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Effects of Mannanoligosaccharide on Different Cecal Parameters and on Cecal Concentrations of Enteric Pathogens in Poultry

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Dedicated to my parents

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CONTENTS

PART II: Trials **Page**

ABSTRACT

The ability of different enteric pathogens and coliforms to trigger agglutination of a yeast cell wall preparation (MOS), a yeast culture (*Saccharomyces cerevisiae*, NYCC 1026) and haemagglutination was studied. Three strains which agglutinated MOS (*Salmonella typhimurium* 29E, *Salmonella dublin* and *Escherichia coli* 15R) and one non-agglutinating strain (*S. typhimurium* 27A) were then selected as challenge organisms. The effects of dietary MOS on cecal concentrations of these challenge organisms and on activities and concentrations of the cecal microflora were evaluated in chicks under controlled conditions. Furthermore, the effects of MOS on ileal morphology were investigated.

Five of seven strains of *E. coli* and seven of ten strains of *S. typhimurium* and *Salmonella enteritidis* agglutinated MOS and *S. cerevisiae* NYCC 1026. Strains of *Salmonella choleraesuis, Salmonella pullorum* and *Campylobacter* did not lead to agglutination. Agglutination of both yeast products could be inhibited by 25 mM of mannose. All strains that agglutinated the yeast products also triggered haemagglutination. However, haemagglutination of *E. coli* K99 could not be inhibited by mannose. Mannose sensitive agglutination of the yeast products and mannose resistant haemagglutination by *E. coli* K99 suggest that these strains express both a mannose sensitive (type-1) and a mannose resistant (K 99) type of fimbriae.

In a series of three trials in which 3 d old chicks were orally challenged with 10^4 CFU of *S. typhimurium* 29E, birds receiving 4000 ppm of dietary MOS had reduced cecal *S. typhimurium* 29E concentrations (5.40 vs. 4.01 log CFU/g; $P < 0.05$) at d 10. In a second series of three trials with *S. dublin* as challenge organism the number of birds that tested salmonella positive in the ceca at d 10 was lower when MOS was part of the diet (90 % vs. 56 %; $P < 0.05$). Mannanoligosaccharide also reduced the number of birds from which the challenge organism *E. coli* 15R could be recovered (75 % vs. 15 %). In order to test the effect of MOS on concentrations of bacteria that do no express type-1 fimbriae, a challenge trial was conducted with *S. typhimurium* 27A. However, strain 27A did not colonize the birds sufficiently to evaluate if MOS affected its

cecal concentration. Mannanoligosaccharide showed a tendency to reduce the concentrations of cecal coliforms in both series of trials with *S. typhimurium* 29E and *S. dublin*. The statistical analyses combining data from all experiments showed a significant reduction in cecal coliforms (8.80 vs. 8.54 log CFU/g; $P < 0.05$). Mannanoligosaccharide had no effect on cecal concentrations of lactobacilli, enterococci, anaerobic bacteria, lactate, VFA or on cecal pH.

Dietary mannanoligosaccharide led to changes in ileal morphology. Average villi length was increased by 18% ($P < 0.05$) and crypt depth was increased by 22 % ($P < 0.05$) with dietary MOS. Villi width and number of goblet cells were not affected by treatment.

ZUSAMMENFASSUNG

Verschiedene enteropathogene und koliforme Bakterien wurden auf ihre Fähigkeit untersucht, ein Hefezellwandpräparat (MOS) und eine Hefekultur (*Saccharomyces cerevisiae* NYCC 1026) zu agglutinieren und Haemagglutination zu verursachen. Drei agglutinierende Stämme (*Salmonella typhimurium* 29E, *Salmonella dublin* und *Escherichia coli* 15R) und ein nicht-agglutinierender Stamm (*S. typhimurium* 27A) wurden zur weiteren Untersuchung in Tierversuchen ausgewählt. Unter kontrollierten Umweltbedingungen wurde dann die Wirkung von MOS auf die Konzentration dieser Organismen, sowie auf die Aktivität und Konzentration der Dickdarmflora in Küken untersucht. Zusätzlich wurde der Einfluss von MOS auf die ileale Morphometrie getestet.

Fünf von sieben *E. coli* Stämmen und sieben von zehn *S. typhimurium* und *Salmonella enteritidis* Stämmen agglutinierten MOS und *S. cerevisiae* 1026. *Salmonella choleraesuis, Salmonella pullorum* und *Campylobacter* Stämme agglutinierten die Hefeprodukte nicht. Agglutination beider Hefeprodukte konnte mit 25 mM Mannose blockiert werden. Alle Bakterienstämme die Hefeprodukte agglutinierten, führten auch zu Haemagglutination. Im Gegensatz zu Agglutination der Hefeprodukte konnte Haemagglutination von *E. coli* K99 aber nicht mit Mannose blockiert werden. Mannosesensitive Agglutination von Hefeprodukten und mannoseresistente Haemagglutination von *E. coli* K99 deuten darauf hin, dass diese Stämme sowohl mannosesensitive (Typ-1) als auch mannoseresistente K99 Fimbrien exprimieren.

In einer ersten Versuchsreihe, in welcher 3-tägigen Küken 10^4 KBE *S. typhimurium* 29E verabreicht wurden, verminderte MOS Supplementierung (4'000 ppm) die Konzentration von *S. typhimurium* 29E im Blinddarm (5,40 vs. 4,01 log KBE/g; $P < 0,05$) am zehnten Lebenstag. In einer zweiten Reihe mit *S. dublin* Verabreichung reduzierte der MOS-Zusatz den Anteil der Küken, von welchen der verabreichte Salmonellenstamm isoliert werden konnte (90 % vs. 56 %; $P < 0,05$). Zusätzlich reduzierte MOS den prozentualen Anteil der Küken, aus deren Verdauungstrakt *E. coli* 15R kultiviert werden konnte (75 % vs.

15 %). In einem nächsten Versuch wurde der Einfluss von MOS auf die Blinddarmkonzentration eines Bakterienstammes, der keine Typ-1 Fimbrien exprimiert (*S. typhimurium* 27A), untersucht. Stamm 27A kolonisierte die Küken aber nur ungenügend, wodurch eine Aussage über die Wirkung von MOS auf dessen Konzentration unmöglich war. Der Zusatz von Mannanoligosaccharid führte in beiden Versuchsreihen *mit S. typhimurium* 29E und *S. dublin* zu leicht niedrigeren Konzentrationen der koliformen Keime. Die statistische Auswertung über alle Experimente ergab signifikant tiefere Koliformkonzentrationen mit MOS Zusatz (8,80 vs. 8,54 KBE/g; $P < 0,05$). Supplementierung mit MOS hatte keinen Einfluss auf die Konzentration der Laktobazillen, Enterkokken, anaeroben Keime sowie auf Laktat- und flüchtige Fettsäurenkonzentrationen und Dickdarm pH.

Zusatz von MOS führte zu Veränderungen in der ilealen Morphometrie. Darmzotten waren 18 % höher ($P < 0,05$) und Darmkrypten waren 22 % tiefer ($P < 0,05$) mit MOS Zusatz. Darmzottenbreite und Anzahl der Becherzellen wurde durch die Behandlung nicht beeinflusst.

RÉSUMÉ

Cette étude montre la capacité des différentes bactéries entérique pathogènes et coliforme de provoquer l'agglutination d'une préparation de parois cellulaires de levures (MOS: mannan-oligosaccharides), d'une culture de levures (Saccharomyces cerevisiae NYCC 1026) et de provoquer une hémagglutination. Trois souches qui agglutinent le MOS (*Salmonella typhimurium* 29E, *Salmonella dublin* and *Escherichia coli* 15R) et une souches non-agglutinante (*S. typhimurium* 27A) sont ensuite sélectionnées comme micro-organismes infectants. L'effet du MOS dans la ration sur les concentrations caecales de ces micro-organismes et sur l'activité et les concentrations de la microflore caecale est évalué sur des poussins dans des conditions contrôlées. Les effets du MOS sur la morphologie de l'iléon sont également étudiés.

Cinq de sept souches d'*E. coli* ainsi que sept de dix souches de *S. typhimurium* et *Salmonella enteritidis* agglutinent les MOS et *S. cerevisiae* NYCC 1026. Les souches de *Salmonella choleraesuis, Salmonella pullorum* et *Campylobacter* n'ont par contre montré aucune agglutination. L'agglutination des deux produits de levures peut être inhibée par une solution de mannose (25 mM). Toutes les souches qui agglutinent les produits de levures provoquent aussi une hémagglutination. Cependant l'hémagglutination d'*E. coli* soit inhibée par le mannose et que l'hémagglutination de ces même bactéries soit résistante au mannose suggère que ces souches possèdes deux types de cils: un type sensible au mannose (type-1) et un type résistent au mannose (K99).

Dans une série de trois essais, des poussins de trois jours sont infectés par voie orale avec 10^4 CFU de *S. typhimurium* 29E. Les poussins reçoivent 4'000 ppm de MOS dans leur ration ont des concentrations caecales de *S. typhimurium* 29E réduites (4,01 par rapport à 5,40 log CFU/g; $P < 0,05$) à l'âge de 10 jours. Dans une seconde série de trois essais avec S. dublin comme agent infectant, le nombre de poussins contaminés par la salmonelles dans le caecum, à l'âge de 10 jours, est moins élevé lorsque le MOS est inclus dans la ration (90 % par raport à 56 %; $P < 0,05$). Le mannan-oligosaccharide réduit également le nombre de poussin contaminés par *E. coli* 15R après infection

expérimentale (75 % par rapport à 15 %). Afin de tester l'effet du MOS sur les concentrations de bactéries ne possédant pas des cils de Type-1, un essai est conduit avec *S. typhimurium* 27A. Mais cette souches n'a pas suffisamment colonisé les poussins pour pouvoir déterminer si le MOS affecte les concentrations caecales en *S. typhimurium* 27A. L'utilisation du MOS tend à réduire les concentrations de coliformes caecaux dans les deux séries d'essais avec *S. typhimurium* 29E et *S. dublin*. L'analyse statistique combinant tous les essais montre une réduction significative du nombre de coliformes caecaux (8,54 par rapport à 8,80 log CFU/g; $P < 0,05$). Le mannan-oligosaccharide n'a aucun effet sur les concentrations caecales en lactobacilles, entérocoques, bactéries anaérobies, le lactate, le AGV et le pH ceacal.

Le mannan-oligosaccharide induit des changements de morphologie de l'iléon. La longueur moyenne des villosités est augmentée de 18% ($P < 0,05$) et la profondeur des cryptes de 22 % ($P < 0,05$) chez les poussins recevant le MOS dans leur ration. La largeur des villosités et le nombre de cellules caliciformes ne sont pas modifiés par le traitement MOS.

LIST OF TABLES

LIST OF TABLES (continued)

LIST OF FIGURES

ABBREVIATIONS

AGV	Acide Gras Volatile
BGA	Brilliant Green Agar
CE	Competitive Exclusion
CFU	Colony Forming Units
FOS	FructoOligoSaccharide
GI	GastroIntestinal
GOS	GalactoOligoSaccharide
HACCP	Hazard Analysis and Critical Control Points
KBE	Kolonie Bildende Einheit
KFA	KF Streptococcal Agar
LS	LactoSucrose
MacA	Mac Conkey Agar
MOS	MannanOligoSaccharide
NCTC	National Collection of Type Cultures
NYCC	North American Yeast Culture Collection
OD_{660}	Optical Density measured at 660 nm
PAS	Periodic Acid Shiff
PBS	Phosphate Buffered Saline
RCA	Reinforced Clostridial Agar
RoA	Rogosa Agar
TSB	Triptic Soy Broth
VFA	Volatile Fatty Acids
VLA(M)	Viande Levure Agar (Media)
VRBA	Violet Red Bile Agar
WHO	World Health Organization

PART I:

LITERATURE REVIEW

1. INTRODUCTION

The twentieth century has seen important successes in the fight against infectious diseases, especially due to the discovery of antibiotics and other microbial agents. Nevertheless, most industrialized countries are experiencing an increase in reported cases of foodborne disease (Figure 1). Reported cases may represent no more than 10 % of the true number. Buzby and Roberts (1995) estimated the true number of salmonellosis cases in the US to be between 700,000 - 4,000,000 in 1993. They estimated medical costs and lost productivity due to salmonellosis of 0.6 to 3.5 billion dollars.

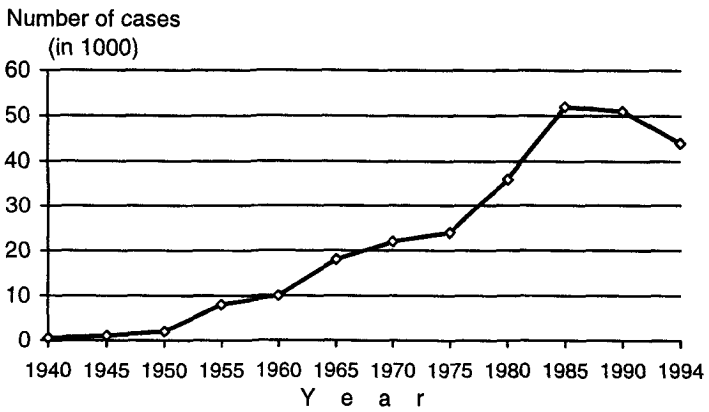


FIGURE 1: Number of reported cases of human salmonellosis in the US from 1940 to 1994 (Maurice, 1994)

Factors contributing to increased incidence of salmonellosis include better epidemiology and improved microbiological detection and identification methods (Kvenberg and Archer, 1987) as well as improved data collection. Experts, however, agree that improved detection methods alone do not fully account for the augmentation and that a real increase is actually occurring (Kvenberg and Archer, 1987). The factors that contribute to increased incidence of foodborne diseases are not fully known, which makes it difficult to apply effective control measures.

Many different food groups including meat, seafood, dairy products, vegetables and fruits have been traced as vectors for foodborne diseases. Bacterial pathogens are the leading causative agents with salmonellae accounting for over 40% of all reported outbreaks in the United States. About half of all salmonella outbreaks in the United States cannot be linked to a specific vector. If the vector can be traced, beef, dairy products and poultry have been determined as the leading sources (Bean and Griffin, 1990). Most outbreaks from poultry products are caused by improper food storage and handling. Informing consumers on how to handle food safely can therefore help to reduce the risk. Nevertheless, each foodborne disease outbreak clearly demonstrates that the responsibility for safe food handling cannot be delegated to consumers. The poultry producers and processors have to take strict measures to improve the safety of their products. At this time eradication of salmonellae in food from animal origin is not attainable without the use of irradiation. However, concerns about the safety of irradiation raised by consumer organizations and regulatory agencies are keeping the food industry from intensive use of the procedure. Concerns arise from fears that irradiation would increase the level of free radicals in the end products and would lead to generally lower hygienic production standards because irradiation would allow to cover for low standards (Teuber, 1994). Scientific data suggest no measurable increase in free radicals with irradiation doses as proposed by the WHO (Teuber, 1994), however, it remains questionable how well production standards and misuse of irradiation could be controlled. Unfortunately, no other control measure has been shown to be particularly effective when used alone. This underscores the need for a comprehensive approach in the control of salmonellae in poultry production and processing systems (Jones *et al.*, 1991). Approaches must be designed to minimize the risk of product contamination while keeping the control costs low to keep the price increase of the end product tolerable.

During the last decade, poultry industry and governmental control agencies have been working together to make poultry products safer. The enforcement of HACCP (Hazard Analysis and Critical Control Points) in all US poultry processing plants will help to reduce the risk of foodborne diseases (USDA, 1995). This system requires the processing plants to evaluate potential hazards, and to devise and implement appropriate control measures to reduce these hazards. Continuous

monitoring of the controls, end product testing and record keeping assure that the implemented control measures are working effectively. Today, HACCP programs are not enforced at the production facilities, however, producers are taking a HACCP-like approach to control enteric pathogens. This approach has shown some success in reducing the prevalence of causative agents of foodborne diseases (Cox *et al.,* 1996). However, there is still room for improvement.

For a HACCP program to be effective, critical control points have to target steps during production or processing which are known to present a considerable risk of product contamination. Since the newly hatched bird is especially prone to salmonella colonization (Milner and Schaffer, 1952), HACCP programs for poultry production have included control measures to protect young chicks from colonization with enteric pathogens. One of the ways to help control colonization of salmonellae has been through competitive exclusion (CE). The CE-concept aims to create a gastrointestinal (GI) environment that is unfavorable for colonization of enteric pathogens. Complex mixtures of bacterial cultures have been applied for several years to achieve this goal. Another approach to exclude salmonellae from the GI tract is the use of mannose or mannose-like sugars which have been shown to inhibit adherence of pathogenic bacteria in the GI tract by blocking mannose sensitive lectins (Ofek *et al.,* 1977; Oyofo *et al.,* 1989b). Unfortunately, relatively high concentrations of mannose are required to control colonization of pathogenic bacteria, and the cost of using pure mannose is prohibitive even for a short regimen. However, mannose based carbohydrates naturally occur in different compounds, such as yeast cell wall or different gums, which are available at reasonable price. The lower cost of these compounds makes them attractive as feed additives if they show similar CE effects as pure mannose.

Preliminary observations indicate that a commercial preparation of mannanoligosaccharide (MOS) from the cell wall of *Saccharomyces cerevisiae* (Jacques and Newman, 1994; Sisak, 1994) can reduce colonization of enteric pathogens. The present study investigates the effect of MOS on the GI environment, on the composition of the GI microflora and on salmonella colonization in chicks.

2. SALMONELLAE

2.1. *Salmonella*: The Organism

Salmonellae are named after D.E. Salmon, an American bacteriologist and veterinarian (Minor, 1984). These organisms are classified as Genus III in the Family of *Enterobacteriaceae* (Minor, 1984). Salmonellae are Gram-negative, non-endospore- and non-microcyst-forming bacilli. Most strains are motile by peritrichous flagella. Like all other enterobacteriaceae, salmonellae are facultative anaerobes. They are non-fastidious chemoheterotroph organisms, which can be grown easily on ordinary media such as peptone or meat extract. Most strains are aerogenic and produce gas in the acid fermentation of carbohydrates. They do not ferment lactose but can use citric acid as sole carbon source. These are important metabolic characteristics which distinguish them from other genera of the same family. Although many members of the genus *Salmonella* have species-like names, such as *Salmonella typhimurium* or *Salmonella pullorum*, no individual species are recognized. Instead, this genus is taxonomically divided into serovars, based on cell wall or intracellular antigens (O antigens), capsular antigens (Vi or K antigens) and flagellar antigens (H antigens) using the Kauffmann-White Scheme (Minor, 1984). Currently, over 2,300 serovars have been described and new ones are being added every year (Langlois, 1993).

Salmonellae grow at temperatures between 5 and 45°C (Guthrie, 1988; Matches and Liston, 1968). They can multiply in a wide pH range of 4 to 9 with a pH of 6.5 to 7.5 being optimal for cell multiplication (Chung and Goepfert, 1970; Guthrie, 1988, Thomas *et al.*, 1992,). Growth is generally inhibited in a 4 to 6 % saline solution (Thomas *et al.*, 1992). They are sensitive to heat. Pasteurization at 71.2°C for 15 seconds is sufficient to kill salmonellae (Guthrie, 1988).

The primary habitat of salmonellae is the intestinal tract of vertebrates and occasionally insects (Langlois, 1993). Although their primary habitat is the intestinal tract, they may also be found in other parts of the body. As facultative anaerobic organisms, which can grow in a relatively wide temperature and pH

range and can use a wide variety of nutritional substrates, salmonellae are able to survive under many different environmental conditions. Salmonellae are found worldwide in soil, water and on many different foodstuffs (Guthrie, 1988). Since all strains of *Salmonella* are potentially pathogenic to humans and vertebrates, they are of great medical and economic importance. For epidemiology purposes, the salmonellae can be classified into three groups according to their pathogenic properties or host specificity (WHO, 1988; Langlois, 1993).

1. Serovars primarily adapted to humans: These serovars include *Salmonella typhi* and *Salmonella paratyphi*, which are the agents of typhoid and paratyphoid fevers and are associated with the most severe diseases caused by salmonellae.
2. Serovars adapted to particular animal hosts: This group includes the serovars *S. pullorum* and *Salmonella gallinarum* which are especially adapted to poultry. It also includes several serovars adapted to different mammals. Other animals or humans can serve as secondary hosts for these serovars.
3. Unadapted serovars: This group includes most serovars involved in foodborne illness (e.g. *Salmonella enteritidis*, *S. typhimurium*).

Most *Salmonella* serovars are members of the third group, which means they can be carried by many different animal species including humans. Many different approaches have been used to control salmonellae, however, their abilities to colonize a wide variety of animals and to survive under extreme environmental conditions for longer periods of time, make it difficult to restrain them.

