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***In vitro* Prebiotic Bacterial Growth Properties of Xylooligosaccharides Produced by Autohydrolysis of Corn Fiber**

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Abstract: Xylooligosaccharides (XOS) are considered to be prebiotics. Prebiotics are defined as the non-digestible food ingredients that benefit the host by stimulating the growth and activity of a limited number of bacteria, such as the *Bifidobacterium* genus, in the intestine. The objective of this study was to evaluate prebiotic properties for XOS produced by autohydrolysis of corn fiber. We compared commercial XOS (XOS-C), fructo-oligosaccharides (FOS), inulin, monosugars (xylose and glucose) and control (no sugars), with autohydrolysis liquor containing XOS (XOS-D) for the growth of *Bifidobacterium breve*, *B. adolescentis* and *Lactobacillus brevis*. Optical density at 550 nm (OD₅₅₀) was normalized by taking logarithm of ratio of OD on particular day to OD on day 0, for each organism/substrate/media combination. Normalized optical density is referred to as specific growth. Growth on commercial XOS (XOS-C) was comparable with growth on other prebiotics (FOS and inulin). XOS-D promoted growth more than that of the control. For XOS-D, highest growth recorded was for *L. brevis* (0.461) followed by *B. breve* (0.267) and *B. adolescentis* (0.263). XOS-D performance was comparable to FOS and inulin for *L. brevis*. XOS produced from corn fiber exhibit the potential to be used as a prebiotic in poultry.

Key words: Prebiotics, corn fiber, bioproducts, xylooligosaccharides, XOS

INTRODUCTION

Consumers have become concerned about the consequences of feeding antibiotics to livestock for both human and animal health. The overuse of antibiotics could lead to drug resistance in pathogens of significance to humans. The nature of the problem has been addressed by the Health Council of Netherlands, 1998 and many researchers in their reviews (SOU, 1997). The need to substitute antibiotic growth promoters has led researchers and producers to investigate alternatives. Several alternatives such as probiotics, prebiotics and other modifiers of microbial activity are being considered by the livestock industry. Prebiotics are defined as non-digestible food ingredients that benefit the host by stimulating the growth and activity of a limited number of commensal bacteria in the intestine (Gibson and Roberfroid, 1995). The gastrointestinal tract (GIT) of animals contains up to 500 different types of commensals, of which lactic acid bacteria constitute approximately 10%. Commensals contribute to numerous biological functions, such as maintaining a well-balanced flora (Kontula *et al.*, 2000). Like soluble dietary fiber, oligosaccharides such as xylooligosaccharides (XOS), inulin, fructooligosaccharides (FOS) and oligofructose

are not digested in the small intestine, but digested in the large intestine. They are selectively fermented by intestinal flora, resulting in promotion of optimal intestinal function. Along with FOS, oligofructose and inulin, XOS are considered to be emerging prebiotics. Sugar molecules with a degree of polymerization ranging from 2 to 10 are defined as oligosaccharides (Nakakuki, 1993). XOS are xylose based oligomers derived from xylan rich hemicelluloses. Xylan are β -1,4 linked polymers of D-xylose with D-glucuronic acid, L-arabinose, acetyl or phenolic substituents substituted at positions C2 and C3 (Ebringerova and Heinze, 2000). Previous researchers have demonstrated that the intestinal microbiota plays pivotal role in the maintenance of health and in the prevention of disease (Holzapfel and Schillinger, 2002). Zhenping *et al.* (2013) reported 9% increase in body weight gain of broilers fed straw-derived XOS. Yong *et al.* (2005) reported that feeding XOS to layers improved egg shell quality and did not affect the egg shape index and egg yolk rate. Courtin *et al.* (2008) reported that feeding commercial XOS to chickens increased the number of cecal bifidobacteria after two weeks of feeding while treatments did not impact the number of enterobacteria and lactobacilli in ceca.

