THE EFFECT OF ISOMALTOOLIGOSACCHARIDE ON *BIFIDOBACTERIUM* SPP. POPULATION IN YOUNG BROILER CHICKENS

by

SUTAWEE NARINT THITARAM

(Under the Direction of Gregory R. Siragusa)

ABSTRACT

Chicken ceca contain a large and diverse population of bacteria. *Bifidobacterium* spp. have been considered to promote health effects to the host. The broiler starter diet was supplemented with isomaltooligosaccharide (IMO) at 1%, 2%, 4% (w/v), and a control group without IMO supplementation. Forty one-day-old chicks were randomly divided into four groups and provided assigned treatment diets. All groups were challenged with 0.25 ml of a culture of *Salmonella enterica* ser. Typhimurium 200 µg/ml nalidixic acid resistant (*S.* ser. Typhimurium Nal^r), containing 10^8 cells, after seven days of placement. IMO supplemented diets resulted in significantly higher cecal bifidobacteria compared to the control diet ($P<0.05$). Chickens fed diets with 1% w/v IMO had a significant two log reduction in the level of inoculated *S.* ser. Typhimurium Nal^r ($P<0.05$) present in the ceca compared to the control group. No significant improvement in weight gain was observed when compared to the control groups. It may be suggested that feeding the optimum level of IMO in the diet of chickens promoted the growth of *Bifidobacterium* spp. and reduced *Salmonella* colonization in young chickens in addition to promoting *Bifidobacterium* spp. growth.

In the process, a bifidobacteria selective medium was formulated and tested for efficacy in selective enumerating of chicken cecal samples. Transoligosaccharide propionate agar medium (TOS) was improved by addition of mupirocin (0.1 mg/ml) and glacial acetic acid (1%v/v). Four media were evaluated to determine their efficiency and selectivity for *Bifidobacterium* spp. in chicken cecal sample; TOS without modification, TOS-A supplemented with glacial acetic acid (1%v/v), TOS-M supplemented with mupirocin (0.1 mg/ml), and TOS-AM supplemented with glacial acetic acid (1%v/v) and mupirocin (0.1 mg/ml). The addition of mupirocin and acetic acid contribute to the inhibition of the growth of non-bifidobacteria. TOS-AM was found to have the most *Bifidobacterium* spp. selectivity among media tested. However the number of *Bifidobacterium* spp. recovered from chicken cecal samples enumerated on TOS with mupirocin supplementation (TOS-M and TOS-AM) was significantly lower ($P<0.05$) than those of media without mupirocin supplementation (TOS and TOS-A). TOS-A was found to have more *Bifidobacterium* spp. selectivity compared to TOS without modification and without reducing *Bifidobacterium* spp. population from chicken cecal samples compared to TOS with mupirocin supplementation. TOS-A has a simple preparation and could be potentially used for isolation and enumeration of *Bifidobacterium* spp. from chicken cecal samples.

INDEX WORDS: Isomaltooligosaccharide, *Salmonella*, Chicken, Colonization, Competitive Exclusion, *Bifidobacterium*, Chicken ceca, Selective Media

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For my loving parents

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INTRODUCTION

The microflora of the chicken's gastrointestinal tract (GIT) has received increased attention as the focus of efforts to minimize foodborne illness in humans and to improve animal nutrition (Mulder, Havenaar and Huis in't Veld 1997; Bezkorovainy 2001; Ishibashi and Yamazaki 2001; Isolauri *et al*. 2001; Marteau *et al*. 2001; Gong *et al*. 2002; Kieessen *et al*. 2003). In healthy chickens, a balance of microorganisms in the intestine helps in the efficient digestion and optimal absorption of nutrients and increases the body's resistance to infectious diseases (Salter and Fulford 1974; Macfarlane and Cummings 1991). An increase in potential pathogens, such as *Salmonella enterica* ser. Typhimurium, may give way to clinically apparent GIT disorders or result in decreasing feed efficiency, bird body weight, and eventually chicken flock.

Salmonellas are not native members of the microbiota in poultry, but they readily colonize intestines of young chicks and persist there during rearing (Stavric 1992). The intestinal tract of chicks at the time of hatching is essentially sterile (Stavric 1992). However, it rapidly becomes colonized by bacteria from the environment (Jayne-Williams and Fuller 1971; Fuller 1989; Isolauri *et al*. 2001; van der Wielen *et al*. 2002).

The importance of the early establishment of a normal microflora in young chicks was first recognized by Nurmi and Rantala (1973), who demonstrated that oral inoculation of intestinal bacterial flora from healthy adult birds protected chicks against colonization by

salmonellas. Since then, this phenomenon has since been termed "competitive exclusion (CE)" or the "Nurmi Concept" (Nurmi, Nuotio and Schneitz 1992).

The lower parts of the chicken gastrointestinal tract harbors a diverse community of bacteria comprised of many genera (Fernandez, Hinton and van Gils 2002). These include bifidobacteria and lactic acid bacteria (Barnes *et al*. 1979; Mitsuoka, Hidaka and Eida 1987). Bifidobacteria and lactic acid bacteria are considered beneficial microorganisms, being thought to create conditions unfavorable to the growth of pathogens, such as *Salmonella* (Isolauri *et al*. 2001), Consequently, an increase in growth of bifidobacteria and lactic acid bacteria with a decrease of *Salmonella* would appear to be of benefit to the host animal.

Evidence has been presented that many oligosaccharides, when fed to animals or humans can reach the colon undegraded and provide a carbohydrate substrate particularly suited to the growth of bifidobacteria (Playne and Crittenden 1996; Tomomatsu 1994). Isomaltooligosaccharide (IMO) was developed by Chung and Day (2002) using *Leuconostoc mesenteroides* ATCC 13146 fermentation with sucrose and maltose by an acceptor reaction. Interestingly, the IMO showed the preferential utilization by bifidobacteria and lactobacilli but not by pathogenic organisms (Chung and Day 2002).The use of this oligosaccharide in poultry feed may favor the growth of probiotic bacteria and promote the growth of healthy, *Salmonella*-free birds without using antibiotics (Day, 2001). This study did not allow direct prediction of *in vivo* effects but indicated that this type of oligomer could be a potential prebiotic for intestinal microflora (Chung and Day 2002).

However, enumerating bifidobacteria from the faecal and cecal contents of broilers continues to be a difficult task (Farnworth, Modler and Chambers 1996). A number of media for isolation and enumeration of bifidobacteria have been developed (Tanaka and Mutai 1980;

Munoa and Pares 1988; Hartemink *et al*. 1996; Nebra and Blanch 1999; Arroyo, Cotton and Martin 1995; Martineau 1999; Rada, Sirotek and Petr 1999; Rada and Petr 2000), but most media are used for enumerating bifidobacteria from human feces and dairy products. In addition they are supplemented with several antibiotics and difficult to prepare.

Purpose of the Study

This study was aimed to test the hypothesis that IMO would increase a number of *Bifidobacterium* spp. in a chicken cecum and concomitantly reduce the colonization by *S.* ser. Typhimurium. In the process, a bifidobacteria selective medium was formulated and tested for efficacy in selective enumerating of chicken cecal samples.

Ultimately it is hoped that the addition of IMO will lead to microbiological control, allowing for more consistent production responses in the absence of antibiotics. In order to develop and better understand these three constraints, the following three main objectives were reached:

1. To evaluate the effects of IMO on *Bifidobacterium* spp. populations in young broiler chickens.
2. To determine the effect of the IMO on weight gain, feed conversion, and feed efficiency of young broiler chickens.
3. To develop a new selective media for isolating and enumerating bifidobacteria from chicken cecal samples using commercially available media by adding selective agents.

Hypotheses and Experiments

First, IMO is expected to increase the number of bifidobacteria and lactic acid bacteria concomitantly reduce the colonization of *S.* ser. Typhimurium in chicken gastrointestinal tract.

The simplest hypothesis to test here is whether or not IMO effect the population of *Bifidobacterium* spp.

$H\text{-}1_O$: IMO does not increase the number of *Bifidobacterium* spp.

$H\text{-}1_A$: IMO increases the number of *Bifidobacterium* spp.

Secondly, IMO is expected to improve feed efficiency and increase bird weight. The simplest hypotheses to test here are whether or not IMO will improve feed efficiency and increase body weight of young broiler chickens.

$H\text{-}2_O$: IMO does not improve feed efficiency.

$H\text{-}2_A$: IMO improves feed efficiency.

$H\text{-}3_O$: IMO does not increase bird weight.

$H\text{-}3_A$: IMO increases bird weight.

One hundred twenty broiler chickens (Ross-Ross) were fed four different types of diet; control (broiler starter without IMO added); 1% IMO; 2% IMO; and 4% IMO (broiler starter with 1%, 2%, and 4% IMO [w/v] added, respectively. Utilizing randomized complete block design with three replications and different time of experiment as a blocking factor. At the end of the experiment, the population of bifidobacteria, *S.* ser. Typhimurium, total anaerobic bacteria, total lactic acid bacteria, bird weight, cecal weight, cecal pH, and feed consumed were recorded. Both qualitative and quantitative analyses of bacterial populations were done. The feed conversion, feed efficiency and bird body weight gain were determined.

Lastly, selective agents may improve the selectivity of the media by inhibiting the growth of non-bifidobacteria. The simplest hypothesis to test here is whether or not the selective agents will improve the selectivity of the media.

$H\text{-}4_O$: Selective agents do not improve the selectivity of the media.

$H\text{-}4_A$: Selective agents improve the selectivity of the media.

Several commercially available media and selective agents were evaluated with bifidobacteria strains and chicken cecal samples.

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LITERATURE REVIEW

1. Antibiotics used in Poultry

An antibiotic is defined as a substance produced by microorganisms that inhibits or kills other microorganisms. Synthetic antimicrobial substances are referred to as chemotherapeuticals. The word "antimicrobial" (as a noun) is often used to encompass any substance of natural, semi synthetic or synthetic origin that kills or inhibits the growth of a microorganism (Edqvist and Pedersen 2002). Antibiotics have been used in animal agriculture since shortly after their discovery, in the 1950s (Fuller 1989; Revington 2002). They are used for treatment and prevention of bacterial infections in domestic and food-producing animals. If bacteria become resistant, the antibiotics become ineffective. In food-producing animals, some antibiotics are used for growth promotion and improving feed efficiency, including poultry (Turnidge *et al*. 1999; Mathews 2001). However, the use of antibiotics, particularly as growth promotants, has received increasing attention as a contributory factor in the international emergence of antibiotic-resistant bacteria in human health and agriculture (Fuller 1989; Wegener *et al*. 1998; Wegener *et al*. 1999a; Turnidge *et al*. 1999; Aarestrup 1999; Aarestrup, Bager and Anderson 2000; Wray and Davies 2000; World Health Organization; WHO 2003; Turnidge 2004). In some countries, such as Sweden and Denmark, the use of antimicrobials as growth promoters (AGPs) is prohibited (Ewing and Cole 1994a). Sweden banned all growth promoting antibiotics used for farm animal in 1986 (WHO 2003). Denmark banned avoparcin and virginiamycin in 1995 and withdrawn AGPs from use in cattle, broilers and finisher pigs in February 1998 (WHO 2003).

Virtually no AGPs have been used in Denmark since the end of 1999 (WHO 2003). The Danish Integrated Antimicrobial Resistance Monitoring and Research Programme (DANMAP) defined AGPs as "antimicrobials for growth promotion include only those agents approved by the European Union (EU) as feed additives which currently are avilamycin, flavomycin and the ionophores salinomycin and monensin" (DANMAP 2002). In 2002, the EU has ordered member countries to end the use of AGPs in farm animals by 1 January 2006 (Ferber 2003). The emergence of resistant bacteria and resistance genes following the use of antimicrobial agents is relatively well documented (Aarestrup 1999).

Resistance to antimicrobial drugs can arise either from new mutations in the bacterial genome or through the acquisition of genes coding for resistance. These genetic changes alter the defensive functions of the bacteria by changing the target of the drug, by detoxifying or ejecting the antibiotic, or by routing metabolic pathways around the disrupted point (Davies 1994).

The greatest threat to the use of antibiotics for therapy of human bacterial infections is the development of resistance in pathogenic bacteria (Aarestrup 1999). Mead (2000) indicated that administration of antibiotic alone may eliminate the infection, but leaves the birds open to re-infection once the treatment has ceased, this indicating the resistance. Wegener *et al*. (1999b) also found a close association exists between the amounts of antibiotics used and the levels of resistance observed. The potential risks of selecting resistant bacteria among food producing animals was first discussed in 1955 (National Research Council 1956) but no data on selection of resistance were available (Aarestrup 1999). Most classes of antibiotics used in animals have human analogues, and are capable of selecting for resistance to human antibiotics. In response to continued pressure, the EU adopted the precautionary principle and suspended the use of the growth promoter in-feed antibiotics: avoparcin, virginiamycin, spiramycin, tylosin and bacitracin

because of their ability to select for resistance to antimicrobials of human importance. The United States has taken a different approach. The Center for Veterinary Medicine at the Food and Drug Administration (FDA) has preferred instead to apply the principle of proof, gathering evidence that a problem has emerged before taking action. The center has recently withdrawn a fluoroquinolone from use in poultry based on this principle (Turnidge 2004). A consequence of this decision has been a loosening of some of the constraints on intestinal bacterial growth rates which will benefit from the use of slowly digested ingredients, because undigested feed is a potential substrate for bacterial fermentation. However, the use of poorly digestible ingredients runs the risk not only of poor performance but now there is an additional danger from bacterial overgrowth and subsequent disease/intestinal disorders (Bedford 2000).

Foodborne *Salmonella* infections are major problem in industrialized countries (Helms *et al*. 2002; Wilkins et al. 2002; Kimura *et al*. 2004; Voetsch *et al*. 2004; Vugia *et al*. 2004). Glynn *et al*. (1998) reported that multidrug-resistant *Salmonella enterica* serotype Typhimurium definitive type (DT) 104 has become a major cause of illness in humans and animals in the United States which increased from 0.6 % in 1979-1980 to 34 % in 1996. The strain of *S.* ser. Typhimurium DT104 is usually resistant to five drugs: ampicillin, chloramphenicol, streptomycin, sulfonamides, and tetracycline. An increasing proportion of DT104 isolates also have reduced susceptibility to fluoroquinolones (Molbak 1999). During July 1995, an outbreak of *S.* ser. Typhimurium DT170 occurred in South Wales. A case-control study found that illness was associated with eating kebabs, and kebabs with yoghurt based relish. These foodborne outbreaks linked to cross-contamination of undercooked meat and unsealed yoghurt (Evans *et al*. 1999). The *Morbidity and Mortality Weekly Report* (MMWR) from Center for Disease Control and Prevention (CDC) of April 14, 2000 indicated the outbreaks of salmonellosis associated with

handling chicks and ducklings occurred in Michigan and Missouri. There were 21 case-patients reported with onset of illness and three patients were hospitalized in Michigan with *S.* ser. Infantis. Forty case-patients were identified with onset of illness and three patients were hospitalized in Missouri with *S.* ser. Typhimurium. Both states reported that infected patients came into direct and/or indirect contact with young fowl (MMWR, 2000). The annual report from OzFoodNet network, Australia (2002) reported 92 foodborne illness outbreaks from *S*. ser. Typhimurium affecting 1,819 persons, of whom 103 persons were hospitalized and 2 people died. The risk factors were included undercooked chicken and egg consumption, bakeries as a source of *Salmonella* infection and problems associated with spit roast meals served by mobile caterers (The OzFoodNet network 2002). Daniels *et al*. (2002) indicated that *Salmonella* was the most commonly cause of foodborne disease outbreaks (36%) in United States during 1973-1997.

Wegener *et al*. (1999a) reported that the use of the glycopeptide avoparcin as a growth promoter for poultry has created a major reservoir of *Enterococcus faecium* in food animals. Furthermore, glycopeptide-resistant strains, as well as resistance determinants, can be transmitted from animals to human gut flora (Turnidge 2004). Aarestrup *et al*. (2000) also found that the use of avoparcin as a growth promoter for poultry in Denmark, Finland, and Norway and were frequently observed.

2. The Competitive Exclusion Concept (CE)

The Need for CE. Poultry meat continues to be associated with numerous outbreaks of *Salmonella* food poisoning in most of the developed countries (Mead and Impey 1986; Kimura *et al*. 2004). Colonization of *Salmonella* in poultry is enhanced by the ubiquitous and antibiotic resistant nature of the members of this genus. Most commonly, an infection is encountered as a chronic intestinal condition with the salmonellas colonizing the caecum where they multiply to

high levels and are shed intermittently in the feces (Schleifer 1985). Todd (1980) reported that *Salmonella* spp. outnumber other etiologic agents as the cause of poultry-associated foodborne disease on a world wide basis as well as in the United States. Recently a study by the CDC indicated that poultry products continue to be one on the most important sources of human salmonellosis out-breaks in the United States (Anonymous 1982; Daniels *et al*. 2002; Voetsch *et al*. 2004; Vugia *et al*. 2004). Many measures have been designed to eliminate *Salmonella* organisms from poultry. They include: treatment with chemotherapeutic agents and antibiotics, immunization with bacterins, improving hygiene of the birds with extensive monitoring of the status of the flock coupled with appropriate depopulation procedures as outlined under the National Poultry Improvement Program (Anonymous 1976), the production and maintenance of *Salmonella*-free breeding stock, and heat treatment of feed (Mead and Impey 1986). However these measures have only resulted in limited success so *Salmonella* contamination of the hatching environment continues to be a problem (Schleifer 1985). Also, the concern that the use of antibiotics as a therapeutic agent and growth promoter in food animals has resulted in the development of resistant bacteria which has made subsequent use of antibiotics for therapy difficult (Fuller 1989) and leading to human health problems (Wegener *et al*. 1998; Fuller 1999; Wegener *et al*. 1999a; Turnidge *et al*. 1999; Aarestrup 1999; Aarestrup, Bager and Anderson 2000; Turnidge 2004).

Development of the CE Concept. In 1973, Nurmi and Rantala introduced a technique to increase the resistance of newly hatched chicks to *Salmonella* infection by inoculating them orally with adult fowl intestinal content. In their experiment, the chicks were produced and reared, having no contact with the mother hen and being placed in a clean, sanitized environment, with little opportunity for rapid development of an intestinal microflora that could

successfully compete with any ingested salmonellas. The deficiency could be overcome by oral administration of a saline suspension of the contents of the alimentary tract from adult birds in which a mature microflora was present. In this way, an adult-type microflora was established and newly hatched chicks were protected from *Salmonella* infection (Nurmi and Rantala 1973). Later in 1973, they found that the protective material could be cultured anaerobically prior to use, thus providing a more convenient treatment preparation for commercial use. (Rantala and Nurmi 1973). This phenomenon has since been termed "competitive exclusion (CE)" or the "Nurmi Concept" (Nurmi, Nuotio and Schneitz 1992).

The Poultry Gastrointestinal Tract (GIT) as an Ecosystem. The GIT is a complex and delicately balanced ecosystem (Holzapfel *et al*. 1998) that associates a resident microbiota and cells of various phenotypes lining the epithelial wall expressing complex metabolic activities (Servin 2004). Fuller (1989) stated that within such a complex system are many interrelationships between different microorganisms and between microorganisms and the host. The mucosal surface provides a large area for microbial adherence and colonization (Holzapfel and Schillinger 2002). The resident microbiota in the digestive tract is a heterogeneous microbial ecosystem containing up to 10^{14} colony-forming units (CFUs) (Fuller 1989) and consisting of 400 different species of bacteria (Moore and Holdman 1974). Van der Wielen *et al*. (2002) found that chickens of the same age, raised under the same condition, receiving the same feed, and living in contact with each other have its own unique dominant intestinal bacterial community and suggested that host-specific factors are important in the establishment of the intestinal bacterial community. The intestinal microbiota plays an important role in normal gut function and maintaining host health (Mulder, Havenaar, and Huis in't Veld 1997; Bezkorovainy 2001; Ishibashi and Yamazaki 2001; Isolauri *et al*. 2001; Marteau *et al*. 2001; Gong *et al*. 2002). The

host is protected from potentially harmful microbial microorganisms attack by the physical and chemical barriers created by the gastrointestinal epithelium (Fuller 1989; Servin 2004) which depends on the establishment of indigenous microflora (Marteau *et al*. 1993; Isolauri *et al*. 2001) and host health (Fuller, 1989).

