

**The effect of yeast cell wall preparations  
on salmonella colonisation,  
gastrointestinal health and performance  
of broiler chickens**

**by**

**Mieke Brümmer**

**Submitted in partial fulfillment of the  
requirements for the degree M.Sc. (Agric) in the  
Faculty of the Natural & Agricultural Science  
University of Pretoria  
January 2007**

I, Mieke Brümmer, declare that this dissertation for the degree M.Sc. (Agric) at the University of Pretoria has not been submitted by me for a degree at any other university.

## Acknowledgements:

Appreciation is extended to the following persons for their contributions to this study:

- Dr Christine Jansen van Rensburg, my promoter, for countless hours of dedication
- Dr Colm Moran for his inspirational guidance as my supervisor at Alltech
- Alltech: Dr T. Pearse Lyons, Mrs Deirdre Lyons, Dr Karl Dawson and Dr Ronan Power for making the internship available and allowing me to conduct the research at the Alltech North American Biosciences Center, Kentucky, USA
- The Alltech laboratory technicians, especially Ursula Thielen, for their assistance and help in the completion of this research

A special thank you to Vaughn, Pablo, my parents and Wichard for all the moral support and understanding

# Table of contents

<b>Abstract.....</b>	<b>i</b>
<b>Frequently used abbreviations.....</b>	<b>iii</b>
<b>List of Tables.....</b>	<b>iv</b>
<b>List of Figures.....</b>	<b>vii</b>

**Chapter 1:** Literature review – The application of mannan oligosaccharides in animal health and nutrition

1.1) Introduction.....	1
1.2) What are mannan oligosaccharides?.....	2
1.3) How does mannan oligosaccharides function?.....	2
1.4) The use of MOS as a replacement for antibiotics.....	6
1.5) The effect of MOS on chickens.....	9
1.6) The effect of MOS on other animal species.....	15
1.7) Conclusion.....	21
1.8) Motivation for conducting this study.....	22

**Chapter 2:** The effect of feeding Bio-Mos, mannose or a soluble mannan preparation on the colonization of *Salmonella* Typhimurium in broiler chickens

2.1) Materials and methods.....	25
2.1.1) Chickens.....	25
2.1.2) Standard inoculums.....	25
2.1.3) Bacteria.....	25
2.1.4) Experimental design.....	26
2.1.5) Husbandry.....	27
2.1.6) Sampling and sample analysis.....	27
2.1.7) Procedures for autoclaved shavings.....	29

2.1.8) Procedures for autoclaved feed.....	29
2.1.9) Procedures for fresh caecal culture preparation.....	29
2.1.10) Treatments used in the different trials.....	29
2.1.11) Data analysis.....	31
2.2) Results.....	32
2.2.1) Trial 1; 05-006.....	32
2.2.2) Trial 2; 05-013.....	34
2.2.3) Trial 3; 05-019.....	36
2.2.4) Trial 4; 05-025.....	37
2.2.5) Trial 5; 05-031.....	39
2.2.6) Trial 6; 05-033.....	41
2.2.7) Trial 7; 05-044.....	43
2.2.8) Trial 8; 05-048.....	45
2.3) Discussion.....	49
2.4) Conclusion.....	53

**Chapter 3:** The effect of Bio-Mos, with or without the addition of a soluble mannan preparation, on the performance and gastrointestinal health of broiler chickens

3.1) Materials and methods.....	54
3.1.1.) General experimental procedures.....	54
3.1.1.1) Experimental design .....	54
3.1.1.2) Chickens .....	54
3.1.1.3) Husbandry .....	55
3.1.1.4) Treatments .....	55
3.1.1.5) Sampling and sample analysis.....	55
3.1.1.6) Response variables.....	56
3.1.1.7) Data analysis.....	58

3.1.2) Histology.....	59
3.1.2.1) Processing of fresh tissue samples.....	59
3.1.2.2) Alcian-Blue/ Periodic Acid Schiff's staining technique.....	59
3.2) Results.....	62
3.2.1) Feed conversion ratios.....	62
3.2.2) Histology.....	64
3.2.2.1) Villus height, villus width, crypt depth and muscle thickness.....	64
3.2.2.2) Goblet cell measurements.....	65
3.2.3) VFA analysis.....	66
3.2.4) VRBA Plates.....	68
3.3) Discussion.....	70
3.4) Conclusion.....	74
<b>Chapter 4:</b> Conclusion.....	75
<b>Chapter 5:</b> References.....	76
<b>Appendix:</b> a) Preparation of Microbial media.....	88
b) Preparation of staining media.....	90

## Abstract

The main aim of the studies was to evaluate the modes of actions of Bio-Mos and the effect that it has on intestinal health as well as performance in broiler chickens. For the purpose of this study there were 2 main objectives. The first was to determine the effect of Bio-Mos as well as soluble mannan on salmonella colonization and to do this it was necessary to develop an *in vivo* pathogen challenge model, specifically designed for salmonella, using the chicken as animal model. The aim with this salmonella assay was to design a model that could accurately determine the efficacy of different components of the yeast cell wall at reducing or eliminating salmonella colonisation in chickens. The second objective was to evaluate the effect of Bio-Mos with or without the addition of a soluble mannan, fed at different inclusion levels, on chicken health. Specific parameters measured included feed conversion ratios (FCR), volatile fatty acid (VFA) analysis, antibiotic resistance amongst coliform populations, immunoglobulin quantification and gut morphology. Gut morphology measurements included villi height and width, crypt depth, muscularis thickness, goblet cell size and goblet cell density.

The salmonella assay trial was not able to yield positive results for either the cell wall preparations or the positive control, indicating that there are some external factors that have to be addressed before this assay can be used to draw any accurate conclusions from. The second section of this study did show FCR differences between some of the treatments, but did not show numerically large differences for VFA production or antibiotic resistance, however the histological evaluation did yield interesting results. Measurements based on the villi height and width, crypt depth and muscularis thickness showed no significant differences between treatments but there was a treatment effect on the goblet cells. The goblet cells of chickens receiving cell wall preparations were statistically significantly larger and present at a higher density than those of the control treatment birds.

In an attempt to develop the salmonella assay several aspects of the existing assay model were altered or eliminated. It is possible that the assay can work with some more

adjustments, but due to time constrictions it was not possible to further explore alternative approaches. Little research has been done on the effect of nutrition on the goblet cells in chicken intestines. The results noted in this report warrant a more in-depth investigation into the exact modes of action resulting in the differences in goblet cells observed. The use of cell wall preparations on a commercial level holds many advantages, as cell wall preparations appear to affect animal health in a positive way.



## Frequently used abbreviations

MOS: Mannan oligosaccharide

MRF: Mannan rich fraction

H & E stain: Hematoxylin and Eosin stain

AB/PAS: Alcian Blue and Periodic Acid Schiff's stain

FCR: Feed conversion ratio

VFA: Volatile fatty acid

CFU: Colony forming unit

CE: Competitive exclusion

FCC: Fresh caecal culture

## List of tables

<b>Table 2.1.</b> Raw material composition of the basal starter diet.....	<b>26</b>
<b>Table 2.2.</b> Raw material composition of the mannose basal diet balanced for the mannose treatment group.....	<b>27</b>
<b>Table 2.3.</b> Specific alterations made to the standard method between different trials.....	<b>29</b>
<b>Table 2.4.</b> The different treatments applied for the various trials.....	<b>30</b>
<b>Table 2.2.1.</b> Log <sub>10</sub> CFU/g wet caecal contents <sup>1</sup> and number of birds infected per pen (n=10) comparing mannose against methyl manno-pyranoside in their efficacy at reducing salmonella colonisation in the caeca.....	<b>32</b>
<b>Table 2.2.2.</b> Log <sub>10</sub> CFU/g wet caecal contents <sup>1</sup> and number of birds infected with <i>Salmonella typhimurium</i> per pen (n=10) with mannose as positive control and Bio-Mos as third treatment.....	<b>34</b>
<b>Table 2.2.3.</b> Log <sub>10</sub> CFU/g wet caecal contents <sup>1</sup> and number of birds infected with <i>Salmonella typhimurium</i> per pen (n=10) for broilers receiving diets containing no additive, mannose or Bio-Mos.....	<b>35</b>
<b>Table 2.2.4.</b> Log <sub>10</sub> CFU/g wet caecal contents <sup>1</sup> and number of birds infected with <i>Salmonella typhimurium</i> per pen (n=10), with mannose as positive control treatment, a negative control with no additive, and Bio-Mos as the third treatment.....	<b>37</b>

**Table 2.2.5.** Log<sub>10</sub> CFU/g wet caecal contents<sup>1</sup> and number of birds infected with *Salmonella typhimurium* per pen (n=10) indicating the efficacy of mannose as positive control and a soluble mannan (mannan rich fraction /MRF) in the reduction of salmonella colonisation in chicken caeca..... **38**

**Table 2.2.6.** Log<sub>10</sub> CFU/g wet caecal contents<sup>1</sup> and number of birds infected with *Salmonella typhimurium* per pen (n=10) with a negative control with no additives, mannose as positive control and a soluble mannan (mannan rich fraction /MRF)...**40**

**Table 2.2.7.** Log<sub>10</sub> CFU/g wet caecal contents<sup>1</sup> and number of birds infected with *Salmonella typhimurium* per pen (n=10) testing the efficacy of Bio-Mos against salmonella colonisation in the caeca, with a negative control treatment with no additives, mannose as positive control and Bio-Mos..... **41**

**Table 2.2.8.** Log<sub>10</sub> CFU/g wet caecal contents<sup>1</sup> and number of birds infected with *Salmonella typhimurium* per pen (n=10). A negative control with no additives, a positive control as mannose and Bio-Mos were used as the treatments..... **43**

**Table 2.2.10.** Summary of the Bio-Mos and MRF trials presented as log<sub>10</sub> CFU/g wet caecal contents and the colonisation percentages for the specific treatments in brackets..... **46**

**Table 3.1.** Raw material composition of the basal starter diet..... **54**

**Table 3.2.1.** Feed conversion ratio values obtained by the broilers for the respective treatments, as well as the statistical analysis results..... **61**

**Table 3.2.2.1.** Summary of villi measurements (µm), including villi height (VH), villi width (VW), crypt depth (CD), muscularis thickness (MT) and villi height to crypt depth ratio (VH: CD)..... **62**

**Table 3.2.2.2.** Measurements representing goblet cell (GC) size ( $\mu\text{m}^2$ ) as well as goblet cell (GC) density (number goblet cells per  $100\mu\text{m}^2$ )..... **64**

**Table 3.2.3.1.** VFA (mM) results measured from the caeca contents of birds for each of the treatments..... **64**

**Table 3.2.4.1.** Antibiotic resistance measured as the number of *coliform* colonies that grow on a Violet Red Bile Agar (VRBA) plate and the number of colonies that survives when transferred to a tetracycline containing VRBA plate (30 mg/kg)..... **67**

## List of Figures

<b>Figure 2.2.1.</b> Colonisation percentages <sup>1</sup> comparing the efficacy of mannose against that of methyl manno-pyranoside to determine which can be used as positive control.....	<b>33</b>
<b>Figure 3.2.2.</b> Colonisation percentages <sup>1</sup> comparing Bio-Mos against the positive control, mannose.....	<b>34</b>
<b>Figure 2.2.3.</b> Colonisation percentages <sup>1</sup> comparing the percentage of birds colonized by Salmonella typhimurium for the Bio-Mos treatment group against that of the mannose treatment and control group.....	<b>36</b>
<b>Figure 2.2.4.</b> Colonisation percentages <sup>1</sup> showing the similar salmonella colonisation observed in the for each of the 3 treatment groups.....	<b>37</b>
<b>Figure 2.2.5.</b> Illustration of the colonisation percentages <sup>1</sup> showing the soluble mannan product, MRF, as a more efficient product than the positive control.....	<b>39</b>
<b>Figure 2.2.6.</b> Colonisation percentages <sup>1</sup> indicating the difference between the colonisation percentages for mannose vs. MRF.....	<b>40</b>
<b>Figure 2.2.7.</b> Colonisation percentages <sup>1</sup> indicating no differences between the various treatments.....	<b>42</b>
<b>Figure 2.2.8.</b> Colonisation percentages <sup>1</sup> for mannose vs Bio-Mos indicating the ability of mannose to reduce colonisation.....	<b>44</b>
<b>Figure 2.2.9.</b> Mannose vs. Bio-Mos trials <sup>1</sup> comparing the variation in colonisation percentage as changes were applied to the standard protocol.....	<b>45</b>

**Figure 3.2.1.** Effect of dietary inclusion of Bio-Mos and mannan rich fraction, alone or in combination, on the FCR<sup>1</sup> of broilers at 14 days of age..... **60**

**Figure 3.2.2.1.** Pictures taken at 400 X magnifications illustrating the differences observed between treatments for both goblet cell size and density..... **63**

**Figure 3.2.3.1.** Effect of dietary inclusion of Bio-Mos and mannan rich fraction, alone or in combination, on volatile fatty acids composition in the caeca contents of broilers..... **65**

**Figure 3.2.4.1.** Antibiotic resistant populations as measured across treatments in comparison to total amount of coliforms present..... **66**

# Chapter 1

## Literature review: The application of mannan oligosaccharides in animal health, intestinal development and nutrition

### 1.1. Introduction

Many different oligosaccharides exist and the effect that they have on animal health and performance, when added to animal diets, depends greatly on their chemical structure (Iji & Tivey, 1998). Non-digestible oligosaccharides such as mannan oligosaccharides (MOS) and fructo oligosaccharides withstand hydrolysis by enzymes in the gastrointestinal tract (O'Carra, 1998). Mannan oligosaccharides' mode of action differs from other oligosaccharides (e.g. transgalacto oligosaccharides and fructo oligosaccharides) as it functions from within the gastrointestinal tract in an indirect manner as opposed to changing the natural intestinal microflora directly (Flickinger, 2003).

Mannan oligosaccharides possess several positive characteristics, which make it more attractive as an animal feed additive. Tests conducted indicate that heat treatment does not influence the ability of MOS to function in its normal manner, making it possible to include MOS in pelleted feeds (Shane, 2001, Hooge, 2004a). Spring *et al.* (2000) also stated that when used as an adsorbent of enteropathogens such as salmonella, it could be more feasible to use MOS, than for example mannose, which have been shown to have salmonella adsorbing qualities (Oyofa *et al.* 1989a). MOS can be used effectively in much smaller quantities and as Van Immerseel *et al.* (2002) pointed out, it is present as a naturally occurring oligosaccharide in the yeast cell wall.

## 1.2. What are mannan oligosaccharides?

Mannan oligosaccharides are complex sugars (Newman, 2005), and are extracted from the cell wall of either *Saccharomyces cerevisiae* or *Saccharomyces boulardii* yeasts (Hofacre *et al.* 2003).

The yeast cell wall consists of 4 main, covalently linked, components of which mannoproteins and  $\beta(1,3)$  glucans, which functions as the backbone of the cell wall, account for the largest portion (Lipke & Ovalle, 1998). The other two yeast cell wall components are chitin, which is important for cell wall insolubility, and  $\beta(1,6)$  glucans, responsible for forming the link between the helix shaped  $\beta(1,3)$  glucan and some of the mannoproteins (Lipke & Ovalle, 1998). The mannoproteins are carried outside the cell wall and consist of 50 - 90% carbohydrates as the mannoproteins support N-linked glycans, which in turn comprise of 50-200 mannose molecules (Moran, 2004).

According to Pettigrew *et al.* (2005) Bio-Mos (Alltech, Inc.) is the “most thoroughly researched of the mannan oligosaccharide products available”. Shane *et al.* (2001) declared “Bio-Mos contain modified cell wall fragments of *Saccharomyces cerevisiae* which are obtained by lysis followed by centrifugation and subsequent washing and spray drying”.

## 1.3. How does mannan oligosaccharides function?

Hooge (2004a) indicated that MOS has three main modes of action, which includes its ability to adsorb enteropathogenic bacteria, to improve gastrointestinal health and finally its ability to modulate the immune system.

Kocher *et al.* (2004a) acknowledged that MOS can influence the utilisation of nutrients in the intestines, and was capable of stimulating specific microbial populations resulting in improved fibre fermentation with a reduction in starch and sugar utilising bacterial populations.



### *Mannan oligosaccharide as an adsorbent*

Research showed that fimbria, expressed on bacterial surfaces, were involved in the occurrence of infection in both the gastrointestinal tract and urinary pathway (Firon *et al.*, 1987). These fimbriae are composed of proteins that vary in length (Kelly, 2004). The majority of fimbriae capable of binding with sugars, and such fimbriae are classified as lectins (Kelly, 2004). Kelly (2004) also stated that these sugar molecules to which fimbria bind are glycoconjugates. In order to invade a host, colonize and cause an infection in the gastrointestinal tract, it is said to be essential for the bacteria to adhere to the mucosa of the intestines (Oyofu *et al.*, 1989a). If bacteria fail to adhere to the epithelial cells of the intestinal lining, they are expelled from the gastrointestinal tract by peristaltic movements as well as the mucus secreted by the intestines (Oyofu *et al.*, 1989b).

The enterocytes in the lining of the gastrointestinal tract are targeted by colonising enteropathogenic bacteria, which are able to attach to specific carbohydrate residues present on the glycoproteins carried on the surface of the host cells, via fimbria (Shane, 2001). The presence of toxins or high levels of ammonia produced by microflora results in an acceleration in the process of enterocyte replacement, leaving less energy and protein available for growth and tissue development (Shane, 2001). Pathogens expressing mannose sensitive fimbria readily bind with the mannose carried on the mannoproteins of yeast cell walls (Moran, 2004). MOS then functions as bait for the enteropathogenic bacteria and prevents these bacteria from reaching the tissues of the intestinal lining (Sharon & Lis, 1993). Of 30 salmonella species and 77 *E.coli* strains tested, 70% of the *E.coli* and 53% of the salmonella strains expressed mannose sensitive Type 1 fimbria and thus have the ability to bind with MOS (Shane, 2001). MOS reportedly also appears to reduce *Clostridium perfringes* in turkeys (Sims *et al.*, 2004).

In all of the articles reviewed, Cotter *et al.* (2002) was one of a few reporting that MOS could also function as an adsorbent of immunosuppressive mycotoxins, while Zaghini *et*

*al.* (2005) noted in trials conducted by them, that MOS was capable of binding both zearalenone and aflatoxin B<sub>1</sub>.

### *Mannan oligosaccharides and gastrointestinal health*

Enteric disease is one of the focal points where poultry production is concerned, not only because of the economic losses incurred as a result of production inefficiency, but also out of concern for consumer health (Patterson & Burkholder, 2003). Enteropathogens are resisted by the body through intestinal microflora, immune response and mucosal epithelium (Patterson & Burkholder, 2003).

The microflora colonising the chicken gut under standard conditions have been identified as a crucial part in the normal digestion, health and well being of the bird (Amit-Romach *et al.*, 2004). Microbial populations were found to affect broiler health and production through effects exerted on the gut environment as well as the manner in which the animal responded to immune system challenges (Oviedo- Rondón *et al.*, 2006).

At the same time it was found that microflora also exert some undesirable effects such as toxin synthesis; high rates of gut epithelium turnover and competing with host for nutrients (Dibner & Richards, 2005). Mucus was found to protect the intestines against microflora, enteropathogenic activity and normal digestive processes in the gut (Smirnov *et al.*, 2006). Mucus secreting cell development reportedly increased in the embryo at day 17 pre-hatch (Smirnov *et al.*, 2006).

A stable microflora took two to three weeks to establish in the chicks intestines (Amit-Romach *et al.*, 2004; Dibner & Richards, 2005). Amit-Romach *et al.* (2004) experimented with the use of new molecular techniques to identify bacterial populations, and found that the chicks' intestines were predominantly colonised by *Lactobacilli* species, while bifidobacteria only became significant as the chick matured.

The establishment, as well as the maintenance, of a healthy gut in poultry appeared to be critical in achieving a stable microflora population (Collett, 2004). Further, the earlier such a stable microflora could be established, the better for gut health (Collett, 2004).

Collett (2004) reported that yeast cell wall products of specific yeast strains, such as Bio-Mos (Alltech Inc.), could be used to sustain the gastrointestinal tract environment as well as to modulate the microflora inhabiting the gut, together with the elimination of enteropathogenic bacteria.

The development of the morphology of the gastrointestinal tract is influenced by the diet of the animal (Santin *et al.*, 2001). Several studies where MOS was added to animal diets indicated improved intestinal morphology (Moran, 2004), such as lengthened villi, which are associated with superior gut health as well as improved nutrient absorption (Sims *et al.* 2004).

#### *Influence on immune modulation*

Flickinger (2003) agreed with Newman (1999) stating that MOS was involved in immune modulation. Newman (1999) further reported that MOS affected both the humoral response by increasing the level of initial cytokine release; and the cell mediated response by increasing the phagocytic ability of the white blood cells. This caused acceleration in the initial reaction time of the immune system during a pathogen challenge (Newman, 1999). MOS also assisted with antigen processing in order to initiate the early stages of the immune response (Moran, 2004).

Shortening the immune response activation time could result in more energy being available for growth and tissue development (Davis *et al.*, 2004). Collett (2004) reported that yeast cell wall had been shown to subdue the fever response observed as part of an immune response. Chicks from broiler breeders where both the breeder hens and roosters were fed MOS had a better innate immunity than control chicks (Shashidhara & Devagowda, 2003).