Corn fiber could be a good source for producing XOS. Corn fiber is a rich source of branched polymer hemicellulose, which is the second most abundant polysaccharide after cellulose in the plant cell wall (Schadel *et al.*, 2010). The hemicelluloses constitute an important group of polysaccharides, linked to microfibrils of cellulose and pectins, the most important are: xylans, arabinoxylans, mannans, galactomannans, glucomannans, arabinogalactan II, beta-1,3-glucan and beta-1,3-beta-1,4-glucans (Heredia *et al.*, 1995). Recently, we demonstrated the production of XOS from corn fiber using an autohydrolysis method, wherein corn fiber was treated with water at high temperatures in an enclosed vessel (Samala *et al.*, 2012). Samala *et al.* (2012) showed that autohydrolysis performed at 180°C with 15 min hold time were the optimum conditions for XOS production. No previous study has evaluated the *in vitro* prebiotic properties of XOS produced from DDGS fiber. The objective of this study was to evaluate and compare prebiotic properties of XOS produced from DDGS fiber (XOS-D) with other substrates such as FOS, commercial XOS (XOS-C), xylose, glucose and inulin, on intestinal bacteria, *B. dolescentis*, *B. breve* and *Lactobacillus brevis*.

MATERIALS AND METHODS

Corn fiber: Corn fiber was obtained from distillers dried grains with solubles (DDGS) by using the Elusieve process, a combination of sieving and air classification. DDGS is a by product of fuel ethanol production from corn. DDGS was procured from a local feed mill and processed to separate fiber using the Elusieve pilot-plant at Mississippi State University (Srinivasan and Columbus, 2009). The large size fiber fraction (size >868 µm) was used in this study and was stored in vacuum sealed bags at 5°C until use.

Xylooligosaccharide production by auto-hydrolysis of fiber at 180°C: Autohydrolysis procedure was same as that described by Samala *et al.* (2012). The auto-hydrolysis of fiber was conducted in a 750 mL Parr reactor (model 4843, Parr Instruments Co., Moline, Illinois, USA) (Fig. 1) heated with temperature control. In each batch, the Parr reactor was filled with 10 g of corn fiber and 90 mL of deionized water. The mixture was carried out at 180°C and held for 15 min. The reaction mixture was filtered by gravity filtration using filter paper (Fisher brand, USA, catalogue no. 09-801E, particle retention 5-10 µm) and a size P5 funnel. The filtrate was further filtered by a vacuum filtration system using a glass fiber prefilter (Millipore, USA) on a Buchner funnel. The reaction mixture was filtered twice to obtain a particle-free solution for HPLC analysis. The solid residue was thoroughly washed with deionized water ranging from 100-120 mL, to ensure complete oligosaccharides removal and dried at room

temperature. The washing was collected in a bottle, labeled as liquor and stored at 0°C.

Characterization of liquor and commercial XOS: The XOS in the liquor and commercial XOS were analyzed by HPLC equipped with a Bio-Rad HPX 42 A column at 80°C and a guard column (Bio-Rad Laboratories, USA) by eluting the column with HPLC grade water (Sigma Aldrich, USA) at a flow-rate of 0.6 mL/min. The XOS standards used were xylobiose, xylotriose, xylotetrose, xylopentose, xylohexose and monomeric xylose (Megazymes, Ireland). The acidic components and sugar degradation products were analyzed by HPLC equipped with a Bio-Rad HPX 87 H (300 X 7.8 mm) column at 80°C and a guard column (Bio-Rad Laboratories, USA) by eluting with 0.005M H₂SO₄ at a flow rate of 0.6 mL/min. Standard acids used were: acetic acid, formic acid and levulinic acid. Degradation compounds analyzed were hydroxymethyl furfural (HMF) and furfural (Sigma Aldrich, USA).

Lyophilization of liquor solution: The autohydrolysis liquor, obtained from corn fiber separated from DDGS at 180°C, was lyophilized using Labanco Lyophilization Equipment (Kansas City, MO) equipped with a high vacuum pump JAVAC (Brook Crompton Betts Pty.) to obtain the xylo-oligosaccharides in powdered form. The conditions were as follows: vacuum pressure of 0.5 mbar and temperature of -40°C.

