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Prebiotic index evaluation of crude laminaran of *Sargassum* sp. using feces of wistar rats

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Abstract. Today, prebiotics are often added to food. This compound is a food substance which could not be digested, yet benefiting the host by selectively stimulating the growth or activity of one or more bacteria in the colon to improve the health of the host. One of the foodstuffs derived from algae, which could not be digested is laminaran (β-Glucan). The relationship between microflora with the added prebiotics was tested by measuring their prebiotic index, which is supported by total sugars and laminaran levels. The results showed that total sugar content of Laminaran Acid Extract (LAE) (9.075 %) was higher than that of Laminaran Modified Extract (LME) (7.355 %), while the laminaran level of LME (42.23 %) was higher than that of LAE (30.92 %). HPLC test result confirmed the presence of laminaran. The obtained prebiotic index values of LAE and LME were 1.29 and 2.10, respectively, with a negative index score for LAE prebiotic, yet positive one for LME in terms of probiotic from *Lactobacillus* group. Laminaran extract, especially LME, deserves to be regarded as a prebiotic candidate.

1. Introduction

Prebiotic has developed rapidly as result of consumer awareness growth about a link between health and diet. Diet is a major factor in treating digestive tract of healthy human [1], since many physiological processes related to health and disease of the host starts from here, resulting in the development of the concept of prebiotics [2]. Prebiotics are nondigestible compounds having some physiological effects on humans by selective stimulation of growth or bioactivity of beneficial microorganisms that are present in the intestines or lead therapeutical [3]. Prebiotics are food that cannot be digested, which benefits the host by selectively stimulating the growth or activity of one or more bacteria in the colon to improve the health of the host [4].

The higher demand for prebiotic results in the emergence of new prebiotics, one of the potential ones is algae. Polysaccharide polymers in algae are largely (approximately 40 %) comprised of non-starch polysaccharide (NSP) [5]. Typical carbohydrates in varieties of brown algae consist of laminaran [β-(1.3)-glucan], fucoidan, cellulose, alginate and mannitol [6]. Laminaran, also called β-glucan, is one of macroalgae food reserves located within the cell (cytoplasm) [7]. It is a polysaccharide reserving in some brown algae with varied structures, similar to laminaran in terrestrial plants and mushrooms cell wall [8].

One way to evaluate a prebiotic candidate is by looking at the prebiotic index value. Prebiotic index (PI) describes the relationship between changes in beneficial microflora with those in unwanted microbes associated with the initial concentration. The PI gives a quantitative score that describes the prebiotic effect [9]. Prebiotic index of crude laminaran could be tested using methods of Sanz et al.

[10]. The purpose of this study was to evaluate the prebiotic index of Laminaran Acid Extract (LAE) and Laminaran Modified Extract (LME), which is produced from brown algae *Sargassum crassifolium*.

2. Materials and Methods

2.1. Raw materials and medium

The raw material used in this study was brown algae *Sargassum crassifolium*, clumps having talli with length from 40–100 cm. It was obtained from Garut, West Java, in February 2009 which was harvested from the wild with unknown age. Its natural habitat was at a depth of 50–250 cm of tidal area. Chemicals used were Laminaran and Laminarinase (Sigma Chemical Co), sodium acetate buffer and DNS. Various selective media (Columbia agar + cysteine HCl, and propionic acid for Bifidobacteria; Rogosa agar and glacial acetic acid supplements for Lactobacilli; reinforced clostridial agar + novobiocin and colistin for Clostridium; TSA + kanamycin, haemin, vancomycin and lake horse blood for Bacteroides) were from Oxoid Ltd.

2.2. Preparation of LAE and LME

First algae powder was defatted (using ethanol, chloroform and final solution with acetone), then extracted using 0.9 M (1:16) H_2SO_4 solution, 70 °C for 2.5 h using a water bath shaker. Then performed filtration, resulting in supernatant and residue. The supernatant was precipitated with 96 % ethanol (1:2) then centrifuged resulting in filtrate and residue (pellet). The pellets were dried and ground as LAE. The H_2SO_4 extract residue was extracted using aquadest (1:10) with the same temperature and time, and the process was the same until the pellet was produced. The pellets were dried and ground as LME.

2.3. Preparation of fermentation medium

The fermentation medium was prepared by mixing 0.3 g trypticase, 30 mL solution of trace minerals, 30 mL of buffer solution, 30 mL of macromineral and 4.02 mL of reducing solution, and then suspended in distilled water to reach a total volume of 120 mL [11]. The obtained fermentation medium was then divided into two: a-100 mL used to dissolve the substrate (LAE, LME, Inulin and control) and a-20 mL used to dissolve the inoculum.

2.4. Preparation of inoculum

The inoculum was derived from the cecum contents of Wistar rats (200–250 g). Rats were anesthetized in a closed container, which has been saturated with vapor of sodium pentobarbital (60 $mg \cdot kg^{-1}$). Surgery was then performed to the treated rats to take their cecum contents, which were then weighed by 2.0 g and added to 20 mL of fermentation medium to reach sterile inoculum concentration of 100 $g \cdot L^{-1}$. The mixture was finally homogenized for 10 min in a stomacher.

2.5. Substrate preparation

Each of substrates (LAE, LME and Inulin) was weighed as much as 0.312 g and then diluted with 25 mL of the fermentation medium. The final concentration of substrate solution was 0.0125 %. Fermentation medium without additional carbon source was used as a control. Next, 4 mL of each substrate solution and control was transferred into 15-mL-test screw tubes. The number of tubes was according to with the number of measured hours, i.e. four tubes for each treatment. Then, tubes were covered and sterilized at 121 °C for 15 min.

