shank length at Week 15 (93.944mm). These differences were not significant. The mean shank lengths over 15 weeks are shown in Table 2.7.2. According to Table 2.7.2 the mean shank length of the coccidiostat treatment was significantly shorter than the 1.0% EM level but differed not from the rest of the EM levels and the control.

The effect of EM and coccidiostat supplementation on average weekly body weights from 1 – 16 weeks of age is given in Table 2.7.3. From Week 1 to 7 no significant differences existed between all the treatments. However, a tendency for the 1.0% EM level to have higher body weights than all the other EM levels and the control was observed from Week 9 until Week 15. At the end of the trial period at Week 16 the EM 1.5% level had the highest body mass (1277.61g) followed by the 1.0% level (1248.55g), the control (1233.09g) and the EM 0.5% level (1232.49g). These differences were not significant. From Week 7 until the end of the trial at Week 16 the chicks receiving the coccidiostat in the diets had highly significant lower body weights than all the EM levels and the control. EM supplementation did not improve the mean body weight over 16 weeks over the control (Table 2.7.4). According to Table 2.7.4 the mean body weight over 16 weeks of the coccidiostat was significantly lower than all the EM treatments and the control. Details of the regression equations used to describe the response in body

weight to dietary EM and coccidiostat treatments are given in Table 2.7.5. Predicted body mass values for the treatments against time are shown in Figure 2.1.

There were no significant differences in average daily gain between the EM treatments and the control (Table 2.7.6). From Week 7 to 16 the coccidiostat treatment had a significant lower average daily gain than all the EM treatments and the control (Table 2.7.6). Measured from Week 1 to 16, the EM 1.5% level had the highest average daily gain (11.811g / d) followed by the 1.0% level (11.535g / d), the control (11.390 g / d) and the 0.5% level (11.375g / d). The overall average daily gain (Week 1 to 16) was the lowest for the coccidiostat treatment (10.397 g / d) which differed significantly from all the EM treatments and the control (Table 2.7.6).

Mean weekly feed intake between chicks fed different levels of EM, the coccidiostat and the control are shown in Table 2.7.7. Chicks fed the diets containing the coccidiostat had a significant higher intake than the EM 0.5% treatment in Week 4. However, from Week 10 onwards the coccidiostat treatment tended to maintain lower feed intakes (Table 2.7.7). In some cases this lower feed intake differed significantly from some of the EM treatments and the control (Weeks 10, 11, 12 and 13). No significant differences in the mean feed intake per chick over 15 weeks were, however, observed between all the treatments (Table 2.7.8). For the mean cumulative feed intake per chick from Week 1 to 16, no significant differences were obtained between all the treatments (Table 2.7.9). At the end of the trial period at Week 16, the EM 1.0% level consumed the most feed per chick (5440.32g), followed by the control (5438.34g), EM1.5% (5424.57g), EM 0.5% (5377.61g) and the coccidiostat treatment (5243.74g). These differences were not significant (Table 2.7.9).

The cumulative feed conversion ratio for the different treatments are shown in Table 2.7.10. Since the coccidiostat had significant lower body weights from Week 7 onwards (Table 2.7.5) but not significant lower feed intakes (Tables 2.7.8 & 2.7.9), feed conversion ratios were significantly influenced. From Week 8 until the end of the trial period at Week 16 the coccidiostat treatment had significantly worse feed conversion

ratios than all the other treatments. According to Table 2.7.10 the EM 1.5% level had the best feed conversion at Week 16 (4.380), followed by the EM 1.0% (4.493), EM 0.5% (4.505) and the control (4.547). These differences were not significant. The coccidiostat treatment had a significant worse feed conversion ratio than all the other treatments at Week 16 (4.804).

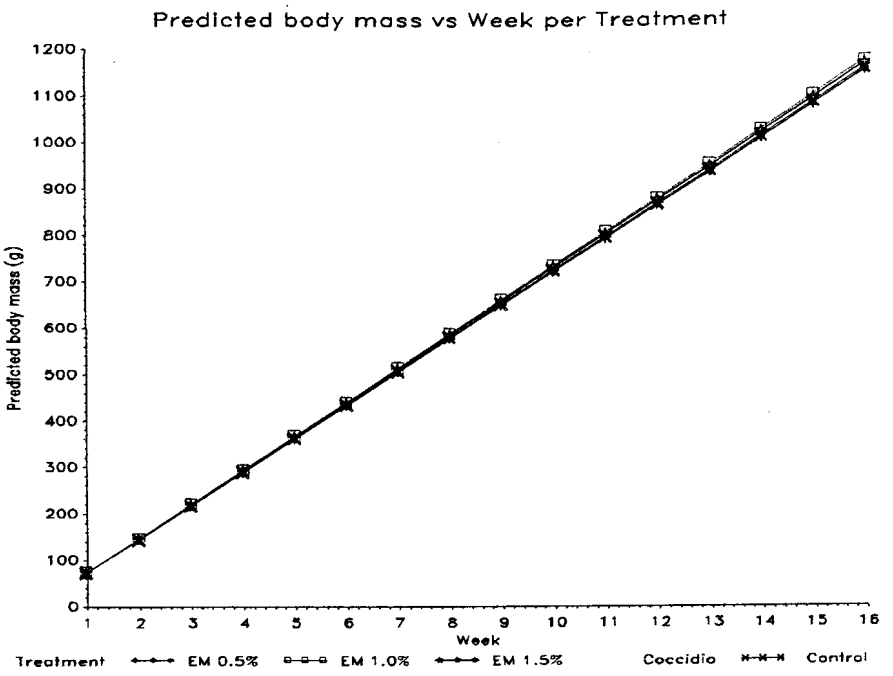

Figure 2.1 Predicted body mass vs week per treatment

Table 2.7.1. Average bi-weekly shank length (means in mm)

Week	Treatment						
	EM 0.5%	EM 1.0%	EM 1.5%	Coccidio-Stat	Control	Type III P-value	SEM
1	26.389^{ab}	25.722^{b}	26.278^{ab}	26.444^{ab}	27.056^{a}	0.0944	0.318
3	39.111^{a}	38.667^{a}	39.333^{a}	38.056^{a}	39.611^{a}	0.4552	0.628
5	53.500^{a}	53.389^{a}	53.944^{a}	52.056^{a}	53.056^{a}	0.6465	0.894
7	64.722^{a}	66.556^{a}	64.056^{a}	63.944^{a}	65.667^{a}	0.2327	0.908
9	74.833^{a}	75.000^{a}	74.778^{a}	73.833^{a}	74.611^{a}	0.8511	0.788
11	83.667^{ab}	84.833^{a}	82.889^{ab}	81.000^{b}	84.056^{ab}	0.0343	0.832
13	92.889^{a}	93.222^{a}	93.500^{a}	90.722^{a}	92.556^{a}	0.3306	0.996
15	95.556^{a}	95.278^{a}	96.167^{a}	93.944^{a}	94.389^{a}	0.0962	0.602

* Means with different superscripts within weeks are significantly different ($P < 0.05$) according to Tukey's Studentized Range (HSD) Test.

Table 2.7.2. Mean shank length over 15 weeks

Treatment	Mean Shank Length (mm)	SEM
EM 0.5%	66.33^{ab}	0.365
EM1.0%	66.58^{a}	0.365
EM1.5%	66.37^{ab}	0.365
Coccidiostat	65.00^{b}	0.365
Control	66.38^{ab}	0.365
Type III P-value	0.0344	

* Means with different superscripts are significantly different ($P < 0.05$) according to Tukey's Studentized Range (HSD) Test.

Table 2.7.3. Average weekly body weight (Means in grams)

Week	Treatment						
	EM 0.5%	EM 1.0%	EM 1.5%	Coccidio-Stat	Control	Type III P-value	SEM
1	38.07[a]	37.43[a]	37.47[a]	37.88[a]	37.10[a]	0.3218	0.348
2	62.86[a]	61.94[a]	62.36[a]	62.77[a]	62.82[a]	0.9195	0.822
3	110.01[a]	109.41[a]	109.24[a]	110.66[a]	111.05[a]	0.8367	1.303
4	170.01[a]	170.78[a]	172.04[a]	171.82[a]	173.36[a]	0.8782	2.354
5	235.78[a]	239.76[a]	243.94[a]	237.35[a]	240.15[a]	0.5281	3.441
6	306.89[a]	311.56[a]	317.31[a]	310.74[a]	313.83[a]	0.7051	5.226
7	403.64[a]	406.19[a]	413.38[a]	407.66[a]	411.95[a]	0.7545	5.873
8	500.77[a]	510.25[a]	509.71[a]	485.30[a]	511.37[a]	0.1254	7.786
9	574.46[a]	587.11[a]	586.99[a]	541.07[b]	583.78[a]	0.0005	7.217
10	646.90[a]	664.89[a]	656.63[a]	603.20[b]	656.28[a]	0.0001	7.255
11	785.01[a]	797.65[a]	780.94[a]	703.91[b]	793.57[a]	0.0001	9.200
12	889.17[a]	913.24[a]	896.31[a]	791.03[b]	894.58[a]	0.0001	9.634
13	995.18[a]	1009.02[a]	998.57[a]	879.90[b]	991.83[a]	0.0001	10.034
14	1086.32[a]	1107.28[a]	1088.20[a]	969.63[b]	1088.83[a]	0.0001	9.695
15	1180.14[a]	1209.17[a]	1191.15[a]	1068.36[b]	1181.88[a]	0.0001	11.145
16	1232.49[a]	1248.55[a]	1277.61[a]	1129.56[b]	1233.09[a]	0.0001	14.890

* Means with different superscripts within weeks are significantly different ($P < 0.05$) according to Tukey's Studentized Range (HSD) Test.

Table 2.7.4. Mean body weight over 16 weeks

Treatment	Mean Body Weight (g)	SEM
EM 0.5%	576.11[a]	5.371
EM1.0%	586.51[a]	5.371
EM1.5%	583.87[a]	5.371
Coccidiostat	531.11[b]	5.371
Control	580.34[a]	5.371
Type III P-value	0.0001	

* Means with different superscripts are significantly different ($P < 0.05$) according to Tukey's Studentized Range (HSD) Test.

Table 2.7.5. Regression relationship between predicted cumulative body weight (Y) and time (X)

Regression relationship		
Treatment	**R- square**	**Regression equations[1]**
EM 0.5%	0.9731	Y = 71.8836x
EM 1.0 %	0.9387	Y = 73.2390x
EM 1.5 %	0.9615	Y = 72.8566x
Coccidiostat	0.9471	Y = 65.6542x
Control	0.8711	Y = 72.2752x

[1] Graphs were fitted to pass through the origin of both axes, for all equations the intercept is therefore zero.

Table 2.7.6. Average daily gain (means in g / day)

Weeks	Treatment						
	EM 0.5%	EM 1.0%	EM 1.5%	Coccidio-Stat	Control	Type III P-value	SEM
01 to 04	6.283[a]	6.350[a]	6.408[a]	6.378[a]	6.488[a]	0.7545	0.110
04 to 07	11.125[a]	11.210[a]	11.492[a]	11.231[a]	11.362[a]	0.7530	0.209
01 to 07	8.704[a]	8.780[a]	8.950[a]	8.804[a]	8.925[a]	0.6881	0.137
07 to 10	11.584[a]	12.319[a]	11.583[a]	9.312[b]	11.635[a]	0.0001	0.192
01 to 10	9.664[a]	9.960[a]	9.828[a]	8.973[b]	9.828[a]	0.0001	0.114
10 to 13	16.584[a]	16.387[a]	16.283[a]	13.176[b]	15.978[a]	0.0001	0.317
01 to 13	11.394[a]	11.567[a]	11.442[a]	10.024[b]	11.366[a]	0.0001	0.119
13 to 16	11.301[a]	11.406[a]	13.288[a]	11.888[a]	11.489[a]	0.1728	0.622
01 to 16	11.375[a]	11.535[a]	11.811[a]	10.397[b]	11.390[a]	0.0001	0.141

* Means with different superscripts within weeks are significantly different ($P < 0.05$) according to Tukey's Studentized Range (HSD) Test.

Table 2.7.7. Mean weekly feed intake per chick (means in g)

Week	Treatment						
	EM 0.5%	EM 1.0%	EM 1.5%	Coccidio-Stat	Control	Type III P-value	SEM
1	81.37[a]	83.62[a]	82.18[a]	77.03[a]	74.15[a]	0.3052	3.488
2	140.51[a]	130.14[a]	129.64[a]	140.74[a]	129.01[a]	0.5004	6.522
3	144.38[a]	146.38[a]	151.39[a]	143.89[a]	149.56[a]	0.5705	3.786
4	186.30[b]	204.57[ab]	198.09[ab]	211.73[a]	195.50[ab]	0.0348	5.471
5	231.91[a]	239.03[a]	246.78[a]	241.17[a]	235.96[a]	0.6636	7.188
6	273.25[a]	275.12[a]	290.58[a]	279.92[a]	277.07[a]	0.7358	9.662
7	359.80[a]	369.95[a]	374.32[a]	359.16[a]	376.97[a]	0.5391	9.207
8	340.15[a]	351.37[a]	354.33[a]	340.19[a]	352.42[a]	0.4158	6.876
9	541.83[a]	542.11[a]	524.82[a]	506.38[a]	522.14[a]	0.5516	17.045
10	438.30[ab]	446.06[ab]	450.41[a]	411.69[b]	459.22[a]	0.0135	9.170
11	475.75[ab]	487.99[a]	468.85[ab]	444.78[b]	483.94[a]	0.0094	8.287
12	529.71[a]	525.89[a]	516.19[ab]	489.43[b]	531.55[a]	0.0042	7.749
13	546.82[a]	541.39[ab]	532.77[ab]	513.88[b]	535.79[ab]	0.0273	6.924
14	557.41[a]	565.72[a]	565.96[a]	558.06[a]	570.30[a]	0.8867	10.478
15	530.13[a]	530.99[a]	538.26[a]	525.70[a]	544.75[a]	0.6111	9.116

* Means with different superscripts within weeks are significantly different ($P < 0.05$) according to Tukey's Studentized Range (HSD) Test.

