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Prebiotic Properties of Levan in Rats

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Abstract Generally, two different types of fructose polymer are found in nature. One is inulin, whose fructosyl residues are linked mainly by a β-(2,1)-linkage, while the other is high-molecular-weight levan, whose fructosyl residues are linked mainly by a β-(2,6)-linkage. In contrast to the extensive studies on the prebiotic properties of inulin, there has been no report on the effect of levan on the large bowel microflora *in vivo*. Therefore, to examine whether dietary levan can be used as a prebiotic, Sprague-Dawley male rats were fed one of two diets for 3 weeks: 1) basal diet plus sucrose; 2) basal diet plus 10% (wt/wt) levan. The cecal bowel mass, cecal and colon short-chain fatty acids (SCFAs), pH, and microflora were then compared. The intake of the levan-containing diet significantly increased the total cecal weight and wall weight. The analyses of the SCFAs in the cecal and colonic contents revealed that levan was converted into acetate, butyrate, and lactate, which resulted in acidic conditions. The intake of levan also significantly increased the total number of microorganisms by 5-fold and lactic acid-producing bacteria (LAB) 30-fold in the feces. Accordingly, the current work shows that levan can be used as a prebiotic for stimulating the growth of LAB in an animal model.

Key words: Levan, prebiotics, lactic acid-producing bacteria

The production of functional foods that provide health benefits is one of the fastest developing fields in the food industry [14]. Of the most popular functional foods, non- or partially-digestible inulin-type fructooligosaccharides (FOS), has been shown to stimulate the growth of lactic acid-producing bacteria (LAB) and limit the growth of competing pathogenic organisms [14, 18]. Fructan, a widely distributed biopolymer in nature, is a homopolysaccharide composed of D-fructofuranosyl residues joined by β-(2,6) and β-(2,1) linkages, and inulin and levan are two types of fructan, distinguishable by the type of linkage present. Chemically, levan consists of β-D-fructofuranosyl residues linked predominantly by β-(2,6) as a 6-kestose of the basic trisaccharide, with extensive branching through β-(2,1) linkages. In contrast, inulin is composed of β-D-fructofuranose attached by β-(2,1) linkages [5].

Levan is found in plants and especially in the bioproducts of microorganisms. Plant levans, graminans and phleins, have shorter residues (varying from 10 to approximately 200 fructose residues) than microbial levans, which have molecular masses of up to several million daltons with multiple branches. Microbial levans are produced from a wide range of taxa, such as bacteria, yeasts, and fungi [5, 10, 13, 14]. The production and utilization of levan at an industrial level are not well developed, and only a few papers have reported on the production of levan using fermentation techniques [5, 6, 12, 19]. Recently, the enzymatic process for the efficient production of levan was developed using a levansucrase (sucrose-6-fructosyltransferase, EC 2.4.1.10) isolated from *Zymomonas mobilis* [26].

One advantage of fructose polymers is their ability to be fermented by gut microflora [27], which in turn improves the intestinal flora and increases the mineral absorption [20, 21]. Characteristically, these compounds pass through the small intestine without being digested and are fermented in the large intestine. Specifically, dietary inulin and its fructooligosaccharides have been shown to markedly increase *Bifidobacterium* and *Lactobacillus* in healthy humans and animals [8, 23, 25, 29].

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Levan is naturally present in various food products and thus is regularly consumed by humans. However, levan has been generally ignored as a functional food ingredient until recently, because of limited resources [5]. Furthermore, it has been argued that, unlike inulin, levan can not be used as a carbon source in animals and humans due to its high molecular weight and branched structure [15]. This hypothesis is supported by the observation that LAB do not utilize levan as a carbon source in *in vitro* experiments [15, 28]. Preliminary experiments by the current authors determined the effects of pH, enzymes, and various bacterial species on the size of levan *in vitro*, and demonstrated active hydrolysis of levan at an acidic pH, suggesting that the *in vivo* fermentation of levan may be different from that under *in vitro* conditions [15, 28]. To date, there have been no previous studies on the effect of ingested levan on the large bowel microflora. Accordingly, we undertook to evaluate the *in vivo* effects of levan on the cecal weight, cecal and colon SCFAs (short-chain fatty acids) concentration, pH, and level of intestinal microbiota in rats.