Franklin *et al.* (2005) stated that cattle have 3 serum collectins namely conglutin, collectin 43 and mannose binding protein. These collectins have the ability to bind with particles that contain mannan (Franklin *et al.* 2005). This facilitates superior phagocytic ability of the innate immune system. As several viruses contain mannan, this could possibly be the reason for viruses being targeted by the innate immune system (Franklin *et al.* 2005). Franklin *et al.* (2005) further hypothesised that the addition of MOS to cattle diets stimulates collectin production, resulting in an improved immune system.

A number of studies conducted showed that MOS had the ability to stimulate elevated antibody levels, especially IgG and IgA levels (Santin, 2001; Shane, 2001). According to Shashidhara & Devagowda (2003), the antigen stimulating effect of the cell wall can be attributed to the mannan chain component of the cell wall. Mannans are known to affect the immune system by elevating IgA levels in rat caeca, IgA (bile) and IgG (systemic) in turkeys as well as fish, and dog neutrophil activity (Swanson *et al.*, 2002). The secretion of IgA is very important as it causes an increase in mucus secretion and prevents bacteria from attaching and penetrating the gastrointestinal tract lumen (Swanson *et al.*, 2002).

Franklin *et al.* (2005) suggested that a less likely, but possible, mode of action of MOS surrounds the theory of natural antimannan-antibody production at gastrointestinal tract level, reportedly observed in cattle. These antibodies could possibly enter the blood system where it might improve response to virus vaccinations and enhanced engulfment of antimannan-antibody bound viruses by macrophages (Franklin *et al.*, 2005).

#### 1.4. The use of MOS as a replacement for antibiotics

The growth promoting effects caused by the addition of antimicrobials to animal diets were recognised in 1946 by researcher Moore (Jones & Ricke, 2003). Although a certain degree of antimicrobial resistance was noticed at the time, it was considered rare and the rapid rate at which antibiotic resistance would increase was not expected (Spring, 1999). It is currently hypothesised that the antibiotics that have been added to animal feed at

subtherapeutical levels, have resulted in human pathogens' resistance to specific antibiotics (Heinrichs *et al.*, 2003).

In 1999 the Animal Health Institute indicated that nearly 20.42 million pounds (approximately 9.89 million kg) of antibiotics were used annually in the USA alone. Its uses included disease treatment in the animal production as well as companion animal industries, growth promotant and coccidiosis control (Jones & Ricke, 2003). In 2000 the World Health Organisation advised national governments to lower antimicrobial use for enhanced production purposes in animals, with immediate exclusion of any antibiotics also used in human medicine (Dibner & Richards, 2005).

Effects of antibiotic growth promoters reported include direct effects on the gut microbes, such as reduced competition for available substrates and fewer quantities of growth inhibiting secondary metabolites, as well as direct effects on the gut, such as thinner gut walls which reportedly enhance nutrient digestibility (Dibner & Richards, 2005). Gastrointestinal tract alterations due to antibiotic additions to chicken diets were believed to be due to a reduced rate of cell proliferation, a reduction in mucosa thickness and absorptive surface area increase (Miles *et al.*, 2006).

Despite the general characteristic of antibiotics to control microbial growth, the mechanism of action between antibiotics differ, which explains the observation that not all antibiotics were equally effective at enhancing growth and feed efficiency in animals (Miles *et al.*, 2006).

Microflora were identified as being crucial for immune system, organ and tissue development, but at the same time microflora competed with the host for nutrients, synthesised toxins and caused inflammatory responses (Dibner & Richards, 2005). Inflammatory response resulted in protein surface shedding of up to 20%, but protein shedding was reportedly reduced by antibiotic additives (Miles *et al.*, 2006). Further, Miles *et al.* (2006) reported that this increase in cell proliferation caused by the

microflora in the gastrointestinal tract allowed for less energy available for production purposes such as growth.

Patterson & Burkholder (2003) stated that microflora played a vital role in the body's defence against pathogens by occupying the binding sites, and thus reducing binding sites available for pathogen binding, and competing with pathogens for nutrients.

The increased awareness and concern voiced by the consumer against antibiotic fed animal products (Patterson and Burkholder, 2003), as well as the drastic increase in the ionophore anticoccidial salinomycin utilisation observed in response to antibiotic restrictions in Denmark, indicated the need for alternatives to antibiotics in the animal production industry (Dibner & Richards, 2005). Sun *et al.* (2005) showed that the performance of broiler trials without the addition of antibiotics to the feed were poor, emphasising the need for non- antibiotic alternatives that can improve the broiler performance without being of a health concern to the consumer.

MOS was originally studied as a substitute for antibiotic growth promoters in calf milk replacers, but the improvement in the health and growth of the calves fed MOS initiated additional research on the effect of MOS in the pig and poultry production industry (Moran, 2004). Further, the rise in antibiotic resistance, as well as the quest to reduce the current status of antibiotic resistance, led to the implementation of restrictions regarding the use of antibiotics in European countries, forcing researchers to look for alternatives (Newman, 2002).

According to Kocher *et al.* (2004a) MOS promotes growth mainly as a result of its ability to lower enteropathogenic Type 1 bacteria colonisation and improved gut health, combined with its effects on nutrient utilisation. Santin *et al.* (2001) also reported that MOS was able to reduce the FCR and increase the rate of body weight gain in experiments conducted on broilers. Shane *et al.* (2001) concluded from several broiler and turkey experiments that MOS can possibly be used to produce poultry meat for the antibiotic free food market. This conclusion is supported by Newman (2002), who stated

that MOS has the ability to replace antibiotic growth promoter use, as well as to reduce morbidity and mortality numbers in poultry production systems. Newman (2002) also observed that the addition of MOS to animal feed rations had been shown to lower the number of specific Gram-negative bacteria, noted to be antibiotic resistant, in pigs.

Interestingly, in studies where both MOS and antibiotics were combined in a single treatment, the results indicated a synergistic or additive effect (Hooge, 2004a). This finding is supported by that of Sims *et al.* (2004) who observed the highest 18 week body weight in turkeys in the treatment group where bacitracin methelene disalicylate and MOS were combined, especially under stress conditions.

### 1.5. The effect of MOS on chickens

#### *The influence of MOS on general production parameters*

MOS significantly improved the 42-day-old weight of broilers, which was statistically comparable to birds receiving antibiotics applied as a growth-promoting additive (Hooge, 2004a). It also improved FCR in a similar manner and proved superior to antibiotic supplements in its ability to reduce mortality percentages (Hooge, 2004a). MOS supplemented diets resulted in a faster weight gain in broilers than that of control broiler groups receiving a basal diet only (Iji & Tivey, 1998). However, these observations were questioned by Waldroup *et al.* (2003), who conducted a study in which they compared antibiotic supplemented diets with diets formulated to contain either MOS or copper. From the results of this experiment Waldroup *et al.* (2003) concluded that MOS was not able to increase the 42-day-old body weight, and that it did not improve feed conversion ratios or lowered mortality rates.

Chickens are known to naturally produce a high level of antibodies against many pathogens in the environment, but when MOS was added to the diet of chickens, an elevated plasma level of antibodies was produced upon immunisation (Cotter *et al.*,

2002). Shane (2001) reported a better FCR as well as a higher final body weight at 42 days for broilers fed MOS. The study also indicated improved crude fibre utilisation increasing from 6.23% in control groups to 12.8% in treatment groups (Shane, 2001). Santin *et al.* (2001) also reported improved gut health as well as improved FCR and body weight gain when including *Saccharomyces cerevisiae* cell wall in the diet of broilers.

A unique observation was that the addition of MOS to a broiler diet increased the amino acid uptake in the ileum in addition to the previously reported increase in ileum villi height (Iji *et al.* 2001). It is hypothesised that FCR improvement effects observed in poultry supplemented with MOS, is a result of not only enhanced nutrient utilisation, but also a nutrient sparing effect (Shashidhara & Devagowda, 2003).

Hooge (2004a) observed that MOS affected fibre digestibility in a positive manner and also lowered the water to feed intake ratio. The lower water intake resulted in superior litter quality, which is an vital factor in the poultry industry (Hooge, 2004a).

#### *The effect of MOS on salmonella in chicken production*

*Salmonella enteritidis* and *Salmonella typhimurium* are considered the two most hazardous salmonella serotypes (Palmu & Camelin, 1997). Huang *et al.* (2006) stated that, worldwide, *Salmonella typhimurium* is one of the most frequently isolated salmonella serovars in cases of human salmonellosis.

Salmonella contamination of poultry products can only be controlled and eliminated through the implementation of strict control programmes, addressing the various components of poultry production (Corrier *et al.*, 1998). Two types of salmonella exist – the invasive and non-invasive type (Van Immerseel *et al.*, 2002). Invasive salmonella can move from the intestines into the other tissues, and should the follicles in the ovary be infected, the developing egg will be infected (Van Immerseel *et al.*, 2002). Infestation of the egg is only a small part of a larger cycle of infection starting at the hen house,



commonly infected as a result of rodent pests (Guard-Petter, 2001). As hens can be infected with salmonella without exhibiting any clinical symptoms, it is possible to produce salmonella contaminated table eggs and thereby putting people at risk of contracting salmonellosis (Guard-Petter, 2001). Doyle & Erickson (2006) found when evaluating salmonella, eggs or egg containing products were the greatest culprits for spreading salmonella organisms, particularly *Salmonella typhimurium*, which in turn could be especially dangerous due to the high number of multi antibiotic resistant strains that exists. Salmonella is still regarded as one of the most dangerous of all human food borne pathogens (Huang *et al.*, 2006).

Salmonellosis has always been linked to contaminated poultry products, but if it was possible to eliminate salmonella contamination prior to the processing plant, carcasses could be salmonella free (Stern *et al.*, 2001). Pre-slaughter feed withdrawal had been reported to considerably increase the level of salmonella contamination in the crop (Chambers *et al.*, 1998; Doyle & Erickson, 2006). Tearing of crops during the processing of chicken carcasses then contributed to the contamination of such carcasses with salmonella (Chambers *et al.*, 1998). Corrier *et al.* (1999) were also able to show with their studies that the crop was one of the main sources of carcass contamination. Observations showed that feed withdrawal prior to slaughter resulted in the consumption of house litter, which contributed to the elevated levels of salmonella found in the crop (Corrier *et al.*, 1999). This also proved problematic when molt was induced in layers through feed withdrawal, although egg dip treatments have been shown effective against salmonella (Doyle & Erickson, 2006). The caeca have long been known as being a target site for salmonella colonisation and the position of the ileum with regards to the caeca explained some of the reports that salmonella moved from the caeca into the ileum upon food withdrawal (Thompson & Applegate, 2006). This increased the odds of carcass contamination in the event of rupturing an ileum during processing (Thompson & Applegate, 2006). Palmu & Camelin (1997) concluded from their experiments that reducing the number of salmonella positive birds that are processed were the key to reducing overall salmonella contamination.

Competitive exclusion (CE) refers to the inoculation of young chicks with adult microflora, in the form of a standard inoculum, and has been used to provide chicks with some protection against salmonella. It is however a very short-lived protection (Hume *et al.*, 1996; Corrier *et al.*, 1998; Hume *et al.*, 1998; Fernandez *et al.*, 2000; Davies & Breslin., 2003). In addition, it takes up to 48 hours before the maximum protection level, provided by the standard inoculum, is reached (Stavric *et al.*, 1987). Also, antimicrobials may render CE treatments ineffective as it affects the microbial population of the CE treatment product (Palmu & Camelin, 1997). Fernandez (2000) found that treating chicks with adult microflora obtained from hens fed MOS, as well as feeding the chicks MOS as part of their diets provided a better protection against salmonella colonisation than competitive exclusion alone. Palmu & Camelin (1997) recorded a statistically significant lower percentage of salmonella positive broilers arriving on the farm when treated with a CE product in the hatchery than in the untreated broilers, even though the treated broilers still had a number of salmonella positive birds. The salmonella positive birds were retested on day 45 and tested negative, while 4 out of the 8 control flocks that tested positive upon arrival remained salmonella positive (Palmu & Camelin, 1997). In cases where hatcheries were greatly contaminated with salmonella, CE was less successfully applied (Baily *et al.*, 1998). Generally, the researchers found that out of 10 000 eggs, only 1 egg was normally contaminated with salmonella, but the ease with which salmonella was capable of spreading between chicks made it difficult to control the spread of salmonella through the hatchery (Baily *et al.*, 1998).

Spring *et al.* (2000) found that the addition of MOS to broiler diets reduced the amount of salmonella colonising the caeca. The caeca are the major site for salmonella colonisation in the chicken (Corrier *et al.*, 1999; Thompson & Applegate, 2006). This discovery followed the research conducted by Oyoyo *et al.* (1989a,b), showing that mannose was able to block the binding of bacteria expressing Type 1 fimbria. When mannose was included in the drinking water of broilers, colonisation of salmonella in the broiler gut was drastically reduced (Oyoyo *et al.*, 1989a).

### *The effect of MOS on gastro intestinal tract development*

Experiments indicated that the degree of development of the small intestine was directly responsible for the extent of nutrient absorption and digestion which in turn determined the growth of the chick as well as whether it could reach its genetic potential (Tako *et al.*, 2004).

The broiler chick's small intestine undergoes maximum growth between the first six and ten days after hatching, independent of the availability of food (Sklan, 2004), yet Smirnov *et al.* (2006) stated that the presence of feed in the small intestine was essential for the development of enteric cells. The enterocytes of the small intestine of the chick develops rapidly, elongating and forming a brush border. At the same time the crypts develop and villi lengthen (Sklan, 2004). The intestinal lining only has to be exposed to a specific dietary factor for a short period of time in order to observe changes in the structure of the mucosa due to this normal rate of cell turnover (Iji *et al.*, 2001).

Previous studies conducted showed that immediate post hatch feeding of chicks elevated the rate at which the small intestine developed, as well as the rate of intestinal crypt development (Tako *et al.*, 2004). Tako *et al.* (2004) found that when feeding late-term embryos *in ovo* with carbohydrates and/or  $\beta$ -hydroxy- $\beta$ -methylbuterate (HMB) it took only 48 hours to observe increased villi width. In addition chicks fed HMB also had a 45% greater intestinal surface area than control birds at 3 days post-hatch. Yet, the standard management of hatcheries and delayed transport of the chicks to farms meant that some chicks would not be fed for 24 – 48 hours post hatching, which in turn could interrupt mucosa development or lead to abnormalities such as clustered microvilli (Uni *et al.*, 1998). Zhang *et al.* (2005) observed greater villi height for birds kept on a yeast cell wall supplemented diet as well as superior ileal mucosa development.

Including MOS in chicken feed rations resulted in increased villi height and reduced crypt depth, which changed positively when increasing amounts of cell wall were included in the feed ration, allowing for greater absorption area and improved nutrient

absorption (Santin *et al.*, 2001). The inclusion of MOS in poultry diets caused a reduction in crypt depth, which is a function of the rate of enterocyte replacement, with deeper crypts usually an indication of a high turnover rate (Shane, 2001). Researchers hypothesised that the more rapid growth observed in their experiment in male broilers were due to the faster development of the gastro intestinal tract (Miles *et al.*, 2006).

#### *The effect of MOS on reproduction and egg characteristics*

Fertility and hatchability experiments conducted on broiler breeder chickens indicated that MOS had the ability to affect production in a number of ways (Devegowda, 2004). Breeder hens aged 60-65 weeks fed MOS produced eggs that proved superior in terms of egg specific gravity as well as hatchability (through a reduction in infertile eggs and dead embryos, and possibly as a result of better egg shell quality) and were reported to induce a greater deposition of essential nutrients in the egg contents (Devegowda, 2004). Devegowda (2004) also found that MOS had a positive effect on the semen quality of breeder males, measured as an increase in spermatozoa density. The amount of spermatozoa reaching the oviduct seems to play a key role in embryonic death in chickens and of course fertility (Devegowda, 2004). MOS fed to broiler breeders housed in cages improved hatchability by reducing the total number of dead-in-shell and infertile eggs (Shashidhara & Devagowda, 2003). Further, a higher sperm density in MOS fed male broilers was observed, although the percentage of live sperm remained unaltered. The higher sperm density was possibly the result of higher antioxidant activity (glutathione peroxidase – GSH-Px – and superoxide desmutase- SOD) in the testes, which are of great importance in production and maturation of spermatozoa (Shashidhara & Devagowda, 2003). An experiment conducted by Zaghini *et al.* (2005) showed that although the egg weight was lower in MOS fed layer hens, the eggshells were heavier, together with higher albumin protein content.

## 1.6. The effect of MOS on other animal species

There is a large body of research supporting the use of MOS in different animal species:

### *Poultry: Turkeys*

Experiments, conducted with turkeys, comparing the effects of MOS with those of antibiotics, showed similar results to that obtained for broilers, indicated by a better FCR and liveability, together with a higher live weight (Shane, 2001). It was found that the body weight of the male birds was mostly improved when MOS was added for a minimum period of the first 8 weeks of age (Shane, 2001). An elevation in the IgG and IgA antibody levels was observed in turkeys fed MOS treated diets (Santin *et al.*, 2001; Cotter *et al.*, 2002) and an improvement in the carcass quality was also noted (Iji & Tivey, 1998).

Dose dependent trials indicated that 0.11% MOS inclusion in turkey diets resulted in maximum weight gain and improved the 8-week body weight significantly. It also lowered the feed conversion ratio (Sims *et al.*, 2004). Even when challenged with *Eschericia coli*, MOS supplemented poult had superior weight gain as well as higher 3-week body weight (Sims *et al.*, 2004).

When compared to control diets, MOS was able to improve body weight significantly while reducing mortalities (Hooge, 2004b). Further, when compared with antibiotic treatments applied as growth promoters, MOS was not able to exceed the effects of the antibiotics, but seeing that the results obtained for the MOS did not differ significantly, MOS could still be recommended as a non-antibiotic growth promoter (Hooge, 2004b).

## *Pigs*

The European ban and limitations on the use of several antibiotics was followed by increased piglet mortalities as well as the increased occurrence of diarrhoea in young piglets (Spring, 1999). In trials conducted, MOS was able to outperform the negative controls as well as the antibiotic treatment groups in terms of both FCR and mortality rates (Spring, 1999).

Over the years, copper had become a known additive in pig diets, added for its performance promoting effects (improved average daily gain, average daily feed intake and gain to feed ratio's), but feeding higher levels of copper increased the level of copper excreted in manure, which in turn contributed to environmental pollution (Davis *et al.*, 2002). Although MOS was not able to affect the performance parameters to the same extent as copper, it was concluded that it could be used as an alternative to copper due to the moderate effects that it showed and the possibility that the mode by which copper and MOS affect growth, are similar (Davis *et al.*, 2002).

Zinc is added to piglet diets during the early weaning phase in an attempt to reduce diarrhoea as well as for growth improvement, but with the EU legislation limiting zinc inclusion in pig diets to 500 mg/kg and the minimum effective inclusion rate at 1000 mg/kg, it is necessary to look for alternatives (Davis *et al.*, 2004). In experiments conducted by Le Mieux *et al.* (2003), the researchers found that MOS can potentially be used as a substitute for the high levels of zinc used in piglet diets, probably because of its immune modulating and enteropathogen adsorbing properties. Le Mieux *et al.* (2003) also stated that an increase in growth rate could be observed in young nursing piglets when the sows received MOS supplementation prior to farrowing or in their lactation diet.

When testing the response of nursery pigs to MOS supplementation, it was determined that piglets appearing less healthy showed a greater response in growth rate to MOS additives than the apparently more healthy piglets (Pettigrew & Miguel, 2003).

In another article, Pettigrew *et al.*, (2005) reported on 6 different experiments conducted, investigating the effects of MOS when included in the pre-farrowing diet and post-farrowing (or lactation) diet. It was concluded that MOS increased birth weight, pre-weaning weight and post-weaning growth. MOS also elevated the levels of immunoglobulins in the sows' colostrums and reduced the period from weaning until the next estrus observed in the sow.

### *Ruminants: Cattle*

In a paper written by Corless (2003), the author described several different fields, in both ruminant nutrition and management, where MOS inclusion in the diet of cattle was experimented with. The first trial showed that the inclusion of MOS in the diet of late pregnant cows elevated the level of immunoglobulins present in their colostrum, and when combined with vaccinations against the rotavirus, increased IgG and IgM serum concentrations in the 24-hour-old calf. This indicated that MOS improved the transfer of vaccine antibodies from cow to calf. This finding was supported by a similar experiment conducted by Franklin *et al.* (2005). These researchers observed that when cows were vaccinated against the rotavirus (four and two weeks prior to parturition) the serum rotavirus neutralisation titres at parturition were higher in cows that received MOS (Franklin *et al.*, 2005). Corless (2003) also reported that when combining MOS with the colostrum fed to calves, the immunoglobulin uptake was improved and the immunoglobulin levels in the calves' serum were increased. The experiments also showed that the use of MOS as an adsorbent of enteropathogenic bacteria resulted in a greater growth rate response and also decreased the recovery period necessary for calves to recover from scours induced by nutritional factors (Corless, 2003). Several trials support the use of MOS as a method to effectively reduce and control salmonella infestations in dairy cattle, but MOS had also been used successfully in beef cattle to control the prevalence of diarrhoea caused by *E. coli* (Corless, 2003). MOS additions to dairy rations

also reduced the incidence of dairy heifers testing positive for the presence of salmonella (Newman, 2002).

An unexplainable observation made by Franklin *et al.* (2005) was that when calves were fed colostrums containing similar levels of IgA, calves born from cows fed MOS diets had lower IgA levels in their serum than the calves born from the control cows. It is possible that MOS affects the absorption of IgA across the intestinal tract (Franklin *et al.*, 2005).