Table 2.7.8. Mean feed intake per chick over 15 weeks

Treatment	Mean Feed Intake (g)	SEM
EM 0.5%	358.51[a]	3.961
EM1.0%	362.69[a]	3.961
EM1.5%	361.64[a]	3.961
Coccidiostat	349.58[a]	3.961
Control	362.56[a]	3.961
Type III P-value	0.1339	

* Means with different superscripts are significantly different ($P < 0.05$) according to Tukey's Studentized Range (HSD) Test.

Table 2.7.9. Mean cumulative feed intake per chick (means in g)

Week	Treatment						
	EM 0.5%	EM 1.0%	EM 1.5%	Coccidio-Stat	Control	Type III P-value	SEM
00 to 01	81.37[a]	83.62[a]	82.18[a]	77.03[a]	74.15[a]	0.3052	3.488
01 to 02	221.88[a]	213.76[a]	211.82[a]	217.77[a]	203.17[a]	0.4287	7.055
01 to 03	366.26[a]	360.14[a]	363.20[a]	361.66[a]	352.73[a]	0.8332	8.391
01 to 04	552.56[a]	564.71[a]	561.30[a]	573.39[a]	548.23[a]	0.5610	11.427
01 to 05	784.47[a]	803.73[a]	808.07[a]	814.56[a]	784.20[a]	0.4649	14.490
01 to 06	1057.72[a]	1078.85[a]	1098.66[a]	1094.47[a]	1061.26[a]	0.4267	18.657
01 to 07	1417.51[a]	1448.80[a]	1472.98[a]	1453.63[a]	1438.23[a]	0.6369	25.451
01 to 08	1757.67[a]	1800.18[a]	1827.32[a]	1793.83[a]	1790.65[a]	0.6289	30.768
01 to 09	2299.50[a]	2342.29[a]	2352.14[a]	2300.21[a]	2312.80[a]	0.6845	32.244
01 to 10	2737.80[a]	2788.35[a]	2802.55[a]	2711.89[a]	2772.01[a]	0.4092	36.576
01 to 11	3213.54[a]	3276.33[a]	3271.40[a]	3156.67[a]	3255.95[a]	0.2507	41.837
01 to 12	3743.25[a]	3802.23[a]	3787.58[a]	3646.09[a]	3787.50[a]	0.1412	46.321
01 to 13	4290.06[a]	4343.61[a]	4320.35[a]	4159.97[a]	4323.29[a]	0.1130	51.115
01 to 14	4847.47[a]	4909.33[a]	4886.31[a]	4718.04[a]	4893.59[a]	0.1493	57.012
01 to 15	5377.61[a]	5440.32[a]	5424.57[a]	5243.74[a]	5438.34[a]	0.1339	59.412

* Means with different superscripts within weeks are significantly different ($P < 0.05$) according to Tukey's Studentized Range (HSD) Test.

Table 2.7.10. Cumulative feed conversion ratio (means in g/g)

Week	Treatment						
	EM 0.5%	EM 1.0%	EM 1.5%	Coccidio-Stat	Control	Type III P-value	SEM
2	3.290[a]	3.439[a]	3.392[a]	3.108[a]	2.893[a]	0.3664	0.212
3	3.085[a]	2.970[a]	2.962[a]	3.000[a]	2.749[a]	0.2523	0.104
4	2.774[a]	2.701[a]	2.706[a]	2.702[a]	2.589[a]	0.2601	0.056
5	2.794[a]	2.791[a]	2.723[a]	2.877[a]	2.701[a]	0.1222	0.049
6	2.918[a]	2.932[a]	2.890[a]	2.988[a]	2.835[a]	0.0891	0.037
7	2.894[a]	2.926a	2.923[a]	2.960[a]	2.831[a]	0.0996	0.037
8	3.064[b]	3.064[b]	3.121[ab]	3.249[a]	3.033[b]	0.0009	0.033
9	3.277[b]	3.275[b]	3.325[b]	3.566[a]	3.275[b]	0.0001	0.042
10	3.778[b]	3.734[b]	3.800[b]	4.070[a]	3.735[b]	0.0001	0.040
11	3.666[b]	3.670[b]	3.773[b]	4.072[a]	3.664[b]	0.0001	0.045
12	3.776[b]	3.742[b]	3.811[b]	4.191[a]	3.797[b]	0.0001	0.038
13	3.911[b]	3.913[b]	3.941[b]	4.330[a]	3.970[b]	0.0001	0.042
14	4.093[b]	4.060[b]	4.112[b]	4.465[a]	4.110[b]	0.0001	0.032
15	4.245[b]	4.190[b]	4.235[b]	4.579[a]	4.275[b]	0.0001	0.036
16	4.504[b]	4.493[b]	4.380[b]	4.804[a]	4.547[b]	0.0002	0.054

* Means with different superscripts within weeks are significantly different ($P < 0.05$) according to Tukey's Studentized Range (HSD) Test.

2.8. Discussion

Feeding different levels of a multiple strain probiotic (EM) in the starter and grower diets of commercial laying pullets during the growing phase did not improve body weights over the control significantly from Week 1 to 16. Abdulrahim *et al.* (1999) found a significant improvement in body weight over the control with *Lactobacillus* addition to the diet of broilers at the beginning of week 8 (2.456kg vs. 2.303kg). Arends *et al.* (1981) found an average 25.5g increase in body weight over the control in broilers raised in four different areas and supplemented with a strain of *Lactobacillus acidophilus* – no indication of statistical significance was given. In this study no significant differences in body weights existed between the EM treatments and the control at the end of the trial. This is in accordance to Watkins & Kratzer (1984) and Buenrostro & Kratzer (1983) who found no significant improvement in body weight at week 7 in broilers supplemented with *Lactobacillus*.

Although the EM 1.0% had the highest mean body mass over 16 weeks, the EM 1.5% level had the highest body mass (1277.61g) followed by the 1.0% level (1248.55g), the control (1233.09g) and the EM 0.5% level (1232.49g) at the end of the trial period at week 16. This slight, but insignificant difference in body weight is in accordance with the work of Mohan *et al.* (1996) and Crawford (1979 as cited by Jernigan & Miles, 1985). Mohan *et al.* (1996) found a slight improvement in body weight over a control at week 8 when supplying 75 mg / kg of a multiple strain probiotic in the diet of broilers (1272.0±27.10g vs. 1204.0±31.18g). Crawford (1979 as cited by Jernigan & Miles, 1985) found a mean weight gain from hatch to marketing of 1.88kg in broilers fed a probiotic culture at 454g per ton. This was higher than the control that had a mean weight gain of 1.83kg. This difference, favouring the probiotic, was not significant. In contrast Watkins & Kratzer (1983) found that chick growth was numerically depressed when *Lactobacillus* strains were dosed although these values were not significant. This depression in growth suggested to Watkins & Kratzer (1983) that there is possibly a proper level of probiotic supplementation (in their case lactobacilli) required by the chicken that provides the most benefits. Dosing below or above this level may cause undesirable effects such as bacterial competition for nutrients when dosing too much. In

this study the dosage levels may have been too low as no significant depression in growth was obtained with EM supplementation levels of 0.5%, 1.0% and 1.5%.

The insignificant differences found in average daily gain between the EM treatments and the control from week 1 to 4 are in contrast with Yeo & Kim (1997) who found an increase in the average daily gain in broilers fed a probiotic over the control during the first 3 week period (30.7g vs. 28.7g). Yeo & Kim (1997) ascribed the increased growth with probiotic supplementation to the suppression of growth of bacteria that produce urease by the probiotic. Mohan *et al.* (1996) reported an increase in the average daily gain in favour of probiotic supplementation from the beginning of the fourth week onwards suggesting a lag phase of 21 days before the effect of probiotic supplementation is seen. In this study no significant trend between the three EM levels and the control was seen during the first 4 weeks suggesting a possible lag phase but subsequent insignificant differences between the different EM levels and the control indicated that EM supplementation at these levels did not influence average daily gain significantly during the whole of the trial period. Measured from week 1 to 16, the EM 1.5% level had the highest average daily gain (11.811g / d) followed by the 1.0% level (11.535g / d), the control (11.390 g / d) and the 0.5% level (11.375g / d).

Although the EM 1.0% level consumed the most feed over 16 weeks (5440.32g / chick), followed by the control (5438.34g / chick), the EM1.5% (5424.57g / chick), the EM 0.5% (5377.61g / chick) and the coccidiostat treatment (5243.74g / chick), these differences were not significant. This is in agreement with Watkins & Kratzer (1983) who found no significant differences in feed consumption between broilers dosed with *Lactobacillus* and a control. In contrast, Yeo & Kim (1997) found an increase in feed intake in broilers supplemented with 0.1% *Lactobacillus casei.*

EM supplementation in the diets of growing pullets did not improve the cumulative feed conversion ratio over the control significantly. At week 16 the EM 1.5% level had the best feed conversion ratio (4.380), followed by the EM 1.0% (4.493), EM 0.5% (4.505), the control (4.547) and the coccidiostat treatment (4.804). Similar results were found by

Mohan *et al.* (1996) in broilers. In the study of Mohan *et al.* (1996) broilers supplemented with 100 mg / kg of a multiple strain probiotic had a better feed conversion ratio over the control (2.26 vs. 2.30) but this was also not significant. Arends *et al.* (1981) found an average improvement of 0.0625 units in the feed conversion ratio of broilers raised in four different areas fed a strain of *Lactobacillus acidophilus* – no indication of statistical significance was given. Watkins & Kratzer (1983) and Watkins & Kratzer (1984) found no improvement in feed utilization when chicks were dosed with high numbers of lactobacilli. In contrast, Buenrostro & Kratzer (1983) found that feed efficiency was decreased when chicks were inoculated with *Lactobacillus acidophilus*. Decreased performance was attributed to the fact that chicks were housed in a clean environment where exposures to enteropathogenic microorganisms were minimal. Under such conditions probiotic supplementation to chickens could produce inferior performance due to competition with the host for nutrients especially when a diet with a deficiency in one or more nutrients are fed. In this study, however, the slight improvement in the feed conversion ratio with the highest EM supplementation level indicated that there was either minimal competition with the host for nutrients or that the diet was sufficient to provide in all the requirements of the host despite the competition effect of the probiotic.

EM supplementation did not influence the uniformity of commercial laying pullets as no significant differences between the EM treatments and the control were observed regarding the average bi-weekly shank length. The coccidiostat treatment had numerical shorter shank lengths than all the EM treatments and the control throughout weeks 5 to 15 suggesting that these birds were somewhat smaller than the rest of the treatments. Although the EM 1.0% had the longest mean shank length over 15 weeks, the EM 1.5% level had the longest shank length (96.167mm) followed by the 0.5% level (95.556mm), the 1.0% level (95.278mm) and the control (94.389mm) at the end of the trial period. The coccidiostat treatment had the shortest shank length at week 15 (93.944mm).

The significant lower body weights, average daily gains and the worse feed conversion ratios obtained with the coccidiostat treatment should be apprehended with caution. The

pullets were never challenged with an outbreak of coccidiosis or any other disease due to the very clean environment they were raised in and the low bird density per cage. Should this have been the case the results obtained may have been different.

It was expected, with modern methods of perinatal care where the egg is hatched in a clean incubator and where the chick acquires its flora from the incubator environment (Fuller, 1989), that supplementation with a multiple strain probiotic from day-old age until point–of–lay might benefit the growing pullet.

However, EM supplementation in the diets of growing pullets did not improve their performance over the control significantly. Although not significant, the EM 1.5% level had the highest body mass at week 16, best average daily gain, best feed conversion ratio and the longest shank length at week 15. This insignificant better performance could be due to the fact that the EM levels in the diets were too low as Watkins & Kratzer (1983) suggested that there is possibly a proper level of probiotic supplementation required by the chicken that provides the most benefits and that dosing below or above this level may cause undesirable effects. However, Barrow (1992) states that lack of significance does not necessarily imply biological insignificance. For example, a small but consistent weight gain may be economically significant for a large number of birds. Difficulties in demonstrating statistically significant probably result largely from genetic heterogeneity in poultry or microbiological variation in the gut flora of individuals and different groups of animals (Barrow, 1992).

It is known that in healthy chickens reared on a complete diet administration of probiotics is unlikely to give any substantial direct nutritional benefit because the gut flora would be in a state of equilibrium (Barrow, 1992). In this study chicks were never subjected to stress situations such as a challenge from disease or large bird densities and results obtained would therefore tend to confirm this statement. Results obtained with the coccidiostat treatment would also tend to support this statement. Housing under commercial circumstances and higher levels of EM in the diet may give a better response.

2.9. Conclusion

The hypothesis that EM has a potential positive influence on the growth of commercial laying pullets could not be substantiated by the results obtained in this study.

