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Different effects of difructose anhydride III and inulin-type fructans on caecal microbiota in rats

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Abstract
The effects of different kinds of inulin-type fructans on caecal microbiota were evaluated in rats. Four groups of male Wistar rats were fed either a control diet, or diets containing 5% inulin, 5% fructooligosaccharides (FOS), or 5% difructose anhydride III (DFAIII) for two weeks. In the DFAIII group, caecal propionate, butyrate, counts of bifidobacteria, and total anaerobes were lower than in the inulin group, while caecal propionate, succinate, counts of bifidobacteria, and total anaerobes were lower than in the FOS group. Compared to controls, in the DFAIII group the counts of clostridia in caecum were increased by 3 log units. However, this change was statistically not significant. There were no differences between inulin and FOS groups for the pool of short chain fatty acids in caecum and bacterial counts. Results indicate that DFAIII has different effects on caecal microbiota compared to inulin and FOS and that these differences are most likely due to the $\alpha(3\rightarrow2)$ bonds in DFAIII.

Keywords: *Difructose anhydride III, fructooligosaccharides, inulin, short chain fatty acids, caecum*

1. Introduction

Prebiotic effects of non-digestible saccharides have been extensively evaluated. Inulin is a mixture of molecules consisting of fructose moieties linked to each other by $\beta(2\rightarrow1)$ bonds, which are called fructans. Glucose molecules may be linked to the end of chain by a $\beta(1\rightarrow2)$ bond, such as occurs in sucrose. The commercial inulin product has an average degree of polymerization (DP) of 25 and a molecular distribution ranging from 11 – 60. Fructooligosaccharides or oligofructose (FOS) are inulin-type fructans with a smaller DP than seen in commercial inulin products (Niness, 1999; Roberfroid, 2005). Inulin and FOS have been shown to have prebiotic effects, and in addition, they especially increase bifidobacteria *in vitro* (Kaplan & Hutkins, 2000; Roberfroid et al., 1998), in rats (Campbell et al., 1997; Sakai et al.,

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2001), in rats associated with human faecal microbiota (Djouzi & Andrieux, 1997; Klessen et al., 2001), and in humans (Bouhnik et al., 1999; Kruse et al., 1999).

Difructose anhydride III (DFAIII) is a non-digestible disaccharide that has recently been manufactured from inulin by microbial fermentation (Yokota et al., 1991; Saito & Tomita, 2000). Ingestion of DFAIII stimulates calcium absorption from the rat intestine more effectively than ingestion of FOS (Suzuki et al., 1998). However, there have been no reports evaluating the difference in the effect of caecal microbiota between DFAIII and other inulin-type fructans. DFAIII has a $\beta(2\rightarrow1)$ bond within two fructose moieties; however, it also has an extra $\alpha(3\rightarrow2)$ bond between the moieties. Using a breath hydrogen test, which is an indicator of intestinal bacterial fermentation, we have shown that DFAIII was slightly fermentable during 8 h after ingestion in humans (Tamura et al., 2003). Furthermore, prolonged ingestion of DFAIII did not influence the low fermentability of DFAIII (Tamura et al., 2004). These results suggest that DFAIII may be resistant to intestinal bacterial fermentation in humans. Meanwhile, it has been shown that DFAIII lowered the pH of caecal contents in rats (Suzuki et al., 1998; Mitamura et al., 2002; Afsana et al., 2003), implying that DFAIII was fermented by caecal microbiota. Additionally, a DFAIII fermenting bacterium, *Ruminococcus sp. M-1*, has been isolated from the caecum of rats fed DFAIII (Minamida et al., 2005). These diverse results in humans and rats may indicate that DFAIII has a potential prebiotic effect with a unique fermentability.

The aim of this study was to determine the effect of DP and the extra glycoside linkage of the inulin-type fructan on the caecal microbiota in rats. In addition, to determine if the compound has a potential as prebiotic food material, the study also examined whether DFAIII has any beneficial effect on caecal microbiota.

