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# Inhibitory effects of laminaran and alginate on production of putrefactive compounds from soy protein by intestinal microbiota *in vitro* and in rats

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ABSTRACT

Soybean is one of the major components of the Japanese diet. In traditional Japanese cuisine, soybean-based food items are often consumed with brown algae. In this study, we examined the effect of water-soluble and fermentable polysaccharides, laminaran and sodium alginate, from brown algae, on putrefactive compound production, by human faecal microbiota in broth containing 3% (w/v) soy protein. We also investigated the effect of 2% laminaran or alginate diet on caecal putrefactive compounds in rats maintained on diets containing 20% (w/w) soy protein. The caecal microbiota was also analysed using denaturing gradient gel electrophoresis and pyrosequencing with primers targeting the bacterial 16S rRNA gene. The polysaccharides, particularly laminaran, inhibited ammonia, phenol, and indole production by human faecal microbiota. Both the algal polysaccharides lowered the caecal indole content. Laminaran was found to increase the number of *Coprobacter*, whereas *Helicobacter* was found to decrease in the presence of both laminaran and sodium alginate.

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## 1. Introduction

The adult human intestine contains $10^{13}$–$10^{14}$ bacteria that belong to at least 500 different species or strains (Dethlefsen, Huse, Sogin, & Relman, 2008). This intestinal microbiota plays an important role in host health (Sears, 2005). Intestinal bacteria, including probiotics, have both beneficial and harmful effects on the host. For example, some lactic acid bacteria prevent pathogen colonisation, stimulate immune responses (Kawahara et al., 2015; Kuda, Kanno, Kawahara, Takahashi, & Kimura, 2014; Kuda, Kawahara, Nemoto, Takahashi, & Kimura, 2014; Nakamura et al., 2012) and control processes involved in digestion and absorption (Kuda, Yazaki, Ono, Takahashi, & Kimura, 2013; Willing & Kassei, 2010). Harmful effects include the production of intestinal putrefactive compounds, such as ammonia, hydrogen sulphide, amines, phenols, and indoles, due to protein breakdown (An, Kuda, Yazaki, Takahashi, & Kimura, 2014a). In excess quantities, these putrefactive compounds are considered putative carcinogens and toxins (Windey, Preter, & Verbeke, 2012). The composition of intestinal microbiota in an individual depends on various factors such as age, stress, climate, infective agents, disease, drugs, and diet (Sekirov, Russell, Antunes, & Finlay, 2010). In addition, diet composition is dependent on the geographical location and cultural background of the individual (De Filippo et al., 2010).

Soybeans are part of the traditional food consumed in Asian countries, including Japan. In Japan, for instance, soybeans are used in traditional fermented food products such as soy sauce, *miso*, and *natto*, as well as in popular processed food items such as *tofu*. Soy protein has been proven to be beneficial for health. For example, compared to products of milk-digestion, soy protein digestion products reportedly suppress the rate of pathogen invasion in human enterocyte-like Caco-2 cells as well as in A/J mice (Kuda, Nakamura, An, Takahashi, & Kimura, 2012). However, we have previously shown that the indole content in the caecum of Wistar rats fed soy protein was greater than that in rats fed milk casein (An, Kuda, Yazaki, Takahashi, & Kimura, 2013). Indole is synthesised from tryptophan by the intestinal microbiota, and soy protein is rich in tryptophan.