Chapter 3

The Influence of EM (Effective Microorganisms) Supplementation during the Growing and the Laying Phase on Egg Production and Egg Quality of the Commercial Laying Hen

3.1. Introduction

In South Africa it is projected that the consumption of eggs will increase from currently 306 000 tons to 464 000 tons in the year 2020. The numbers of layers that are needed to provide in this demand will have to increase from the current 17.40 million to 22.51 million in the year 2020. The feed consumption to provide for the egg production requirement is projected to increase from a current 854 00 tons to 1102 097 tons in the year 2020 (Briedenhann, 1999). It has been shown that the production area to produce animal products can increase but not significantly enough to meet this huge demand (Briedenhann, 1999). This will inevitably lead to denser bird populations to increase the production per unit area. Confinement of layers can be a predisposing factor to increased susceptibility to disease (Nahashon *et al.*, 1994a). To add to the problem a growing human population will compete for the consumption of feed raw materials, such as maize, on an even bigger scale. Therefore, in the future more emphasis will be put on production and the efficiency of utilization of feeds in layer flocks.

Some of the existing problems in the egg industry will not go away in the future. Formation of eggs with inferior shell quality is a major source of economic loss to the poultry industry on a global level. It is estimated that due to inferior shell quality about 6 to 8% of eggs are lost in different phases of the egg handling system from the point of production to the point of consumption (Keshavarz, 1994).

The demands of the consumer will also play an even bigger role in the future. Egg yolk colour has always been regarded as an important egg quality characteristic and recently has had an even more important role in the marketing of eggs. The consumer associate

strong yolk colour with good quality. Marketing companies are therefore requesting their producers with intensively kept hens to produce eggs with a rich yolk colour in order that it can be promoted with a good country image (Belyavin & Marangos, 1989).

Legislation against the use of antibiotics and other pronutrients (including antifungals and anticoccidials), whether it be currently in place or an event that may occur in the future, has placed greater reliance on management, husbandry and biotechnology to create alternatives to improve the efficiency of poultry production. Such alternatives should keep birds healthy, improve productivity and efficiency and at the same time improve the quality of the marketable product, in this case the egg.

Numerous studies have been done on the use of probiotic supplementation in the laying hen and its effect on performance, productivity and egg quality. Most studies dealt with the use of single-strain *Lactobacillus* cultures and a few studied the use of yeasts. Some found no improvement in hen-day egg production, feed efficiency, body weight gain, internal egg quality or egg size (Goodling *et al.*, 1987; Nahashon *et al.*, 1994b, Cerniglia *et al.*, 1983; Day *et al.*, 1987). Some found improvements in egg production, body weight gains, feed conversion, egg weight, egg mass, internal egg quality, egg size, nutrient retention, shell quality and a reduction in the cholesterol values of egg yolk (Nahashon *et al.*, 1994a; Nahashon *et al.*, 1994b, Miles *et al.*, 1981; Haddadin *et al.*, 1996; Abdulrahim *et al.*, 1996; Grimes *et al.*, 1997; Bolden & Jensen, 1985). None of these studies investigated the effect of probiotic supplementation in the growing phase on subsequent performance during the laying phase.

It has been proposed that multiple-strain probiotic cultures are active against a wider range of conditions and may therefore be more successful in improving performance (Fuller, 1989). Mohan *et al.* (1995) found an improvement in egg production and shell thickness when supplementing the diet of layers with a multiple-strain probiotic containing *Lactobacillus acidophilus*, *Lactobacillus casei*, *Bifidobacterium bifidum*, *Aspergillus oryzae* and *Torulopsis*. Internal egg quality, yolk colors and mean egg weights were not measured in that study.

In view of these results a study was undertaken to determine several hypotheses. The first hypothesis tested was if different dietary inclusion levels of a multiple strain probiotic named EM (composition as described by Phillips & Phillips, 1996) during the laying phase has a beneficial effect on the performance, egg production and various egg quality indices of the commercial laying hen in comparison to an antibiotic and a control. The secondly if EM supplementation during the laying phase has any additional beneficial effect to EM supplementation during the growing phase alone.

3.2. Trial design and diets

Five hundred and eighty three DeKalb (Amber-Link®) Single Comb White Leghorn pullets raised on different experimental diets as described in Chapter 2 were involved in this experiment. The same experimental design as described in Chapter 2 was continued in this phase whereby thirty replicate groups, each consisting of 20 pullets, were assigned to the same five different treatments they received during the growing phase. Each treatment was represented therefore by six replicates. However, to account for possible differences between EM supplementation during the growing phase and EM supplementation during the laying phase on performance during the laying phase, a split-plot experimental design was implemented in the original design. All the replicate groups of the five treatments from the growing phase were halved into two half-replicates, each containing approximately 10 pullets. One half-replicate from each of the six halved replicates per treatment was then randomly chosen and assigned the letter "B". These six half-replicates per treatment continued the treatments they received in the growing phase (Chapter 2) during the laying phase.

Treatment diets consisted of a control basal layer diet, diets containing 0.5% EM bokashi, 1.0% EM bokashi and 1.5% EM bokashi respectively and a layer diet containing 15 ppm of a commercial antibiotic which substituted the coccidiostat treatment. The EM0.5% (B) half-replicates therefore received 0.5% EM bokashi in the layer diet, the EM1.0% (B) half-replicates received 1.0% EM bokashi and the EM1.5% (B) half replicates received 1.5% EM bokashi in the layer diet. The half-replicates from the original coccidiostat

treatment from the growing phase which were chosen to continue with the treatment in the laying phase now received the layer diet containing 15 ppm of a commercial antibiotic and were named Antibiotic (B).

The half-replicates of each treatment not chosen to continue with the treatments served as controls for the original treatments to account for the effect of supplementation during the growing phase on performance during the laying phase and were assigned the letter "A". EM0.5% (A) served as a control for EM0.5% (B), EM1.0% (A) for EM1.0% (B), EM1.5% (A) for EM1.5% (B) and Antibiotic (A) for Antibiotic (B). These half-replicate controls received a basal layer diet containing no feed additives. The control treatment from the growing phase served as the overall control during the laying phase and received a basal layer diet containing no feed additives. A summary of the partition of the various treatments is given in Table 3.2.1.

From the age of 18 weeks until the end of the study on 32 weeks, the pullets that were chosen to continue with the treatments received the various treatments in the layer diet. The layer diets were composed to comply with the calculated composition as described in Table 3.2.2. The EM bokashi was compiled as described in Chapter 2. Diets were formulated to take into account the nutritional contribution from the bokashi but not from the EM *per se*. The antibiotic treatment diet contained 15 ppm of a commercial antibiotic. Diets were fed in a mash form and were provided for *ad libitum* consumption. No treatments were provided in the drinking water. Drinking water was provided for *ad libitum* consumption. All feeds and bokashi were formulated and supplied by Rainbow Feeds (Pty.) Ltd.

3.3. Housing

Before the onset of this phase of the study, the layer house was properly cleaned. At the age of 18 weeks, pullets were caged individually in laying cages (21 × 46 × 46cm) in a convection house on the premises of the experimental farm of the University of Pretoria. The laying cages had sloping wire floors. Treatments were randomly distributed throughout the laying house. Pullets of the same half-replicates were housed next to each

other and to eliminate environmental factors, "A" and "B" half replicates were also housed next to each other. One cage was each time left open between half-replicates to prevent contamination and mixing of experimental diets. A regimen of 16 hours light : 8 hours dark per day was followed throughout the experimental period which lasted 14 weeks. A conventional vaccination program was followed.

3.4. Parameters recorded and chemical analysis

Beginning at 19 weeks of age, hen day egg production, mean egg weight and egg mass production per hen were recorded weekly. Mean egg weights were determined by bulk weighing each replicate's eggs gathered in 7 days. The incidence of uncollectable (soft, cracked, dwarf, malformed and double-yolked) eggs was recorded weekly. Mortalities were noted daily but were so few that it was of no significant importance and therefore were not treatment-linked. At the end of the trial period at week 32 all the hens were weighed and the mean body mass per treatment were determined. Due to the split plot design, mean body weight gain could not be determined. Right shank lengths were also measured to determine the average shank length at week 32.

Various parameters of internal egg quality were measured once during the trial period. At the beginning of week 31, five eggs were randomly collected from each replicate, weighed individually and broken on a glass break-out stand with a reflective mirror to detect blood and meat spots on the under side of the egg. The incidence of bloodspots and meat spots were recorded and treated as categorical variables. The albumin height of each egg was then measured with an electronic Haugh meter (Technical Services and Supplies QCD Haugh Gauge, ARC Irene). A software program calculated the Haugh units of the various treatments (Eggware Version 1.02 release 04J7, ARC Irene, e-mail: support@tss-york.com).

Yolk colour was compared to the Roche colour fan with the colour ranging from 1 being light yellow to 15 being orange colour. To be more objective colour values ranging from 4 to 5 and colour values ranging from 6 to 7 were grouped into two separate groups to

give a better indication of lighter and darker egg yolks. Yolk colour was treated as a categorical variable. The eggshells of the same eggs were allowed to dry for one week at 25°C. Shell thickness was then measured in each shell with a micrometer. Measurements were taken at three random points at the blunt side of the egg with the shell membranes still attached. Average shell thickness was then determined.

Breaking strength of eggs was measured at the beginning of week 32. Five eggs from each replicate were randomly collected and breaking strength measured with an Inströn tensile strength measuring device.

Representative samples of each experimental diet were collected and chemically analyzed according to the methods of the AOAC (1990). Feed nitrogen levels were determined by Kjeldahl analysis. Crude fat was extracted using the Soxhlet apparatus. Crude fibre levels were determined using the Wijkström method (Application note of tecator AN02/78A). For the determination of calcium and total phosphorus in the diet, samples were first digested using the wet digestion method as described by Heckman (1971). Calcium levels were then determined by atomic absorption spectrophotometry (Perkin-Elmir model 2380). Phosphorus levels were determined using a Technikon Auto analyzer (Industrial method nr 334-74W/B^{+}, March 1977). The analyzed compositions of the layer experimental diets are shown in Table 3.4.1.

3.5. Statistical analysis

Data were analyzed using the SAS® system. This system is an integrated system of software providing complete control over data management, analysis, and presentation and is marketed by SAS Institute South Africa (Pty) Ltd, 1st Floor North Wing, President Place, 1 Hood Avenue, Rosebank, P. O. Box 3469, Parklands 2121, South Africa. Data were subjected to analysis of variance using the General Linear Models (GLM) and the ANOVA procedures. Tukey's Studentized Range (HSD) Test and Fisher's t-test were used to determine the significance of differences between treatment means at the $P<0.05$

level. The Frequency procedure of SAS® analyzed categorical variables by means of chi-square testing.

Table 3.2.1. Partition of treatments during the laying phase in a split-plot design[1]

Treatment replicates continuing on the original treatments from the growing phase[2]	**Treatment replicates serving as controls to correct for the growing phase[3]**
EM0.5% B (6 replicates with ± 10 pullets each)	EM0.5% A (6 replicates with ± 10 pullets each)
EM1.0% B (6 replicates with ± 10 pullets each)	EM1.0% A (6 replicates with ± 10 pullets each)
EM1.5% B (6 replicates with ± 10 pullets each)	EM 1.5% A (6 replicates with ± 10 pullets each)
Antibiotic B (6 replicates with ± 10 pullets each)	Antibiotic A (6 replicates with ± 10 pullets each)

[1] the control treatment from the growing phase served as overall control during the laying phase and is not shown in the table

[2] these treatments continued the treatments they received during the growing phase. EM0.5% B received 0.5% EM bokashi in the diet, EM1.0% B received 1.0% EM bokashi, EM1.5% B received 1.5% EM bokashi and Antibiotic B received 15 ppm of a commercial antibiotic in the layer diet.

[3] all these treatments received the same basal layer diet the control treatment received and discontinued EM supplementation during the laying phase

Table 3.2.2. Calculated composition of the basal experimental diets for laying hens (19 to 32 weeks)[1]

Calculated Composition	**(Dry Basis)**
ME (MJ / kg)	11.30
Crude Protein (g / kg)	150.02
Lysine (g / kg)	7.43
Methionine (g / kg)	3.62
Fat (g / kg)	39.97
Crude Fibre (g / kg)	47.78
Calcium (g / kg)	35.99
Available Phosphorus (g / kg)	3.43
Sodium (g / kg)	1.69

[1] For the different EM treatment diets, 0.5%, 1.0% and 1.5% EM bokashi were added to the diet. Diets were formulated to account for nutritional contributions from the bokhasi *per se*. Possible nutritional contributions from the EM *per se* could not be taken into account. The antibiotic treatment diet contained 15 ppm a commercial antibiotic.

Table 3.4.1. Analyzed composition of the layer experimental diets (19 - 32 Weeks)

Analyzed Composition (Dry Basis)	**EM0.5%**	**EM1.0%**	**EM1.5%**	**Antibiotic**	**Control**
% Dry	91.585	90.987	91.766	92.337	90.850
Crude Protein (%)	13.959	13.905	14.173	13.894	14.657
Ether Extract (%)	7.978	7.616	6.488	6.818	7.078
Crude Fibre (%)	7.286	8.269	9.135	8.148	7.188
Ash (%)	12.089	12.019	10.172	9.662	11.561
Ca (%)	2.617	2.472	2.547	2.530	2.902
Total P (%)	1.030	0.976	1.053	1.035	1.033

3.6 Results

The treatments that received EM during the growing and the laying phase (the B replicates) were compared to EM supplementation in the growing phase alone (the A replicates) to observe if EM supplementation during the laying phase had had any additional effect to EM supplementation during the growing phase. The treatments receiving EM during the growing and the laying phase (the B replicates) were then compared to the control (overall) to observe if the different levels of supplementation during the laying phase has had any effect on performance during the laying phase. The EM (B) treatments were then compared to the antibiotic to see if EM supplementation during the laying phase differed from antibiotic supplementation.

