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Effect of Prebiotics on Intestinal Microflora and Fermentation Products in Pig *In Vitro* Model

Dong Woon Kim[1]*, Su Jin Chae[1], Sung Back Cho[1], Ok Hwa Hwang[1], Hyun Jeong Lee[1], Wan Tae Chung[1], Jun Cheal Park[1], In Cheul Kim[1] and In Ho Kim[2]

[1]National Institute of Animal Science, R.D.A.,

[2]Department of Animal Resource & Science, Dankook University

ABSTRACT

The objective of this study was to evaluate the effect of the different types and levels of prebiotics on intestinal microflora and fermentation products in the *in vitro* fermentation model. The prebiotcs used in this study were IMO (iso-malto oligosaccharide), CI (partially digested chicory-inulin), RA (raffinose) and CD (cyclodextrin). Experimental diet for growing pigs was predigested by digestive enzymes and this hydrolyzed diet was mixed with buffer solution containing 5% fresh swine feces. Then, the mixture was fermented with or without prebiotics at the concentrations of 0.5 and 1.0% for 24 h. Samples were taken at 24 h, and viable count of micoflora, gas, pH, volatile organic compounds and short-chain fatty acids were determined. The viable count of *Enterobacteriaceae* was significantly decreased ($p<0.001$) in all treatments added with prebiotics in comparison to control without prebiotics. However, the increase of lactic acid bacteria was observed in the prebiotics treatment. Gas production increased as the level of prebiotics increased. The pH values in the fermentation fluid decreased in a dose-dependent manner with increasing the concentration of prebiotics. The fermentation with prebiotics resulted in the reduction of malodorous compounds such as ammonia, hydrogen sulfide, indole and skatole. The increase in short-chain fatty acid (SCFA) production was observed in the treatments with prebiotics. In conclusion, the results of this study demonstrated that the fermentation with prebiotics was effective in reducing the formation of malodorous compounds and increasing lactic acid bacteria and SCFA. These effects depended on the concentration of prebiotics. Moreover, further study is needed to determine whether the *in vitro* efficacy on the reduction of malodorous compounds and increase of SCFA would also be observed in animals.
(**Key words :** *In vitro* fermentation, Prebiotics, Volatile organic compounds, Short-chain fatty acid)

INTRODUCTION

The gut represents a complex and dynamic microbial ecosystem in which microorganisms in the intestine have important and specific metabolic and protective functions (Massi et al., 2006). The main source of substrate for the growth of the gastrointestinal microflora comes from the diet. In addition to diet, the transit time of digestive residues in the large intestine is an important determinant of bacterial metabolism. It is generally accepted that the microflora in the small intestine competes with the host animal for absorbing digestible nutrients and at the same time produces a variety of compounds. Odorous volatile organic compounds (VOC), short-chain volatile fatty acid, and other volatile carbon-, nitrogen-, and sulfur-containing compounds from microbial fermentation in the gastrointestinal tract of pigs can be emitted immediately after feces are excreted. Furthermore, the release of urinary ammonia from the enzymatic conversion of urea can occur within a short time after excretion. Microorganisms may utilize a variety of potential substrates, including starch, proteins, lipids and non-starch polysaccharides, to produce odorous compounds (Mackie et al., 1998).

A different approach to exploit the advantages of probiotics bacteria can be done by administration of prebiotics. Prebiotics are not viable organisms but specific substrates which are selectively metabolized by the potentially beneficial bacteria which have already colonized the intestines. As a result, they enhance the growth of these specific bacterial populations. Non-digestible carbohydrates include miscellaneous compounds such as resistant starch, non-starch polysaccharides, and non-digestible oligosaccharides (Delzenne and Roberfroid, 1994). All of these non digestible carbohydrates are expressed as non digestible polysaccharides

* Corresponding author : Dong Woon Kim, Swine Science Division, National Institute of Animal Science, R.D.A. #9 Eoryong-ri Seonghwan-eup Cheonan-si Chungnam 330-801 Korea. Tel: 82-41-580-3449, Fax: 82-41-580-3459, E-mail: dwkim9405@korea.kr

because they are not hydrolyzed by endogenous enzymes in the small intestine, but hydrolyzed by colonic bacteria in the large intestine.