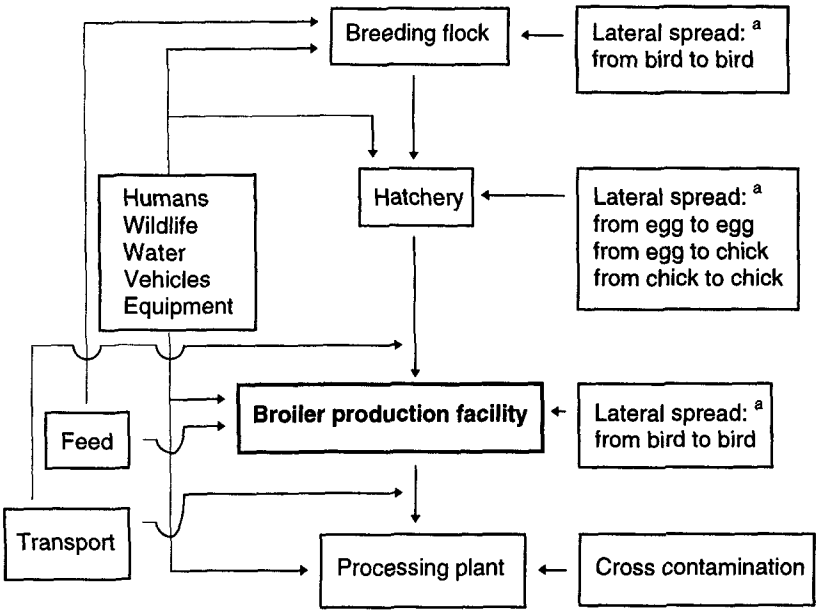
2.2. Salmonellae as Pathogen in Poultry

Salmonellae related diseases have been a major problem to the poultry industry throughout this century. Early in the century losses to the industry were mainly caused by *Salmonella pullorum* and *Salmonella gallinarum* the causative organism of pullorum disease and fowl typhoid, respectively. The enforcement of strict control measures against these diseases allowed the poultry industry to

reduce the incidence of these serovars to a point where they are rare in most advanced poultry producing areas today (Snoeyenbos, 1991). Presently, the major economical losses to the poultry industry due to salmonellae are caused by non-host-specific paratyphoid serovars, such as *Salmonella typhimurium* and *Salmonella enteritidis* (Nagaraja *et al.,* 1991; Snoeyenbos, 1991). These serovars are the causative organisms of salmonellosis. Economic losses to the poultry industry due to salmonellosis occur at the production level and through human food-poisoning caused by poultry and egg consumption.

Salmonella pullorum and *S. gallinarum* are relatively host-specific organisms and have therefore quite simple cycles of infection. Pullorum disease is seen predominately in chicks under 3 wk of age (Severens *et al.,* 1944). Its causative agent is primarily spread from an infected parent via the ovary to the newly hatched chick (Snoeyenbos, 1991). Infected chicks then spread the disease by lateral transmission throughout the flock i.e. by direct animal contact or by fecal contamination of drinking water, feed or litter. *Salmonella pullorum* can be controlled by monitoring breeder flocks and by strictly eliminating infected flocks (Ashton, 1990). Fowl typhoid is usually seen in growers or adult birds, although chicks can also be affected (Snoeyenbos, 1991). The causative agent is passed from the hen to its progenies via infected egg (Hall *et al.,* 1949, Gordeuk *et al.,* 1949). Clinical signs can be reduced with antimicrobial agents, but it is unlikely that *S. gallinarum* infections can be eliminated with such treatments (Ashton, 1990). Birds can also be protected from *S. gallinarum* infections by vaccination (Pomeroy, 1984). Continuous monitoring of breeder flocks combined with high hygienic standards in the production facilities and proper management practices, allow some control of this disease without the application of antibiotics and vaccines (Pomeroy and Nagaraja, 1991).

While pullorum disease and fowl typhoid have almost been eradicated by putting effective control measures in place, control programs for efficient elimination of non-host specific salmonellae are still lacking. Due to the non-host specific nature of the causative organisms the routes of transmission are very complex (Figure 2).



[a] Spread by direct contact or via contamination of vectors such as water, feed, litter or dust.

FIGURE 2: Cycle of non-host specific salmonella infection in broiler production (adapted from Ashton, 1990)

Many different non-host specific serotypes have been isolated from poultry. In the US alone, over 200 serotypes have been reported (Nagaraja *et al.,* 1991). The prevalence of different serotypes varies regionally, with *S. typhimurium* being the predominant serotype in the US (Nagaraja *et al.,* 1991) and *S. enteritidis* the predominant serotype in Europe (Cooper *et al.,* 1994). Clinical signs of salmonellosis are unspecified and resemble those observed in pullorum disease, fowl typhoid and some other bacterial infections. Signs include diarrhea, depression, reluctance to move, standing with closed eyes, ruffled feathers and drooping wings (Ashton, 1990). Clinical signs and mortality are most frequently observed during the first days of life (Fagerberg *et al.,* 1976; Gordon, 1977). Mortality rates vary depending on the virulence of the

Salmonella strain. With *S. enteritidis* phage type 4, the predominant phage type associated with salmonellosis in Europe today (Gast and Benson, 1995), mortality of up to 20 % has been reported (Copper *et al.*, 1994). Adult birds infected with non-host specific organisms usually show no clinical signs (Olesiuk *et al.*, 1969). In fact, the relationship between the colonizing salmonellae and the host appears to be commensal. As the birds health is not impaired, it does little to rid itself of the organism (Bailey, 1987a). Birds that are colonized by paratyphoid salmonellae may serve as carriers of the infection over longer periods of time and may also contaminate eggs and meat. The lack of clinical signs of carrier birds and the complex infection cycle of non-host specific salmonellae make the control of those salmonella serovars difficult.

2.3. Salmonella Control at the Broiler Production Facility

The complex infection cycle of paratyphoid, non-host specific salmonellae requires many different control measures in order to be successful in eliminating the causative organism. A HACCP concept, which combines different preventive control measures to minimize the contamination of the end product with food-poisoning causing microorganism, seems to be the control concept of choice (USDA, 1995). In order to minimize salmonella colonization in broiler flocks, measures have to be taken to minimize the introduction of salmonellae into the flocks. Such measures have to target all routes of salmonella entry (Figure 2). Of particular importance is the management and control of breeder flocks and hatcheries to assure that each broiler production cycle is started with salmonella free day-old chicks. Further, the risk of salmonella contamination during the production cycle has to be minimized. Measures to achieve this goal have to aim at: eliminating salmonella contamination of feed, water and litter; denying access of domestic and wild animals to the production facilities; avoiding the introduction of pathogenic microorganisms into the flock via production workers; minimizing transmission of salmonellae to subsequent flocks by effectively cleaning and disinfecting the equipment and rearing house after use, as well as by allowing an appropriate

resting period for each house between crops of birds and by avoiding the spread of slurry or manure near the premises (Oosterdom, 1991).

Beside minimizing the risk of entry of salmonellae into the production facilities, salmonellae can be curbed by improving the resistance of the birds to salmonella infections. Numerous factors affect the susceptibility of chickens to salmonella colonization. These include age, stress level of the bird, health of the bird, genetics of the bird as well as type and amount of feed additives (Bailey, 1987a). It is particularly important to reduce the susceptibility during the first days of life, since birds are most prone to salmonella colonization at early ages (Milner and Schaffer, 1952).

Keeping the stress level of the birds low is important in controlling salmonellae since different stress factors have been shown to increase the birds susceptibility to colonization with enteric pathogens. Temperature stress (Thaxton *et al.*, 1971), stress through feed and water deprivation (Bierer and Eleazer, 1965) as well as health stress through coccidiosis infections (Morishima *et al.*, 1984; Fukata *et al.*, 1984) have all been shown to increase the birds susceptibility to salmonella infections.

While genetic resistance to *Salmonella pullorum* has been used as a selection criteria in poultry breeding, little has been done to improve the genetic resistance against infections with non-host specific serovars. Bailey (1987b) determined apparent differences in the susceptibility of different commercial strains of chickens to colonization by non-host specific salmonellae. Such differences between strains indicate a potential for genetic improvement.

Research has focused on ways to improve the resistance of birds to enteric pathogen infections through the use of feed additives. Antibiotics are often added to poultry feed at low levels (15-25 ppm) to improve weight gain and feed conversion and have been used at therapeutic levels (100-200 ppm) against salmonella and other bacterial infections (Bailey, 1987b). Antibiotic treatment has been shown to suppress salmonella infections. However, this does not provide long term protection since after termination of treatment, salmonellae are again secreted, often at higher levels than before treatment (Smith and Tucker, 1975a,b, 1978, 1980). Incorporation of organic acids into poultry diets has been shown to reduce prevalence of salmonellae in broiler

flocks in some instances. Formic acid or mixtures of formic and propionic acid added to salmonella contaminated feed have been shown to reduce the isolation rate of salmonellae from broiler chicks (Hinton *et al.*, 1985; Hinton and Linton, 1988) and in both laying hens and their progeny (Humphrey and Lanning, 1988). These acids did not affect the salmonella level in the feed, which suggests that they are active at reducing colonization in the digestive tract of the birds. However, Hinton *et al.* (1991) did not find any effect of feeding propionic and formic acid on the incidence of salmonella positive cecal samples of birds challenged with *Salmonella enteritidis* phage 4 from an environmental source. Differences in challenge organism, challenge dose and challenge method might account for the conflicting results.

There are no easy answers on how to efficiently improve the resistance of chicks to salmonella infections. Maintaining the health of the flock and keeping the stress level low are two important measures that have to be taken to improve the resistance of the flock. Additionally, the use of bacterial cultures containing a wide variety of beneficial intestinal bacteria has been shown to be a useful means to reduce salmonella colonization in poultry (Hirn *et al.*, 1992).

3. THE CONCEPT OF COMPETITIVE EXCLUSION AND THE AVIAN GASTROINTESTINAL MICROFLORA

3.1. Competitive Exclusion and its History

The theory of survival of the fittest was established by Darwin in the 19th century (Darwin 1872). The theory proposes that individuals best adapted to live in a given ecosystem will survive and outcompete less adapted individuals. This theory does not only hold true for individuals of the same species but also for populations of different species. A population well adapted to live in a given ecosystem can control other populations in this ecosystem. The extinction of the dinosaurs, the suppression of legumes by grass in a nitrogen fertilized pasture or the exclusion of salmonellae by the indigenous microflora from the gastrointestinal (GI) tract are examples of such control. Different terms have been used to describe these control mechanisms. Greenberg (1969) used the term competitive inhibition to describe the control of one population by others. Competitive inhibition is now commonly referred to as competitive exclusion (CE). Competitive exclusion implies the prevention of entry or establishment of one entity into a given environment because that niche is already occupied by a competing entity. The latter is better suited to establish or maintain itself in that environment or is producing a compound inhibitory to its competition (Bailey, 1987b).

Competitive exclusion in the GI tract was first described at the beginning of this century by Metchnikoff (1903). He reported that feeding lactobacilli in fermented milk was beneficial to the host. Metchnikoff suggested that the presence of lactic acid bacteria is inimical to the growth of bacteria of putrefaction and this inhibition would account for the beneficial effects of fermented milk. In the 1930's, strong evidence accumulated that not only bacteria added daily via the diet, but also the indigenous microflora has an inhibitory effect on undesirable microorganisms (Meynell, 1963). However, it was not until gnotobiotic techniques and antibiotics became available that the protective effect of the indigenous microflora could be more closely investigated. These tools allowed for the production of germ-free animals and animals with a suppressed GI microflora. Comparisons of gnotobiotic animals to

animals with a healthy microflora allowed for evaluation of the protective effects of the innate microflora against certain pathogens. It soon became clear that antibiotics not only suppressed certain microorganisms but in turn could promote the growth of resistant bacteria by eliminating sensitive bacteria and therefore reducing bacterial competition. Streptomycin was shown to increase the susceptibility of the host to pathogenic microorganisms such as *Salmonella typhimurium* by disturbing the normal intestinal microflora of mice (Bohnhoff *et al.*, 1964). Inhibition of enteric pathogens through CE is one of the most important functions of the GI microflora. Imbalances in the GI ecosystem, however, can weaken the protection and give enteric pathogens a better chance to colonize the GI tract. Imbalances in the ecosystem also occur during periods of stress, changes in the diet and in the young animal. Failures of the natural protection mechanisms have motivated researchers to work on better understanding the exact mechanisms involved in CE and to develop products which strengthen the natural defense mechanisms in such situations. Many of the regulatory mechanisms associated with CE have been identified over the last 40 y. However, these very complex bacterial interactions are still not fully understood.

3.2. Control Factors Involved in Competitive Exclusion

The animal host and its intestinal microflora constitute an enormously complex ecosystem in which many different mechanisms affect the composition of the microbial flora. Control mechanisms can be associated with either allogenic or autogenic factors (Savage, 1987). Allogenic factors are exerted by the host's tissue, the diet and the GI environment, whereas autogenic factors are exerted by components of the microflora on one another. Most factors interact with other factors in a complex, but not well understood manner (Morishita and Mitsuoka, 1976; Savage, 1986). The complexity of these interactions makes it extremely difficult to study the effects of one isolated factor. Based on findings from many different studies, bacterial populations impact one another through four main mechanisms (Table 1).

TABLE 1: Autogenic regulatory mechanism which affect the composition of the microbial flora in the GI tract [a]

Regulatory mechanism	Control factors
Nutrient utilization	- Competition for nutrients or growth factors
	- Synergistic nutrient utilization
Attachment	- Competition for receptor sites
	- Stimulation of epithelial cell turnover
Creation of a restrictive environment	- pH
	- Lactic acid
	- VFA
	- Hydrogen sulfide
	- Eh
	- Modification of bile salts
	- Induction of immunologic process
Production of antimicrobial substances	- Ammonia
	- Hydrogen peroxide
	- Hemolysin
	- Bacterial enzymes
	- Bacteriophage
	- Bacteriocins
	- Antibiotics

[a] Adapted from Miles (1993), Rolfe (1991) and Savage (1987)

The effect of nutrient competition is difficult to assess in relation to other inhibitory factors which may function in the environment (Rolfe, 1991). Investigation on the inhibitory activity of coliforms against *Salmonella flexneri* in an *in vitro* culture showed that the inhibition could be reversed by the addition of glucose. This demonstrates that the inhibition of *S. flexneri* by coliforms was due to the competition for carbon or energy sources (Rolfe, 1991). Inhibition due to competition for substrates can exist for any limiting nutrient or growth factor, however, this has not yet been studied in the avian alimentary tract.

Numerous genera of microorganisms have been associated with the epithelia in various areas of the GI tract (Savage, 1983). The ability to adhere to mucosal surfaces is important for some bacteria in order to establish or maintain colonization in the GI tract (Eshdat *et al.*, 1978; Costerton, *et al.*, 1978; Fuller *et al.*, 1981). Microorganisms associate with epithelial surfaces either by adhering to the glycocalyx or by using motility mechanisms to enter mucous gels (Savage, 1987). Adhesion is achieved through non-specific and specific mechanisms. Non-specific adhesion is mediated through different interactive forces, such as ionic, dipolar, H-bonds and hydrophobic attraction (Vanbelle *et al.*, 1989). Specific adhesion is usually a two-component mechanism involving a lock and key mechanism. Different fimbrial adhesins on the cell surface of the bacteria which attach to specific receptors on the intestinal epithelium have been described (Klemm, 1985). Since the mucosal surface area is limited, the introduction of bacteria into the GI environment creates competition for mucosal attachment sites. Competition between bacteria for adherence sites leading to the exclusion of intruding pathogens is one of the most likely mechanisms of CE. Davidson and Hirsh (1975) showed that pigs orally inoculated with a non-pathogenic *Escherichia coli* possessing the K88 antigen could be protected from subsequent oral challenge with an enterotoxin producing strain of *E. coli* possessing the K88 antigen. Since no protection was obtained with strains not containing K88 antigens, the protection was attributed to the blocking of K88 antigen receptors. Schneitz *et al.* (1993) convincingly demonstrated that the effect of inhibiting colonization of enteric pathogens depends on the adhering ability of the bacterial culture used to protect the animal. A wild strain of *Lactobacillus acidophilus* that adhered strongly to chicken epithelial cells, was tested for its ability to displace *Salmonella infantis.* When the same strain was subcultured several times on a selective agar it lost its adhering ability. This change was accompanied by a considerable loss of protective activity against colonization of newly hatched chicks with *S. infantis.*

Organic acids, the major end-products of fermentation, play a key role in bacterial antagonism in the GI tract. Tramer (1966) showed that the inhibition of *E. coli* by *L. acidophilus* could be related to the strong germicidal action of lactic acid at a low pH. While pH *per se* is a factor, lower pH values also increase the

antimicrobial activity of organic acids. At low pH, organic acids are mainly present in their undissociated forms. Undissociated organic acids penetrate bacterial cell walls more easily because they are uncharged and therefore exert a stronger antimicrobial activity than their negative charged dissociated forms (Bohnhoff *et al.*, 1964; Barnes *et al.*, 1979; Hinton *et al.*, 1990, Adams and Hall, 1988). Barnes *et al.* (1979) showed that different anaerobic bacteria inhibited salmonella *in vitro* and attributed the inhibition to high VFA concentrations coupled with a low pH. Nisbet *et al.* (1993) determined that birds containing higher cecal concentrations of undissociated propionic acid had lower numbers of salmonellae. Other restricting factors include the concentrations of oxygen, unconjugated bile acids and hydrogen sulfide (Rolfe, 1991). Low oxygen concentrations can restrict the proliferation of bacteria requiring oxygen for growth (Savage, 1987). Unconjugated bile acids have been shown to inhibit many anaerobic bacteria *in vitro*, however, *in vivo* data on the inhibitory effects of these compounds are inconclusive (Floch *et al.*, 1972). Hydrogen sulfide has been shown to reduce the range of substrates which a given bacterial species can utilize under anaerobic conditions (Freter *et al.*, 1983).

The antimicrobial metabolites produced by the native gut microorganisms include a wide range of inhibitory substances. Lactobacilli have been extensively studied for production of antagonists. Several antibiotic-like substances with activity against Gram-positive and Gram-negative bacteria have been isolated (Gilliland, 1989). Reuterin for example is produced by *Lactobacillus reuteri* under anaerobic conditions, at a pH and temperature similar to those in the small intestine of animals (Juven *et al.*, 1991). In addition, *L. acidophilus* also produces bacteriocins with antimicrobial activity against closely related species of bacteria (Gilliland, 1989). Wide range inhibitory compounds have also been demonstrated in pediococci (Juven *et al.*, 1991) However, the significance of wide and narrow range antibiotic-like substances as regulators of microbial populations in the GI tract is still uncertain (Rolfe, 1991). Other metabolites with antibacterial activity include ammonia, hydrogen peroxide, hemolysin, bacterial enzymes and bacteriophages (Rolfe, 1991).

Many complex bacterial control mechanisms are involved in regulating the composition of the GI microflora and in excluding pathogens from the digestive

tract. Profound knowledge of the composition of the microflora and its regulatory forces is essential to understand failures of the innate microflora to exclude pathogens and to take appropriate measures to minimize the occurrence of such failures.

3.3. The Avian Gastrointestinal Microflora

3.3.1. The Composition of the Adult Gastrointestinal Microflora

From the microbiological standpoint, the avian GI tract can be divided into five sections: crop; proventriculus and gizzard; small intestine; ceca and colon and cloaca (Figure 3). The microbial populations mainly differ due to differences in pH, Eh and flow rate in these sections.

Although a proportion of the nutrients ingested may pass to the proventriculus, most of the food enters the crop where it may remain for up to 6 hours (Barnes, 1979; Jayne-Williams and Fuller, 1971). The crop pH generally lies between 4.5 and 6.0 with extreme values as low as 2.7 (Barnes, 1979; Bolton, 1965; Herpol and van Grembergen, 1961; Smith, 1965a, Wieseman *et al.*, 1956). Oxygen concentrations in the crop are high compared to concentrations found in the lower GI tract. Many workers (Sieburth, *et al.*, 1954; Lev and Briggs, 1956; Wiesman *et al.*, 1956) have shown that lactobacilli predominate in the crop at concentrations of 10^9 cells/g, forming a close association with the epithelial lining of the crop (Fuller and Brooker, 1974; Fuller and Turvey, 1971). Enterococci (streptococci) and coliform bacteria (up to 10^5 CFU/g) and occasionally small numbers of micrococci, staphylococci and yeast are also present (Jayne-Williams and Fuller, 1971). Strict anaerobes do not normally occur in the crop because the redox potential, the type of substrate and the predominance of lactobacilli are unfavorable for their growth (Jayne-Williams and Fuller, 1971).