The three EM (B) treatments did not improve hen day egg production, egg production / hen / week, weekly egg weight and weekly egg mass production / hen over the control (overall). No significant differences in all the mentioned parameters were observed between all the EM (B) treatments and their respective controls (A). No significant differences between the antibiotic (B) and its control (A) were observed for all the above-mentioned parameters. No significant differences were observed between the EM (B) treatments and the antibiotic (B) treatment for all the mentioned parameters (Table 3.6.1).

No significant differences in the average hen day egg production over 14 weeks and the mean egg weight over 14 weeks were observed between the different EM (B) levels and the control (overall). The various EM (B) levels and the antibiotic (B) treatment did not differ significantly from their respective controls (A) for the same parameters, and no significant differences existed between the three different EM (B) and the antibiotic (B) (Table 3.6.2 & 3.6.3). Although both the antibiotic (B) and its control (A) had fewer eggs laid per hen over the trial period of 14 weeks, this did not differ significantly from the EM (B) treatments and the control (overall). There was no significant difference in the total amount of eggs laid per hen over the production period between the various EM (B) levels and their respective controls (A). For the same parameter no significant differences were obtained between the EM (B) treatments and the control (overall) and the antibiotic (B) (Table 3.6.4).

The mean percentage soft, cracked and DDM (double yolked, dwarf or malformed) eggs laid from Week 20 to Week 32 did not differ between the various EM (B) levels and their controls (A). No significant differences were observed between the EM (B) treatments and the control (overall). No significant differences were observed between the antibiotic (B) and the three EM (B) levels (Table 3.6.5).

No significant differences existed between all the EM (B) treatments and their controls (A) for the average body mass and shank length at Week 32 (Table 3.6.6). The antibiotic (B) treatment also did not differ from its control (A). The antibiotic (B) did not differ significantly from all the EM (B) treatments and the control (overall). The EM (B) treatments did not differ from the overall control for these two parameters (Table 3.6.6).

No significant differences existed in Haugh units between the EM (B) treatments and their controls (A). The EM (B) treatments also did not differ from the antibiotic (B) and the overall control. The antibiotic treatment (B) did not differ from its control (A) (Table 3.6.7). The EM 1.5% (B) level had significant thicker eggshells than its control (A) and the antibiotic (B) treatment. The control (overall) had significant thicker eggshells then the antibiotic (B) treatment (Table 3.6.7). No significant differences in shell thickness existed between the rest of the EM (B) treatments, their controls and the overall control. Also, no significant differences were observed between the rest of the EM (B) treatments and the antibiotic (B) (Table 3.6.7).

The antibiotic (B) treatment had a significant lower breaking strength than its control (A), the overall control and the EM 0.5% (B) level (Table 3.6.8). No significant differences in breaking strength existed between the rest of the EM (B) treatments, their controls (A) and the overall control. Also, no significant differences were obtained between the rest of the EM (B) treatments and the antibiotic (B) (Table 3.6.8).

The EM 0.5% (B) and EM 1.5% (B) had significantly more yolks classified in the lighter egg yolk group than all the other treatments (Table 3.6.9). Significant differences were

observed between the EM 0.5% (B) and its control (A) and the EM 1.5% (B) level and its control (A), the EM 0.5% (B) having more yolks classified as being light than its control and the EM 1.5% (B) level having slightly fewer yolks classified as light than its control. Although not significant the EM 1.0% (B) had also more yolks classified as being light than its control (A). In contrast, the antibiotic (B) treatment had a higher frequency of darker yolks than its control (A), the overall control and all the EM (B) treatments. Except for EM 0.5% (B) and EM 1.5% (B) and the antibiotic (B) treatment, no other significant differences existed between the rest of the EM (B) treatments and the overall control in terms of yolk colour (Table 3.6.9).

No significant differences were observed between the different EM (B) levels and their controls (A) regarding the incidence of bloodspots at Week 31. Also, no significant difference existed between the antibiotic (B) and its control (A). No significant differences existed between the EM (B) levels, the antibiotic and the overall control (Table 3.6.10). The EM 1.0% (B) treatment had the highest incidence of meat spots for all the treatments at week 31 (Table 3.6.10). This incidence was significantly higher than its control (A), all the EM treatments and the antibiotic (B) (Table 3.6.10). The antibiotic control (A) had the lowest incidence of meat spots of all the treatments.

Table 3.6.1. Egg production indices and egg weights (Week 19 to Week 32)

Week 19					
Treatment		**Hen Day Egg Production (%)**	**Egg Production / Hen / Week**	**Egg Weight (g)**	**Egg Mass Production / Hen (g)**
EM 0.5%	A	-	0.100[a]	-	-
	B	-	0.112[a]	-	-
EM 1.0%	A	-	0.172[a]	-	-
	B	-	0.152[a]	-	-
EM 1.5%	A	-	0.090[a]	-	-
	B	-	0.138[a]	-	-
Antibiotic	A	-	0.000[a]	-	-
	B	-	0.019[a]	-	-
Control	A	-	0.035[a]	-	-
	B	-	0.134[a]	-	-
Type III P-value		-	0.9269	-	-
Week 20					
EM 0.5%	A	29.307[a]	2.052[a]	39.854[a]	81.137[a]
	B	35.688[a]	2.498[a]	40.071[a]	99.840[a]
EM 1.0%	A	25.079[a]	1.756[a]	39.661[a]	70.077[a]
	B	21.825[a]	1.528[a]	40.840[a]	62.819[a]
EM 1.5%	A	25.971[a]	1.818[a]	39.838[a]	72.588[a]
	B	23.853[a]	1.670[a]	40.061[a]	65.358[a]
Antibiotic	A	18.030[a]	1.262[a]	40.877[a]	51.747[a]
	B	15.291[a]	1.070[a]	33.230[a]	41.819[a]
Control	A	30.040[a]	2.103[a]	40.428[a]	85.012[a]
	B	21.544[a]	1.508[a]	41.073[a]	61.735[a]

Type III P-value		0.7555	0.7555	0.3198	0.7245
Week 21					
EM 0.5%	A	60.433[a]	4.230[a]	43.647[a]	184.307[a]
	B	65.886[a]	4.612[a]	43.389[a]	200.200[a]
EM 1.0%	A	52.460[a]	3.672[a]	42.513[a]	155.698[a]
	B	49.233[a]	3.446[a]	42.656[a]	146.769[a]
EM 1.5%	A	63.874[a]	4.471[a]	42.886[a]	191.386[a]
	B	60.577[a]	4.240[a]	42.926[a]	182.177[a]
Antibiotic	A	55.584[a]	3.891[a]	44.712[a]	173.947[a]
	B	47.037[a]	3.293[a]	43.090[a]	142.461[a]
Control	A	61.574[a]	4.310[a]	43.746[a]	188.708[a]
	B	55.356[a]	3.875[a]	43.670[a]	169.393[a]
Type III P-value		0.6603	0.6603	0.5198	0.6467
Week 22					
EM 0.5%	A	66.169[a]	4.632[a]	46.207[a]	212.523[a]
	B	64.940[a]	4.546[a]	45.530[a]	206.808[a]
EM 1.0%	A	63.175[a]	4.422[a]	46.206[a]	204.319[a]
	B	59.947[a]	4.196[a]	48.750[a]	202.860[a]
EM 1.5%	A	65.083[a]	4.556[a]	46.103[a]	210.068[a]
	B	63.706[a]	4.459[a]	46.109[a]	205.303[a]
Antibiotic	A	59.670[a]	4.179[a]	46.914[a]	195.725[a]
	B	56.276[a]	3.939[a]	45.490[a]	179.100[a]
Control	A	64.828[a]	4.538[a]	47.284[a]	214.452[a]
	B	65.115[a]	4.558[a]	46.042[a]	209.761[a]
Type III P-value		0.9674	0.9674	0.1850	0.9291

Week 23					
EM 0.5%	A	92.987[a]	6.509[a]	47.398[a]	308.675[a]
	B	90.820[a]	6.357[a]	46.927[a]	298.320[a]
EM 1.0%	A	91.772[a]	6.424[a]	48.375[a]	309.745[a]
	B	88.413[a]	6.189[a]	48.355[a]	298.781[a]
EM 1.5%	A	93.318[a]	6.532[a]	48.121[a]	314.231[a]
	B	92.633[a]	6.484[a]	46.793[a]	302.918[a]
Antibiotic	A	91.364[a]	6.395[a]	48.597[a]	309.932[a]
	B	90.397[a]	6.328[a]	46.047[a]	289.449[a]
Control	A	87.474[a]	6.123[a]	48.868[a]	299.475[a]
	B	93.785[a]	6.565[a]	48.104[a]	315.718[a]
Type III P-value		0.5429	0.5429	0.3879	0.4692
Week 24					
EM 0.5%	A	91.494[a]	6.405[a]	49.177[a]	315.175[a]
	B	95.999[a]	6.720[a]	49.706[a]	333.401[a]
EM 1.0%	A	92.566[a]	6.480[a]	50.271[a]	324.085[a]
	B	88.968[a]	6.228[a]	49.743[a]	309.018[a]
EM 1.5%	A	96.266[a]	6.739[a]	50.356[a]	337.664[a]
	B	93.143[a]	6.520[a]	49.871[a]	324.482[a]
Antibiotic	A	93.788[a]	6.565[a]	50.444[a]	330.263[a]
	B	91.085[a]	6.376[a]	49.575[a]	314.601[a]
Control	A	92.434[a]	6.470[a]	50.169[a]	324.714[a]
	B	95.224[a]	6.666[a]	49.816[a]	332.091[a]
Type III P-value		0.1890	0.1890	0.4018	0.1754

Week 25					
EM 0.5%	A	94.004[a]	6.580[a]	50.322[a]	331.205[a]
	B	93.611[a]	6.553[a]	50.596[a]	331.341[a]
EM 1.0%	A	97.116[a]	6.798[a]	52.215[a]	350.999[a]
	B	95.265[a]	6.669[a]	51.233[a]	341.656[a]
EM 1.5%	A	98.628[a]	6.904[a]	51.064[a]	352.462[a]
	B	98.716[a]	6.910[a]	51.148[a]	352.528[a]
Antibiotic	A	98.203[a]	6.874[a]	51.421[a]	353.541[a]
	B	96.964[a]	6.788[a]	51.117[a]	346.115[a]
Control	A	93.856[a]	6.570[a]	51.297[a]	333.776[a]
	B	98.266[a]	6.879[a]	51.018[a]	349.994[a]
Type III P-value		0.5051	0.5051	0.4515	0.3013
Week 26					
EM 0.5%	A	93.810[a]	6.567[a]	51.518[a]	337.528[a]
	B	95.880[a]	6.712[a]	51.592[a]	345.213[a]
EM 1.0%	A	95.212[a]	6.665[a]	52.360[a]	348.676[a]
	B	95.873[a]	6.711[a]	52.601[a]	353.045[a]
EM 1.5%	A	96.413[a]	6.749[a]	52.168[a]	351.906[a]
	B	99.091[a]	6.936[a]	52.100[a]	361.369[a]
Antibiotic	A	92.511[a]	6.476[a]	52.086[a]	337.382[a]
	B	97.057[a]	6.794[a]	52.612[a]	356.263[a]
Control	A	95.119[a]	6.659[a]	52.279[a]	348.354[a]
	B	95.986[a]	6.719[a]	51.730[a]	347.704[a]
Type III P-value		0.9455	0.9455	0.5832	0.8791

Week 27					
EM 0.5%	A	93.983[a]	6.579[a]	52.026[a]	342.477[a]
	B	93.214[a]	6.525[a]	52.211[a]	340.712[a]
EM 1.0%	A	93.175[a]	6.522[a]	52.773[a]	343.060[a]
	B	95.714[a]	6.700[a]	52.766[a]	353.511[a]
EM 1.5%	A	97.430[a]	6.820[a]	52.689[a]	359.513[a]
	B	98.182[a]	6.873[a]	53.066[a]	363.788[a]
Antibiotic	A	94.286[a]	6.600[a]	53.751[a]	354.361[a]
	B	96.124[a]	6.729[a]	53.559[a]	360.588[a]
Control	A	92.632[a]	6.484[a]	52.738[a]	342.430[a]
	B	94.704[a]	6.629[a]	52.426[a]	347.701[a]
Type III P-value		0.9495	0.9495	0.9586	0.9720
Week 28					
EM 0.5%	A	95.931[a]	6.715[a]	52.613[a]	352.469[a]
	B	90.919[a]	6.364[a]	52.304[a]	332.719[a]
EM 1.0%	A	94.259[a]	6.598[a]	53.085[a]	349.414[a]
	B	98.942[a]	6.926[a]	53.698[a]	371.992[a]
EM 1.5%	A	95.525[a]	6.687[a]	52.885[a]	353.423[a]
	B	92.525[a]	6.477[a]	52.929[a]	343.050[a]
Antibiotic	A	94.437[a]	6.611[a]	53.180[a]	351.642[a]
	B	93.307[a]	6.531[a]	53.775[a]	349.714[a]
Control	A	95.417[a]	6.679[a]	53.002[a]	354.746[a]
	B	94.774[a]	6.634[a]	52.761[a]	347.943[a]
Type III P-value		0.5867	0.5867	0.5748	0.4672