2. Materials and methods

2.1. Test substances

DFAIII (>97%) was purchased from Nippon Beet Sugar Mfg. Co. Ltd. (Tokyo, Japan). Inulin (Raftilin HP, >99% inulin with a DP between 5 and 60 and $<0.5\%$ glucose, fructose and sucrose) was a gift from Nippon Beet Sugar Mfg. Co. Ltd. (Tokyo, Japan). FOS (Meioligo P, >95% FOS with a main DP of 2–4) was purchased from Meiji Seika Kaisha, Ltd. (Tokyo, Japan).

2.2. Experimental design

The study was approved by the Nayoro City College Animal Use Committee, and animals were maintained in accordance with the guidelines for the care and use of laboratory animals, Nayoro City College.

Male Sprague-Dawley rats ($n=30$) were obtained from Japan SLC, Inc. (Hamamatsu, Japan) at four weeks of age. They were housed in individual stainless steel cages with screen bottoms in a room maintained at $23 \pm 1^{\circ}$C with lighting from 07:00 to 19:00 h. For the first nine days, all rats were fed the basal diet (Table I) *ad libitum*. After this dietary treatment, rats were divided into 5 groups ($n=6$), with one group killed by bleeding from the abdominal aorta for the purpose of obtaining baseline data. The other 4 groups were assigned to one of following diets (Table I); control (basal), DFAIII, FOS or inulin diets. Each test diet contained the specific inulin-type fructan at 5% of the total weight. Tap water was given *ad libitum* throughout the experimental period. After 14 d experimentally feeding rats were killed.

Table I. Composition of the diet.

Constituent	Amount [g/kg]
Casein	200
Corn starch	360
α-Corn starch	170
Sucrose	99.486
Soybean oil	70
Mineral mixture*	35
Vitamin mixture#	10
Cholin bitartrate	2.5
L-Cystine	3
Cellulose	50
tert-butylhydroquinone	0.014

*Mineral mixture was prepared as AIN-93G [mg/100 g of mixture]: $CaCO_3$, 37 000; KH_2PO_4, 19 600; $K_3C_6H_5O_7 \cdot H_2O$, 7078; NaCl, 7400; K_2SO_4, 4660; MgO, 2400; $FeC_6H_5O_7 \cdot H_2O$, 606; $ZnCO_3$, 165; $MnCO_3$, 63; $CuCO_3$ $Cu(OH)_2 \cdot H_2O$, 32.4; $NaSiO_3 \cdot 9H_2O$, 145; $CrK(SO_4) \cdot 12H_2O$, 27.5; LiCl, 1.74; H_3BO_3, 8.15; NaF, 6.35; $NiCO_3 \cdot 2Ni(OH)_2 \cdot 4H_2O$, 3.06; NH_4VO_3, 0.66; sucrose ad 100 g; #Vitamin mixture was prepared as AIN-93 [mg/100 g of mixture]: Nicotinic acid, 300; calcium pantothenate, 160; pyridoxine hydrochloride, 70; thiamine hydrochloride, 60; riboflavin, 60; folic acid, 20; D-biotin, 2.0; cyanocobalamin, 250; α-tocopherol, 1500; cholecalciferol, 25; phylloquinone, 7.5; sucrose ad 100 g. In experimental diets difructose anhydride III, fructoseoligosaccharides, or inulin were substituted for sucrose at 5%.

2.3. Sampling and analysis

At the time of sacrifice, 1 g of caecal contents were immediately collected into a microtube under a stream of CO_2 gas and put in an anaerobic bag (AneroPouch Kenki; Mitsubishi Gas Chemical Company, Inc., Tokyo, Japan) at 4°C. All samples were used for analysis of microbiota and were analysed concurrently within 48 h in one laboratory. All staff was blinded to sample identity. Analyses of microbiota were performed using the methods of Mitsuoka (1980). The analysed bacteria were as follows (medium listed in parentheses): *Streptococcus* (Triphenyltetrazolium chloride-acridine orange-thallous sulphate esculin crystal violet), *Staphylococcus* (Phenylalcohol-egg yolk), *Lactobacillus* (Lactobacillus selective agar), *Bifidobacterium* (Bifidobacterium selected agar), *Bacteroidaceae* (Neomycin-brilliant green taurochlolate blood), anaerobic gram positive cocci (Eggerth Gagnon agar), Clostridium, total anaerobes, anaerobic gram positive cocci (Glucose blood liver agar), total aerobes (trypticase soy agar). The remaining caecal samples were stored at −30°C until subsequent analyses. Caecal short chain fatty acids (SCFA) were measured by an HPLC method, as previously described (Afsana et al., 2003). Direct measurements of the pH of caecal contents were made using a pH meter with a glass electrode.

