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Inhibitory effects of laminaran and low molecular alginate against the putrefactive compounds produced by intestinal microflora in vitro and in rats

Takashi Kuda [a,*], Toshihiro Yano [a], Noriko Matsuda [a], Makoto Nishizawa [b]

[a] Department of Food Science, Ishikawa Agricultural College, Nonoichi, Ishikawa 921-8836, Japan
[b] Department of Food Science, Tokyo University of Agriculture, Abashiri, Hokkaido 099-2493, Japan

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Abstract

The inhibitory effects of laminaran and low molecular weight sodium alginate (MW = 49,000) against formation of ammonia, indole compounds and phenol compounds, putrefactive and harmful compounds, induced by human fecal microflora, were examined in vitro. Laminaran was fermented to acetate, propionate, *n*-butyrate and lactate. The alginate was fermented to acetate and propionate. Both of these polysaccharides inhibited formation of the putrefactive compounds. In the case of rats fed diet containing 2% (w/w) laminaran or low molecular alginate, the fermentation pattern agreed with that of the in vitro experiment. Laminaran suppressed indole, *p*-cresole and sulfide, significantly. These putrefactive compounds, in rats fed low molecular alginate, also tended to be lower. These results suggest that the fermentation of laminaran by intestinal bacteria suppresses the putative risk markers for colon cancer.

Keywords: Laminaran; Alginate; Intestinal microflora; Putative risk markers; Rats

1. Introduction

It is believed that dietary factors play a major part in intestinal, particularly colon, carcinogenesis. For example, ammonia, indole and phenol are putative risk markers for colon cancer produced from undigested or endogenous compounds of proteins by colonic bacteria (Mitsuoka, 2000). Epidemiological studies have reported a significant positive association between meat and risk of colon cancer (Reddy, 1995) and reduction in risk in individuals and populations consuming high amounts of dietary fibre (Cummings, Bingham, Heaton, & Eastwood, 1992). It is considered that dietary fibres suppress risk of colon cancer by diluting carcinogens, mutagens and promoters (Bach Knudsen, Johansen, & Glitso, 1997).

On the other hand, some water-soluble fibres, such as resistant starch from potatoes and undigested oligosaccharides, are metabolized to short-chain fatty acids (SCFA; acetic acid, propionic acid and butyric acid) and lactic acid by colonic bacteria (Cherbut, Michel, & Lecannu, 2003). Butyric acid is the preferred fuel for colonocytes, especially in the distal colon, and may be a protective factor in colon caricinogenesis (Weaver, Krause, Miller, & Wolin, 1988). Seaweeds have a large quantity of insoluble dietary fibre and soluble dietary fibre (Suzuki, Ohsugi, Yoshie, Shirai, & Hirano, 1996). Edible brown algae have soluble polysaccharides, such as alginate and fucoidan in intercellular space and laminaran (β-1,3 glucan) as storage compounds (Kuda, Taniguchi, Nishizawa, & Araki, 2002). Alginate and laminaran are fermented by some strains of *Bacteroides*

* Corresponding author. Tel.: +81 76 248 3135; fax: +81 76 248 8402.
E-mail address: kuda@ishikawa-c.ac.jp (T. Kuda).

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and *Clostridium* (Kuda, Goto, Yokoyama, & Fujii, 1998) but most intestinal strains of microflora cannot ferment fucoidan.

There are many reports of the beneficial effects of oligosaccharides, such as prebiotics to increase intestinal bifidobacteria, on the putative risk markers (Gibson & Roberfroid, 1995). On the other hand, although *Bifidobacterium* cannot utilize laminaran, we reported that the degraded products from laminaran produced by *Clostridium ramosum*, a general human intestinal bacterium, are utilized by *Bifidobacterium* strains (Kuda, Fujii, Hasegawa, & Okuzumi, 1992). Therefore, we believe that dietary laminaran and also alginate have effects, similar to dietary oligosaccharides, on the organic acid fermentation and production of putrefactive compounds by intestinal microflora. However, the effects of laminaran and alginate are still not clear.

In this study, we examined the effects of laminaran and sodium alginate on the production of SCFA, ammonia, indole compounds and phenol compounds by human fecal microflora in vitro. Furthermore, effects of dietary laminaran and alginate on the cecal microbial metabolic products in rats were also investigated.

