Research Note

Efficacy of *Leuconostoc mesenteroides* (ATCC 13146) Isomaltooligosaccharides as a Poultry Prebiotic

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ABSTRACT The complex dietary carbohydrates, called prebiotics, have been used to control *Salmonella* and improve intestinal bacterial balance in broilers. *Leuconostoc mesenteroides* (ATCC 13146) isomaltooligosaccharides (IMO) stimulate growth of *Bifidobacterium* and *Lactobacillus* and are not used by *Salmonella* or *Escherichia coli*. We tested the efficacy of these IMO as a prebiotic. IMO, compared with fructooligosaccharides (FOS) as sole carbon source, promoted growth of chicken cecal isolates and *Bifidobacterium*. Cecal isolates and *Salmonella typhimurium* grown in mixed culture on IMO reduced the *Salmonella* population. Cecal isolates grown on IMO showed higher viable counts and faster growth than *Salmonella*, indicating a potential value for these oligomers for poultry intestinal microflora modification.

(*Key words*: branched glucooligosaccharide, *Leuconostoc*, prebiotic, *Salmonella*)

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INTRODUCTION

The presence of *Salmonella* in broilers is a major source of human salmonellosis (Coyle et al., 1988; St. Louis et al., 1988, Perales and Audicana, 1989; Henzler et al., 1994; Bryan and Doyle, 1995). Competitive exclusion (CE) and the use of complex dietary carbohydrates such as fructooligosaccharides (FOS) have shown promise in controlling *Salmonella* (Nurmi and Rantala, 1973; Bailey et al., 1991; Oyarzabal and Conner, 1995). FOS reduce susceptibility of poultry to *Salmonella* colonization (Bailey et al., 1991), increase *Bifidobacterium* levels, and reduce the level of *Salmonella* present in the cecum. Chambers et al. (1997) studied *Salmonella typhimurium* colonization in 3-, 5-, and 6- wk-old broilers, fed a control ration or rations with added FOS or lactose derivatives (LD). Both FOS and LD reduced cecal pH and *Salmonella* cell numbers. In addition, broilers fed refined FOS had a lower rate of cross-infection by *S. typhimurium* than control birds. Fukata et al. (1999) reported that FOS inhibited *Salmonella* colonization of chicks. The mean number of *Salmonella enteritidis* in chicks fed FOS was ($P < 0.05$) lower than in the control group. Branched isomaltooligosaccharides (IMO), another type of oligosaccharide, have been shown to alter rat and humans intestinal bacterial balances (Kohmoto et al. 1988; Valette et al., 1993; Djouzi et al., 1995). We previously reported on a method for production of branched α-IMO through use of a modified *Leuconostoc mesenteroides* fermentation. These IMO in vitro were used for growth by *Bifidobacterium* and *Lactobacillus* but not by *Salmonella* spp. or *Escherichia coli* (Chung and Day, 2002). This report details some characteristics of these oligosaccharides relative to their potential as a poultry prebiotic.

MATERIALS AND METHODS

Organism, Culture Medium, and Inoculum Preparation

The strains of bacteria used in this study were either obtained from the American Type Culture Collection[2] (ATCC) or were isolated from chicken ceca (4 passages from 6- to 8-wk-old broilers). They were maintained at 4°C on agar slants and transferred monthly. Anaerobes were subcultured weekly. *S. typhimurium* ATCC 14028 and *E. coli* B ATCC 23226 were maintained on tryptic soy agar[3] (TSB). *Bifidobacterium bifidum* ATCC 35914, *Bifidobacterium longum* ATCC 15708, and *Leuconostoc mesenteroides* ATCC 13146 were maintained anaerobically on lactobaccilli MRS[3] slants containing 0.05% (wt/vol) cysteine. Chicken ceca were kindly supplied by G. Siragusa.[4] Screening and isolation of bacteria from chicken ceca were conducted following the method described by Hartemink and Rombouts (1999). Ceca, in a plastic bag, were homog-

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Abbreviation Key: FOS = fructooligosaccharides, IMO = isomaltooligosaccharides.

enized by kneading the bag, and a subsample of ~10 g was transferred to a preweighed glass container containing 90 mL of anaerobic buffered peptone water[5] with 0.5 g/L L-cysteine-HCl. The container was then closed and weighed to determine the actual sample size. Mixed samples were diluted further with reduced physiological salt solution (rps; peptone, 1 g/L; L-cysteine-HCl 0.5 g/L; and NaCl 8 g/L) or test medium[3] (MRS broth). Finally, the samples were plated on the media and incubated at 37°C for 48 h. Unless otherwise stated, mixing, diluting, plating, and incubation were carried out anaerobically. Six colonies out of the hundreds of isolates were selected randomly and designated as chicken cecal isolates 1 to 6. Detailed identification of 6 cecal isolates was not conducted, but they were consistent with lactic acid bacteria, being gram positive, catalase negative, producing lactic acid, and having a small creamy-white colonial morphology.