2.4. Statistical analysis

Values are presented as means ± SEM. After a log conversion, the counts of bacteria in the caecum were expressed as mean ± SEM. A Student's *t*-test was used to determine the difference between the basal data and control. A one-way ANOVA test was used to determine the effect of the diet and the dose on each parameter. Tukey's test was used to determine significant differences among the means of control, DFAIII, FOS and inulin groups. Differences were considered significant at $p < 0.05$ for all analyses.

3. Results and discussion

This study investigated the influence of the chain length and extra $\alpha(3\rightarrow2)$ linkage of inulin-type fructans on their effects on the caecal microbiota.

As shown in Table II, body weight gain and food intake were not different among the groups. The weights of caecal contents and walls were higher in all test groups compared to the control group, which was similar to previously reported studies (Campbell et al., 1997; Sakai et al., 2001; Djouzi & Andrieux, 1997; Suzuki et al., 1998; Mitamura et al., 2002; Afsana et al., 2003; Le Blay et al., 1999). While the caecal pH was lower in the inulin, FOS and DFAIII groups as compared to the control, the difference was only significant in the DFAIII group. Compared to the control, rats fed test diets had higher caecal acetate, propionate, butyrate or succinate levels (Table III). Furthermore, the total SCFA was twice as high in the DFAIII group, and threefold as high in the FOS and inulin groups. Lowered caecal pH via an increase in SCFA or other organic acids implied a promoted fermentation in the caecum when undigestible saccharides were ingested. Organic acids, especially succinic acid, have been shown to predominantly contribute to a higher caecal proton concentration in rats fed undigestible saccharides (Hoshi et al., 1994). In the DFAIII group, caecal pH was the lowest among all groups; however, caecal SCFA and succinate pools and concentrations (data not shown) were lower than estimated in the FOS and inulin groups. The reason for this lowered caecal pH in the DFAIII group was not evaluated in this study. Undigestible saccharides have been demonstrated to decrease caecal ammonia concentration (Zdunczyk et al., 2004), which possibly contributes to the lowering of the pH in the caecum in rats. Thus, in rats fed DFAIII, the lower pH in the caecum might be due to a decrease in ammonia concentrations.

There was no difference between FOS and inulin groups in caecal acetate, propionate, butyrate and succinate (Table III). In contrast, rats fed the DFAIII diet had significantly lower propionate and succinate as compared to rats on the FOS diet, and lower propionate and butyrate as compared to rats on the inulin diet. As shown in Table IV, rats fed the DFAIII diet had lower counts of caecal bifidobacteria and total anaerobes than in the FOS and inulin diets. The counts of lactobacilli and clostridia were higher in the FOS and inulin groups compared to the control group. These results support the previous *in vitro* study that showed that DFAIII was not assimilated by specific kinds of *Bifidobacteria* or *Clostridium butyricum* (Saito & Tomita, 2000). Also, there was no difference between the FOS and inulin groups in the count of all evaluated caecal bacteria. These results suggest that the effect of DFAIII on caecal microbiota and SCFA profile is different from FOS and inulin groups, which exhibit similar effects. FOS used in the present study was a mixture of fructan with the main DP of

Table II. Body weight, food intake, caecal weights, and caecal pH.

Groups	Body weight gain [g/d]	Food intake [g/d]	Caecal wall [g]	Caecal content [g]	Caecal pH
Baseline[†]	8.4 ± 0.2	16.4 ± 0.5	0.51 ± 0.02	1.78 ± 0.08	7.48 ± 0.08
Control	7.7 ± 0.3*	20.6 ± 0.6	0.65 ± 0.06^{a}	2.56 ± 0.29^{a}*	7.01 ± 0.29^{b}*
DFAIII[#]	8.0 ± 0.2	20.7 ± 0.4	1.46 ± 0.11^{b}	5.97 ± 0.39^{b}	5.68 ± 0.33^{a}
FOS[§]	8.2 ± 0.5	20.8 ± 0.9	1.32 ± 0.12^{b}	6.58 ± 0.92^{b}	6.14 ± 0.18^{ab}
Inulin	8.1 ± 0.4	21.2 ± 1.0	1.37 ± 0.15^{b}	6.42 ± 0.51^{b}	6.57 ± 0.23^{ab}

[†]Killed after the first 9 days; [#]Difructose anhydride III; [§]Fructooligosaccharides; Values are means $\pm$ SEM, $n = 6$. Means in a column not sharing a common superscript letter are significantly different. *Significantly different from basal group at $p < 0.05$.