Newman (2002) believed that the respiratory problems associated with the young calf could be solved by feeding MOS because of an increase in macrophage activity, which should assist in the removal of respiratory stressors more efficiently.

In an experiment conducted comparing the difference between antibiotics and MOS in calf milk replacers of neonatal calves, the data indicated that both treatments reduced the prevalence of scours in calves to the same extent (Heinrichs *et al.*, 2003). The calves that received the MOS supplemented diet were, however, able to consume larger quantities of the calf starter grain that was offered on an *ad lib* but monitored basis, and that the rate of increased grain consumption was also higher (Heinrichs *et al.*, 2003).

#### *Equine: Horses*

Information on the effect of MOS on horses proved to be limited, as little research has been conducted in this field. Still, every year, the horse stud farms spend large amounts of money on veterinary bills for the treatment of diarrhoea in young foals (Ott, 2002). Ott (2002) conducted experiments in which it was observed that the inclusion of MOS in the diets of mares, prior to foaling, possibly increased the antibody levels in the colostrum of the mares, which in turn resulted in a smaller number of cases of severe diarrhoea in foals when compared to the control groups.



A more recent experiment (Ott, 2005) conducted by the same author found that mares kept on a diet that included MOS supplementation prior to foaling, had higher IgG and IgA levels in their colostrums, and again the occurrence of diarrhoea in the young foals was reduced.

*Companion animals: Canine*

Grieshop (2003) stated that feeding MOS to canines have several different effects, all of which contribute to an improved health and immune status, including the exclusion of enteropathogenic bacteria, immune stimulation and mycotoxin neutralisation. Increased serum lymphocytes and IgA levels were also observed (Grieshop, 2003). These findings are in agreement with that reported by O'Carra (1998) who observed that feeding dogs MOS improved the feed's digestibility, reduced the prevalence of pathogenic enterobacteria and improved the immune system, as indicated by elevated neutrophil levels in the dogs receiving MOS together with vaccinations, as opposed to vaccinations only (O'Carra, 1998).

Bifidobacteria is a species of bacteria found in the intestines of the dog and is regarded as beneficial to the intestinal microflora. This specific bacterium is only present in small quantities, but research showed that providing dogs with MOS increased the percentage of these bacteria in the intestines (Kotcher & Tucker, 2005). Bifidobacteria are lactate producers, and increased lactate production levels reduce pH, making it impossible for some of the pathogenic bacteria to survive and proliferate in the gastrointestinal tract (Swanson *et al.*, 2002).

Kotcher & Tucker (2005) hypothesised that less healthy dogs or dogs subjected to stress factors might obtain greater benefit from MOS than healthy dogs. Certain effects on the intestinal morphology of the dogs, such as an increased goblet cell density, have been noticed in MOS supplemented dogs (Kotcher & Tucker, 2005).

### *Aquaculture*

Data investigated by Spring (2003), supported the theory that MOS was capable of pathogen exclusion as well as immune modulation in African catfish. This was a very important discovery as the cold-water fish industry is prone to infections caused by the bacteria *Aeromonas hydrophila* (Spring, 2003).

Lysozymes appeared to be an important component of the immune system of fish, as any form of pathogen challenge or environmental stress factor resulted in a subsequent change in lysozyme activity (Staykov *et al.*, 2005). In two trials conducted with rainbow trout (*Salmo gairdneri irideus* G) and a single trial conducted with the common carp (*Cyprinus carpio* L), groups given MOS had a higher mean body weight, a faster growth rate and a lower mortality than the control treatment groups, resulting in a 12% increase in the overall fish production. It also improved the lysozyme levels in the fish by up to 40% (Staykov *et al.*, 2005).

In a separate study, the addition of MOS to the diet of Coho and Atlantic salmon reduced mortalities incurred as a result of salmonide rickettsia syndrome (SRS) and improved the overall weight gain of the salmon. (Spring, 2003)

### *Other: Rabbits*

Commercial rabbit production can be faced with post weaning mortalities of as high as 20% as a result of the presence of enteropathogenic bacteria such as *E. coli* (Kocher *et al.* 2004b). With legislation and public opinion against the use of antibiotics, MOS have been recommended as a possible substitute for antibiotics, with experiments showing that MOS increased body weight gain, and reduced feed conversion ratio as well as lowered mortalities (Kocher *et al.* 2004 b).

Feeding MOS as an additive in the daily feed ration of rabbits improved the villi length in the ileum, increasing the area that absorbs nutrients (Pinheiro *et al.*, 2004). Volatile fatty

acids were measured as a function of microbial activity, and the greater amount of VFAs (associated with a reduction in caeca pH) present in the caeca of MOS-fed rabbits is associated with a higher level of protection against *E. coli* (Pinheiro *et al.* 2004).

#### *Other: Ostriches*

Ostrich farms face mortalities of 30-40% during the initial 3 months of raising chicks, with enteropathogenic bacteria (specifically *E. coli*) as the main cause of these mortalities. (Verwoerd *et al.*, 1998). In a study conducted over a period of 1 year, several South African ostrich farms included MOS in the diets of their ostrich chicks and mortality rates decreased with an average of 5 - 20%, with a 60% improvement observed in one specific case (Verwoerd *et al.*, 1998).

### 1.7. Conclusion

Mannan oligosaccharides are components of the yeast cell wall, forming part of the structure that protects the interior of the cell. Only when isolated from the cell wall and placed in animal feeds are its true properties revealed, as it improves growth parameters beyond that of most other commonly used growth promoters. It influences the immune system to respond quicker during pathogen challenges and at the same time it is capable of improving and maintaining the integrity of the intestinal morphology for enhanced nutrient absorption. Apart from the well-known modes of action, MOS has also been implicated in reproductive performance improvement.

A key characteristic of MOS, that makes it so unique, is its flexibility in use across the different species, irrespective of the digestive tract type. However, the key to the successful application of MOS does not lie in its ability to function in a similar manner between species, but its ability to elicit production responses that are comparable to that

of antibiotic growth promoters, which are currently being phased out in interest of human health.

The future of animal production is based on the ability of the farmer to provide the consumer with guaranteed safe and wholesome products. With the vast amount of research to support its efficacy, mannan oligosaccharides can play a pivotal role in the future of antibiotic free animal production.

### 1.8 Motivation for conducting this study

The addition of antibiotics to poultry diets remains an area of concern, with public opinion against the use of antibiotics in production animal diets. Yet, there is a need to control pathogen levels in poultry houses and poultry products. Oyofa *et al.* (1989a, b, c) conducted several trials, which indicated that mannose (included at 2.5% w/v in drinking water) could be used as a feed additive to reduce salmonella colonisation in broiler chickens. However, the problem is that the use of mannose at the recommended inclusion rate, at a large scale for commercial poultry production, is far too costly and more feasible natural alternatives are needed. Studies undertaken by Spring *et al.* (2000) indicated that feeding mannan oligosaccharides, located in the cell wall of the yeast *Saccharomyces cerevisiae*, had a similar effect as mannose on colonisation of salmonella while the dose required was substantially lower (4kg /tonne inclusion). The aim of the salmonella study conducted was initially to develop a salmonella assay using broiler chicks based on the work done by Spring *et al.* (2000). This assay could then be used to test other yeast cell wall derived preparations for their efficacy against salmonella colonisation in broiler chicks.

Reports on the positive effects of yeast cell wall on the gastrointestinal tract morphology (Santin *et al.*, 2001; Zhang *et al.*, 2005) initiated a study on the effects of Bio-Mos or a yeast cell wall derivative, mannan rich fraction (MRF), on the morphology of the ileum. From the studies already conducted it was expected that Bio-Mos and MRF would increase crypt depth, villi height and width, and muscularis thickness. Goblet cell

measurements were also made as it was hypothesised that the addition of Bio-Mos and/or MRF would affect the goblet cell density and the goblet cell size.

## Chapter 2

### The effect of feeding Bio-Mos, mannose or a soluble mannan preparation on the colonisation of *Salmonella typhimurium* in broiler chickens

The aim of this study was to investigate the effect of Bio-Mos (Alltech Inc. Nicholasville, KY), a mannan rich fraction (MRF) (Alltech Inc. Nicholasville, KY) on the colonisation of *Salmonella typhimurium* in the intestines of broiler chickens. To do this it was necessary to construct a salmonella model that could be used to test the efficacy of various products, against a negative and positive control, at reducing salmonella colonisation. Oyoyo *et al.* (1989c) investigated the use of various carbohydrates such as glucose, lactose, sucrose and mannose to reduce the colonisation of salmonella in broiler chickens. Mannose proved to be the most successful at reducing salmonella colonisation of all the carbohydrates tested. Oyoyo *et al.* (1989a) and Fernandez *et al.* (2000) also found a reduction in salmonella colonisation with the use of mannose in broiler diets. In another study conducted, Oyoyo *et al.* (1989b) reported that methyl- $\alpha$ -D-mannoside (MMS) reduced salmonella colonisation in *in vitro* studies conducted. Based on the results reported by Oyoyo *et al.* (1989a,b,c) it was decided to select a positive control for the salmonella assay from either mannose or methyl- $\alpha$ -D-mannoside. The first salmonella trial was then conducted to compare the efficacy of mannose and MMS at reducing the colonisation of *Salmonella typhimurium* in the caeca of broiler chickens. Mannose was chosen as the positive control for the salmonella assay, while the negative control used in this assay was the basal diet without any additives.

MRF was also included in the later trials undertaken; as it was hypothesised that soluble mannan could possibly also have salmonella colonisation reduction properties. Soluble mannan was fed to the chickens in the form of a mannan rich fraction produced by Alltech Inc. (Nicholasville, KY).

## 2.1) Materials and methods

The study was conducted in the Poultry Isolation room at the North American Biosciences Centre, Alltech Inc., Nicholasville, Kentucky, USA.

The assay was based on the following standard method as described by Spring *et al.* (2000). Changes were made to subsequent individual trials after evaluating the results of each trial. These specific changes are noted in Table 2.3. All of the animal studies conducted followed an animal care protocol approved by the Alltech Institutional Animal Care and Use Committee.

*2.1.1) Chickens:* A total of 100-day-old male broiler chicks (Cobb) were obtained from a credible commercial poultry-breeding farm. Chicks were randomly assigned to 9 different isolation chambers (Fiberglass Unlimited Inc., Roanoke, Alabama, USA), 11 chicks per chamber. On the day of arrival, every chick received 0.25ml hatchery waste inoculums via oral gavage to establish an intestinal bacterial population of similar composition for each of the chicks. The chicks were screened for salmonella by means of a faecal sample taken on day 3 of the trial, prior to the administration of the salmonella culture.

*2.1.2) Standard inoculum:* The standard inoculum was prepared by adding 20g hatchery waste to 80g 0.1% sterile peptone water in a bladed blender jar. The contents were blended (Oster kitchen blender) for 30 seconds for use as an inoculum. All hatchery waste used was screened for the presence of salmonella.

*2.1.3) Bacteria:* The challenge culture (Naladixic Acid Resistant strain *Salmonella enterica* subsp. *enterica* serovar *Typhimurium* 29E) was obtained from Dr K.E. Newman, Venture Labs, Lexington KY. The salmonella culture was administered on day 3 of the trial via oral gavage. All chicks received 0.25ml of the salmonella culture ( $1 \times 10^5$  CFU/ml).

2.1.4) *Experimental design*: Three different treatments with 3 replications each were randomly allocated to the various isolation chambers. Treatment 1 was a control treatment and the chicks received a standard broiler starter diet. Treatment 2 and 3 received a standard chick starter diet with the added feed additives as required for the trial, at the correct inclusion rates. The composition of the standard broiler starter diet is noted in Table 2.1. Due to the effect of mannose inclusion on the energy balance of the diet a separate basal diet was used for the feed containing mannose in trials 6 and 7. The composition of this diet is noted in Table 2.2.

**Table 2.1. Raw material composition of the basal starter diet**

Ingredient	% inclusion
Maize	53.34
Soybean meal, dehulled	38.00
Maize oil	4.37
Dicalcium phosphate	2.04
Limestone	1.25
Salt, ionized	0.50
DL-methionine	0.25
UK poultry vit-TM	0.25
Calculated Analysis	%
ME	13.4MJ/kg
CP	22.92
Ca	1.00
P	0.50



**Table 2.2. Raw material composition of the mannose basal diet balanced for the mannose treatment group**

Ingredient	% inclusion
Maize	50.59
Soybean meal, dehulled	38.3
Maize oil	4.30
Mannose	2.50
Dicalcium phosphate	2.06
Limestone	1.25
Salt, ionized	0.50
DL-methionine	0.25
UK poultry vit-TM	0.25
Calculated Analysis	%
ME	13.4MJ/kg
CP	22.92
Ca	1.00
P	0.50

2.1.5) *Husbandry*: Each replicate of treatment were kept in an individual isolation chamber. The isolation chambers were equipped with removable wire platforms, placed inside the chambers to act as a wire floor system. The chambers were all fitted with fibreglass air filter systems. Chicks received feed and autoclaved water on an *ad libitum* basis. Temperature was initially kept at approximately 32°C for the first two days after which it was gradually reduced to reach approximately 28°C by day 10. Light was continuously provided for the duration of the 10-day trial period. On completion of each trial the facility as well as all the implements used during the trial was sterilised with bleach, oxonia and a bactericidal product as indicated in the experimental protocol.

2.1.6) *Sampling and sample analysis*: On day 10, ten chicks from each pen (i.e. 30 chicks per treatment) were sacrificed by asphyxiation with argon gas, and a single caecum of each bird was aseptically removed. The contents of the caecum were placed in a sterile test tube, weighed and diluted 1:10 with maximum recovery diluent (MRD). The emptied caecum was cut longitudinally and placed in lactose broth (Difco, Sparks, MD)(a). Decimal dilutions were prepared of caecal contents in MRD, and salmonella was

enumerated on Brilliant Green Agar-Naladixic acid plates ( $10^1$  to  $10^5$  dilutions) after 24 h at 37°C(b).

(a) After 24 h growth in lactose broth for enrichment purposes, 1 ml aliquots were added to 9 ml of Rappaport Vassiliadis R10 broth (RV broth) and incubated at 37°C overnight. These cultures were then streaked on Xylose Lysine Deoxycholate (XLD) agar and incubated at 37°C overnight. Putative salmonella colonies on these media were 'streaked and stabbed' on Triple Sugar Iron (TSI; Difco, Sparks, MD) agar slants (Hajna, 1945). Salmonella appeared as black colonies, with evident gas production in the TSI slants and could also be seen on the XLD plates as black colonies. Black colonies were in some cases further tested by biochemical test analysis (API 20E, BioMerieux) according to manufacturers instructions to confirm presence of salmonella.

(b) From the BGA-Nal plates used for the determination of original colony count, randomly selected colonies showing characteristic salmonella colony morphology were inoculated in TSI agar slants to confirm the presence of salmonella. Subsequent testing for the presence of salmonella was performed according to the AOAC standard method for the isolation of salmonella.

In order to improve on the standard salmonella assay model, several changes were made to the 7 trials that followed the first standard assay trial that was conducted. These changes are tabulated below in Table 2.3.

**Table 2.3. Specific alterations made to the standard method<sup>1</sup> between different trials**

Trial	Experiment number	Specific changes to methods
1	05-006	Standard method
2	05-013	A higher challenge inoculum of $6.25 \times 10^4$ cfu/bird
3	05-019	A higher challenge inoculum of $6.25 \times 10^4$ cfu/bird Autoclaved sawdust as litter
4	05-025	A higher challenge inoculum of $6.25 \times 10^4$ cfu/bird Autoclaved sawdust as litter No standard inoculum
5	05-031	A higher challenge inoculum of $6.25 \times 10^4$ cfu/bird Autoclaved sawdust as litter
6	05-033	A higher challenge inoculum of $6.25 \times 10^4$ cfu/bird Autoclaved sawdust as litter
7	05-044	A higher challenge inoculum of $6.25 \times 10^4$ cfu/bird Autoclaved sawdust as litter Autoclaved feed
8	05-048	A higher challenge inoculum of $6.25 \times 10^4$ cfu/bird Autoclaved sawdust as litter Autoclaved feed Fresh caecal culture as standard inoculum

<sup>1</sup>Standard method included a wire grid flooring system, a challenge inoculum of  $2.5 \times 10^4$  cfu/bird, a standard inoculum made of hatchery waste and non-autoclaved feed.

*2.1.7) Procedures for autoclaving shavings (Trials 3 - 8):* The quantity of litter required for each chamber was calculated to be 1.2kg. This amount was placed in autoclaveable bags and autoclaved at 121°C for 40 minutes. Shavings were left to cool and dry in the bags prior to use in the isolation chambers.

2.1.8) *Procedures for autoclaved feed (Trials 7 and 8)*: Feed requirement for each chamber was calculated and said amount was placed in autoclaveable bags. The cell wall preparation was added to the basal diet prior to autoclaving (Spring *et al.*, 2000) but the mannose was only added after autoclaving as mannose melts when autoclaved due to poor heat resistance. The pure mannose was Gram stained as well as wet mounted and tested negative for bacteria when observed under the microscope. The feed was autoclaved at 121°C for 20 minutes (Spring *et al.*, 2000).

2.1.9) *Procedures for the Fresh Caecal Culture preparation (Trial 8)*: Fresh caecal culture was obtained from specific pathogen free (SPF) chickens (Charles River Laboratories; SPAFAS Avian Products and Services, Wilmington, MA). After euthenization with argon gas, the caeca of 3 SPF chickens were removed. The caeca contents was extracted and placed in a test tube using CO<sub>2</sub> probes to create an anaerobic environment inside the test tube. Caeca contents were diluted with maximum recovery diluent (MRD) by multiplying the total caeca contents weight with 9 and adding the calculated amount of MRD to the caeca contents. An anaerobic serial dilution was made and the 10<sup>6</sup> dilutions were used as the standard inoculum of which the day old chicks received 0.25ml.

The fresh caecal culture was plated on Plate Count Agar (PCA; Difco, Sparks, MD) and the total number of colony forming units determined to be  $2.8 \times 10^7$  CFU/ml.

2.1.10) *Treatments used in the different trials*: The treatments used varied between individual trials. All of the trials had a negative control and mannose treatments as the positive control. Quantities of mannose used in the trials were changed between trials to determine the exact amount required for favourable results. All the different treatments are tabulated in Table 2.4.

**Table 2.4. The different treatments applied for the various trials**

Trial	Experiment number	Treatments
1	05-006	T1: Control, no additives T2: Methyl $\alpha$ -D-manno-pyranoside (1kg/T) T3: Mannose (50kg/T)
2	05-013	T1: Control, no additives T2: Mannose (50kg/T) T3: Biomos (4kg/T)
3	05-019	T1: Control, no additives T2: Mannose (50kg/T) T3: Biomos (4kg/T)
4	05-025	T1: Control, no additives T2: Mannose (50kg/T) T3: Biomos (4kg/T)
5	05-031	T1: Control, no additives T2: Mannose (50kg/T) T3: Mannan Rich Fraction (5kg/T)
6	05-033	T1: Control, no additives T2: Mannose (25kg/T) T3: Mannan Rich Fraction (5kg/T)
7	05-044	T1: Control, no additives T2: Mannose (25kg/T) T3: Bio-Mos (4kg/T)
8	05-048	T1: Control, no additives T2: Mannose (50kg/T) T3: Bio-Mos (4kg/T)

2.1.11) *Data analysis*: Salmonella counts were log transformed to fit a normal distribution. Colony counts were recorded after incubation as CFU/g wet caecal contents. Caecal counts that tested salmonella negative on BGA-Nal but positive in the enrichment

tests were assigned a concentration of 1.5 log<sub>10</sub> CFU/g salmonella. Samples that were confirmed as negative after the enrichment test were assigned a concentration of 1 CFU/g salmonella (0 log CFU/g) (Nisbet *et al.*, 1993). Due to a lack of results showing obvious differences between different treatments no statistical analysis was done for any of the salmonella trials.

## 2.2) Results:

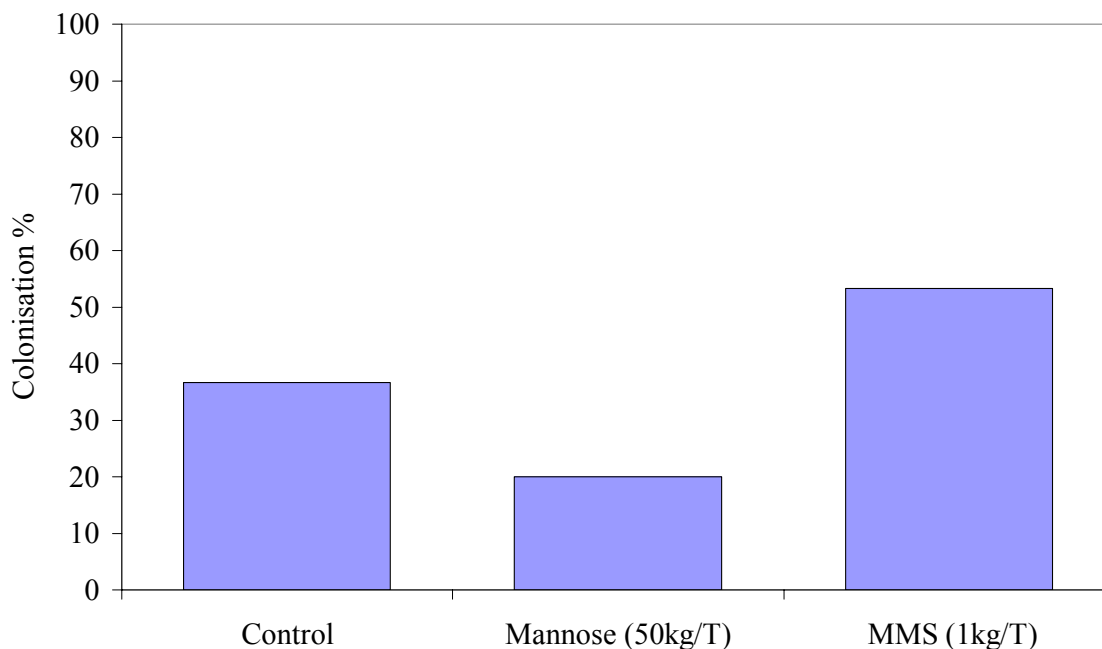
Below are results for each of the trials. The means for all treatments are given in the summary table (Table 2.2.10.), which follows after the results of Trial 8.