Week 29					
EM 0.5%	A	96.104[a]	6.727[a]	52.103[a]	350.623[a]
	B	95.589[a]	6.691[a]	52.963[a]	354.306[a]
EM 1.0%	A	96.878[a]	6.781[a]	53.138[a]	359.246[a]
	B	96.958[a]	6.787[a]	53.009[a]	358.877[a]
EM 1.5%	A	97.203[a]	6.804[a]	52.961[a]	360.548[a]
	B	96.635[a]	6.764[a]	52.901[a]	356.996[a]
Antibiotic	A	92.922[a]	6.505[a]	53.501[a]	346.900[a]
	B	97.024[a]	6.792[a]	53.964[a]	366.537[a]
Control	A	97.388[a]	6.817[a]	51.427[a]	348.895[a]
	B	96.758[a]	6.773[a]	52.570[a]	355.163[a]
Type III P-value		0.8051	0.8051	0.7568	0.5950
Week 30					
EM 0.5%	A	94.957[a]	6.647[a]	53.226[a]	352.968[a]
	B	97.811[a]	6.847[a]	53.618[a]	366.853[a]
EM 1.0%	A	97.593[a]	6.831[a]	53.691[a]	365.858[a]
	B	96.614[a]	6.763[a]	54.316[a]	367.366[a]
EM 1.5%	A	96.058[a]	6.724[a]	53.202[a]	357.820[a]
	B	98.593[a]	6.902[a]	53.302[a]	367.874[a]
Antibiotic	A	97.446[a]	6.821[a]	54.041[a]	366.179[a]
	B	92.976[a]	6.508[a]	54.746[a]	356.435[a]
Control	A	95.476[a]	6.683[a]	53.253[a]	356.264[a]
	B	97.335[a]	6.813[a]	53.723[a]	365.059[a]
Type III P-value		0.1197	0.1197	0.9463	0.3323

Week 31					
EM 0.5%	A	91.126[a]	6.379[a]	54.184[a]	345.644[a]
	B	92.956[a]	6.507[a]	54.114[a]	351.943[a]
EM 1.0%	A	93.545[a]	6.548[a]	54.184[a]	353.956[a]
	B	94.418[a]	6.609[a]	54.538[a]	360.609[a]
EM 1.5%	A	94.059[a]	6.584[a]	54.236[a]	356.995[a]
	B	92.292[a]	6.460[a]	55.855[a]	359.860[a]
Antibiotic	A	93.290[a]	6.530[a]	54.457[a]	354.703[a]
	B	91.283[a]	6.390[a]	54.942[a]	351.291[a]
Control	A	91.468[a]	6.403[a]	54.639[a]	349.974[a]
	B	93.860[a]	6.570[a]	54.051[a]	355.239[a]
Type III P-value		0.5185	0.5185	0.5997	0.9027
Week 32					
EM 0.5%	A	92.338[a]	6.464[a]	54.024[a]	349.133[a]
	B	92.507[a]	6.475[a]	53.451[a]	343.935[a]
EM 1.0%	A	94.233[a]	6.596[a]	53.767[a]	347.386[a]
	B	93.757[a]	6.563[a]	54.268[a]	356.238[a]
EM 1.5%	A	93.141[a]	6.520[a]	53.965[a]	350.708[a]
	B	95.373[a]	6.676[a]	54.184[a]	360.876[a]
Antibiotic	A	93.810[a]	6.567[a]	54.758[a]	358.687[a]
	B	94.021[a]	6.581[a]	54.652[a]	360.069[a]
Control	A	93.836[a]	6.569[a]	54.068[a]	354.207[a]
	B	94.007[a]	6.580[a]	52.945[a]	348.482[a]
Type III P-value		0.9108	0.9108	0.4296	0.5255

* Means with different superscripts within weeks are significantly different ($P < 0.05$) according to Tukey's Studentized Range (HSD) Test.

Control A and B is the same treatment – the Overall Control

Table 3.6.2. Mean egg weight over 14 weeks

Treatment		Egg Weight (g) (Means)	SD
EM 0.5%	A	49.715[a]	0.603
	B	49.729[a]	0.796
EM 1.0%	A	50.172[a]	0.637
	B	50.521[a]	0.832
EM 1.5%	A	50.036[a]	1.000
	B	50.096[a]	0.592
Antibiotic	A	50.672[a]	1.302
	B	49.754[a]	1.640
Control	A	50.246[a]	1.035
	B	49.994[a]	0.414
Type III P-value		0.5561	

* Means with different superscripts are significantly different ($P < 0.05$) according to Tukey's Studentized Range (HSD) Test.
Control A and B is the same treatment – the Overall control

Table 3.6.3. Average hen day egg production over 14 weeks

Treatment		Hen day Egg Production (Means)	SD
EM 0.5%	A	84.049[a]	3.626
	B	85.063[a]	4.518
EM 1.0%	A	83.620[a]	3.884
	B	82.764[a]	2.463
EM 1.5%	A	85.613[a]	2.096
	B	85.025[a]	3.081
Antibiotic	A	82.721[a]	2.430
	B	81.449[a]	2.854
Control	A	83.965[a]	5.134
	B	84.363[a]	1.924
Type III P-value		0.9169	

* Means with different superscripts are significantly different ($P < 0.05$) according to Tukey's Studentized Range (HSD) Test.
Contol A and B is the same treatment – the Overall Control

Table 3.6.4. The total number of eggs laid per hen from Week 19 to Week 32

Treatment		Number of Eggs / Hen	SD
EM 0.5%	A	76.58[a]	3.33
	B	77.52[a]	4.09
EM 1.0%	A	76.27[a]	3.60
	B	75.47[a]	2.49
EM 1.5%	A	77.78[a]	1.95
	B	77.51[a]	2.92
Antibiotic	A	75.28[a]	2.21
	B	74.02[a]	2.52
Control	A	75.80[a]	4.98
	B	76.90[a]	1.89
Type III P-value		0.8777	

* Means with different superscripts are significantly different ($P < 0.05$) according to Tukey's Studentized Range (HSD) Test.
Control A and B is the same treatment – the Overall Control

Table 3.6.5. The mean percentage soft, cracked and DDM (double yolked, dwarf or malformed) eggs laid from Week 20 to Week 32

Treatment		Soft Eggs		Cracked Eggs		DDM	
		Means (%)	Amount	Means (%)	Amount	Means (%)	Amount
EM 0.5%	A	0.493[a]	23	1.812[a]	84	0.386[a]	18
	B	0.530[a]	23	0.817[a]	34	0.302[a]	13
EM 1.0%	A	0.426[a]	19	0.995[a]	44	0.227[a]	10
	B	0.305[a]	13	1.098[a]	47	0.294[a]	13
EM 1.5%	A	0.491[a]	20	0.803[a]	34	0.099[a]	4
	B	0.429[a]	20	1.128[a]	51	0.152[a]	8
Antibiotic	A	0.282[a]	13	1.004[a]	46	0.502[a]	23
	B	0.427[a]	18	0.910[a]	39	0.211[a]	9
Control	A	0.288[a]	12	0.819[a]	35	0.368[a]	16
	B	0.259[a]	12	0.794[a]	35	0.147[a]	6
Type III P-value		0.6554		0.6113		0.3582	

* Means with different superscripts significantly different ($P < 0.05$) according to Tukey's Studentized Range (HSD) Test.

Table 3.6.6. Average body weight and shank length at Week 32

Treatment		Body Weight Means (kg)	SD	Shank Length Means (mm)	SD
EM 0.5%	A	2.012[a]	0.091	95.611[a]	1.960
	B	1.941[a]	0.038	96.722[a]	3.130
EM 1.0%	A	1.958[a]	0.067	96.222[a]	2.297
	B	1.973[a]	0.068	96.722[a]	2.728
EM 1.5%	A	2.017[a]	0.090	95.167[a]	2.588
	B	1.990[a]	0.072	96.444[a]	1.656
Antibiotic	A	1.960[a]	0.087	96.167[a]	1.206
	B	1.981[a]	0.069	96.889[a]	2.934
Control	A	2.002[a]	0.060	95.444[a]	1.940
	B	1.960[a]	0.097	98.278[a]	1.143
Type III P-value		0.5313		0.7395	

* Means with different superscripts are significantly different ($P < 0.05$) according to Tukey's Studentized Range (HSD) Test.
Control A and B is the same treatment – the Overall Control

Table 3.6.7. Haugh units and shell thickness of eggs at Week 31

Treatment		Egg Mass LSmeans (g)	Haugh Unit LSmeans (HU)	Shell Thickness LSmeans (mm)
EM 0.5%	A	54.077	96.570	0.319
	B	52.805^{ab}	94.722	0.311
EM 1.0%	A	54.767	93.963	0.318
	B	55.620^{a}	95.090	0.312
EM 1.5%	A	55.287	95.520	0.303^{a}
	B	54.203	94.573	0.318^{ab}
Antibiotic	A	53.850^{c}	98.147	0.309
	B	55.793^{bc}	95.630	0.306^{bc}
Control		54.198	94.877	0.322^{c}
Type III P-value		0.0371	0.8597	0.0021

* Means with the same superscripts are significantly different ($P < 0.05$) according to Fisher's t-test.

Table 3.6.8. Mean breaking strength of eggs at Week 32

Treatment		Breaking Strength LSmeans (-N)	Egg Mass LSmeans (g)
EM 0.5%	A	35.560	56.690
	B	34.361[a]	55.341
EM 1.0%	A	31.325	54.222
	B	33.323	55.422
EM 1.5%	A	33.022	54.384[a]
	B	31.967	56.308[a]
Antibiotic	A	35.422[b]	55.369
	B	29.387[abc]	56.593
Control		33.453[c]	55.327
Type III P-value		0.1284	0.2140

* Means with the same superscripts are significantly different ($P < 0.05$) according to Fisher's t-test.

Table 3.6.9.Yolk color at Week 31

Treatment		Roche value (4 – 5)			Roche value (6 – 7)		
		Amount	Expected Frequency	Cell chi-square	Amount	Expected Frequency	Cell chi-square
EM 0.5%	A	13	16.068	0.586	17	13.932	0.676
	B	24	16.604	3.294	7	14.396	3.800
EM 1.0%	A	15	16.068	0.071	15	13.932	0.082
	B	18	16.068	0.232	12	13.932	0.268
EM 1.5%	A	24	16.068	3.915	6	13.932	4.516
	B	22	16.068	2.190	8	13.932	2.526
Antibiotic	A	14	16.068	0.266	16	13.932	0.307
	B	7	16.068	5.118	23	13.932	5.903
Control		26	32.137	1.172	34	27.863	1.352
Likelihood ratio chi-square		0.001			0.001		

Table 3.6.10. The incidence of bloodspots and meat spots at Week 31

Treatment		Bloodspots			Meat spots		
		Amount	Expected Frequency	Cell chi-square	Amount	Expected Frequency	Cell chi-square
EM 0.5%	A	10	4.957	5.130	3	5.385	1.056
	B	5	5.123	0.003	7	5.564	0.371
EM 1.0%	A	5	4.957	0.000	5	5.385	0.028
	B	5	4.957	0.000	9	5.385	2.428
EM 1.5%	A	2	4.957	1.764	5	5.385	0.028
	B	4	4.957	0.185	4	5.385	0.356
Antibiotic	A	6	4.957	0.219	0	5.385	5.385
	B	4	4.957	0.185	4	5.385	0.356
Control		10	9.915	0.000	10	10.769	0.055
Likelihood ratio chi-square		0.552			0.009		

3.7. Discussion

EM supplementation during the laying phase did not improve hen day egg production, egg production / hen / week, mean weekly egg weight and mean weekly egg mass production / hen over supplementation during the growing phase alone and the control. EM supplementation during the laying phase also did not increase the total amount of eggs laid over the 14 week experimental period over supplementation during the growing phase alone and the control. The mean percentage soft, cracked and DDM (double yolked, dwarf or malformed) eggs laid from week 20 to week 32 did not differ significantly between the hens that received EM during the laying phase and hens that received EM during the growing phase alone and the control. Nahashon *et al.* (1994a) found an increased body weight gain and an increase of 25% in nitrogen retention in layers fed a diet supplemented with 2200 ppm (8.8×10^7 cfu / mg of culture) of a *Lactobacillus* culture. In this study, EM treatment during the laying phase did not improve the average body mass and shank length at week 32 over supplementation during the growing phase alone and the control.

The results regarding EM supplementation in the laying phase on the production performance of the commercial laying hen are in agreement with several studies. Grimes *et al.* (1997) found no significant effect of probiotic supplementation on egg production and egg weight. Miles *et al.* (1981a) found no influence of probiotic supplementation on egg weight. Cerniglia *et al.* (1983) included either liquid or dried nonviable *Lactobacillus* cultures in the diet of laying hens and found no significant increase in hen day egg production. Goodling *et al.* (1987) found no improvement in hen day egg production when feeding various levels of a viable *Lactobacillus* product. Reasons cited by Goodling *et al.* (1987) as to why no improvement in egg production were found are two-folded. Siriwar (1977 as cited by Goodling *et al.*, 1987) reported that *Lactobacillus* cultures are host-specific as well as being specific in their by-product production. Goodling *et al.* (1987) speculates that perhaps the cultures used in that study were not host-specific. Secondly, because lactobacilli become established in the gut of most animals soon after birth (Savage *et al.*, 1968 as cited by Goodling *et al.*, 1987), *Lactobacillus* supplementation under relatively ideal conditions may not show benefits.