Preparation of Oligosaccharides

Batch fermentations were conducted in a 2-L BioFlo II fermentor[6] with a working volume of 1.0 L. The medium had the following composition: sucrose (100 g/L), maltose (50 g/L), yeast extract (5 g/L), $MgSO_4 \cdot 7H_2O$ (0.2 g/L), $FeSO_4 \cdot 7H_2O$ (0.01 g/L), NaCl (0.01 g/L), $MnSO_4 \cdot 7H_2O$ (0.01 g/L), $CaCl_2$ (0.05 g/L), and KH_2PO_4 (3 g/L) at pH 7.2. Fermentors were inoculated from late log phase flask cultures at 1.0% of the working volume. Fermentations were maintained at pH 6.5, 28°C, and 200 rpm. After being harvested, cells were removed by centrifugation[7] at 10,400 × *g* for 20 min. Activated charcoal[8] (5 g/L) and Celite 545[9] (1 g/L) were added to cell-free culture broths and mixed at 50°C for 20 min. The broths were then filtered through no. 6 filter paper[10] to remove the carbon. The filtered broths were desalted using ion-exchange columns filled with an anion-exchange resin in the hydroxide form and a cation-exchange resin in the hydrogen form.[11] The eluents were concentrated by vacuum evaporation to 65% solids. Mannitol crystallized upon cooling the concentrates and was removed by decantation. Mannitol-free oligosaccharides were separated from the concentrates using a cation-exchange column (in calcium form); the oligosaccharide fractions were concentrated by vacuum evaporation.

Analytical Methods

Bacterial growth was measured by turbidimetry at 660 nm, calibrated against cell dry weight. Cells from a known volume were harvested by centrifugation at 10,400 × *g* for 2 min, washed with deionized water, resuspended in a minimum volume of water, and dried (initially overnight at 95°C and then at 105°C) to constant weight. An absorbance of 1.0 at 660 nm was equivalent to 0.51 g of dry matter/L.

HPLC Analysis. High-performance ion chromatography with a CarboPac MA1 column[12] and a pulsed amperometric detector[12] were used for quantitative analysis of glucose, fructose, sucrose, mannitol, and maltose concentrations in solution. The samples were eluted at 0.4 mL/min with a solution of 0.48 *M* NaOH. Oligosaccharide concentrations were calculated from peak areas of HPLC on an Aminex-HPX-87K Bio-Rad column[13] run at 85°C with K_2HPO_4 as eluent, a constant flow rate of 0.5mL/min, and glucose as a standard.

Oligosaccharide Use by Selected Microorganisms

The growth of selected bacteria in the presence of oligosaccharides was compared by measuring absorbances over time at 660 nm. The media used for both *B. longum* was of the same composition as *Lactobacillus* MRS broth with 0.05% (wt/vol) cysteine, except the carbon source was replaced by various oligosaccharide preparations. The growth medium for *S. typhimurium* and *E. coli* was TSB, and the carbon source replaced by various oligosaccharide preparations. Carbon sources were supplied at a final concentration of 0.5% (wt/vol). All carbon sources were filter sterilized (0.2 μm). The following carbon sources were compared: glucose,[8] commercial FOS[14] (>97.5%), and oligosaccharide preparations. Anaerobic growth tests for individual cultures were conducted in sealed glass test tubes. Each tube was inoculated from an overnight culture with *S. typhimurium* or *E. coli* and a 24- to 48-h culture of a *B. longum* The experiments was conducted under anaerobic conditions using the Oxyrase plate system.[15] MRS broth containing 0.05% (wt/vol) cysteine with IMO as sole carbon source was used for mixed cultures of *S. typhimurium* and individual cecal isolates. Total viable counts were conducted on MRS agar, and the cell numbers of *S. typhimurium* were determined from growth on MacConkey agar[3] plates. The cell numbers for chicken cecal isolates were determined as the difference between total viable count and *S. typhimurium* numbers. For determining oligosaccharide consumption patterns of various strains, the media were MRS broth for *B. longum* and cecal isolates and TSB for *S. typhimurium* and *E. coli* containing 0.5% (wt/vol) of *Leuconostoc* glucooligosaccharides instead of glucose as the carbon source. Media pH were adjusted to 6.0 using 10 *N* HCl, and 0.1% (vol/vol) inoculum grown overnight in MRS broth was used.