2. Materials and methods

2.1. Materials

Laminaran (from *Eicenia bicyclis*) was purchased from Tokyo Kasei Kogyo Co., Ltd. (Tokyo). Low molecular (MW = 49,000) sodium alginate was obtained from Kyosei Pharmceutical Co. Ltd. (Otaru, Hokkaido, Japan). High molecular (MW = 780,000) sodium alginate was purchased from Wako Pure Chemical Ind., Ltd. (Osaka, Japan). GAM Semisolid without dextrose "Nissui" (Nissui Pharmaceutical Co., Ltd., Tokyo) was used as basal medium in this study.

2.2. In vitro experiment

Fresh feces from three normal adult human males were collected and obtained immediately prior to this experiment. The feces were mixed and diluted with nine volumes of GAM 1/4 (2.5 g of peptone, 0.75 g of soy-peptone, 2.5 g of proteose–peptone, 4.35 g of digested serum powder, 1.25 g of yeast extract, 0.55 g of meat extract, 0.3 g of liver extract, 0.625 g of NaH_2PO_4, 8.5 g of NaCl, 0.075 g of L-cysteine–HCl and 0.075 g of sodium thioglycolate per 1000 ml of distilled water, pH 7.1). The fecal dilution (0.5 ml) was inoculated to 4 ml of GAM 1/4 containing urea (1 mg/ml) and tyrosine (5 mg/ml) with or without 10 mg/ml of laminaran or low molecular sodium alginate ($n = 3$). The inoculated cultures were incubated at 37 °C for 48 h by the steel-wool method (Mitsuoka, 1984). In this experiment, we used a N_2 atmosphere instead of CO_2, in order to prevent pH falling in the medium.

After 0, 12, 24 and 48 h of incubation, the pH value was determined by a pH electrode (twin pH, B-211, Horiba, Kyoto, Japan). Organic acids were determined by high-pressure liquid chromatographic (HPLC) procedures described by Miller and Wolin (1996). Levels of ammonia-N and phenol compounds were determined by the indophenol method and aminoantipyrine method (Reagent set for water analyzer No. 7 and 17, Kyoritsu Chemical-Check Lab., Co., Tokyo). The level of indole compounds was measured using Kovacs' reagent (Lombard & Dowell, 1983).

2.3. Animal experiment

This study was conducted in conformity with the policies and procedures detailed in the "Guide for the Care and Use of Laboratory Animals" (NIH Publication No. 86-23, 1985). Twenty-four male Wistar 4 week-old rats (Ninox Labo Supply Inc., Ishikawa, Japan) were individually housed in stainless wire cages in a room maintained at 20–24 °C with 50–60% relative humidity. Rats were fed a diet containing no dietary fibre (CD in Table 1) with tap water. After seven days, the rats were divided at random into four groups ($n = 6$) and fed CD, diet containing 2% (w/w) laminaran (LD), high molecular alginate (HAD) or low molecular alginate (LAD) for 14 days. At the end of the experiment, the rats were bled from the abdominal aorta under diethyl ether anesthesia. The cecum was removed, and then weighed. The cecal contents were stored at −20 °C prior to their chemical analysis.

Cecal pH, organic acid composition and ammonia-N were determined as above, after dilution with nine volumes of distilled water. Water-soluble saccharide and polysaccharide contents were determined by the phenol–sulfuric acid method (Dubois, Gilles, Hamilton, Rebers, & Smith, 1956) and alcohol precipitation method (Kuda et al., 1998). Glucose content was measured by the glucose oxidase method (Glu B-test Wako). Water-soluble protein was determined by a test kit (*DC*-Protein Assay, Bio-Rad, Hercules, CA). Levels of indole, skatole, phenol and *p*-cresole were determined by HPLC procedures after dilution with nine volumes of methanol. After centrifuging, the supernatant was passed through a 0.45 μm pore size filter. The column was TSK-gel, Octyl 80Ts (Tosoh Co., Tokyo) type, heated to 40 °C. The eluent was 40% methanol, and the flow rate was 1 ml/min. Eluting compounds were detected at UV 280 nm. Sulfide content was determined by the methylene blue method (Reagent set for water analyzer No. 53).

Table 1
Composition of test diets (g/100 g)

	CD	LD	HAD	LAD
Corn starch	20	18	18	18
Laminaran		2.0		
High molecular alginate			2.0	
Low molecular alginate				2.0
Sucrose	50	50	50	50
Casein	20	20	20	20
Corn oil	5.0	5.0	5.0	5
Mineral mix (AIN-93)	3.5	3.5	3.5	3.5
Vitamin mix (AIN-93)	1.0	1.0	1.0	1.0
DL-Methionine	0.3	0.3	0.3	0.3
Choline bitartrate	0.2	0.2	0.2	0.2

2.4. Statistical analysis

Data were expressed as the mean and SD or SE. Statistical analysis for the animal experiment was performed using the EXCEL Statistic 5.0 (Esumi Co., Ltd., Tokyo). One-way ANOVA was used to assess the effects of treatments. Then, individual means of results in rats fed CD and other diets were compared by Dunnet's multiple-range test. Significant difference was accepted at $p < 0.05$.