The complexities of the GIT differ from species to species (Fuller 1989; Isolauri *et al*. 2001; van der Wielen *et al*. 2002), site to site within the gut (van der Wielen *et al*. 2002) and on the health of the animal (Ewing and Cole 1994b). However, from the study of van der Wielen *et al*. (2002), they found that every chicken as well as every compartment of the intestinal tract within one chicken has its own specific dominant bacterial community except for the left and right ceca. Furthermore, they concluded that the crop, duodenum, and ileum in very young broilers have a similar dominant bacterial community but the bacterial community becomes intestinal compartment specific with broiler age. Immediately after hatching the intestinal tract of broiler chickens is germfree (Stavric 1992) and subsequently will be colonized by bacteria from the surrounding environment (Jayne-Williams and Fuller 1971; Fuller 1989; Isolauri *et al*. 2001; van der Wielen *et al*. 2002). Poultry are non-ruminant, simple stomached animal, and do not rely on a symbiotic relationship with the microbial flora within their GIT to survive to the same degree as animal possessing a rumen (Ewing and Cole 1994b). A further major difference is that the chicken has a crop (diverticulum) which is a pear-shaped reservoir in the esophagus for holding food. There is some microbial activity, mainly lactobacilli (Table 1), with the production of lactic and acetic acids (Barrow 1992). The crop, which is a storage organ, is linked by the proventriculus to the gizzard. Starch from feed that is consumed is hydrolyzed by bacterial activity in the crop. The production of volatile fatty acids in the crop indicates that some of the bacterial activity is from anaerobes. Lactobacilli are the major bacterial inhabitants and are

involved in the correct maintenance of microbial balance. Antibiotics can affect both the *Lactobacillus* level and the total microbial biomass, with a reduction in the total level being related to improvement in performance. The small intestine of poultry is short but still has a significant microflora. Poultry have large ceca and a small colon. The ceca assume the role of mixing the digesta together with fermentation and absorption (Ewing and Cole 1994b). The microbial content of the ceca and feces is influenced by whether they contain material from the small intestine. Cecal droppings are discharged two to four times every day (Barrow 1992).

The different bacteria have specific niches within the gut (Table 1). For example, in the crop, predominate organisms are lactobacilli (Barrow 1992; Freter 1992; Schrezenmeir and de Vrese 2001). These lactobacilli not only influence the crop but also the small intestine and form a symbiotic relationship with the host animal (Smith 1965; Dubos *et al.* 1965; Fuller and Turvey 1971). The chicken crop is also home to streptococci although they are in smaller numbers than the lactobacilli (Smith 1965). The diversity of the dominant bacterial community in the intestinal tract also increases with age (van der Wielen *et al*. 2002).

Table 1. Major groups of bacteria in the digestive tract of poultry†

Organ	Organisms	Population level*
Crop	lactobacilli	10^9
	enterococci	10^4
	streptococci	10^4
	yeasts	10^3
	E. coli	10^2

*CFU g^{-1} of organ contents (wet weight)

Table 1.(Con't) Major groups of bacteria in the digestive tract of poultry†

Organ	Organisms	Population level*
Gizard	lactobacilli	10^7
	enterococci	10^4
Small intestine	lactobacilli	10^8
	enterococci	10^4
	E. coli	10^2
	yeasts	10^2
Ceca	streptococci	10^{10}
	bifidobacteria	10^{10}
	clostridia	10^9
	lactobacilli	10^8
	enterococci	10^6
	E. coli	10^5
	salmonellae	10^6
	yeasts	10^2

*CFU g^{-1} of organ contents (wet weight)

†Adapted from Smith (1965b); Tannock (1992); Ewing and Cole (1994c); Rada and Petr (2000)

Mechanism of Competitive Exclusion. The immediate effect of CE treatment on a subsequent *Salmonella* challenge is to prevent the organisms from multiplying in the ceca, so that they are gradually eliminated from the bird (Impey and Mead 1989).

Competitive exclusion agents are considered to exert their effect by one or more of four general principle actions, namely the creation of a restrictive physiological environment, competition for bacterial receptor sites, elaboration of antibiotic-like substances (for example, bacteriocins) and/or depletion of essential substrates (Schneitz and Mead 2000; Fuller and Turvey 1971).

In the normal intestinal microflora, Mead (2000) described that volatile fatty acids (VFA) are produced mainly as a result of the metabolism of sporulating and non- sporulating anaerobic bacteria. The VFA can be inhibitory to other organisms present; especially in the undissociated state below pH 6.0. VFA that are inhibitory to salmonellas include acetic, propionic and lactic acid to suppress the salmonellas.

The precise mechanism of the protective effect is unknown, and may never be determined because of the complexity of the gut as a habitat for micro-organisms and the variety of host-microbe and microbe-microbe interactions that can occur (Rolfe 1991).

Efficacy of Competitive Exclusion. The prevention or reduction of *Salmonella* in young chickens by CE has been recognized for many years, and the method has received wide spread use in some Scandinavian countries. Large scale trials with a CE preparation in the Netherlands found that the rate of *Salmonella* carriage in CE-treated birds was 0.9% as compared with 3.5% for untreated birds (Wray and Davies 2000).

Nurmi, Nuotio and Schneitz (1992) reported that the protection is less effective towards the end of the growing period in countries where the *Salmonella* colonization is higher and suggested that CE-treatment is no panacea or compensation for unsatisfactory production hygiene. For prophylactic use, the chicks beings treated should be *Salmonella*-free; otherwise the effect is likely to be minimal (Mead 2000).

3. Probiotics and Prebiotics as Functional Foods

Definition of Functional Foods. Functional foods are also known as pharmafood, vitafood, nutraceutical, food for specific health use, or designer food (Gibson and Collins 1999) may be defined as “a dietary ingredient that affects the functions of the body in a targeted

manner so as to exert positive effects that may, in due course, justify certain health claim" (Gibson and Roberfroid 1995).

Definition of Probiotics. The word "probiotic" is derived from the Greek meaning "for life" (Fuller 1992). The term probiotic was introduced by Lilley and Stillwell (1965) to describe substances produced by one microorganism that stimulated the growth of other microorganisms. Since then, probiotics have been given several different definitions over the years (Fuller 1992; Holzapfel *et al*. 1998; Ouwehand *et al*. 1999a). Havenaar, Ten Brink, and Huis in't Veld, (1992) defined probiotics as "mono- or mixed cultures of live microorganisms which, when applied to animal or man, beneficially affect the host by improving the properties of the indigenous microflora". Marteau *et al*. (2001) defined probiotics as nonpathogenic microorganisms that, when ingested, exert a positive influence on the health or physiology of the host. However, Tannock (1997) described probiotics as "a phenomenon observed when two organisms were cultured together, in which substances produced by one organism stimulated the growth of the other organism. The most common definition currently used is that of Fuller who redefined probiotics as "a live microbial feed supplement which beneficially affects the host animal by improving its intestinal microbial balance" Fuller (1989).

Composition of Probiotics. Probiotics can be presented to the animal in various ways. The type of preparation will depend on the sort of use intended. They can be produced in the form of capsules, paste, powder or granules which can be used for dosing animals directly or through their food; feed and water (Fuller 1989). Probiotic preparations may consist of single microbial strains or may contain any number up to eight strains (Fuller 1989). Some species currently being used in probiotic preparations are shown in Table 2. The genera most frequently used as probiotics are *Lactobacillus* and *Bifidobacterium* (Isolauri *et al*. 2001; Isolauri and

Yamazaki 2001). Some bacteria that do not normally inhabit the intestinal tract may also come under the category of probiotics. They are used as starters in dairy products and include mainly *Lactobacillus bulgaricus*, *Streptococcus thermophilus*, *Leuconostoc* and *Lactococcus* species (Isolauri and Yamazaki 2001).

Table 2. Example of microorganisms applied in probiotic products*.

Lactobacillus species	*Bifidobacterium* species	Non-latics	Other Lactic Acid Bacteria
L. acidophilus	*B. adolescentis*	*Bacillus cereus (*toyoi*)*[a,d]	*Enterococcus farcalis*[a]
L. casei	*B. animalis*	*Escherichia coli (*Nissle*1917)*[d]	*E. faecium*
L. crispatus	*B. bifidum*	*Propionibacterium freudenreichii*[a,d]	*Lactococcus lactis*[c]
L. gallinarum[a]	*B. breve*	*Saccharomyces cerevisiae (boulardii)*[d]	*Pedicoccus acidilactici*[c]
L. gasseri	*B. infantis*		*Sporolactobacillus inulinus*[a]
L. johnsonii	*B. lactis*[b]		*Streptococcus thermophilus*
(L. paracasei)	*B. longum*		*Leuconostoc mesenteroides*[c]
L. plantarum	*B. thermophilum*[a]		
L reuteri	*B. pseudolongum*[a]		
L. rhamnosus			
L. bulgaricus			
L. salivarius			
L. helveticus			

[a] Mainly used for animals.

[b] Probably synonymous with *B. animalis*.

[c] Little known about probiotic properties.

[d] Mainly as pharmaceutical preparations.

* Adapted from Fuller (1989), Holzapfel *et al*. (1998), Fooks, Fuller and Gibson (1999), and Ishibashi and Yamazaki (2001).

Mechanisms and Established Effects of Probiotics. The balanced colonic microflora normally presents a barrier to invading organisms, but pathogens often become established when the integrity of the microbiota is impaired through stress, illness, antibiotic treatment, changes in

diet, or physiological alterations in the gut (Macfarlane and Cummings 1999). Probiotic bacteria are shown to be involved in resisting the colonization of microbial flora and promoting the endogeneous host defense mechanisms through the CE process (Isolauri *et al*. 2001). Attachment of probiotics to the gut epithelium is an important determinant of their ability to modify host immune reactivity (Macfarlane and Cummings 1999). Isolauri *et al*. (2001) described possible mechanisms of probiotic therapy by the promotion of a nonimmunologic gut defense barrier, which includes increased intestinal permeability and improved gut microecology balance. Another possible mechanism is the improvement of the intestine's immunologic barrier, particularly through intestinal immunoglobulin A responses and alleviation of intestinal inflammatory responses, which produce a gut-stabilizing effect. Fuller (1989) summarized possible modes of action of probiotics into 3 categories; (1) Suppression of viable count by production of antibacterial compounds, competition for nutrients, and competition for adhesion sites; (2) Increasing the activity of useful enzymes (such as β-galactosidase); (3) Stimulation of immunity by increasing antibody levels or macrophage activity.

Different strains of probiotic bacteria may exert different effects even within one species (Bernet *et al*. 1993; Ouwehand *et al*.1999b). Bacterial colonizing such high-transit-rate sites must adhere firmly to the mucosal epithelium (Savage 1972; Fuller 1973; Beachey 1980). The competition for adhesion receptors between probiotic and pathogenic microorganisms, therefore, is dependent on specific gut characteristic (Schrezenmeir and de Vrese 2001). Although probiotic microorganisms are considered to promote health, the actual mechanisms involved have not yet been fully elucidated (Holzapfel *et al*. 1998; Chung and Day 2002).

Characteristics of an effective Probiotic. Some features of an effective probiotic are shown in Table 3. The bacteria must survive the acidic conditions of the upper GIT and then

proliferate in and/or colonizes the intestine (Fuller 1989; Ziemer and Gibson 1998). There must be no pathogenic, toxic, mutagenic, or carcinogenic reaction to the host, its fermentation products or cell components. Furthermore, the bacteria should be antagonistic towards carcinogenic and pathogenic microorganisms and it must also be genetically stable with no plasmid transfer mechanism (Ziemer and Gibson 1998). For an organism to be a suitable probiotic it must be easily reproducible and remain viable during processing and storage (Fuller 1989; Ziemer and Gibson 1998). Such a probiotic with all these features has considerable advantages over antibiotics currently in use. Probiotics do not induce resistance to antibiotics which will compromise therapy. Probiotics are not toxic and therefore will not produce undesirable side-effects when being fed and, in the case of food animals, will not produce toxic residues in the processed carcass (Fuller 1989).

The evidence suggested that even with careful strain selection based on epithelial adhesion, growth rate and in-vitro bacterial antagonism, the effects produced after cessation of treatment are only of a limited duration (Fuller 1989; Kullen *et al*. 1997). The production of a probiotic which would permanently colonize the gut and thus would require only a limited administration would be ideal, yet it may be difficult to practically achieve.

The effective method of administration to farm animal is continuous feeding. This would ensure that the probiotic was present in the gut in large numbers and able to metabolize and produce its probiotic effect. However, even with continuous administration it is important to select strains with the maximum ability to survive in the intestine, and attention to colonization factors such as epithelial adhesion and growth rate is still recommended (Fuller 1989).

Table 3. Features of an effective probiotic*

1. Should be a strain which is capable of exerting a beneficial effect on the host animal, e.g. increased growth or resistance to disease.
2. Should be non-pathogenic and non-toxic.
3. Should be present as viable cells, preferably in large numbers, although we do not know the minimum effective dose.
4. Should be capable of surviving and metabolizing in the gut environment, e.g. resistant to low pH and organic acids.
5. Should be stable and capable of remaining viable for long periods under storage and field conditions.

* Fuller (1989).

Probiotics used in Poultry. Probiotic and competitive exclusion approaches have been used as one method of control of endemic and zoonotic agents in poultry (La Ragione and Woodward 2003). In the case of farm animals, faster weight gain for the same amount of food consumed (feed efficiency) have been of primary importance (Tannock 1997).

From the work of Mulder, Havenaar, and Huis in't Veld (1997), it was concluded that the administration of probiotic products, at the given dosage during a 4-week trial period, did not influence feed efficiency and body weight gain. The initial numbers of lactobacilli and other organisms present in the probiotic products seemed to exert no effect on the level of *Salmonella* colonization in ceca. Additional pH measurements in several parts of the gastrointestinal tract showed a large variation and no effect related to the administration of probiotic products could be observed when compared to the control.

Farnworth, Modler, and Chambers (1996) found that incorporating probiotic growth factors into poultry feeds is possible. However, finding suitable carriers for viable microbial preparations is more problematic in the case of poultry. Freeze-dried *Bifidobacterium*

thermophilium ATCC 25525 was applied at a rate of 10^6-10^7/g of feed material, after pelleting. Results showed that *B. thermophilium* decreased very rapidly in commercial feed samples (10.52% moisture; $a_w = 0.64$) and only freezing (-18 ^oC) or refrigeration at 5 ^oC would preserve the bacterial counts. Probiotics, when included in feeds, have a limited protective effect on its survival. It was concluded from this work that the addition of *B. thermophilium* and other oxygen-sensitive probiotics to feed materials is not a suitable technique in poultry rations.

Definitions of Prebiotics. The term prebiotic was introduced by Gibson and Roberfroid who exchanged "pro" for "pre", which means "before" or "for" (Schrezenmeir and de Vrese 2001). They defined prebiotics as "a nondigestible food ingredient that beneficially affects the host by selectively stimulating the growth and/or activity of one or a limited number of bacteria in the colon, and thus improves host health" (Gibson and Roberfroid 1995). Food ingredients classified as prebiotics must not be hydrolyzed or absorbed in the upper GIT, need to be a selective substrate for one or a limited number of colonic bacteria, must alter the microbiota in the colon to a healthier composition and should induce luminal or systematic effects that are beneficial to host health (Gibson and Roberfroid 1995). Such resistant short-chain carbohydrates are also referred to as nondigestible oligosaccharides (Cummings, Macfarlane, and Englyst 2001), or low-digestible carbohydrates (Marteau and Flourie 2001).

Because the viability of live bacteria and during transit through the gastrointestinal tract may be variable, the prebiotic idea has been developed. A selective growth of indigenous gut bacteria through the diet is required. Thus, the prebiotic approach advocates administration of non-viable entities and aims to overcome survival problems in the upper gastrointestinal tract. Certain oligosaccharides which cannot be digested, except through bacterial activity, are prebiotics (Fooks, Fuller, and Gibson 1999). An alternative to direct ingestion of live bacteria

into the colon through dietary supplementation is to increase population of natural bifidobacteria and lactobacilli in the intestinal microbiota through the use of prebiotics (Ziemer and Gibson 1998; Chung 2002).

Oligosaccharides. Oligosaccharides are carbohydrates generally consisting of two to ten monomeric residues linked by glycosidic bonds (Fig. 1). They may be produced synthetically by microbial fermentative and enzymatic processes, or they may be derived from naturally occurring sources such as plant matter (Playne and Crittenden 1996; Eggleston and Cote 2003).

Since 1980, their use as components of "functional foods" has been recognized. Evidence has been presented since that date that many oligosaccharides, when fed to animals or humans, can reach the colon undegraded and provides a carbohydrate substrate particularly suited to the growth of bifidobacteria (Playne and Crittenden 1996). However, another possible health claim of oligosaccharides is the ability to act as anti-infective agents. Zopf and Roth (1996) concluded that many pathogens use carbohydrate-binding proteins to attach to cells and initiate diseases. The first line of defense against these infectious diseases consists of decoy oligosaccharides in the mucous layer that lines all exposed epithelial cells. Decoy oligosaccharides bind to the microbe's carbohydrate-binding proteins and pathogens are cleared by the physiological mechanism. Some oligosaccharides have recently been commonly referred to as "prebiotics" (Playne and Crittenden 1996).

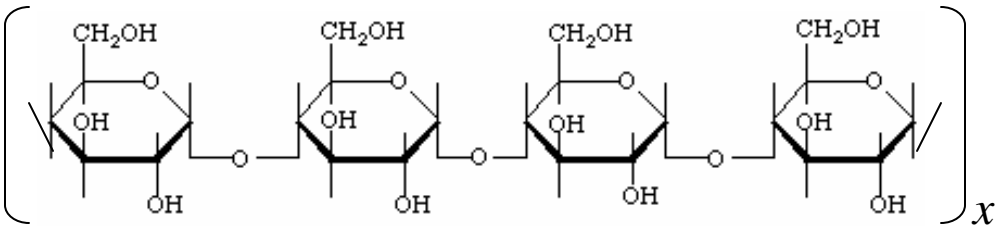

Figure 1. A general structure of oligosaccharide

Oligosaccharides as Bifidogenic Factors. Prebiotic oligosaccharides are able to traverse the upper GIT and arrive intact in the lower GIT because of specific linkages between monomers that resist cleavage by host enzymes. The presence of microbial enzymes able to act on these bonds allows these carbohydrates to be selectively degraded. Further selectivity is conferred by the fact that not all colonic microorganisms have the appropriate enzymes to degrade all polysaccharide linkages (Ziemer and Gibson 1999). Oligosaccharides passing through to the lower portions of the GIT are preferentially utilized by bifidobacteria as carbon and energy sources (Tomomatsu 1994). These compounds are called "bifidogenic factors" (Modler, McKellar, and Yaguchi 1990; Modler 1994). Bifidobacteria selectively fermented the fructans, in preference to other carbohydrate sources such as starch, fructose, pectin and polydextrose (Fooks, Fuller, and Gibson 1999).