### 2.2.1 Trial 1; 05-006

**Table 2.2.1. Log<sub>10</sub> CFU/g wet caecal contents<sup>1</sup> and number of birds infected per pen (n=10) comparing mannose against methyl manno-pyranoside in their efficacy at reducing salmonella colonisation in the caeca**

Treatment	Replicate	CFU/g	Birds infected/pen
Control	1	5.56	1
	2	6.69	3
	3	7.67	7
Mannose	1	6.44	3
	2	0.00	0
	3	7.13	3
Methyl mannopyranoside	1	7.54	7
	2	7.02	1
	3	7.50	8

<sup>1</sup>Statistical analysis using Duncan's Multiple Range Test revealed that there were no significant differences between CFU/g reported for each of the treatments ( $p = 0.3723$ )



**Figure 2.2.1. Colonisation percentages<sup>1</sup> comparing the efficacy of mannose against that of methyl manno-pyranoside to determine which can be used as positive control**

<sup>1</sup> Colonisation percentages were calculated as the number of birds that tested positive for salmonella per treatment, divided by the total number of birds in the treatment (Oyofa *et al.* 1989c)

Trial 1 followed the standard procedures as outlined in the materials and methods section. The low number of chickens infected in the control treatment was interpreted as a too low and thus ineffective inoculum size (see Figure 2.2.1 and Table 2.2.1). Despite the low inoculum size, the mannose treatment showed lower numbers of birds infected than the methyl  $\alpha$ -D-manno-pyranoside treatment (MMS). The MMS treatment replicates in general showed higher numbers of CFU/g wet caecal content than observed in the mannose group. Based on the colonisation percentages, number of birds infected per pen as well as the  $\log_{10}$  CFU/g wet caecal contents values obtained for the treatments it was decided to use mannose as the positive control for the salmonella assay.

The salmonella culture was tested by Dr Newman (Venture Labs, Lexington, KY) to have an aggregation rate of 1.4 minutes with 25% aggregation.

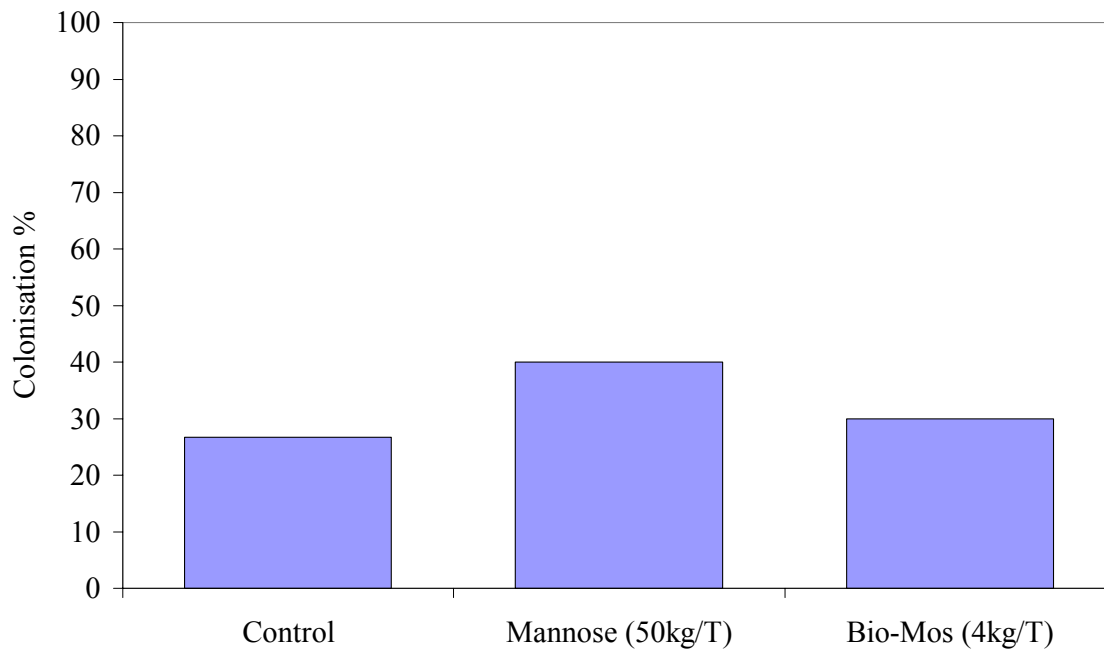


2.2.2 Trial 2; 05-013

**Table 2.2.2. Log<sub>10</sub> CFU/g wet caecal contents<sup>1</sup> and number of birds infected with *Salmonella typhimurium* per pen (n=10) with mannose as positive control and Bio-Mos as third treatment**

Treatment	Replicate	CFU/g	Birds infected/pen
Control	1	5.28	1
	2	6.32	4
	3	6.43	3
Mannose	1	7.80	5
	2	5.95	4
	3	6.18	3
Bio-Mos	1	6.83	1
	2	7.38	7
	3	4.91	1

<sup>1</sup>Statistical analysis was done by means of a Duncan's Multiple Range Test. No significant differences were observed between the various treatments (p = 0.7560)



**Figure 2.2.2. Colonisation percentages<sup>1</sup> comparing Bio-Mos against the positive control, mannose**

<sup>1</sup> Colonisation percentages were calculated as the number of birds that tested positive for salmonella per treatment, divided by the total number of birds in the treatment (Oyofa *et al.* 1989c)

Trial 2 was conducted using a higher inoculum size based on the results obtained from the first trial. Again the results showed poor salmonella colonisation in the caeca of the control group. The lowest number of CFU/g wet caecal content is observed in one of the Bio-Mos chambers while the highest number is in the mannose group. The Bio-Mos treatment also showed the most variation between the different Bio-Mos groups in terms of CFU/g wet caecal content (Table 2.2.2). The mannose treatment showed the highest level of colonisation (Figure 2.2.2).

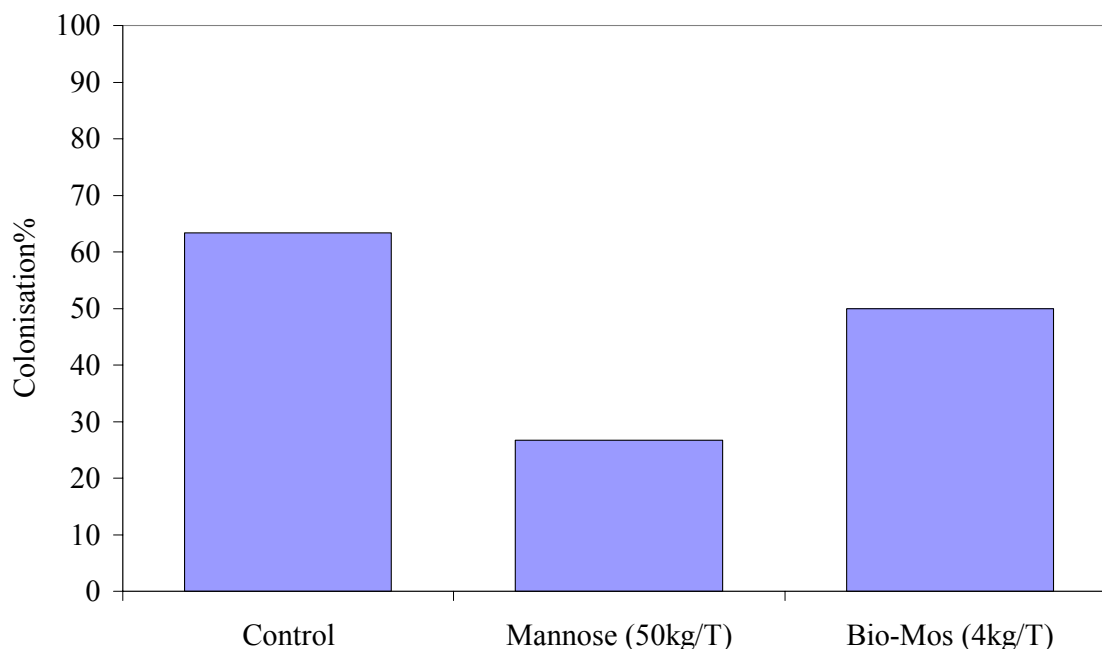
Salmonella culture obtained from Dr Newman (Venture Labs, Lexington KY) had a concentration of  $2.9 \times 10^5$  CFU/ml.

### 2.2.3 Trial 3; 05-019:

**Table 2.2.3. Log<sub>10</sub> CFU/g wet caecal contents<sup>1</sup> and number of birds infected with *Salmonella typhimurium* per pen (n=10) for broilers receiving diets containing no additive, mannose or Bio-Mos**

Treatment	Replicate	CFU/g	Birds infected/pen
Control	1	6.23	9
	2	6.50	7
	3	6.60	3
Mannose	1	5.09	1
	2	5.84	3
	3	6.03	4
Bio-Mos	1	6.50	3
	2	6.25	6
	3	7.01	6

<sup>1</sup>Statistical analysis (Duncan's Multiple Range Test) did show a significant difference ( $p = 0.0482$ ). CFU/g reported for Mannose was significantly lower than Control treatment or Bio-Mos treatment. Control and Bio-Mos did not differ significantly



**Figure 2.2.3. Colonisation percentages<sup>1</sup> comparing the percentage of birds colonized by *Salmonella typhimurium* for the Bio-Mos treatment group against that of the mannose treatment and control group**

<sup>1</sup> Colonisation percentages were calculated as the number of birds that tested positive for salmonella per treatment, divided by the total number of birds in the treatment (Oyofu *et al.* 1989c)

The colonisation percentage for Trial 3 was in favour of the mannose treatment (see Figure 2.2.3). Not only were the salmonella colonisation percentages lower in the mannose group but also the level of colonisation appeared lower, as reflected by the CFU/g wet caecal content values ( $p = 0.0482$ ), which were the lowest for the respective chambers. Bio-Mos was the third treatment and also represented the chamber with the highest level of CFU/g wet caecal content (Table 2.2.3). The introduction of autoclaved sawdust as a flooring system instead of the wire grids did not appear to induce secondary infection problems over the time period that the chicks were kept.

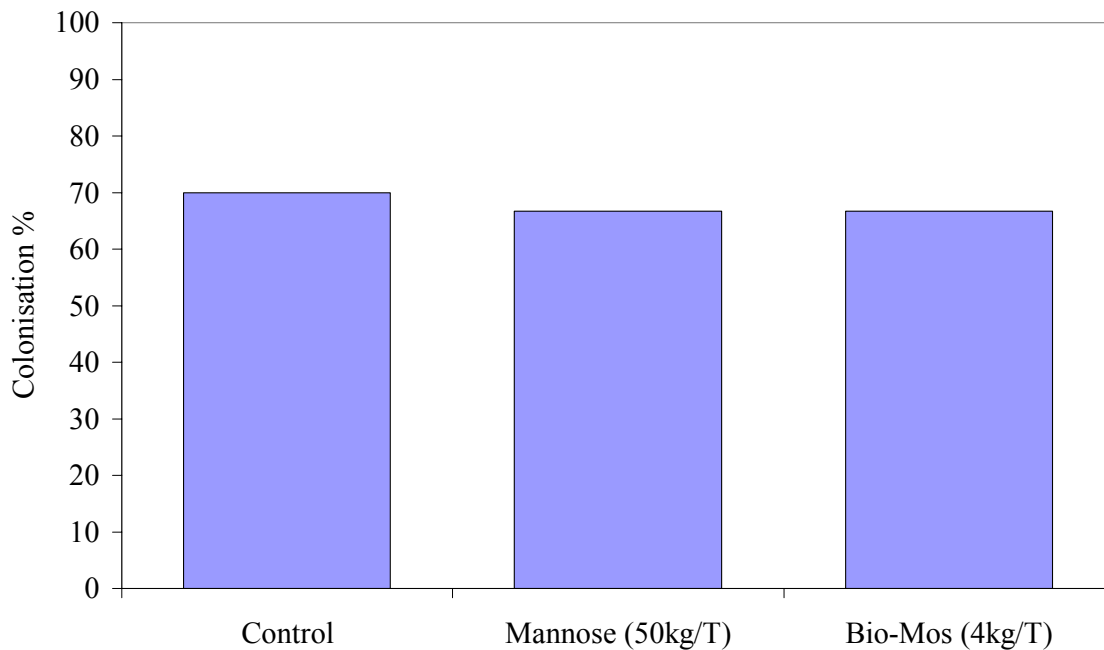
Salmonella culture was tested by Dr Kyle Newman (Venture Labs, Lexington KY) and had an aggregation rate of 0.9 minutes with an aggregation of 26%.

## 2.2.4 Trial 4; 05-025

**Table 2.2.4. Log<sub>10</sub> CFU/g wet caecal contents<sup>1</sup> and number of birds infected with *Salmonella typhimurium* per pen (n=10), with mannose as positive control treatment, a negative control with no additive, and Bio-Mos as the third treatment**

Treatment	Replicate	CFU/g	Birds infected/pen
Control	1	6.67	4
	2	7.57	10
	3	7.56	7
Mannose	1	7.39	6
	2	6.76	8
	3	7.01	6
Bio-Mos	1	7.09	8
	2	7.41	9
	3	7.45	3

<sup>1</sup>Duncan's Multiple Range Test revealed no significant differences between CFU/g reported for the treatments (p = 0.6674)



**Figure 2.2.4. Colonisation percentages<sup>1</sup> showing the similar salmonella colonisation observed in the for each of the 3 treatment groups**

<sup>1</sup> Colonisation percentages were calculated as the number of birds that tested positive for salmonella per treatment, divided by the total number of birds in the treatment (Oyofa *et al.* 1989c)

Trial 4 was conducted with the aim to investigate the effect of the use of a standard inoculum on day 1 by excluding the standard inoculum from the protocol. As reflected in the results the colonisation percentages were above 50% for all three of the treatments (Figure 2.2.4). The level of CFU/g wet caecal content was similar for all the different chambers except for one of the control and one of the mannose replicates which showed slightly lower levels of colonisation (Table 2.2.4).

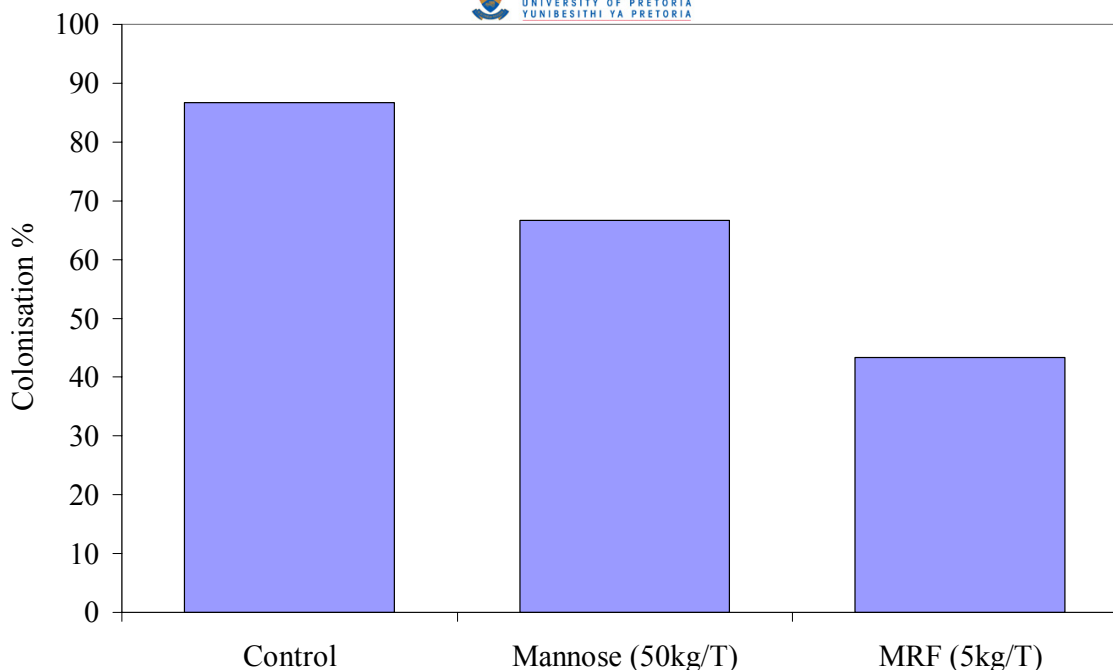
Salmonella culture obtained from Dr Newman (Venture Labs, Lexington KY) had a concentration of  $2.5 \times 10^5$  CFU/ml.

### 2.2.5 Trial 5; 05-031

**Table 2.2.5. Log<sub>10</sub> CFU/g wet caecal contents<sup>1</sup> and number of birds infected with *Salmonella typhimurium* per pen (n=10) indicating the efficacy of mannose as positive control and a soluble mannan (mannan rich fraction /MRF) in the reduction of salmonella colonisation in chicken caeca**

Treatment	Replicate	CFU/g	Birds infected/pen
Control	1	7.56	8
	2	7.52	8
	3	7.90	10
Mannose	1	7.52	10
	2	8.24	2
	3	7.71	8
MRF	1	7.66	4
	2	7.26	9
	3	0.00	0

<sup>1</sup>Trial 5 showed no significant differences between the treatments. Duncan's Multiple Range test was applied (p = 0.3573)



**Figure 2.2.5. Illustration of the colonisation percentages<sup>1</sup> showing the soluble mannan product, MRF, as a more efficient product than the positive control**

<sup>1</sup> Colonisation percentages were calculated as the number of birds that tested positive for salmonella per treatment, divided by the total number of birds in the treatment (Oyoyo *et al.* 1989c)

Trial 5 yielded positive colonisation percentages (Figure 2.2.5). As the third treatment group a mannan rich fraction (MRF) was used. The colonisation percentages show that MRF was able to lower the colonisation percentages below that of the mannose treatment, which was the positive control. When the CFU/g caecal content is taken into account the favourably low colonisation percentages that can be observed for the MRF treatment is the result of one of the replicates of the MRF treatment that has 0% colonisation. Based on CFU/g all the treatments and the replicates for each treatment had similar levels of CFU/g, with the exception of one mannose chamber that had a relatively high colonisation and the MRF replicate that tested negative (see Table 2.2.5).

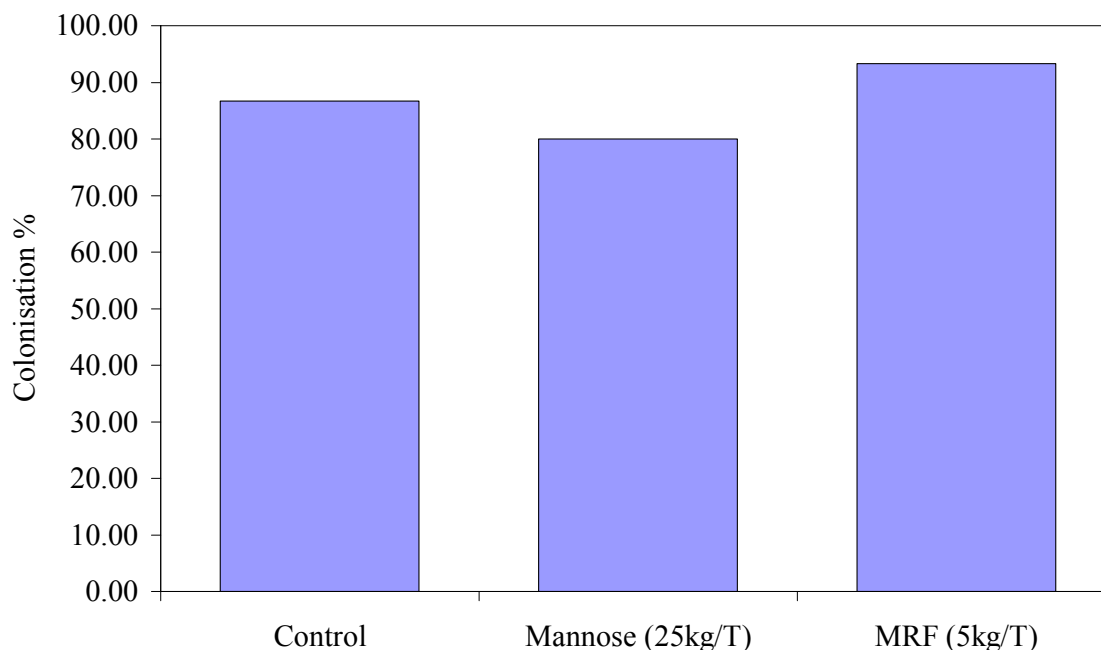
The salmonella culture had an aggregation rate of 0.7 minutes with 26% aggregation, as tested by Dr Kyle Newman (Venture Labs, Lexington KY).