Table III. Caecal pool of short chain fatty acids (SCFA) and succinic acid in rats fed a diet supplemented with difructose anhydride III (DFAIII), fructooligosaccharides (FOS) or inulin.

Groups	Total SCFA[#]	Acetate	Propionate	n-Butyrate	Succinate
	[μmol/caecum]				
Baseline	76.9 ± 6.9	48.9 ± 5.3	17.8 ± 1.1	10.2 ± 0.9	5.59 ± 2.20
Control	164 ± 31^{a}*	109 ± 20^{a}*	34.2 ± 6.3^{a}*	21.0 ± 5.1^{a}	4.31 ± 1.75^{a}
DFAIII	308 ± 33^{ab}	254 ± 27^{b}	43.3 ± 8.6^{a}	10.5 ± 10.5^{a}	42.2 ± 20.9^{ab}
FOS	506 ± 118^{b}	270 ± 57^{b}	197 ± 58^{b}	39.2 ± 19.1^{ab}	252 ± 51^{c}
Inulin	432 ± 53^{ab}	208 ± 24^{ab}	151 ± 26^{ab}	72.8 ± 12.5^{b}	189 ± 65^{bc}
Ratio to total SCFA					
Baseline	100	63	24	13	
Control	100	67	21	12	
DFAIII	100	83	14	3	
FOS	100	56	36	8	
Inulin	100	49	35	17	

[#]Total SCFA was calculated as the sum of acetate, propionate and n-butyrate. Values are means ± SEM; values in a column not sharing a common superscript letter are significantly different. *Significantly different from basal group at $p < 0.05$.

Table IV. Caecal microbiota in rats fed diets supplemented with difructose anhydride III (DFAIII), fructooligosaccharides (FOS), or inulin.

Bacteria	Baseline	Control	DFAIII	FOS	Inulin
	[log10 cfu/g ceacal contents]				
Streptococcus	6.2 ± 0.4	6.9 ± 0.3	6.6 ± 0.3	6.9 ± 0.4	6.4 ± 0.3
Staphylococcus	4.6 ± 0.3	4.3 ± 0.3	3.3 ± 0.2	3.0 ± 0.7	3.7 ± 0.4
Lactobacillus	9.1 ± 0.3	7.7 ± 0.5^{a}*	8.3 ± 0.3^{ab}	9.2 ± 0.2^{b}	9.1 ± 0.2^{b}
Bifidobacterium	7.2 ± 1.4	7.3 ± 0.3^{ab}	5.0 ± 1.6^{a}	9.6 ± 0.2^{b}	9.3 ± 0.1^{b}
Bacteroidaceae	7.5 ± 0.9	4.4 ± 1.2	7.0 ± 0.8	5.0 ± 0.6	5.8 ± 0.7
Anaerobic gram positive cocci	8.6 ± 0.2	5.7 ± 1.8	8.1 ± 0.4	9.2 ± 0.2	9.1 ± 0.8
Clostridium	4.6 ± 1.6	3.3 ± 1.5^{a}	6.6 ± 0.8^{ab}	8.4 ± 0.4^{b}	8.2 ± 0.4^{b}
Total aerobes	9.1 ± 0.3	8.2 ± 0.3^{a}*	8.3 ± 0.3^{ab}	9.2 ± 0.2^{b}	9.1 ± 0.2^{ab}
Total anaerobes	9.2 ± 0.2	8.1 ± 0.5^{a}	8.4 ± 0.4^{a}	9.8 ± 0.2^{b}	9.8 ± 0.1^{b}
Total bacteria	9.6 ± 0.1	8.6 ± 0.4^{a}	8.8 ± 0.4^{ab}	10.0 ± 0.2^{b}	9.9 ± 0.1^{b}

Values are means ± SEM after a log transduction; Values in a column not sharing a common superscript letter are significantly different; *Significantly different from basal group at $p < 0.05$.