The equilibration of the colonic biotope has been demonstrated for dietary fructans with other oligosaccharides in many studies in animals and in humans (Delzenne and Williams, 2002). Recent data obtained with fructo-oligosaccharides demonstrate that the dose and the duration of oligosaccharide intake are important factors influencing the extent of the prebiotic's effect (Rao, 2001; Tuohy et al., 2001a, 2001b). Oligosaccharide fermentation products, namely the SCFA, such as acetate, propionate, and butyrate are beneficial to the host (Buddington, 2001). Stewart et al. (1993) summarized the potential beneficial effects of prebiotics as being: antagonism towards pathogens, competition with pathogens, stimulation of enzyme reactions, decreases in ammonia and phenol production, increased colonization resistance. The supplementation of indigestible dietary fiber components in the form of non-starch polysaccharides (NSP) reduced the fecal loss of odorous components (Mroz et al., 2000). We are very interested in nutritional manipulation on the formation of odorous compounds.

Therefore, the purpose of this study was to evaluate the effect of the different types and levels of prebiotics on intestinal fermentation products and microflora in the pig *in vitro* fermentation model.

MATERIALS AND METHODS

1. Experimental design and substrates

The prebiotics used in the *in vitro* fermentation study were IMO (iso-malto oligosaccharide), CI (partially digested chicory-inulin), RA (raffinose) and CD (cyclo-dextrin). The concentrations of prebiotics in fermentation fluid were 0.5 and 1.0%, respectively.

2. Predigestion of diet

Experimental diet for growing pigs (Table 1) was predigested as described by Boisen and Fernandez (1995). This was a stepwise procedure with a 1 g of ground feed in 25 ml of phosphate buffer (0.1 M, pH 6.0) were mixed by vortex. The mixture was adjusted to pH 2 with a 1 M HCl or a 1 M NaOH solution. One ml of prepared pepsin solution (containing 10 mg pepsin) was then added to the mixture, and incubated in a shaking waterbath at 37°C for 4 h. In the second step, 10 ml of phosphate buffer (0.2 M, pH 6.8) plus 5 ml of a 0.6 M NaOH solution was added. The pH was then adjusted to pH 6.8 with a 1 M HCl or a 1 M NaOH solution. One ml of prepared pancreatin solution (containing 50 mg pancreatin) was then added to the mixture, and incubated in a shaking waterbath at 37°C for 18 h. Samples were prepared by freeze drying after heated at 80 °C for 15 min. The predigested diet was used as substrate in an *in vitro* fermentation study.

Table 1. Composition of the diet used for *in vitro* fermentation

Ingredients	%
Corn	70.45
Soybean meal	17.91
Wheat	8.09
Soybean oil	1.00
Limestone	0.97
Dicalcium phosphate	0.61
Vitamin/mineral premix[1)]	0.35
L-lysine HCL	0.27
Salt	0.25
Antibiotics	0.10

[1)] Provided per kg diet: 20,000 IU of vitamin A, 4,000 IU of vitamin D_3, 80 IU of vitamin E, 16 mg of vitamin K_3, 4 mg of thiamine, 20 mg riboflavin, 6 mg of pyridoxine, 0.08 mg of vitamin B_{12}, 20 mg niacin, 50 mg of Ca-pantothenate, 2 mg of folic acid and 0.08 mg biotin, 140 mg of Cu, 179 mg of Zn, 12.5 mg of Mn, 0.5 mg of I, 0.25 mg of Co and 0.4 mg Se.

3. *In vitro* fermentation system

Fresh feces (5%) collected from the rectum of growing pigs were added to sterile buffer containing 1% of predigested feed and prebiotics. The buffer composition (McDougall, 1948) was as follows : 9.8 g $NaHCO_3$, 9.3 g Na_2HPO_4, 0.67 g NaCl, 0.57 g KCl, 0.12 g $MgSO_4 \cdot 6H_2O$, and 0.079 g $CaCl_2 \cdot 6H_2O$ in 1 L of distilled water. Buffer pH was then adjusted to 7.0 by adding 4 M HCl. The buffer solution was kept at 37°C and flushed with CO_2 for 15 min. The incubation temperature was kept at 37°C for 24 h for determination of intestinal fermentation products and microflora. Each substrate was subjected to *in vitro* fermentation at three replicates, and all samples were stored at −20°C until analysis.