Commercially Available Oligosaccharides. Oligosacchrides currently produced (Table 4) for commercial markets include cyclomaltodextrins, maltodextrins, fructooligosaccharides, galactooligosaccharides, soybean oligosaccharides, and others produced mainly for the prebiotic markets in Japan and Europe (Eggleston, and Cote 2003). Inulin and fructooligosaccharides (FOS) are probably the most commonly used prebiotics (Ziemer and Gibson 1998; Fooks, Fuller, and Gibson 1999; Holzapfel and Schillinger 2002). Estimated productions of oligosaccharides in the year 1995 are shown in Table 5 (Playne and Crittenden 1996).

Table 4. Example of oligosaccharide products and their manufacturers.*

Oligosaccharides	Product name	Manufacturers
Lactulose	MLS-50	Morinaga Milk Industry Co., Japan
	Lactulose syrup	Solvay, Germany
	Lactulose powder	Solvay, Germany
Lactosucrose	Pet-Oligo P55	Ensuiko sugar Refining Co., Japan
	Nyuka-Origo LS-40L	Ensuiko sugar Refining Co., Japan
	Newka-Oligo LS-35	Hayashibara Shoji Inc., Japan
Fructooligosaccharides	NutraFlora	Golden Technologies Co., USA
	Meioligo P	Meiji Seika Kaisha, Japan
	Oligo-Sugar	Cheil Foods and Chemicals, Korea
Inulin	Raftilose L95	ORAFTI, Belgium
Palatinose	ICP	Mitsui Sugar Co., Japan
Maltooligosaccharides	Fuji-Oligo #350	Nihon Shokuhin Kako Co., Japan
	Tetrup	Hayashibara Shoji Inc., Japan
Galactooligosaccharides	Oligomate 55	Yakult Honsha Co. Ltd., Japan
	TOS-100	Yakult Honsha Co. Ltd., Japan
	Cup-Oligo P	Nissin Sugar Mfg. Co. Ltd., Japan
	P7L+others	Snow Brand Milk Products, Japan
	TOS-Syrup	Borculo Whey Products, The Netherlands
Cyclodextrin	Celdex α-CD	Nihon Shokuhin Kako Co., Japan
	Dexy Pearl	Ensuiko Sugar Refining Co., Japan
Gentiooligosaccharides	Gentose #45	Nihon Shokuhin Kako Co., Japan
Soybean oligosaccharides	Soya-oligo	The Calpis Food Industry Co., Japan
Xylooligosaccharides	Xylo-oligo 70	Suntory Ltd., Japan

*Adapted from Playne and Crittenden (1996), Tanaka and Matsumoto (1998) and Teruo (2003).

Table 5. Estimated production of oligosaccharides in 1995*

Oligosaccharide type	Tons
Galactooligosaccharides	15,000
Lactulose	20,000
Lactosucrose	1,600
Fructooligosaccharides (FOS)	12,000
Palatinose-oligosaccharides	5,000
Glycosyl sucrose	4,000
Maltooligosaccharides	10,000
Cyclodextrins	4,000
Gentiooligosaccharides	400
Soy oligosaccharides	2,000
Xylooligosaccharides	300
Total	85,300

* Adapted from Playne and Crittenden 1996.

Isomaltooligosaccharides (IMO). IMO was developed by Chung (2002) using *L. mesenteroides* ATCC 13146 fermentation with sucrose and maltose by an acceptor reaction. IMO has been developed previously by Lee *et al*. (1997) using *Streptococcus sobrinus*.

IMO have as yet not been extensively investigated (Fooks, Fuller, and Gibson 1999). Although they are thought to be bifidogenic, one study fed gnotobiotic rats a diet of 40 g/day of IMO and found little effect on the bacterial groups. However, they did modify certain glycolytic activities (Djouzi and Andrieux 1997).

Composition and Structure of IMO. From the work of Chung (2002), IMO are branched polymers ranging in size from degree of polymerization (DP) 2 to 7. By HPLC peak area, there was 6.9% DP 2, 28.4% panose, 36.7% branched DP 4, 19.1% branched DP 5, 7.4%

branched DP 6, and 1.2% branched DP 7, In the pure form, there was only a trace amount of monosaccharides (<0.2%) present and no polysaccharides larger than DP 7. Structural analysis of IMO by C^{13} NMR showed that the IMO are linked mainly by α-1,4 and α-1,6 glycosidic bond linkages.

IMO as Prebiotics. Chung (2002) observed utilization of IMO, commercial FOS, and glucose by six bacterial isolates (they were identified as lactic acid bacteria; all showed gram positive, catalase negative and lactic acid formation form glucose) from chicken ceca. Three of the six bacteria showed more growth after 24 hr on media with IMO as a carbon source than on media with FOS as a carbon source (Table 6). Growth of selected bacteria on the same three types of media was also determined. Both types of oligosaccharide media produced significantly reduced growth of *S.* ser. Typhimurium and *E. coli* compared with growth on media with glucose as a carbon source (Table 7).

Table 6. Growth comparison of lactic acid bacteria from six chicken cecas on the media with glucose, IMO, and FOS as a carbon source*

Ceca	Relative growth to glucose as a carbon source at 24 hr incubation ([Absorbance unit of Glc at 24 hr/Absorbance unit of x100] per hr)		
	glucose[a]	IMO	FOS[b]
1	100.00	22.77	61.20
2	100.00	99.34	79.29
3	100.00	75.85	48.01
4	100.00	36.72	61.40
5	100.00	87.23	50.25
6	100.00	87.17	87.66

[a] Growth level at stationary phase (at 24 hr) on glucose was calculated as 100.

[b] Samyang Genex Co., Seoul, Korea

* Adapted from Chung (2002)

Table 7. Growth comparison of selected bacteria on the media with glucose, IMO, and FOS as a carbon source*

Organism	Growth rate in exponential growth rate ([Absorbance unitx100] per hr)			(Growth rate on IMO/glucose)
	glucose	IMO	FOS[a]	
S. ser. Typhimurium	9.89	3.64	3.48	36.8
E. coli	9.35	2.68	2.44	28.7
B. bifidum	13.30	9.81	13.10	73.8
L. johnsonni	11.06	10.74	10.70	97.1
B. longum	11.72	11.69	11.70	99.7

[a] Samyang Genex Co., Seoul, Korea

* Adapted from Chung (2002).

Although the ability of probiotic bacteria to ferment oligosaccharides may be an especially important characteristic (Gibson and Roberfroid 1995), there is no clear answer as to why prebiotics stimulate growth of probiotic strains such as *Lactobacillus* and *Bifidobacterium* (Chung 2002). However, the IMO showed the preferential utilization by probiotic strains but not by pathogenic organisms (Chung and Day 2002). The use of this oligosaccharide in poultry feed may favor the growth of probiotic bacteria and promote the growth of healthy, *Salmonella*-reduced birds without using antibiotics (Day, 2001). Nevertheless, this study did not allow direct prediction of *in vivo* effects but indicated that this type of oligomer could be a prebiotic for intestinal microflora (Chung and Day 2002).

Prebiotics used in Poultry. A variety of compounds have been incorporated in poultry feed rations for the purpose of improving weight gain and reducing the growth of pathogenic bacteria, such as *Salmonella*. The results have been highly variable, but some positive results have been achieved using feed additives which have included FOS (crude and purified), lactose,

lactulose, cyclodextrins and transgalactosylated oligosaccharides such as lactosucrose (Farnworth, Modler and Chambers 1996).

The earlier work with FOS (0.25-0.50% of the diet) demonstrated some advantages in terms of feed efficiency (Ammerman, Quarles and Twining 1989), live body weight (Ammerman 1990). Others have demonstrated that FOS components, at a dietary level of 0.75%, are capable of reducing *Salmonella* colonization in 48-day-old broiler by 3 or 4 fold (Izat *et al*. 1990). Choi, Namkung and Paik (1994) demonstrated that FOS could be utilized to suppress *Salmonella typhimurium* in broiler chickens. In this work, broiler chicks were exposed to two level of challenge (10^4 and 10^6 CFU/ml) at 3 days of age and four FOS levels (0, 0.5, 1.0, and 2%) in the 2 by 4 factorial design. Results showed that the challenge level had no significant effect on weight gain and feed efficiency. However, all FOS groups showed a numerical but not a statistically significant improvement over the control group in terms of weight gain, particularly at the lower level of challenge. The *S.* ser. Typhimurium counts in the cecal contents of the FOS-supplemented groups were low compared to the control but not statistically significant.

Waldroup *et al*. (1993) evaluated the effects of dietary FOS (0.375%) in combination with bacitracin methylene disalicylate (BMD) administered at a lever of 55 mg/kg of diets. Broiler were grown to 49 days of age and then sacrificed to determine the most probable number of *Salmonella* on prechill carcasses. Other parameters which were evaluated included dressing percentage, abdominal fat content, growth rate, and feed efficiency. Results showed that FOS had no effect on any of the parameters evaluated. The combination of FOS and BMD was slightly antagonistic towards *Salmonella* growth but was not highly significant.

Safety of Pro- and Prebiotics. The reports of a harmful effect of probiotics to the host are rare (Ishibashi and Yamazaki 2001). Adams and Marteau (1995) also concluded that there is

no evidence that lactic acid bacteria except of the pathogenic species and the opportunistic enterococci have a pathogenic potential. The ecological consideration also has significance for the efficiency as well as the safety of pre- and probiotics. With regard to their safety evaluation, one has to consider the inherent properties of the probiotic strain or prebiotic compound as well as their effects on the microbial flora altogether. As long as prebiotic compounds are traditional food or food components, toxicological aspects may not be of concern (Hammes and Hertel 2002).

4. Synbiotics

Definitions. The term synbiotics is used when a product contains both probiotics and prebiotics. Because the word alludes to synergism, this term should be reserved for products in which the prebiotic compound selectively favors the probiotics. In this strict sense, a product containing oligofructose and probiotic bifidobacteria would fulfill the definition, whereas a product containing oligofructose and a probiotic *L. casei* strain would not (Schrezenmeir and de Vrese 2001).

Bielecka, Biedrzycka and Majkowska (2002) demonstrated the influences of fructans-type oligosaccharides (as prebiotics) on growth and acidifying activity of *Bifidobacterium* strains (as probiotics) in vitro, using minimal nutrition media. Daily, $>10^9$ live cells of bifidobacteria strains and/or 5% (w/w) of oligofructose in the diet were orally administered to Wistar rats. After a 14-day feeding experiment, they found that incorporation of oligofructose into the diet stimulated the proliferation of fecal bifidobacteria by 1.6 log CFU/g in comparison to the control. However, administration of bifidobacteria together with the oligofructose (as synbiotics) improved the bifidogenic effect by 1.4 log CFU/g of feces. They concluded that the proper

selection of probiotics and prebiotics for synbiotics showed higher effectiveness in relation to probiotics.

5. Lactic Acid Bacteria (LAB)

Lactic acid is the main acid in sour milk. It was first identified as a fermentation product in 1847 by Blondeau, and in 1877, a pure culture of lactic acid producing bacteria, *Streptococcus lactis*, was isolated (Ewing and Cole 1994).

Characteristics of LAB. LAB are gram-positive, nonsporing, catalase-negative organisms, although pseudo-catalase can be found in rare cases, (Schleifer and Ludwig 1992) that are devoid of cytochromes and of nonaerobic habit but are aerotolerant, fastidious, acid-tolerant, and strictly fermentative (Holzapfel *et al*. 2001). They occur as cocci or rods and grow only in complex media (Schleifer and Ludwig 1992). Lactic acid is the major end product of carbohydrate fermentation (Axelsson 1998).

LAB are comprised of the following genera: *Aerococcus*, *Alloiococcus*, *Carnobacterium*, *Dolosigranulum*, *Enterococcus*, *Globicatella*, *Lactobacillus*, *Lactococcus*, *Lactosphaera*, *Leuconostoc*, *Oenococcus*, *Pediococcus*, *Streptococcus*, *Tetragenococcus*, *Vagococcus*, and *Weissella* (Axelsson 1998). The genus *Bifidobacterium*, often considered as LAB (Axelsson 1998) but has a unique mode of carbohydrate fermentation (Scardovi 1986).

A further classification has resulted in two subdivisions depending on whether they are able to ferment glucose solely to lactate or to other products (Ewing and Cole 1994). As described below:

Homofermentative. Homofermentative lactic acid bacteria metabolize glucose via the fructose-bisphosphate pathway, having all the necessary enzymes, including aldolase, and being

able to use the hydrogen obtained from the dehydrogenation of glyceraldehydes-3-phosphate (to 1,3-bisphosphoglycerate) to reduce pyruvate to lactate.

Heterofermentative. In the case of heterofermentative LAB, fifty percent of the end products of glucose metabolism are lactic acid. In addition, CO_2 (20-25%), acetic acid and ethanol are also produced per molecule of glucose. Other characteristics of this group include mannitol production from fructose, gas from glucose and gluconate, ribose fermentation without gas production, thiamine growth requirements and glucose-6-phosphate dehydrogenase activity.

Probiotic Properties of LAB. LAB used as probiotics are commonly defined as viable microorganisms that exhibit a beneficial effect on the health of the host when digested (Mattila-Sandholm, Matto and Saarela 1999). LAB, among them many probiotics, have been observed to produce anti-microbial substances (Ouwehand *et al*. 1999a) and have the longest history of use as probiotics and are still the most common ingredients of those intended for consumption by farm animals, notably pigs and poultry (Tannock 1997). The abundant antimicrobial substances produced by LAB are organic acids, especially lactic and acetic acids, and hydrogen peroxide (Fuller 1989; Ouwehand *et al*. 1999a).

Lactic acid bacteria are known to be associated to the gut wall of chickens (Fuller and Turvey 1971). Bibiloni, Perez and de Antoni (1999) also suggested that adhesion to intestinal epithelia is an important trait for LAB strains because adherent strains would be capable to resist washout due to intestinal motility and thus have greater probiotic effects.

6. *Bifidobacterium* spp.

The first organism of the bifidobacteria group was isolated from the feces of breast fed infants (Mitsuoka and Kaneuchi 1977). Since that time bifidobacteria have been supposed to play an important role in nutrition and resistance to infection in breast fed infants. These organisms

are also found in the human adult intestine, vagina, and mouth, and in the gastrointestinal tract of various kinds of animals (Mitsuoka and Kaneuchi 1977). Their name comes from the observation that these organisms often exist in a Y-shaped (Fig. 2) or "bifid" form (Lim, Hun, and Baek 1993; Arunachalam 1999) or club-shaped or spatulated extremities (Scardovi 1986).

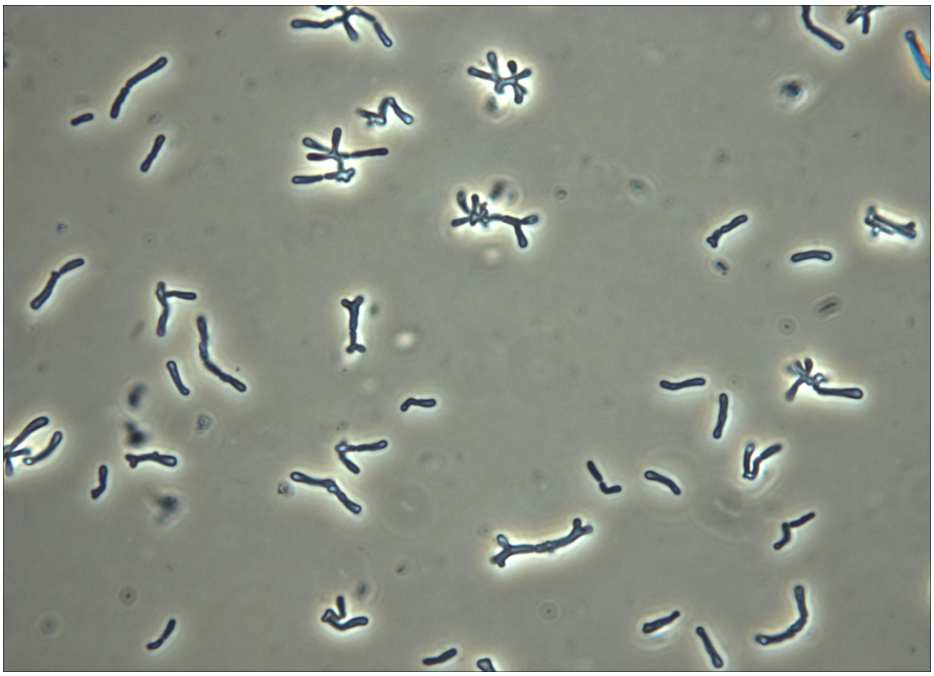

Figure 2. Distinct bifurcation Y-shaped morphology of *B. longum* on MTPY under 1000X light Microscope (image by Thitaram, S.N.)

For many years bifidobacteria were included in the genus Lactobacillus as *Lactobacillus bifidus* (Mitsuoka and Kaneuchi 1977), *Bacillus* (Lim, Hun, and Baek 1993), *Bacterroides*, *Nocardia*, and *Corynebacterium*, before being classified in a separate genus in 1974 (Modler, McKellar, and Yaguchi 1990). At present, the genus *Bifidobacterium* includes 34 species (Biavati *et al*. 2000; Hoyles *et al*. 2002). Approximately 20 species have been recovered from fecal sources, of which only 10 appear to have been recovered from GIT samples (O'Riordan and Fitzgerald 1997; Biavati *et al*. 2000).

Characteristics of Bifidobacteria. Bifidobacteria are rods, gram-positive, strictly anaerobic, non-acid-fast, nonspore forming, nonmotile, catalase-negative (except that *B. indicum* and *B. asteroids* are catalase-positive when grown in the presence of air with or without added hemin). Cells often stain irregularly with methylene blue and often have a bifurcating morphology characteristic. Some species can tolerate O_2 only in the presence of CO_2. Optimum growth temperatures are 37-45 ^{o}C. Optimum pH for initial growth is 6.5-7.0, no growth below 4.5-5.0 or above 8.0-8.5 (Scardovi 1986).

Acetic and lactic acid are formed primarily in the molar ratio of 3:2. CO_2 is not produced. Small amounts of formic acid, ethanol and succinic acid are produced. Butyric and propionic acid are not produced. Glucose is degraded exclusively and characteristically by the fructose-6-phosphate shunt in which fructose-6-phosphoketolase (F6PPK-EC 4.1.2.22) cleaves fructose-6-phosphate into acetylphosphate and erythrose-4-phosphate (Scardovi 1986; Bibiloni, Perez and de Antoni 2000). Fructose-6-Phosphate Phosphoketolase is the characteristic key enzyme of the "bifid shunt" (Scardovi 1986) and is considered to be the most reliable indication that gram-positive rod-shaped bacteria belong to the genus *Bifidobacterium* (Gavini *et al.* 1996). Fructose-6-phosphate is apparently absent in anaerobic gram-positive bacteria of "pseudobifid" morphology, such as *Arthrobacter*, *Propionibacterium*, *Corynebacterium* and *Actinomyces* (Scardovi and Trovatelli 1965). End products are formed through the sequential action of transaldolase (EC 2.2.1.2), transketolase (EC 2.2.1.1), xylulose-5-phosphate phosphoketolase (EC 4.1.2.9) and enzymes of EMP acting on glyceraldehydes-3-phosphate. Additional acetic and formic acid may be formed through the cleavage of pyruvate (Scardovi 1986). Glucose-6-phosphate dehydrogenase (EC 1.1.1.49) is generally not detectable in *Bifidobacterium* while it is found in *Lactobacilusi* (Arunachalam 1999).