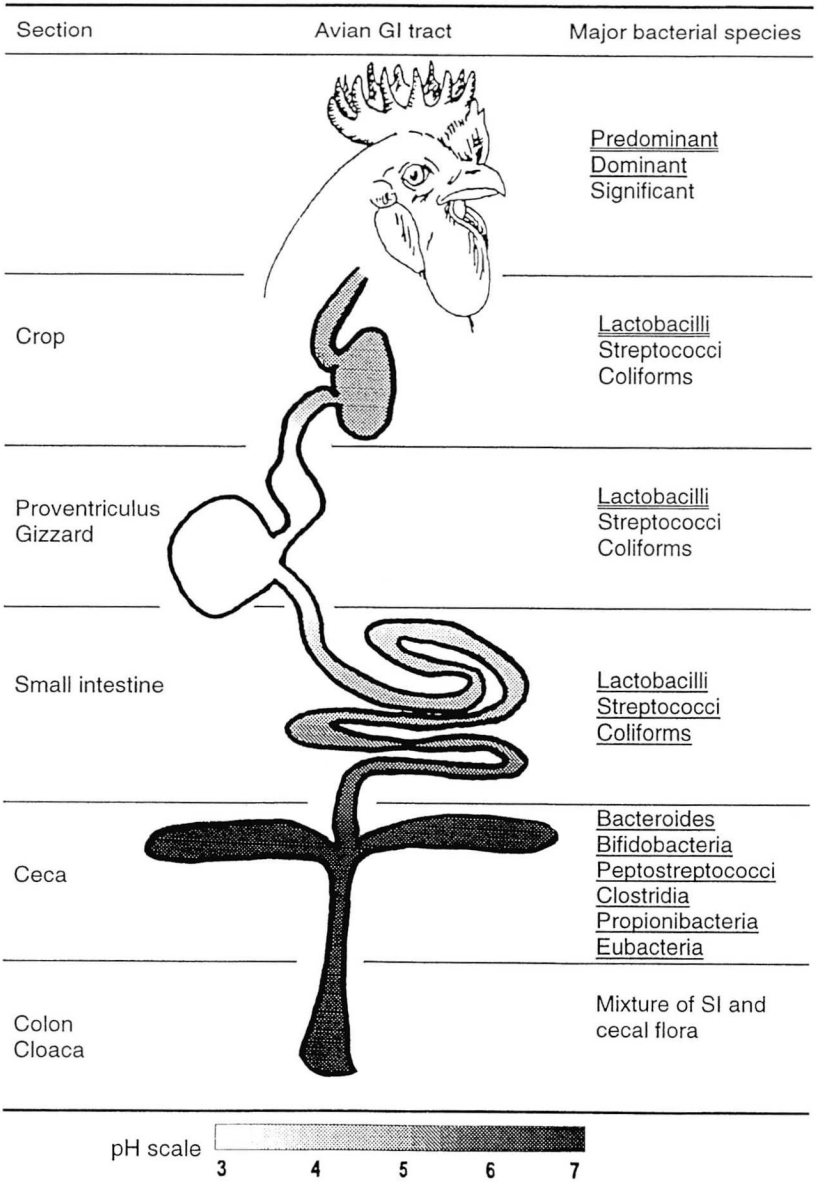


FIGURE 3: Avian GI tract divided in 5 sections based on microbiological flora: crop; proventriculus and gizzard; small intestine; ceca; colon and cloaca

Organisms discharged from the crop pass through the proventriculus to the gizzard where they are subjected to acidity between pH 2.0 to 4.0 (Jayne-Williams and Fuller, 1971; Wieseman *et al.*, 1956). Bacterial numbers are reduced 10- to 100-fold by the bactericidal effect of acidity (Jayne-Williams and Fuller, 1971).

The environment in the small intestine, the main site of chemical digestion and absorption, is limited by a fast flow rate and the presence of high concentrations of endogenous enzymes and bile salts. At the posterior end of the small intestine, the flow rate decreases and the pH increases to values close to neutrality (Barnes, 1979; Wiseman *et al.*, 1956). This environment is associated with greater bacterial concentrations (Jayne-Williams and Fuller, 1971). The microbial population in the anterior part of the small intestine is merely a transient flora with a composition similar to the one in the crop. Lactobacilli which adhere to the columnar epithelial cells are the predominant species in the small intestine (Fuller and Turvey, 1971). The main lactobacilli present are *Lactobacillus acidophilus, Lactobacillus salivarius* and *Lactobacillus fermentum* (Morishita *et al.*, 1971). The composition of the GI microflora in the lower portion of the small intestine is more complex and mainly consists of lactobacilli, enterococci and enterobacteriaceae (Timms, 1968). The bacterial concentrations found in the small intestine are rather low when compared to the concentrations normally found in the ceca and are generally less than 10^8 cells/g (Barnes, 1979).

Neutral pH (Smith, 1965a; Timms, 1968) and anaerobic conditions in the ceca provide ideal conditions for bacterial proliferation. The retention time of the digesta is relatively long and allows for substantial bacterial growth. Bacterial concentrations are about 10^{10}-10^{11} cells/g (Barnes *et al.*, 1972; Hutanen and Pensack, 1965). Anaerobic bacteria form the major part of cecal flora and most of the microorganisms present in the ceca are able to decompose uric acid (Barnes and Impey, 1974; Hutanen and Pensack, 1965). These bacteria help the host recycle nitrogen from uric acid which flows back into the ceca after its entry into the cloaca from the kidneys. The bacterial population is mainly composed of bacteroides, bifidobacteria, peptostreptococci, clostridia, propionibacteria and eubacteria. The relative proportions of the different

species can vary greatly. Barnes and Impey (1970) indicated that Gram-negative non-sporing rods (*Bacteroidaceae*) and Gram-positive non-sporing rods were present in almost equal proportions and formed about 80% of the cecal flora. The rest of the flora consists mainly of peptostreptococci. On the other hand Salanitro *et al.* (1974) reported lower levels of *Bacteroidaceae*. Ten percent of the total population were clostridia in their study. Cellulolytic organisms have never been isolated from the chicken ceca at levels above 10^3 CFU/g (Barnes *et al.*, 1972).

The colon is relatively short and contains the digesta coming from the ceca or the small intestine. The retention time of the digesta is quite short with about six fecal droppings daily (Roesseler, 1929). The colon pH is very similar to the pH in the ceca (Wiseman *et al.*, 1956). The bacterial population of the large intestine is subject to large fluctuations in concentrations of specific groups of bacteria (Smith and Crabb, 1961). These fluctuations occur because the ceca does not provide a continuous flow of digesta into the large intestine. Jayne-Williams and Fuller (1971) stated that the ceca evacuate themselves only every 6 to 8 h whereas Olson and Mann (1935) reported values from 24 to 48 h. The proportion of material entering the large intestine from the ceca and the small intestine therefore varies as well as the composition of the bacterial flora.

3.3.2. The Development of the Gastrointestinal Microflora in the Newly Hatched Bird

The alimentary tract of the newly hatched, healthy chicken is usually sterile (Mead and Adams, 1975, Savage, 1987). Soon after hatching, the young birds naturally develop a mature intestinal microflora through contamination with fecal material from mature birds. With coprophagic birds, such as the domestic fowl, the transfer of bacteria from the parents to the chicks occurs very efficiently and allows the young animal to establish a protective intestinal flora within the first 5 d of life. With young animals such as rats or puppies the colonization of the alimentary tract with microorganisms takes longer as they are not active at birth and are kept in the nest under comparatively clean conditions (Smith, 1965b). The same delay in establishing a protective

microflora is observed in chickens reared in a modern system of mass production under hygienic conditions. These birds establish their microflora through contact with bacteria in hatching debris, in the hatchery environment, on transport, in the production facility and through ingestion of bacteria in feed and water.

The bacterial population in the different segments of the avian GI tract reach concentrations very similar to the concentrations found in adult birds within the first 3 d of life. However, the early floras are very simple and the change in complexity, especially the establishment of obligate anaerobic bacteria takes several weeks. The typical adult flora of the duodenum and the small intestine is established within the first 2 wk, but the cecal microflora takes much longer to develop (Ochi *et al.*, 1964; Smith, 1965b). Non-fastidious organisms such as enteric Gram-negative bacilli and streptococci are normally the first organisms to colonize the GI tract of young animals (Rolfe and Iaconis, 1983; Lee and Gemmell, 1972). They are then partially or completely replaced by other microorganisms, which slowly establish themselves until a stable climax population develops.

Escherichia coli and enterococci are the dominant organisms found in the crop of the newly hatched chick. They reach concentrations of 10^8 CFU/g within 12 h after hatching. Lactobacilli then start to colonize the crop so that *E. coli* and enterococci concentrations decrease below 10^5 CFU/g within the first week of life (Smith, 1965b).

Bacterial colonization in the duodenum is slower than in the other segments of the GI tract (Hutanen and Pensack, 1965). Concentrations are below 10^5 cells/g the first day and reach concentrations normally found in adult animals by day 3 with *Enterococcus faecalis* being the dominant species. Clostridia and enterobacteriaceae were also reported to be present at the first days on life (Ochi *et al.*, 1964; Smith, 1965b). Lactobacilli establish themselves more slowly, but start to outnumber the quick invaders such as *E. coli* and enterococci. By day 14, the flora of the duodenum consists almost entirely of lactobacilli while that of the lower portion of the small intestine consists of lactobacilli with enterococci and enterobacteriaceae (Ochi *et al.*, 1964; Barnes *et al.*, 1972).

The cecal bacterial concentration reaches 10^{10} cells/g within the first day of life and remains at this level in a healthy bird (Barnes *et al.*, 1972; Hutanen and Pensack, 1965). As in the upper GI tract, the cecal population of the 1 d old chick is predominated by enterococci and coliform bacteria. Clostridia are often present in lower numbers (Hutanen and Pensack, 1965; Impey *et al.*, 1982; Shariro and Sarles, 1949; Smith, 1965b). Hutanen and Pensack (1965) reported that by day 6 enterococci were greatly outnumbered by other aerobic types. However, Barnes (1972) and Smith (1965b) mentioned that enterococci remained the predominate species until they were outnumbered by obligate anaerobes and established themselves at about 10 % of the total flora. Reports are conflicting regarding the time frame when obligate anaerobes outnumber the facultative anaerobic bacteria. Hutanen and Pensack (1965) and Barnes (1977) reported that by day 14 cecal organisms were mainly obligate anaerobes. The proportion of anaerobes increased until the age of 4 wk, when bifidobacteria, bacteroides, eubacteria, peptostreptococci and clostridia predominated (Barnes, 1977, Salanitro, *et al.* 1974). Mead and Adams (1975) indicated that the flora became dominated by anaerobic bacteria, as soon as day 4. Smith (1965b) isolated bacteroides not earlier than day 6. These differences might be due to different environmental rearing conditions of the chicks. The proportion of uric acid-utilizers in the ceca is high starting from day 1. *Enterococcus faecalis* is such a uric acid utilizer (Barnes *et al.*, 1974; Mead and Adams, 1975).

Bacterial concentrations in the GI tract of the newly hatched chick reach values similar to those found in the adult bird within the first few days of life. However, the GI microflora of the young bird is simple in composition containing only a limited number of bacterial species and bacterial strains. This lack in complexity of the microflora weakens bacterial exclusion mechanisms and makes the young bird more prone to be colonized with enteric pathogens.

3.4. Competitive Exclusion Products

3.4.1. Undefined Competitive Exclusion Cultures

Nurmi and Rantala (1973) first associated the high incidence of salmonella infections in broiler chicks with the hampered development of the intestinal flora in abnormal rearing conditions. They showed that oral pretreatment of chicks with ingesta of healthy adult cocks decreased susceptibility to *Salmonella infantis* infection. They assigned this increased resistance to competition between the newly-established intestinal flora and the invading pathogen, *S. infantis*. These positive results stimulated the development of commercial CE cultures produced under standardized conditions, which would meet the requirements for registration and licensing. Rantala (1974) developed a CE culture grown on Viande-Levure-Medium (VL-Medium) under anaerobic conditions which demonstrated a protective effect similar to fresh ingesta. Similar protection against *Salmonella typhimurium*, *S. infantis* and *Salmonella kedougou* have been obtained on numerous occasions using the cecal or fecal contents of adult birds or the same type of material cultured anaerobically in laboratory scale trials involving small groups of birds (Hinton *et al.*, 1991; Impey and Mead, 1989; Impey *et al.*, 1984; Lloyd *et al.*, 1977; Pivnick *et al.*, 1982; Rantala, 1974; Salvat *et al.*, 1992; Stavric *et al.*, 1985; Stersky *et al.*, 1984). Reductions in cecal salmonella concentrations from 1 million to less than 10 CFU/g and reductions in percentage of salmonella positive birds from 80 - 100 % to 0 -25% have been reported from such trials. Treatment with CE cultures is primarily prophylactic and it should be applied as early as possible to avoid salmonella contamination prior to treatment. Little effect can be expected from treating birds which are already infected with salmonellae (Mead and Impey, 1987). Protection against *Escherichia coli* O157:H7, *Campylobacter jejuni* and *Salmonella gallinarum* through CE culture application has also been reported (Stavric *et al.*, 1992; Soerjadi *et al.*, 1982; Silva *et al.*, 1981). However, protection against these microorganisms was lower than protection against *S. typhimurium*, *S. infantis* or *S. kedougou*.

Reduced salmonella colonization with undefined CE cultures has also been reported from field studies. Results, however, were not as convincing as

results obtained in small scale trials. Differences in hygienic conditions, housing, water quality as well as the method of administering the treatment material between the two test methods might account for the different success rates. Surveys in Finland including 600 flocks showed a reduction in the percentage of salmonella positive flocks from 21 % to 6.5 % and from 55 % to 11% with CE treatment (Hirn *et al.,* 1992). Reduced salmonella prevalence in the field with CE cultures has also been reported from Sweden (Wierup *et al.,* 1992). Dutch data (Goren *et al.,* 1988) using spray application of CE culture at the hatchery showed a reduction in salmonella positive flocks (38.6 % to 7.6 %) as well as a reduction in the incidence of infected boilers within a positive flocks from 15.9 % to 5.2 % with CE treatment. The percentage of salmonella positive flocks increased again after withdrawal of the treatment culture. A reduction of the percentage of salmonella positive birds with CE application was noticed only at the farm level but not in the processing plant. Cross contamination during transport and slaughter might have reversed the reduction on the farm achieved with CE. This demonstrates the CE treatment can only be successful in combination with other measures, such as reduction of cross contamination during production and processing.

Considerable progress has been made in the development of undefined CE products and application methods over the past 20 y. Culturing cecal content in anaerobic VL-Media allows production of a safe product with a protective effect similar to fresh cecal content from a healthy adult bird. The culture inoculum can be effectively screened for presence of enteropathogenic bacteria and advanced technology allows to avoid contamination during the production process. In addition, subculturing decreases the transmission of non-bacterial pathogens such as viruses and protozoa which are unable to grow in culture media. The production of lyophilized cultures (Mead and Impey, 1987; Pivnick *et al.,* 1982; Hollister *et al.,* 1994a,c) makes it possible to distribute commercial products with a satisfactory shelf life. Furthermore drinking water application (Hirn et al., 1992) and spray application in the hatchery (Goren *et al.,* 1984) have been developed. No undesirable side effects from CE treatments have been reported from laboratory scale and field trials and CE products have been used intensively for almost 20 years in

Scandinavia. Nevertheless safety concerns about undefined CE products still exist. Therefore, different research groups are developing defined cultures to eliminate any safety concerns.

3.4.2. Defined Competitive Exclusion Cultures

Different researchers have tested cultures combining different concentrations and strains of bacteria attempting to develop defined CE cultures (Table 2). The application of pure cultures of *Enterococcus faecalis* or a *Clostridium* sp. showed a protective effect against *Salmonella typhimurium* colonization in chicks (Soerjadi *et al.*, 1978; Rigby *et al.*, 1977). However, cultures of *Lactobacilli* sp. *Bacteroides* sp. and *Bifidobacterium* sp. alone or in combination with or without the addition of a *Eubacterium* sp. provided no protection against *S. typhimurium* or even led to higher cecal pathogen concentrations (Barnes *et al.*, 1979 and 1980). Application of pure cultures can disturb the ecological balance of the GI microflora, exclude beneficial bacteria and, therefore, decrease the complexity of the innate microflora. This can weaken the indigenous flora and allow colonization of enteric pathogens. In general, CE preparations containing single strains or mixtures of a limited number of strains have not consistently protect chicks against subsequent challenges with salmonellae (Stavric, 1992).

Similar protection against salmonellae as with undefined cultures has been achieved with mixtures containing large numbers of bacterial strains. Blanchfield *et al.* (1984) inoculated *in vitro* cultures with different amounts of cecal content and determined the minimal number of strains required to give maximal protection. As little as 0.1 µg of cecal content as an inoculum was sufficient to produce a protective culture. This indicates that only strains which are present at concentrations of at least 10^7 CFU/g in the cecum are required in an CE culture to be effective. In an attempt to maintain the ecological balance of the cecal flora, Stavric *et al.* (1985) developed a relatively complex defined mixture of 50 bacterial strains containing species of *Escherichia, Enterococcus, Bacteroides, Fusobacterium, Lactobacillus, Eubacterium, Propionibacterium, Clostridum, Bifidobacterium* and unidentified Gram-positive rods. This mixture gave protection against *S. typhimurium* comparable to that obtained with

undefined cecal culture. When the number of cultures in the mixture of 50 organisms was gradually reduced by deletion of individual strains the protective activity of the mixture decreased. Impey *et al.* (1982 and 1984) achieved protection against salmonella infection in chicks with a culture combining 48 strains. However, this culture was not effective in turkey poults.

TABLE 2: Effect of defined cultures combining different number and strains of bacteria on salmonella colonization of chicks

Species	No of strains	Protection	Reference
Enterococcus faecalis	1	++[a]	Soerjadi *et al.*, 1978
Clostridium sp.	1	-[b]	Rigby *et al.*, 1977
Bacteroides hypermegas	1	-	Barnes *et al.*, 1979
Bifidobacterium sp.	1	-	Barnes *et al.*, 1979
Lactobacillus ssp.	Several	-	Barnes *et al.*, 1980
Lactobacillus ssp. *Bifidobacterium* sp. *Bacteroides vulgatus*	Several 1 1	-	Barnes *et al.*, 1980
Lactobacillus ssp. *Bifidobacterium* sp. *Eubacterium* sp.	Several 2 1	-	Barnes *et al.*, 1980
Obligate and facultative anaerobes[c]	11	+[d]	Nisbet *et al.*, 1993b
Obligate and facultative anaerobes[c]	29	+	Corrier *et al.*, 1995a,b
Obligate and facultative anaerobes[c]	10	+	Stavric *et al.*, 1985
Obligate and facultative anaerobes[c]	25	+	Stavric *et al.*, 1985
Obligate and facultative anaerobes[c]	40	+	Stavric *et al.*, 1985
Obligate and facultative anaerobes[c]	50	++	Stavric *et al.*, 1985
Obligate and facultative anaerobes[c]	65	++	Impey *et al.*, 1982
Obligate and facultative anaerobes[c]	48	++	Impey *et al.*, 1982

[a] Protection similar to that of an undefined culture
[b] No protection (similar salmonella concentrations as in untreated birds
[c] Combination of strains belonging to at least 6 genera
[d] Lower protection compared to protection given by an undefined culture

The lack of a sound scientific basis on which to select potentially protective strains or combinations of strains makes the development of defined

cultures difficult (Mead and Impey, 1987). Two different selection procedures have shown some promising results. First, Schoeni and Doyle (1992) used the growth properties of the pathogen to be excluded and the ability to produce metabolites which inhibit the pathogen as criteria for isolation of CE cultures. This selection criteria was expected to enhance the chances of finding CE bacteria that occupy niches similar to pathogens. Since *Campylobacter jejuni* colonizes the crypt mucus and can use mucus as its sole substrate, mucus utilization and the production of anti-campylobacter metabolites have been used as criteria for isolation of CE cultures against *C. jejuni* (Schoeni and Wong, 1992). A culture combining strains that contain these properties showed considerable protection against *C. jejuni*. Nisbet and coworkers (1993) used continuous culture techniques to select a defined mixture of 11 strains of bacteria containing species of *Enterococcus, Lactobacillus, Citrobacter, Escherichia, Bifidobacterium* and *Propionibacterium*. Protection against *S. typhimurium* with this culture was improved by the addition of dietary lactose but did not reach the protection level seen with undefined cultures. Using the same selection techniques a culture combining 29 strains representing 10 genera provided better protection against salmonella than the culture combining 11 strains (Corrier *et al.*, 1995a,b and c; Droleskey *et al.*, 1995).

Up-to-date consistent protection against salmonella challenge has only been obtained by introducing complete cecal cultures or cultures combining large number of bacterial strains. Of the many species of bacteria present in the cecum those that are important for protection are presently unknown. It might be possible that some key components were missing in cultures combining only a few strains. However, the attempts to develop defined cultures suggest that defined cultures must be very complex to achieve full protection. These somewhat unsatisfactory results with defined cultures ask for further approaches to target salmonella contamination. One possibility is to strengthen the innate GI microflora instead of introducing bacterial cultures into the birds.

4. CARBOHYDRATES AS COMPETITIVE EXCLUSION PRODUCTS

4.1. History

Metchnikoff's observation (1903) that fermented milk products improve animal performance and health caught the interest of other researchers and stimulated studies on the secrets of sour milk. Pioneer work by Rettger (1915) revealed that not only sour milk but also fresh milk improved growth and reduced mortality in chicks. Improvements were noticed under normal conditions and in birds challenged with *Bacterium* (*Salmonella*) *pullorum*. Rettger demonstrated that feeding sour milk fermented with *Bacillus (Lactobacillus) bulgaricus* did not bring better results than feeding sweet or regular sour milk and he therefore questioned Metchnikoff's theory that milk acids or milk bacteria were the active ingredients. He concluded that the beneficial properties of milk exist in the product as such. Rettger hypothesized that dietary milk might improve performance and health by improving the composition of the intestinal microflora. And in fact, he observed a change from a mixed bacterial flora to a flora dominated by *Bacillus (Lactobacillus) acidophilus* and *Bacillus (Bifidobacterium) bifidus* in rats fed milk. The fact that these two microorganisms both ferment carbohydrates indicated that the secret ingredient in milk might be lactose. This hypothesis was proven true since feeding pure lactose brought similar results to feeding milk (Rettger, 1915), whereas feeding other sugars showed little improvement. Rettger's work clearly demonstrated that the composition of the gastrointestinal (GI) microflora can be altered and improved by adding lactose. His findings showed that diets should not only be composed to cover the animal's nutritional requirements, but can also be designed to improve the composition of the GI microflora and with it the health of the host.