4. Sampling and measurements

(1) Gas production and pH

Gas production in the fermentation fluid was analyzed using water displacement apparatus (Ferorak and Hrwdey, 1983). The pH values in fermentation fluid were measured at room temperature as the mixture was stirred on a magnetic stirring plate using an electronic pH meter (HANNA 212, Italia).

(2) Malodor gas emission

Ammonium ions (NH_4^+) in the samples were analyzed with spectrophotometer at 630 nm as described by Chaney and Marbach (1962). Volatile organic compound concentration in the fermentation fluid was analyzed with gas chromatography procedure (Willing et al., 2005). The fermentation liquid was centrifuged and 2.0 ml of the supernatant was mixed with 4 drops of 4 M NaOH, and 1.0 ml of chloroform. The sample was then centrifuged at 3,500 rpm for 10 min and then analyzed on Gas chromatography (Agilent, 6890N, USA) with DB-5ms column. Hydrogen sulfide of the fermentation fluid was determined by Gastec detector (Model GV-100, Gastec, Japan) and Gastec detector tube No. 4LL (Gastec, Japan).

(3) SCFA and microflora

SCFA concentration in the fermentation fluid was analyzed by Gas chromatography (Agilent, 6890N, USA) with HP-INNOW WAX column. The fermentation liquid was centrifuged and 5.0 ml of the supernatant was mixed with 0.05 ml of saturated $HgCl_2$, and 1.0 ml of 25% HPO_3. The mixture was centrifuged at 12,000 rpm for 10 min, after catalysis at room temperature for 20 min. Subsequently, the supernatant was additionally filtrated through a 0.2 mm syringe filter and then analyzed on Gas chromatography. Viable counts of microflora were measured by plating serial 10-fold dilutions onto MRS agar plates for lactic acid bacteria, DHL agar plates for *Enterobacteriaceae*. MRS agar plates were incubated for 48 h at 37°C under anaerobic conditions. DHL agar plates were incubated for 24 h at 37 °C under aerobic conditions.

5. Statistical analysis

All experimental data were analyzed in accordance with the General Linear Model procedure established by the Statistics Analysis Systems Institute (1996). The model included the effects of block (replication) and treatment. Orthogonal contrasts were used to separate treatment means and consisted of 1) CON (control) vs. PRE (prebiotics) and 2) PRE 0.5 vs. PRE 1.0. The variability of all of the data was expressed as the standard error (SE). Significant differences between control and treated groups were expressed at a significant level of $p<0.05$.

RESULTS AND DISSCUSSION

1. Microflora

Counts of viable bacteria after 24 h of *in vitro* incubation of swine fecal microflora with prebiotics or without prebiotics are shown in Table 2. The viable counts of *Enterobacteriaceae* was significantly decreased ($p<0.001$) in all treatments added with prebiotics when compared with CON. However, the number of lactic acid bacteria was higher in all treatments with prebiotics in comparison to CON. Buddington et al. (1996) studied the influence of oligofructose supplementation on the fecal flora of adult humans. The number of *bifidobacteria* was increased after supplementation of oligofructose, and the proportion of total anaerobes represented by *bifidobacteria* was increased from 3.5% to 9.5% during the supplemental period (Wang and Gibson, 1993). The feeding of galacto-oligosaccharides to rats with a human-type microflora has been shown to significantly increase populations of *bifidobacteria* and *lactobacilli*, while decreasing enterobacteria (Rowland and Tanaka, 1993), indicating their potential as prebiotics. Our

Table 2. Counts of viable bacteria after 24 h of *in vitro* incubation using swine fecal microflora with prebiotics or without prebiotics