RESULTS AND DISCUSSION

Growth of Bacteria Isolated from Chicken Ceca on IMO

Maximum growth achieved on IMO and FOS is shown in Figure 1. Three isolates grew better on IMO carbohy-

[5]Oxoid, Basingstoke, Hampshire, England.
[6]New Brunswick Scientific Co., Edison, NJ.
[7]Sorvall RC5C, Dupont, Newtown, CT.
[8]Sigma Chemical Co., St. Louis, MO.
[9]Fisher Scientific, Fair Lawn, NJ.
[10]Whatman International Ltd., Maidstone, England.
[11]Rohm and Haas, Philadelphia, PA.
[12]Dionex, Sunnyvale, CA.
[13]Bio-Rad Laboratories, Hercules, CA.
[14]Samyang Genex Co., Seoul, Korea.
[15]Oxyrase, Inc., Mansfield, OH.

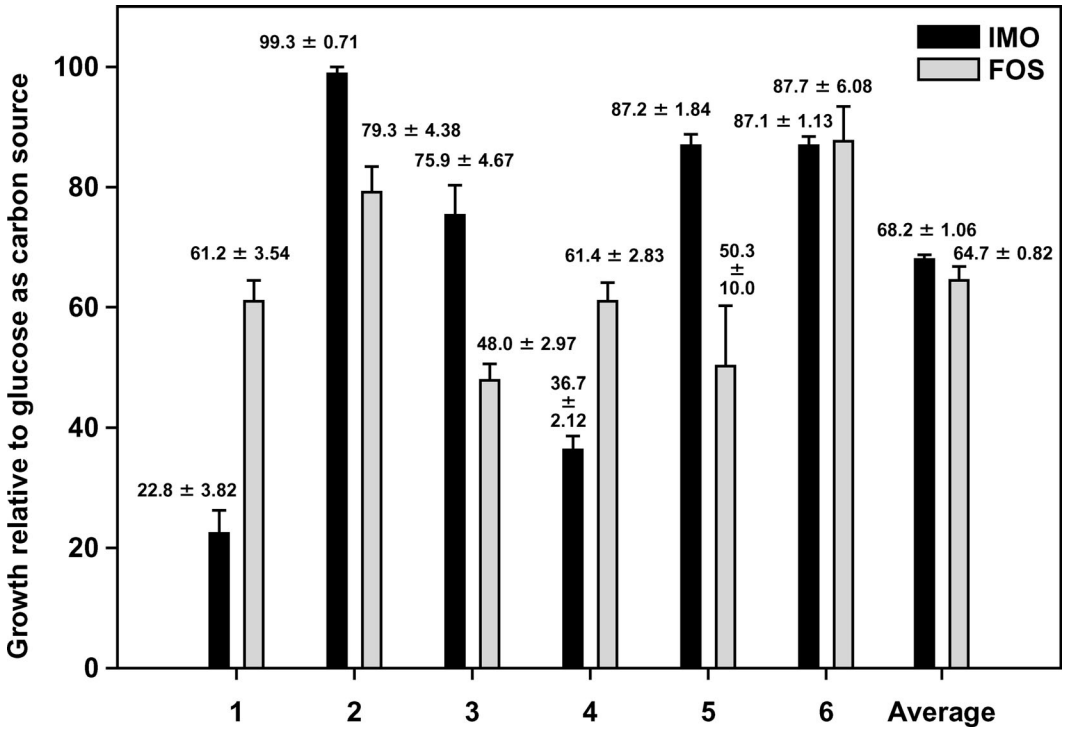

FIGURE 1. Comparison of maximum growth achieved in 24 h by chicken ceca isolates on *Leuconostoc* isomaltooligosaccharide (IMO) and commercial fructooligosaccharide (FOS; Samyang Genex Co., Seoul, Korea) preparations. Growth determined at stationary phase (24 h) on glucose was calculated as 100 {[absorbance unit of glucose (Glc) at 24 h/absorbance unit of IMO (or FOS) × 100]/h}. Bars show the mean of duplicate samples, and the error bars are standard error of the mean.

drate supplement than on FOS. Two isolates grew better on FOS; however, the percentage of growth increase was smaller than for those isolates that grew on IMO. Based on the averages for all 6 isolates, growth on IMO was equivalent to that on FOS. The use of a complex carbohydrate as an avian prebiotic requires passage to the microflora in the ceca. According to Valette et al. (1993), an oligosaccahride similar to the IMO used in this study was not hydrolyzed in the digestive tract of germ-free rats and was fermented to short-chain fatty acids in the intestines. Similar results can be expected for IMO passing through poultry digestive system. It appears that IMO is used by some of microflora present in chicken ceca.