Under normal, commercial conditions, factors that could disturb or destroy an ideal gut flora include intestinal damage due to coccidiosis and mycosis or the feeding of additives known to alter the flora. Under this normal, but not ideal situations, a beneficial effect of probiotic feeding could result in improved performance (Goodling *et al.*, 1987).

Results obtained in this study are also in agreement with results obtained in other poultry species. Damron *et al.* (1981) found that the inclusion of 625 mg / kg of a mixed, dried *Lactobacillus* culture to the diets of Broad Breasted Large White turkeys did not significantly affect egg production. Miles *et al.* (1981b) found that the addition of 625 mg / kg of a *Lactobacillus* culture to the diet of Bobwhite quail breeders did not influence egg production. The conclusion made in that study was that the addition of the *Lactobacillus* culture was neither detrimental nor significantly beneficial to the production performance of quail breeders.

However, the result obtained in this study regarding egg production is also in contrast to several studies. Krueger *et al.* (1977 as cited by Jernigan and Miles, 1985) found an increase of 3.03% in egg production when supplying *Lactobacillus* at a rate of 2.27 kg / ton in the layer diet. Miles *et al.* (1981a) incorporated a living *Lactobacillus acidophilus* culture in the diet of two strains of laying hens housed at three different geographical locations. An across trial analysis of all the subsequent egg production data provided a statistical advantage to adding the probiotic to the diet. Abdulrahim *et al.* (1996) found that the addition of a liquid *Lactobacillus acidophilus* culture at concentrations exceeding 1×10^6 cfu / g of the diet significantly improved egg production over the control (81.6±3.16% vs. 73.7±3.36%). A marginal elevation in egg weight was also seen. Mohan *et al.* (1995) supplied a multiple stain probiotic in the diet of laying hens and found that egg production was improved by 5% and 2.6% respectively in groups receiving 100mg / kg and 150 mg / kg of the probiotic. Nahashon *et al.* (1994a) found that hen day egg production was significantly higher than the control in pullets fed 1100 ppm (4.4×10^7 cfu / mg culture) of a living *Lactobacillus* culture (90.4% vs. 88.9%). Egg mass and egg weights were also significantly increased over the control for the pullets fed the diet containing 1100 ppm (4.4×10^7 cfu / mg culture) *Lactobacillus*.

EM supplementation during the laying phase did not improve internal egg quality over supplementation during the growing phase alone and the control. This is in contrast to Nahashon *et al.* (1994a) who found that internal egg quality was significantly improved in pullets fed a layer diet containing 2200 ppm (8.8×10^7 cfu / mg of culture) of a living *Lactobacillus* culture over the control (84.5 HU vs. 83.1 HU). However, this is in agreement with Miles *et al.* (1981a) who found no influence of probiotic supplementation on internal egg quality.

EM supplementation at 1.5% during the laying phase resulted in eggs with significant thicker shells than supplementation at 1.5% during the growing phase alone suggesting that supplementation at this level in the laying phase was beneficial over supplementation during the growing phase alone. However, no significant differences in eggshell thickness were observed between supplementation at 1.5% in the laying phase and the two other two treatments that received EM in the layer diet and the control. This is in agreement with Abdulrahim *et al.* (1996) who found no increase in the average shell thickness with the addition of a *Lactobacillus acidophilus* culture (1×10^6 cfu / g of diet) in the diet of laying hens. In contrast, Mohan *et al.* (1995) found a marginal improvement in shell thickness when supplying a multiple strain probiotic in the diet of laying hens. EM supplementation during the laying phase did not influence egg breaking strength over supplementation in the growing phase alone and the control.

EM supplementation at 0.5% and 1.5% in the laying phase caused the production of eggs with lighter yolks than supplementation at this levels during the growing phase alone and the control. This is in contrast to Miles *et al.* (1981a) who found that yolk colour was not affected in hens housed at three different geographical locations when the diets were supplemented with three levels of a *Lactobacillus* culture. Supplementing 1.5% EM in the growing phase alone resulted in the production of eggs with lighter yolk colours in the subsequent laying phase suggesting that this highest form of supplementation during the growing phase had an effect on yolk colour in the laying phase even when supplementation at this level was discontinued in the laying phase. These results should

be apprehended with great care as yolk colour was subjectively appraised in a relatively small amount of eggs.

EM supplementation in the laying phase did not influence the incidence of bloodspots significantly over supplementation in the growing phase alone and the control. This is in agreement with Nahashon *et al.* (1994a) who found that the incidence of bloodspots was not affected by *Lactobacillus* supplementation. EM supplementation at 1.0% during the laying phase resulted in eggs with a higher incidence of meat spots than supplementing at this level in the growing phase. This was also higher than the control and the rest of the EM treatments. These results should be apprehended with great care as these two parameters were subjectively appraised in a relatively small amount of eggs.

Antibiotic supplementation did not improve egg production, egg weights and did not increase the total amount of eggs laid over the experimental period over EM supplementation and the control. Also no significant differences were obtained in the percentage soft, cracked and DDM (double yolked, dwarf or malformed) eggs laid over the experimental period with antibiotic supplementation. Antibiotic supplementation did not improve internal egg quality but resulted in eggs with significantly thinner eggshells than the treatment receiving 1.5% EM during the laying phase and the control (overall). Thinner shells were reflected in the breaking strength of the eggs as the breaking strength from the antibiotic treatment was significantly lower than its control (which received the coccidiostat during the growing phase), the overall control and the treatment that received 0.5% EM in the layer diet. Although the mean egg weight of the antibiotic treatment was higher than its control, the overall control and the EM 0.5% level, this was not significant. The lower breaking strength of the antibiotic treatment could therefore not be solely attributed to larger eggs that would theoretically have had thinner shells. In contrast to the different EM levels, antibiotic supplementation improved yolk color. Antibiotic treatment during the laying phase did not influence the incidence of bloodspots. Besides the improvement in yolk colour the results obtained with antibiotic supplementation tend to support the conclusion made by Abdulrahim *et al.* (1996) that attempting to control the intestinal microflora with an antibiotic is too crude an approach

to produce a desirable effect. The treatment that received the coccidiostat during the growing phase but no supplementation during the laying phase had a significantly lower incidence of meat spots than the antibiotic treatment and the treatments that received EM during the growing and the laying phase. Both the antibiotic and its control did not differ significantly from all the EM treatments and the control regarding the average body weight and shank length. Both these treatments received the coccidiostat treatment during the growing phase (Chapter 1) which resulted in significantly lower body weights at the end of that phase (Chapter 1). This may be taken as an indication that both these treatments compensated for lost body mass in the growing phase during the laying phase. As both these treatments compensated for lost body mass it could not have been an affect due to antibiotic supplementation alone.

Therefore, EM supplementation during the growing and the laying phase did not influence the production performance of the commercial laying hen in this experiment, and where results were obtained in egg quality (yolk colour, meat spots) it was mostly in subjectively appraised parameters. Several factors may have influenced the results obtained in this study. Most of the studies that have obtained significant results on the production performance of the commercial laying hen with probiotic supplementation included either viable strains in their experimental diets or included the probiotic in a liquid form in the diet which would have sustained viability to a point (Miles *et al.*, 1981a & Nahashon *et al.*, 1994a, Abdulrahim *et al.*, 1996). Viability may have played a role in this study as the survival of the EM strain was determined by the layer diet that had a high dry-matter content. A second factor that may have played a role is the degree to which the microorganisms had adhered to the gut epithelia. Goodling *et al.* (1987) included a viable *Lactobacillus* strain in the diet of laying hens and attributed the lack of significant results to the fact that the strain used may not have been host–specific. In the present study host-specificity of the *Lactobacillus* in EM may have played a role. A third factor is housing under ideal, stress-free conditions. In this study pullets were housed individually and were not exposed to stress factors associated with dense bird populations such as an outbreak of a disease. The experimental conditions may therefore not have been "normal" for the probiotic to exert its effect. This was also seen regarding the

antibiotic as no significant improvements in the production performance, except in yolk colour, were seen regarding antibiotic supplementation furthermore suggesting that a stress situation should exist before any beneficial effect would be seen. It can be concluded that more benefits may be seen regarding production performance and egg quality with EM supplementation under more commercial circumstances.

3.8. Conclusion

In view of these results, the hypotheses that EM supplementation during the growing and laying phase has a beneficial effect on the performance, egg production and various egg quality indices of the commercial laying hen in comparison to an antibiotic and a control could not be accepted.

Chapter 4

The Influence of the Method of EM (Effective Microorganisms) Supplementation on the Egg Quality of the Commercial Laying Hen

4.1. Introduction

A prerequisite for a good probiotic is that it should be capable of being prepared as a viable product on an industrial scale and that it must remain stable and viable for long periods under storage and field conditions (Fuller, 1989). This may not always be achievable. The dry matter content of poultry diets may be too high for viable organisms of an administered probiotic to remain viable for long periods. This would bring about a number of management problems regarding the provision of feed to a layer flock as fresh batches of feed would then need to be prepared daily or even more regularly to ensure the desired response from probiotic supplementation.

As an alternative to supplementation in the feed, a probiotic may be administered in the drinking water. Watkins & Kratzer (1984) supplied a *Lactobacillus* culture in the drinking water of broilers but found no change in body weights and feed conversion. No studies have been done on the effect of probiotic supplementation in the drinking water on the performance and product quality of the commercial laying hen. In addition, it has been proposed that multiple-strain probiotic cultures are active against a wider range of conditions and may therefore be more successful in improving performance (Fuller, 1989). A viable multiple strain probiotic may be able to improve eggshell thickness, internal egg quality, egg yolk colour and a number of other egg quality indices. In addition it may be possible to increase the shelf life of eggs which would be advantageous to both the producer and consumer. A longer shelf life may even influence the marketing of eggs, as producers would be able to keep their produce for a while longer until the demand ensures a good price.

In view of these remarks a study was undertaken to determine if there was a difference in various egg quality parameters when a multiple-strain probiotic named EM (composition as described by Phillips & Phillips, 1996) is supplemented in the feed or the drinking water of the commercial laying hen.

Three hypotheses were tested in the first experiment of this study. The first hypothesis tested was if EM supplementation in the drinking water had a beneficial effect on egg quality. The second hypothesis tested was if there is a difference in egg quality between EM supplementation in the drinking water or in the diet and the third hypothesis tested was if there was an improvement in egg quality if EM was supplied in both the feed and in the water. In the second experiment the hypothesis tested was if EM supplementation in the food or water had a beneficial effect on the deterioration of internal egg quality (as an indication of shelf life) over a fixed period of time.

4.2. Trial design and housing

At the onset of this phase of the study, 50 of the 669 DeKalb Single Comb White Leghorn (Amber-link®), day-old pullet chicks obtained from a commercial hatchery as described in Chapter 2 were randomly selected and reared in two separate floor-pens in an environmentally controlled broiler house to 18 weeks of age. During the growing phase, which occurred during the same time and in the same broiler house as the growing phase described in Chapter 2, chicks received basal starter and grower diets containing no feed additives whatsoever. Pullets were not de-beaked and all general management procedures were performed as described in Chapter 2. At 18 weeks of age, birds were randomly placed individually in laying cages (21 × 46 × 46cm) in a convection house on the premises of the experimental farm of the University of Pretoria. Pullets were housed in the same convection house at the same time the laying phase, which tested different dietary EM inclusion levels in the layer diet as described in Chapter 3, commenced.

During the laying phase (19 - 32 weeks), pullets were subjected to two different treatments. An experimental design was followed whereby ten replicate groups, each consisting of 5 pullets, were randomly assigned to a basal diet containing no feed

additives (the control diet) or a diet containing 1.5% EM bokashi. Each treatment was therefore represented by five replicates. Treatment replicates were randomly distributed throughout the laying house. Hens of the same replicates were, however, housed next to each other. Diets were formulated as described in Chapter 3. The calculated and analyzed compositions of both experimental diets were the same as described in Chapter 3 as these pullets received the same experimental diets used in the laying phase described in Chapter 3.

In addition to the dietary treatments, all the replicates from both these treatments received EM stock solution in the drinking water for the duration of the trial period. The EM stock solution was included in the drinking water at a concentration of 1: 1000 as specified by the APNAN user's guide (Phillips & Phillips, 1996). Drinking water and feed were supplied for *ad libitum* consumption. Comparisons in recorded parameters were then done in two separate experiments.

Experiment 1

In this experiment the two treatments receiving different diets but the same EM level in the water were compared to two different dietary treatments receiving no EM supplementation in the drinking water. These treatments were the ones receiving 1.5% EM bokashi in the layer diet and the overall control receiving only a basal layer diet as described in Chapter 3. These treatments had access to drinking water containing no EM supplementation whatsoever.

Experiment 2

In this experiment the two treatments receiving EM in the drinking water were compared to the three different dietary EM treatments as well as the treatment receiving 15 ppm of a commercial antibiotic in the layer diet as described in Chapter 3. These treatments had access to drinking water that contained no EM supplementation whatsoever.