MATERIALS AND METHODS

Materials and Media Preparation

The levan, synthesized by the enzyme levansucrase from *Z. mobilis* [26], was obtained from RealBioTec Co. Ltd. (Taejon, Korea). The estimated molecular mass of the levan was about 6×10^6 dalton [11]. To identify the anaerobic microbes in the feces, two media were used: GAM agar and LAB agar. The GAM agar (Nissui Pharm. Co., Ltd., Tokyo, Japan) was used for the total cell counts. For the LAB enumeration, the cells were grown in an LAB agar containing 10 g of peptone, 10 g of lactose, 10 g of yeast extract, 25 g of sodium acetate, 0.02 g of L-cysteine hydrochloride, 1 g of ascorbic acid, 5 g of calcium carbonate, 0.06 g of bromocresol purple, and 15 g of agar per liter, at pH 6.8.

Experimental Animals and Sampling Procedures

Sixteen Sprague-Dawley male rats (Korea Center for Experimental Animals, Seoul, Korea), weighing about 210 g each, were divided in to two groups according to their body weight. The animals were given free access to food and water during the adaptation period. The rats were divided into two groups (n=8) and fed with a control diet or 10% levan diet for 3 weeks (Table 1). The rats were housed individually in stainless steel cages with mesh bottoms. The cages were placed in a room with controlled temperature (22±2°C), relative humidity (50±5%), and 12-h light/dark cycle. At night on the final day of the experiment, the food was removed for 12–14 h. The fresh feces were collected and immediately used for viable cell counts. Blood was drawn from the heart under anesthetization with pentobarbitol, and cecum and colon samples were removed. The cecal and colonic contents were transferred into two microfuges; one was immediately used for an SCFA and lactate analysis, while the pH of the cecal and colonic contents was measured in the other one. Following the removal of the appropriate samples, the tissues were cleaned with water, blotted dry, and weighed to determine the cecal and colonic wall weight.

Table 1. Composition of control and levan diets (g/kg).

Constituents	Control diet	Levan diet
Casein	250	250
Corn oil	50	50
Cellulose	50	50
Sucrose	600	500
Levan	0	100
Choline chloride	4	4
Mineral mix[a]	10	10
Vitamin mix[b]	36	36

[a]AIN-76 Mineral mix; Dyets, Bethlehem, Pennsylvania, U.S.A.
[b]AIN-76A Vitamin mix; Dyets, Bethlehem, Pennsylvania, U.S.A.

In Vivo Fermentation of Levan by Lumen Microbes

To determine the distribution of the intestinal bacteria, fresh fecal specimens were collected at the end of the experiment. After homogenization of the individual samples, the specimens (1 g) were dissolved in 9 ml of an anaerobic diluent [16], mixed thoroughly, and then a series of 10-fold dilutions (10^{-1} to 10^{-8}) were prepared. According to the method of Mitsuoka [16], 0.1 ml of appropriate dilutions was spread onto plates (GAM agar and LAB agar plates) and anaerobic incubation was carried out. The viable cells were counted after 2–3 days of incubation, and the colony forming units (CFU) per gram of wet sample were calculated. The numbers of LAB were calculated as the number of cells forming a clear zone around the colony.

Analysis of Short-Chain Fatty Acids

For the SCFA analysis, approximately 0.5 g of the cecal and colonic contents was acidified with 1% phosphoric acid (3×0.33 ml) and centrifuged at 12,000 ×g for 10 min. The supernatants were then combined and immediately used for the SCFA and lactate analysis. The SCFA concentrations were analyzed using a Hewlett-Packard (Wilmington, DE, U.S.A.) 5890A series II gas chromatograph (GC) equipped with a 30 m Qmegawax 320 column (0.25 μm film, 0.32 mm, i.d., Supelco, Bellefonte, PA, U.S.A.). Helium was used as the carrier gas at a flow rate of 25 ml/min. The detector temperature was 260°C and injector temperature 250°C. The oven temperature was initially set at 70°C for 1 min, then increased to 200°C by 10°C/min. Finally, the temperature was maintained at 200°C for 5 min. The split ratio was 10:1. The identity of the individual SCFAs was established by comparing the retention times with those of standard SCFAs containing a known amount of the SCFA

(Sigma). To quantify the acetate and lactate in the cecal and colonic contents, the supernatant obtained above was analyzed by HPLC using a UV detector (set at 204 nm) and column (PRP-X300, 250×4.1 mm, 7 μm, Alltech). Five mN H_2SO_4 was used as the mobile phase at 2 ml/min.