Nutrient broth media preparation: Three different broths were used in this study: Haas Bushnell Broth (HB), Tryptone Polypeptone Broth (TP) and Polypeptone Soybean Meal Broth (PS). HB Broth was prepared as follows: 15 g Noble agar; 1 g KH₂PO₄; 1 g K₂HPO₄; 1 g NH₄NO₃; 0.2 g MgSO₄ 7 H₂O; 0.05 g FeCl₃; 0.02 g CaCl₂ 2H₂O and glucose (dextrose) 10 g (Sigma-Aldrich, USA) suspended in one liter dH₂O. TP Broth preparation was modified from Wang *et al.* (2010) and consisted of: tryptone (Difco Laboratories, USA), 10 g; polypeptone (Becton Dickinson Microbiology Systems, USA), 5 g; glucose, 5 g; Tween 80, 1 g; yeast extract, 2.5 g (Sigma-Aldrich, USA); cysteine (Sigma Chemical Company, MO, USA), 0.5 g; dipotassium phosphate, 2 g; magnesium chloride hexahydrate, 0.5 g; zinc sulphate heptahydrate, 0.25 g; calcium chloride, 0.15 g; ferric chloride traces (Sigma-Aldrich, USA) suspended in one liter dH₂O (Wang *et al.*, 2010). PS Broth preparation was modified from Moura *et al.* (2007) and contained: polypeptone (Becton Dickinson Microbiology Systems, USA), 22 g; soybean meal papaic digest (Acumedia Manufacturers, Inc, USA), 3 g; sodium chloride, 5 g; yeast nitrogen base (Sigma-Aldrich, USA), 5 g; cysteine hydrochloride (Sigma Chemical Company, MO, USA), 0.5 g; dipotassium phosphate, 2.5 g; ammonium chloride, 0.16 g; calcium chloride, 0.08 g; magnesium chloride hexahydrate, 0.08

g; ferric chloride traces (Sigma-Aldrich, USA) suspended in one liter dH₂O (19). Prepared media were autoclaved at 121°C and 15 psi for 30 min and were cooled to approximately 50°C in a water bath. XOS-C (Dalian Honglu Chemicals Company Ltd., China), XOS-D, Inulin and FOS (Sigma-Aldrich, USA) were added at 10 g per L of prepared broth after cooling.

Organisms and culture conditions: *Bifidobacterium* are the most studied reference genus for prebiotic work in poultry (Patterson *et al.*, 1997). *Lactobacillus* is another genus that plays a major role in poultry (Kizerwetter-Swida and Binek, 2005; Noohi *et al.*, 2014; Jin *et al.*, 1998). Three different organisms were used in this study: *B. adolescentis*, *B. breve* and *Lactobacillus brevis*. The selection of species (*B. adolescentis*, *B. breve* and *L. brevis*) was based on previous research (Crittenden *et al.*, 2002; Garde *et al.*, 2002; Jaskari *et al.*, 1998). *B. adolescentis* ATCC 15703, *B. breve* ATCC 15700 and *Lactobacillus brevis* ATCC 8287 were obtained from American Type Culture Collection (ATCC; Manassas, VA). The bacteria were cultured at 37°C under anaerobic conditions established by an Anoxomat Anaerobic Culture System using the default anaerobic gas mixture (MART Microbiology, Drachten, Netherlands). Cultures were prepared in Clostridial broth (Oxoid) two days prior to performing experiments. Cultures were brought to stationary state at approximately 10⁸ cfu/ml titer (colony forming unit).

The prebiotic properties of XOS-C, XOS-D, Inulin and FOS was evaluated through separate *in vitro* incubations using *B. adolescentis*, *B. breve* and *Lactobacillus brevis*. Each strain was anaerobically grown in separate incubations using XOS-C, XOS-D, Inulin, FOS, glucose, xylose and control in triplicate wells. Each experiment was performed in flat bottom polystyrene 96-well plates in which 150 µL media with XOS-C, XOS-D, inulin, glucose, xylose and FOS was inoculated with 25 µL of bacterial culture. Nutrient base medium with no bacteria was used as a negative control for microplate readings. A TECAN GENios Automated Microplate reader was run with Magellan v. 2.0 software (Tecan; Durham, NC). Optical density readings were measured at 0-7 days post-inoculation at 550 nm. Bacterial growth was recorded in triplicate. 96-well plates were incubated anaerobically with moisture in an Anoxomat Anaerobic Culture System using the default anaerobic gas mixture. In summary, seven different substrates (control, FOS, XOS-C, XOS-D, xylose, glucose and inulin) were evaluated for growth of three different organisms (*B. adolescentis*, *B. breve* and *L. brevis*) on three different growth broths (HB, TS and PS) in three replicates. Growth was reported in terms of maximum optical density at 550 nm and specific growth achieved during a period of 6 days. Optical density at 550 nm (OD₅₅₀) was normalized by taking logarithm of ratio of OD on

Table 1: Substrate (control, FOS, XOS-C, XOS-D, xylose, glucose and inulin) ranking using maximum specific growth for *B. adolescentis*, *L. brevis* and *B. breve* in HB broth, PS Broth and TP Broth