B. globosum has a preferred "animal" habitat, and is often isolated from feces of animals and rumen of cattle. *B. dentium* is harbored in "human" habitats, including human feces and dental caries of man. Two electrophoretic types of phosphoketolase (F6PPK) were previously distinguished and called "animal" and "human" type according to the habitat of the bifid organism (Sgorbati, Lenaz and Casalicchio 1976). The two phosphoketolases display a very different optimum pH range, and molecular weight. An outstanding difference was found in the substrate specificity; the enzyme from *B. globosum* was able to cleave xylulose-5-phosphate as well as fructose-6-phosphate whereas the phosphoketolase from *B. dentium* appeared to be specific for fructose-6-phosphate (Sgorbati, Lenaz and Casalicchio 1976).

Acetic acid is produced predominantly by *Bifidobacterium* (Scardovi 1986). In addition, acetic acid has been found to be more effective antimicrobial agent than lactic acid (Rasic and Kurman 1983). The antimicrobial effect of the organic acids is thus principally produced by the undissociated molecules through the acidification of cytoplasm, destruction of the transmembrane of the proton motive force, and loss of active transport of nutrients through the membrane. Acetic acid was highly lipophilic and able to neutralize transmembrane pH gradients efficiently (Yusof *et al*. 2000). As compared to lactic acid, with a lower pK value, has less effect on neutralizing the proton motive force than acetic acid (Eklund 1989). The mechanisms of action of these organic acids are most likely based on the toxicity of low pH value, due to partly to the penetration of undissociated weak acids into the cells of the pathogens. When the internal pH changes, amino acid transferase RNA is inhibited and protein synthesis stops, resulting in the death of cells (Banwart 1989).

Fluorescence *In Situ* Hybridization (FISH) of Bifidobacteria. *Bifidobacterium* spp. can be enumerated either by using selective agar media (Munoa and Pares 1988; Talwalkar and

Kailasapathy 2004) or turbidimetric techniques using a standard curve (Bibiloni, Perez and de Antoni 2000). A FISH assay has been developed for an accurate method for their detection and enumeration, in contrast of culturing (Langendijk *et al*. 1995). The *in situ* identification of fixed whole bacterial cells using fluorescently labeled, ribosomal RNA (rRNA)-targeted oligonucleotide, originally described by DeLong *et al*. (1989) has found numerous applications in microbiology. By using the rRNA as a nucleic acid target of hybridization, fluorescent oligonucleotide probes can specifically detect whole bacterial cells that contain the target sequence among a heterogeneous population of non-target cells (Amann *et al*. 1992). FISH is a powerful tool to study microbial population structures and dynamics (Amann, Ludwig and Schleifer 1995).

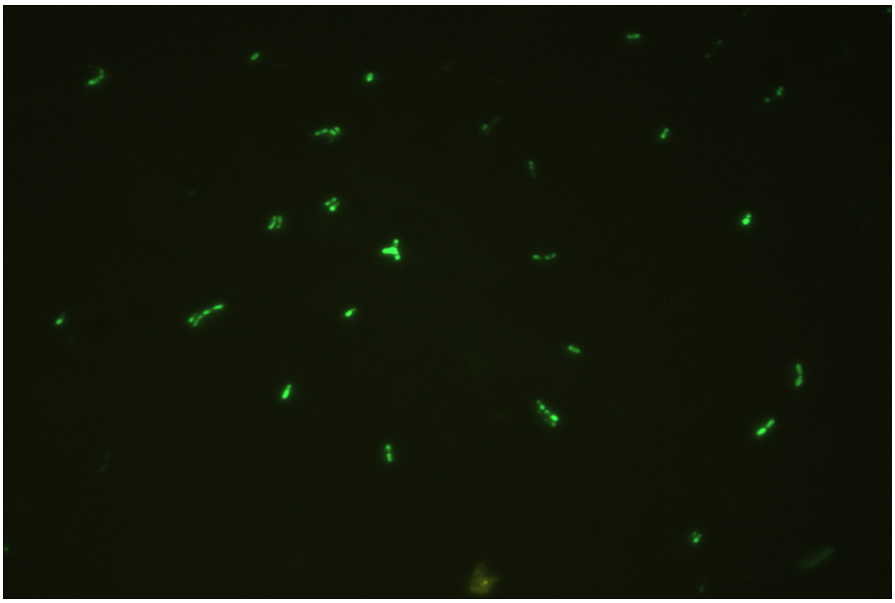

Figure 3. *B. longum* hybridized with bifidobacteria specific 16S rRNA DNAprobe with Cy3 label on a glass slide, under 1000X fluorescent microscope
(image by Thitaram, S.N.).

Development of Selective Media for Bifidobacteria. A large variety of media have been devised for isolating or enumeration bifidobacteria in natural habitats and from foods

(Table 8). By using antibiotics (Lim, Huh and Baek 1993) or low pH (Beerens 1990; Payne, Morris and Beers 1999) to inhibit the growth of non-bifidobacteria are the key to developing the media (Lim, Huh and Baek 1993). Differentiation of bifidobacteria from lactobacilli is difficult in the routine laboratory because cultural and biochemical properties of both genera are largely overlapped. Lactobacilli are susceptible to the antibiotic mupirocin while bifidobacteria are resistant. Thus, the resistance to mupirocin may serve as a simple criterion distinguishing *Bifidobacterium* spp. from *Lactobacillus* spp. (Rada 1997; Rada and Petr 2000).

However, enumerating bifidobacteria from the fecal and cecal contents of broilers continues to be a difficult task; difficult in differentiating bifidobacteria colonies from other non-bifidobacteria colonies, and requiring strictly anaerobic condition, particularly when the bacterial number is less than 10^4/g content. Additional understanding and study are required in the area of selective enumeration of these organisms (Farnworth, Modler and Chambers 1996).

Table 8. Example of media used for the isolation and enumeration of bifidobacteria from various habitats.

Medium	Selectivity based on	Used for	References
MPN	riboflavin, nucleic acid bases, pyruvic acid, nalidixic acid	feces	Tanaka and Mutai 1980
RB-agar	propionate, lithium chloride, raffinose	dairy products, human	Hartemink *et al.* 1996
MTPY	acetic acid, mupirocin	chicken cecas	Rada and Petr 2000
BFM	lactulose, methylene blue, propionic acid, lithium chloride	environmental, food samples	Nebra and Blanch 1999
BIM-25	nalixidic acid, kanamycin sulfate, polymycin B sulfate, iodoacetic acid,	sewage	Munoa and Pares 1988

Table 8.(Con't) Example of media used for the isolation and enumeration of bifidobacteria from various habitats.

Medium	Selectivity based on	Used for	References
AMC-agar	nalixidic acid, polymycin B sulfate, kanamycin sulfate, iodoacetic acid, lithium chloride, 2,3,5-triphenyl-tetrazolium chloride propionate	*B. longum*	Arroyo, Cotton, and Martin 1995
BS-MCA	neomycin sulfate, propionic acid, lithium chloride, paromycin sulfate	feces	Martineau 1999
MW	acetic acid, mupirocin	chicken cecas	Rada, Sirotek, and Petr 1999

The success of bifidobacteria detection and enumeration in an optimal culture medium is mainly dependent upon the following factors: (1) if the culture medium has no selective agent, non-bifidobacteria may outgrow bifidobacteria; (2) the macroscopic identification of bifidobacteria colonies may be facilitated using chromogenic selective agar media; (3) the optimal growth of bifidobacteria is dependent upon the freshness of the ingredients of the medium; and (4) the composition of the culture medium allow the growth of different biotypes present in the investigated material (Rasic and Kurmann 1983).

7. Salmonella

Poultry products contaminated with *Salmonella* are a major source of foodborne disease in most developed countries (WHO 1988). It has been reported that poultry-borne salmonellosis constitutes a major health problem in many countries (Persson and Jendteg 1992). During the last two decades the number of reported cases of human salmonellosis, predominantly caused by

S. ser. Typhimurium, in Denmark has increased from 300 to 3,000 per year (Bisgaard 1992). Daniels *et al*. (2002) also reported that *Salmonella* was the most commonly cause of foodborne disease outbreaks (36%) in United States during 1973-1997.

S. ser. Typhimurium belongs to genus *Salmonella* in family *Enterobacteriaceae* (Le Minor 1984). *Salmonella* are gram-negative, facultatively anaerobic, straight rods, usually motile (peritrichous flagella). Colonies are generally 2-4 mm in diameter on a Brilliant Green Sulfa agar medium. The biochemical reactions of genus *Salmonella* are shown in Table 9.

Table 9. Biochemical reactions of genus *Salmonella*[*]

Reactions	
Nitrates reduced to nitrites	Yes
Gas formation from glucose	Yes
Utilization of Citrate as a sole carbon source	Yes
Indole reaction	No
Urease reaction	No
Lysine reaction	Yes
Ornithinedecarboxylase reaction	Yes
Sucrose fermentation	No
Salicin fermentation	No
Inositol fermentation	No
Amygdalin fermentation	No
Lipase production	No
Deoxyribonuclease production	No

[*]Adapted from Le Minor (1984).

The Impact of *Salmonella* on Poultry and Human. *Salmonella* is generally of fecal origin and can be found in the feces of almost all food animals. Broiler chickens can become contaminated by several routes. A key route of infection is from infected parent flocks to

progeny (Notermans and Hoogenboom-Verdegaal 1992). This is especially so for serotypes which can invade organ such as the ovaries, as well as vertical transmission, horizontal transmission (via environment and feed) is also an important route of infection (Notermans and Hoogenboom-Verdegaal 1992).

Savage (1987) stated that if a pathogen is present in the gastrointestinal tract of an animal, whether or not it is an indigenous member of the microbiota, and if the animal's products are used as food source for humans and are contaminated with intestinal contents, then the pathogen may be transmitted to humans. In most cases, salmonellae are present in animals without causing illness. Certain types of *Salmonella* can cause contamination in poultry organs. An example is *S.* ser. Enteritidis in chickens. This organism, as well as *S.* ser. Typhimurium, is invasive and can penetrate the reproductive organs. In consequence, egg contents may become contaminated with these organisms. Because of the fecal origin of *Salmonella*, meat, raw milk and other foods which can become contaminated with fecal material may contain salmonellas (Notermans and Hoogenboom-Verdegaal 1992).

The impact of colonizing chicks in the hatchery is far reaching and should not be underestimated. The exposure of young chicks to high levels of salmonellae, early in grow-out by ingesting waster and feed contaminated by droppings from an infected flockmate, can override the beneficial effect of a treatment, such as CE, or negate intervention efforts aimed at environmental sources of salmonellae (Cox *et al*. 1990).

Control of *Salmonella* in Poultry. Salmonellas are not part of indigenous microbiota in poultry, but they readily colonize intestines of young chicks and persist there during rearing (Stavric 1992). The intestinal tract of chicks at the time of hatching is essentially sterile (Stavric 1992). However, it rapidly becomes colonized by bacteria from the environment (Jayne-

Williams and Fuller 1971; Fuller 1989; Isolauri *et al*. 2001; van der Wielen *et al*. 2002). On the other hand, from the study of Cox *et al*. (1990), it has been suggested that the vulnerable day-of-hatch chick may be at a greater colonization risk in the hatchery than during grow-out. Contamination and penetration of the shell of hatching eggs may constitute the most important link in the transmission of salmonellae to young birds and eventually the consumer. An effective intervention method may have to be employed at this point to break the transmission link and significantly impact the overall problem of *Salmonella* colonization in poultry.

Nurmi concept or CE concept opened a new, promising approach to the control of *Salmonella* in poultry (Nurmi, Nuotio and Schneitz 1992), and had been confirmed by a number of studies in various countries (Oyofo *et al*. 1989; Baba *et al*. 1991; Bailey, Blankenship and Cox 1991; Nisbet *et al*. 1993; Terada *et al*. 1994; Orban *et al*. 1997; Patterson *et al*. 1997; Fernandez, Hinton and van Gils 2000; Henriksson and Conway 2001; Fernandez, Hinton and van Gils 2002; Kleessen *et al*. 2003). It has been suggested that native gut bacteria and *Salmonella* spp. complete for the sites of adherence on the intestinal wall of young chicks, and that protection of chicks is achieved by a CE process (Lloyd, Cumming and Kent 1977). CE of *S.* ser. Typhimurium was reported in gnotobiotic chickens administered fecal suspensions from healthy adult hens or bacteria isolated from cecum of young chickens (Hudault *et al*. 1986). Fukata, Baba and Arakawa (1987) also found similar result. The population of *S.* ser. Typhimurium was suppressed when fed the gnotobiotic chickens with cecal content of healthy adult chicks.

The effective control of *Salmonella* in poultry flocks and minimization of public health risks from poultry and poultry products is dependent on a supply of uninfected breeder stock at the production level and preventing the introduction of infection (Dawson 1992).

From the economic point of view, Persson and Jendteg (1992) concluded that prevention of poultry-borne salmonellosis by using the CE concept is definitely worthwhile both from individuals and producers and seems to be the best alternative for reducing Salmonellae in poultry (Bailey 1987). The CE concept combined with a certain level of surveillance biosecurity could be an alternative to more ambitious control and prevention of *Salmonella* colonization in poultry (Persson and Jendteg 1992).

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CHAPTER 1

THE EFFECT OF ISOMALTOOLIGOSACCHARIDE ON *BIFIDOBACTERIUM* SPP. POPULATION IN YOUNG BROILER CHICKENS[1]

[1]Thitaram, S. N., G. R. Siragusa., J.S. Bailey and A. Hinton, Jr. To be submitted to Poultry Science.

Abstract

The broiler starter diet was supplemented with isomaltooligosaccharide (IMO) at 1% (w/v), 2% (w/v), 4% (w/v), and a control group without IMO supplementation. Forty one-day-old chicks were randomly divided into four groups and provided the treatment diet. All groups were challenged with 0.25 ml of a culture of *Salmonella enterica* ser. Typhimurium 200 µg/ml nalidixic acid resistant (*S*. ser. Typhimurium Nalr), containing 10^8 cells, after seven days of placement. The experiment was done in three replications. IMO supplemented diets resulted in significantly higher cecal bifidobacteria compared to the control diet ($P<0.05$). However, there was no significant difference in bifidobacteria counts among the treatment group. Chickens fed diets with 1% w/v IMO had a significant two log reduction in the level of inoculated *S.* ser. Typhimurium Nalr ($P<0.05$) present in the ceca compared to the control group. No significant improvement in weight gain was observed between any treatment groups. It may be suggested that feeding the optimum level of IMO in the diet of chickens may reduce *Salmonella* colonization in young chickens.

Key words: isomaltooligosaccharide, *Salmonella*, chicken, colonization, competitive exclusion.

Introduction

Non-therapeutic antibiotic growth promotants (AGPs) are used for growth promotion improving feed efficiency of food-producing animals and poultry (Turnidge *et al*. 1999; Mathews 2001). However, the use of antibiotics, particularly as growth promotants, has received increasing attention as a contributory factor in the international emergence of antibiotic-resistant bacteria in human health and agriculture (Fuller 1989; World Health Organization, WHO 1997; Wegener *et al*. 1998; Wegener *et al*. 1999a; Turnidge *et al*. 1999; Aarestrup 1999; Aarestrup, Bager and Anderson 2000; Wray and Davies 2000; Turnidge 2004).

Nurmi concept or the competitive exclusion (CE) concept opened a new, promising approach to the control of *Salmonella* in poultry (Nurmi, Nuotio and Schneitz 1992), and had been confirmed by a number of studies in various countries (Oyofo *et al*. 1989; Baba *et al*. 1991; Bailey, Blankenship and Cox 1991; Nisbet *et al*. 1993; Terada *et al*. 1994; Orban *et al*. 1996; Patterson *et al*. 1996; Fernandez, Hinton and van Gils 2000; Henriksson and Conway 2001; Fernandez, Hinton and van Gils 2002; Kleessen *et al*. 2003).

The prebiotic concept has been supported by evidence that many oligosaccharides, when fed to animals or humans can reach the colon undegraded and provide a carbohydrate substrate particularly suited to the growth of bifidobacteria (Playne and Crittenden 1996; Tomomatsu 1994) and some lactic acid bacteria (Flickinger *et al*. 2000). Bifidobacteria and lactic acid bacteria are beneficial microorganisms, thought to create conditions unfavorable to the growth of pathogens, such as *Salmonella* (Isolauri *et al*. 2001). Consequently, an increase in growth of *Bifidobacterium* and lactic acid bacteria with a decrease of *Salmonella* would appear to be of benefit to the host animal.

From the study by Chung and Day (2002), isomaltooligosaccharide is utilized preferentially by probiotic strains (*Bifidobacterium bifidum*, *B. longum* and *Lactobacillus johnsonii*). The use of oligosaccharides as prebiotics should lead to the production of intestinal lactic acid, an increase in short-chain fatty acid production and lowering of pH in the large intestine (Chung and Day 2002). Thereby being a useful feed additive to help prevent colonization by pathogenic bacteria. Ultimately it is hoped that the addition of IMO will lead to microbiological control and improve bird performances, allowing for more consistent production responses in the absence of antibiotics.

Materials and Methods

Experimental design. Completely randomized block design was used as an experimental design utilizing chickens and time difference as a blocking factor. Treatments were four levels of IMO (0%, 1%, 2%. and 4% w/v) with 3 replications. Experimental units were 12 groups of 3 chicks.

Experimental birds. Forty, one-day-old broiler chicks (*Gallus domesticus*) were obtained from a local commercial hatchery (Gold Kist Hatchery, Commerce, GA). The chicks were randomly allocated to 4 isolator units (Controlled Isolation Systems Inc., San Diego, California, measuring 0.3 m x 0.48 m, containing nipple drinkers, pan feeder and a filtered negative pressure ventilation system; Fig. 4) containing 10 birds per units and each unit was randomly assigned one of four dietary treatments; control (0% IMO), 1% IMO, 2% IMO, and 4% IMO of the diet (w/v). All chicks were placed at room temperature maintained at approximately 35 ^{o}C for the first week and at approximately 32 ^{o}C thereafter.

Water and assigned feed were provided *ad libitum* for the duration of the experiment. Feed consists of a commercial unmedicated diet (Feed mill at Department of Poultry Science,

The University of Georgia, Athens, Georgia) based on corn and soybean meal (crude protein 22.5%, crude fat 5.28%, crude fiber 2.53, calcium 0.95%, available phosphorus 0.45% and estimated energy 3080 kcal/kg) that contains or exceeds levels of critical nutrients as recommended by the National Research Council (1984).

Chicks were inoculated orally, through a gavage needle (Animal Feeding Needles, Popper and Sons Inc., New Hyde Park, New York; 1 ml Syringe, size 0.4 mm x 13 mm, Becton Dickinson and Co., Franklin Lakes, New Jersey) inserted approximately 2.5 cm into the esophagus, with 0.25 ml (containing 10^8 cells) of a culture of *Salmonella enterica* ser. Typhimurium nalidixic acid strain (*S.* ser. Typhimurium Nalr) after seven days of placement. Twenty one days after placement, chicks were euthanized by cervical dislocation.

The experiment was done in 3 replications. All animal care procedures were done in compliance with the local animal care and use committee.