### 2.2.6 Trial 6; 05-033

**Table 2.2.6. Log<sub>10</sub> CFU/g wet caecal contents<sup>1</sup> and number of birds infected with *Salmonella typhimurium* per pen (n=10) with a negative control with no additives, mannose as positive control and a soluble mannan (mannan rich fraction /MRF)**

Treatment	Replicate	CFU/g	Birds infected/pen
Control	1	4.74	8
	2	6.61	10
	3	4.73	8
Mannose	1	4.38	7
	2	5.40	8
	3	4.88	9
MRF	1	6.65	10
	2	5.30	8
	3	6.31	9

<sup>1</sup>Statistical analysis (Duncan's Multiple Range Test) revealed no significant difference in CFU/g reported for treatments (p = 0.2587)



**Figure 2.2.6. Colonisation percentages<sup>1</sup> indicating the difference between the colonisation percentages for mannose vs. MRF**

<sup>1</sup> Colonisation percentages were calculated as the number of birds that tested positive for salmonella per treatment, divided by the total number of birds in the treatment (Oyoyo *et al.* 1989c)

MRF was again used in this trial to test the efficacy of MRF at reducing salmonella colonisation in the caeca of chickens. The only change made was a lower dietary inclusion of mannose in the mannose treatment group diet. The CFU/g wet caecal weight indicates that a lower level of colonisation occurred in the control group, mannose group and the MRF group. However, the colonisation percentages show that nearly all the birds were colonized by the salmonella. The highest level of colonisation were observed in the MRF treatment, indicating that for this trial, MRF was not able to reduce colonisation of salmonella in the caeca below that levels observed for the control groups (Figure 2.2.6 and Table 2.2.6). The colonisation percentage of the mannose treatment was lower than for the other treatments. Also, a large amount of variation was observed between the different chambers for the various treatments, especially in the control and mannose groups with both having two replicates with lower infection levels and one higher infection level.

Venture Labs (Lexington KY) tested the salmonella culture and it had an aggregation rate of 1.2 minutes with a colonisation of 26%.

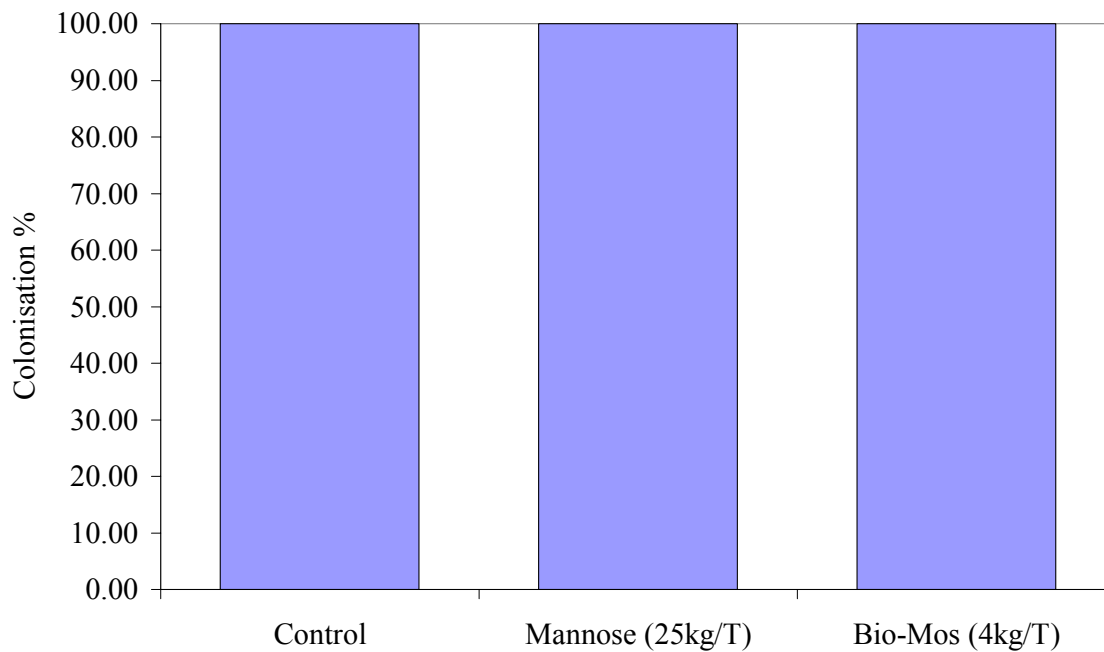
#### 2.2.7 Trial 7; 05-044

**Table 2.2.7. Log<sub>10</sub> CFU/g wet caecal contents<sup>1</sup> and number of birds infected with *Salmonella typhimurium* per pen (n=10) testing the efficacy of Bio-Mos against salmonella colonisation in the caeca, with a negative control treatment with no additives, mannose as positive control and Bio-Mos**

Treatment	Replicate	CFU/g	Birds infected/pen
Control	1	7.91	10
	2	7.29	10
	3	7.39	10
Mannose	1	7.93	10
	2	7.91	10
	3	8.11	10
Bio-Mos	1	8.35	10
	2	8.41	10
	3	8.08	10

<sup>1</sup>Duncan's Multiple Range Test indicates that the control treatment had significantly ( $p = 0.0185$ ) lower levels of CFU/g than the other two treatments, which in turn did not differ from each other





**Figure 2.2.7. Colonisation percentages<sup>1</sup> indicating no differences between the various treatments**

<sup>1</sup> Colonisation percentages were calculated as the number of birds that tested positive for salmonella per treatment, divided by the total number of birds in the treatment (Oyofu *et al.* 1989c)

Trial 7 showed 100% salmonella colonisation in all 3 treatments (Figure 2.2.7). The mannose and Bio-Mos products were not able to reduce the colonisation. The CFU/g wet caecal content indicated that the highest level of infection was amongst the Bio-Mos replicates as the greatest numbers of salmonella colonies were enumerated from the caecal contents of the birds in this treatments' replicates (Table 2.2.7). The laboratory that provided the salmonella culture indicated that there was a possibility that the salmonella culture used to infect the birds was of a higher concentration than initially indicated by them due to an error in their dilution process. There appeared to be little variation between the different chambers, especially between the control and Bio-Mos treatments.

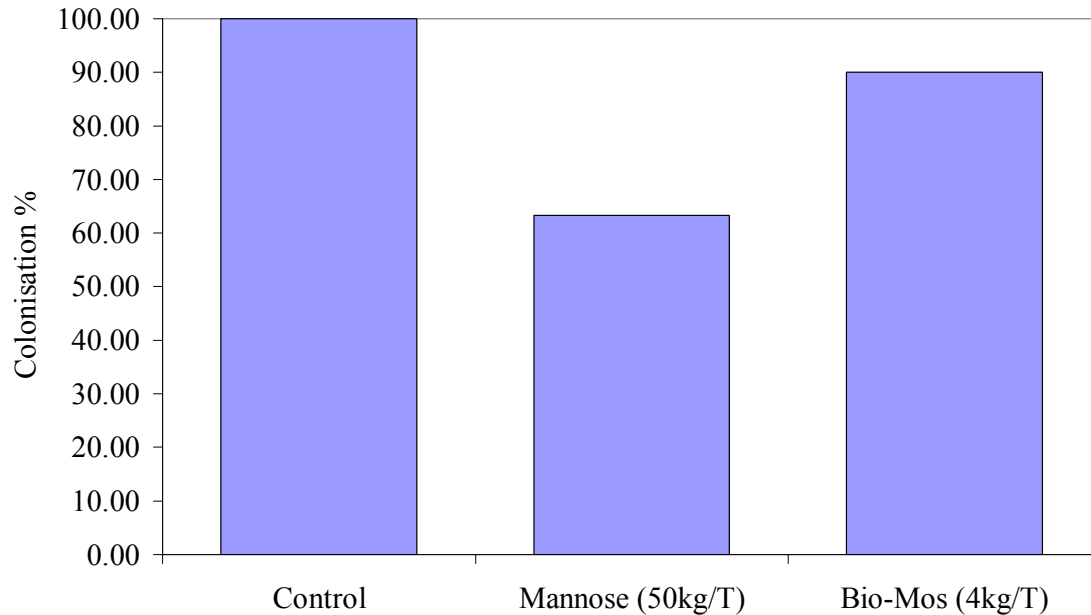
The salmonella culture was tested by Dr Kyle Newman (Venture Labs, Lexington KY) and had an aggregation rate of 0.5 minutes with a 29% aggregation. The salmonella culture also had a concentration of  $1.2 \times 10^8$  CFU/ml.

### 2.2.8 Trial 8; 05-048

**Table 2.2.8. Log<sub>10</sub> CFU/g wet caecal contents<sup>1</sup> and number of birds infected with *Salmonella typhimurium* per pen (n=10). A negative control with no additives, a positive control as mannose and Bio-Mos were used as the treatments**

Treatment	Replicate	CFU/g	Birds infected/pen
Control	1	7.47	10
	2	7.92	10
	3	7.79	10
Mannose	1	6.59	6
	2	7.46	5
	3	6.07	8
Bio-Mos	1	7.36	10
	2	8.13	10
	3	6.12	7

<sup>1</sup>No significant difference ( $p = 0.2990$ ; Duncan's Multiple Range Test) was observed for CFU/g between the treatments

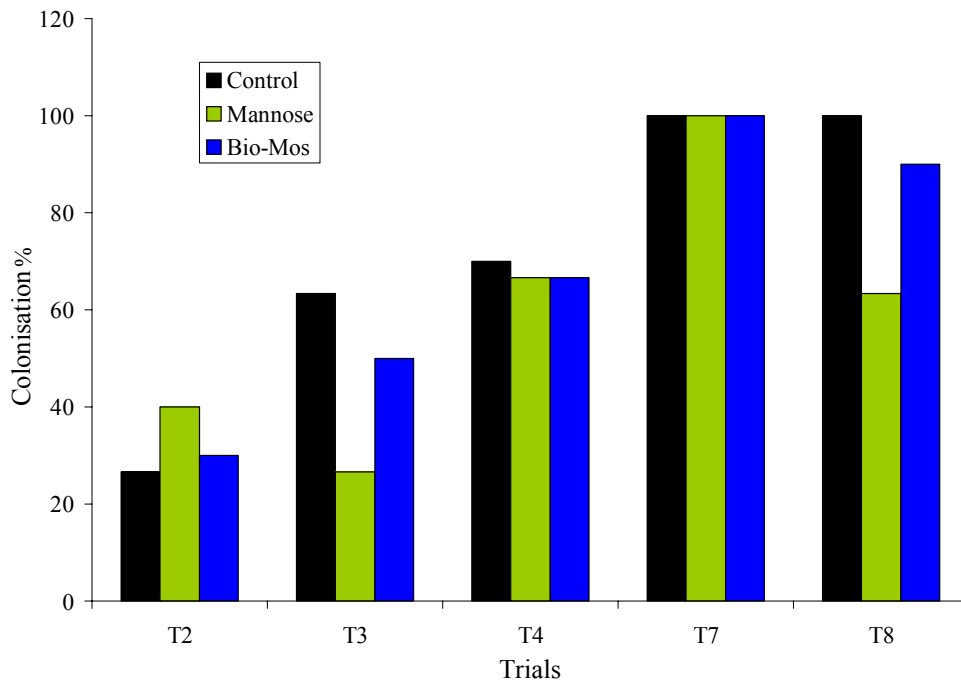


**Figure 2.2.8. Colonisation percentages<sup>1</sup> for mannose vs Bio-Mos indicating the ability of mannose to reduce colonisation**

<sup>1</sup> Colonisation percentages were calculated as the number of birds that tested positive for salmonella per treatment, divided by the total number of birds in the treatment (Oyoyo *et al.* 1989c)

Trial 8 produced a 100% colonisation in the control group (Fig 2.2.8). Mannose colonisation was lower than that of the control group as well as the Bio-Mos group, however, when CFU/g wet caecal content is taken into consideration (Table 2.2.8), all the treatments showed high levels of CFU/g. Mannose CFU/g wet caecal content numbers were slightly lower than the other treatments. The Bio-Mos treatment showed variation between the different replicates, and the pen with the highest level of infection for this trial could be found within this treatment.

Figure 2.2.9 is a summary of the colonisation percentages obtained across the treatments. The results from these 8 trials were not able to display the reported pathogen absorbing qualities of cell wall preparations. At the same time the mannose treatment that was used as the positive control for this assay was also not able to show any consistently positive results. These findings are not consistent with those reported by Spring *et al.* (2000) who found reductions in salmonella colonisation when cell wall products were used.



**Figure 2.2.9. Mannose vs. Bio-Mos trials<sup>1</sup> comparing the variation in colonisation percentage as changes were applied to the standard protocol**

- <sup>1</sup>T2: Mannose (50kg/T); Bio-Mos (4kg/T); Control feed (basal diet with no added additives)  
 T3: Mannose (50kg/T); Bio-Mos (4kg/T); Control feed (basal diet with no added additives)  
 T4: Mannose (50kg/T); Bio-Mos (4kg/T); Control feed (basal diet with no added additives)  
 T7: Mannose (25kg/T); Bio-Mos (4kg/T); Control feed (basal diet with no added additives)  
 T8: Mannose (50kg/T); Bio-Mos (4kg/T); Control feed (basal diet with no added additives)

Table 2.2.10. Summary of the Bio-Mos and MRF trials presented as log<sub>10</sub> CFU/g wet caecal contents and the colonisation percentages for the specific treatments in brackets

Study #	Change	Control	Mannose	Bio-Mos	MRF
		Log <sub>10</sub> cfu/g (Colonisation %)	Log <sub>10</sub> cfu/g (Colonisation %)	Log <sub>10</sub> cfu/g (Colonisation %)	Log <sub>10</sub> cfu/g (Colonisation %)
2	Shavings	6.01	6.64	6.37	*
	Higher salmonella inoculum	(26.70)	(40.00)	(30.00)	*
3	Shavings	6.44	5.65	6.58	*
	Higher salmonella inoculum	(63.30)	(26.70)	(26.70)	*
4	Shavings	7.27	7.05	7.32	*
	Higher salmonella inoculum no standard inoculum	(70.00)	(66.70)	(66.70)	*
5	Shavings	7.66	7.78	*	4.97
	Higher salmonella inoculum	(86.70)	(66.70)	*	(43.30)
6	Shavings	5.36	4.89	*	6.09
	Mannose 2.5% Higher salmonella inoculum	(86.70)	(80.00)	*	(93.30)
7	Shavings	7.53	7.98	8.28	*
	Mannose 2.5% Higher salmonella inoculum	(100.00)	(100.00)	(100.00)	*
8	Shavings	7.73	6.70	7.20	*
	Higher salmonella inoculum Fresh caecal culture	(100.00)	(63.30)	(90.00)	*

### 2.3) Discussion:

Several enterobacteria have Type I fimbria, which could be characterised by its sensitivity to mannose (Firon *et al.*, 1984). Type I fimbria binds to the mannoside glycoprotein receptors located on numerous different epithelial cells (Thorns *et al.*, 2000). Type I fimbria are rod-shaped organelles and can be up to 100nm in length and 7-8nm in diameter (Thorns *et al.*, 2000).

Colonising salmonella populations have been found to target the caeca, as it is the most stable section of the intestinal tract for microorganisms to populate (Schneitz & Mead, 2000). The crop has also been identified as a major area for salmonella contamination. During the processing phase of chicken carcasses it often happened that one or both of these organs ruptured, and if it was colonised with salmonella, contamination of the carcass occurred (Hume *et al.*, 1996). Murase *et al.* (2000) investigated a salmonella food borne outbreak, and reported salmonellosis symptoms to occur between 10.5 and 121 hours post ingestion of contaminated food. Symptoms observed included diarrhoea, fever and vomiting and treatment varied from antidiarrheal drugs to antimicrobial drugs, depending on the severity of the symptoms (Murase *et al.*, 2000).

Intensive production systems tend to prevent chicks from acquiring a protective microflora within the first few days post hatch (Schneitz & Mead, 2000) by restricting the chicks from accessing the outer eggshell bacteria or faecal material from adult birds, thus reducing the number of naturally protective bacteria in the chicks' digestive tract (Hume *et al.*, 1996). Hume *et al.* (1996) further reported that the Nurmi concept was based on this phenomenon and involved the use of a standard inoculum in the form of competitive exclusion bacteria. The protective properties expressed by these microbial populations was observed as the protective bacteria bound to the binding sites available in the intestines which in turn reduced the number of sites that were available for the salmonella to adhere to (Hume *et al.*, 1998). Pathogenesis of the salmonella population depends on the ability of salmonella to adhere to the epithelium cell surface of the gastrointestinal tract mucosa. It should be obvious then that preventing adhesion to the

epithelium cells could prevent colonisation (Droleskey *et al.*, 1984). Schneitz & Mead, (2000) also stated that competitive exclusion bacteria were effective against enteropathogenic bacteria because it helped to create a “physiologically restrictive environment” and utilized certain substrates thought to be essential for the survival and proliferation of the enteropathogen.

The objective of the salmonella trials was to design a model that could be used to test the effectiveness of various different yeast products, such as the different components of the yeast cell wall, for their ability to reduce and control salmonella colonisation in broiler chickens.

The results for the 8 trials that were undertaken did not show any significantly positive results for the cell wall preparations that were used as treatments. At the same time, with the exception of one trial, mannose, which was used as the positive control in all of the trials, did not respond favourably either. These results contradict the results reported by Spring *et al.* (2000) who found that the inclusion of MOS in the diets of the chicks reduced salmonella colonisation. The results observed for the mannose treatments also contradicted literature which showed that mannose had the ability to reduce salmonella colonisation in chickens (Oyofa *et al.*, 1989a,c; Fernandez *et al.*, 2000). These studies were the reason for the inclusion of mannose as the positive control in the salmonella assay model. Yet the high level of mannose that has to be included in the diet of chickens to reduce the salmonella colonisation, made it economically less feasible on a commercial level than the use of a natural alternative such as cell wall preparation, which is included at a lower level (Spring *et al.*, 2000).

The ineffectiveness of the positive control treatment in these trials indicated that there were various additional factors that played a critical roll in the success of this model. Some of the problem areas that were addressed included the specific floor system that was used in each of the trials, the validity of the use of a standard inoculum, the material used to produce the standard inoculum and the effect of the sterility of feed. It was also

noted that the quality of the chicks used in these trials were of utmost importance. Chicks that appeared weak upon arrival were not able to withstand the pathogenic insult.

One of the most important factors that have been identified to play a key role in the outcome in the salmonella assay results was the administration of a standard inoculum on day one of the trial to establish a stable intestinal micro flora population for each of the chicks. Day of hatch chicks do not have the stable intestinal microbial populations found in older chickens, making these young chicks most susceptible to salmonella insult during this early phase (Hume *et al.*, 1996). Administering a standard inoculum was also a way to ensure that all of the chicks across the experiment had a comparable micro flora profile and a similar level of protection.

For the first seven trials (with the exclusion of trial 4) hatchery waste was finely ground in a peptone water solution and then used as the standard inoculum. The hatchery waste used in each of the trials was screened for the presence of salmonella but only the first few batches of hatchery waste used proved to be adequate for our trials as, at a specific point, the hatchery waste started testing positive for the presence of salmonella, possibly due to the change in season. Although the hatchery waste did not have an excessive amount of salmonella, this salmonella could possibly have competed for adhesion sites with the laboratory mutated salmonella strain that was used in this trial and could have resulted in inaccurate salmonella enumeration on day 10. It was decided to seek alternative methods of preparing a standard inoculum. Protective bacteria can be found in the crop, caeca, intestines, faecal material and used litter of adult chickens (Hume *et al.*, 1998). Preparing the fresh caecal culture from specific pathogen free birds made it possible to eliminate the probability of inoculating the birds with unknown quantities of foreign salmonella organisms. The use of the fresh caecal culture as standard inoculum proved to be a success in the last of the pathogen trials, as the chicks appeared to have enough protection against the salmonella to prevent excessive colonisation, as was observed in trials 4 and 7, but were still vulnerable enough to allow for 100% colonisation in the control treatment. Obtaining high colonisation levels in the control group (as was achieved in the last 4 trials) is important for two reasons: Firstly, it



indicates that the salmonella culture was effective and that reduction in colonisation observed for treatment groups can be attributed to the efficacy of the treatment product used. Secondly, it excludes the possibility that it is the standard inoculum protecting the birds from the salmonella culture, so that reduction seen in colonisation percentages in the treatment groups could be accredited to the effectiveness of the treatment products.

Autoclaving all the feed used in the trials helped to reduce the growth that was observed on some of the BGA/Nal plates. This bacteria, which are resistant to Naladixic acid, was identified as *E. Coli* I with the use of an API 20 E V4.0 strip (BioMerieux, France). The bacteria not only interfered with the accuracy of enumerating the CFU/g wet caecal contents but it possibly also targeted the same receptor sites as our salmonella strain, which in turn could have affected the ability of the salmonella to colonise in the gut. Subjecting the chicken feed to heat treatment resulted in a reduction in the total amount of bacterial contamination of the feed. It is important to control the amount of bacteria that the birds are exposed to in pathogen studies (hence the careful sanitation of the facilities), as various bacterial interactions can affect the outcome of results.

The Alltech Poultry Isolation Facilities were designed in such a way that made it possible to experiment with the effect of 2 different types of flooring systems. At first, a wire grid floor was used as it was thought that the wire grid system would remove the birds from infected faecal matter and reduce the effects of constant re-infection. Contradicting this theory, it was found that the best results came from the sawdust litter system where the birds were housed on autoclaved sawdust for the duration of the 10-day experiment. One possible theory for this effect: As the salmonella agglutinated with Bio-Mos, the salmonella was inactivated. Re-infection was then eliminated to a great extent. The control birds however were constantly re-infected. Compared to the wire system then, the birds were removed from the infected faecal material, which meant that there was no re-infection. Thus, if the salmonella culture was not able to aggressively colonise from the immediate point of administration, low colonisation was seen in all groups concerned.