In traditional Japanese cuisine, soybean-based food items are often consumed with brown algae. Brown algae contain water-soluble polysaccharides, such as laminaran and alginate, that are regarded as dietary fibres (An, Yazaki, Takahashi, Kuda, & Kimura, 2013; Kuda, Yano, Matsuda, & Nishizawa, 2005). Alginates are viscous compounds found in the algal cell wall matrix, and are polymers of glucoronic acid and mannuronic acid (Kuda, Taniguchi, Nishizawa, & Araki, 2002; Kuda et al., 2015). Laminaran,

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composed of $\beta$ (1 → 3) glycosidic bonds with $\beta$ (1 → 6) branching linkages, is found in the algal cells as a storage polysaccharide (Kuda, Enomoto, & Yano, 2009; Usui, Toriyama, & Mizuno, 1979). On consumption, these polysaccharides are not digested by intestinal enzymes; instead, they are fermented to organic acids by the intestinal microbiota. These processes are similar to those that prebiotics undergo (An, Kuda, et al., 2013; An, Yazaki, et al., 2013; Kuda et al., 2005). Several intestinal bacteria such as *Parabacteroides distasonis*, *Bacteroides ovatus*, and *Clostridium ramosum* are reportedly alginate and/or laminaran-fermenting strains (Fujii, Kuda, Saheki, & Okuzumi, 1992). In addition, oligosaccharides from laminaran promote the growth of *Bifidobacterium* strains (Kuda, Fujii, Hasegawa, & Okuzumi, 1992). Furthermore, supplementation with these fermentable fibres has been shown to suppress the formation of intestinal putrefactive compounds in rats fed milk casein (An, Kuda, et al., 2013; Kuda et al., 2005).

In this study, we examined the effect of laminaran and sodium alginate on indole production by human faecal microbiota *in vitro*. We also investigated the effect of dietary laminaran and alginate on the caecal indole content in rats fed diets containing 20% (w/w) soy protein. The caecal microbiota was also analysed using denaturing gradient gel electrophoresis and pyrosequencing with primers targeting the bacterial 16S rRNA gene.

## 2. Materials and methods

### 2.1. Laminaran and alginate

Laminaran extracted from a brown alga *Eisenia bicyclis* was purchased from Tokyo Chemical Industry, Tokyo, Japan. It is known that laminaran from *E. bicyclis* consists of glucose residues linked by $\beta$-1,6-branched $\beta$-1,3-glycosidicbonds and the ratio of the 1,6 and 1,3 bonds is about 1:2 (Menshova et al., 2014; Shin, Oh, Kim, Won Kim, & Son, 2009). Approximate degree of polymerisation (DP) of the laminaran is between 22 and 25 and the molecular weight is about 5 kDa (Alderkamp, van Rijssel, & Bolhuis, 2007). Sodium alginate (80–120 cp) was purchased from Wako Pure Chemical Industries Ltd., Osaka, Japan. Weight-average molecular weight of the alginate analysed by HPLC method (Shinohara, Kammono, Aoyama, Bando, & Nishizawa, 1999) was 1325 kDa. The ratio of Guluronate content (G%) andguluronate–guluronate diad frequency (GG%) evaluated by a $^1$H NMR method (Kuda et al., 2002) were 34.4 and 18.9%, respectively.

### 2.2. *In vitro* experiment

Fresh faecal samples from normal human adult males were collected immediately prior to the experiment (Kuda et al., 2005). The faecal samples were diluted 1:9 with Gifu Anaerobic Medium (GAM 1/4; 2.5 g peptone, 0.75 g soy-peptone, 2.5 g proteose–peptone, 4.35 g digested serum powder, 1.25 g yeast extract, 0.55 g meat extract, 0.3 g liver extract, 0.625 g $NaH_2PO_4$, 8.5 g NaCl, 0.075 g L-cysteine–HCl, and 0.075 g of sodium thioglycolate per 1000 ml distilled water; pH 7.1). The diluted faecal samples (50 μl) were inoculated in 1 ml of GAM 1/4 containing 30 mg/ml soy protein (Fuji Oil, Izumisano, Japan) with or without 10 mg/ml laminaran or sodium alginate ($n$ = 3). The inoculated cultures were incubated at 37 °C for up to 48 h under anaerobic conditions, by using the AnaeroPack system (Mitsubishi Gas Chemical, Tokyo, Japan) (An, Kuda, Yazaki, Takahashi, & Kimura, 2014b).