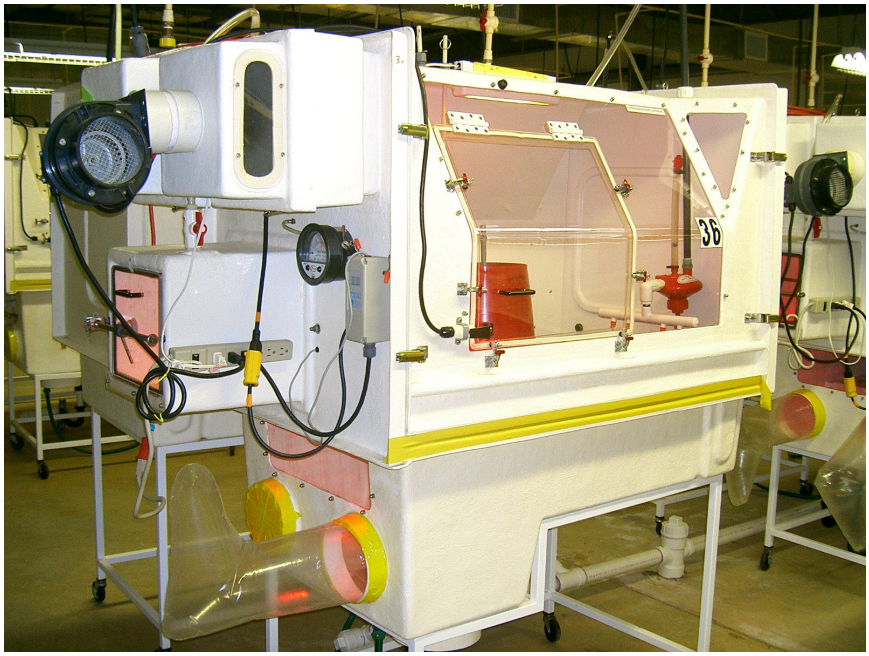

Figure 4. Isolator units (Controlled Isolation Systems Inc., San Diego, California) used in the experiment (image by Thitaram, S.N.)

Weight determination. Body weights of chickens in all groups were measured on day 1 and day 21 to ascertain if the addition of IMO to the diet had any effect on body weight gain. The mean body weight per treatment group was calculated from thirty pooled samples per group.

Feed conversion and feed efficiency determination. All feed and bird body weight from all groups were monitored through all the experiment to ascertain if the addition of IMO to the diet had any effect on feed conversion and feed efficiency. The mean feed conversion and feed efficiency per treatment group was calculated from thirty pooled samples per group.

***Salmonella enterica* ser. Typhimurium Nal^r.** A culture of 200 µg/ml nalidixic acid *S.* ser. Typhimurium resistant strain was obtained from Agricultural Research Service, Russell Research Center; Athens, Georgia. Inocula for infectious challenge exposure was prepared from brilliant green sulfa agar (BGS; Difco, Detroit, Michigan) supplemented with 200 µg/ml of nalidixic acid. Cultures were incubated at 37 ^oC for 24 hr. The culture was diluted in sterile isotonic saline solution to obtain challenge inoculate containing $1x10^8$ *S.* ser. Typhimurium Nalr per 0.25 ml.

Microbiological media. The Transoligosaccharide propionate agar medium (TOS; Yakult Pharmaceutical, Japan) supplemented with glacial acetic acid; 1% v/v (TOS-A) was used as the enumerating media for *Bifidobacterium*. Samples for bifidobacteria analysis were first diluted in pre-reduced Wilkins-Chalgren broth (WC; Oxoid, Hampshire, England). Trypticase phytone yeast extract broth (TPY, Scardovi 1986) was used to culture presumptive *Bifidobacterium* subsequent to isolation from TOS-A, for fructose-6- phosphate phosphoketolase (F6PPK) assay and maintaining the *Bifidobacterium* cultures. BGS (Difco, Detroit, Michigan) supplemented with 200 µg/ml of nalidixic acid (BGS+Nal) was used to enumerate *S.* ser. Typhimurium Nalr. Modified lysine iron agar (MLIA; Oxoid, Hampshire, England), buffered

peptone water (BPW; Difco, Detroit, Michigan), Rappaport-Vassiliadis R10 broth (RV; Difco, Detroit, Michigan), tetrathionate broth (TT; Difco, Detroit, Michigan), triple sugar iron agar (TSI; Difco, Detroit, Michigan), and lysine iron agar (LIA; Difco, Detroit, Michigan) slants were used as a confirmational media for *Salmonella*. Reinforced clostridial agar (RCA; Oxoid, Hampshire, England) and Lactobacillus MRS agar (MRS; Oxoid, Hampshire, England) were used to enumerate total anaerobic bacteria and total lactic acid bacteria, respectively.

All media were autoclaved at 121 ^{o}C for 15 min prior to use and used within 2 weeks.

Cecal bacterial population determination. Randomly three birds were picked from each isolator unit and chickens were euthanized by cervical dislocation. Ceca from individual birds was removed, weighed and kept under anaerobic condition in an anaerobic jar (Anaerobic Plus System, Oxoid, Hampshire, England) equipped with AnaeroGen sachets (Oxoid, Hampshire, England) until analyses. Immediately, each cecal content was blended in WC (1:9, w/v) and further serial dilutions were made in the same media for enumeration. A 100 µl aliquot of appropriate dilutions were spread plated on the TOS-A, BGS+Nal, RCA and MRS agar. All steps were performed under anaerobic condition (except for *Salmonella*) in an anaerobic chamber (Bactron Anaerobic, Model BacII, Sheldon Manufacturing; 5% hydrogen, 5% CO_2, balanced nitrogen). All media (except for BGS+Nal) were incubated anaerobically in anaerobic jars (Anaerobic Plus System, Oxoid, Hampshire, England) at 37 ^{o}C for 72 hr, 48 hr, and 24 hr for *Bifidobacterium*, total lactic acid bacteria-total anaerobic bacteria, and *S. ser. Typhimurium Nalr*, respectively. Anaerobic jars were equipped with AnaeroGen sachets (Oxoid, Hampshire, England) to create anaerobic condition. All bacterial population counts were calculated as colony forming units (CFU) per g wet weight of ceca content. Bacterial populations were enumerated in duplicate at two different dilutions.

pH determination of cecal content. Immediately after cecas were removed, each cecal content was placed in individual 50 ml plastic centrifuge tubes. Twenty ml of sterile, distilled water were added to each container, and the contents were vortexed thoroughly (Vortex Genie 2, Bohemia, New York). The pH of each suspension was measured electronically (Corning pH meter 430, Corning, New York) (The technique was adapted from Hinton, Buhr and Ingram 2000).

Confirmation of bifidobacteria. For each sample, the colonies were identified as members of the genus *Bifidobacterium* by the following criteria: (1) they were gram positive, pleomorphic rods with characteristic bifurcated *Bifidobacterium* cell morphology, (2) they were unable to grow under aerobic conditions, (3) they were catalase-negative, (4) they showed fructose-6-phosphate phosphoketolase (F6PPK) activity, and (5) they were positive in the *Bifidobacterium* genus specific fluorescence *in-situ* hybridization (FISH) assay.

Fructose-6-phosphate phosphoketolase (F6PPK) assay. Presumptive bifidobacteria were cultured anaerobically in TPY at 37 ^{o}C for 72 hr prior to the assay. The procedure was as followed:

Reagents: 1) 0.05 M phosphate buffer pH 6.5 plus cysteine 500 mg/liter; 2) a solution containing NaF, 6 mg/ml, and K or Na iodoacetate, 10 mg/ml; 3) hydroxylamine HCl, 13.9 g/100 ml of water, freshly neutralized with NaOH to pH 6.5; 4) trichloroacetic acid (TCA), 15% (w/v) in water; 5) 4 M HCl; 6) $FeCl_3 \cdot 6H_2O$ 5% (w/v) in 0.1 M HCl; 7) fructose-6-phosphate (Na salt; 70% purity), 80 mg/ml in water (Scardovi 1986).

Procedure: Cells harvested from 10 ml TPY broth were washed twice with buffer (1) and resuspended in 2.0 ml of the same buffer. The cells were disrupted by sonication (Microtip Sonicator, Model W-370, Heat Systems-Ultrasonics, Inc., Plainview, New York) for 2 min in the

cold, and 0.25 ml each of reagents (2) and (7) were added to the sonicate. After 30 min incubation at 37 ^{o}C (Thermolyne type 16500 Dri-Bath, Dubuque, Iowa), the reaction was stopped with 1.5 ml of reagent (3). After 10 min at room temperature, 1.0 ml each of reagents (4) and (5) were added. Tubes were inverted to mix contents. Any reddish-violet color that developed was immediately recorded as a positive result (Scardovi 1986).

Fluorescence *in-situ* Hybridization (FISH) assay. The *Bifidobacterium* specific FISH assays were performed using the Microscopic Enumeration FISH Kit (FITC or Cy3 labeled, RiboTechnologies, The Netherlands). The procedure was as followed: Buffers were prepared; pre-warmed the MCW5-buffer and the hybridization mixture at 45 ^{o}C in a water bath (VWR brand Model1235, Sheldon Manufacturing Inc., Cornelius, Oregon) to ensure complete dissolution of components. The MCW1-buffer was prepared by dissolving the MCW5-buffer 5 times with sterile water and pre-warming the solution at 45 ^{o}C. Five ml of MCPX1-buffer was prepared by mixing 0.5 ml of MCPX10-buffer and 4.5 ml sterile water. The sample was prepared by suspending 0.5 g of cecal material in 4.5 ml of MCPX1-buffer and approximately 10 sterile glass beads were added (diameter = 3 mm) and vortexed at highest speed (10) for 1-2 min (Vortex Genie 2, Bohemia, New York). The suspension was centrifuged for 1 min at 80 x g to remove debris (the supernatant contains the bacteria). One hundred µl of the supernatant was transferred into a clean centrifuge tube, 10 µl of Fixative solution was added and incubated for 1 hr at 4 ^{o}C. After fixation, collected and washed the cells twice by centrifugation at 15,800xg for 5 min (Microcentrifuge 1236V, National Labnet Co., Edison, New Jersey). The cells were permeabilized for 1 hr at -20 ^{o}C by resuspending the pellet into 100 µl of Solution A. Ten µl of the permeabilized cells was mixed with 100 µl of hybridization mixture. Then the cells were hybridized in the killing block overnight at 45 ^{o}C in the dark (Dri-Bath Thermolyne, Model

D816525, Dubuque, Iowa). After hybridization, the cells were washed in pre-warmed MCW1-buffer for 20 min at 45 ^{o}C. The hybridized cells were washed and filtered onto a 0.22 µm diameter polycarbonate filter. The filter was placed on a glass slide and overlaid with a drop of mounting fluid before it was observed using fluorescence microscopy.

Confirmation of *S.* ser. Typhimurium Nalr. Negative samples from BGS+Nal media (no viable count on BGS+Nal) were pre-enriched, from original dilution, overnight aerobically at 37 ^{o}C. The 0.5 ml of pre-enriched samples were transferred to 10 ml TT (Difco, Detroit, Michigan) and 0.1 ml to 10 ml RV (Difco, Detroit, Michigan), and incubated aerobically at 37 ^{o}C for 24 hr. Then, one loopful each of TT and RV was streaked on MLIA, BGS, and BGS+Nal incubated aerobically at 37 ^{o}C for 24 hr. After incubation, a well-isolated purple colony with or without black center was picked from MLIA agar; a well-isolated opaque colony with a smooth appearance and entire edge surrounded by a red color in the medium was picked from BGS+Nal, TSI and LIA slants were inoculated with the isolate by stabbing the agar butts and streaking the slants. Following the incubation aerobically at 37 ^{o}C overnight, the results were determined.

Statistical analysis. The data obtained from this study were analyzed as a randomized complete block design. All data were analyzed using SAS, PROC GLM with lsmeans procedure (Version 8.0, SAS Institute 2001). Bacterial populations were analyzed after $\log_{10}$ transformation. Differences in the mean were compared by using the Student *t* Test (Least Significant Difference; LSD). All statements of statistical significance were based on $P < 0.05$.

Results and Discussion

Feed conversion, feed efficiency and body weight gain. In this study, body weight gain, feed consumed, feed conversion and feed efficiency were similar for birds fed treated IMO diet compared to the control diet (Table 10). IMO did not have any significant improvement on

bird body weight gain when compared to control diet (Table 10). Birds that were fed 4% IMO diet were found to have the greatest weight gain but the difference was not significant. However, the result showed significant reduction of weight in birds fed 1% IMO diet. The results of this study are similar to Oyofo *et al*. (1989), Patterson *et al*. (1997) and Iji, Saki and Tivey (2001). Oyofo *et al*. (1989) found no significant difference on weight gain between chickens fed several types of carbohydrates (dextrose, lactose, sucrose, mannose, and maltose; all at 2.5%) when compared to control group in a 10 days trial period. Patterson *et al*. (1997) found that weight gain, feed consumption, feed efficiency, and feed digestibility were similar for 4-week-old birds fed thermally produced kestoses (at 2%) compared to control group and birds fed other sugars (8% sucrose and 8%glucose). Iji, Saki and Tivey (2001) found no significant effects of mannanoligosaccharide (at 0, 1.0, 3.0, and 5.0 g/kg diet) on feed consumption and weight gain when fed to broiler chickens in a 28 days trial period. It may be suggested that undetermined factors such as stress, temperature, animal health, and others may influence the efficacy of IMO on broiler chicken performance. High environmental temperature is known to cause reduction in feed consumption and body weight of broilers (Orban and Roland 1992). Waldroup *et al*. (1993) found body weight gain from birds fed fructooligosaccharides (FOS) but the differences were not significant. However they found significant body weight gain from birds fed fructooligosaccharides (FOS) together with bacitracin methylene disalicylate (BMD). The chickens of this study were raised at 32 ^{o}C, whereas chickens in the study by Waldroup *et al*. (1993) were raised at a lower temperature (32.2 ^{o}C for the first week and reduced 2.8 ^{o}C /wk to 21.1 ^{o}C).

Cecal pH and cecal weight. There was no significant difference between cecal pH observed among the treated diets when compared to the control diet (Table 11). This finding is

similar to Chambers, Spender and Modler (1997) who studied the effect of FOS and lactose derivatives (LD) on *S.* ser. Typhimurium colonization, pH, and density of broiler ceca. Chambers, Spender and Modler (1997) found that mean cecal pH of broiler fed FOS and LD were significantly lower than those of control broiler at 5 and 6 week of age but there was no significant difference among treatment groups in broiler at 3 week of age. This may suggest that the changes of cecal pH level are influenced by bird age.

While, the cecal weights of all groups were similar, the chickens fed with 1% IMO diet were found to have the largest ceca (Table 11) albeit this was no significant difference when compared to control diet.

Total anaerobic bacteria population. There were no significant changes in the number of total anaerobic bacteria for all treatment group compared to control group (Table 12).

Lactic acid bacteria and *Bifidobacterium* population. All the treated IMO diets significantly showed an increase number of bifidobacteria compared to the control diet (Table 12). However, there was no significant difference among the treatment groups (1, 2, and 4% IMO diets). The diet supplemented with 1% and 2% IMO had increases in the number of lactic acid bacteria when compared to the control diet (Table 12). However, the differences were not significant. The increase in bifidobacterial numbers was similar to that of other oligosaccharide products. For example, Patterson *et al*. (1997) studied the effects of thermally produced kestoses on broiler performance and selective enrichment of bifidobacteria in the intestinal tract of 4-week old broilers. They found that dietary administration of thermally produced kestoses at 2% of the diet significantly increased the number of bifidobacteria and lactobacilli in the chicken cecas. They found a smaller increase of *Lactobacillus acidophilus* and *L. salivarius* number when compared to bifidobacteria numbers (bifidobacteria; control=8.98, kestoses=10.36;

lactobacilli; control=9.56, kestoses=10.43; numbers were expressed as $\log_{10}$ CFU/g cecal contents wet weight). Orban *et al*. (1997) found a significant increase in the number of bifidobacteria in the cecas of broilers fed diet supplemented with 7.5% Sucrose Thermal Oligosaccharide Caramel (STOC) in a 4-week trial period. The study by Rada *et al*. (2001) found a highly significant one log increase number of bifidobacteria ($P<0.01$) in inulin-treated diet (at 5%) in one-week-old laying hens.

Cecal colonization by *S.* ser. Typhimurium Nalr. Chickens treated with 1% IMO diet had a significant two log reduction in the level of *S. ser. Typhimurium Nalr* present in the ceca compared to the control group (Table 12). However, the higher percentage of IMO did not show a reduction in the colonization by *S.* ser. Typhimurium Nalr in the chicken gut. Reduction of *Salmonella* colonization in the chicken provided IMO diet was associated with the higher number of *Bifidobacterium* and lactic acid bacteria presented in the cecas. Bailey *et al*. (1991) found that FOS had minimal effect on *Salmonella* colonization in 2-week-old broiler chicks when fed a supplemented FOS diet at 0.375% and 0.75%. Fernandez, Hinton and van Gils (2002) studied the effects of mash diet, or mash supplemented with either 2.5% mannose oligosaccharide (MOS) or palm kernel meal (PKM), on the microflora of the hen cecal contents for a 4-week trial period. They found the diet supplemented with MOS and PKM affected the bird intestinal microflora by increasing the number of *Bifidobacterium* spp. and *Lactobacillus* spp., while decreasing the *Enterobacteriaceae* groups. The diets also found to reduce the susceptibility in 4-week-old hens to colonization by *S*. ser. Enteritidis.

As oligosaccharides and related carbohydrate are neither degraded nor hydrolyzed in the upper intestinal tract of animals, and hence reach the ceca (Hidaka *et al*. 1986; Oku 1986). Because of its low digestion and absorption, IMO passes into the lower portions of the intestine

and ceca and is subsequently available for bifidobacteria and lactic acid bacteria to utilize as a growth substrate. The importance of normal intestinal flora in the gastrointestinal tract (GIT) in reducing pathogen colonization has been documented in animals (Mulder, Havenaar and Huis in't Veld 1997; Bezkorovainy 2001; Ishibashi and Yamazaki 2001; Isolauri *et al*. 2001; Marteau *et al*. 2001; Gong *et al*. 2002) and by the Nurmi concept of CE in poultry (Oyofo *et al*. 1989; Baba *et al*. 1991; Bailey, Blankenship and Cox 1991; Nisbet *et al*. 1993; Terada *et al*. 1994; Orban *et al*. 1997; Patterson *et al*. 1997; Fernandez, Hinton and van Gils 2000; Henriksson and Conway 2001; Fernandez, Hinton and van Gils 2002; Kleessen *et al*. 2003). However, the mechanisms by which probiotic strains prevent colonization by enteropathogens are not known (Chung 2002). The CE are considered to exert their effect by one or more of four general principle actions, namely the creation of a restrictive physiological environment, competition for bacterial receptor sites, and elaboration of antibiotic-like substances (for example, bacteriocins) and/or depletion of essential substrates (Fuller and Turvey 1971; Schneitz and Mead 2000). In the normal intestinal microflora, Mead (2000) described that volatile fatty acids (VFA) are produced mainly as a result of the metabolism of sporulating and non- sporulating anaerobic bacteria. The VFA can be inhibitory to other organisms present; especially in the undissociated state below pH 6.0. VFA that are inhibitory to salmonellas include acetic, propionic and lactic acid to suppress the salmonellas. The precise mechanism of the protective effect is unknown, and may never be determined because of the complexity of the gut as a habitat for microorganisms and the variety of host-microbe and microbe-microbe interactions that can occur (Rolfe 1991).

When chicks are colonized with salmonellae, the cecum is the area of the intestine most likely to be colonized (Fanelli *et al*. 1971). It has been reported that bifidobacteria were present in poultry ceca at 10^9-10^{10} cfu/g of content (Mead 1997). Therefore, sampling ceca maximizes

the chances of detecting even low levels of colonized *Salmonella* and recovering *Bifidobacterium* population. Most salmonellae do not infect or cause pathogenesis in chickens. Rather, they have a commensal relationship in the gut of the chick where the salmonellae colonize and multiply with little or on apparent effects to the host chicks (Bailey *et al*. 1988).