The key to designing diets that support a healthy GI microflora was to gain knowledge on the effect of different feed ingredients on the microbial composition of the GI tract and to understand the modes of action involved. The first clues on how lactose changes the GI microflora came from challenge trials with coccidia. Dietary lactose was shown to decrease the cecal pH and this was

suggested to be part of the mode of action of lactose to exclude coccidia (Ashcraft, 1933; Beach, 1925; Beach *et al.*, 1925a,b). The importance of a low gut pH in controlling enteric pathogens has been confirmed in various trials (Tramer, 1966; Barnes *et al.*, 1979; Nisbet *et al.*, 1993). Many different sugars have been tested and differences have been revealed in their effects on the GI microflora and their modes of action. Researchers have been working not only to better understanding the effects of existing sugars but have also used their knowledge to synthesize new carbohydrate oligomers, so called neo-sugars, with new and superior properties.

4.2. Carbohydrates and their Modes of Action

Dietary carbohydrates have been shown to improve the composition of the GI microflora through two main mechanisms. Some support the growth of specific beneficial bacterial populations, while others selectively block attachment of detrimental bacteria to the gut wall.

The majority of GI microorganisms use carbohydrates as their main carbon and energy sources. Since carbohydrates are selectively used by different bacterial species, they have the ability to support the growth of distinct bacterial groups and as a result their addition to the diet can change the composition of the GI microflora. Improvements in the composition of the GI microflora with carbohydrate addition depend mainly on two factors: First, the host competes for carbohydrates with the GI microflora. If a carbohydrate can be digested by host enzymes, end products are subsequently absorbed and the carbohydrate is not fully available to support bacterial growth. This will lower its ability to alter the GI microflora. Second, bacterial populations compete among one another for added sugars. It is desirable for a carbohydrate to selectively support the growth of beneficial bacteria, which will give these bacteria a competitive advantage over pathogenic microorganism (Newman, 1993). This competitive advantage strengthens the beneficial populations which can lead to reduction or even complete exclusion of pathogens.

More recently it has been observed that certain carbohydrates can bring changes in the composition of the GI microflora through specifically blocking

bacterial attachment to epithelial cells (Oyofo *et al.*, 1989a,c). Although a number of attachment mechanisms which are specific for sugars such as galactose and fucose are involved (Oyofo *et al.*, 1989a; Cinco *et al.*, 1984) binding proteins (lectins) specific for mannose predominate in intestinal pathogens (Mirelman *et al*, 1980). Mannose and mannose-like sugars are therefore of particular interest as blocking agents.

4.3. Competitive Exclusion Effect of Readily Fermentable Carbohydrates

4.3.1. Mono- and Disaccharides

Among mono- and disaccharides which are readily fermented by intestinal bacteria, lactose and mannose have proven to be more effective in protecting chicks from salmonella colonization than other sugars (Table 3). Due to the lack of the enzyme lactase the avian digestive system can not utilize lactose, and it is therefore fully available for bacterial fermentation. Furthermore lactose is not fermented by salmonellae. This may partially explain why lactose is more efficient at controlling *Salmonella typhimurium* colonization than glucose, sucrose or maltose which are utilized by the avian digestive system and can serve as a substrate for salmonellae. In contrast, mannose is fermented by a wide range of GI bacteria (Holdeman *et al.*, 1977). In particular, many different pathogens such as *E. coli,* salmonellae or clostridia are able to use mannose as their sole carbon and energy source (Holdeman *et al.*, 1977; Brennerr, 1984). This makes it very unlikely that the reduction of salmonella colonization seen with mannose addition is through mannose serving as substrate for beneficial bacteria. Mannose seems mainly to work by blocking bacterial attachment (Oyofo *et al.*, 1989a,b).

The effect of lactose on salmonella colonization has been intensively studied in laboratory scale trials. Inclusion rates of 5 % in the diet or 2.5 % in the drinking water of chicks or turkey poults challenged shortly after hatching with different strains of *S. typhimurium* have been shown to decrease cecal concentrations of the challenge organisms by 2 to 4 $\log_{10}$ - units by 10 d of age (Corrier *et al.*, 1990b; Hinton *et al.*, 1990; Hollister *et al.*, 1994a,b; Nisbet *et al.*,

1993; Oyofo *et al.*, 1989a). Low protection was achieved in chicks challenged with high doses of *S. typhimurium* (Hinton *et al.*, 1990) and in turkey poults (Hollister *et al.*, 1994b). In trials conducted in birds grown to market age, the protective effect of dietary lactose was less pronounced. In chicks housed in brooder batteries, continuous lactose application significantly reduced, but did not eliminate the number of broilers whose cecal content tested positive for *S. typhimurium* (Corrier *et al.*, 1990a). However, when chicks were housed on litter and prevalence of salmonellae on the prechilled carcass was used as a test parameter to more closely simulate commercial conditions, no protection was achieved with lactose (Waldroup *et al.*, 1992). Since lactose never showed complete elimination of salmonellae, lactose treatment still allows continual salmonella shedding into the litter. This might lead to contamination of the external surface of the bird and subsequent carcass contamination.

TABLE 3: Effect of different dietary carbohydrates on the prevalence of cecal *Salmonella typhimurium* in salmonella challenged broiler chicks [a]

Treatment	% of chicks colonized	Concentration Log CFU/g
Negative control	0	0
Positive control	100	6.6
Dextrose	93.3	5.9
Lactose	53.3	3.6
Sucrose	96.6	6.6
Mannose	26.6	2.9
Maltose	86.6	6.0

[a] adapted from Oyofo *et al.*, 1989b

Combined application of both lactose and a CE culture has been shown to protect chicks better against salmonella colonization than either of the treatments used alone (Hinten *et al.*, 1990; Hollister *et al.*, 1994a,b, Nisbet *et al.*, 1993). This combination assures that the bacterial populations which benefit most from lactose addition are present in the GI tract. Lactose fermenting populations assure high cecal VFA and lactate concentrations as well as a low

cecal pH (Corrier *et al.*, 1990b). Investigations in anaerobic continuous flow cultures inoculated with the cecal flora from an adult chicken, showed a preference of fermenting lactose to lactic acid with subsequent conversion to acetic and propionic acid (Hume *et al.*, 1992ab). Increased VFA concentrations in combination with lower cecal pH seem to be a major mechanism of salmonella exclusion with lactose addition (Corrier *et al.*, 1990a).

More recently synthetic disaccharides such as lactulose and lacitol have been discussed as potential GI microflora modifiers in animal diets (Küther, 1994). These neo-sugars are derived from lactose through several isomerization and hydrogenation processing steps. Lactulose contains galactose and fructose whereas lacitol is composed of galactose and sucrose. These compounds are linked with beta bonds and their enzymatic degradation in the GI tract of mammals is slow compared to lactose (Harju, 1991). This could make them more efficient GI modifiers in mammals than lactose. However, since lactose is not utilized in the avian GI tract and the neo-disaccharides are not more selectively used by beneficial bacteria than lactose, these synthetic compounds offer little advantage over lactose in poultry diets.

4.3.2. Oligosaccharides

Oligosaccharides are complex carbohydrates composed of at least three carbohydrate monomers (Belitz and Grosch, 1987). They may contain similar or different building blocks, different linkage structures and may be linear or branched. With the exception of malto-dextrins, derived from starch, most oligosaccharides have a composition that can not be degraded by digestive enzymes of mammals or birds. This allows complex carbohydrates to avoid enzymatic digestion by the host and they serve as nutrients for the GI microflora. However, many oligosaccharides promote the growth of beneficial bacteria more selectively than lactose (Hidaka *et al.*, 1986).

Oligosaccharides include a wide range of molecules which are natural constituents of plants and microorganisms. Some of the complex sugars, such as raffinose or stachyose are abundant in beans, peas and soybeans and contain anti-nutritive value for the animal (Coon *et al.*, 1990). Others such as fructooligosaccarides (FOS) galactooligosaccharides (GOS) and lactosucrose

(LS), have shown the potential to improve health and performance when being added to animal diets.

Over the last decade, the main interest in fermentable oligosaccharides has been in FOS. Fructooligosaccharides were originally investigated as a possible low calorie sweeting agent for humans and have been widely used for this purpose for many years in Japan. Fructooligosaccharides are found in plants such as Jerusalem artichoke, onions, garlic, asparagus and different grains and are also synthesized enzymatically through the action of fructosyltransferase on sucrose (Mitsuoka *et al.,* 1987; Spiegel *et al.,* 1994). They consist of a sucrose molecule to which one, two or three additional fructose units have been linked by a β-(2-1)-gycosidic linkage to the fructose unit of sucrose.

Fructooligosaccharides are selectively used by beneficial bacteria such as bifidobacteria (Table 4, not all strains shown). Fructooligosaccharides were utilized as well as glucose by all tested strains of *Bifidobacterium* with the exception of *Bifidobacterium bifidus* (Hidaka *et al.,* 1986). These are also used by many strains of *Enterococcus* and *Bacteroides.* On the other hand, none of the tested strains of *Clostridium* were shown to utilize FOS. Results of FOS supporting growth of salmonellae depend on the purity of FOS. Pure FOS does not support growth of salmonellae (Bailey *et al.*, 1991; Oyarzabal *et al.,* 1995), however, commercial FOS products do contain variable concentrations of glucose and fructose and therefore support growth of salmonellae (Oyarzabal *et al.* 1994, 1995).

Bacterial species, such as bifidobacteria, which are able to utilize FOS efficiently *in vitro* considerably increase in concentration in the GI tract with FOS addition. This increase occurs at the expense of other species (Hidaka *et al.,* 1986). Among all bacteria able to utilize FOS, bifidobacteria seem to utilize FOS most rapidly. In humans, FOS has been shown to significantly increase the stool concentration of bifidobacteria, while lactobacilli concentrations tended to increase and concentrations of *Clostridium perfringens* tended to decrease with FOS addition (Hidaka *et al.,* 1986; Mitsuoka, *et al.*, 1987).

TABLE 4: Utilization of glucose, lactose and FOS by different GI bacteria as well as avian and mammalian GI enzymes [a]

Bacterial species	Lactose	FOS
Bifidobacterium longum	++ [b]	++
Bifidobacterium infantis	++	++
Bifidobacterium bifidum	++	- [c]
Lactobacillus acidophilus	++	-
Enterococcus faecium	++	+ [d]
Bacteroides fragilis	++	++
Eubacterium lentum	-	-
Clostridium perfringens	++	-
Clostridium difficile	-	-
Escherichia coli	++	-
Salmonella ssp.	-	-
Avian enzymes	= [e]	=
Mammalian enzymes	# [f]	=

[a] adapted from Bailey *et al.*, 1991; Hidaka *et al.*, 1986; Mitsuoka, *et al.*, 1987; Oyarzabal *et al.*, 1994)
[b] Same level of growth compared to glucose
[c] No growth
[d] Weaker growth compared to glucose
[e] Not hydrolyzed by indigenous enzymes
[f] Efficient utilization only in the young animal

If sufficient fermentable carbohydrates are available in the hindgut, they can help to prevent the development of a predominantly proteolytic flora and subsequent toxic effects of the protein degradation products. Decreased concentrations of noxious putrefactive products such as indoles, skatoles, *p*-cresols and phenols have been observed in different species (Bunce *et al.*, 1995; Hidaka *et al.*, 1986). In rabbits which are known to be especially sensitive to digestive disorders, decreased cecal ammonia concentrations have been noted in diets containing 0.25% FOS (Morisse *et al.*, 1992). In the same trial FOS reduced the cecal pH and increased the level of VFA. These improvements in the microflora were associated with reduced morbidity

following an artificial oral inoculation with pathogenic *Escherichia coli* and increased production efficiency. As part of an anti-salmonella program in chicks, FOS showed promising results when used in combination with CE-cultures (Bailey *et al.*, 1991). However, FOS showed little ability to control either *Salmonella typhimurium* or *Campylobacter jejuni*, when used as a sole prevention tool (Bailey *et al.*, 1991; Schoeni and Wong, 1994). Effects of FOS on broiler growth rate, feed utilization and mortality have been inconsistent (Ammerman *et al.*, 1988a,b, 1989; Waldroup *et al.*, 1993).

Increased bifidobacteria concentrations accompanied by a reduction in putrefactive substances, ammonia, *C. perfringens* and *Pseudomonas* have also been reported with LS addition in poultry diets (Terada *et al.*, 1994). Lactosucrose also tended to improve animal performance. Galactooligosaccharides have not been intensively tested in poultry but have been shown to reduce putrefactive substances and improve performance of piglets (Kuether, 1994, Mathew *et al.*, 1994).

Oligosaccharides in particular FOS, have been shown to improve the GI environment. However, these sugars when used alone showed little ability to improve salmonella resistance in chicks.

4.4. Competitive Exclusion Effect of Carbohydrates which Selectively Block Bacterial Attachment

4.4.1. Fimbrial Attachment of Enteric Pathogens and its Control

The adherence of bacterial cells to the gut lining is mediated by many different mechanisms. These different mechanisms are the result of the large variety of surface structures which are present on the bacterial cell and interact with the host tissue. Many of these structures have still not been completely defined and bacterial : host cell interactions are still poorly understood. Over the last two decades, a large amount of research has focused on fimbriae-mediated attachment of enteric pathogens, which is the most thoroughly investigated mechanism today. Fimbriae-mediated attachment is of particular interest because gaining a foot-hold in the gut is an essential step for many enteric diseases. The control of bacterial attachment is one possible means of

keeping enteric pathogens in check and is therefore of great economical interest.

The adhesive properties of *Enterobacteriacae* were first recognized by Guyot (1908), who observed that some strains of *Escherichia coli* possessed the ability to agglutinate red blood cells from different animal species (Parry and Rooke, 1984). However, the first clues about the mechanisms of attachment were not revealed until the 1950s, when Duguid and coworkers (Duguid *et al.*, 1955) discovered that bacterial strains agglutinating red blood cells had microscopic appendages on their surface. The structures were named fimbriae. Almost simultaneously, C. Brington described the same structures and named them pili (Sharon and Lis, 1993). Both terms are interchangeable and still in use. Fimbriae are 5 to 10 nm in diameter and several hundreds of nanometers long. To learn how bacteria bind to the cells, Duguid and coworkers exposed agglutinating cells to a wide range of compounds and found that only mannose and similar sugars could inhibit haemagglutination. The isolation of a surface protein (lectin) with mannose binding properties, shed further light into this complex adherence mechanism (Eshdat *et al.*, 1978). Other types of fimbriae have been isolated over the last two decades of which some are presented in Table 5.

As bacterial adhesion is so critical to infection, different approaches to inhibit bacterial attachment have been taken. Fimbrial mediated attachment can be restrained in four different ways: blocking intestinal receptor sites, blocking bacterial lectins, eliminating intestinal receptors and inhibiting lectin expression.

Elimination of intestinal receptors has been successfully used to prevent adherence of K88-fimbriae of *E. coli*. The K88-fimbriae is found on many enterotoxigenic strains of *E. coli* which are known to cause diarrhea in piglets. Adult pigs and other mammals are not affected by K88-positive *E. coli* since they do not express the required receptors for attachment. It has also been shown that K88-positive *E. coli* do not adhere to the brush borders of all piglets, as some piglets lack the K88-receptor (Sellwood *et al.*, 1975). Since K88-resistance arises as a result of simple Mendelian inheritance, homozygote recessive piglets which do not express the K88-receptors can be bred. These piglets are resistant to K88-infections.

TABLE 5: *Fimbrial adhesins of enteric bacteria and organs and receptors* to which they can bind [a]

Fimbrial species	Organ specificity	Receptors
Type-1	Broad host and organ specificity	Mannosides
K88	Porcine intestinal epithelium	Galactoside
K99	Bovine, porcine and ovine intestinal epithelium	Sialoglycoconjugate
P987	Porcine intestinal epithelium	ND[b]
CFA 2	Human intestinal epithelium	Sialoglycoconjugate

[a] adapted from Klemm, 1985; Parry and Rooke, 1984; Seignol *et al.*, 1991; Ofek and Doyle, 1994
[b] Not determined

To date, inhibition of lectin expression, as a measure to control bacterial attachment, has not been thoroughly investigated. However, exposure of different strains of *E. coli* to different antibiotics has been shown to suppress the expression of mannose sensitive surface lectins *in vitro* (Sharon and Ofek, 1985). Therefore, it might be possible that some of the growth promoters currently used in the feed industry affect bacterial attachment.

4.4.2. Blocking of Type-1 Fimbriae

Type-1 fimbriae which specifically bind to mannose can bind to yeast cells which results in agglutination of the yeast similar to haemagglutination of erythrocytes (Mirelmann *et al.*, 1980). A positive agglutination of yeast that can be prevented by D-mannose is an indication for the presence of mannose sensitive lectins. This reaction is now commonly used to test bacterial cultures for the presence of the type-1 fimbriae (Ofek and Beachy, 1978), which are widespread among enteric pathogens (Table 6). Most of the serotypes of *Salmonella* with the exception of *Salmonella pullorum* and *Salmonella gallinarum* express type-1 fimbriae (Duguid *et al.*, 1964 and 1966). Its presence is also high among *Escherichia coli, Klebsiella pneumoniae, Citrobacter*

freundii, *Proteus morganii*, *Serratia marcescens* and *Aeromonas hydrophila* (Mirelmann *et al.*, 1980). On the other hand, strains of *Shigella* sp., *Staphylococcus aureus*, *Nessieria meningitidis*, *Pseudomonas aeruginosa* and *Proteus* ssp. other than *Proteus morganii* did not agglutinate yeast cells.

D-mannose has been shown to inhibit adherence of type-1 fimbriae not only to yeast and red blood cells, but also to epithelial tissue (Ofek *et al.*, 1977). This blocking ability makes the sugar a potential tool to inhibit bacterial attachment in the GI tract (Figure 4). Oyofo *et al.* (1989c) tested the effect of different sugars on the adherence of *Salmonella typhimurium* to epithelial cells from 1 d old chicks *in vitro* and found that mannose and methyl-α-D-mannoside were the most efficient in inhibiting adherence. They reported that mannose addition decreased the number of adherent bacterial cells to a defined intestinal surface area by more than 90 % when compared to a control with no carbohydrate addition. Other tested sugars were less effective at inhibiting adherence. Mannose and methyl-α-D-mannoside have also been reported to inhibit the adherence of *S. typhimurium* to enterocytes of rats (Lindquist *et al.*, 1987).

TABLE 6: Presence of mannose sensitive lectins on different bacterial isolates

Organism	Mirelmann *et al.*, 1980		Duguid *et al.*, 1964, 1966	
	No. of strains tested	No. of strains with mannose-sensitive lectins	No. of strains tested	No. of strains with mannose-sensitive lectins
Salmonella typhimurium	13	4	775	668
Salmonella typhi	6	4	150	122
Salmonella gallinarum			14	0
Salmonella pullorum			30	0
Salmonella enteritidis	4	4	21	21
Salmonella paratyphi A	2	0	78	2
Other salmonellae			250	220
Salmonella paratyphi B			135	106
E. coli	118	54	108	80

The situation in the GI tract differs in many ways from the situation of *in vitro* adherence studies. Mannose can be fermented by some bacteria and the complex bacterial population offers other possible binding sites for mannose. Despite these differences, mannose has been shown to be effective in decreasing enteric pathogen concentrations in the avian GI tract. In a laboratory scale salmonella challenge trial involving 30 chicks per treatment, Oyofo, *et al.* (1989a) compared the effect of 2.5 % of lactose, sucrose, dextrose, maltose and mannose added to the drinking water on cecal *S. typhimurium* concentrations in chicks. Mannose was the most effective of the tested sugars at decreasing cecal salmonella colonization when measured 7 d after salmonella application. Mannose reduced cecal salmonella concentrations by 4 log_{10}-units when compared to the control and also reduced the number of salmonella positive birds from 100 to 25 %. These positive results were confirmed in several similar trials (Oyofo *et al.* 1989b). When chicks were housed on litter and salmonella contamination was monitored on the prechilled carcass at the processing plant, no protection was achieved with mannose (Izak *et al.*, 1990). Protective effects associated with mannose have also been reported with *Campylobacter jejuni* (Schoeni and Wong, 1994).