Statistical Analysis

Data analyses were performed using the Statistical Analysis System (SAS) program (SAS Institute, Cary NC, U.S.A.). All statistical analyses were performed using a one-way analysis of variance, and the differences between the means were tested using a paired t-test. The correlations were analyzed according to the Pearson correlation (CORR) procedure of SAS. A *P*-value of 0.05 was considered significant.

Results

Food Intake, Body Weight, and Various Cecal Characteristics

The levan diet had no influence on the food intake, final body weight, and organ (heart, liver, and kidney) weights of the rats (Table 2). In contrast, the levan diet significantly increased the total weight of the cecal and cecal wall weight. Generally, in the rats fed the levan diet, the cecum was 3-4 fold heavier than that of the control group (1.76±0.29 g for control diet and 6.56±0.91 g for levan diet) (Table 2).

SCFA Concentrations in Cecal and Colon Contents of Rat

Four different approaches were used to explore the putative prebiotic effects of levan: GC analysis of SCFA (acetate, propionate, butyrate, valerate) concentrations, HPLC analysis of acetate and lactate concentrations, pH measurements of cecal and colonic contents, and enumeration of LAB and total anaerobes in the feces.

In the GC analysis of the SCFAs from the cecal and colonic contents, acetate, butyrate, and propionate were identified as the major SCFAs, while valerate was detected as a minor component (Table 3). The most noticeable difference between the control and the 10% levan diet was an increase in the proportion of acetate, which was more dramatic in the cecum (24.3 to 259.8 μmol/cecum content), with a smaller degree of increment in the colon (34.9 to 123.2 μmol/colon content) (Table 3). The levan diet also increased the level of butyrate in both the cecum and colon compared to the control diet.

In the cecum and colon, lactate and acetate can be produced by intestinal bacteria, particularly by LAB [27]. In general, the molar ratio of lactate to acetate in the colon was higher than that in the cecum, suggesting that as fermentation progressed through the cecum to the colon, lactate production increased. The levan diet enhanced the

Table 2. Intake of levan increased total cecal weight and wall weight.

	Control diet	Levan diet
Food intake[a], g/d	19.37±1.72	19.17±1.97
Body weight, g	238±22	236±25
Organ weight, g		
Heart	0.88±0.15	0.86±0.09
Liver	8.66±0.72	8.79±1.43
Kidney	2.09±0.34	2.06±0.29
Total weight, g		
Small intestine	6.97±0.98	6.87±0.91
Cecum	1.76±0.29	6.56±0.91***
Colon	2.60±0.42	2.74±0.79
Wall weight, g		
Small intestine	4.46±1.05	4.35±1.23
Cecum	0.45±0.14	1.03±0.31***
Colon	1.12±0.33	1.14±0.64

Each value is the mean±SD for 8 rats. ***, $P<0.0001$, significantly different from the control diet based on the t-test.
[a]Data are from last 3 d results.

Table 3. Intake of levan increased level of acetate and butyrate in the cecum and colon.

	Control diet	Levan diet
Cecum (μmol per gram of cecum content)		
Acetate	24.3±3.7	259.8±43.4***
Propionate	9.4±1.9	23.3±14.8*
Butyrate	3.2±0.6	15.5±5.5**
Valerate	0.5±0.3	-
Total	37.4±5.0	298.6±4.7***
Lactate	0.5±0.0	14.6±2.4**
Colon (μmol per gram of colon content)		
Acetate	34.9±7.5	123.2±21.1***
Propionate	4.8±2.2	2.9±1.5
Butyrate	1.9±0.6	9.5±2.0***
Valerate	-	-
Total	41.6±8.2	135.6±21.9***
Lactate	1.4±0.3	12.1±2.1*

[a]Each value is the mean±SD for 8 rats. *, $P<0.05$; **, $P<0.001$; ***, $P<0.0001$, significantly different from the control diet based on the t-test.

Table 4. Correlation analysis of cecal and colon data for rats fed two different diets.