2–4 linked to a single glucose moiety at its terminal end. The DP and the sugar composition of the FOS are different from those of DFAIII. In a previous human study, FOS with a DP of 2–6 increased faecal bacteria (Gibson et al., 1995). FOS enriched by fructose-type fructan, which is composed of only fructose moieties, also increased faecal *Bifidobacteria* in humans (Menne et al., 2000). These previous reports imply that a glucose moiety linked to the terminal of the fructan or the chain length of fructan within the range of FOS has little influence on its prebiotic effect. Therefore, the extra α(3→2) bond between the two fructose moieties but not the chain length may contribute to the difference seen in the caecal environment when inulin-type fructans are ingested. Only one strain of DFAIII fermenting intestinal bacteria, which has a high similarity to *Ruminococcus productus*, has been identified

(Minamida et al., 2005). This is in contrast to many strains of FOS and inulin fermenting bacteria, especially bifidobacteria that have been identified (Roberfroid et al., 1998). These results may indicate that some specific minor strains of caecal microbiota are able to break the extra $\alpha(3\rightarrow2)$ bond between the two fructose moieties.

There have been some studies that compared the probiotic effects of inulin-type fructan within the same test (Roberfroid, 2005; Klessen et al., 2001; Wang & Gibson, 1993). However, previous separate studies on inulin (Kruse et al., 1999) and FOS (Campbell et al., 1997; Sakai et al., 2001; Djouzi & Andrieux, 1997; Bouhnik et al., 1999) have shown the molecules to be bifidogenic. Both inulin and FOS increased specific bifidobacteria when they were compared within the same model *in vitro* (Roberfroid et al., 1998), and they tended to increase bifidobacteria when tested in a batch culture (Wang & Gibson, 1993). When rats associated with human faecal microbiota were fed inulin or FOS diets, an increased total content of SCFA and butyrate in caecum were observed in both groups, while only the FOS group exhibited increased caecal propionate as compared to control. In the same study, counts of caecal bifidobacteria were only lower in the inulin group and counts of caecal clostridia were only lower in the FOS group when compared to the control group (Klessen et al., 2001). The present results, which showed no significant difference in the caecal microbiota between rats fed FOS and inulin, do not agree with results that have been observed for rats associated with human faecal microbiota. This difference may be related to the difference in initial microbiota.

Previous studies have shown that DFAIII is fermented in the caecum of rats (Suzuki et al., 1998; Mitamura et al., 2002; Afsana et al., 2003; Minamida et al., 2005). The results of the present study also indicated that DFAIII is fermentable; however, we observed a different effect on the caecal microbiota as compared to the inulin-type fructans with a linear chain. The detailed effect of DFAIII on caecal microbiota in rats cannot be described, as there was no difference between the control and DFAIII groups for each of the caecal bacterial counts (Table IV). However, in the present study we observed that there was a tendency for bifidobacteria to have decreased counts in rats fed the DFAIII diet compared to the control group. These results show that DFAIII has no bifidogenic effect, which is a well-described prebiotic effect. Additionally, clostridia tended to increase in the DFAIII group. While clostridia comprise also pathogenic species such as *Clostridium perfringens* (Mitsuoka, 1980), it also contains many non-pathogenic species. Further studies are needed to determine which strains increase with ingestion of DFAIII.

Ingestion of DFAIII increased the proportion of acetate and decreased the ratio of propionate and butyrate as compared to the control group. This result agrees with a previous report (Minamida et al., 2005). There are many H_2/CO_2-using acetogenic bacteria in the related strain of *Ruminococcus productus* (Bernalier et al., 1996). This strain has a high similarity to *Ruminococcus* M-1, a DFAIII fermenting bacterium. Therefore, DFAIII fermenting bacteria may contribute to an increase in caecal acetate after ingestion of DFAIII.

In conclusion, the effect of DFAIII on the caecal environment of rats is different from that of the inulin-type fructans with a linear chain. This difference may be due to the extra $\alpha(3\rightarrow2)$ bond between the two fructose moieties, and thus DFAIII may have an anti-bifidogenic effect. Therefore, results indicate that DFAIII is not applicable as a putative prebiotic food.

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