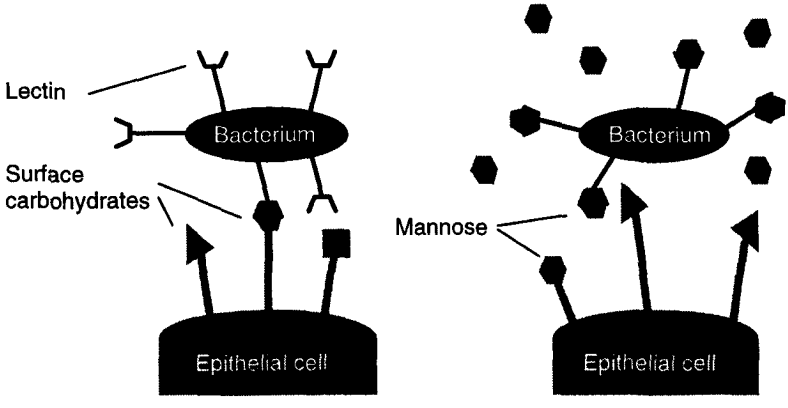


FIGURE 4: *Adherence of an enteric bacteria by means of type -1 fimbriae (left side)* and inhibition of adherence with mannose (right side)

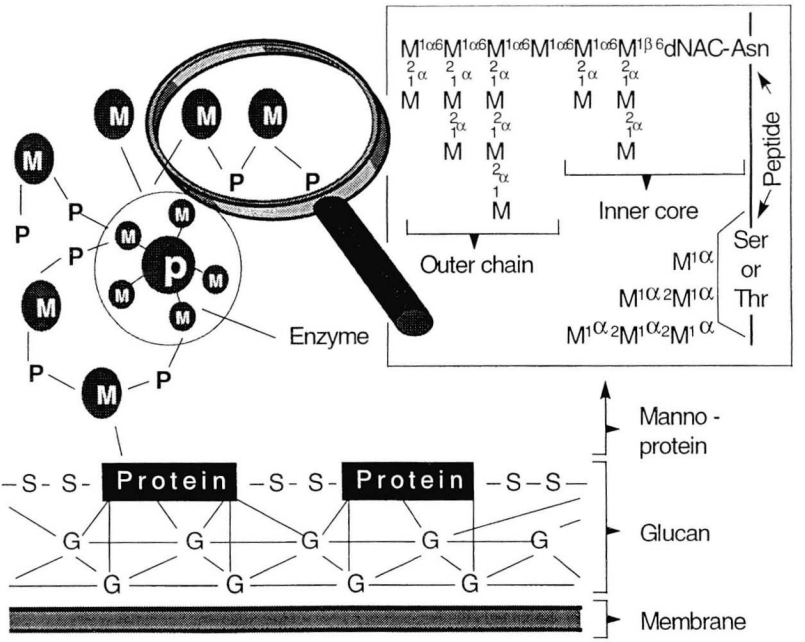
4.4.3. Mannanoligosaccharide derived from Yeast Cell Wall

Relatively high concentrations of mannose are required to control colonization of pathogenic bacteria, and the cost of pure mannose is a prohibitive expense even for just a 10 d regimen. Pure mannose is therefore not used in commercial production units. However, different plant and bacterial feed ingredients, such as certain gums and yeast cell wall contain mannose based carbohydrates in high concentrations (Ballou, 1976; Merck, 1983). These ingredients are available at reasonable costs and are therefore investigated as possible feed additives.

Yeasts have been used for many years as a high quality protein source in animal diets. Their high content of vitamins, enzymes and other important co-factors also made them attractive as a digestive aide (Dawson, 1994). Due to their ability to improve bacterial fermentation the use of yeast cultures as ruminal fermentation modulators has increased over the last decade. Improvements in bovine performance with yeast cultures have mainly been associated with improved rumen fermentation (Dawson *et al.,* 1990; Dawson, 1994). Since live yeast cultures maintain their metabolic activity in the GI tract, they can produce metabolites on site. Evidence accumulates that certain yeast metabolites stimulate growth of ruminal bacteria (Girard, 1996) and that the presence of these compounds improve ruminal fermentation. Yeast cultures have also been used in monogastric animals to stabilize the bacterial populations and improve digestion in the lower GI tract (Bradley *et al.,* 1994; Ignacio and Sefton, 1995). Since changes in bacterial patterns are very similar to the ones seen in the rumen, similar modes of action might bring about these changes (Moore *et al.,* 1994; Spring, 1992). Recently, evidence has accumulated that some of the positive effects of yeasts in monogastrics might be associated with the yeast cell wall.

Yeast cell wall consists almost entirely of protein and homopolysaccharides, which are composed of glucose, mannose and N-acetyglucosamine. Glucans and mannans, the two main sugars, are present in about equal concentrations (Cabib and Roberts 1982). Chitin is present in only small amounts (about 1%) (Cabib and Roberts 1982; van Rinsus *et al.,* 1991). In *Saccharomyces cerevisiae* the glucans contain mainly β-1-3 linkages with

some β-1-6 linkages at the branch points. The yeast mannans are exposed to the external surface and are linked to the cell wall protein fraction (Cabib and Roberts, 1982). The mannan fraction is composed of short and long chains. In *Saccharomyces cerevisiae* the short mannan chains (15-17 units) which are linked via α-1-2 and α-1-3 bonds are attached to threonine and serine of the peptide chains. The long chains (100 - 200 units) are attached to asparagine residues through a di-N-acetylchitobiose unit. These chains are connected by α-1-6 linkages and have branches which are linked via α-1-2 and α-1-3 bonds. Only the di-N-acetylchitobiose unit and the mannose attached to this unit are linked by a β-bond (Ballou, 1976; Cabib and Roberts, 1982; Figure 5).



G = glucose P = peptide Ser = serine
M = mannose dNAC = the di-N-acetylchitobiose Thr = threonine
S - S = di sulfur bonds Asn = asparagine

FIGURE 5: Structure of cell wall of *Saccharomyces cerevisiae* (adapted from Ballou, 1976; Cabib and Roberts, 1982)

The complex structure of yeast cell wall is relatively resistant to degradation by GI enzymes and bacteria (Chandler and Newman, 1994). As sole carbon source, yeast cell wall preparations do not support growth of most GI bacteria. Testing a wide range of enteric bacteria, Chandler and Newman (1994) found that none of the tested strains were able to utilize a cell wall preparation from *S. cerevisiae*. Despite the inability to support bacterial growth, yeast cell wall preparations have been shown to be effective as a feed additive.

Agglutination of yeast cells with bacterial cultures has been used for many years to screen bacteria for the presence of type-1 fimbriae. However, the effects of such agglutination in the GI tract have not been investigated thoroughly. Preliminary results suggest that a commercial preparation of mannanoligosaccharides (MOS) from the cell wall of *Saccharomyces cerevisiae* can decrease concentrations of enteric pathogens in the GI tract (Newman *et al.*, 1994). Supplementation of a commercial milk replacer with mannanoligosaccharide (MOS) has been shown to decrease fecal coliform concentrations in calves (Newman and Jacques, 1994). Mannanoligosaccharides have also been shown to reduce salmonella colonization in chicks during a trial at the Czech Research Institute (Sisak, 1994). One kilogram of MOS per tonne of feed reduced the colonization of an invasive strain of *Salmonella* in the cecum as well as in the liver and the spleen. The number of birds displaying natural infections was reduced in the group given MOS with 76% fewer cecal infections and 66% fewer organ infections compared to the control group. Since the challenge strain used in this study did not colonize the birds, colonization of a wild type of salmonellae which accidentally infected the birds was examined. It is questionable if this strain was distributed evenly throughout control and treatment groups at the onset of the experiment. Therefore, these results have to be interpreted with caution. Lou *et al.* (1995) also reported decreased fecal concentrations of lactose-negative *Entrobacteriaceae* resistant to streptomycin, sulfisoxazole and tetracycline with MOS addition in pigs. These results indicate that MOS selectively alters the concentrations of certain enterobacteriaceae. Possible effects of MOS on the GI environment and on concentrations of other bacteria in the GI tract have not yet been investigated.

In addition to preventing the colonization of enteric pathogens, MOS seem to play a role in animal immunity. *In vivo* stimulation of the specific immune system with MOS has been demonstrated in turkeys. Savage *et al.,* (1996) reported increased concentrations of plasma IgG and bile IgA with dietary MOS. Immune modulation by MOS has resulted in improved animal health. MOS has been shown to reduce respiratory diseases in calves (Newman *et al.,* 1993). This suggests that the immunological benefits with MOS are not species specific. The exact mechanisms involved in immune stimulation are still not fully understood. However, Newman (personal communication) suggests, that a small portion of the MOS might be taken up in the small intestine by M-cells, causing B-cell activation and subsequent activation of T-cells and macrophages.

Yeast cell wall preparations are also used as feed additives because of their reported ability to adsorb toxins. Yeast cultures and yeast cell preparations have been shown to bind zearalenone and aflatoxin but not vomitoxin *in vitro* (Charmley *et al.,* 1995). Park *et al.,* 1996 reported reduction in liver cholesterol and liver fat level with MOS in aflatoxin stressed chicks. Increased liver cholesterol and liver fat are typically observed during aflatoxicoses and their reduction with MOS can be taken as an indicator for a detoxifying effect of MOS.

These different modes of action of MOS have led to improved animal health and performance in various species. Table 7 summarizes trial results from different species. Mannanoligosaccharide has been shown to improve performance of broilers, turkeys and calves. Experimental conditions and with it performance responses with MOS vary considerably.

Mannanoligosaccharides derived from yeast cell wall have been shown to improve animal performance and health. Initial research indicates that these positive effects might be due to changes in the GI microflora and the immune system with dietary MOS. Detailed research is required to better understand the effects of MOS as a GI microflora and immune system modulator.

TABLE 7: Effect of mannanoligosaccharide[a] on performance and health parameters in different farm animals

Species	Weight gain: % change over control	Feed conversion % change over control	Remarks	Reference
Layer	-[b]	-	20.1 % lower egg cholesterol level with MOS	Stanley *et al.*, 1996a
Broiler	-	-	13.4 % lower breast muscle and 23.0 % lower liver tissue cholesterol with MOS; 15.9 % higher breast muscle protein content and 16.2 % higher liver tissue protein content with MOS	Stanley *et al.*, 1996a
Broiler	8.1	-	No change in cecal coliform concentrations with MOS	Stanley *et al.*, 1996b
Broiler			Reduction of liver cholesterol and liver fat content with MOS in aflatoxin stressed chicks	Park *et al.*, 1996
Broiler	- 0.2	2.4	14.9 % Increase bile IgA, reduced mortality from acites with MOS	Savage *et al.*, 1996b
Turkey	8.5[c]	- 1.3	0 - 4 week	Savage *et al.*, 1995
Turkey	14.9[c]	- 6.1[c]	4 - 8 week	Savage *et al.*, 1995
Turkey	6.7[c]	-1.0	30 % increase in bile IgA and 26 % increase in plasma IgG with MOS	Savage *et al.*, 1996a
Emu	0	0	Lower plasma carbonate, higher plasma trigyceride concentrations with MOS	Cunningham *et al.*, 1996
Calves	26.0[c]	-	Less respiratory diseases, starter intake 18 % higher with dietary MOS	Newman *et al.*, 1993
Calves	19.0[c]	-	Less fecal coliforms	Jacques and Newman, 1994

[a] Bio-Mos, Alltech Inc., Nicholasville KY, USA [b] Not determined [c] significant

5. HYPOTHESIS AND OBJECTIVES

Mannose has been shown in several trials to decrease the prevalence of enteric pathogens that express type-1 fimbriae in poultry (Oyofo *et al.*, 1989a,b,c). These authors suggested that mannose affects bacterial concentrations by blocking type-1 fimbriae and therefore keeping bacteria from adhering to the intestinal surface. Yeast cell wall consists of 30 % mannose and might therefore affect bacterial concentrations in the GI tract in a similar way as mannose.

Hypothesis:

- Mannanoligosaccharides have the ability to decrease the concentrations of enteric pathogens that express type-1 fimbriae in poultry.

The objectives of these studies were to:

- screen different bacterial strains for their ability to agglutinate MOS and compare this agglutination patterns with haemagglutination.
- determine the effect of MOS on cecal fermentation parameters, cecal microflora and enteric pathogen and coliform colonization in chicks under controlled conditions.
- evaluate the effect of MOS on ileal morphology in chicks.

PART II:

TRIALS

6. ADHERENCE STUDIES

6.1. Abstract

The abilities of different enteric pathogens and coliforms to trigger agglutination of a yeast cell wall preparation (MOS), a yeast culture and haemagglutination were qualitatively evaluated. Moreover, a titer for agglutination of MOS was determined and inhibition of agglutination by mannose and fructose was quantified.

Seven of nine strains of *Escherichia coli* and eleven of seventeen strains of *Salmonella typhimurium* and *Salmonella enteritidis* agglutinated MOS and cells of *S. cerevisiae* NYCC 1026. No differences were observed between the agglutination patterns of the two yeast products. The tested strains of *Salmonella choleraesuis, Salmonella pullorum* and *Campylobacter* did not lead to agglutination. All agglutinations could be inhibited by mannose, which indicates the presence of type-1 fimbriae on those bacterial strains. Agglutination could also be inhibited by fructose while glucose and galactose had no effect on agglutination. However, concentrations of fructose required to inhibit agglutination were eight to sixteen times higher than those of mannose. Bacterial concentrations required to cause agglutination varied between strains with strains of *E. coli* causing agglutination at lower concentrations than *Salmonella* strains. In general, haemagglutination patterns were similar to agglutination pattern of the yeast products. However, despite causing mannose-sensitive agglutination of the yeast products, haemagglutination of strains of *E. coli* K99 could not be inhibited with mannose.

6.2. Introduction

Adherence of bacteria to host tissue plays a central role in the ability of bacterial pathogens to colonize mucosal and endothelial surfaces (Ofek and Beachy, 1978). Therefore, much research has focused on describing the adherence systems which are involved in bacteria to host cell interactions. The agglutination of erythrocytes in the presence of bacteria has been known since the beginning of the century (Guyot, 1908). Rosenthal (1943) showed that

strains of *Escherichia coli* do not only agglutinate red blood cells, but also leukocytes, thrombocytes, sperm, yeast cells, fungal spores and plant pollen of various kinds. However, it was not until 1955 that agglutination was associated with the presence of fimbriae on the surface of the bacterial cell (Duguid, *et al.*, 1950). Several types of fimbriae have been described. The attachment of type-1 fimbriae can be inhibited by mannose or mannose derivatives. Most of the serotypes of *Salmonella* with the exception of *Salmonella pullorum* and *Salmonella gallinarum* express mannose sensitive fimbriae (Duguid *et al.*, 1964, 1966). Type-1 fimbriae have also been shown to mediate bacterial attachment to epithelial cells, and such attachment can be blocked by mannose *in vitro* (Ofek *et al.*, 1977). Addition of 2.5 % of mannose to the drinking water has been shown to decrease cecal salmonella concentrations and the number of salmonella positive birds 7 d after salmonella challenge (Oyofo *et al.* 1989b). Mannose and mannose derivatives such as MOS have the potential to inhibit bacterial attachment in the GI tract and can specifically decrease bacterial concentrations of strains that express mannose sensitive fimbriae.

The objectives of these studies were the following: screen different enteric pathogens for their ability to agglutinate MOS and yeast culture; quantify the inhibitory effect of different carbohydrates on positive agglutination; quantify the minimal bacterial concentrations required to cause agglutination and compare agglutination of MOS and yeast culture with haemagglutination.

6.3. Materials and Methods

MOS: The mannanoligosaccharide preparation used in these studies was the commercial product Bio-Mos (Alltech Inc., Nicholasville KY). The product contains yeast cell wall fragments derived from *Saccharomyces cerevisiae*. The cell wall fragments are obtained by centrifugation from a lysed culture of *Saccharomyces cerevisiae* (lysing conditions not made available by Alltech). The pellet containing the yeast cell wall fragments is then washed and spray dried.

Agglutination of yeast products: In a first series of tests agglutinations of MOS and of a culture of *Saccharomyces cerevisiae* NYCC 1026 by different enteric pathogens and coliforms were qualitatively examined. The source of the tested bacterial strains is given in Table 9. Analyses were conducted as described in the method for qualitative determination of yeast cell agglutination (Mirelmann *et al.*, 1980). Bacterial cells were grown for 24 h in Media for Enrichment of Type-1 Fimbriae (Table 8). Cells were harvested by centrifugation and suspended in phosphate buffer saline (PBS, pH 7.2) to reach an optical density at 660 nm (OD_{660}) of approximately 1.0. Mannanoligosaccharide was suspended at a rate of 1 g/L in PBS (OD_{660} = 1.60). A 24 h culture of *Saccharomyces cerevisiae* NYCC 1026 grown in tryptic soy broth (TSB) was harvested by centrifugation and cells were suspended in PBS to reach an optical density similar to the one of the MOS suspension (OD_{660} = 1.60). Both yeast and MOS suspension were blended for 1 min in a kitchen blender to separate clumping cells and MOS particles, respectively. Ten microliters of bacteria and MOS or yeast suspension were placed on a microscopic slide and mixed for 2 min on a vortex shaker. Agglutination was then judged under a light microscope (magnification of 250x). Mannanoligosaccharide or yeast suspension mixed with PBS served as a negative control.

In order to determine the inhibitory effect of carbohydrates on agglutination, each agglutinating strain was first incubated for 5 min in PBS containing 50 mM glucose, mannose, galactose or fructose. These sugars were selected since they are the major components of oligosaccharides used as feed additives in the animal feed industry. Agglutination reactions were then performed as described above. A sugar was said to inhibit agglutination if inhibition (free, non clumping yeast cells or MOS particles) could be observed under the microscope. No differentiation was made if inhibition was partial or complete.

TABLE 8: Media for Enrichment of Type-1 Fimbriae [a]

Ingredient	g/L
Peptone	10
NaCl	5
Yeast extract	5

[a] Firon *et al.*, 1987

In a second series of agglutination tests, seven agglutinating strains were selected and an agglutination titer was determined for each strain. For this purpose bacterial cultures were grown and prepared as described (OD_{660} = 1.0). Two-fold serial dilutions were then prepared and agglutination tests were performed as described above. The highest two-fold dilution that showed agglutination to MOS was then determined.

A third series of tests was conducted to quantify the inhibition of agglutination by mannose and fructose. The seven bacterial cultures used in the second series prepared in PBS with an OD_{660} of 1.1 were used for these tests. Two-fold serial dilutions of 2.0 M mannose and fructose solutions were prepared in PBS and added to the bacterial cultures (0.1 ml of sugar solution to 0.9 ml of bacterial culture → 1:10 dilution) to yield suspensions containing 200, 100, 50, 25,....0.39 mM of sugar and an OD_{660} of 1.0. Cultures were then incubated for 5 min at room temperature and agglutination tests were performed. The lowest sugar concentration required to completely inhibit agglutination was then determined under the microscope. Agglutination of MOS caused by *Escherichia coli* K99 could not be completely inhibited with 200 mM fructose solution. Therefore, this strain was harvested into PBS containing 400 mM of fructose and agglutination test was performed with this preparation.

Haemagglutination: Seven MOS agglutinating and non-agglutinating strains were selected and agglutination patterns of MOS was compared to haemagglutination. Blood and bacterial cell suspensions were prepared as described by Ghosh *et al.* (1993). Pig and horse blood were used in the study. The blood suspensions were modified to contain 2 % instead of 3 % (vol./vol.)

of cells. Fifty microliters of each suspension were then placed in wells of microtiter plates. The plates were rotated at 150 rpm for 20 min and kept at 4°C for 2 h before agglutination was evaluated. Agglutination was tested for fructose, mannose, glucose and galactose sensitivity by individually adding 1 g of each sugar to 100 ml of the blood cell preparations prior to the agglutination test. Agglutination was defined as being sensitive to a certain sugar when the agglutination was partially or completely inhibited in its presence.

6.4. Results

As shown in Table 9, five of seven strains of *Escherichia coli* and seven of ten strains of *Salmonella typhimurium* and *Salmonella enteritidis* agglutinated MOS. *Salmonella montevideo, Salmonella give, Salmonella kedougou* and *Salmonella dublin* also triggered agglutination. *Salmonella choleraesuis and Salmonella pullorum* strains did not lead to agglutination. Positive agglutination could be inhibited with both mannose and fructose. However, it was observed that fructose was less effective as a blocking agent. Agglutination patterns were similar to patterns of a culture of *Saccharomyces cerevisiae* NYCC 1026 (data not shown). Different strains of *Campylobacter* were also tested for their abilities to agglutinate MOS. Only two strains showed some agglutination, however, these agglutinations were very weak and were not affected by addition of carbohydrates.

TABLE 9: Ability of different bacterial strains to agglutinate MOS and effect of fructose, galactose, glucose and mannose on agglutination

Strain	Source	Agglutination of MOS	Inhibition of Agglutination by			
			Fructose	Galactose	Glucose	Mannose
E. coli K99	A[a] K99	+[b]	Yes	No	No	Yes
E. coli K99	ATCC[c] 31619	+	Yes	No	No	Yes
E. coli 4157	A 4157	-[d]				
E. coli 15R	UKAS[e] 15R	+	Yes	No	No	Yes
E. coli	A 39639 B2	+	Yes	No	No	Yes
E. coli O157:H7	A 0157H7	-				
E. coli	A 39639 B2	+	Yes	No	No	Yes
S. enteritidis	A 13A	+	Yes	No	No	Yes
S. enteritidis	A 371	+	Yes	No	No	Yes
S. enteritidis	A 3	+	Yes	No	No	Yes
S. enteritidis	A 52	+	Yes	No	No	Yes
S. enteritidis	ATCC 13076	-				
S. typhimurium	UKAS 29E	+	Yes	No	No	Yes
S. typhimurium	ATCC 14028	+	Yes	No	No	Yes
S. typhimurium	ATCC 13311	+	Yes	No	No	Yes
S. typhimurium	ATCC 29630	-				
S. typhimurium	UKAS 27A	-				
S. montevideo	A 95111010 J	+	Yes	No	No	Yes
S. give	A 95111010 F	+	Yes	No	No	Yes
S. kedougou	NCTC[f] 12173	+	Yes	No	No	Yes
S. dublin	ATCC 15480	+	Yes	No	No	Yes
S. pullorum	ATCC 9120	-				
S. pullorum	ATCC 19945	-				
S. choleraesuis	ATCC 13317	-				
S. choleraesuis	ATCC 9150	-				

(Table 9 continued)

Strain	Source	Agglutination of MOS	Inhibition of Agglutination by			
			Fru	Gal	Glu	Man
S. choleraesuis	ATCC 13312	-				
C. jejuni	ATCC 29428	-				
C. jejuni	ATCC 25217	-				
C. jejuni	UKAS 240	weak [g]	No	No	No	No
C. jejuni	UKAS 215	-				
C. jejuni	UKAS W91	-				
C. coli	ATCC 43481	-				
C. coli	ATCC 33559	-				
C. coli	UKAS 237	-				
C. coli	UKAS 216	-				
C. coli	UKAS 218	weak	No	No	No	No
C. lari	UKAS 234	-				

[a] Culture Collection of Alltech Inc., Nicholasville, KY
[b] MOS particles are clumping together
[c] American Type Culture Collection, Rockville, MD
[d] No MOS particles are clumping together
[e] Culture Collection of the Univ. of Kentucky, Dept. Anim. Sci., Lexington, KY
[f] National Collection of Type Cultures, Central Public Health Laboratory, London, UK
[g] A few particles are clumped but the majority are free

The agglutination titer of various strains of bacteria for MOS-agglutination was defined as the highest dilution of a bacterial culture (OD_{660} = 1.0) that agglutinated a given concentration of MOS. As shown in Table 10 the concentration of *E. coli* K99 required to cause agglutination was lower than for any other tested strains of bacteria. *Escherichia coli* K99 caused MOS to agglutinate even after a dilution of 1:128. The agglutination titers of the *Salmonella* strains were lower than those of the *E. coli* strains. Depending on the strain, the minimal salmonella concentration that still led to agglutination was obtained after 8- to 32-fold dilutions.