	Cecum			
	Acetate	Propionate	Butyrate	Lactate
Colon				
Acetate	0.9759**	0.7503*	0.9249*	-0.8648*
Propionate	-0.5246	0.0585	-0.4719	-0.2185
Butyrate	0.9800***	0.8270*	-0.7039*	-0.7310
Lactate	0.7730*	0.9120*	-0.7971*	-0.8002
Cecal and colonic pH correlation=0.9011***				

*, $P<0.05$; **, $P<0.001$; ***, $P<0.0001$.

Table 5. Intake of levan changed cecal and colonic content pH to acidic conditions.

	Control diet	Levan diet
Small intestine	6.97±0.14	6.88±0.22
Cecum	7.15±0.25	5.54±0.38***
Colon	6.71±0.46	4.99±0.26***

Each value is the mean±SD for 8 rats. ***, $P<0.0001$, significantly different from the control diet based on the t-test.

lactate production and ultimately increased the molar ratio of lactate to acetate (2.95 in the cecum contents and 5.21 in the colon contents) (Table 3). A correlation analysis between the cecal and colon data is presented in Table 4. The concentrations of SCFAs (acetate and butyrate) in the cecal and colonic contents were found to be correlated.

pH of the Cecum and Colon

The dramatic increase in acetate and lactate (Table 3) with the intake of levan may be explained by the degradation of levan and following acidification. Thus, to test this hypothesis, the pHs from small intestine, cecal, and colon contents were measured, however, no differences were found in pH of the small intestine contents from the rats fed the two different diets (pH 6.97 with the control diet and pH 6.88 with the 10% levan diet), indicating that the small intestine was not the location of the fermentation of the levan (Table 5). The pH values for the cecum and colon samples from the rats given the levan diet were 5.54±0.38 and 4.99±0.26, respectively, while those for the rats given the control diet were 7.15±0.25 and 6.71±0.46, respectively (Table 5). Overall, the cecal and colon pHs were found to be correlated ($r=0.9011$) ($P<0.0001$) (Table 4).

Bacterial Concentrations

To evaluate the effects of the levan diet on the intestinal microbes and LAB, viable cell counts were performed on GAM and LAB agar plates using the samples collected from the feces. The total number of bacteria counted was 3.3×10^9 for the control diet and 1.7×10^{10} for the levan diet (Table 6). The number of LAB in the control diet and levan diet were 3.0×10^7 and 9.0×10^8 CFU/g of wet feces, respectively, demonstrating that the levan diet significantly increased the number of LAB 30-fold when compared to that with the control diet.

Table 6. Intake of levan increased total anaerobic microbes and lactic acid-producing bacteria in feces.

(log CFU[a]/g wet feces)

	Control diet	Levan diet
Total cells[b]	9.51±0.15	10.17±0.29*
Lactic acid-producing bacteria[c]	7.40±0.25	8.89±0.28*

*, $P<0.05$, significantly different from the control diet based on the t-test.
[a]CFU: colony forming units.
[b]The enumeration of the total viable cells was performed in triplicate on a GAM agar plate.
[c]The cell counts for LAB were performed in triplicate on an LAB agar plate.

Fate of Levan

The presence of levan or its metabolites in the feces of the rats fed levan was investigated by acid hydrolysis of the feces following an HPLC analysis. No or only a trace amount of fructose liberated from levan was detected. In addition, no levan or levan-oligosaccharides was recovered from the urine, indicating no significant absorption. The stool consistency was monitored throughout the study and no visible difference was noted between the groups (data not shown).

DISCUSSION

The majority of previous studies on the prebiotic effects of fructose polymers (fructan) have concentrated on inulin-type fructans [2, 4, 7]. Gibson and Roberfroid [4] defined a prebiotic as a nondigestible and nonabsorbable food ingredient in the upper part of the gastrointestinal tract that beneficially affects the host by selectively stimulating the growth and/or activity of one or a limited number of bacteria in the colon, thereby improving the health of the host. Therefore, nondigestible carbohydrates, in particular inulin-type oligosaccharides, can be classified as authentic prebiotics [3]. Inulin-type fructans are distinct from other polymers in terms of their specific fermentation in human and animal intestines [7]. *In vivo*, inulin and inulin-oligosaccharides are both utilized by LAB, including *Bifidobacterium* species [4]. Generally, fructooligosaccharides mean β-2,1-D-fructans with a DP (degree of polymerization) varying between 2 and 20 (oligofructose) and 20-60 (inulin). The fructan used in the present work was levan, which is commonly found in various natural foods [5, 17, 22]. Levan is a homopolysaccharide composed of D-fructofuranosyl residues joined by β-2,6 (as the major linkages) and β-2,1 linkages, of which molecular mass are up to several million daltons with multiple branches. The physiological effects of fructan are dependent on the size and linkage type; nonetheless, only limited data concerning the fermentability of levan has been published, mainly due to limited resources. Thus, the aim of the current study was to investigate whether levan could modulate the gut microflora. For the control group, levan was replaced with sucrose. Since chemical and physical properties of levan and sucrose are so different, the present observation have to be further evaluated using nonfermentable fiber such as cellulose as the supplement.