Item	PRE1, %									SE[2]	P-value for contrasts	
	−	0.5				1.0						
Log cfu/ml	CON	IMO	CI	RA	CD	IMO	CI	RA	CD		CON vs. PRE	PRE 0.5 vs. PRE 1.0
Enterobacteriaceae	7.75	6.62	6.97	7.15	7.00	6.64	6.60	6.44	6.28	0.06	0.001	0.001
Lactic acid bacteria	8.15	8.49	8.31	8.57	8.27	8.41	8.45	8.60	8.51	0.01	0.001	0.001

[1] CON: control; IMO: CON + iso-malto oligosaccharide CI: CON + chicory-inulin RA: CON + raffinose CD: CON + cyclo-dextrin.
[2] Pooled standard error.

results are in agreement with previous reports that the supplementation of oligosaccharides resulted in the increased *lactobacilli* and decreased enterobacteria *in vitro* or *in vivo* system.

2. Gas production and change in pH

Effects of prebiotics on gas production are presented in Table 3. Fermentation with four different oligosaccharides at the concentration of 0.5 % resulted in increased amount of gas production when compared with CON ($p<0.001$). Also, higher gas production was recorded in increasing level of prebiotics ($p<0.001$). It has been known that intestinal gases like H_2, CO_2, and CH_4 originated form colonic fermentation of non-digestible oligosaccharides (Delzenne and Roberfroid, 19994). Our data indicate that the increase in gas production resulting from fermentation of prebiotics tested may provide indirect evidence that genera other than bifidobacteria and lactobacilli were stimulated since they do not produce gas during homolactic fermentation.

The pH in the fermentation fluid was significantly decreased ($p<0.001$) in the prebiotics treatments, particularly with the addition of 1.0% prebiotics ($p<0.001$). Flickinger et al. (2000) reported a 17% decrease in pH values when short-chain fructo-oligosaccharides were fermented with canine fecal microflora. The decrease of pH in the caecal contents was shown to favour the growth of lactic acid producing bacteria such as *Lactobacillus* and *Bifidobacterium* species (Gottschalk, 1979; Hill, 1983). Our data indicate that the decrease in pH with prebiotcs addition results from increased population of lactic acid producing bacteria and their metabolites such as lactic acid and acetic acid.

3. Malodor gas emission

The effects of prebiotics on the production of malodor gas emission compounds are shown in Table 4. The reduction of ammonia, hydrogen sulfide, indole and skatole emissions was observed in the prebiotics treatment groups ($p<0.001$), particularly in the 1.0% prebiotics treatment ($p<0.001$). It was shown that the beneficial intestinal bacteria synthesize their fermentation products that provide energy to the colon epithelium, and decrease the formation of major malodorous compounds (Rideout et al., 2004). Our results indicate that the decrease of malodorous compounds might be probably due to the low pH, which may depress the metabolic activity of specific microbial species producing enzymes such as tryptophanase and cystein desulfunase which catalyze the

Table 3. Gas production and pH after 24 h of *in vitro* incubation using swine fecal microflora with prebiotics or without prebiotics

Item	PRE[1], %									SE[2]	P-value for contrasts	
	−	0.5				1.0						
	CON	IMO	CI	RA	CD	IMO	CI	RA	CD		CON vs. PRE	PRE 0.5 vs. PRE 1.0
Gas production (ml)	16.33	26.68	27.33	24.00	25.33	30.68	29.33	28.68	29.68	0.67	0.001	0.001
pH	6.63	6.25	6.22	6.30	6.16	5.86	5.77	5.55	5.87	0.01	0.001	0.001

[1] CON: control; IMO: CON + iso-malto oligosaccharide CI: CON + chicory-inulin RA: CON + raffinose CD: CON + cyclo-dextrin.
[2] Pooled standard error.