Even though the higher percentage of GOS did not show a greater effect in reducing the colonization by *S.* ser. Typhimurium Nalr in the chicken gut, it may be suggested that feeding the optimum level of GOS (1%) in the diet of chickens may reduce *Salmonella* colonization in young chicken.

Table 10. Effect of isomaltooligosaccharide (IMO) supplementation on broiler performance.

Parameter	Treatment*			
	Control	1% IMO	2% IMO	4% IMO
Body weight gain (kg)‡	6.85 ± 0.36[a]	6.17 ± 0.26[b]	6.51 ± 0.26[ab]	6.91 ± 0.27[a]
Feed consumption (kg)‡	10.95 ± 1.58[a]	9.32 ± 0.96[a]	9.98 ± 0.53[a]	11.36 ± 1.90[a]
Feed conversion (kg:kg)‡	1.60 ± 0.22[a]	1.51 ± 0.09[a]	1.53 ± 0.12[a]	1.65 ± 0.22[a]
Feed efficiency (kg:kg)‡	0.63 ± 0.08[a]	0.67 ± 0.04[a]	0.65 ± 0.05[a]	0.62 ± 0.08[a]

*Isomaltooligosaccharide was added to the diet at 1%(v/w), 2%(v/w), and 4%(v/w), respectively.

‡Mean ± SD of 30 chicks. Values within a row followed by different lower-case superscripts differ significantly ($P < 0.05$).

Table 11. Cecal pH and cecal weight of 3-week-old broiler chicks at different percentage of IMO.

Treatment*	Cecal pH‡	Cecal weight‡ (g)
Control diet	6.09 ± 0.39	11.24 ± 2.61
1% IMO	6.09 ± 0.65	11.28 ± 3.15
2% IMO	6.24 ± 0.56	11.20 ± 3.29
4% IMO	6.07 ± 0.43	10.74 ± 3.95

*Isomaltooligosaccharide was added to the diet at 1%(v/w), 2%(v/w), and 4%(v/w), respectively.

‡Mean ± SD of 9 chicks. Values within a column are not significant difference ($P < 0.05$).

Table 12. Bacterial population of the cecal microflora in 3-week-old broiler chicks at different percentage of isomaltooligosaccharide (IMO).

	Bacteria (log CFU g-1 ceca wet weight)*			
Treatment‡	*S.* ser. Typhimurium Nalr §	Bifidobacteria	Total anaerobic bacteria	Total Lactic Acid Bacteria
Control diet	7.19 ± 2.27[a]	9.07 ± 0.69[a]	10.40 ± 0.86[a]	9.88 ± 1.43[ab]
1% IMO	5.42 ± 1.93[b]	10.09 ± 1.54[b]	10.66 ± 0.73[a]	10.28 ± 1.31[a]
2% IMO	7.61 ± 2.28[a]	10.04 ± 1.46[b]	10.58 ± 1.02[a]	10.05 ± 1.50[ab]
4% IMO	6.74 ± 1.47[a]	9.98 ± 1.21[b]	10.81 ± 0.71[a]	9.67 ± 1.67[b]

*Mean ± SD of 9 chicks. Values within a column followed by different lower-case superscripts differ significantly ($P < 0.05$).

‡ Isomaltooligosaccharide was added to the diet at 1%(v/w), 2%(v/w), and 4%(v/w), respectively.

§ *Salmonella enterica* ser. Typhimurium nalixidic acid resistant strain (200 μg/ml).

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CHAPTER 2

INCORPORATION OF MUPIROCIN INTO TOS SELECTIVE AGAR ENHANCES ENUMERATION OF *BIFIDOBACTERIUM* SPP. FROM CHICKEN CECA SAMPLES[1]

[1]Thitaram, S. N. and G. R. Siragusa. To be submitted to Letters in Applied Microbiology.

Abstract

Chicken ceca contain a large and diverse population of bacteria. Bifidobacteria has been considered to promote health effect to the host. Transoligosaccharide propionate agar medium (TOS) was improved by addition of mupirocin (0.1 mg/ml) and glacial acetic acid (1% v/v). Four media were evaluated to determine their efficiency and selectivity for bifidobacteria in chicken cecal sample; TOS without modification (control), TOS-A supplemented with glacial acetic acid (1% v/v), TOS-M supplemented with mupirocin (0.1 mg/ml), and TOS-AM supplemented with glacial acetic acid (1% v/v) and mupirocin (0.1mg/ml). The presumptive colonies of bifidobacteria were screened for the presence of fructose-6-phosphate phosphoketolase activity. The addition of mupirocin and acetic acid did not inhibit the growth of bifidobacteria compared to the control media (7.74; 7.57; 7.66; 7.56 for TOS, TOS-A, TOS-M, and TOS-AM, respectively; log CFU/ml). Colony size of bifidobacteria was larger (>1 mm in diameter) on all supplemented media compared to colonies on the control media (TOS; < 0.5 mm in diameter). TOS-M and TOS-AM inhibited the growth of *Lactobacillus acidophilus*, *L. gallinarum*, *L. helveticus*, and *Streptococcus gordonii*. TOS-AM was found to have the most bifidobacteria selectivity among media tested (61.0%). However the number of bifidobacteria recovered from chicken cecal samples enumerated on TOS-M and TOS-AM was significantly lower ($P<0.05$) than those of media without mupirocin supplementation (TOS and TOS-A). TOS-A had more bifidobacteria selectivity compared to the control media and without reducing bifidobacteria population from chicken cecal samples compared to TOS with mupirocin supplementation. TOS-A has a simple preparation and could be potentially used for isolation and enumeration of bifidobacteria from chicken cecal samples.

Keywords: *Bifidobacterium*, chicken ceca, selective media

Introduction

The microflora of the chicken's gastrointestinal tract (GIT) has received increased attention as the focus of efforts to minimize foodborne illness in humans and to improve animal nutrition (Mulder, Havenaar, and Huis in't Veld 1997; Bezkorovainy 2001; Ishibashi and Yamazaki 2001; Isolauri *et al*. 2001; Marteau *et al*. 2001; Gong *et al*. 2002; Kleessen *et al*. 2003). In poultry ceca, bifidobacteria are present at 10^9-10^{10} CFU/g of content (Rada and Petr 2000). Bifidobacteria are considered beneficial microorganisms and thought to create conditions unfavorable to the growth of pathogens, such as *Salmonella* (Isolauri *et al*. 2001).

Differentiation of bifidobacteria from lactobacilli is difficult in the routine laboratory because cultural and biochemical properties of both genera overlapped. Also bifidobacteria are strictly anaerobes. Many of media have been devised for isolating or enumeration bifidobacteria in natural habitats (Tanaka and Mutai 1980; Munoa and Pares 1988; Hartemink *et al*. 1996; Nebra and Blanch 1999; Arroyo, Cotton and Martin 1995; Martineau 1999; Rada, Sirotek and Petr 1999; Rada and Petr 2000). The key to developing the media are using antibiotics (Lim, Huh, and Baek 1993) or lowering pH of media (Beerens 1990; Payne, Morris and Beers 1999) to inhibit the growth of non-bifidobacteria.

Rada (1997) reported that bifidobacteria were resistant to inhibition by the antibiotic mupirocin which may serve as a simple criterion distinguishing *Bifidobacterium* spp. from *Lactobacillus* spp. (Rada 1997; Rada and Petr 2000). This same agent inhibits several other genera of gram positive bacteria including other closely related lactic acid bacteria commonly found in the poultry ceca. While it was reported that mupirocin could be extracted from antimicrobial discs (200 µg, Oxoid) (Rada and Petr 2000), the inconsistent extraction efficiency of that process, as well as, lack of availability of mupirocin antimicrobial discs in the

United States led us to the use of 2% mupirocin (in a polyethylene glycol ointment base; TEVA Pharmaceuticals, Sellersville, PA) as a source of the compound for this experiment.

Transoligosaccharide propionate agar medium (TOS) (Yakult Pharmaceutical, Japan) uses galactooligosaccharides as a bifidobacteria carbon source. This commercially available medium is easy to prepare and stable.

This study was conducted to evaluate the selectivity of mupirocin on TOS media for enumeration of *Bifidobacterium* from chicken cecal samples.

Materials and Methods

Selective media, cultural media and diluents. The TOS agar medium (Yakult Pharmaceutical, Tokyo, Japan) has the following composition (g/l or ml/l): trypticase peptone 10.0; yeast extract 1.0; KH_2PO_4 3.0; K_2HPO_4 4.8; $(NH_4)_2SO_4$ 3.0; $MgSO_4 \cdot 7H_2O$ 0.2; L-cysteine $HCl \cdot H_2O$ 0.5; sodium propionate 15; galactooligosaccharides 10; agar 15; and distilled water to 1 l; pH 6.3 $\pm$. TOS-A was supplemented with glacial acetic acid (1% v/v), TOS-M was supplemented with mupirocin (0.1 mg/ml), and TOS-AM was supplemented with glacial acetic acid (1% v/v) and mupirocin (0.1 mg/ml).

Wilkins-Chalgren broth (WC; Oxoid) was used to prepare sample dilutions has the following composition (g/l): tryptone 10; gelatin peptone 10; yeast extract 5; glucose 1; NaCl 5; L-Arginine 1; sodium pyruvate 1; menadione 0.0005; and haemin 0.005; and distilled water to 1 l; pH 7.1 $\pm$ 0.2.

Trypticase phytone yeast extract broth (TPY, Scardovi 1986) was used to culture presumptive bifidobacteria for the fructose-6-phosphate phosphoketolase (F6PPK) assay and maintaining the cultures, has the following composition (g/l or ml/l): trypticase (BBL) 10; phytone (BBL) 5; glucose 5; yeast extract (Difco) 2.5; Tween 80 1; cysteine hydrochloride 0.5;

K_2HPO_4 2; $MgC_{l2} \cdot 6H_2O$ 0.5; $ZnSO_4 \cdot 7H_2O$ 0.25 $CaCl_2$ 0.15; $FeCl_3$ a trace; and distilled water to 1 l; pH 6.5.

All media were autoclaved at 121 ^oC for 15 min and used within 2 weeks.

Microbiological assay. *Bifidobacterium longum* ATCC 15708 was kindly provided by Dr. Donal F. Day and Dr. Chang-Ho Chung (Louisiana State University, Baton Rouge, Louisiana). All other strains (Table 13) were isolated from chicken cecal samples. Twelve chickens were killed by cervical dislocation, cecas were collected and kept under anaerobic condition in an anaerobic jar (Anaerobic Plus System, Oxoid) which were equipped with AnaeroGen sachets (Oxoid) before analyzing. Immediately, each cecal content was blended in WC broth (1:9, w/v) and serially diluted in the same medium. Appropriate 100 µl of dilutions were spread plated on the various selective media in duplicate. All steps were performed under anaerobic condition using anaerobic chamber (Bactron Anaerobic, Model BacII, Sheldon Manufacturing Inc. under an atmosphere of 5% hydrogen, 5% CO_2 and balanced nitrogen). All media were incubated anaerobically in anaerobic jars (Anaerobic Plus System, Oxoid) at 37 ^oC for 72 hr. Anaerobic jars were equipped with AnaeroGen sachets (Oxoid) to create anaerobic condition. Bifidobacteria strains were handled the same as chicken cecal samples after cultured anaerobically in TPY broth at 37 ^oC for 72 hr.

Four strains of lactic acid bacteria (*Lactobacillus acidophilus, L. gallinarum, L. helveticus* and *Streptococcus gordonii*) were streaked on four types of TOS media, to evaluate the resistance of mupirocin. All lactic acid bacteria were isolated from chicken cecal samples and identified by fatty acid analysis (Hewlett-Packard 5890A gas chromatograph with a HP 5971A mass selective and set up with microbial identification software; MIDI, Inc.).

All bacterial population counts were calculated as colony forming unit (CFU) per gram wet weight for cecal samples or CFU/ml for bifidobacterial and lactic acid cultures.

Confirmation of bifidobacteria. For each sample, the colonies were identified as members of the genus *Bifidobacterium* by the following criteria: (1) they were gram positive, pleomorphic rods with characteristic bifidobacteria morphology, (2) they were unable to grow under aerobic conditions, (3) they were catalase-negative, (4) they showed F6PPK activity, and (5) they were positive for Fluorescence *in-situ* Hybridization (FISH) assay.

Fructose-6-Phosphate Phosphoketolase (F6PPK) assay. Presumptive bifidobacteria were cultured anaerobically in TPY at 37 ^{o}C for 72 hr prior to the assay. The procedure was as followed:

Reagents: 1) 0.05 M phosphate buffer pH 6.5 plus cysteine 500 mg/liter; 2) a solution containing NaF, 6 mg/ml, and K or Na iodoacetate, 10 mg/ml; 3) hydroxylamine HCl, 13.9 g/100 ml of water, freshly neutralized with NaOH to pH 6.5; 4) trichloroacetic acid (TCA), 15% (w/v) in water; 5) 4 M HCl; 6) $FeCl_3 \cdot 6H_2O$ 5% (w/v) in 0.1 M HCl; 7) fructose-6-phosphate (Na salt; 70% purity), 80 mg/ml in water (Scardovi 1986).

Procedure: Cells harvested from 10 ml TPY broth were washed twice with buffer (1) and resuspended in 2.0 ml of the same buffer. The cells were disrupted by sonication (Microtip Sonicator, Model W-370, Heat Systems-Ultrasonics, Inc., Plainview, New York) for 2 min in the cold, and 0.25 ml each of reagents (2) and (7) were added to the sonicate. After 30 min incubation at 37 ^{o}C (Thermolyne type 16500 Dri-Bath, Dubuque, Iowa), the reaction was stopped with 1.5 ml of reagent (3). After 10 min at room temperature, 1.0 ml each of reagents (4) and (5) were added. Tubes were inverted to mix contents. Any reddish-violet color that developed was immediately recorded as a positive result (Scardovi 1986).

Fluorescence *in-situ* Hybridization (FISH) assay. The *Bifidobacterium* specific FISH assays were performed using the Microscopic Enumeration FISH Kit (FITC or Cy3 labeled, RiboTechnologies, The Netherlands). The procedure was as followed: Buffers were prepared; pre-warmed the MCW5-buffer and the hybridization mixture at 45 ^{o}C in a water bath (VWR brand Model1235, Sheldon Manufacturing Inc., Cornelius, Oregon) to ensure complete dissolution of components. The MCW1-buffer was prepared by dissolving the MCW5-buffer 5 times with sterile water and pre-warming the solution at 45 ^{o}C. Five ml of MCPX1-buffer was prepared by mixing 0.5 ml of MCPX10-buffer and 4.5 ml sterile water. The sample was prepared by suspending 0.5 g of cecal material in 4.5 ml of MCPX1-buffer and approximately 10 sterile glass beads were added (diameter = 3 mm) and vortexed at highest speed (10) for 1-2 min (Vortex Genie 2, Bohemia, New York). The suspension was centrifuged for 1 min at 80 x g to remove debris (the supernatant contains the bacteria). One hundred µl of the supernatant was transferred into a clean centrifuge tube, 10 µl of Fixative solution was added and incubated for 1 hr at 4 ^{o}C. After fixation, collected and washed the cells twice by centrifugation at 15,800xg for 5 min (Microcentrifuge 1236V, National Labnet Co., Edison, New Jersey). The cells were permeabilized for 1 hr at -20 ^{o}C by resuspending the pellet into 100 µl of Solution A. Ten µl of the permeabilized cells was mixed with 100 µl of hybridization mixture. Then the cells were hybridized in the killing block overnight at 45 ^{o}C in the dark (Dri-Bath Thermolyne, Model D816525, Dubuque, Iowa). After hybridization, the cells were washed in pre-warmed MCW1-buffer for 20 min at 45 ^{o}C. The hybridized cells were washed and filtered onto a 0.22 µm diameter polycarbonate filter. The filter was placed on a glass slide and overlaid with a drop of mounting fluid before it was observed using fluorescence microscopy.

Statistical analysis. All bacterial counts were analyzed after $\log_{10}$ transformation. Data were analyzed using SAS, PROC GLM with lsmeans procedure (Version 8.0, SAS Institute 2001). Differences in the mean recoveries on the various media were compared by using the Student *t* Test. All statements of statistical significance are based on $p < 0.05$.

Result and Discussion

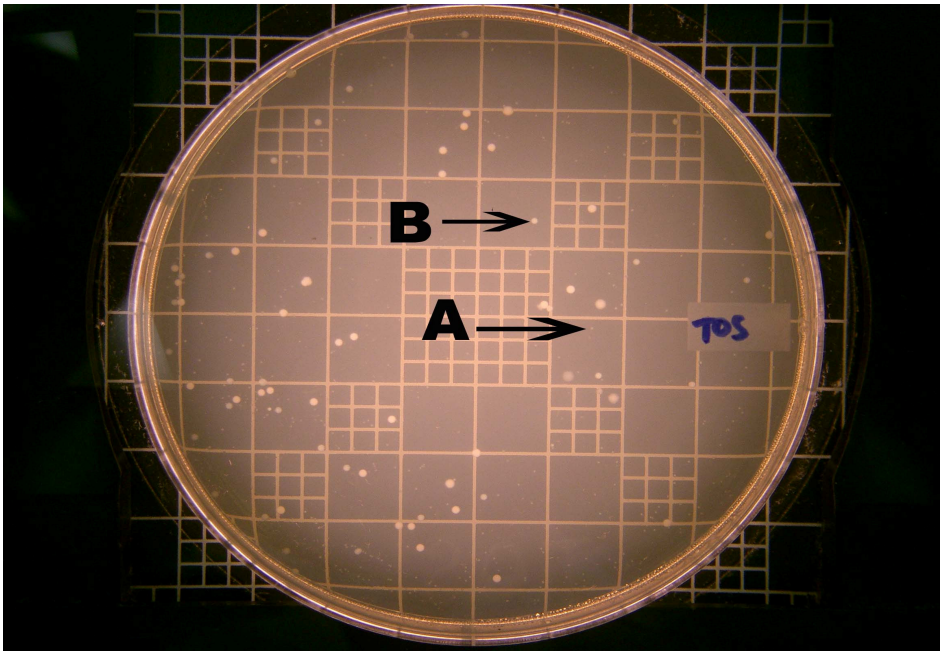
All types of TOS media tested were efficient (no significant difference among media tested, $P<0.05$) for enumerating pure cultures of bifidobacteria from chicken cecal samples (Table 13). All bifidobacteria strains isolated from chicken cecal sample were verified by F6PPK activity. Four strains of lactic acid bacteria (*Lactobacillus acidophilus, L. gallinarum, L. helveticus* and *Streptococcus gordonii*) did not grow on TOS media supplemented with mupirocin (Table 14). Consistent with other worker (Rada 1997; Rada and Petr 2000), our data indicated that the addition of acetic acid and mupirocin did not inhibit the growth of bifidobacteria but prevented lactobacilli from forming colonies. The colony size of bifidobacteria observed from TOS media supplemented with mupirocin (TOS-M and TOS-AM) were clearly larger than that of TOS media without mupirocin supplementation (Fig 5-8).

TOS-AM had the most selectivity among media tested (Table 15). The addition of mupirocin and acetic acid contribute to the inhibition of the growth of non-bifidobacteria (Table 14-15). However, there was no difference between the selectivity for bifidobacteria of TOS-M and TOS-AM. The bifidobacteria number observed from TOS-M and TOS-AM was significantly less than those of TOS and TOS-A (Table 16). It appeared that the growth of bifidobacteria was suppressed by the concentration of mupirocin. Lowering the concentration of mupirocin may allow for better proliferation of all *Bifidobacterium* species.