Microbe	<i>L. brevis</i>			<i>B. adolescentis</i>			<i>B. breve</i>		
Rank	TP Broth	PS Broth	HB Broth	TP Broth	PS Broth	HB Broth	TP Broth	PS Broth	HB Broth
1	XOS-C 0.974 ^a	Xylose 0.814 ^a	XOS-C 0.631 ^a	FOS 0.787 ^a	FOS 0.856 ^a	Inulin 0.310 ^a	Glucose 0.916 ^a	Glucose 0.771 ^a	Glucose 0.326 ^a
2	Glucose 0.730 ^{ab}	Glucose 0.751 ^a	Xylose 0.608 ^{ab}	XOS-C 0.718 ^a	XOS-C 0.718 ^a	Control 0.238 ^{ab}	FOS 0.805 ^a	FOS 0.584 ^b	Inulin 0.310 ^a
3	Xylose 0.675 ^{ab}	XOS-C 0.607 ^b	Glucose 0.512 ^b	Inulin 0.502 ^b	Xylose 0.596 ^b	XOS-C 0.208 ^{ab}	XOS-C 0.762 ^a	Xylose 0.502 ^{bc}	FOS 0.213 ^{ab}
4	FOS 0.465 ^{bc}	XOS-D 0.416 ^c	Inulin 0.330 ^c	XOS-D 0.415 ^b	Glucose 0.573 ^b	FOS 0.208 ^{bc}	Xylose 0.471 ^b	XOS-C 0.460 ^{cd}	XOS-C 0.196 ^{ab}
5	XOS-D 0.461 ^{bc}	FOS 0.315 ^c	XOS-D 0.250 ^{cd}	Glucose 0.328 ^{cd}	Inulin 0.362 ^d	Glucose 0.083 ^d	Inulin 0.341 ^b	Control 0.388 ^d	XOS-D 0.128 ^b
6	Control 0.204 ^d	Inulin 0.233 ^d	FOS 0.207 ^d	Xylose 0.244 ^{cd}	XOS-D 0.263 ^d	XOS-D 0.082 ^d	XOS-D 0.267 ^{cd}	Inulin 0.224 ^e	Control 0.108 ^b
7	Inulin 0.177 ^d	Control 0.210 ^d	Control 0.010 ^e	Control 0.207 ^e	Control 0.007 ^e	Xylose 0.062 ^d	Control 0.093 ^e	XOS-D 0.186 ^e	Xylose 0.091 ^b

Values are means of three replicates.

^{a-e} Means within a column with no common superscript differ significantly (p<0.05).

Means in the same column followed by same letter are not significantly different (p<0.05).

The values obtained were mean value of three replicates.

Specific growth is logarithm of the ratio of OD on particular day to OD on day 0, for each organism/substrate/media combination

particular day to OD on day 0, for each organism/ substrate/media combination. Normalized optical density is referred to as specific growth.

Statistical analysis: Analysis of Variance (ANOVA) and Duncan's test (SAS Institute, Cary, NC) were used to compare maximum optical density means of *B. adolescentis*, *L. brevis* and *B. breve* using substrates (control, FOS, XOS-C, XOS-D, xylose, glucose and inulin) on the broths (TP, PS and HB) and for ranking of the substrates. Statistical significance level was 5% ($p < 0.05$). Standard error is calculated as standard deviation of replicates by the square root of number of replicates.

RESULTS AND DISCUSSION

Characterization of XOS-D and XOS-C: Degradation products (furfural and HMF) which act as microbial inhibitors, such as that observed by Carvalho *et al.* (2004) were not present in the liquor solution. The liquor had no traces of formic acid and levulinic acid, which are formed on the degradation of HMF and furfural compounds (Dunlop, 1948; Ulbricht and Thomas, 1984). The liquor contained mainly xylotriose (16.4% yield) and xylopentose (2.2% yield). The liquor also contained monosugars: glucose (14.2% yield), xylose (13.1% yield), arabinose (15.8% yield) and galactose (7.0% yield) (Samala *et al.*, 2012). XOS-C composition was: xylobiose (26.9%), xylotriose (40.0%), xylotetrose (19.6%), xylopentose (7.6%) and xylohexose (5.7%).

Prebiotic properties-three media types: Figures 1-3 are representative growth curves for three different combinations of broth and microbe on all seven substrates. Most microbial growth reached a maximum by day 2; growth thereafter was stationary (Fig. 1-3). The figures demonstrate that growth does occur on XOS-D.

Among the three broths, HB broth resulted in the lowest growth for all tested microbes (Table 1). This can be attributed to the lack of supportive nutrients in HB broth, though, any growth noted in this media could be attributed to the addition of substrate. PS broth and TP broth contain additional nutrients in the form of glucose or soybean meal. PS broth was favorable for monosugars compared to other broths.