Use Pattern of IMO

E. coli and *S. typhimurium* did not use IMO, whereas *B. longum* used all components (Table 1). When carbohydrates are used as an energy source, they must be degraded enough that they can be transported inside bacterial cell. Structural analysis of IMO by C^{13} NMR has shown that IMO is a glucan linked mainly by α-1,4 and α-1,6, similar to those found in dextran. An α-1,4 linkage is located at the reducing end of isomaltosyl residues containing α-1,6 linkages (Day, 2002). This structure is not readily degraded by *E. coli* or *S. typhimurium*. However, some strains of bifidobacteria produce a dextranase (Rasic and Kurmann, 1983), and an intracellular enzyme, α-1,6 glucosidase (isomaltodextranase) has been found in extracts obtained rumen strains of *Bifidobacterium* cultured on dextran (Bailey and Roberton, 1962; Rasic and Kurmann, 1983). *Lactobacillus johnsonii* and *B. longum* have shown no differences between growth on glucose and on these oligosaccharides (Chung and Day, 2002). It appears that IMO is used preferentially by some probiotic strains. It is probable that dextranases in the bifidobacteria and

TABLE 1. Use of specific isomaltooligosaccharides by selected bacteria

	Oligosaccharide used (%)		
Bacterium	Panose	Branched[1] DP4	Branched DP5
Escherichia coli	0.4 ± 0.9[2]	0.0 ± 0.4	0.0 ± 0.2
Salmonella typhimurium	0.0 ± 1.4	3.3 ± 0.7	0.0 ± 0.4
Bifidobacterium longum	100.0 ± 1.2	86.5 ± 3.2	89.5 ± 2.1
Cecal isolate 5	88.0 ± 2.8	12.3 ± 1.7	10.4 ± 0.8
Cecal isolate 6	88.0 ± 1.6	8.6 ± 0.8	1.3 ± 0.3

[1]Degree of polymerization.

[2]Percentage of oligosaccharide was calculated from the ratio of the oligosaccharide peak areas at 24 to 0 h. Media pH was adjusted to 6.0 with HCl. Each numbers is the mean of duplicate samples and standard errors.