A possible explanation for the fermentation of levan in the current work could be that the acid hydrolysis of levan

in the stomach produced a smaller size of levan or levan-oligosaccharides, which was then fully utilized by lumen bacteria. One of the important beneficial properties of lumen bacteria would seem to be the formation of SCFAs in the intestines. These SCFAs limit the growth of harmful lumen bacteria and are an important energy source for the host [27]. We demonstrated that the ability of the 10% levan-containing diet to elevate the cecal total SCFA pool relative to the control diet might have been mainly due to the formation of acetate and butyrate (Table 3). Similar effects have also been demonstrated by Campbell *et al.* [2] in rats fed inulin-type oligosaccharides, cellulose, and xylooligosaccharides at 6% of the dietary weight. The butyrate formation rate was higher in the inulin-type oligoaccharides-fed rats, compared to the rats fed xylooligosaccharides, with acetate being the primary SCFA followed by butyrate. In the present study, the level of butyrate was higher in the rats fed the levan diet compared to those fed the control diet. This was anticipated due to the higher amount of available fermentable fiber produced by the levan diet. The SCFA concentrations in the colonic contents were lower than those in the cecal contents from the rats fed the levan diet (Table 3), which was also expected, because SCFAs are rapidly absorbed via intestinal mucosa [27]. Furthermore, the composition of the SCFAs and lactate in the cecal and colonic contents was different in the current work, particularly propionate and lactate. This might have been due to the differences in the SCFA absorption rates, as lactate is absorbed much more slowly than other SCFAs [9, 27]. Because of this fluctuation in the SCFAs in the colon and cecal contents, it has been suggested that the pH value is the most reliable index for inferring intestinal fermentation [9]. In Table 5, the acidic cecal and colonic pH resulting from ingestion of the levan diet was probably caused by the greater production of total SCFAs (Table 3). Although the identity of the bacteria responsible for the levan fermentation was not clarified, it is quite possible that the levan was degraded, then fully fermented by lumen bacteria in the cecum and colon of the rats.

Therefore, since the levan was fully utilized by the lumen bacteria, as with inulin [24], no fructose, levan-oligosaccharides, or levan was detected in the feces from the rats fed with the 10% levan diet. Based on the results of human fecal slurries together with *in vitro* analyses and fermentation studies, it has been demonstrated that inulin and its oligosaccharides all disappear rapidly (4–5 h) and are quantitatively fermented in the large intestine [24]. However, this would not seem to be the case with levan. Results from *in vitro* fermentations [7, 15, 28] using various bacteria and/or human fecal slurries have shown that the enzymatic hydrolysis (or bacterial fermentation) of levan is negligible.

It has well been known that acetate and lactate are the major products from the fermentation of carbon sources by LAB [15]. In the present study, the level of lactate and number of LAB were both significantly higher in the rats fed the levan diet, thereby potentially changing the microbial ecology in the colon. No previous analyses of the SCFAs, lactate, and bacterial enumeration resulting from the dietary supplement of levan have been reported, therefore the present data need to be evaluated by further investigations. It has been suggested that the maximum specific growth rate of Bifidobacteria was high on oligofructose, followed by inulin, and low on glucose [4]. This selectivity appears to be typically due to the specific β(2-1) bond between the fructose units in the fructan chain. Therefore, the high level of lactate in the present study might be attributed to the fermentation of levan by lumen LAB.

From the current observation, it is not clear whether intakes of levan also increase the number of LAB in humans. Follow-up investigations to identify the bacteria responsible for levan hydrolysis and the active substances for the proliferation of LAB in animal model and human subjects are also needed.

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