Prebiotic properties-bacterium and substrate: Among the three organisms, *L. brevis* performed best (Table 1). There was no clear trend in terms of performance between *B. breve* and *B. adolescentis*. Moura I. (2007) also reported that among the tested strains, *L. brevis* displayed the highest growth and XOS consumption. *B. breve* grew more effectively on monomers (xylose and glucose) in all three broths (Table 1). *B. adolescentis* displayed a high preference for FOS followed by XOS-C over xylooligosaccharides produced from XOS-D (Table 1). *B. breve* grew better on glucose followed by FOS and XOS-C, while *B. Breve* demonstrated significant growth on xylooligosaccharides produced from XOS-D. *L. brevis* displayed highest growth on the XOS-C and similar growth on both XOS-D and FOS. For XOS-D, highest growth recorded was for *L. brevis* (0.461) followed by *B. breve* (0.267) and *B. adolescentis* (0.263). The results of ranking for specific growth rates of *B. adolescentis*, *L. brevis* and *B. breve* using substrates (control, FOS, XOS-C, XOS-D, xylose, glucose and inulin) in the broths (TP, PS and HB) are summarized in Table 1. Among the substrates, all performed better than or equal to the control for all tested bacteria in all three broths, except for *B. adolescentis* in HB Broth (Table 1). Thus, bacteria growth was negligible in the absence of substrate. Monosugars (xylose and glucose) typically enhanced growth over all other substrates for *B. breve* and *L. brevis*; the exception was *B. breve* in HB broth (Table 1).

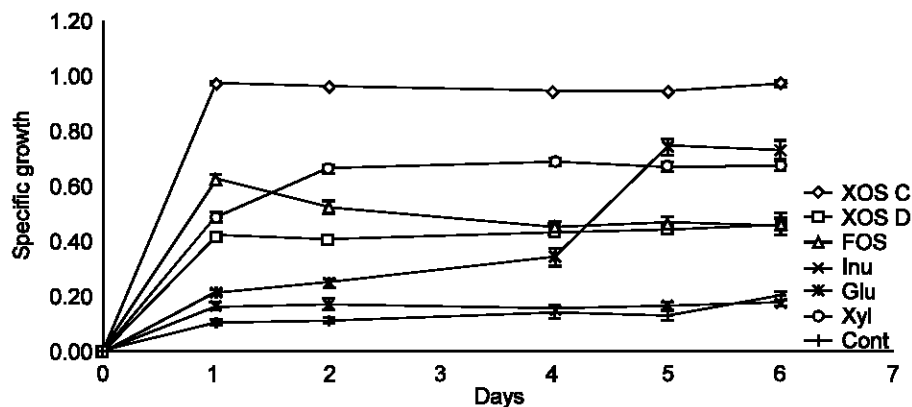


Fig. 1: *Lactobacillus brevis* growth on commercial XOS, XOS-D, FOS, Inulin, Glucose, Xylose and Control (without sugars) in TP broth. Values are means of three replicates. Standard error is calculated as standard deviation of replicates divided by the square root of number of replicates. Specific growth is logarithm of the ratio of OD on particular day to OD on day 0, for each organism/substrate/media combination

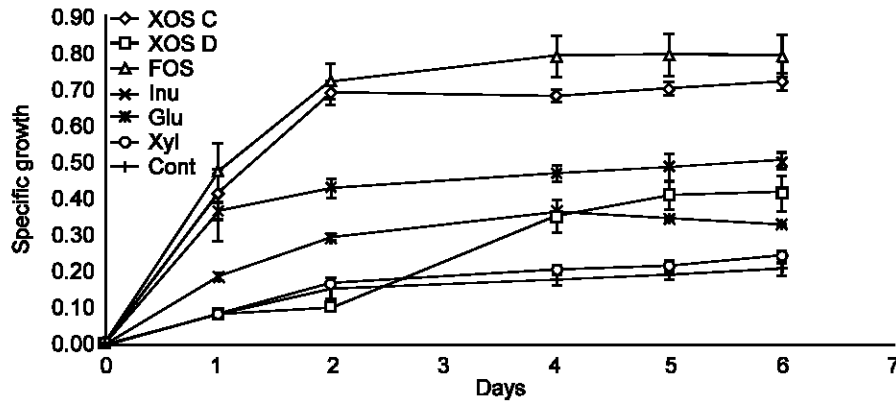


Fig. 2: *Bifidobacterium adolescentis* growth on commercial XOS, XOS-D, FOS, Inulin, Glucose, Xylose and Control (without sugars) in PS broth. Values are means of three replicates. Standard error is calculated as standard deviation of replicates divided by the square root of number of replicates. Specific growth is logarithm of the ratio of OD on particular day to OD on day 0, for each organism/substrate/media combination