TABLE 10: Agglutination titer of various enteric pathogens and coliforms

Bacterial strain	Agglutination titer [a]
Escherichia coli K99	128
E. coli K99 O101	128
E. coli 15R	64
Salmonella typhimurium 29E	8
Salmonella dublin	32
Salmonella enteritidis 13A	32
Salmonella kentucky	16

[a] Highest dilution factor that caused agglutination

The ability of mannose and fructose to inhibit agglutination of MOS by different enteric bacteria was quantified on seven bacterial strains, which have previously been shown to agglutinate MOS. As shown in Table 11, agglutination caused by bacterial strains with a high agglutination titer (Table 10) required higher concentrations of both mannose and fructose for inhibition. As observed in the qualitative agglutination test, mannose was more effective than fructose inhibiting agglutination. Mannose concentrations required to inhibit agglutination were 8 to 16 times lower than fructose concentrations. *Escherichia coli* K99 required the highest concentration for inhibition with 25 mM mannose and 400 mM fructose.

As shown in Table 12 bacterial strains that agglutinated MOS also triggered haemagglutination. Most agglutination of horse and pig blood could be inhibited with both mannose and fructose. However, agglutination triggered by *E. coli* K99 were not affected by either of those two carbohydrates.

TABLE 11: Concentrations of mannose and fructose required to inhibit agglutination of MOS by different enteric pathogens and coliforms

Bacterial strain	Mannose (mM)	Fructose (mM)	Difference Factor[a]
Escherichia coli K99	25	400.00	16
E. coli K99 O101	25	200.00	8
E. coli R15	6.25	50.00	8
Salmonella typhimurium 29E	0.39	3.13	8
Salmonella dublin	0.39	3.13	8
Salmonella enteritidis 13A	0.78	6.25	8
Salmonella kentucky	0.78	6.25	8

[a] Fructose concentration / mannose concentration

TABLE 12: Haemagglutination of enteric pathogens and coliforms to pig and horse erythrocytes and inhibitory effects of fructose and mannose on agglutination

Bacterial strain	Haemagglutination with erythrocytes of		Inhibition	
	Pig	Horse	Fructose	Mannose
E. coli K99	+[a]	+	No	No
E. coli K99 O101	+	+	No	No
E. coli 15R	+	+	Yes	Yes
E. coli O157:H7	-[b]	-		
S. typhimurium 29E	+	+	Yes	Yes
S. typhimurium 27A	+	+	Yes	Yes
S. choleraesuis 9150	-	-	No	No

[a] Erythrocytes are clumping in the center of the well
[b] Erythrocytes are homogeneously distributed in the well

6.5. Discussion

The colonization of bacteria on mucosal tissues is recognized as an important step in the infectious process. To colonize the mucosal surfaces bacteria must first bind to the epithelial cells of these tissues. One way of binding to epithelial cells is through attachment of type-1 fimbriae (Ofek *et al.*, 1977). Type-1 fimbriae which specifically bind to mannose can bind to yeast cells or erythrocytes which results in agglutination. A variety of strains of *Escherichia coli*, *Salmonella* and *Campylobacter* were tested for the presence of type-1 fimbriae by means of yeast and MOS agglutination. A positive agglutination of yeast that can be inhibited with mannose can be taken as an indicator for the presence of type-1 fimbriae (mannose-specific fimbriae) on the surface of the tested bacteria (Mirelmann *et al.*, 1983). No differences were determined between agglutination pattern of the two yeast products. The MOS particles seem to have similar agglutination properties as the intact yeast cells. However, due to the hydrolysis and smaller particle size more surface might be exposed with MOS than intact yeast. The majority of the tested strains of *Escherichia coli* and paratyphoid *Salmonella* agglutinated both the yeast culture and MOS. All agglutinations were mannose sensitive. This is in agreement with findings by Duguid *et al.* (1964, 1966) who reported high frequency of type-1 fimbriae expression in *Salmonella typhimurium, Salmonella typhi* as well as *Salmonella enteritidis*. On the other hand Mirelmann *et al.* (1980) only found 4 of 13 strains of *S. typhimurium* to express type-1 fimbriae. The poultry specific salmonella types, *Salmonella pullorum* and *Salmonella gallinarum* did not trigger agglutination in the work of Duguid *et al.* (1964) or in the study presented here. Four out of seven tested strains of *E. coli* agglutinated MOS. Mirelmann *et al.* (1980) reported that about half of the tested strains of *E. coli* expressed type-1 fimbriae. Agglutination could not only be inhibited by mannose but also by fructose. However, minimal inhibitory sugar concentrations were 8 to 16 times lower for mannose than for fructose. This indicates that fructose has lower affinity than mannose to bind to type-1 fimbriae. Fructose is also efficiently used for bacterial growth which reduces its concentrations down the digestive tract. This suggests that the concentrations

of fructose or its oligosaccharides required to inhibit bacterial attachment would be higher than required mannose concentrations. Therefore, it is unlikely that inhibition of bacterial attachment would be a major mode of action of FOS.

The agglutination titer is a quantitative measure for the ability of a bacterial culture to cause yeast or MOS agglutination. Values between strains varied from 8 to 128, meaning that bacterial cultures of an OD_{660} of 1.0 caused agglutination after 8 and 128-fold dilutions, respectively. Further dilutions did not triggered agglutination. Strains of *E. coli* showed higher agglutination titers than *Salmonella* strains. Mirelmann *et al.* (1980) found similar differences between strains (8 - 256), however, absolute values can not be compared due to differences in the methods used to measure agglutination. It is questionable if the *in vitro* agglutination titer has much relevance on the effect of MOS on bacterial attachment *in vivo,* since expression of fimbriae can vary with the growth conditions of a bacterial culture (Firon *et al.,* 1983). Fimbriae expression *in vivo* might therefore greatly vary from *in vitro* measurements.

In general, haemagglutination patterns were similar to the agglutination patterns observed with the two yeast products. However, haemagglutination of strains of *E. coli* K99 could not be inhibited by mannose. This may be due to the presence of a non-mannose sensitive fimbriae (Klemm, 1985). The same cultures of *Escherichia coli* K99 agglutinated MOS and were mannose sensitive which indicates the presence of type-1 fimbriae. Therefore, screening bacterial isolates for the presence of type-1 fimbriae should be conducted with yeast products, since haemagglutination does not allow detection of type-1 fimbriae if other haemagglutinating types of fimbriae are present on the bacterial cell surface.

None of the tested *Campylobacter* strains caused a mannose sensitive agglutination of MOS. Mannose was reported to inhibit adherence of *Campylobacter jejuni* to epithelial cells *in vitro* (McSweegan and Walker, 1980). Protective effects with dietary mannose was also reported against *Campylobacter jejuni* colonization in chickens (Schoeni and Wong, 1994). However, these authors did not determine if the *Campylobacter* strains used in their study expressed mannose sensitive fimbriae. Therefore, it is not possible to say if inhibition of attachment or other mechanisms were involved in their

studies. Inhibition of *Campylobacter* colonization has also been reported with fucose addition (Cinco *et al.,* 1983), which is a constituent of the mucus the primary site of *Campylobacter* colonization.

The majority of strains of *E. coli* and paratyphoid *Salmonella* showed agglutination of MOS, while *S. pullorum* and *Campylobacter* strains did not trigger agglutination. It remains to be tested if MOS can affect the concentrations of agglutinating strains in the GI tract, by keeping them from adhering to the gut wall.

7. CHALLENGE TRIALS

7.1. Abstract

The effects of a preparation of mannanoligosaccharide (MOS) from *Saccharomyces cerevisiae* on the prevalence of three strains of enteric bacteria that expressed type-1 fimbriae (*Salmonella typhimurium* 29E, *Salmonella dublin* and *E. coli* 15R) and one strain that did not express type-1 fimbriae *(S. typhimurium* 27A) were studied in chicks. Additionally, changes in the activities and concentrations of the cecal microflora with dietary MOS was determined. To control experimental conditions, the gastrointestinal (GI) microflora of each chick was standardized at the beginning of the experiment and birds were housed in bacterial isolation chambers. Birds received 10^4 CFU/bird of the challenge organisms at day 3 and the following cecal parameters were analyzed at day 10: Concentrations of challenge organisms, coliforms, lactobacilli, enterococci, anaerobic bacteria, lactate, and VFA and cecal pH.

In two series of three trials, MOS treated chicks had reduced cecal concentrations of *S. typhimurium* 29E (5.40 vs. 4.01 log CFU/g; $P < 0.05$), and the number of birds that tested positive for *S. dublin* in the ceca was lower when MOS was part of the diet (90 % vs. 56 %; $P < 0.05$). Dietary addition of MOS also reduced the number of birds from which *E. coli* 15R could be recovered (75 % vs. 15 %). Mannanoligosaccharide tended to reduce the concentrations of cecal coliforms in the trials with *S. typhimurium* 29E and *S. dublin.* Statistical analysis over all experiments showed a significant reduction in cecal coliforms (8.80 vs. 8.54 log CFU/g $P < 0.05$). Mannanoligosaccharide had no effect on cecal concentrations of lactobacilli, enterococci and anaerobic bacteria. Lactate concentrations, VFA concentrations and cecal pH were also not affected by treatment. In order to test the effect of MOS on concentrations of bacteria that do no express type-1 fimbriae, a trial was conducted with *S. typhimurium* 27A as challenge organism. However, strain 27A did not colonize the birds sufficiently to evaluate if MOS affected its cecal concentration.

7.2. Introduction

Many enteric pathogens must attach to the mucosal surface of the gut wall to establish themselves in the GI tract (Costerton *et al.*, 1978; Fuller *et al.*, 1981). Due to the simple composition of the microflora, little competition for bacterial attachment sites on the gut wall exists in the newly hatched chick. This lack of competition makes the young bird very susceptible to colonization with enteric pathogens such as salmonellae. Since attachment of enteric pathogens is often mediated through binding of bacterial lectins to D-mannose containing receptors (Eshdat *et al.*, 1978), blocking these lectins with mannose or similar sugars offers an approach to restrain bacterial attachment and therefore reduce bacterial colonization.

Oyofo *et al.* (1989c) tested the effects of different sugars on the adherence of *Salmonella typhimurium* to epithelial cells from 1 d old chicks *in vitro* and found mannose and methy-α-D-mannoside to be the most efficient in inhibiting adherence. They reported that their addition decreased the number of adhered bacterial cells to a defined intestinal surface area by more than 90 % compared to a control with no carbohydrate addition. Similar effects were reported on inhibition of the adherence of *S. typhimurium* to enterocytes of rats (Lindquist *et al.*, 1987). The inhibition of adherence of enteric pathogens with mannose might be the reason for decreased colonization of *S. typhimurium* when mannose was added to the diet of young chicks (Oyofo *et al.*, 1989a,b). However, mannose is not used commercially due to its high cost.

Mannanoligosaccharide derived from yeast cell wall may offer a source of mannose at a lower cost. Most unadapted paratyphoid salmonella-isolates have been shown to agglutinate MOS *in vitro*, by means of type-1 fimbriae (Chapter 6). In the GI tract, this bacteria to MOS interaction would not result in agglutination, since the MOS fragments are not in close contact with each other. However, the interaction could result in adsorption of the bacteria to the MOS. The effect of MOS on concentrations of pathogens has not yet been investigated under controlled laboratory conditions, however, an initial trial indicates that MOS might decrease salmonella concentrations in the cecum, liver and spleen of chicks (Sisak, 1994). Therefore, MOS might decrease the

prevalence of enteric pathogens and coliforms that express type-1 fimbriae in the cecum of chicks.

The objectives of these studies were to: determine the effects of MOS on the cecal concentrations of different strains of *Salmonella* and *Escherichia coli* and evaluate the effects of MOS on the activities and concentrations of different populations of cecal bacteria.

7.3. Materials and Methods

Experimental design: All animal trials were conducted following the animal care protocol IACUC 94-0004A approved by the University of Kentucky. Each experiment consisted of two control and two treatment groups containing ten birds each. Birds were hatched and housed in bacterial isolation chambers. Feed and water was provided *ad libitum*. Gastrointestinal microflora of birds was standardized at the beginning of each experiment. Birds were given 10^4 CFU/bird of one of the challenge organisms on day 3 and cecal parameters were determined on day 10.

Bacterial isolation chambers: Five bacterial isolation chambers (Standard Safety Equipment Co., Palatine, IL) were used for these studies. One chamber containing the incubator was outfitted with a bacterial trap filled with disinfecting solution, while the four others containing plastic housing boxes (3840 cm^2 → 320 cm^2 per bird) were equipped with regular inlet ports (Figure 6). The bacterial trap allowed entry or removal of material in or from the chamber without exchanging bacteria between the chamber and the environment. Each chamber had two bacterial filters (one as air inlet and one as air outlet) to prevent cross contamination of the bacterial populations between the different treatment groups, and to prevent the escape of pathogenic microorganism into the environment. These filters were autoclaved before each experiment. The chambers were then sanitized by spraying 500 ml of a 2 % solution of peracetic acid in each inflated chamber (Makin and Tzipori, 1980). Twenty-four hours after fumigation the chambers were vented for 24 h.

Feed, water and 500 g of wood shavings were placed in autoclave bags, sterilized for 20 min at 121 °C and placed in the chambers prior to fumigation.

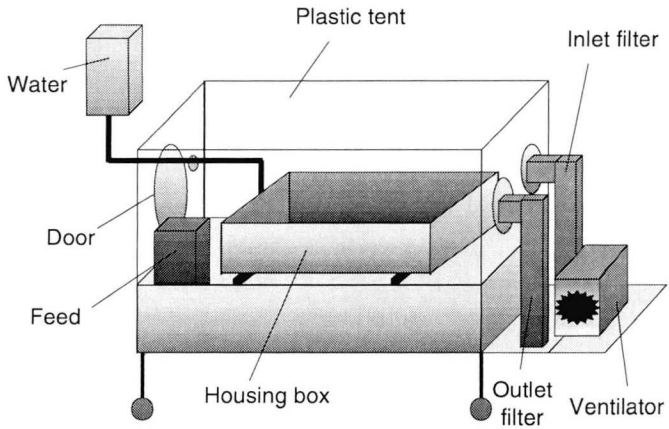


FIGURE 6: Bacterial isolation chamber fitted with air inlet and outlet filter to avoid cross contamination of bacterial populations between treatment groups

Birds: Sixty eggs (Line-24) obtained from Avian Farms (Sommerset, KY) were used to produce the chicks for each study. Eggs were sanitized by spraying with a disinfectant solution (2.0% H_2O_2, 0.8% quaternary ammonium, 0.25% acetic acid) and were then incubated in a commercial incubator which was previously sanitized with the same disinfectant. Eggs were misted daily with the disinfectant. On day 19, eggs were candled and fertile eggs were dipped for 30 sec in disinfectant solution (37°C) and transferred into the sterile incubator which was placed in one bacterial isolator. The bacterial trap at the chamber inlet was filled with the same disinfectant solution. Six hours after standard inoculation, chicks were randomly assigned to four groups of ten chicks and were transferred into the bacterial isolation chambers.

Standard inoculum: Newly hatched chicks were inoculated with a standard inoculum to provide similar basic microflora for each bird at the beginning of each experiment. The debris originated from a commercial hatchery (Hudson Foods, Coydon, IN). After the newly hatched chicks were removed from the hatching trays, the debris (without unhatched or dead birds) of 5 trays were combined in a sterile bag and the particle size of the combined sample was reduced. The debris were then mixed and stored in 100 g aliquots at - 80 °C. Four 100 g samples were tested for the presence of salmonellae according to the AOAC standard method for isolation of salmonellae (Wallace *et al.,* 1992). No salmonellae could be isolated from any of the four samples. Microbial counts on the debris revealed the following bacterial concentrations: Total bacteria, 2.5 x 10^7 CFU/g (determined on Viand Levure Agar (VLA) at pH 6.7; Barnes *et al.,* 1979); Coliforms, 8.03 x 10^6 CFU/g (determined on MacConkey Agar (MacA), Difco Laboratories, Detroit, MI); Enterococci, 7.50 x 10^6 CFU/g (determined on KF Streptococcal Agar (KFA), Difco Laboratories, Detroit, MI); Lactobacilli, < 1000 CFU/g (determined on Rogosa Agar (RoA), Difco Laboratories, Detroit, MI). The defrosted debris was mixed 1:5 with 0.1 % peptone water, blended in a sterile kitchen blender and each bird was given 0.25 ml from the tip of a pipette (1.25 x 10^6 CFU/bird).

Husbandry: Room temperature was adjusted to 34 °C for the first 2 d and was then gradually lowered to 25 °C at day 10. Light was on for 24 h after the chicks were transferred into the chamber and for 20 h each of the following days. Feed and water were supplied *ad libitum.* Tables 13 and 14 show the composition and the calculated nutrient concentrations of the unmedicated broiler starter diet.

TABLE 13: Composition of unmedicated broiler starter diet

Ingredient	Amount (kg/t)
Corn	563.0
Soybean meal 48	351.5
Blended fat	43.0
NaCl	5.0
Limestone	11.0
Dical (23 -18)	18.0
Vitamin-Mineral Pak [a]	5.0
DL methionine	2.0
Choline CL 60 %	1.5

[a] to provide the following concentrations per kg finished diet:
Zn: 75 ppm; Fe: 40 ppm; Mn 64 ppm; Cu: 10 ppm; I: 1.85; Se: 0.3 ppm;
vitamin A: 10,000 IU; vitamin D_3: 3,500 IU; vitamin E: 30 IU;
vitamin K activity: 2.8 IU; vitamin B_{12}: 0.03 mg; menadione: 0.9 mg;
riboflavin: 7.7 mg; thiamine: 2.2 mg; d-pantothenic acid: 18 mg;
niacin: 55 mg; vitamin B6: 5 mg; folic acid: 1.1 mg;
choline chloride: 550 mg; choline: 500 mg; d-biotin: 0.22 mg

TABLE 14: Nutrient concentration of unmedicated broiler starter diet

Item		Concentration	Item		Concentration
Energy	(MJ/kg)	12.96	Protein	(%)	21.88
Fiber	(%)	2.60	Lysine	(%)	1.21
Calcium	(%)	0.94	Methionine	(%)	0.55
Available P	(%)	0.47	Meth + Cys	(%)	0.91
Total P	(%)	0.72	Isoleucine	(%)	1.10
Sodium	(%)	0.22	Leucine	(%)	1.88
Fat	(%)	6.72	Threonine	(%)	0.89
Xanthophylles	(mg/kg)	13.18	Arginine	(%)	1.55
Linoleic acid	(%)	1.72	Valine	(%)	1.10
			Choline	(%)	0.19

Treatment: Treatments in all trials included an unsupplemented diet (control) and a diet supplemented with 4000 ppm of dietary MOS (Bio-Mos, Alltech Inc., Nicholasville, KY). Mannanoligosaccharide was added to the diet prior to autoclaving.

Challenge cultures and their application: Challenge cultures were grown for 18 h on Media for Enrichment for Type-1 Fimbriae (Firon *et al.*, 1987). The cultures were then diluted based on optical density in sterile 0.1 % peptone solution (Bacto Peptone, Difco Laboratory, Detroit, MI) to a final concentration of approximately $4x10^4$ CFU/ml. Concentrations of salmonella were determined on Brilliant Green Agar (BGA) (Difco Laboratories, Detroit, MI) using spread plate technique. *Escherichia coli* concentrations were determined on Violet Red Bile Agar with MUG (VRBA) (Difco Laboratories, Detroit, MI). One quarter of a milliliter of the diluted culture ($1x10^4$ CFU/bird) was given to each bird from the tip of a pipette.