## 2.4 Conclusion

No statistically significant positive results were observed for the yeast cell wall product or mannan rich fraction in these trials. These results are in contradiction to the work done by Spring *et al.* (2000). At the same time the positive control (mannose) was also not able to significantly reduce salmonella colonisation, indicating that there are still some external factors that controlled the outcome of this assay, and these factors would have been identified and corrected before this specific model will function in the way it was designed to. Most of the advances that were made during this study were based on a trial and error approach, making it difficult to point out any factor that will change the results favourably. Due to time constrictions it was not possible to replicate the trial that was conducted with fresh ceecal culture (FCC). It is recommended that the possibility of using the FCC be further explored.

## Chapter 3

### The effect of Bio-Mos, with or without the addition of a soluble mannan preparation, on the performance and gastrointestinal health of broiler chickens

The objective of this study was to determine how Bio-Mos, with or without the addition of a soluble mannan product, would affect the intestinal health and performance of broiler chickens. The main focus of the study was on the intestinal health of these broiler chickens and involved the morphology of the ileum. Reports on the positive effects of yeast cell wall on the gastrointestinal tract morphology (Santin *et al.*, 2001; Zhang *et al.*, 2005) initiated a study on the effects of Bio-Mos or a yeast cell wall derivative, mannan rich fraction (MRF), on morphology parameters such as villi height and width, crypt depth and muscularis thickness. Observations made on goblet cell differences between treatments resulted in the inclusion of measurements of goblet cell size and density across the treatments.

### 3.1) Materials and Methods

#### 3.1.1. General experimental procedures:

This trial was undertaken with the approval of the Alltech Institutional Animal Care and Use committee. The trial was conducted in the starter cages located at the Poultry Research Centre on the Alltech Research Farm.

*3.1.1.1) Experimental design:* The experiment was conducted as a complete randomised design. It was designed to accommodate 7 treatments. Each treatment consisted of 7 replications, and 5 birds were placed in each replicate. The time frame of the trial was 15 days.

3.1.1.2) *Chickens*: A total of 245, day-old male broiler chicks (Cobb) were obtained from a credible commercial poultry-breeding farm. Chicks were randomly assigned to the 49 pens. 5 chicks were placed in each of the pens. 7 different treatments were applied in this experiment, which meant 7 pens per treatment and thus 35 chicks per treatment. Chicks remained in these pens for the entire 15-day experimental period. Pens were completely constructed out of wire grids, and similar to the battery system used for layer hens. Individual pens were equipped with collection trays, attached underneath the wire floor, for faecal collection purposes. Pens were large enough to comfortably house 5 chicks, up to 15 days of age.

3.1.1.3) *Husbandry*: Each pen was fitted with a nipple drinker to allow *ad libitum* access to drinking water. Feed was provided on a continuous basis from tube feeders. The room temperature was initially adjusted to approximately 32°C and then gradually lowered to reach approximately 26°C by day 10, after which it was kept at this temperature for the remainder of the experiment. Temperature was checked and recorded twice daily to minimize possible temperature fluctuations. Light was continuously provided for the duration of the experiment.

3.1.1.4) *Treatments*: Chicks received a maize-soybean starter meal, which was fortified with minerals and vitamins (See Table 3.1)

**Table 3.1. Raw material composition of the basal starter diet**

Ingredient	% inclusion
Maize	53.34
Soybean meal, dehulled	38.00
Maize oil	4.37
Dicalcium phosphate	2.04
Limestone	1.25
Salt, ionized	0.50
DL-methionine	0.25
UK poultry vit-TM	0.25
Calculated Analysis	%
ME	13.4MJ/kg
CP	22.92
Ca	1.00
P	0.50

The different treatments constituted the basal diet with the treatment supplement added to the basal diet. The different treatments were as follows:

Treatment 1: Negative control – no additives

Treatment 2: Bio-Mos (2kg/T)

Treatment 3: Bio-Mos (4kg/T)

Treatment 4: MRF (100g/T)

Treatment 5: MRF (200g/T)

Treatment 6: Bio-Mos (2kg/T) + MRF (100g/kg)

Treatment 7: Bio-Mos (4kg/T) + MRF (200g/kg)

#### *3.1.1.5) Sampling and sample analysis:*

a) On day 15 all birds from each of the pens were sacrificed by asphyxiation with argon gas. 2 chicks were randomly selected from each pen and the caeca aseptically removed (i.e. 14 birds per treatment). The contents of one caecum (from each bird) were placed in a sterile test tube, weighed and diluted 1:10 with maximum recovery diluent (MRD). Decimal dilutions were prepared of the caecal contents in MRD, and Coliforms was enumerated on Violet Red Bile agar (VRBA) plates ( $10^1 - 10^5$  dilutions). Plates were incubated at 37°C for 20 hours. Colony counts were recorded as CFU/g. After counting colonies, the plates were replicated onto VRBA containing 30 mg/kg tetracycline, using the velvet plating technique.

b) Crypt depth; villus height and width; muscularis externa:

Villus height was measured as the length between the villus-crypt axis and the tip of the villus. The villus width was measured at the midpoint between the villus-crypt axis and the tip of the villus. Crypt depth was measured from the villus-crypt axis to the base of the specific crypt. The thickness of the muscularis externa was measured from the base of the crypt to the base of the muscularis externa. These measurements were made from the slides stained with the H&E stain. Villi were photographed with a Nikon Spot Insight

Colour camera model #3.2.0. The software used for the measurements was Spot Software version 4.5.

c) Goblet cell measurements:

Goblet cell size was measured as the “cup” area size of the goblet cells ( $\mu\text{m}^2$ ). Only perfect cross sections of the goblet cells were measured i.e. goblet cell had to show the cup and the tail area to be measured and also had to touch the villi edge. Goblet cell density was determined as the number of goblet cells per 100 $\mu\text{m}$  length of the villus. All density measurements were taken from the midsection of the villus to make measurements comparable with one another. Only goblet cells found on the edge of the villus were counted for the density determination calculations and goblet cells also had to show the “cup and tail portion” to be counted. These measurements were made from the slides stained in the Alltech laboratory with the Alcian Blue/PAS stain. Villi were photographed with a Nikon Spot Insight Colour camera model #3.2.0. The software used for the measurements was Spot Software version 4.5.

d) Antibiotic resistance:

Antibiotic resistance was measured by plating caecal culture on violet red bile agar plate, allowing it to incubate for 24 hours before doing replicate plating onto a tetracycline containing violet red bile agar plate (30 mg/kg).

*3.1.1.6) Response variables:*

a) Feed conversion ratio: Live body weights were recorded for each pen on a weekly basis. The final weight recorded was done 24 hours prior to asphyxiation of the chicks. Feed intake was recorded simultaneously with body weight by weighing back of all feed not consumed at that point. Feed spillage was minimal with frequent checking and refilling of feed containers.

b) VFA production: VFA production was measured from caecal samples, collected on the final day of the trial. Caecal samples were treated with 2.5% meta-phosphoric acid and frozen until processed. VFA levels were measured by means of gas chromatography.

c) Ileum sample for gut morphology analysis: As explained previously, as well as in section 3.2.1, ileum samples were taken and specific morphological measurements made. These measurements included villi width and length, crypt depth and muscularis thickness. Goblet cell measurements included goblet cell size and goblet cell density.

d) Antibiotic resistance: Coliforms and Coliform (Tet) counts: See section 3.1.1.5a & d.

*3.1.1.7) Data Analysis:* Coliforms was be log transformed to fit a normal distribution prior to statistical analysis. Experimental data (gut morphology measurements, plate count data) was analysed as a complete randomised design using the general linear model procedure of SAS (SAS, 1988).

### 3.1.2) Histology

*3.1.2.1) Processing of fresh tissue samples:* The intended section of the terminal ileum was removed and immediately rinsed with saline solution to remove any intestinal contents from the ileum section. The tissue sample was then placed in an Omnisette Tissue Cassette, which was in turn placed in buffered formalin (10% Neutral buffered formalin; Sigma-Aldrich, St Louis MO) for a period of 18 hours. The cassettes were then rinsed 3 times with deionised water and placed in 70% (v/v) ethanol. Samples were sent to the University of Louisville where the samples were placed in paraffin blocks. Of each bird two samples were cut and placed on slides. One of each of these slides (i.e. one per bird) was stained with H&E stain by the University of Louisville for the purpose of villi length, width, crypt depth and muscle thickness determination. The others were received in the unstained form for specific staining procedures in the Alltech Laboratory.

*3.1.2.2) Alcian-Blue/ Periodic Acid Schiffs staining technique:* Alcian-Blue is a copper stain. Alcian-Blue forms salt linkages with the acidic groups of acid mucopolysaccharides. Expected results: Nuclei = blue; Acid mucosubstances = light blue; Neutral mucopolysaccharides; polysaccharides = purple. The Periodic Acid Schiffs (PAS) stain gives a positive (pink) reaction in the presence of 1-2 glycol, amino or alylamino groupings, and is used to stain numerous tissue components, including mucins of the intestinal tract. The combination of Alcian Blue and PAS stain was used as it was found to give a perfectly clear image of the goblet cells, increasing the accuracy with which the measurements could be conducted.



Method: The following unique staining method was developed and adapted during this experiment, based on the work done by Uni *et al.* (2003).

**A**     *Deparaffinize and hydrate:*

- i. Fisher Protocol Safeclear II (Fisher Scientific, Fair Lawn, NJ) repeat 3 times, using fresh solution, for 5 minutes each
- ii. 100% Ethanol for 1 minute. Repeat once, using fresh solution, for 1 minute
- iii. 70% Ethanol for 1 minute. Repeat once, using fresh solution, for 1 minute
- iv. 30% Ethanol for 1 minute. Repeat once, using fresh solution, for 1 minute
- v. DI water for 1 minute. Repeat once, using fresh solution, for 1 minute

**B**     *Stain:*

<i>Solution</i>	<i>Time</i>
Alcian-Blue solution	5min
Running tap water	3min
DI water	Rinse
Periodic Acid solution	10min
Running tap water	3min
DI water	Rinse
Schiffs reagent*	15min
Running tap water	3min
DI water	Rinse
Hematoxylin**	20sec
Running tap water	3min
DI water	Rinse

\*Schiffs Reagent (Sigma-Aldrich, St Louis, MO)

\*\*Hematoxylin Gill 3X (Fisher Scientific, Kalamazoo, MI)

***C*** ***Rehydrate and clear:***

- i. DI water for 1 minute. Repeat once, using fresh solution, for 1 minute
- ii. 30% Ethanol for 1 minute. Repeat once, using fresh solution, for 1 minute
- iii. 70% Ethanol for 1 minute. Repeat once, using fresh solution, for 1 minute
- iv. 100% Ethanol for 1 minute. Repeat once, using fresh solution, for 1 minute
- v. Fisher Protocol Safeclear II (Fisher Scientific, Fair Lawn, NJ) repeat 3 times, using fresh solution, for 5 minutes each

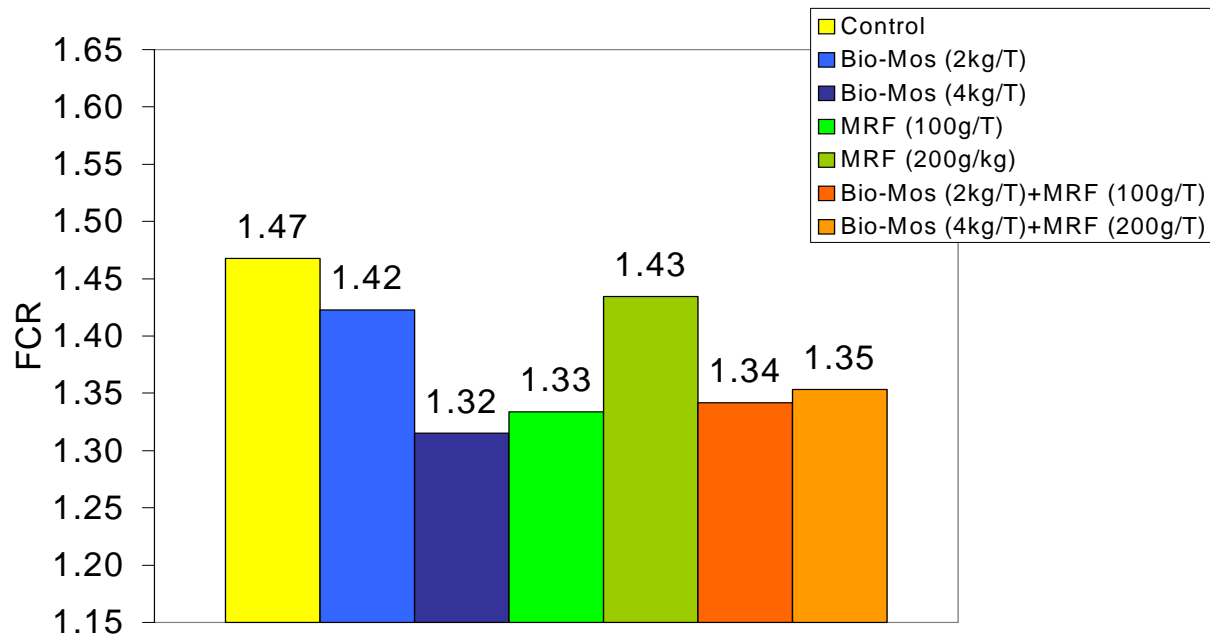
***D*** ***Mounting:***

Mount the slide (Fisher Protocol Mounting Media; Fisher Scientific, Kalamazoo, MI) and allow drying for 2 hours.

### 3.2) Results:

#### 3.2.1. Feed conversion ratios

Bird and feed intake was recorded throughout the study. Feed conversion ratios were calculated and are presented in Figure 3.2.1.



**Figure 3.2.1. Effect of dietary inclusion of Bio-Mos and mannan rich fraction, alone or in combination, on the FCR<sup>1</sup> of broilers at 14 days of age**

<sup>1</sup>FCR refers to feed conversion ratio

**Table 3.2.1. Feed conversion ratio values obtained by the broilers for the respective treatments, as well as the statistical analysis results**

Treatment	Feed Conversion Ratio
Control	1.47 a
Bio-Mos (2kg/T)	1.42 ab
Bio-Mos (4kg/T)	1.32 b
MRF (100g/T)	1.33 ab
MRF (200g/kg)	1.43 ab
Bio-Mos (2kg/T)+MRF (100g/T)	1.34 ab
Bio-Mos (4kg/T)+MRF (200g/T)	1.35 ab
P-Value	0.2149

The p - value indicated was obtained using the stringent Duncan's Multiple Range Test. However, when a Fisher Test was applied, FCR values without a common letter differ significantly ( $p=0.0327$ )

The feed conversion ratios revealed that the control group had the poorest FCR, while the Bio-Mos (4kg/T) treatment was able to attain the best FCR value for this experiment. As indicated in Table 3.2.1., these two treatments also differed significantly. The differences observed for the FCR values between Bio-Mos (4kg/T), MRF (100g/T), Bio-Mos (2kg/T) + MRF (100g/T) and Bio-Mos (4kg/T) + MRF (100g/T) were small and statistically not significant. MRF (200g/T) and Bio-Mos (2kg/T) had similar FCR values, and it appeared as if the FCR's of these two treatments were less favourably affected when compared to the other treatments.

### 3.2.2. Histology

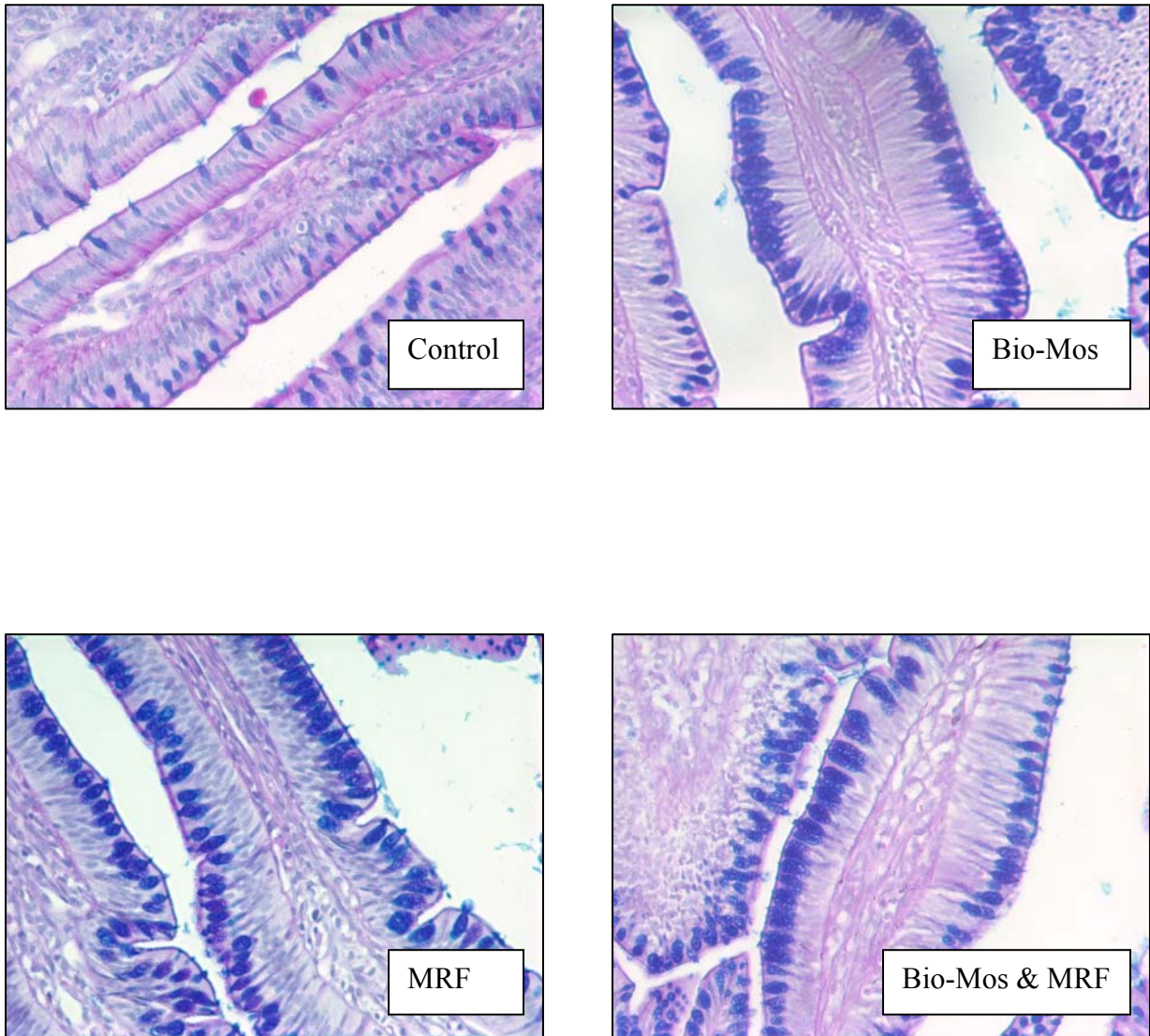
### 3.2.2.1. Villus height, villus width, crypt depth and muscle thickness

**Table 3.2.2.1. Summary of villi measurements ( $\mu\text{m}$ ), including villi height (VH), villi width (VW), crypt depth (CD), muscularis thickness (MT) and villi height to crypt depth ratio (VH: CD)**

Treatment	V H	V W	C D	M T	VH:CD
Control	417.44	123.70	86.34	337.34	4.83
Bio-Mos (2kg/T)	413.04	126.08	97.28	341.92	4.25
Bio-Mos (4kg/T)	403.67	128.00	90.96	322.05	4.44
MRF (100g/T)	406.25	118.00	85.04	325.25	4.78
MRF (200g/T)	411.86	132.35	91.88	331.62	4.48
Bio-Mos (2kg/T) + MRF (100g/T)	423.03	124.29	92.29	338.48	4.58
Bio-Mos (4kg/T) + MRF (200g/T)	431.71	137.42	93.67	340.47	4.61
SED	20.76	12.46	3.836	28.31	
P- value	0.386	0.220	0.910	0.731	

From the slides stained with the H&E stain, measurements were made to determine the given parameters. No statistical differences were found for any of these measurements between the control treatment and any of the other treatments or between the various differences. This indicated that there were no changes in villi morphology between treatments, and as all treatment birds had similar villi morphology, there should have been similar absorptive surface area in the small intestine of all birds across treatments.

3.2.2.2. Goblet Cell measurements



**Figure 3.2.2.1. Pictures taken at 400 X magnifications illustrating the differences observed between treatments for both goblet cell size and density**

From Figure 3.2.2.1. a lower number of goblet cells, as well the smaller size of the goblet cells in the control treatment can clearly be observed when compared to the rest of the treatment birds. A summary of the actual measurements taken of the goblet cells can be seen in Table 3.2.2.2.