Table 4. Malodor gas emission after 24 h of *in vitro* incubation using swine fecal microflora with prebiotics or without prebiotics

Item	PRE[1], %									SE[2]	P-value for contrasts	
	−	0.5				1.0						
ppm	CON	IMO	CI	RA	CD	IMO	CI	RA	CD		CON vs. PRE	PRE 0.5 vs. PRE 1.0
NH_4^+	253.83	114.83	62.67	120.83	121.33	17.83	5.00	49.17	26.83	2.07	0.001	0.001
H_2S	135.25	140.00	86.00	120.00	117.00	113.25	28.75	105.75	29.68	1.63	0.001	0.001
4-methyl phenol	6.48	6.53	5.32	5.72	6.23	6.36	5.22	6.48	5.65	0.23	0.030	0.030
Indole	3.49	3.44	2.86	2.90	3.34	3.43	2.94	2.97	2.93	0.06	0.001	0.001
Skatole	8.56	6.41	5.65	5.70	6.04	4.92	2.86	4.72	3.84	0.14	0.001	0.001

[1] CON: control; IMO: CON + iso-malto oligosaccharide CI: CON + chicory-inulin RA: CON + raffinose CD: CON + cyclo-dextrin.
[2] Pooled standard error.

Table 5. Short-chain fatty acid production after 24 h of *in vitro* incubation using swine fecal microflora with prebiotics or without prebiotics

Item	PRE[1], %									SE[2]	P-value for contrasts	
	−	0.5				1.0						
ppm	CON	IMO	CI	RA	CD	IMO	CI	RA	CD		CON vs. PRE	PRE 0.5 vs. PRE 1.0
Acetic acid	740.82	1096.86	1068.36	938.34	1052.62	1334.43	1193.79	1212.63	1111.62	19.39	0.001	0.001
Propionic acid	831.67	1382.92	1457.99	1367.69	1668.85	1837.42	2073.96	2116.15	1921.98	31.54	0.001	0.001
Iso-butyric acid	43.94	33.36	31.65	26.83	33.24	31.98	28.95	31.54	31.67	0.64	0.001	0.001
Butyric acid	458.25	590.61	525.08	473.52	550.16	851.79	768.69	707.52	813.43	15.98	0.001	0.001
Iso-valeric acid	87.83	62.64	61.12	52.71	62.47	59.22	63.70	59.78	68.70	1.38	0.001	0.001
Valeric acid	264.64	342.84	337.22	296.85	302.52	487.90	546.90	475.93	498.27	7.94	0.001	0.001

[1] CON: control; IMO: CON + iso-malto oligosaccharide CI: CON + chicory-inulin RA: CON + raffinose CD: CON + cyclo-dextrin.
[2] Pooled standard error.

formation of indole, skatole, hydrogen sulfide and ammonia.

4. SCFA production

Effects of prebiotics on the SCFA production are presented in Table 5. The concentrations of acetic acid, propionic acid, butyric acid and valeric acid in the prebiotics treatment group were significantly higher ($p<0.001$) than CON, while the fermentation with prebiotics resulted in the reduction of iso-butyric acid and iso-valeric acid production. SCFA play several important roles in animal metabolism. Roediger (1982) reported that butyrate was the preferential energy source of colonocytes in rats. Additionally, SCFA can contribute up to 28% of the total maintenance energy requirement of pigs (Imoto and Namioka, 1978). Inulin consumption generally yields high levels of SCFA in the rat model (Levrat et al., 1993; Younes et al., 2001). The pH in the colon was lower in pigs fed 3% fructo-oligosaccharide (FOS) diet and this is probably due to an increase of SCFA production in the proximal colon (Gibson et al., 1995). It was suggested that FOS given at the high level may stimulate fermentation in the large intestine, and then the production of SCFA may exceed the absorptive capacity. Flickinger et al. (2000) reported an increase in acetate, propionate and butyrate production after 11 h of short-chain fructo-oligosaccharide fermentation. The SCFA production data presented by Flickinger et al. (2000) are similar with our data for *in vitro* fermentation of CI which is partially digested chicory inulin including about 20% of fructo-oligosaccharide. Cyclodextrins are cyclic oligosaccharides consisting of six α-cyclodextrin, seven β-cyclodextrin, eight ɣ-cyclodextrin or more glucopyranose units. They are not metabolized in the upper intestinal tract but broken down only by intestinal bacteria in caecum and colon after oral administration to rats (Martin Del Valle, 2004). It is interesting to note that the fermentation of CD resulted in the decrease in malodor gas emission compounds and increase of lactic acid bacteria and SCFA, and thus, CD could be utilized to serve as fermentative substrate in the large intestine of swine.

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