4.3. Parameters recorded

Different egg quality parameters were measured once in the trial period from eggs collected from these two treatments receiving different diets but receiving the same treatment in the drinking water. Results were then compared in two experiments to measured parameters of various dietary treatments from the laying phase as described in Chapter 3.

Experiment 1

The two treatments receiving different diets but the same EM level in the water were compared to two different dietary treatments receiving no EM supplementation in the drinking water. These treatments were the ones receiving 1.5% EM bokashi only in the layer diet and the overall control receiving only a basal layer diet as described in Chapter 3.

Various egg quality parameters were measured once during the trial period. At the beginning of Week 31, five eggs were randomly collected from each replicate, weighed individually and broken on a glass break-out stand. The incidence of bloodspots and meatspots were recorded and treated as categorical variables. The albumin height of each egg was then measured and Haugh units calculated as described in Chapter 3. Yolk colour was compared to the Roche colour fan as described in Chapter 3. To be more objective colour values ranging from 4 to 5 and colour values ranging from 6 to 7 were grouped into two separate groups to give a better indication of lighter and darker egg yolks. Yolk colour was treated as a categorical variable. The eggshells of the same eggs were allowed to dry for one week at 25°C. Shell thickness was measured in each egg with a micrometer in the same way as described in Chapter 3. Breaking strength of eggs was measured at the beginning of Week 32. Five eggs from each replicate were randomly collected and breaking strength measured with an Inströn tensile strength measuring device.

Results from all above-mentioned parameters were then compared to the treatment receiving 1.5% EM bokashi in the layer diet and the overall control receiving only a basal layer diet as described in Chapter 3.

In addition five eggs from each replicate from all four aforementioned treatments were collected at Week 32, broken and the shells collected. Shells were allowed to dry at 25°C for one week. The five shells from each replicate were pooled, mixed and ground with the shell membranes still attached. Representative samples from each replicate were collected for the determination of the calcium and phosphorus contents of eggshell. Samples were first digested using the wet digestion method as described by Heckman (1971). Calcium levels were then determined by atomic absorption spectrophotometry (Perkin-Elmir model 2380). Phosphorus levels were determined using a Technikon Auto Analyzer (Industrial method nr 334-74W/B^{+}, March 1977).

Experiment 2

In this experiment the two treatments receiving EM in the drinking water were compared to the three different dietary EM treatments as well as the treatment receiving 15 ppm of a commercial antibiotic in the layer diet as described in Chapter 3. At the beginning of Week 35, eggs laid were allowed to accumulate over two days. Eight eggs per replicate were then randomly collected. On the same day the eggs were collected, four eggs were randomly selected from each collection of eight eggs, broken on a glass break-out stand and albumin height measured. Haugh units were calculated as described in Chapter 3. The four remaining eggs per replicate were incubated in a hatcher at 37°C for seven days. On the seventh day these eggs were broken on a glass break-out stand and albumin height measured. Haugh units were again calculated as described in Chapter 3. The Haugh units after incubation were then subtracted from the Haugh units before incubation. The difference was taken as an indication of the deterioration of egg (albumin) quality over time.

4.4. Statistical analysis

In both experiments data were analyzed using the SAS® system. Data were subjected to analysis of variance using the General Linear Models (GLM). Fisher's t-test was used to determine the significance of differences between treatment means at the $P<0.05$ level. The Frequency procedure of SAS® analyzed categorical variables by means of chi-square testing.

4.5. Results

Experiment 1

EM supplementation in the drinking water of commercial laying hens did not alter the phosphorus and calcium content or the calcium to phosphorus of eggshells. No significant differences existed in the phosphorus and calcium content or the calcium to phosphorus ratio of eggshells between hens receiving EM supplementation in the drinking water and hens receiving no supplementation in the drinking water and both receiving a basal layer diet containing no EM (3 & 4 in Table 4.5.1 & 4.5. 2 & 4.5.3). EM supplementation in the drinking water did not increase the phosphorus and calcium content or the calcium to phosphorus ratio of eggshell significantly above EM supplementation in the diet alone (1 & 4 Table 4.5.1 & 4.5.2 & 4.5.3). No significant differences in calcium and phosphorus content and the calcium to phosphorus ratio were obtained between EM supplementation in both the diet and drinking water and the control receiving no supplementation. Although not significant, the group of hens that received EM in both the diet and the drinking water had the highest phosphorus content in eggshell whilst the group that received EM supplementation only in the drinking water had the highest calcium content (Table 4.5.1& 4.5.2). The treatment that received EM only in the diet had the highest calcium to phosphorus ratio. The unsupplemented control had the lowest calcium to phosphorus ratio in eggshell. This was not significant (Table 4.5.3).

Mean breaking strength of eggs at Week 32 is presented in Table 4.5.4. EM supplementation in the drinking water did not affect the breaking strength of eggs (3 & 4

Table 4.5.4). Method of EM supplementation had no effect on egg breaking strength as no significant difference in breaking strength was observed between hens receiving EM only in the diet and hens receiving EM only in the drinking water (1 & 4 Table 4.5.4). EM supplementation in both the diet and the drinking water did not improve egg breaking strength over the control significantly (2 & 3 Table 4.5.4). The treatment receiving EM in both the diet and drinking water had the highest breaking strength whilst the treatment receiving EM only in the drinking water had the lowest breaking strength (Table 4.5.4). These differences were not significant.

Mean shell thickness is presented in Table 4.5.5. Hens receiving EM in the drinking water alone produced eggs with significantly thinner shells than hens receiving EM in the diet alone (1 & 4 Table 4.5.5). These shells were also significantly thinner than the shells of hens receiving no EM supplementation at all (3 & 4 Table 4.5.5). Hens receiving EM supplementation in both the feed in water produced the thickest shells but this did not differ significantly from shells of hens receiving no EM supplementation at all (2 & 3 Table 4.5.5).

EM supplementation in the feed or the water or both did not influence internal egg quality significantly. The treatments receiving EM in the drinking water, regardless of diet, had numerically higher mean Haugh values (Table 4.5.5). EM supplementation in both the feed and the drinking water did not improve internal egg quality over the control significantly; there was, however, a numerical difference in favour of the EM treatment (2 & 3 Table 4.5.5).

No significant differences were found for the incidence of bloodspots between EM supplementation in the drinking water and the control (3 & 4 Table 4.5.7). Supplementation in the drinking water alone or in the feed alone exerted no effect on the incidence of bloodspots (1 & 4 Table 4.5.7). EM supplementation in both the feed and the water also did not increase the incidence of bloodspots significantly (2 & 3 Table 4.5.7). Treatments receiving EM supplementation in the drinking water, regardless of

diet, had a significantly higher incidence of meat spots than hens receiving only EM in the diet and hens receiving no supplementation at all (1,2,3 & 4 Table 4.5.7).

Hens receiving EM supplementation in the diet alone produced eggs with significantly lighter yolks than hens receiving EM in the drinking water (regardless of diet) and the control (Table 4.5.8).

Experiment 2

Haugh units of eggs before and after incubation at 37°C are shown in Table 4.5.6. The eggs taken as a sample from each treatment to represent the mean Haugh units before incubation showed significant differences between treatments. The treatment that received EM in the feed alone showed the highest mean Haugh unit value. This value differed significantly from the treatments receiving 0.5% and 1.0% EM in the diet and the treatment that received EM only in the drinking water. The antibiotic treatment had the second highest Haugh unit value that differed significantly from both the treatments receiving EM in the water regardless of the diet. Both the treatments that received EM in the drinking water had the lowest Haugh units, which was significantly lower than the control which received no supplementation whatsoever.

After incubation at 37°C for seven days significant differences existed between the treatments. The treatment that received EM in the layer diet and in the drinking water had the highest mean Haugh unit value. This value differed significantly from the treatments that received 0.5%, 1.0% and 1.5% EM bokashi in the layer diet. Although higher, this value did not differ significantly from the antibiotic treatment, the control receiving no additives and the treatment that received EM only in the drinking water. The treatment receiving 1.5% EM in the diet had the lowest mean Haugh unit value after incubation.

The difference in Haugh units is also shown in Table 4.5.6. The two treatments that received EM in the drinking water had the lowest difference. The treatment receiving EM in the drinking water and the layer diet had the lowest mean difference. This

difference was highly significant and differed from all the treatments except from the treatment receiving EM only in the drinking water. The treatment receiving only EM in the drinking water had the second lowest difference. This differed significantly from the treatments receiving 1.0% and 1.5% EM in the diet but not from the 0.5% EM level, the antibiotic treatment, the control and the treatment receiving EM in both the layer diet and the drinking water.

Table 4.5.1. Mean percentage Phosphorus in eggshell at Week 32

Treatment		Phosphorus LSmeans (%)	SEM
1	EM 1.5% (feed) + 0 EM (water)	0.1023[a]	0.0020
2	EM 1.5% (feed) + 1 : 1000 EM (water)	0.1072[b]	0.0022
3	Control (feed) + 0 EM (water)	0.1040[c]	0.0020
4	Control (feed) + 1 : 1000 EM (water)	0.1054[d]	0.0022
Type III P-value		0.4354	

* Means with the same superscripts are significantly different (P < 0.05) according to Fisher's t-test.

Table 4.5.2. Mean percentage Calcium in eggshell at Week 32

Treatment		Calcium LSmeans (%)	SEM
1	1.5% EM (feed) + 0 EM (water)	34.4201[a]	1.4944
2	1.5% EM (feed) + 1 : 1000 EM (water)	33.9059[b]	1.6371
3	Control (feed) + 0 EM (water)	31.8114[c]	1.4944
4	Control (feed) + 1 : 1000 EM (water)	34.7266[d]	1.6371
Type III P-value		0.3825	

* Means with the same superscripts are significantly different (P < 0.05) according to Fisher's t-test.

Table 4.5.3. Mean Calcium to Phosphorus ratio in eggshell at Week 32

Treatment		Calcium : Phosphorus LSmeans (%)	SEM
1	1.5% EM (feed) + 0 EM (water)	346.508[a]	15.779
2	1.5% EM (feed) + 1 : 1000 EM (water)	316.380[b]	17.285
3	Control (feed) + 0 EM (water)	307.696[c]	15.779
4	Control (feed) + 1 : 1000 EM (water)	329.949[d]	17.285
Type III P-value		0.3634	

* Means with the same superscripts are significantly different (P < 0.05) according to Fisher's t-test.

Table 4.5.4. Mean breaking strength of eggs at Week 32

Treatment		Breaking Strength LSmeans (-N)	Egg Mass LSmeans (g)
1	EM 1.5% (feed) + 0 EM (water)	31.967[a]	56.308[a]
2	EM 1.5% (feed) + 1 : 1000 EM (water)	34.045[b]	54.869[b]
3	Control (feed) + 0 EM (water)	33.453[c]	55.327[c]
4	Control (feed) + 1 : 1000 EM (water)	31.168[d]	55.558[d]
Type III P-value		0.1284	0.2140

* Means with the same superscripts are significantly different (P < 0.05) according to Fisher's t-test.

Table 4.5.5. Haugh units and shell thickness of eggs at Week 31

Treatment		Egg Mass LSmeans (g)	Haugh Unit LSmeans (HU)	Shell Thickness LSmeans (mm)
1	EM 1.5% (feed) + 0 EM (water)	54.203^{a}	94.573^{a}	0.318^{a}
2	EM 1.5% (feed) + 1 : 1000 EM (water)	55.876^{b}	95.664^{b}	0.325^{b}
3	Control (feed) + 0 EM (water)	54.198^{c}	94.877^{c}	0.322^{c}
4	Control (feed) + 1 : 1000 EM (water)	54.780^{d}	95.916^{d}	0.302^{abc}
Type III P-value		0.0371	0.8597	0.0021

Means with the same superscripts are significantly different ($P < 0.05$) according to Fisher's t-test.

Table 4.5.6. Haugh units of eggs (Week 35) before and after incubation at 37°C for seven days

Treatment	Haugh Units (Before incubation)	Haugh Units (After incubation)	Haugh Units (Difference)
EM 0.5% (feed)	100.446^{a}	70.096^{a}	30.350^{a}
EM 1.0% (feed)	102.300^{e}	69.621^{b}	32.679^{bf}
EM1.5% (feed)	103.442^{abf}	69.613^{c}	33.829^{cg}
Antibiotic (feed)	102.900^{cg}	72.746	30.154^{d}
Control (feed) + 1 : 1000 EM (water)	99.445^{efgh}	72.710	26.735^{fg}
EM 1.5% (feed) + 1 : 1000 EM (water)	99.515^{bcd}	74.745^{abc}	24. 770^{abcde}
Control (feed)	102.754^{dh}	72.363	30.392^{e}
Type III P-value	0.0520	0.1881	0.0150

* Means with the same superscripts are significantly different ($P < 0.05$) according to Fisher's t-test.