Sampling and Sample Analysis: At day 10 the chicks from each group were sacrificed by asphyxiation with CO_2 and both ceca were aseptically removed. The content of one cecum was placed in a sterile test tube, weighed and diluted 1:10 with a sterile 0.1 % peptone solution. The empty cecum was cut longitudinally and placed in lactose broth. Decimal dilutions of each sample were prepared in 0.1 % peptone solution. Salmonellae were enumerated on BGA containing 30 mg of naladixic acid per L after 24 h incubation at 37 °C. Naladixic acid was added to the media to facilitate the selection of the challenge organisms (*S. typhimurium* 29E and 27A, *S. dubin* and *E. coli* 15R). Randomly selected colonies showing characteristic salmonella colony morphology were inoculated on Triple Sugar Iron Agar to confirm the presence of salmonellae. The tube containing the empty cecum in lactose broth was incubated at 37°C for 24 h for enrichment of salmonellae. Subsequent testing for the presence of salmonellae was performed according to the AOAC standard method for isolation of salmonellae (Wallace *et al.*, 1992). Cecal samples that were salmonella negative on BGA plates, but positive in the

enrichment test were assigned as 1.50 log CFU/g (Nisbet *et al.*, 1993). Samples that were confirmed negative in the enrichment test were assigned as 0 log CFU/g (1 CFU/g) (Nisbet *et al.*, 1993). *Escherichia coli* challenge organisms were recovered in VRBA containing 30 mg of naladixic acid. Four birds per treatment were randomly selected for analysis of the concentrations of different groups of intestinal bacteria. Further decimal dilutions of the samples were prepared in 0.1 % peptone solution. Lactobacilli, enterococci, coliforms and anaerobic bacteria were enumerated in duplicate using pour plate technique as follows:

- Lactobacilli: RoA plated at 10^{-6} to 10^{-8} ml
- Enterococci: KFA plated at 10^{-6} to 10^{-8} ml
- Coliforms: VRBA plated at 10^{-5} to 10^{-7} ml
- Anaerobic bacteria: Reinforced Clostridial Agar (RCA) plated at 10^{-6} to 10^{-9} ml

RoA and RCA plates were incubated anaerobically in an incubator placed in an anaerobic chamber (Coy Laboratories, Ann Arbor, MI). KF Streptococcus Agar and VRBA plates were incubated aerobically for 48 h at 37 °C.

Determination of pH, VFA and lactate concentrations: The content of the second cecum was diluted 1:10 in distilled water and the pH was determined with a combined electrode (Orion Research Inc., Boston, MA). Samples were stored at – 20 °C until VFA and lactate analyses. Volatile fatty acid analysis was conducted on a Hewett-Packard model 5890 gas chromatograph (Hewlett-Packard, Palo Alto, CA), fitted with a 180 cm x 4 mm glass column, containing 10 % SP 1000/1 % H_3PO_4 on 100/120 Chromosorb WAW (Supelco Inc., Bellofonte, PA) using a modification of the method described by Erwin *et al.* (1961). D- and L-lactate were determined spectrophotometrically with D- and L-lactate dehydrogenase, respectively, in the presence of NAD (Brandt *et al.*, 1980).

Statistical analysis: Data were analyzed by Analysis of Variance appropriate for the randomized complete block design, with chambers (10 birds) considered to be the experimental unit. Differences between treatment

means were assessed by Least Significant Difference Test. Bacterial concentrations were subject to log 10 transformation prior to analysis. Statistical analyses were conducted with the General Linear Models Procedure of SAS (SAS, 1985).

7.4. Challenge Trial with *Salmonella typhimurium* 29E

7.4.1. Introduction

This first series of three trials was conducted to evaluate the effects of MOS on activities and concentrations of different cecal bacteria and on the cecal concentration of *Salmonella typhimurium 29E* in chicks reared under controlled environmental conditions. *Salmonella typhimurium 29E* was chosen as challenge organism for four reasons. First, it has been shown to express type-1 fimbriae and was expected to adsorb to MOS; second, it has been shown in a preliminary experiment to colonize chicks at high concentrations; third, it has been shown to colonize the ceca of chicks at a rate of more than 90 % and fourth, it is naladixic resistant which facilitates its enumeration from a mixed cecal culture.

7.4.2. Materials and Methods

Trials were conducted using a naladixic acid resistant strain of *Salmonella typhimurium* 29E (Table 9; Jones *et al.*, 1983) grown on Media for Enrichment for Type-1 Fimbriae (Firon *et al.*, 1987). Table 15 shows the challenge doses and the time tables of trials 1 to 3. Trials 1 and 2 were conducted as described in chapter 7.3. Trial 3 was designed to study the development of *S. typhimurium* 29E colonization over time. Therefore, this trial involved 104 birds. Eight birds per group were sacrificed 2 and 4 d after challenge and 10 birds per group 7 d after challenge. The challenge doses in trials 1 to 3 were between 7.81×10^3 and 1.33×10^4 CFU of *S. typhimurium* 29E per bird. More than 90 % of all birds were salmonella positive, therefore, cecal salmonella concentration was used to evaluate salmonella colonization.

TABLE 15: Challenge culture, challenge dose and time schedule of experimental design used in trials 1 to 3 with *Salmonella typhimurium* 29E as challenge organism

	Trial 1	Trial 2	Trial 3
Challenge culture	*S. typhimurium* 29E	*S. typhimurium* 29E	*S. typhimurium* 29E
Dose (CFU/bird)	4.01×10^4	7.81×10^3	1.33×10^4
Number of birds	40 (4 x 10 birds)	40 (4 x 10 birds)	104 (4 x 26 birds)
Number of birds harvested:			
- 2 d after challenge	0	0	32
- 4 d after challenge	0	0	32
- 7 d after challenge	40	40	40

7.4.3. Results

Table 16 shows the effect of dietary MOS on concentrations of different bacterial populations. *Salmonella typhimurium* 29E concentrations were lower in birds fed MOS in all three experiments. Salmonella concentrations were between 4.97 and 5.73 CFU/g for the control and ranged from 3.79 to 4.29 log CFU/g for the MOS treatment. Over the three trials MOS supplemented chicks had lower *S. typhimurium* 29E concentrations by about 25-fold (5.40 vs. 4.01 log CFU/g; $P < 0.05$). Concentrations of coliforms also tended to be lower when MOS was added to the feed (8.71 vs. 8.47 log CFU/g), however, the overall difference was not significant ($P > 0.05$). Concentrations of lactobacilli, enterococci, anaerobic bacteria, VFA, lactate and cecal pH were not affected by treatment and are therefore presented in Table 17 as means of each experiment.

TABLE 16: Effect of dietary MOS on concentrations of different bacterial populations in the ceca of chicks maintained in microbiological isolators and challenged with *Salmonella typhimurium* 29E

Parameter		Trial 1		Trial 2		Trail 3		Overall		SE
		Control	MOS	Control	MOS	Control	MOS	Control	MOS	
Salmonella	log CFU/g	5.73	4.29	5.50	3.95	4.97	3.79	5.40[a]	4.01[b]	0.08
Coliforms	log CFU/g	8.78	8.40	8.87	8.73	8.48	8.29	8.71	8.47	0.05
Lactobacilli	log CFU/g	<6.00	<6.00	<6.00	<6.00	7.41	7.61			
Enterococci	log CFU/g	8.12	8.35	8.26	8.25	8.03	8.24	8.13	8.28	0.05
Anaerobes	log CFU/g	9.37	9.14	9.20	9.11	9.21	8.91	9.26	9.05	0.04

[ab)] Values in same row with different superscripts differ significantly $P < 0.05$

Values in Table 17 represent the average of four groups (2 control and 2 MOS groups) per experiment. Concentrations of anaerobic bacteria were between 9.06 and 9.25 log CFU/g and did not differ between experiments. Both coliform and enterococci concentrations were above 8 log CFU/g in all experiments. Coliform numbers were higher in trial 2 when compared to trial 3 (8.80 vs. 8.38 log CFU/g; $P < 0.05$). On the other hand, lactobacilli concentrations were above 6 log CFU/g only in the third trial. Acetate was the predominant VFA and its concentrations were higher in trials 1 and 2 when compared to trial 3 (71.37 and 66.60 mM vs. 47.15). Concentrations of propionate, butyrate and lactate as well as cecal pH did not differ between experiments.

TABLE 17: Effect of experiment on concentrations of different bacterial populations, VFA, lactate and pH in the ceca of chicks maintained in microbiological isolators and challenged with *Salmonella typhimurium* 29E

Parameter		Trial 1	Trial 2	Trial 3
Coliforms	log CFU/g	8.59[ab]	8.80[a]	8.38[b]
Lactobacilli	log CFU/g	<6.00	<6.00	7.51
Enterococci	log CFU/g	8.23	8.26	8.13
Anaerobes	log CFU/g	9.25	9.15	9.06
pH		5.66	5.64	5.53
Acetate	mM	71.37[a]	66.60[a]	47.15[b]
Propionate	mM	< 5.00	< 5.00	7.77
Butyrate	mM	10.94	11.08	14.48
Lactate	mM	8.04	8.50	10.35

[ab] Values in same row with different superscripts differ significantly $P< 0.05$

Figure 7 shows the development of cecal *S. typhimurium* 29E concentrations over time in trial 3. The concentrations in the control group rose from 3.61 log CFU/g at 2 d to 4.97 log CFU/g at 7 d after challenge. *Salmonella typhimurium* 29E concentrations in the treatment group were lower than those of the control group at every time period. Birds receiving MOS averaged cecal salmonella concentrations of 2.98 log CFU/g at 2 d and 3.79 log CFU/g at 7 d

after challenge. pH was unaffected by treatment (≈ pH 5.5) and did not change over the duration of the experiment (Table 18). Propionate and butyrate concentrations tended to increase with time, but were not affected by treatment. Similarly, treatment did not affect acetate or lactate concentrations at 4 d and 7 d however, acetate concentrations tended to be higher with dietary MOS at 2 d after challenge (39.57 vs. 49.81 mM; Table 18). None of the differences were statistically significant (only two replications).

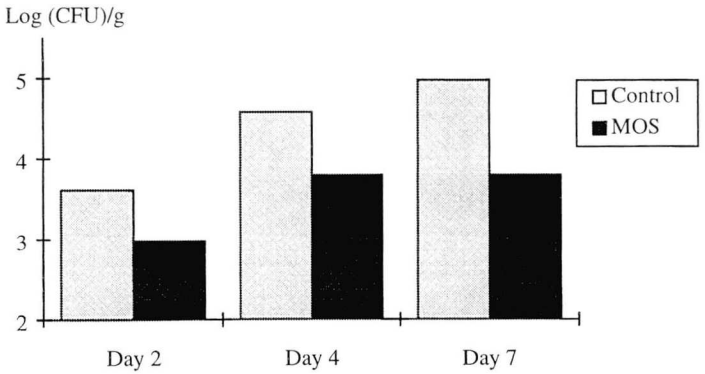


FIGURE 7: Effect of dietary MOS on cecal *Salmonella typhimurium* 29E concentrations of chicks maintained in microbial isolators at 2, 4 and 7 d after salmonella challenge

TABLE 18: Effect of dietary MOS on cecal pH, VFA concentrations and lactate concentrations of chicks maintained in microbiological isolators at day 2, 4 and 7 after *Salmonella typhimurium* 29 E challenge

Parameter		Day 2		Day 4		Day 7	
		Control	MOS	Control	MOS	Control	MOS
pH		5.54	5.57	5.45	5.41	5.47	5.60
Acetate	mM	39.57	49.81	58.21	58.01	48.70	45.60
Propionate	mM	<5.00	<5.00	<5.00	<5.00	8.72	6.81
Butyrate	mM	9.17	9.29	12.84	11.10	13.95	15.01
Lactate	mM	11.60	11.28	9.90	12.22	12.04	8.67

7.5. Challenge Trial with *Salmonella dublin*

7.5.1. Introduction

Mannanoligosaccharide has been shown to reduce cecal concentrations of *Salmonella typhimurium* 29E in trials 1 through 3. Despite a significant reduction from $2.51x10^5$ in untreated chicks to $1.02x10^4$ CFU/g, MOS did not completely exclude them. It was therefore of interest to investigate the effect of MOS on a strain of *Salmonella* that colonizes birds at cecal concentrations lower than 10^4 CFU/g. Therefore, a strain of *Salmonella dublin* (chapter 6) which has been shown to colonize chicks at low concentrations, was chosen for this series of experiments. In addition, this strain has been shown to be naladixic acid resistant, to express type-1 fimbriae and to agglutinate MOS. The objectives of this series of trials were, to evaluate the effects of MOS on activities and concentrations of different cecal bacteria and on the cecal concentration of *Salmonella dublin* in chicks reared under controlled environmental conditions.

7.5.2. Material and Methods

Three trials were conducted using a naladixic acid resistant strain of *Salmonella dublin* (Table 9) grown on Media for Enrichment for Type-1 Fimbriae (Firon *et al.*, 1987). Trial 4 and 5 were conducted as described in chapter 7.3. Due to low hatchability, trial 6 was conducted with only 9 birds per group. Compared to the experiments with *S. typhimurium* 29E, colonization over time was not studied. Challenge doses were as follows: Trial 4: $9.13x10^3$ CFU/bird; Trial 5: $1.22x10^4$ CFU/bird; Trial 6: $1.19x10^4$ CFU/bird. *Salmonella* concentrations were in most birds below the detectable limit with direct plating of 100 CFU/g of cecal content. Therefore, salmonella colonization was expressed as percentage of salmonella positive birds (colonization rate) determined from enrichment cultures.

7.5.3. Results

Table 19 shows the effects of dietary MOS on concentrations of different bacterial populations. The percentage of birds from which *S. dublin* could be recovered, was lower in all three experiments in the MOS treated groups, when compared to the control. The reduction in colonized birds with MOS addition was less in trial 6 (24 %), when compared to trial 4 (47 %) or 5 (44 %). This was because in one of the groups receiving MOS 8 of 9 birds were colonized with *S. dublin*. Over the three trials MOS decreased the number of salmonella positive birds from 89.8 to 55.7 % ($P < 0.05$). Concentrations of coliforms, lactobacilli, enterococci, anaerobic bacteria, VFA, lactate and cecal pH were not affected by treatment and are therefore presented in Table 20 as means of each experiment.

Values in Table 20 represent the average of four groups (2 control and 2 MOS groups) per experiment. Concentrations of indigenous cecal bacteria were not affected by experiment. Cecal concentrations of anaerobic bacteria were above 9.5 log CFU/g in all trials. Coliform and enterococci concentrations ranged from 8.53 to 9.23 and from 8.46 to 9.16 log CFU/g, respectively. Concentrations of lactobacilli were lower than both coliform and enterococci concentrations ranging from 6.67 to 7.48 log CFU/g. pH was lower in trial 6 when compared to trial 4 (5.21 vs. 5.77; $P < 0.05$). This decrease in pH was accompanied by a significant ($P < 0.05$) increase in lactate concentrations in trial 6 when compared to trial 4 and 5. Acetate was the main VFA with cecal concentrations averaging 41.85 mM. Average propionate and butyrate concentrations were both below 5 mM in all experiments.

TABLE 19: Effect of dietary MOS on concentrations of different bacterial populations in the ceca of chicks maintained in microbiological isolators and challenged with *Salmonella dublin*

Parameter		Trial 4		Trial 5		Trail 6		Overall		SE
		Control	MOS	Control	MOS	Control	MOS	Control	MOS	
Salmonella	% colonization	95.0	50.0	80.0	45.0	94.4	72.2	89.8[a]	55.7[b]	8.15
Coliforms	log CFU/g	9.50	8.97	9.16	8.69	8.45	8.62	9.03	8.76	0.28
Lactobacilli	log CFU/g	7.40	7.57	7.38	7.31	7.25	6.09	7.34	6.99	0.50
Enterococci	log CFU/g	7.91	9.01	8.71	9.29	9.19	9.13	8.60	9.14	0.41
Anaerobes	log CFU/g	9.70	9.68	9.79	9.78	9.58	9.71	9.69	9.72	0.06

[ab] Values in same row with different superscripts differ significantly $P < 0.05$

TABLE 20: Effect of experiment on cecal concentrations of different bacterial populations, VFA, lactate and pH in the ceca of chicks maintained in microbiological isolators and challenged with *Salmonella dublin*

Parameter		Trial 4	Trial 5	Trial 6
Coliforms	log CFU/g	9.23	8.92	8.53
Lactobacilli	log CFU/g	7.48	7.34	6.67
Enterococci	log CFU/g	8.46	8.99	9.16
Anaerobes	log CFU/g	9.69	9.79	9.64
pH		5.77^{a}	5.61^{ab}	5.21^{b}
Acetate	mM	40.88	46.75	37.92
Propionate	mM	<5.00	<5.00	<5.00
Butyrate	mM	<5.00	<5.00	<5.00
Lactate	mM	9.87^{a}	10.35^{ab}	16.63^{b}

[ab)] Values in same row with different superscripts differ significantly $P< 0.05$

7.6. Challenge Trials with *E. coli 15R and Salmonella typhimurium 27A*

7.6.1. Introduction

Mannanoligosaccharide was previously shown to reduce the cecal concentrations of two strains of *Salmonella* expressing type-1 fimbriae, *Salmonella typhimurium* 29E and *Salmonella dublin*. If MOS works through blocking bacterial adhesion mediated by type-1 fimbriae, its addition should not affect concentrations of enteric bacteria that do not express type-1 fimbriae. Trial seven was conducted to evaluate the effect of MOS on *S. typhimurium* 27A colonization, a naladixic acid resistant strain of *Salmonella* which does not express type-1 fimbriae. In addition, dietary MOS has also shown a tendency to reduce the total concentration of cecal coliforms in previous trials. Therefore, it was of interest to evaluate how MOS affects the cecal concentration of a strain of *E. coli* that expresses type-1 fimbriae. Trial eight was conducted to evaluate the effect of MOS on *E. coli* 15R colonization, a naladixic acid resistant strain of *Escherichia* which expresses type-1 fimbriae.

7.6.2. Materials and Methods

Two trials, with naladixic acid resistant strains of *Salmonella typhimurium* 27A (Table 9) and *E. coli* 15R (Table 9) as challenge organisms were conducted. A naladixic acid resistant strain of *Escherichia coli* 15R was available form the culture collection while, *S. typhimurium* 27A was selected for naladixic acid resistance by growing it in the presence of increasing concentrations of naladixic acid. Similar to the original strain, the naladixic acid resistant strain of *S. typhimurium* 27A did not cause agglutination of MOS. The trials were conducted as described in 7.3. The challenge doses were as follows: Trial 7: *S. typhimurium* 27A, 1.43×10^4 CFU/bird; Trial 8: *E. coli* 15R: 9.75×10^3 CFU/bird. No bacterial populations except the challenge organism were enumerated in trial 7.

7.6.3. Results

Salmonella typhimurium 27A colonized the chicks at a very low rate. *Salmonella* could only be recovered from the ceca of 3 out of 40 birds. Two control chicks and one bird receiving MOS were salmonella-positive at day 10.

Table 21 summarizes the effects of MOS on activities and concentrations of the cecal microflora and on the cecal concentration of *E. coli* 15R. *Escherichia coli* 15R colonized birds at fairly low concentrations ($< 10^4$ CFU/g). Dietary MOS reduced the rate of birds that were colonized with *E. coli* 15R from 75 % in the control groups to 15 % in the treatment groups. Positive birds were defined as birds, which showed at least one colony on VRB-plates containing naladixic acid at the 10^{-2} dilution. As in previous trials, coliform and enterococci concentrations were in a similar range (8.15 - 8.39 log CFU/g), while lactobacilli concentrations were around 7.5 log CFU/g. Neither cecal pH (5.53 vs. 5.44) nor organic acid concentrations were affected by treatment. The cecal fermentation was predominately an acetic acid fermentation with acetic acid concentrations averaging 72.00 and 66.76 mM for the control and the treatment group, respectively.

TABLE 21: Effect of MOS on concentrations of *E. coli* 15R, concentrations of different bacterial populations and pH in the ceca of chicks maintained in microbiological isolators

Parameter		Control	MOS
E. coli 15R	% positive birds	75.00	15.00
Coliforms	log (CFU)/g	8.39	8.21
Lactobacilli	log (CFU)/g	7.44	7.52
Enterococci	log (CFU)/g	8.15	8.28
Anaerobes	log (CFU)/g	9.08	8.71
pH		5.53	5.44
Acetate	mM	72.00	66.76
Propionate	mM	<5.00	<5.00
Butyrate	mM	17.03	16.76
Lactate	mM	7.67	8.80

7.7. Effect of Mannanoligosaccharide on Cecal Coliforms

In the two trial series with both *Salmonella typhimurium* 29E and *Salmonella dublin* as challenge organism, birds receiving MOS tended to have lower cecal coliform concentrations. Since coliform concentrations were not affected by challenge organism, comprehensive statistical analysis of the effect of MOS on coliform concentrations were conducted. Figure 8 summarizes the effects of dietary MOS on coliform concentration of all seven trials. Statistical analyses show that MOS significantly reduced coliform concentrations (8.80 vs. 8.54 log CFU/g).

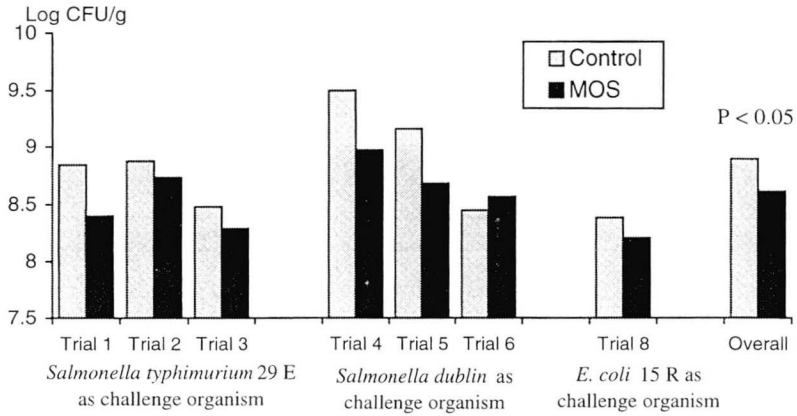


FIGURE 8: Effect of dietary MOS on cecal coliform concentrations in chicks

7.8. Discussion

Standardization of the experimental condition is important to achieve reproducible results. Initial results obtained with birds from a commercial hatchery were not reproducible. It was hypothesized that the birds might have been exposed to different bacterial flora at the hatchery, which could explain differences between trials. Birds which would have been exposed to a more complex microflora at the hatchery would exert a stronger CE-effect in the GI tract and would, consequently, be colonized at lower rates with the challenge organisms (Stavric and D'Aouts, 1993). Therefore, the experimental setup was modified to standardize each bird's microflora at the beginning of the experiment by removing the native microflora of the egg through sanitation and then exposing the newly hatched bird to a constant level of microorganisms derived from hatching debris. In combination with housing the birds in bacterial isolation chambers, the standardization of the microflora at the beginning of the experiment helped to keep the composition of the cecal GI microflora, cecal pH as well as cecal acid concentrations of the birds similar between experiments.