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 acid (lactic acid, acetic acid, propionic acid, and $n$-butyric acid) levels were determined by high-pressure liquid chromatographic (HPLC) methods that have been described previously (An et al., 2014a). Levels of ammonia and phenol compounds were determined by the indophenol and aminoantipyrine methods, respectively, using reagent sets for water analysis (nos. 7, and 17A, respectively; Kyoritsu Chemical-Check Lab., Co., Tokyo, Japan). The absorbances were read using a grating microplate reader (SH-1000 Lab; Corona Electric, Ibaraki, Japan) after dilution of the reaction mixture with four volumes of distilled water (An et al., 2014b). The level of indole compounds was measured using Kovacs reagent (Lombard & Dowell, 1983).

**Table 1**
Chemical composition of the control and test diets (g/100 g).

| | Control | Laminaran | Alginate |
|---|---|---|---|
| Sucrose | 50.0 | 50.0 | 50.0 |
| Soy protein | 20.0 | 20.0 | 20.0 |
| Corn starch | 20.0 | 18.0 | 18.0 |
| Sodium alginate | | | 2.0 |
| Laminaran | | 2.0 | |
| Corn oil | 5.0 | 5.0 | 5.0 |
| AIN-76 Mixed minerals | 3.5 | 3.5 | 3.5 |
| AIN-76 Mixed vitamins | 1.0 | 1.0 | 1.0 |
| DL-Methionine | 0.3 | 0.3 | 0.3 |
| Choline bitartrate | 0.2 | 0.2 | 0.2 |

### 2.3. Animal care

The animal experiments were performed in compliance with the relevant fundamental guidelines specified by the Ministry of Education, Culture, Sports, Science and Technology in Japan (MEXT), and were approved by the animal experiment committee of Tokyo University of Marine Science and Technology (approval no. 2013-10). Four-week-old male Wistar rats (Tokyo Laboratory Animals Science Co., Ltd., Tokyo, Japan) were housed separately in metal-wire cages and allowed free access to water and food. After acclimatisation with AIN-76-based diet (control diet in Table 1) for 7 days, the animals were divided into three groups ($n$ = 6) and provided an experimental diet containing 2% (w/w) of laminaran or sodium alginate or control diet for 14 days.

The rats were then anaesthetised with diethyl ether and exsanguinated from the abdominal aorta. The liver, kidneys, spleen, thymus, and cecum were excised and weighed. We used a portion of the caecal mass for direct total cell counts by using the Gram staining method (Mitsuoka, 2014) and stored the remaining portion at −80 °C for use in subsequent experiments. After dilution with 5 volumes of distilled water, caecal pH, organic acid composition, and the contents of ammonia, indole, and phenol were determined as mentioned above.

### 2.4. DNA extraction and DGGE analysis

Bacterial DNA from each caecal sample was extracted using NuCleo Spin Tissue (Takara Bio, Shiga, Japan). Impurities in the extracts were removed by centrifugation, and the purified DNA was dissolved in Tris–EDTA buffer (TE, pH 8). The purified extracts were used as DNA templates for denaturing gradient gel electrophoresis (DGGE) analysis, which was performed as described previously (An, Takahashi, Kimura, & Kuda, 2010; An et al., 2014a). We selected the following primer pairs for the amplification of the V3 region (~220 bp) of the 16S rRNA gene: forward primer with GC clamp GC-339f (5′-CGC CCG CCG CGC CCC GCG CCC GTC CCG CCG CCC CCG CCC GCT CCT ACG GGA GGC AGC AG-3′) and reverse primer V3-53r (5′-GTA TTA CCG CGG CTG CTG G-3′). Polyacrylamide gels [8% (w/v); acrylamide-bisacrylamide, 37.5:1] were prepared in 1X Tris-acetate–EDTA buffer (TAE, pH 8), with a denaturing gradient using 30–60% denaturant [100% denaturation corresponds to 7 mol/l urea and 40% (v/v) formamide] The gels were loaded and

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