**Table 3.2.2.2. Measurements representing goblet cell (GC) size ( $\mu\text{m}^2$ ) as well as goblet cell (GC) density (number goblet cells per  $100\mu\text{m}^2$ )**

Treatment	Average GC size	Average GC Density
Control	47.48 a	14.10 a
Bio-Mos(2kg/T)	77.77 b	20.50 b
Bio-Mos(4kg/T)	75.58 b	19.41 b
MRF(100g/T)	76.49 b	19.90 b
MRF(200g/T)	83.36 b	20.00 b
BM(2kg/T)+MRF(100g/T)	77.11 b	20.76 b
BM(4kg/T)+MRF(200g/T)	77.89 b	19.83 b
Standard Error Difference	2.198	1.196
P-Value	0.0015	0.0066

Values within a column with a common letter, a-b, are not significantly different

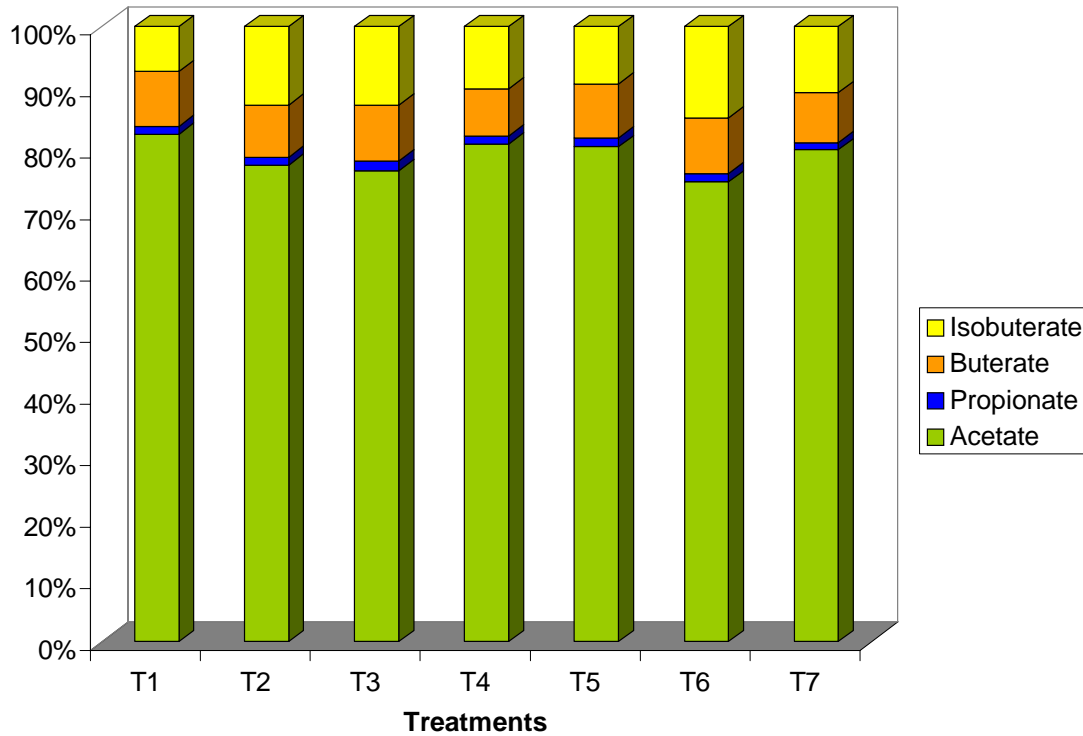
The measurements showed that the goblet cell size as well as the goblet cell density of the treatment birds was much greater than that observed in the control birds. These differences were also statistically significant ( $P < 0.01$ ). No statistical differences were found between the treatment groups. Variance homogeneity of the measurements was tested with the F-Max test, which revealed variances to be homogenous at  $\alpha = 0.05$ ;  $t = 7$  and  $v = 2$ ; value of 128 obtained was smaller than the critical value of 333.

### 3.2.3) VFA Analysis

**Table 3.2.3.1. VFA (mM) results measured from the caeca contents of birds for each of the treatments**

VFA	Control	Bio-Mos (2kg/T)	Bio-Mos (4kg/T)	MRF (100g/T)	MRF (200g/T)	BM(2kg/T)+ MRF(100g/T)	BM(4kg/T)+ MRF(100g/T)	P-value
Acetate	20.87	19.94	19.80	19.13	20.58	19.91	18.78	0.9379
Propionate	0.33	0.33	0.42	0.30	0.36	0.36	0.26	0.7166
Buterate	2.25	2.18	2.34	1.83	2.25	2.42	1.92	0.8388
Isobuterate	1.85	3.28	3.30	2.39	2.39	3.95	2.53	0.6269

No statistically significant values



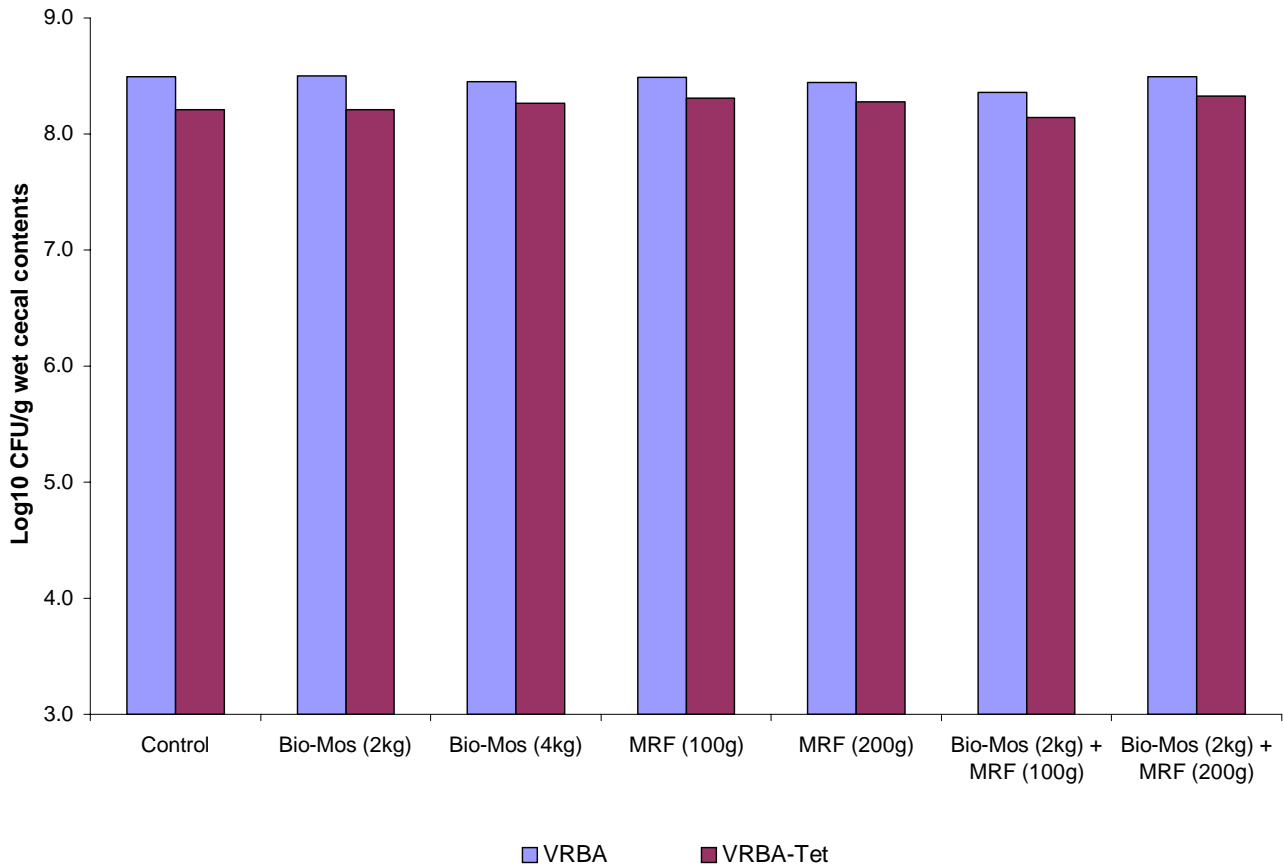
**Figure 3.2.3.1. Effect of dietary inclusion of Bio-Mos and mannan rich fraction, alone or in combination, on volatile fatty acids composition in the caeca contents of broilers**

- T1: Negative control – no additives**
- T2: Bio-Mos (2kg/T)**
- T3: Bio-Mos (4kg/T)**
- T4: MRF (100g/T)**
- T5: MRF (200g/T)**
- T6: Bio-Mos (2kg/T) + MRF (100g/kg)**
- T7: Bio-Mos (4kg/T) + MRF (200g/kg)**

Table 3.2.3.1 shows the VFA numbers as average percentage per treatment. The VFA analysis was done on the caeca contents of the chickens. As can be observed in the graph (see Figure 3.2.3.1), the VFA profile proved to be similar for all the different treatments. Statistical analysis also revealed no significant differences between the birds on a treatment diet and control birds or birds fed different treatment diets.



### 3.2.4: Antibiotic resistance amongst coliform populations



**Figure 3.2.4.1. Antibiotic resistant populations as measured across treatments in comparison to total amount of coliforms present**

It was hypothesised that the antibiotic resistance of bacteria found in the caeca of yeast cell wall fed birds would be lower than that of bacteria found in the control fed birds. Results from the antibiotic resistance calculations indicated that there was a high level of antibiotic resistance in the *coliform*, but according to this trial the level did not change for birds fed different years cell wall preparations. No significant difference were observed between the number of coliforms growing on a tetracycline containing VRBA plate after the colonies were transferred VRBA plate containing no form of added antibiotic (see Table 3.2.4.1.)

**Table 3.2.4.1. Antibiotic resistance measured as the number of *coliform* colonies that grow on a Violet Red Bile Agar (VRBA) plate and the number of colonies that survives when transferred to a tetracycline containing VRBA plate (30 mg/kg)**

Treatment	VRBA (Log <sub>10</sub> CFU/ml)	VRBA-Tet(Log <sub>10</sub> CFU/ml)
Control	8.50	8.21
Bio-Mos (2kg)	8.50	8.21
Bio-Mos (4kg)	8.45	8.26
MRF (100g)	8.49	8.31
MRF (200g)	8.45	8.28
Bio-Mos (2kg) + MRF (100g)	8.36	8.14
Bio-Mos (2kg) + MRF (200g)	8.49	8.33
P-value	0.9423	0.7655

### 3.3) Discussion

A poultry study was conducted to investigate the effect of Bio-Mos with or without the addition of a soluble mannan product (fed as mannan rich fraction) on the intestinal health and performance of broiler chickens. The objective of this study was to determine if adding Bio-Mos or a soluble mannan product, or a combination of both, could affect the intestinal tissue morphology as well as certain performance parameters over the 15-day study period.

Feed conversion ratios were measured for the individual treatments and showed small but not significant differences between the various treatments. The FCR calculations indicated that the control birds had the poorest FCR while Bio-Mos and MRF or the combination of the two was capable of affecting the FCR favourably. An observation made from these calculations was that there seemed to be a dose response amongst the MRF treatments, as MRF influenced the FCR values less favourable when it was included at a higher dose (200g/T) as opposed to the lower MRF inclusion (100g/T), which was the second best FCR value for this trial. No significant differences were found in terms of weight and feed intake for the different treatments.

VFA content of the caeca was measured as it was hypothesised that the VFA profile would differ between the yeast cell wall preparation supplemented and the unsupplemented treatment, based on the digestion of the yeast products. VFA results did not show any specific difference for the VFA profile that was observed across the treatments. Antibiotic resistance calculations also did not indicate that there was a difference in the level of resistance observed for any of the individual treatments. This is possibly an effect that can only be observed in studies conducted over a longer study period.

The most interesting results came from the histology section of this trial, justifying a more in-depth discussion.

In order to fully understand the results found in the histology section it is necessary to have a better understanding of the goblet cell and the way it functions.

Goblet cells can be found in the crypts as well as on the villi of the intestinal tract (Padykula, 1977). Goblet cells are produced in the crypts of the intestinal tract and over a period of about 3 days the goblet cells move up along the sides of the villi, a process referred to as migration, towards the villi tip where they will eventually be sloughed and released into the intestinal lumen (Uni *et al.*, 2003). These goblet cells are replaced in a continuous process (Uni *et al.*, 2003). The main function of the goblet cell is the production of mucus, which forms a protective layer on the villus and gut mucosa (Padykula, 1977). The secreted mucus comprises mostly of mucin glycoproteins and has several different functions (Smirnov *et al.*, 2004). Smirnov *et al.* (2006) stated, “mucin consists of a peptide backbone with attached polysaccharide chains”. Most of the mucus production and mucus release occurred within the second and third day of the goblet cell’s life cycle (Padykula, 1977).

The thickness of the mucus layer could be described as the difference between the amount of mucus released and the rate of mucus degradation, which take place as a result of enzymatic activity and physical abrasion (Smirnov *et al.*, 2004). Apart from the protective properties against direct bacterial adhesion, mucus was found to also assist with transportation between the lumen contents and the epithelial cells; formed an environment in which certain digestive processes could occur; and served as lubrication against irritants such as bile salts (Smirnov *et al.*, 2004). Uni *et al.*, (2003) and co-workers reported an additional function. They found that mucin played an important role in cation absorption such as  $\text{Ca}^{2+}$ , which first binds to anions in goblet cell mucin before it can be absorbed by the enterocytes.

Cytokines, bacterial products, as well as other growth factors, regulates the mucin genes at a transcriptional level (Smirnov *et al.*, 2004). These researchers also calculated the capacity for mucin synthesis by determining the mucin mRNA expression and the actual amount produced by measuring the mucus concentration. In a study conducted on fasted

birds, enhanced mRNA expression for mucin synthesis together with up-regulation of intestinal transporters and enzymes was observed (Smirnov *et al.*, 2004).

Experiments conducted involving fasting of chickens for specified periods of time resulted in enlarged goblet cells as well as an increase in the density of goblet cells (Uni *et al.*, 2003). Enlarged goblet cells were found to have an enhanced ability to store mucus (Smirnov *et al.*, 2005). This indicated possible evidence of a superior mucus secretion response (Uni *et al.*, 2003). Similarly, enlarged goblet cells were observed in chickens receiving feed supplemented with the probiotic *Lactobacillus* and *Bifidobacterium* species (Smirnov *et al.* 2005). The microbial population composition of the intestine plays an important role in mucin degradation, as some microbial species, such as *Bifidobacterium* species, possess what is referred to as mucin-degrading glycosidase and glycosulfatases.

As described, under section 3.1.2, the ileum was used for all the histology work done for this trial, in order to establish a point for comparison. Villi height and width, crypt depth and muscle thickness was determined but no differences were found between the negative control and the treatment groups, or even between the different treatments. From these results it was concluded that, for this specific trial, the cell wall preparations did not have any effect on the villus size, crypt depth or muscularis thickness. A second set of slides were cut from the ileum of the same treatment birds and stained with an Alcian Blue/PAS stain. This stain made it possible to evaluate the goblet cells. The size of the goblet cells as well as the density of the goblet cells were determined for each bird and the results showed a statistically significant difference between the goblet cells of the negative control treatment and the birds receiving the cell wall preparations. The measured difference showed that the yeast cell wall preparation fed birds had larger goblet cells and also a higher density of goblet cells. Measurements made for the villi as well as crypt depth and muscularis thickness revealed that all of the villi were of similar size and results obtained for the goblet cells can thus not be related to stunted villi growth or abnormal morphology of the intestine.

From the research conducted by Smirnov *et al.*, (2005); Smirnov *et al.*, (2004) and Uni *et al.*, (2003) the results obtained in this study could possibly be explained as follows: Mucus is reported to function as a protective barrier against enteropathogens. At the same time mucus was also found to facilitate the absorption of certain minerals, and importantly, nutrients have to cross this layer of mucus in order to reach the enterocytes for absorption. If this mucus layer is too thick it could act as a barrier to nutrient absorption. The enlargement of the goblet cells possibly indicated a greater storage capacity for mucin in the goblet cell together with a superior mucus secretion response, but did not necessarily mean consistent increased mucus secretion. Yeast cell wall preparations increased the goblet cell size as well as the density of goblet cells. This could be interpreted as change in the gut morphology, which improves the capacity of the gastrointestinal tract to respond to pathogenic insult, by rapid response and increased mucus secretion. However, while the pathogenic insult is minor, preference was given to the absorption of nutrients by storing the excess mucin in the goblet cells, instead of secreting it. Due to time restrictions no measurements were made to determine thickness of the mucus layer and all assumptions made is theoretical only, based on goblet cell size and the feed conversion ratio's that were observed in this trial.

Smirnov *et al.* (2004) reported a lack of information on the control mechanisms for mucin synthesis and secretion as well as response to dietary changes and the research that has been conducted appears to be limited to only a few research groups. More research is needed in order to fully understand the significance of the results obtained in this trial.

### 3.4) Conclusion:

Results from this section indicated that cell wall preparations are capable of changing the morphology of the small intestine. The addition of cell wall and soluble mannan preparations lead to an increase in both goblet cell size and goblet cell density. This in turn means greater protection of the epithelial lining of the small intestine against pathogenic insult, harsh digestive enzymes as well as abrasive feed components.

The data also showed that Bio-Mos as well as soluble mannan affects the feed conversion ratio of broiler chickens in a favourable manner.

## Chapter 4

### General conclusion

Although it was not possible to get the pathogen assay on a fully functional level in the given time period, several advances were made in identifying and improving some of the key influencing factors for this model. It is difficult to make any suggestions for future alterations to this model as all of the previous advances were made on a trial and error basis.

Based on the results for the histology work done in this study, there is a large quantity of research that has to be done in terms of gaining a better understanding behind the obvious effect that the cell wall preparations had on the intestinal lining tissue. There are several questions that have to be answered to fully understand the results, such as why the intestinal tissue responds to cell wall preparations in this manner, and at what level of inclusion this effect would be most pronounced in the chicken. Further investigations could also include determining whether or not this effect on the goblet cells is only visible when the treatments are applied from day one onwards, and if not, how long it will take before the tissue cells respond to the cell wall preparations included in the diet, seeing that the cell turnover in intestine is rapid. It might also be applicable in such a case to try and measure the thickness of the mucus layer in the measured area. Another area for investigation could be to determine if the goblet cells along the entire length of the small intestinal tract respond in a similar manner, as these reported measurements were restricted to the ileum only. The small intestine is a very important site for nutrient absorption and if the health of the small intestine can be improved, the nutrients of the feed can be utilized in a more efficient way, which allows for more economically feasible production.



## Chapter 5

### References

- Amit-Romach, E., Sklan, D. and Uni, Z. 2004. Microflora ecology of the chicken intestine using 16S ribosomal DNA primers. *Journal of Poultry Science*. Vol. 83:1093 – 1098.
- Baily, J.S., Cason, J.A. and Cox, N.A. 1998. Effect of salmonella in young chicks on competitive exclusion treatment. *Journal of poultry science*. Vol 77:394 – 399.
- Chambers, J.R., Bisailon, J.-R., Labbe', Y., Poppe, C. and C. F. Langford, C.F. 1998. Salmonella prevalence in crops of Ontario and Quebec broiler chickens at slaughter. *Journal of Poultry Science*. Vol 77: 1497-1501.
- Collett S.R., 2004. Controlling gastrointestinal disease to improve absorptive membrane integrity and optimise digestion efficiency. *Interfacing immunity, gut health and performance*. Ed. Tucker L.A. and Taylor-Pickard, J.A. Nottingham University Press. 77-88.
- Corless, J. 2003. Five applications for mannan oligosaccharides in ruminants and pre-ruminants. *Feeding Times*. Vol.8 no2.
- Corrier, D.E., Byrd, J.A., Hume, M.E., Nisbet, D.J. and Stanker, L.H. 1998. Effect of simultaneous or delayed competitive exclusion treatment on the spread of salmonella in chicks. *Journal of Applied Poultry Research*. Vol. 7:132-137.
- Corrier, D.E, Byrd, J.A., Hargis, B.M, Hume, M.E., Bailey, R.H. and Stanker, L.H.1999. Presence of salmonella in the crop and caeca of broiler chickens before and after pre-slaughter feed withdrawal. *Journal of Poultry Science*. Vol 78:45-49.

- Cotter, P.F., Sefton, A.E. and Lilburn, M.S. 2002. Manipulating the immune system of layers and breeders: Novel applications of manno oligosaccharides. Proceedings of Alltech's 18th Annual Symposium. Ed. Jacques, K.A. and Lyons, T.P. Nottingham University Press: 21-28
- Davies, R.H. and Breslin, M.F. 2003. Observations on the distribution and persistence of salmonella enterica serovar enteritidis phage type 29 on a cage layer farm before and after the use of competitive exclusion treatment. British Poultry Science. Vol. 44:551-557.
- Davis, M.E., Maxwell, C.V., Brown, D.C., de Rodas, B.Z., Johnson, Z.B.,Kegley, E.B., Hellwig, D.H. and Dvorak, R.A. 2002. Effect of mannan oligosaccharides and (or) pharmacological additions of coppersulfate on growth performance and immunocompetence of weanling and growing/ finishing pigs. Journal of Animal Science. 80:2887- 2894.
- Davis, M.E., Brown, D.C., Maxwell, C.V., Johnson, Z.B., Kegley, E.B. and Dvorak, R.A. 2004. Effect of phosphorylated mannans and pharmacological additions of zinc oxide on growth and immunocompetence of weanling pigs. Journal of Animal Science. 82:581-587.
- Devegowda, G. 2004.Fertility and hatchability- the role of MOS. International Hatchery Practice. Vol.18 no.7:15-17.
- Dibner, J.J. and Richards, J.D. 2005. Antibiotic growth promoters in agriculture: History and mode of action. Journal of Poultry Science. 84: 634 – 643.
- Doyle, M.P and Erickson, M.C. 2006. Reducing the Carriage of Foodborne Pathogens in Livestock and Poultry Journal of Poultry Science. Vol 85:960-973.