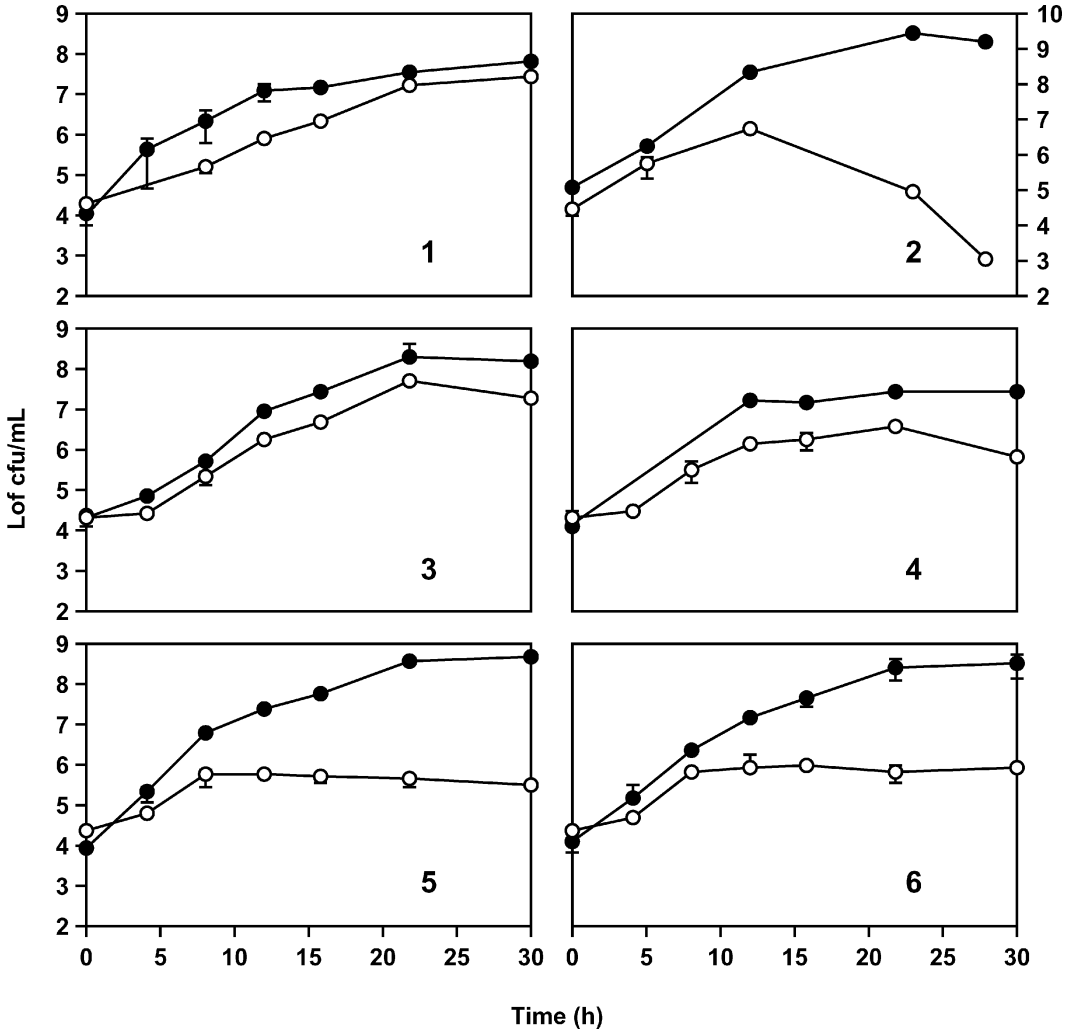

FIGURE 2. Anaerobic growth of mixed cultures of *Salmonella typhimurium* (o, dotted line) and cecal isolates (solid line) on isomaltooligosaccharide preparations at 37°C. Data are presented as mean log of duplicate samples ± standard error.

lactobacilli are responsible for the hydrolysis of IMO into simple sugars.

Two cecal isolates (5 and 6) used primarily the DP3 polymer (panose; linked by a α-1,4 and a α-1,6; Table 1). Our poultry cecal isolates also used IMO larger than DP3 but far less efficiently. Kaplan and Hutkins (2000) investigated the fermentation of FOS by 28 strains of lactic acid bacteria and bifidobacteria. They found that 19 strains used FOS up to DP4, and none were able to use higher than DP5. It is interesting that, even though there are structural differences between IMO and FOS, there is a size limit on the use of both of these oligosaccharides by lactic acid bacteria. The use pattern of IMO is similar to that of FOS.

Mixed Cultures

All cecal isolates had higher viable counts and a faster rate of growth than *Salmonella*. Three isolates (2, 5, and 6) suppressed the growth of *S. typhimurium* (Figure 2). These 3 isolates also had high use, over 85%, of IMO (Figure 1). When *L. johnsonii* and *S. typhimurium* are grown together, oligomers stimulate the growth of *Lactobacillus* but are not readily used by *Salmonella* (Chung and Day, 2002). As reported earlier, some pathogenic microorganisms do not grow well on *Leuconostoc* IMO (Yoo, 1997). When *S. typhimurium* or *E. coli* was grown on IMO, there was less than 37% of the equivalent growth on glucose. This growth response was similar to that observed for commercial FOS (less than 35%). With FOS as the sole carbon source, *Bifidobacterium bifidum*, *Enterococcus faecium*, *Lactobacillus* sp., and *Pediococcus* sp. inhibit growth of 6 different *Salmonella* serotypes (Oyarzabal and Conner, 1995).

The low pH produced by these cecal isolates when grown on IMO may be responsible for the suppression of *S. typhimurium* (Chung and Day, 2002). Although other antagonistic substances, such as bacteroicins and hydrogen peroxide, can be produced that will inhibit *S. typhimurium*, high concentrations of lactic acid bacteria must be present for this to occur.

The efficacy of IMO as a prebiotic for organisms isolated from chicken ceca and as an inhibitor of *S. typhimurium* was comparable to the efficacy of FOS. When used

as sole carbon source, it promoted growth of both cecal isolates and *Bifidobacterium*. Mixed cultures of *S. typhimurium* and individual cecal isolates grown on IMO preparations favored the isolates. They grew to greater concentration and had a faster rate of growth than *Salmonella*. These studies do not directly predict in vivo effects but do indicate that this type of oligomer has potential as avian prebiotic.

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