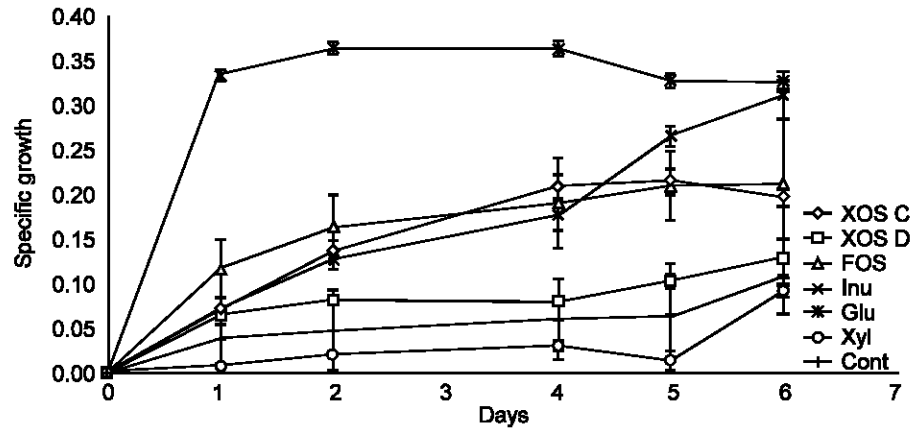


Fig. 3: *Bifidobacterium breve* growth on commercial XOS, XOS-D, FOS, Inulin, Glucose, Xylose and Control (without sugars) in HB broth. Values are means of three replicates. Standard error is calculated as standard deviation of replicates divided by the square root of number of replicates. Specific growth is logarithm of the ratio of OD on particular day to OD on day 0, for each organism/substrate/media combination

Monosugars were not among the top three performing substrates for *B. adolescentis* (in all three broths). Commercial oligosaccharides (FOS, XOS-C and inulin) outperformed other substrates in *B. adolescentis*; however they did not show consistently good performance for *B. breve* and *L. brevis* (Table 1). The exception was FOS, which performed well for *B. breve*. Performance of XOS-C was comparable with other prebiotics (FOS and inulin). The lower performance of XOS-D compared to XOS-C is attributed to the lower concentration of oligosaccharides in XOS-D compared to that in XOS-C. XOS-D showed better performance than the control for all tested bacteria/broth combinations except for *B. breve* in PB broth and *B. adolescentis* in HB broth. XOS-D performance was comparable to FOS and inulin for *L. brevis*, which outperformed other bacteria when grown on XOS-D

(Table 1). Performance of XOS-D substrate and commercial XOS demonstrated that oligosaccharides in XOS-D exhibit potential to be used as prebiotics. Since monosugars were present in XOS-D, the growth of microorganisms specifically from oligosaccharides in XOS-D could not be quantified. Purification of oligosaccharide compounds from XOS-D is required to quantify the performance specifically from oligosaccharides present in XOS-D. Crittenden *et al.* (2002) assessed the prebiotic properties of pure XOS in complex cultures and reported that bacteria from the bifidobacterial strains have ability to utilize XOS. Similar bacterial growth performance on commercial XOS (XOS-C) and XOS-D confirms the validity of this study. Walter *et al.* (2011) discussed symbiotic associations with vast and complex microbial communities of the large intestine, which suggests the complexities of *in vivo*

interactions can't be approached *in vitro* and thus few studies can simulate the many anticipated ecological interactions. An approach for future study would consist of mixed populations mimicking *in vivo* conditions.

Conclusions: XOS-D promoted bacterial growth more than that of control. XOS-D performance was comparable to FOS and inulin for *L. brevis*. Growth on commercial XOS (XOS-C) was comparable with growth on other prebiotics (FOS and inulin). Performance of XOS-D substrate and commercial XOS demonstrated that oligosaccharides in XOS-D exhibit potential to be used as prebiotics. Purification of oligosaccharide compounds from XOS-D is required to quantify the performance specifically from oligosaccharides present in XOS-D. Furthermore, *in vivo* demonstration in poultry is required.

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