- Droleskey, R.E., Oyoyo, B.A., Hargis, B.M., Corrier, D.E. and DeLoach, J.R. 1994. Effect of mannose on salmonella typhimurium – mediated loss of mucosal epithelial integrity in cultured chick intestinal segments. *Avian Diseases*. 38: 275-281.
- Fernandez, F., Hinton, M. and Van Gils, B. 2000. Evaluation of the effect of mannan oligosaccharides on the competitive exclusion of salmonella enteritidis colonization in broiler chicks. *Avian Pathology*. Vol. 29:575-581.
- Firon, N., Ofek, I. And Sharon, N. 1984. Carbohydrate binding sites of the mannose specific fimbrial lectins of enterobacteria. *Infection and immunity*. Mar:1088-1090
- Firon, N., Ashkenazi, S., Mirrelman, D., Ofek, I. and Sahron, N. 1987. Aromatic alpha-glycosides of mannose are powerful inhibitors of the adherence of Type 1 fimbriated *Escherichia coli* to yeast and intestinal epithelial cells. *Infection and Immunity*, Feb:472-476.
- Flickinger, E.A. 2003. Oligosaccharides as functional foods: can we improve gut health? *Proceedings of Alltech's 19th Annual Symposium*. Ed. Jacques, K.A. and Lyons, T.P. Nottingham University Press: 345-354.
- Franklin, S.T., Newman, M.C., Newman, K.E. and Meek, K.I. 2005. Immune parameters of dry cows fed mannan oligosaccharide and subsequent transfer of immunity to calves. *Journal of Dairy Science*. Vol.88: 766-775.
- Grieshop, C.M. 2003. Diet may affect nutrition, immune system of pets. *Feedstuffs*. Vol.76 no.26:1-5
- Guard-Petter, J. 2001. The chicken, the egg and *salmonella enteritidis*. *Environmental microbiology*. Vol.3 no.7:421-430.

- Heinrichs, A.j., Jones, C.M. and Heinrichs, B.S. 2003. Effects of mannan oligosaccharide or antibiotics in neonatal diets and health and growth of dairy calves. *Journal of Dairy Science*. Vol. 86:4064-4069.
- Hofacre, C.L., Beacorn, T., Collette, S. and Mathis, G. 2003. Using competitive exclusion, mannan-oligosaccharides and other intestinal products to control necrotic enteritis. *Journal of Applied Poultry research*. Vol.12:60-64.
- Hooge, D.M. 2004a. Meta analysis of broiler chicken pen trials evaluating dietary mannan oligosaccharide, 1993-2003. *International Journal of Poultry Science*. Vol 3:163-174.
- Hooge, D.M. 2004b. Turkey pen trials with dietary mannan oligosaccharides: Meta analysis, 1993-2003. *International Journal of Poultry Science* Vol.3 no.3: 179-188.
- Huang, D.S, Li, D.F., Xing, J.J., Ma, Y.X., Li, Z.J., and Lv, S.Q. 2006. Effects of feed particle size and feed form on survival of *Salmonella typhimurium* in the alimentary tract and caecal *S. typhimurium* reduction in growing broilers. *Journal of Poultry Science*. Vol 85: 831-836
- Hume, M.E., Corrier, D.E., Nisbet, D.J. and DeLoach. 1996. Reduction of salmonella crop and caecal colonization following by a characterized competitive exclusion culture in broilers during grow-out. *Journal of Food Protection*. Vol. 61:688-693.
- Hume, M.E., Corrier, D.E., Nisbet, D.J. and DeLoach. 1998. Early salmonella challenge time and reduction in chick caecal colonization following treatment by a characterized competitive exclusion culture. *Journal of Food Protection*. Vol. 61:673-676.
- Iji, P.A. and Tivey, D.R. 1998. Natural and synthetic oligosaccharides in broiler chicken diets. *World's Poultry Science Association*. 54:129-143.

- Iji, P.A., Saki, A.A. and Tivey, D.R. 2001. Intestinal structure and function of broiler chickens on diets supplemented with a mannan oligosaccharide. *Journal of the Science of Food and Agriculture*. Vol. 81:1186-1192.
- Jones, F.T. and S. C. Ricke S.C. 2003. Observations on the history of the development of antimicrobials and their use in poultry feeds. *Journal of Poultry Science*. Vol. 86:613 – 617.
- Kelly, D. 2004. Regulation of gut function and immunity. *Interfacing immunity, gut health and performance*. Ed. Tucker L.A. and Taylor-Pickard, J.A. Nottingham University Press:
- Kocher, A., Canolly, A., Zawadzki, J. and Gallet, D. 2004a. The challenge of finding alternatives to antibiotic growth promoters. *International Society for Animal Hygiene-Saint Malo 2004*:227-229
- Kocher, A., Spring, P. and Hooge, D.M. 2004b. Rabbits may respond positively to dietary MOS. *Feedstuffs*. Vol. 76 no. 22:1-3
- Kocher, A. and Tucker, L. 2005. The gut health response to dietary Bio-Mos: Effects on gut microbiology, intestinal morphology and immune response. *Proceedings of Alltech's 21st Annual Symposium*. Ed. Jacques, K.A. and Lyons, T.P. Nottingham University Press:383-388.
- Le Mieux, F.M., Southern, L.L. and Bidner, T.D. 2003. Effect of mannan oligosaccharides on growth performance of weanling pigs. *Journal of Animal Science*. 81:2482-2487.
- Lipke, P.N. and Ovalle, R. 1998. Cell wall architecture in yeast: New structure and new challenges. *Journal of Bacteriology*. Vol.8 no.15:3735-3740.

- Miles, R.D., Butcher, G.D., Henry, P.R. and Littell, R.C. 2006. Effect of antibiotic growth promoters on broiler performance, intestinal growth parameters, and quantitative morphology. *Journal of Poultry Science*. Vol 85: 476 - 485.
- Moran, C.A. 2004. Functional components of the cell wall of *Saccharomyces cerevisiae*: applications for yeast glucan and mannan. *Proceedings of Alltech's 20th Annual Symposium*. Ed. Jacques, K.A. and Lyons, T.P. Nottingham University Press: 283-292.
- Murase, T., Yamada, M., Muto, T., Matsushima, A., Yamai, S. 2000. Faecal excretion of *Salmonella enterica* serovar Typhimurium following a food borne outbreak. *Journal of Clinical Microbiology*. Vol 38. No 9: 3495 – 3497.
- Newman, K.E. 1999. Mannan oligosaccharide - a review of scientific data on this novel ingredient. *Concepts of pig Science 1999. The First Annual Turtle Lake Pig Science Conference*. Ed. Cole, D.J.A. and Lyons, T.P. Nottingham University Press:47-52.
- Newman, K.E. 2002. Bio-Mos: Opportunities for improving calf production? *Feed Compounder*. Jul: 17-20.
- Newman, K.E. 2005. How biotechnology is affecting the reality of Animal nutrition. <http://AnimalScience.ag.utk.edu/nutritionconference/proceedings2005/KyleNewman.pdf>
- Newman, M. 2002. Antibiotic resistance is a reality. Novel techniques for overcoming antibiotic resistance when using growth promoters. *Proceedings of Alltech's 18th Annual Symposium*. Ed. Jacques, K.A. and Lyons, T.P. Nottingham University Press: 97-106.

- O'Carra, R. 1998. Oligosaccharides. Petfood Industry. Sept:16-22.
- Ott, E.A. 2002. Mannan oligosaccharides in diets of mares: effects on mares and foals.  
[www.triplecrownfeed.com/docs/Bio-Mos\\_effects.pdf](http://www.triplecrownfeed.com/docs/Bio-Mos_effects.pdf)
- Ott, E.A. 2005. Influence of Bio-Mos, a mannan oligosaccharide supplement, on the immunessystem of the mare and neonatal foal. Proceedings of Alltech's 21st Annual Symposium. Ed. Jacques, K.A. and Lyons, T.P. Nottingham University Press:447-454.
- Oviedo-Rondón, E.O., Hume, M.E., Hernández, C. and Clemente-Hernández, S. 2006. Intestinal microbial ecology of broilers vaccinated and challenged with mixed *Eimeria* species, and supplemented with essential oil blends. Journal of Poultry Science. Vol. 85:854 – 860.
- Oyofa, B.A., De Loach, J.R., Corrier, D.E., Norman, J.O., Ziprin, R.L. and Mollenhauer, H.H. 1989a. Prevention of *Salmonella typhimurium* colonization of broilers with D-mannose. Poultry Science. Vol. 68:1357-1356.
- Oyofa, B.A., Droleskey, R.E., Norman, J.O., Mollenhauer, H.H., Ziprin, R.L., Corrier, D.E. and De Loach, J.R., 1989b. Inhibition by mannose of in vitro colonization of chicken small intestine by *Salmonella typhimurium*. Poultry Science. Vol. 68:1351-1356.
- Oyofa, B.A., De Loach, J.R., Corrier, D.E., Norman, J.O., Ziprin, R.L., and Mollenhauer, H.H. 1989c. Effect of carbohydrates on *Salmonella typhimurium* colonization in broiler chickens. Avian diseases Vol 33:531-534.
- Padykula, H.A. 1977. Chapter 18. Histology, Fourth Edition. Ed. Weiss, L. and Greep, R.O. McGraw-Hill Book Company, NY, USA. 681 – 686.

- Palmu, L. and I. Camelin. 1997. The use of competitive exclusion in broilers to reduce the level of salmonella contamination on the farm and at the processing plant. *Journal of Poultry Science*. Vol 76:1501 - 1505.
- Patterson, J.A. and Burkholder, K.M. 2003. Application of Prebiotics and Probiotics in Poultry Production. *Journal of Poultry Science*. 82: 627 – 631.
- Pettigrew, J.E. and Miguel, J.C. 2003. Meta analysis of the effect of Bio-Mos on nursery pig performance. *Feeding Times*. Vol.8 no.2.
- Pettigrew, J.E., Miguel, J.C. and Carter, S. 2005. Bio-Mos in sow diets: Performance responses and economics. *Proceedings of Alltech's 21st Annual Symposium*. Ed. Jacques, K.A. and Lyons, T.P. Nottingham University Press:213-220.
- Pinheiro, V., Alves, A., Mourão, J.L., Guedes, C.M., Pinto, L., Spring, P. and Kocher, A. 2004. Effect of mannan oligosaccharides on the ileal morphometry and caecal fermentation of growing rabbits. *Proceedings of the 8th World Rabbit Congress-Puebla*:936-941.
- SAS. (1988) *SAS/STAT™. User's guide*. Cary NC:SAS Institute Inc. Release 6.03.
- Santin, E., Maiorka, A. and Macari, M. 2001. Performance and intestinal mucosa development of broiler chickens fed diets containing *saccharomyces cerevisiae* cell wall. *Journal of Applied Poultry Research*. Vol. 10:236-244.
- Sashidhara, R.D. and Devegowda, G. 2003. Effect of dietary mannan oligosaccharide on broiler breeder production traits and immunity. *Poultry Science*. Vol. 82:1319-1325.
- Schneitz, C. and Mead, G. 2000. Competitive exclusion. Ed. Wray, C., Wray, A. *Salmonella in domestic Animals*. CABI Publishing, NY. 18:301-322



- Shane, S.M. 2001. Mannan oligosaccharides in Poultry nutrition: mechanisms and benefits. Proceedings of Alltech's 17th Annual Symposium. Ed. Jacques, K.A. and Lyons, T.P. Nottingham University Press: 65-77.
- Sharon, N. and Lis, H. 1993. Carbohydrates in cell recognition. Scientific American. Vol. 268:82-89.
- Sims, M.D., Dawson, K.A., Newman, K.E., Spring, P. and Hooge, D.M. 2004. Effects of dietary mannan oligosaccharide, bacitracin methylene disalicylate, or both on the live performance and intestinal microbiology of turkeys. Poultry Science. Vol.83:1148-1154.
- Sklan, D. 2004. Early gut development: the interaction between feed, gut health and immunity. Interfacing immunity, gut health and performance. Ed. Tucker L.A. and Taylor-Pickard, J.A. Nottingham University Press:
- Smirnov, A., Sklan, D. and Uni, Z. 2004. Mucin dynamics in the chick small intestines are altered by starvation. Journal of Nutrition. Vol. 134:736 – 742.
- Smirnov, A., Perez, R., Amit-Romach, E., Sklan, D. and Uni, Z. 2005. Mucin dynamics and microbial populations in the chicken small intestine are change by dietary probiotic and antibiotic growth promoter supplementation. Journal of Nutrition. Vol. 135:187 – 192.
- Smirnov, A., Tako, E., Ferket, P.R. and Uni, Z. 2006. Mucin gene expression and mucin content in the chicken intestinal goblet cells are affected by In Ovo feeding of carbohydrates. Journal of Poultry Science. Vol. 85: 669 – 673.
- Spring, P. 1999. Mannan oligosaccharide as an alternative to antibiotic use in Europe. Zootechnica International. Aug:38-41.

- Spring, P., Wenk, C., Dawson, K.A. and Newman, K.E. 2000. the effects of dietary mannan oligosaccharides on caecal parameters and the concentration of enteric bacteria in the caeca of salmonella challenged broiler chicks. *Poultry Science*. Vol. 79:205-211.
- Spring, P. 2003. Using mannan oligosaccharide in modern nutrition. *Asian Aquaculture Magazine*. Apr;30-31.
- Stavric, S., Gleeson, T.M., Blanchfield, B. and Pivnick, H. 1987. Role of adhering micro flora in competitive exclusion of salmonella from young chicks. *Journal of Food Protection*. Vol. 50 no. 11: 928-932.
- Staykov, Y., Spring, P. and Denev, S. 2005. Influence of dietary Bio-Mos on growth, survival and immune status of rainbow trout (*salmo gairdneri irideus* G.) and common carp (*cyprinus carpio* L.). *Proceedings of Alltech's 21st Annual Symposium*. Ed. Jacques, K.A. and Lyons, T.P. Nottingham University Press:333-344.
- Stern, N.J., Cox, N.A., Bailey, J.S., Berrang, M.E. and Musgrove, M.T. 2001. Comparison of mucosal competitive exclusion and competitive exclusion treatment to reduce *Salmonella* and *Campylobacter* spp. colonization in broiler chickens. *Journal of Poultry Science*. Vol 80:156-160.
- Sun, X., McElroy, A., Webb, Jr., K.E., Sefton, A.E. and Novak, C. 2005. Broiler performance and intestinal alterations when fed drug-free diets. *Journal of Poultry Science*. Vol. 84: 1294 – 1302.
- Swanson, K.S., Grieshop, C.M., Flickinger, E.A., Bauer, L.L., Healy, H., Dawson, K.A., Merchen, N.R. and Fahey, G.C. Jr. 2002. Supplemental fructooligosaccharides and mannanoligosaccharides influence immune function, ileal and total tract nutrient

digestibilities, microbial populations and concentrations of protein catabolites in the large bowel of dogs. *Journal of Nutrition*. Vol.132: 980-989.

Tako, E., Ferket, P.R. and Uni, Z. 2004. Effects of in ovo feeding of carbohydrates and  $\beta$ -Hydroxy- $\beta$ -Methylbutyrate on the development of chicken intestine. *Journal of Poultry Science*. Vol.83:2023 – 2028.

Thompson, K.L. and Applegate, T.J. 2006. Feed withdrawal alters small-intestinal morphology and mucus of broilers. *Journal of Poultry Science*. Vol. 85:1535 – 1540.

Thorns, C.J. and Woodward, M.J. 2000. Fimbriae of salmonella. Ed. Wray, C., Wray, A. *Salmonella in domestic Animals*. CABI Publishing, 10E40th Str suit 3203 NY, NY. 3:35-56

Uni, Z., Ganot, S. and Sklan, D. 1998. Posthatch development of mucosal function in the broiler small intestine. *Journal of Poultry Science*. Vol. 77: 75 – 82.

Uni, Z., Smirnov, A. and Sklan, D. 2003. Pre- and Posthatch development of goblet cells in the broiler small intestine: Effects of delayed access to feed. *Journal of Poultry Science*. Vol. 82: 320 – 327.

Van Immerseel, F., Cauwerts, K., Devriese, L.A., Haesebrouck, F. and Ducatelle, R. 2002. Feed additives to control salmonella in Poultry. *Worlds Poultry Science Journal*. Vol. 58:501-511.

Verwoerd, D.J., Olivier, A.J., Henton, M.M. and van der Walt, M. 1998. *Feed Compounder*, Nov:22-23.

- Waldroup, P., Fritts, C.A. and Yan, F. 2003. Utilization of Bio-Mos mannan oligosaccharide and Bioplex copper in broiler diets. *International Journal of Poultry Science*. Vol.2 no.1:44-52.
- Zaghini, A., Martelli, G., Roncada, P., Simioli, M. and Rizzi, L. 2005. Mannan oligosaccharides and aflatoxin B1 in feed for laying hens: effects on egg quality, aflatoxins B1 and M1 residues in eggs and aflatoxin levels in liver. *Poultry Science*. Vol. 84:825-832.
- Zhang, A.W., Lee, B.D., Lee, S.K., Lee, K.W., An, G.H., Song, K.B. and Lee, C.H. 2005. Effects of yeast (*Saccharomyces cerevisiae*) cell components on growth performance, meat quality, and ileal mucosa development of broiler chicks. *Journal of Poultry Science*. Vol. 84:1015 – 1021.

## Appendix

### a) Preparation of Microbial Media:

Brilliant Green Agar (BGA); (Difco, Sparks,MD) with Naladixic Acid (Sigma, St LouisMO)

- *Prepare the BGA* by combining the correct amount of media concentrate and de-ionized water as indicated on the bottle.
- Bring to the boil to dissolve thoroughly.
- Pour into suitable glass bottles and autoclave at 121°C for 20 minutes.
- Allow to cool to 50°C.
- Add Naladixic Acid Stock Solution at an inclusion of 3ml per liter of media.
- Pour the plates in a sterile hood and allow to set, protected from light.
  
- *Prepare the Naladixic Acid Stock Solution* by combining 100ml 0.05M NaOH (Sigma, St Louis MO) and 1g Naladixic Acid.
- Leave on stirring plate for 30min to dissolve completely, covered with aluminum foil.
- Pour into a centrifuge tube using a sterile filter.
- Store in cold room.

Xylose Lysine Deoxycholate (XLD) (Difco, Sparks,MD)

- Combine the correct amount of media concentrate powder and de-ionized water as indicated on the bottle
- Bring media to the boil and allow to boil for 1 minute
- Pour into sterile glass bottle, using a sterile glass funnel.
- Allow to cool to 55°C and pour plates.

Triple Sugar Agar (TSI) (Difco, Sparks,MD)

- Combine the correct amount of media concentrate powder and de-ionized water as indicated on the bottle.
- Bring media to the boil.
- Distribute 12ml each into test tubes.
- Autoclave at 121°C for 20 minutes.
- Allow to cool at an angle, forming a deep butt.

- Place in cold room as soon as it has set.

#### 0.1% Peptone water

- Per liter of de-ionized water, add 10g peptone (Difco, Sparks,MD) and 5g NaCl (Sigma, St Louis MO)
- Stir for 15 minutes on cold plate
- Autoclave at 121°C for 20 minutes
- Pour 80ml each into bladed jars
- Store in cold room

#### Maximum Recovery Diluent (MRD)

- Combine 8.5g NaCl (Sigma, St Louis MO) and 1g peptone (Difco, Sparks,MD) with 1L de-ionized water.
- Stir for 15 minutes on cold plate.
- Pipette out into test tubes, 9ml per tube.
- Autoclave at 121°C for 20 minutes.
- Store in cold room.

#### Rappaport – Vassiliadis R10 Broth (Difco, Sparks,MD)

- Combine the correct amount of media concentrate powder and de-ionized water as indicated on the bottle.
- Heat gently to dissolve
- Pipette out into test tubes, 9ml per tube.
- Autoclave at 116°C for 15 minutes
- Allow to cool, and then place in the cold room.

#### Lactose broth (Difco, Sparks,MD)

- Combine the correct amount of media concentrate powder and de-ionized water as indicated on the bottle.
- Heat gently without boiling.
- When dissolved, pipette out into test tubes, 9ml each.
- Autoclave at 121°C for 20 minutes.
- Allow to cool, and then place in the cold room.

### Violet Red Bile Agar (Difco, Sparks,MD)

- Combine the correct amount of media concentrate powder and de-ionized water as indicated on the bottle
- Bring media to the boil and allow to boil for 1 minute
- Pour into sterile glass bottle, using a sterile glass funnel.
- Allow to cool to 55°C and pour plates.

### Violet Red Bile Agar (Difco, Sparks,MD) with 30ppm tetracycline(Sigma, St Louis MO)

Prepare tetracycline stock solution:

Add 100mg tetracycline to 10ml deionised water

Stir on stirring plate until dissolved

Filter sterilize (0.22µ Fisherbrand filters) into a 15ml sterile centrifuge tube.

Add stock solution at an inclusion of 3ml per liter VRBA.

### b) Preparation of staining solutions

#### Alcian blue solution:

Make up a 3% acetic acid solution from Acetic Acid Glacial (Fisher Scientific, Fair Lawn, NJ).

Combine:        50ml 3% Acetic Acid  
                      0.5g Alcian Blue 8GX (Sigma-Aldrich, St Louis, MO)

#### Periodic Acid Solution:

Combine:        0.5g Periodic Acid (Sigma-Aldrich, St Louis, MO)  
                      50ml De-ionised water