Collectively these data show that addition of mupirocin (0.1 mg/ml) and acetic acid (1% v/v) to TOS media enhances the selective enumeration of bifidobacteria from poultry cecal samples.

However the number of bifidobacteria recovered from chicken cecal samples enumerated on mupirocin supplemented media (TOS-M and TOS-AM) was significantly lower ($P<0.05$) than those of media without mupirocin supplementation (TOS and TOS-A). TOS-A had more bifidobacteria selectivity compared to the control media and without reducing bifidobacteria population from chicken cecal samples compared to TOS with mupirocin supplementation. TOS-A has a simple preparation and could be potentially used for isolation and enumeration of bifidobacteria from chicken cecal samples.

I



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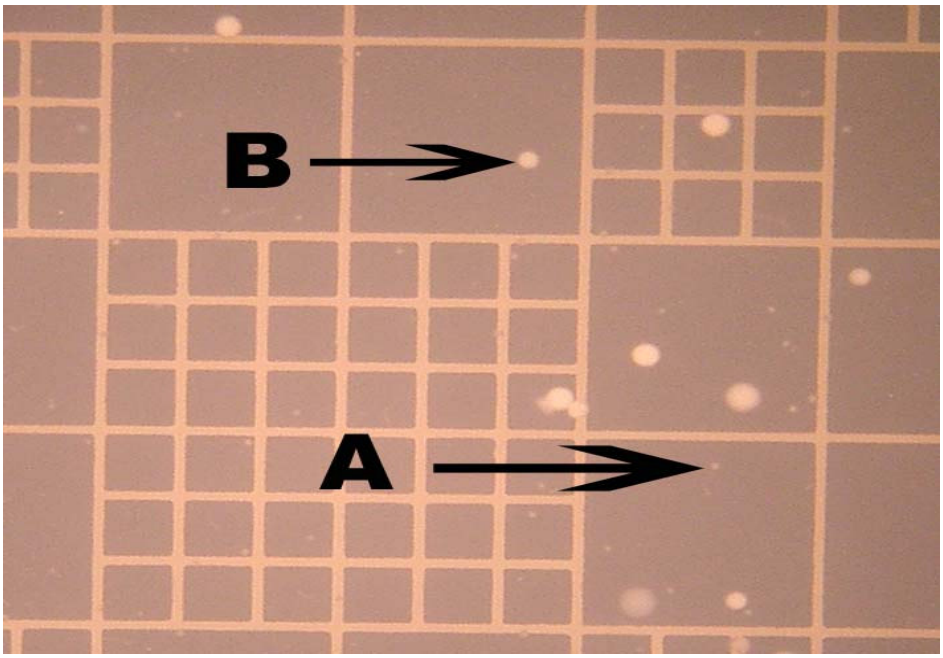


Figure 5. **I**- Cecal sample enumerated on TOS agar media; A indicates bifidobacteria colony; B indicates non-bifidobacteria colony. **II**- Displays enlarged area.

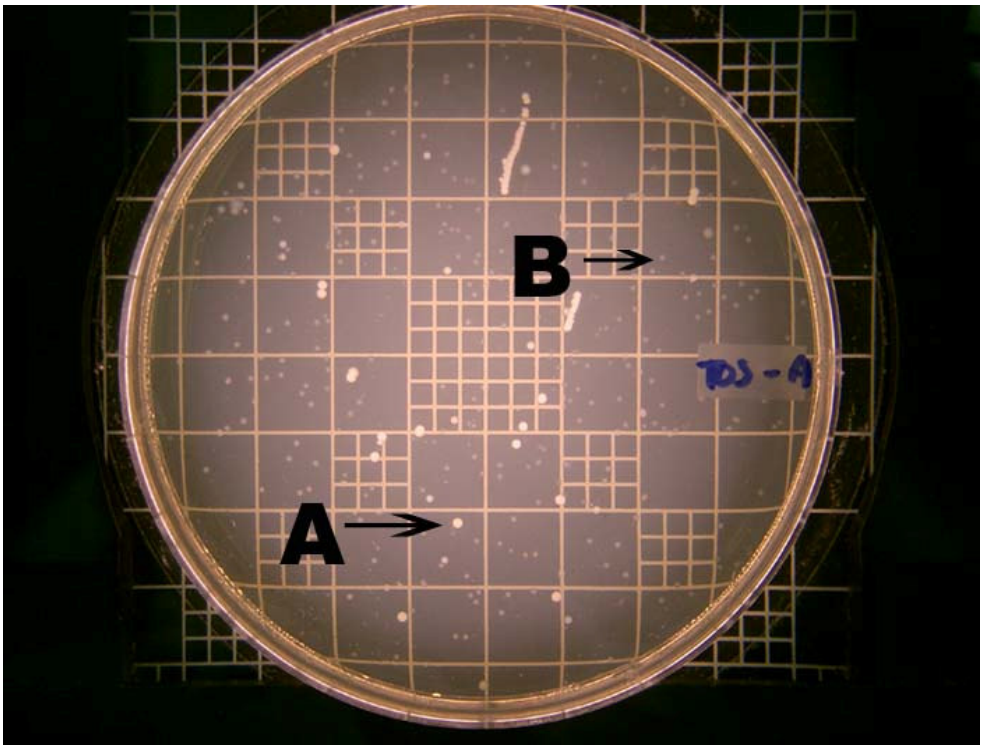
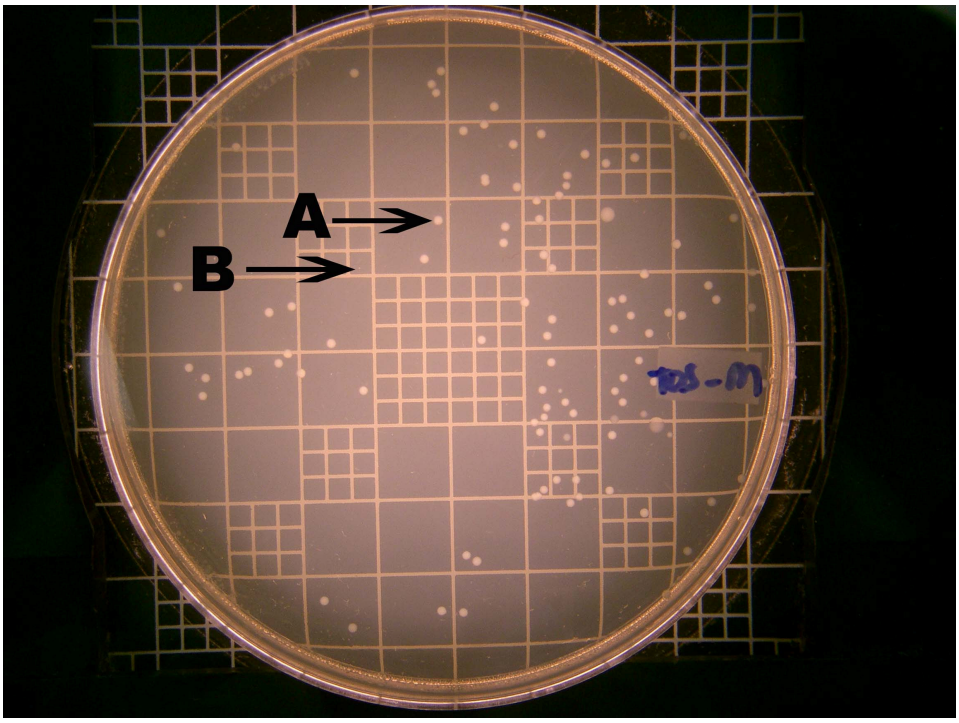


Figure 6. Cecal sample enumerated on TOS-A agar media; A indicates non-bifidobacteria colony; B indicates bifidobacteria colony.

I



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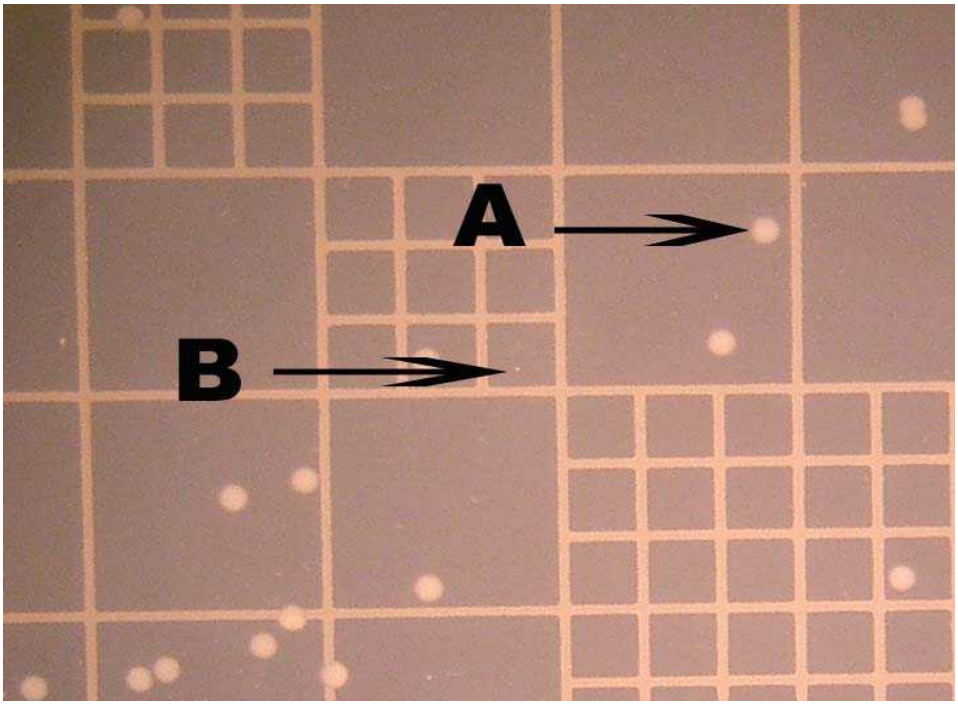
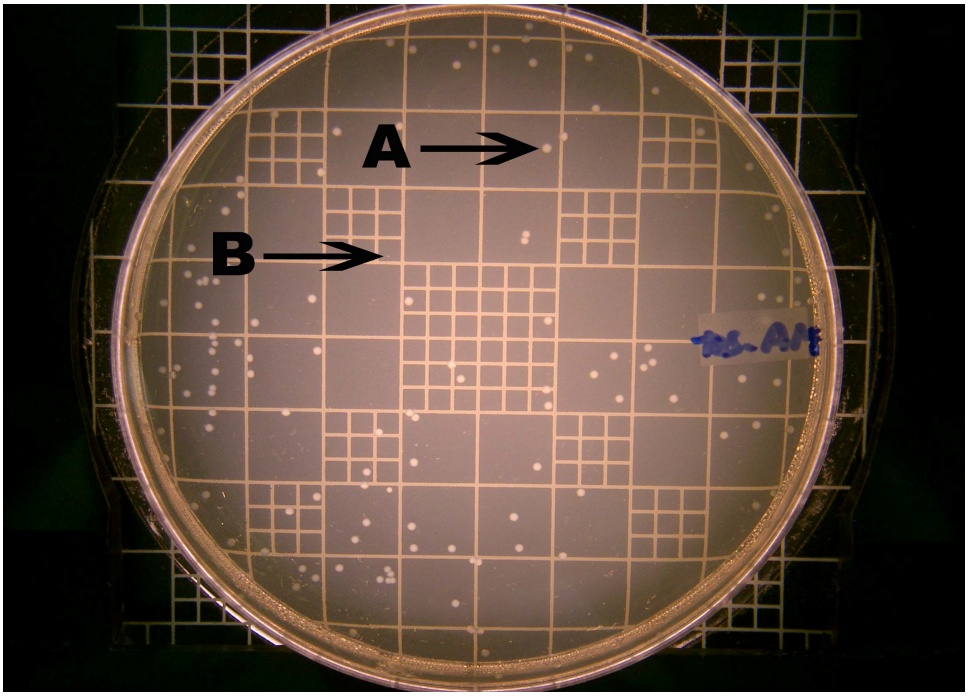


Figure 7. **I**-Cecal sample enumerated on TOS-M agar media; A indicates bifidobacteria colony; B indicates non-bifidobacteria colony. **II**- Displays enlarged area.

I



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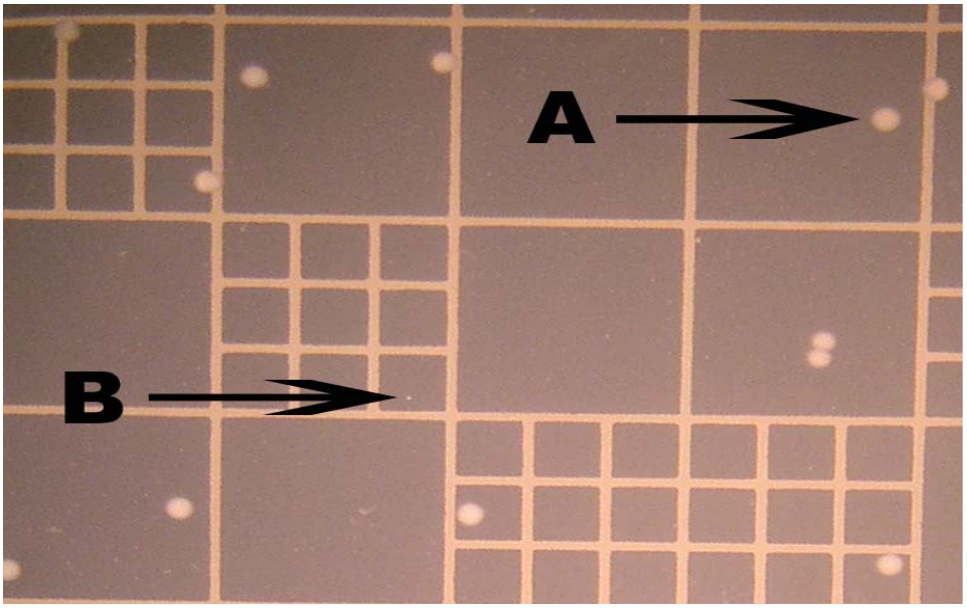

Figure 8. **I**-Cecal sample enumerated on TOS-AM agar media; A indicates bifidobacteria colony; B indicates non-bifidobacteria colony. **II**-Displays enlarged area.

Table 13. Efficiency of different TOS media on several pure culture strains of bifidobacteria.

Strain	Media[§] (log CFU ml^{-1})			
	TOS	TOS-A	TOS-M	TOS-AM
Bifidobacterium longum ATCC 15708	7.18	7.11	7.36	7.11
Bifidobacterium isolate 1-1-4*	8.50	8.51	8.59	8.59
Bifidobacterium isolate 4-2*	8.17	8.01	8.13	7.94
Bifidobacterium isolate 2-1-1*	8.48	8.62	8.58	8.68
Bifidobacterium isolate W1*	6.69	6.91	6.81	6.79
Bifidobacterium isolate W2*	6.82	6.88	7.06	6.85
Bifidobacterium isolate 3*	7.78	6.29	6.30	5.99
Bifidobacterium isolate 5*	8.02	7.83	8.05	8.08
Bifidobacterium isolate 6*	8.02	7.99	8.07	8.02
Mean $\pm$ SD[‡]	7.74 $\pm$ 0.68	7.57 $\pm$ 0.81	7.66 $\pm$ 0.81	7.56 $\pm$ 0.92

* Bifidobacteria *s*trains were isolated from chicken cecal samples and identified as per criteria stated in text.

[‡]No significant difference among media type observed at $P < 0.05$

[§]TOS-A supplemented with glacial acetic acid (1%, v/v), TOS-M supplemented with 0.1 mg Mupirocin/ml, TOS-AM supplemented with glacial acetic acid (1%, v/v) plus 0.1 mg Mupirocin/ml, and TOS with no supplement.

Table 14. Growth of selected strains of lactic acid bacteria on different TOS media*.

Strain	Media[§]			
	TOS	TOS-A	TOS-M	TOS-AM
Lactobacillus acidophilus	+	+	-	-
L. gallinarum	+	+	-	-
L. helveticus	+	+	-	-
Streptococcus gordonii	+	+	-	-

* + indicates growth, - indicates no growth.
[§]TOS-A supplemented with glacial acetic acid (1%, v/v), TOS-M supplemented with 0.1 mg Mupirocin/ml, TOS-AM supplemented with glacial acetic acid (1%, v/v) plus 0.1 mg Mupirocin/ml, and TOS with no supplement.

Table 15. Comparison of selective recoveries of *Bifidobacterium* spp. from chicken cecal sample by different TOS media.

Media§	*Bifidobacterium**	Total anaerobic bacteria*	Selectivity‡
TOS	10.52 ± 1.09[a]	11.56 ± 1.19[a]	26.00
TOS-A	10.39 ± 0.87[a]	11.59 ± 1.16[a]	43.30
TOS-M	9.64 ± 1.25[b]	9.86 ± 1.25[b]	57.40
TOS-AM	9.61 ± 1.36[b]	9.72 ± 1.35[b]	61.00

*Mean number of 12 cecas ± SD, Values within a column followed by different lower-case superscripts differ significantly ($P<0.05$)

‡Expressed as [(mean number of *Bifidobacterium* colonies)/(mean number of total colonies)] x 100.

§TOS-A supplemented with 1 µl glacial acetic acid/ml, TOS-M supplemented with 0.0001 g Mupirocin/ml, TOS-AM supplemented with 1 µl glacial acetic acid/ml plus 0.0001 g Mupirocin/ml, and TOS with no supplement.

Table 16. Bifidobacteria counts from chicken cecal samples on different TOS media*.

	Count‡			
Sample	TOS	TOS-A	TOS-M	TOS-AM
ceca 1	9.40	10.42	6.53	6.34
ceca 2	8.30	9.28	8.31	8.29
ceca 3	9.73	10.03	6.78	6.00
ceca 4	8.86	8.74	7.48	7.70
ceca 5	10.86	10.59	10.48	10.59
ceca 6	10.99	10.82	10.15	9.70
ceca 7	10.93	10.90	9.60	8.60
ceca 8	10.82	10.43	9.51	9.18
ceca 9	9.51	9.72	8.60	8.08
ceca 10	8.60	8.85	8.00	7.00
ceca 11	10.82	10.58	8.48	8.70
ceca 12	8.30	8.48	8.00	9.23
Mean $\pm$ SD§	9.76 ± 1.09^{a}	9.90 ± 0.87^{a}	8.49 ± 1.25^{b}	8.28 ± 1.36^{b}

* TOS-A supplemented with glacial acetic acid (1%, v/v), TOS-M supplemented with 0.1 mg Mupirocin/ml, TOS-AM supplemented with glacial acetic acid (1%, v/v) plus 0.1 mg Mupirocin/ml, and TOS with no supplement.

‡ log CFU g^{-1} wet weight of ceca content

§ Media within a row followed by different lower-case superscripts differ significantly ($P < 0.05$).

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CONCLUSIONS

The effect of isomaltooligosaccharide on *Bifidobacterium* spp. population in young broiler chickens.

Dietary administration of isomaltooligosaccharide at 1, 2, and 4% w/v of the diet significantly increased the number of bifidobacteria and at 4% of the diet significantly increase the number of lactic acid bacteria in cecal contents of 3-week-old broiler chickens when compared to the control diet.

The addition of 1, 2, or 4% w/v of isomaltooligosaccharide to the diet had no significant effect on the body weight gain, feed conversion and feed efficiency in 3-week-old broiler chickens. However, birds that were fed 4% IMO diet were found to have the greatest weight gain but the difference was not significant.