Table 4.5.7. The incidence of bloodspots and meat spots at Week 31

Treatment		Bloodspots			Meat spots		
		Amount	Expected Frequency	Cell chi-square	Amount	Expected Frequency	Cell chi-square
1	EM1.5% (feed) + 0 EM (water)	4	4.957	0.185	4	5.385	0.356
2	EM1.5% (feed) + 1 : 1000 EM (water)	3	4.131	0.310	7	4.487	1.407
3	Control (feed) + 0 EM (water)	10	9.915	0.000	10	10.769	0.055
4	Control (feed) + 1 : 1000 EM (water)	4	4.131	0.004	9	4.487	4.539
Likelihood ratio chi-square		0.552			0.009		

Table 4.5.8. Yolk color at Week 31

Treatment		Roche value (4 – 5)			Roche value (6 – 7)		
		Amount	Expected Frequency	Cell chi-square	Amount	Expected Frequency	Cell chi-square
1	EM1.5% (feed) + 0 EM (water)	22	16.068	2.190	8	13.392	2.526
2	EM1.5% (feed) + 1 : 1000 EM (water)	11	13.390	0.427	14	11.610	0.492
3	Control (feed) + 0 EM (water)	26	32.137	1.172	34	27.863	1.352
4	Control (feed) + 1 : 1000 EM (water)	14	13.390	0.028	11	11.610	0.032
Likelihood ratio chi-square		0.001			0.001		

4.6. Discussion

Experiment 1

Whether EM was supplied in the drinking water or in the diet or in both, no significant effect was exerted on the phosphorus and calcium content and the calcium to phosphorus ratio of eggshell. Although not significant, the treatment receiving EM in both the diet and the drinking water had the highest phosphorus content in eggshell suggesting that this form of supplementation may have exerted a small effect on phosphorus availability.

Regardless of EM supplementation method, egg breaking strength was not significantly influenced. This is in agreement with Day *et al.* (1987) who measured egg breaking strength as an indication of the influence of different phosphorus levels and the addition of a live yeast culture (0.25% and 0.50%) to the diet of laying hens on egg shell quality. Egg breaking strength was not influenced by yeast addition to the diet indicating a non-beneficial effect of adding a live yeast culture to the diet of laying hens. Day *et al.* (1987) speculated that this might be attributed to either low yeast levels in the diet or an adequate total phosphorus level (0.40%) in the diet or to both. In the present study the total phosphorus contents in all the diets were adequate, as the levels were all well above 0.80% (Chapter 3) and thus supports the claim made by Day *et al.* (1987) regarding an adequate total phosphorus level. Junqueira *et al.* (1984) found that eggshell quality declines when the total phosphorus content of the diet are 0.60% and above. In the presence of high levels of phosphorus, precipitation of calcium carbonate is inhibited. Instead of calcium carbonate formation, calcium will react with the phosphorus ions to produce insoluble, non-adsorbent calcium phosphate that inhibits eggshell formation (Junqueira *et al.*, 1984).

In this study, with the high total phosphorus levels (>0.80%) in the diets, it was expected that any additional supply of phosphorus due to the *in vivo* hydrolyses of phytate phosphorus may adversely affect egg breaking strength due to an oversupply of phosphorus. No significant differences were however detected between the breaking strength of eggs of treatments receiving EM supplementation in the drinking water, in the diet or in both. The treatment receiving EM in both the feed and the drinking water had

the highest egg breaking strength. This higher breaking strength was furthermore reflected in the shell thickness that was thicker than all the treatments. Although both these observations were not significant it may be taken as proof that additional hydrolysis of organic phosphorus did not take place due to EM supplementation in any form, whether it was in the feed, the drinking water or both.

The treatment receiving EM supplementation in the drinking water alone produced eggs with significantly thinner shells than the rest of the treatments. Egg weights did not differ significantly between all the treatments therefore differences in eggshell thickness could not be attributed to egg size solely. The production of eggs with thinner shells by the hens receiving EM in the drinking water alone did not reflect in the breaking strength of the eggs. Although this treatment had the lowest breaking strength, it did not differ significantly from the rest of the treatments.

Supplying EM in both the feed and the water resulted in a marginal improvement in eggshell thickness. This is in accordance to Mohan *et al.* (1995) who found a marginal improvement in shell thickness when supplying a multiple strain probiotic to layers in the diet. Mohan *et al.* (1995) attributed this marginal beneficial effect to the creation of a favorable environment in the intestinal tract by the probiotic that may have helped to assimilate more calcium and hence increase mineral deposition to give thicker shells. Robinson (1977 as cited by Haddadin *et al.*, 1996) also suggested that lactic acid secreted by lactobacilli may encourage calcium and phosphorus absorption. All the treatments receiving EM supplementation, regardless of method, had higher calcium contents in the eggshell than the control and although not significant, this tended to support the claims made by Mohan *et al.* (1995) and Robinson (1977 as cited by Haddadin *et al.*, 1996). However, before any definite conclusion can be made, it should be known that analyses of calcium levels in the bone and egg content was not done to verify this speculation.

EM supplementation in the feed or the water or both did not have any significant effect on internal egg quality. This is in contrast to Nahashon *et al.* (1994a) who found a

significant improvement in egg quality when 2200 ppm *Lactobacillus* was added to the diet of laying hens.

EM supplementation in the diet or drinking water or both had no effect on the incidence of bloodspots. This is in agreement to Nahashon *et al.* (1994a) who found that the incidence of bloodspots was not affected by supplementation with *Lactobacillus* in the diet. Regardless of diet, treatments receiving EM in the drinking water had a higher incidence of meat spots in the albumin. These results should be apprehended with great care as both these parameters were subjectively appraised in a small amount of eggs.

Lighter yolk colours in eggs from hens receiving EM in the diet alone are in contrast to Miles *et al.* (1981a). Results from that study indicated that yolk colour was not affected in hens housed at three different geographical locations when the diets were supplemented with three levels of a viable *Lactobacillus* culture. EM supplementation in the drinking water, regardless of diet, had no significant effect on yolk colour. These results should be apprehended with great care as yolk colour was subjectively appraised in a relatively small amount of eggs.

Experiment 2

The low Haugh units of the eggs of the treatments receiving EM in the drinking water (regardless of diet) in this experiment can be attributed to the smaller replicates of these treatments (only five hens) and, because eggs were allowed to accumulate over 2 days, there was a higher probability that older eggs could be collected. The high mean Haugh unit value before incubation of the treatment receiving EM in the diet is in agreement with Nahashon *et al.* (1994a) who found an improvement in egg quality when supplying *Lactobacillus* in the diet of laying hens. According to Nahashon *et al.* (1994a) this improvement in egg quality may have been partly associated with higher nitrogen retention in pullets fed diets containing 2200 ppm *Lactobacillus*.

After incubation at 37°C for seven days the treatment that received EM in both the layer diet and the drinking water had the highest mean Haugh unit value. The two treatments

that received EM in the drinking water, regardless of diet, had the lowest difference in Haugh units before and after incubation. This was taken as an indication that egg quality in these treatments deteriorated significantly less over time. The difference in Haugh units before and after incubation of the treatment receiving EM in both the diet and drinking water differed significantly from all the treatments except from the other treatment that received EM in the drinking water alone.

It was expected in this study that if the viability of a probiotic culture can be maintained by inclusion in the drinking water, more beneficial effects might be seen than with inclusion in the diet alone. In the second experiment this seemed to have been the case because the hypothesis that EM supplementation in the drinking water of laying hens did improve the deterioration quality of eggs could not be rejected with certainty. It seemed that the eggs of treatments receiving EM in the drinking water (regardless of the diet) had a better quality albumin that was not as prone to degradation as the rest of the treatments. Supplementation of EM in the drinking water in combination with a layer diet containing 1.5% EM bokashi gave the best result. Considering that the drinking water treatments had smaller replicates and therefore a greater chance for older eggs to be collected, this difference in Haugh units may even have been smaller had all the eggs been from the same age (between treatments). This may have wide ranging implications for the egg industry regarding the marketing of eggs and consumer satisfaction. Additional studies on this concept are warranted.

In the first experiment, however, the hypothesis that method of EM supplementation exerts a beneficial effect on most of the parameters (calcium and phosphorus content of eggshell, the ratio of these elements in eggshell, breaking strength of eggs, internal egg quality and the incidence of bloodspots) could be rejected with certainty. EM supplementation in the drinking water alone detrimentally affected eggshell thickness and increased the incidence of meat spots regardless of diet whilst EM supplementation in the diet alone produced eggs with lighter egg yolks. Inclusion in both the diet and drinking water exerted some beneficial effect on shell thickness and breaking strength and although this was not significant, the significant improvement in the deterioration quality

of eggs suggests that a combination of EM supplementation could be the best option rather than supplementation in the drinking water or the diet alone.

4.7. Conclusion

In the first experiment of this study, all three hypotheses could be rejected with certainty ($P>0.05$). In the second experiment the hypothesis that EM supplementation in the food or water had a beneficial effect on the deterioration of internal egg quality (as an indication of shelf life) over a fixed period of time could be accepted with 95% certainty ($P<0.05$).

Critical Evaluation

The Study

- The mean feed intake per hen and the mean feed conversion ratio during the laying phase could not be measured because the facilities were unsuitable for the measurement of feed intake. It is a pity that these two important parameters were lost in the second phase of the study (the laying phase).

- Various researchers (Haddadin *et al.,* 1996; Mohan *et al.,* 1995) found a reduction in the serum and egg yolk cholesterol content when feeding probiotic products, especially *Lactobacillus* cultures. They ascribed it to the possible involvement of probiotic organisms in the assimilation of cholesterol. In the EM study a lack of funding and the time limit set by the promoter caused the loss of valuable information regarding the cholesterol content of the eggs – especially where EM was included in the drinking water where it was expected that the viability was even better than with inclusion in the diet alone. The fact that the deterioration quality of eggs was improved with EM supplementation in the drinking water is proof that internal egg quality can be improved and the chances are therefore great that other factors of internal egg quality (such as the cholesterol content) can also be improved.

- Statistical objections made it impossible to test this type of product under commercial circumstances. It is unfortunately so that the most significant results of probiotic supplementation are expected under more commercial circumstances where birds are densely housed and subjected to disease challenges and other stress factors. In this study the birds were never challenged with stress factors associated with commercial conditions because of prescribed experimental conditions.

- It was very difficult to prepare the EM bokashi and to include it in the desired levels in the diet. Because it is a live product, the levels included in the freshly mixed feed and the levels eventually reaching the bird (after some time of storing) could have differed as the microorganisms could have proliferated or died. Where possible,

storage conditions were standardized but because of the complexity of the product, variation could have existed between different batches of feed and this could have overshadowed any significant results.

- No information was available regarding the specie specificity of the various microorganism species included in the EM mixture. As a consequence it could not be established if the bacteria attaches itself to the gut wall or not. Results obtained could therefore not be ascribed to specie specific bacteria. To counteract for this uncertainty of adhesion or not, a continuous dosing regime had to be followed.

- A bigger study could have been done to test the effect of EM supplementation in the drinking water on egg quality – especially albumin quality. In a bigger study, egg production and egg weights could also have been included in the parameters to be measured.

- Goodling *et al.* (1987), Cerniglia *et al.* (1983) and Grimes *et al.* (1997) all found significant increases in the percentage extra large eggs laid with increasing levels of probiotic supplementation in the diet of laying hens. Eggs were not graded in the laying phase of the EM study and if this had been done, some significant results could have been obtained.

- Nahashon *et al.* (1994) found a significant increase in calcium and phosphorus retention in layers supplemented with a *Lactobacillus* product. As the contents of these minerals were measured in the eggshell in the EM study, an additional phase could have been implemented that could have measured the retention of these minerals by means of a digestibility study and analyzing the bone mineral content of sacrificed birds. It could also have been of some value to measure protein digestibility during a digestibility study because Grimes *et al.* (1997) found a significant increase in protein digestibility with probiotic supplementation in laying hens.

- Due to the complexity of the possible mode of action of probiotics, no specific mechanism of how EM actually works could be established. Where significant results were obtained, there could only be speculated on possible modes of action. In future more emphasis could be put on what actually happens in the gut on a microbiological and biochemical level. Other departments in the faculty could also be involved in this.

- In this study a continuous dosage regime was followed whereby EM is included in the diet and fed to the laying hens on a continuous basis. In future, if the mechanisms of attachment of various microorganism species can be established, a single dosage regime may be considered.

- The storage conditions of the EM diets were standardized in accordance with the regulations of the EM suppliers. In future more emphasis could be placed on how probiotic diets should be stored in terms of storage temperature, light intensity etc. For instance, if it is important to maintain the viability of the probiotic over a long time and the product is composed of mainly organisms found naturally in the gut perhaps the product should be stored in similar conditions it will experience in the gut of an animal (i. e. temperature, redoxpotential, amount of light, osmolarity, pH). Gut differences between animal species differences may even be quantified and taken into account in future.

General

- When a MSc study is undertaken, the head of the department should give his written approval of the proposed protocol before any animals and materials for the study are purchased. The promoter should know that when he accepts students he must be aware that he commits himself to both the study and the students for the entire period of time that is allowed for the study to be completed as stipulated by the department. It is unacceptable that a promoter is allowed to leave the department while a specific study is not yet completed. The fact that the promoter of this study (Dr. G. A. Smith) left the university while the study was not yet completed was detrimental to both the

study and the student because it created a lot of communication problems with the new promoter and this led to valuable time being lost.

- Problems encountered during the experimental phase of the study:

1.) Funds were not sufficient to support the study, and some valuable parameters in the proposed protocol (i. e. the cholesterol content of egg yolk) could not be measured.

2.) During the whole of the experimental period, problems were encountered with the labour. The student and a fellow student repaired all the equipment used in the study and also recorded all the parameters (i. e. weighing of birds and feeders).

3.) Lack of facilities prevented the preparation of experimental diets on a daily basis to ensure freshness of the enclosed product.

4.) The laboratory and accompanying equipment were not always accessible for experimental analysis.

5.) The statistical analysis was prolonged due to the fact that the statisticians had to many clients to cope with.

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