3. Results and discussion

3.1. Changes of organic acids and putrefactive compounds in the fecal culture

The pH value of the culture containing laminaran at 24 h incubation was 5.7 (Fig. 1). Though the pH value in the culture containing alginate did not change, the values after 12 h incubation were lower than that of the culture with no added saccharides. In the case of the cultures containing laminaran or alginate, the total organic acids were about 85 and 105 μmol/ml at 24 and 48 h incubation, respectively. Lactic acid, propionic and *n*-butyric acid were higher in the culture containing laminaran than that in the other cultures. At 24 and 48 h incubation time, acetic acid in the culture containing alginate was the highest.

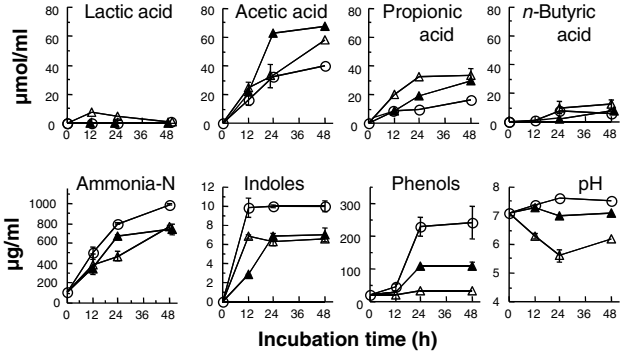

Fig. 1. Organic acid content in the human fecal culture with no added carbohydrate (open circle), laminaran (open triangle) or sodium alginate (closed triangle) at 12, 24 and 48 h of incubation. Values are means and SEs ($n = 3$).

The ammonia concentration of the control culture increased from 100 to 1000 μg/ml in 48 h. In the case of laminaran- or alginate-containing cultures, ammonia was suppressed and the concentration of ammonia was about 800 μg/ml in the cultures at the 48 h incubation time. Concentration of indole compounds of the cultures were suppressed by the dietary fibres from about 10 to 7 μg/ml in the cultures at the 24 and 48 h incubation times. The phenolic compounds in the culture were also suppressed by the dietary fibres, especially laminaran. The content did not increase in the culture containing laminaran.

3.2. Effects on rats

There were no significant differences among the four groups in body weight gain. Fecal frequency and fecal wet weight were increased by HAD (Table 2). Cecal whole weight was increased by the test diets and the wall weight was increased by LD.

Levels of total soluble-saccharide and polysaccharide in the cecum were increased by the all test diets. Furthermore, LD increased cecal glucose. LD increased cecal lactate, propionate, *n*-butyrate and total organic acids, and lowered the pH significantly. On the other hand, LAD increased acetate, propionate and *n*-butyrate. This agrees with the in vitro fermentation results, mentioned above. However, HAD did not affect the cecal organic acids.

In this study, cecal phenol and skatole were not detected in any rats by the HPLC procedure. HAD decreased 32% of cecal soluble protein and 25–60% of putrefactive compounds. On the other hand, though LD did not change cecal protein level, indole, *p*-cresole and sulfide levels were suppressed from 47% to 60% by LD. Furthermore, the results of LAD tended to be similar to those of LD.

Lavrat, Rémésy, and Demigné (1993) compared the effects of the fermentable carbohydrate inulin on protein fermentation in rats fed high- or moderate-protein diets. They showed that inulin in the diets increased the use of ammonia, a source of nitrogen for bacterial growth, in the case of moderate-protein diets. On the other hand, Suzuki et al. (1979) reported that the optimum pH of the urease in most urease-positive anaerobes isolated from human gut, such as *Bacteroides multicidus* and *Proteus mirabilis*, was 8.0. We consider that the suppression of ammonia by the polysaccharides shown in this experiment was affected by both the ammonia utilization of the bacteria for their multiplication and the urease inhibition by the low pH induced by the fermentation.