Chickens treated with 1% IMO diet were found to have a significant two log reduction in the level of *Salmonella enterica* ser. Typhimurium Nal^r present in the ceca compared to the control group. Even though the higher percentage of IMO did not show a greater effect in reducing the colonization by *S.* ser. Typhimurium Nal^r in the chicken gut, it appears that 1% IMO diet has the most economical and beneficial effect to reduce the colonization by *S.* ser. Typhimurium Nal^r in the ceca of young broiler chickens. It may be suggested that feeding the optimum level of IMO in the diet of chickens may reduce *Salmonella* colonization in young chickens.

Incorporation of mupirocin into TOS selective agar enhances enumeration of *Bifidobacterium* spp. from chicken ceca samples.

From this study, we conclude that the addition of acetic acid and mupirocin to a basal agar, TOS, did not inhibit the growth of bifidobacteria from pure culture. TOS base media (Yakult Pharmaceutical, Japan) supplemented with glacial acetic acid (1% v/v) and mupirocin (0.1 mg/ml) (TOS-AM) had the most selectivity for bifidobacteria and inhibition against lactobacilli. There was no significant difference between the selectivity for bifidobacteria of TOS supplemented with mupirocin (0.1 mg/ml) (TOS-M and TOS-AM). It appeared that the growth of bifidobacteria was suppressed by the concentration of mupirocin. Lowering the concentration of mupirocin may allow for better proliferation of all bifidobacteria species.

However the bifidobacteria numbers observed in cecal samples from TOS-M and TOS-AM were significantly less than those of regular TOS and TOS supplemented with glacial acetic acid (1% v/v). TOS-A was found to have more *Bifidobacterium* spp. selectivity compared to TOS without modification and without reducing *Bifidobacterium* spp. population from chicken cecal samples compared to TOS with mupirocin supplementation (TOS-M and TOS-AM). TOS-A has a simple preparation and could be potentially used for isolation and enumeration of *Bifidobacterium* spp. from chicken cecal samples.

Isomaltooligosaccharide as a prebiotic in poultry feed.

Isomaltooligosaccharide, a natural microbial-derived feed amendment produced from sugar cane, is found to have a prebiotic effect to intestinal microflora of young broiler chickens. The use of this oligosaccharide combined with a certain level of surveillance biosecurity could be an alternative to more ambitious control and prevention of *Salmonella* colonization in poultry.

This oligosaccharide is a new compound and currently not commercial available, more research need to be done on their production and their effectiveness on other probiotic strains.

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APPENDIX A

TEMPERATURE, AIR FLOW RECORDS OF ISOLATOR UNITS DATA

Temperature and Air Flow Records for Isolator Units Data

Watkinsville Poultry Facility
Poultry Microbiological Safety Research Unit
ARS, USDA
Watkinsville, GA 30677

March 9 - March 30, 2004
Replication 1
0% IMO (Control Group)

	Ambient	Temp (°C)		Airflow
Date	Temp (°C)	High	Low	Guage
9-Mar	28.3	36.6	16.6	0.4
10-Mar	28.8	28.8	26.6	0.4
11-Mar	28.8	29.4	26.6	0.4
12-Mar	29.4	28.8	28.3	0.4
13-Mar	31.6	29.4	27.7	0.4
14-Mar	30.5	30.0	27.7	0.4
15-Mar	31.6	30.5	28.3	0.4
16-Mar	31.6	29.4	27.7	0.4
17-Mar	31.1	30.5	28.8	0.4
18-Mar	30.5	30.5	28.3	0.4
19-Mar	31.1	30.0	28.3	0.4
20-Mar	30.5	30.0	28.8	0.4
21-Mar	31.6	33.3	28.8	0.4
22-Mar	30.0	34.4	27.7	0.5
23-Mar	31.1	28.8	27.7	0.5
24-Mar	31.1	30.5	28.3	0.5
25-Mar	29.4	30.5	28.8	0.5
26-Mar	29.4	31.1	28.3	0.5
27-Mar	28.3	28.8	27.7	0.5
28-Mar	29.4	32.2	27.7	0.6
29-Mar	28.8	31.6	28.8	0.6
30-Mar	28.8	33.3	30.0	0.5

Watkinsville Poultry Facility
Poultry Microbiological Safety Research Unit
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Watkinsville, GA 30677

March 9 – March 30, 2004
Replication 1
1% IMO Group

	Ambient	Temp (°C)		Airflow
Date	Temp (°C)	High	Low	Guage
9-Mar	28.8	33.8	13.3	0.4
10-Mar	29.4	30.5	26.6	0.4
11-Mar	28.3	28.8	24.4	0.4
12-Mar	30.0	32.2	25.5	0.4
13-Mar	30.0	31.1	25.5	0.4
14-Mar	31.6	31.1	25.5	0.4
15-Mar	31.1	31.1	25.5	0.4
16-Mar	31.1	31.1	26.6	0.4
17-Mar	31.1	31.1	27.2	0.4
18-Mar	30.5	30.0	28.8	0.4
19-Mar	31.1	30.0	26.6	0.4
20-Mar	30.5	30.0	25.5	0.4
21-Mar	28.8	32.2	26.6	0.4
22-Mar	28.3	33.3	26.6	0.4
23-Mar	28.8	31.1	26.1	0.4
24-Mar	31.1	32.2	26.6	0.4
25-Mar	28.8	31.6	27.2	0.4
26-Mar	28.8	31.6	27.7	0.5
27-Mar	28.8	32.2	27.7	0.5
28-Mar	28.8	32.2	26.6	0.5
29-Mar	30.0	32.2	28.8	0.5
30-Mar	29.4	32.2	28.8	0.6

Watkinsville Poultry Facility
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Watkinsville, GA 30677

March 9 – March 30, 2004
Replication 1
2% IMO Group

	Ambient	Temp (°C)		Airflow
Date	Temp (°C)	High	Low	Guage
9-Mar	26.6	31.6	13.8	0.4
10-Mar	26.6	31.1	26.6	0.4
11-Mar	28.8	30.0	25.5	0.4
12-Mar	28.8	32.2	25.5	0.4
13-Mar	30.5	32.2	26.6	0.4
14-Mar	28.3	32.2	26.6	0.4
15-Mar	27.2	32.2	27.2	0.4
16-Mar	27.2	32.2	27.7	0.4
17-Mar	28.3	31.6	27.7	0.4
18-Mar	26.1	32.2	25.5	0.4
19-Mar	27.2	33.8	26.1	0.4
20-Mar	25.5	33.3	25.5	0.4
21-Mar	31.6	31.6	25.5	0.4
22-Mar	26.1	31.6	26.6	0.4
23-Mar	27.7	31.6	27.2	0.4
24-Mar	29.4	33.3	27.7	0.5
25-Mar	30.0	32.2	26.6	0.5
26-Mar	29.4	32.7	26.6	0.5
27-Mar	27.7	32.7	27.7	0.5
28-Mar	29.4	32.2	28.8	0.5
29-Mar	30.5	32.7	30.0	0.5
30-Mar	28.8	32.2	28.8	0.5

Watkinsville Poultry Facility
Poultry Microbiological Safety Research Unit
ARS, USDA
Watkinsville, GA 30677

March 9 – March 30, 2004
Replication 1
4% IMO Group

	Ambient	Temp (°C)		Airflow
Date	Temp (°C)	High	Low	Guage
9-Mar	28.8	28.3	21.6	0.3
10-Mar	28.3	30.5	26.1	0.3
11-Mar	30.0	31.1	24.4	0.4
12-Mar	31.1	32.2	25.5	0.4
13-Mar	31.1	31.1	26.1	0.4
14-Mar	31.6	31.6	25.5	0.4
15-Mar	31.1	31.1	26.1	0.4
16-Mar	31.6	32.2	26.6	0.4
17-Mar	31.1	30.5	26.6	0.4
18-Mar	31.1	31.1	24.4	0.4
19-Mar	31.1	31.1	25.5	0.4
20-Mar	30.5	30.0	25.5	0.4
21-Mar	31.1	33.3	26.6	0.4
22-Mar	30.5	34.4	26.6	0.4
23-Mar	30.5	31.6	27.2	0.4
24-Mar	31.1	31.1	27.7	0.4
25-Mar	29.4	31.6	27.7	0.4
26-Mar	29.4	31.1	28.3	0.4
27-Mar	30.0	30.0	27.7	0.5
28-Mar	29.4	32.2	27.7	0.5
29-Mar	30.0	31.1	27.7	0.5
30-Mar	30.0	33.3	26.6	0.5

Watkinsville Poultry Facility
Poultry Microbiological Safety Research Unit
ARS, USDA
Watkinsville, GA 30677

April 20 - May 11, 2004
Replication 2
0% IMO (Control Group)

	Ambient	Temp (°C)		Airflow
Date	Temp (°C)	High	Low	Guage
20-Apr	29.4	34.4	21.1	0.4
21-Apr	29.4	32.7	28.3	0.4
22-Apr	31.1	31.1	28.8	0.4
23-Apr	31.1	31.1	28.3	0.4
24-Apr	30.0	31.1	27.7	0.4
25-Apr	30.5	28.8	28.8	0.4
26-Apr	31.6	31.1	28.8	0.4
27-Apr	31.6	31.6	28.8	0.4
28-Apr	31.1	31.1	28.8	0.4
29-Apr	30.5	30.5	27.4	0.4
30-Apr	30.0	30.5	28.8	0.4
1-May	29.4	29.4	28.3	0.4
2-May	29.4	28.3	28.8	0.4
3-May	27.7	33.3	27.7	0.5
4-May	28.8	28.8	27.7	0.5
5-May	29.4	31.1	27.7	0.5
6-May	29.4	32.2	27.7	0.5
7-May	27.7	32.7	28.8	0.5
8-May	27.2	33.3	27.7	0.6
9-May	31.6	32.2	31.1	0.6
10-May	28.8	32.2	28.8	0.7
11-May	28.8	32.7	30.0	0.7

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Poultry Microbiological Safety Research Unit
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Watkinsville, GA 30677

April 20 - May 11, 2004
Replication 2
1% IMO Group

	Ambient	Temp (°C)		Airflow
Date	Temp (°C)	High	Low	Guage
20-Apr	29.4	33.3	25.5	0.4
21-Apr	28.8	32.7	28.3	0.4
22-Apr	27.7	31.6	28.3	0.4
23-Apr	31.1	32.2	26.6	0.4
24-Apr	30.5	32.2	26.6	0.4
25-Apr	30.0	30.0	28.8	0.4
26-Apr	31.1	31.6	28.8	0.4
27-Apr	30.0	32.2	28.8	0.4
28-Apr	31.1	33.3	28.8	0.4
29-Apr	28.8	33.3	28.3	0.4
30-Apr	28.8	32.2	27.7	0.4
1-May	30.0	32.2	27.2	0.4
2-May	30.0	32.2	26.6	0.4
3-May	30.0	32.2	27.7	0.4
4-May	27.2	32.2	27.7	0.4
5-May	28.8	32.7	28.3	0.4
6-May	30.0	33.7	28.8	0.4
7-May	27.2	32.2	30.0	0.5
8-May	27.2	36.6	25.5	0.5
9-May	28.8	32.2	25.5	0.5
10-May	29.4	33.3	27.2	0.5
11-May	29.4	32.2	28.8	0.6

Watkinsville Poultry Facility
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Watkinsville, GA 30677

April 20 - May 11, 2004
Replication 2
2% IMO Group

	Ambient	Temp (°C)		Airflow
Date	Temp (°C)	High	Low	Guage
20-Apr	29.4	32.2	20.0	0.4
21-Apr	28.8	32..7	28.8	0.4
22-Apr	26.6	32.2	27.7	0.4
23-Apr	30.5	33.8	26.6	0.4
24-Apr	30.5	32.7	27.2	0.4
25-Apr	28.8	31.1	28.8	0.4
26-Apr	30.5	34.4	27.7	0.4
27-Apr	31.1	33.8	28.8	0.4
28-Apr	29.4	33.3	28.8	0.4
29-Apr	31.1	33.3	26.6	0.4
30-Apr	28.8	33.3	27.7	0.4
1-May	30.0	31.6	28.8	0.4
2-May	30.0	31.6	30.0	0.4
3-May	30.0	32.2	28.8	0.4
4-May	26.1	33.3	30.0	0.4
5-May	27.7	33.3	27.7	0.4
6-May	29.4	33.3	28.3	0.4
7-May	27.7	33.3	32.2	0.5
8-May	27.7	48.8	27.7	0.5
9-May	28.3	43.3	27.7	0.5
10-May	27.7	32.7	27.7	0.5
11-May	28.8	32.2	28.8	0.5

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April 20 - May 11, 2004
Replication 2
4% IMO Group

	Ambient	Temp (°C)		Airflow
Date	Temp (°C)	High	Low	Guage
20-Apr	28.8	33.3	25.5	0.3
21-Apr	29.4	32.7	28..3	0.4
22-Apr	29.4	34.4	27.7	0.4
23-Apr	31.6	32.7	30.5	0.4
24-Apr	31.1	32.2	30.0	0.4
25-Apr	30.5	32.2	27.7	0.4
26-Apr	31.6	32.2	28.3	0.4
27-Apr	30.0	33.3	28.8	0.4
28-Apr	31.6	32.2	27.7	0.4
29-Apr	28.8	32.7	28.3	0.4
30-Apr	28.8	32.2	27.7	0.4
1-May	30.0	32.2	28.8	0.4
2-May	29.4	32.2	27.7	0.4
3-May	30.0	32.2	28.3	0.4
4-May	27.2	32.2	26.6	0.4
5-May	27.7	32.7	27.2	0.4
6-May	30.0	33.3	30.0	0.4
7-May	27.7	32.2	27.7	0.4
8-May	27.2	33.3	25.5	0.4
9-May	28.3	33.3	26.6	0.5
10-May	29.4	33.3	27.2	0.5
11-May	29.4	32.2	28.8	0.5

Watkinsville Poultry Facility
Poultry Microbiological Safety Research Unit
ARS, USDA
Watkinsville, GA 30677

March 9 – March 30, 2004
Replication 3
0% IMO (Control Group)

	Ambient	Temp (°C)		Airflow
Date	Temp (°C)	High	Low	Guage
28-Apr	28.3	36.6	16.6	0.3
29-Apr	30.6	28.8	26.6	0.3
30-Apr	29.4	29.4	26.6	0.3
1-May	28.9	28.8	28.3	0.3
2-May	31.7	29.4	27.7	0.3
3-May	31.7	30.0	27.7	0.3
4-May	30.6	30.5	28.3	0.3
5-May	29.4	29.4	27.7	0.3
6-May	30.6	30.5	28.8	0.3
7-May	30.6	30.5	28.3	0.3
8-May	28.9	30.0	28.3	0.3
9-May	28.9	30.0	28.8	0.3
10-May	28.3	33.3	28.8	0.3
11-May	29.4	34.4	27.7	0.3
12-May	28.3	28.8	27.7	0.4
13-May	28.3	30.5	28.3	0.4
14-May	28.9	30.5	28.8	0.4
15-May	27.2	31.1	28.3	0.4
16-May	26.7	28.8	27.7	0.3
17-May	27.2	32.2	27.7	0.3
18-May	27.8	31.6	28.8	0.3

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March 9 – March 30, 2004
Replication 3
1% IMO Group

Date	Ambient Temp (°C)	Temp (°C) High	Temp (°C) Low	Airflow Guage
28-Apr	30.6	33.8	13.3	0.3
29-Apr	29.4	30.5	26.6	0.4
30-Apr	28.3	28.8	24.4	0.4
1-May	30.0	32.2	25.5	0.4
2-May	30.0	31.1	25.5	0.3
3-May	31.6	31.1	25.5	0.3
4-May	31.1	31.1	25.5	0.3
5-May	31.1	31.1	26.6	0.3
6-May	31.1	31.1	27.2	0.3
7-May	30.5	30.0	28.8	0.3
8-May	31.1	30.0	26.6	0.3
9-May	30.5	30.0	25.5	0.3
10-May	28.8	32.2	26.6	0.3
11-May	28.3	33.3	26.6	0.3
12-May	28.8	31.1	26.1	0.3
13-May	31.1	32.2	26.6	0.3
14-May	28.8	31.6	27.2	0.3
15-May	28.8	31.6	27.7	0.3
16-May	28.8	32.2	27.7	0.3
17-May	28.8	32.2	26.6	0.3
18-May	30.0	32.2	28.8	0.3

Watkinsville Poultry Facility
Poultry Microbiological Safety Research Unit
ARS, USDA
Watkinsville, GA 30677

March 9 – March 30, 2004
Replication 3
2% IMO Group

	Ambient	Temp (°C)		Airflow
Date	Temp (°C)	High	Low	Guage
28-Apr	31.1	33.8	13.3	0.3
29-Apr	26.6	30.5	26.6	0.4
30-Apr	28.8	28.8	24.4	0.4
1-May	28.8	32.2	25.5	0.4
2-May	30.5	31.1	25.9	0.4
3-May	28.3	31.1	25.5	0.4
4-May	27.2	31.1	25.5	0.4
5-May	27.2	31.1	26.6	0.4
6-May	28.3	31.1	27.2	0.4
7-May	26.1	30.0	28.8	0.4
8-May	27.2	30.0	26.6	0.4
9-May	25.5	30.0	25.5	0.4
10-May	31.6	32.2	26.6	0.4
11-May	26.1	33.3	26.6	0.4
12-May	27.7	31.1	26.1	0.4
13-May	29.4	32.2	26.6	0.5
14-May	30.0	31.6	27.2	0.5
15-May	29.4	31.6	27.7	0.5
16-May	27.7	32.2	27.7	0.4
17-May	29.4	32.2	26.6	0.4
18-May	30.5	32.2	28.8	0.4

Watkinsville Poultry Facility
Poultry Microbiological Safety Research Unit
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March 9 – March 30, 2004
Replication 3
4% IMO Group

	Ambient	Temp (°C)		Airflow
Date	Temp (°C)	High	Low	Guage
28-Apr	31.7	33.8	13.3	0.3
29-Apr	29.4	30.5	26.6	0.5
30-Apr	28.3	28.8	24.4	0.5
1-May	30.0	32.2	25.5	0.5
2-May	30.0	31.1	25.9	0.4
3-May	31.6	31.1	25.5	0.4
4-May	31.1	31.1	25.5	0.5
5-May	31.1	31.1	26.6	0.4
6-May	31.1	31.1	27.2	0.5
7-May	30.5	30.0	28.8	0.5
8-May	31.1	30.0	26.6	0.4
9-May	30.5	30.0	25.5	0.4
10-May	28.8	32.2	26.6	0.4
11-May	28.3	33.3	26.6	0.5
12-May	28.8	31.1	26.1	0.5
13-May	31.1	32.2	26.6	0.5
14-May	28.8	31.6	27.2	0.5
15-May	28.8	31.6	27.7	0.6
16-May	28.8	32.2	27.7	0.4
17-May	28.8	32.2	26.6	0.4
18-May	30.0	32.2	28.8	0.5

BIOGRAPHY

Sutawee Narint Thitaram was born on May, 29 1976 in Khon Kaen, Thailand. She is the eldest daughter of Nat and Em-on Narint. She received her elementary education from Sanambin School and secondary education from the Satit Khon Kaen School in Khon Kaen. After finishing high school in 1992, she attended Khon Kaen University in department of plant pathology, faculty of agriculture where she was awarded the student exchange scholarship from the Royal Thai government to work on isolation and identification various soil fungi under the direction of Dr. Richard T. Hanlin in the department of plant pathology at the University of Georgia.

After receiving her Bachelor of Sciences in plant pathology from Khon Kaen University in 1996, she enrolled in the graduate program in the department of food science and technology at the University of Georgia where she conducted her master's research under the guidance of Dr. Gregory R. Siragusa.