Coliform and enterococci were the predominant cecal bacteria at day 10. This is in agreement with Smith (1965) and Hutanen and Pensack (1965) who

stated that these types of bacteria usually predominate in the ceca of chicks the first few days after hatching. These authors also showed, that lactobacilli establish themselves slower than the quick invading coliforms and enterococci. However, compared to Hutanen and Pensack's data, establishment of lactobacilli took slightly longer in these trials. Cecal lactobacilli concentrations reported in their studies were about 1 log-unit higher than concentrations measured here at day 10. Lactobacilli have been shown to quickly increase in concentrations once introduced. Therefore, the slightly different time of introduction of lactobacilli can lead to differences in concentrations as noticed between trial 3 and trials 1 and 2. Lactobacilli concentrations averaged 7.51 log CFU/g in trial 3, but were below 6 log CFU/g in trials 1 and 2. This increase in lactobacilli concentrations might have led to lower *Salmonella* and coliform concentrations in trial 3 when compared to trial 1 and 2. Mead and Adams (1975) showed that coliform concentrations decreased while lactobacilli concentrations increased in the ceca of chicks.

A negative correlation between lactobacilli and salmonella concentrations was shown by Schneitz *et al.* (1993). A slight increase in propionic and butyric acid concentrations might also be an indication for a more complex microflora in trial 3 when compared to trial 1 and 2. A more complex GI microflora would exert a stronger CE effect and therefore lead to lower salmonella concentrations. *Salmonella* concentrations were lower in both treatment groups in trial 3 when compared to trial 1 or 2.

Acetic acid was the predominant VFA with concentrations between 47.15 and 71.37 mM. Hinton *et al.* (1990) reported similar cecal acetate and butyrate concentrations as in these trials, although propionate concentrations in their trials (29.33 - 34.62 mM) were higher than the values reported here. On the other hand, Hollister *et al.* (1994a,b) and Nisbet *et al.* (1993) all reported propionate concentrations below 10 mM for birds that did not receive any CE culture. Lactic acid concentrations (8.04 - 10.35 mM) were in a similar range as values reported by Hinton *et al.* (1990). Decreased concentrations of coliforms might have led to a shift in the cecal acid pattern. Acetic acid concentrations were lower in trial 3 when compared to trials 1 and 2. Cecal pH (5.53 - 5.66) was in a range similar to values reported by Nisbet *et al.* (1993) with 5.5 - 6.2.

Hinton *et al.* (1990) and Corrier *et al.* (1990b) reported higher average values (6.00-6.60) for their control birds from laboratory scale challenge trials.

None of the determined bacterial concentrations were affected by experiments in the series with *Salmonella dublin*. More consistent work techniques due to routine might have led to improvements in reproducibility.

Overall, the additional measures taken to standardize experimental conditions helped to keep the GI microflora fairly constant between experiments. This led to relatively small differences in colonization rate *of Salmonella dublin* (80.0 - 95.0 %) and in cecal concentrations of *Salmonella typhimurium* 29E (4.97 - 5.73 log CFU/g) between experiments. Nisbet *et al.* (1993) in comparison reported cecal salmonella concentrations in the control groups in laboratory scale trials from 4.09 to 7.00 log CFU/g and Hollister *et al.* (1994a) from 4.59 to 6.37 log CFU/g in series of four trials each.

The main goal of these trials was to evaluate the effects of MOS on *Salmonella* colonization in chicks. The focus was put on *Salmonella* strains expressing type-1 fimbriae, since it was hypothesized that MOS might affect colonization of such strains through blocking bacterial attachment to the gut mucosa. In a first series of three trials dietary addition of MOS decreased the cecal concentration of *S. typhimurium* 29E by about 25-fold. Mannanoligosaccharide treated birds were colonized by an average concentration of 10,000 CFU/g of *S. typhimurium* 29E. To further investigate if MOS can decrease the concentration of a strain of *Salmonella,* that colonizes birds with a cecal concentration lower than 10^4 CFU/g, a series of trials was conducted using *S. dublin*, as a challenge organism. Mannanoligosaccharide showed a reduction of the number of birds from which *S. dublin* could be isolated. The challenge trials with *S. dublin* and *S. typhimurium* 29E suggest that MOS can lower cecal salmonella colonization of both, strains that colonize at high and at low concentrations. The study conducted over time showed that MOS reduced cecal *S. typhimurium* 29E concentrations two days after challenge and further stabilized the concentration at a lower level on day 4 as well as on day 7. Following cecal *S. typhimurium* 29E concentration over a longer period of time, with and without withdrawing MOS, might help to further investigate what effect MOS exerts on type-1 fimbriated enteric pathogens. To

the present day, only one other study of the effects of MOS on salmonella colonization in chicks has been conducted. Sisak (1994) determined a decrease in salmonella colonization of a wild type of *Salmonella* with MOS addition. However, the scientific value of these data is somewhat reduced, since it had not been shown that the measured strain of salmonella was distributed evenly throughout the treatment groups at the beginning of the experiment. Live yeast cultures have also shown an inhibitory effect on salmonella colonization in chicks. Line *et al.* (1995) reported decreased colonization of *S. typhimurium* with dietary *Saccharomyces boulardii.* However, different modes of action might be involved with live yeast cells instead of cell wall material. Live yeast cells excrete metabolites which are known to affect the composition of the GI microflora (Girard, 1996) and could, therefore, affect the CE-effect of the indigenous microflora. In addition, the strain of *S. typhimurium* used by Line et al. (1995) has not been screened for presence of type-1 fimbriae. Therefore, it can not be determined, whether the observed reduction was due to yeast cell wall mannan or other mechanisms.

The decrease in concentrations of *Salmonella* expressing type-1 fimbriae was not accompanied by changes in cecal parameters such as a decrease in pH or an increase in propionic acid concentrations, which are known to reduce salmonella concentrations. This shows that other modes of action to decrease salmonella concentrations must be involved with dietary MOS. *In vitro* agglutination results suggest that adsorption of salmonellae by MOS which would keep them from adhering to the intestinal wall could be a possible mode of action. Oyofo *et al.* (1989a) tested the effect of different sugars on the adherence of *S. typhimurium* to epithelial cells of 1 d old chicks *in vitro.* They reported inhibition of adherence by methyl-α-D-mannoside and mannose by more than 90%. Similar effects were reported on inhibition of the adherence of *S. typhimurium* to enterocytes of rats (Lindquist *et al.,* 1987). In different *in vivo* trials, mannose has also been shown to decrease cecal colonization of *S. typhimurium* in young chicks (Oyofo *et al.,* 1989b; Oyofo *et al.,* 1989c).

When birds were challenged with a culture of *E coli* 15R, dietary MOS led to a decrease in the number of birds from which this strain could be recovered from their ceca. *In vitro* agglutination data suggest that MOS affects *E. coli* 15R

concentrations by a similar mode of action as it affects salmonellae. Mannanoligosaccharide was also shown to reduce total coliform concentration in the ceca. Mirelmann *et al.* (1980) reported that type-1 fimbriae are widespread among strains of *E. coli*. Mannanoligosaccharide might have led to a decrease in the concentration of cecal coliforms by adsorbing strains expressing type-1 fimbriae. The fact that MOS had no effect on the concentrations of lactobacilli and enterococci, two bacterial species that are known to not express type-1 fimbriae, further suggests that MOS affects bacterial concentrations in the GI tract by adsorbing bacteria and keeping them from adhering to the gut wall. Having only a slight effect on the major composition of the cecal microflora, MOS, did not affect the concentrations of cecal fermentation end products and cecal pH. This is in agreement with *in vitro* data of Chandler and Newman (1994), who observed that few strains of intestinal bacteria are able to ferment *Saccharomyces cerevisiae* cell wall preparation.

The trial with *S. typhimurium* 27A was conducted to investigate the effects of MOS on a non-agglutinating strain of *Salmonella*. If MOS indeed controls bacterial colonization by blocking attachment, no effect on *S. typhimurium* 27A concentration would be expected. Unfortunately, this hypothesis could not be tested, since strain 27A did not sufficiently colonize the birds to evaluate if MOS affected its concentration.

Data from these challenge trials suggest, that the additional measures taken to standardize experimental conditions helped reduce variation in the composition of the GI microflora and allowed good reproduction of responses of MOS. Mannanoligosaccharide decreased the cecal concentration of pathogens expressing type-1 fimbriae in young chicks. Mannanoligosaccharide also showed a reduction in cecal coliform concentrations. No changes in cecal parameters such as a decrease in pH or an increase in propionic acid concentration, which are known to reduce salmonella concentrations, have been determined with MOS addition. This indicates that MOS might decrease concentrations of enteric bacteria expressing type-1 fimbriae mainly by adsorbing them and, therefore, keeping them from adhering to the intestinal wall.

8. HISTOLOGY STUDY

8.1. Abstract

The objective of this trial was to evaluate the effects of dietary mannanoligosaccharide (MOS) on the structure of the ileal wall of 10 d old chicks. Birds were housed in microbial isolation chambers. Four birds each from two groups of 10 birds were randomly selected per treatment. Treatment included no additive (control) and 0.4 % dietary MOS (Bio-Mos, Alltech Inc., Nicholasville, KY). Ileal samples were stained with Periodic Acid-Shiff (PAS) and were examined under the microscope. Villi length, villi width, crypt depth and concentrations of goblet cells were determined.

The average villus length was 18 % greater ($P < 0.05$) and crypt depth was 22% greater ($P < 0.05$) in animals receiving MOS. Villi width and number of goblet cells were not affected by treatment. These data suggest that dietary MOS can significantly alter the structure of the intestinal wall.

8.2. Introduction

The abilities of MOS to bind enteric pathogens and to stimulate immune systems have been used to explain the beneficial effects of MOS on animal health and performance (Chapter 4.4.3). These modes of action suggest that MOS would be effective in production systems with considerable pathogen challenge. However, Savage *et al.* (1995 and 1996a) reported improved performance in turkeys with dietary MOS under laboratory scale conditions. Normally these conditions do not exert heavy pathogen challenge since small numbers of birds are housed in well controlled environments. Therefore, it might be possible that other, not yet identified mechanisms could explain the effects of dietary MOS.

Changes in structural morphology of the small intestines have been reported with dietary yeast culture in turkeys. Bradley *et al.,* (1994) reported reduced crypt depth and decreased numbers of goblet cells with dietary addition of *Saccharomyces cerevisiae* var. *boulardii.* However, these investigators did not make any attempt to determine if the changes were associated with the

metabolic activity of the yeast or with yeast cell wall components. It might be possible that changes in intestinal morphology might be due to yeast cell wall fractions and the associate MOS. The objective of this trial was to evaluate the effects of dietary MOS on different histological parameters in the ileum of broiler chicks.

8.3. Materials and Methods

The histological investigations were made on birds from trial 3 after challenge with *Salmonella typhimurium* 29E. Experimental conditions were as described in Chapter 7.3. Four birds per group (8 per treatment) were randomly selected for histological examination. Ileal segments were dissected from these birds before the ceca were removed, since changes in ileal morphology *post mortem* were expected to occur faster than changes in activities and concentrations of the cecal microflora. A section of approximately 2 cm was removed cranial to Meckel's diverticulum and was fixed in 10% phosphate buffered (pH 7.0) formalin for 48 h (Nabuurs *et al.*, 1993). Following histological fixation, the tissues were processed through a standard alcohol-dehydration-toluene sequence and embedded in paraffin (Burck, 1988). Three longitudinal sections (5 µm) were then cut parallel to Meckel's diverticulum and subsequently stained with Periodic Acid-Shiff (PAS) (Bradley, *et al.*, 1994). Photographs of each section were then taken with a 35 mm camera attached to a light microscope using a magnification of 100 x (Figure 9). Photographs were projected on a white wall to a size of approximately 1 m and the size of each structure was determined relative to a photographed micrometer (Bradley, *et al.*, 1994).

Three sites were randomly selected on two photographs from each bird to measure the effects of dietary MOS on microscopic structures. The measurements were made only when there was a complete longitudinal section of a villus and its associated crypt. The third photograph served as a replacement if one photograph was not satisfactory for the measurement. The length of the villus was measured from the tip to the crypt-villus junction and the depth of the crypt was measured from the crypt-villus junction to the base

(muscularis mucosae). Villus width was determined 200 µm above the crypt-villus junction. The concentrations of goblet cells were determined on a stretch of villus approximately 100 to 300 µm above the crypt-villus junction.

Statistical analyses were conducted with the General Linear Models Procedure of SAS (SAS, 1985). Birds were considered experimental units. Parameters from each bird were determined by averaging the individual measurements from each photograph. Data were analyzed by Analysis of Variance appropriate for the complete randomized design (Kuehl, 1994). Differences between treatment means were assessed by F-Test.

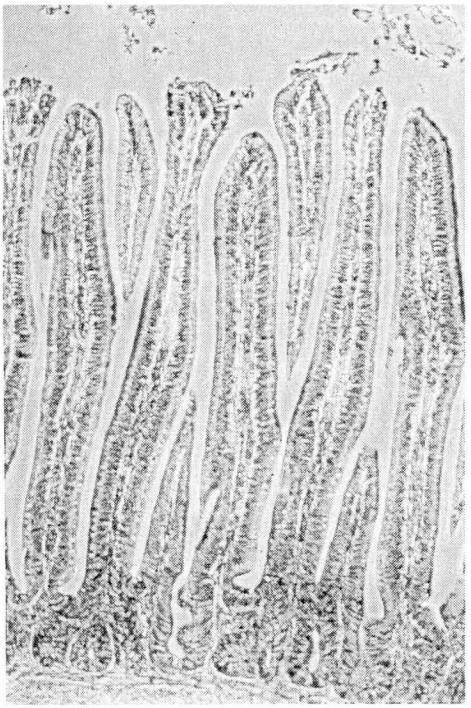


FIGURE 9: Histological preparation of a section of the ileum cranial to Meckel's diverticulum of a 10 d old chick (1:160)

8.4. Results

Average villi length was 18 % greater ($P < 0.05$) in birds receiving dietary MOS (Table 22). However, villi width, another parameter influencing the villi surface, was not affected by treatment (83.8 vs. 79.7 µm), nor was the number of goblet cells. Crypt depth was 22 % greater ($P < 0.05$) in birds receiving dietary MOS.

TABLE 22: Effect of dietary MOS on ileal villus length, villus width, crypt depth and goblet cell concentrations in 10 d old chicks

Parameter	Control group 1	Control group 2	MOS group 3	MOS group 4	Control mean	MOS mean	SE
Villi length (µm)	638.7	626.5	722.3	769.5	632.6[a]	745.9[b]	27.0
Villi width (µm)	85.0	82.5	79.0	80.3	83.8	79.7	3.5
Crypt depth (µm)	122.3	132.8	148.0	164.8	127.6[a]	156.4[b]	7.4
Goblet cells (/mm)	60.6	50.0	52.2	47.9	55.3	50.1	2.4

[ab)] Values in same row with different superscripts differ significantly $P< 0.05$

8.5. Discussion

Changes in intestinal morphology have been associated with animal health and performance. Nabuurs *et al.* (1993) reported shorter intestinal villi and deeper crypts in pigs from herds with postweaning diarrhoea when compared to pigs from healthy herds. The authors suggested that these changes might lead to poor nutrient absorption and increased secretion in the GI tract. The resulting malabsoption syndrome could readily lead to diarrhoea. However, changes in intestinal morphology might not only affect animal performance during disease. Subtherapeutic levels of antibiotics in the diet have been shown to reduce the weight of the intestinal tissue and to decrease the turnover of mucosal cells (Visek, 1978). Slow cell turnover requires low protein synthesis. This could be associated with low rate of energy expenditure

in the GI tract and could explain some of the performance improvements associated with subtherapeutic dietary levels of antibiotics.

Crypt depth gives a general indication of the rate of cell production in the intestinal mucosa. The crypt depth tends to increase as additional cells are produced (Al-Mukhtar, 1982). Crypt cells migrating upwards become villous enterocytes. During the migration they differentiate and develop absorptive capacity (Hampton, 1986). Measurement of crypt depth therefore gives a general indication of the rate of cell production and functional capacity of enterocytes on the villi. In the present study crypt depth was 128 µm in the control group. This is in agreement with Cook and Bird (1973) who reported average crypt depth of 120 µm in 7 d old birds. Crypt depth increased with MOS addition. This might increase protein synthesis and energy expenditure by the GI tract (Radecki et al., 1992). However, the increased cell production with MOS addition led to increased villus length with a corresponding increase in the number of villus cells (Wright, 1982). Since MOS addition decreased the concentrations of salmonellae and coliforms, MOS might have increased villi length by alleviating destruction of intestinal villi by enteric pathogens. Nabuurs *et al.* (1993) reported shorter intestinal villi in pigs from herds with postweaning diarrhoea caused by toxin producing *E. coli* when compared to pigs from healthy herds. Another explanation for the effects noted with MOS addition might be that cell wall polysaccharides in MOS exerted a stimulatory effect on villi development similar to the effect of the innate GI microflora. An increase in both villus length and crypt depth has been reported in conventional chicks when compared to germ free chicks (Cook and Bird, 1973). Since the number of secretory cells per millimeter of villus border was not affected by treatment, MOS addition should have increased the total number of absorptive cells and therefore increased absorption. Cera *et al.* (1988) stated that a large luminal surface area with optimal enterocyte functional maturity is important to young growing animals so they attain maximal digestive and absorptive capacity.

Villus length : crypt depth ratio was similar for both treatments (4.96 vs. 4.77). Bradley *et al.* (1994) reported average villus length : crypt depth ratio of similar magnitude in turkeys (5.42) receiving a corn-soy diet containing no yeast supplementation. However, the yeast product used in their study, a live culture

of *Saccharomyces cerevisiae* var. *boulardii,* led to reduced crypt depth and increased villus length : crypt depth ratio (7.64). These investigators suggest that the energy conserved by reduced turnover rate of the epithelial cells may be utilized for lean tissue mass synthesis and might explain some of the improvements seen in body weight gain with dietary *Saccharomyces cerevisiae* var. *boulardii.* The metabolic activities of the GI tract are known to be high relative to that of other tissues (Ferrell, 1988). Maintenance energy requirements of the liver and GI tract represent a large portion (50 - 75 %) of whole-body maintenance energy requirements (Ferrell and Jenkins, 1985; Ferrell, 1988). Thus, factors that influence the energy efficiency of the digestive system may have a profound influence on the efficiency of energy utilization on the whole body (Jin *et al.,* 1994).

The reasons for different effects of MOS and dietary *Saccharomyces cerevisiae* var. *boulardii* supplementation on intestinal morphology cannot be explained at this point, since the modes of action by which yeast products affect intestinal morphology are unknown. Differences in animal species and experimental conditions, in particular the composition of the diet, might also account for some of the differences. Differences in intestinal morphology due to variations in the composition of the diet have been documented in different experiments (Jin *et al.,* 1994; Dunsford *et al.,* 1989).

Mannanoligosaccharide addition can significantly alter the structure of the intestinal wall. Further research is required to evaluate if MOS exerts a direct effect on the epithelial development or if it works through changing the GI environment. Reduction of toxin concentrations through toxin binding or adsorption of toxin producing enteric pathogens by MOS could explain some of the changes noted in this experiment.

9. CONCLUSIONS

Dietary mannanoligosaccharide (MOS) has been shown to decrease the prevalence of strains of *Salmonella* and *E. coli* expressing type-1 fimbriae and cecal coliform concentrations in young chicks. No changes in cecal parameters such as a major shift in bacterial populations, changes in pH or VFA concentrations, which are known to affect salmonella were observed with MOS addition. This underlines the hypothesis that MOS might decrease concentrations of enteric bacteria expressing type-1 fimbriae mainly by adsorbing them and, therefore, keeping them from adhering to the intestinal wall. Different trials and surveillance studies have shown that most competitive exclusion (CE) products are only effective in the field when combined with other measures. Trials with lactose have shown that a decrease of the prevalence of salmonellae at the flock level will only lead to lower end product contamination if lactose application is combined with high hygienic production standards. This indicates in order to be an effective anti-salmonella tool in the field, MOS would have to be used in combination with proper management practices. Therefore, MOS offers an additional tool which can be included in a HACCP program to the poultry producer.

Furthermore, dietary MOS has been shown to improve animal performance in different animal species. It was hypothesized that changes in intestinal morphology with MOS could account for part of the performance improvements. In fact, MOS affected the intestinal morphology by increasing villi length and crypt depth. However, these changes do not suggest that MOS did improve the energy efficiency of the gastrointestinal tract under the conditions of these experiments.

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