Table 2
Body weight gain, fecal weight, cecal weight, cecal organic acids and cecal putrefactive compounds in rats fed diet containing no carbohydrate (CD), 2% laminaran (LD), 2% high molecular alginate (HAD) or 2% low molecular alginate (LAD) for 14 d[a]

	CD	LD	HAD	LAD
Initial body weight (g)	109 ± 4	112 ± 6	113 ± 3	112 ± 5
Body weight gain (g/14 days)	126 ± 14	127 ± 17	135 ± 8	127 ± 12
Frequency of feces (No./day)	10.3 ± 1.6	12.1 ± 0.7	18.1 ± 2.0**[b]	13.4 ± 1.3
Fecal wet weight (g/day)	0.56 ± 0.16	0.62 ± 0.06	1.18 ± 0.34*	0.68 ± 0.12
Cecal weight (g/100 g body weight)	1.08 ± 0.14	1.38 ± 0.12*	1.62 ± 0.25**	1.40 ± 0.14*
Cecal saccharides (mg/g content)				
Total soluble saccharide	7.1 ± 1.0	22.1 ± 8.3**	20.1 ± 4.0*	17.2 ± 3.2*
Soluble polysaccharides	3.3 ± 1.8	17.0 ± 7.9**	14.3 ± 3.9*	11.7 ± 3.8*
Glucose	0.42 ± 0.03	3.53 ± 1.86**	0.74 ± 0.39	1.09 ± 0.61
Cecal organic acids (μmol/g content)				
Lactic acid	4.87 ± 2.08	8.80 ± 4.13*	2.64 ± 3.49	4.18 ± 2.13
Acetic acid	12.3 ± 1.4	14.7 ± 2.5	12.3 ± 1.4	19.3 ± 3.5**
Propionic acid	4.52 ± 0.76	7.49 ± 1.33**	4.51 ± 1.32	6.45 ± 1.39*
n-Butyric acid	2.09 ± 0.76	4.44 ± 1.50**	1.59 ± 1.21	1.84 ± 0.50
Total organic acid	23.8 ± 2.1	35.5 ± 7.4**	21.1 ± 6.0	31.8 ± 5.7*
Cecal pH	7.2 ± 0.1	6.4 ± 0.2**	7.1 ± 0.3	6.7 ± 0.3**
Cecal soluble protein (mg/g)	49.7 ± 6.8	52.5 ± 3.2	33.7 ± 5.9**	47.3 ± 5.5
Cecal putrefactives (μg/g)				
Volatile basic -N	439 ± 84	433 ± 50	251 ± 60**	372 ± 47
Ammonia-N	333 ± 65	370 ± 31	249 ± 68*	328 ± 43
Indole	15.7 ± 1.4	9.43 ± 5.08*	6.16 ± 4.08**	11.0 ± 3.22
p-Cresole	19.4 ± 6.7	9.12 ± 5.39**	8.74 ± 4.62	13.3 ± 2.6
Sulfide	12.6 ± 1.0	7.06 ± 1.10**	7.45 ± 1.94**	9.29 ± 1.69**

[a] Values are means and SDs ($n = 6$).
[b] Diffrence from CD group, * $p < 0.05$, ** $p < 0.01$.

Although ammonia suppression in rat cecum by laminaran was not shown, we think that ammonia absorption from cecal wall was lowered by the low pH condition.

On the other hand, we have reported that laminaran promoted cecal *Bifidobacteria* in rats (Kuda et al., 1998). Furthermore, degraded products of laminaran can promote the growth of *Bifidobacterium* strains in vitro (Kuda et al., 1992). It is considered that the bacterial groups promoted by laminaran and alginate in rat cecum and also in vitro could suppress the bacteria that can produce the putrefactive compounds.

Ingestion of fermentable oligosaccharides, such as raffinose, decreased intestinal ammonia, indole, phenol and other putrefactive compounds (Nakura, Muraguchi, Uchino, Aritsuka, & Benno, 1999) with high SCFA and low pH. On the other hand, the fermentation of high molecular alginate was difficult with human fecal microflora or rat cecal microflora. So it is considered that the suppression of putrefactive compounds in rat cecum by HAD was caused by physical features, such as swelling and promotion of excretion. It is well known that the alginates have an inhibitory effect on absorbance of harmful compounds from intestines by their physical features (Watanabe et al., 1992).

In conclusion, inhibitory effects of laminaran and alginate, water-soluble polysaccharides from brown algae, on putrefactive compound formation in human fecal culture and rat cecum were shown in this study. These putrefactive compounds were regarded to be harmfully related to colon cancer and aging (Mitsuoka, 2000). The results shown in this study suggest that the fermentation of laminaran and also alginate by intestinal bacteria suppresses the formation of putative risk markers for colon cancer besides the diluting and excretion effects.

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