2.6. Measurement of prebiotic index

Sterile substrate solution and control prepared previously (4 mL) was added to 1 mL of homogenous inoculum. The mixture was then incubated at 37 °C for 48 h. Fermentation medium without an

additional carbon source (control) was used as a zero value, while medium with additional inulin as carbon source was used as a standard (completely fermented substrate).

Plating of bacterial culture was carried out at 0 h and 12 h of fermentation to observe the growth of bacteria on the medium before and after fermentation by using the pour-plate method. From each tube,1 mL of culture was taken and plated on to selective agar media for colony counting. MRS-Raffinose was used for *Bifidobacterium*, Rogosa agar was used for *Lactobacillus*, Tryptone Bile X-glucuronide medium (TBX) was used for *E. coli*, and Perfringens agar was used for *Clostridium*, while Plate Count Agar (PCA) was used for Total Count. Incubation was then conducted for 48 h at 37 °C. Both *Bifidobacterium* and *Clostridium* groups of bacteria were incubated under anaerobic conditions by adding an anaerobic gas kits (Oxoid) in an anaerobic jar. After 48 h, the number of bacterial colonies was counted, and then the prebiotic index of each substrate was calculated using prebiotic index formula [9, 10].

PI= *[(Bif a / Bif b) / (Total a / Total b)] + [(Lab a / Lab b) / (Total a / Total b)] - [(Bac a / Bac b) / (Total a / Total b)] - [(Clos a / Clos b) / (Total a / Total b)]*

Note:
- Bif: the number of *Bifidobacteria* (at sample time) / number at the time of inoculation.
- *E.coli*: the number of *E. coli* (at sample time) / number at the time of inoculation.
- Lac: the number of *Lactobacilli* (at sample time) / number at the time of inoculation.
- Clos: the number of *Clostridia* (at sample time) / number at the time of inoculation.

2.7. Test parameters
The test parameters used in this research included total sugar [12], laminaran levels [13], the content of simple sugars by HPLC and prebiotic index and score prebiotic index.

3. Results and Discussion

3.1. Total sugar
Total sugar is the total content of whole sugars in algae. The total sugar of LAE (9.075 ± 0.01 %) was significantly different ($p < 0.05$) from that of LME (7.355 ± 0.107 %) (table 1). This is likely due to the acid solution used in the extraction process of LAE, which could hydrolyze all parts of the cell including cell walls so that all carbohydrate components possibly including fucoidan are counted. In addition, the produced polysaccharides likely were still crude Laminaran, so it is possible that there were sugars other than glucose which caused the higher value of total sugar. In general, the content of total sugars in algae consists of glucose, sucrose and fructose, as well as some other content in small amounts, such as an alcohol sugar, mannitol, dulcitol and sorbitol. Meanwhile, when the residue was extracted only using water (LME), then only glucose and a small amount of mannitol obtained so that its components were relatively homogeneous.

Table 1. Total sugar and levels of laminaran.

Parameters	Crude Laminaran	
	LAE	LME
Total Sugar (%)	9.075 ± 0.01 a	7.355 ± 0.107 b
Levels of Laminaran (%)	30.92 ± 0.01 a	42.23 ± 0.39 b

The total sugar content of *S. crassifolium* was much lower from *A. nodosum*, *F. veslculosus* and *S. longicruris* by 89.6; 84.1 and 99.1 %, respectively [14]. Likewise, *L. saccharina* using Black, Yvins and News methods by 15.59; 10.60 and 55.56 %, respectively [13]. The content of a polysaccharide is usually influenced by season, population age, species and geographic location [14, 15], while based on

[16], the harvested area was responsible for the discrepancies of polysaccharide along with its structures.

3.2. Levels of laminaran

Brown algae contain polysaccharides such as laminaran, fucoidan and alginate, which are different from those of algae classes. Laminaran is estimated by measuring the concentration of its glucose produced by enzymatic hydrolysis of laminaran [13]. Table 1 shows that the level of LME laminaran is significantly higher ($p < 0.05$) than those of LAE. Higher levels of laminaran at LME were supported by yield and reducing sugar (unreported result). Compared to the levels of commercial laminaran, both LME and LAE laminaran levels (42.23 ± 0.39 and 30.92 ± 0.01 %, respectively) were lower. It was supported by the analysis result by HPLC which also showed a lower value. The low value was caused by the compared compound was patent products, with different raw materials, harvest season and habitat of growing.

The laminaran content of brown algae varies according to season ranging between 0–35 % of the dry weight [17]. *L. japonica* harvested at 1 and 2 years was also contain a very low level of laminaran (< 1 %) [15]. Laminaran structure and composition vary according to the species of algae [18]. In addition, the content and structure of laminaran of *L. cichorioides* vary greatly depending on the season and the age, thus mature *L. cichorioides* is a rich source of laminaran. On the other hand, laminaran content of *F. evanescens* depends on where it grows.

3.3. Simple sugar content of crude laminaran

Analysis of neutral sugars on crude laminaran sample generated by acid hydrolysis process, either total or partial hydrolysis, is required to determine whether the sample contains glucose. Sugar content in the samples was tested qualitatively using HPLC. It was conducted by comparing the retention time of each peak shown by the sample chromatogram with a retention time of the peak of standard compound.

A sample of products obtained by hydrolysis on commercial laminaran showed five peaks on chromatograms (figure 1) with a retention time (tR) 8.61; 10.555; 10.845; 13.414 and 15.512, respectively. By comparing the retention time of these peaks with that of standard LAE and LME compound standard, it could be expected that peak-3 (10.845 min) belong to maltose while peak-4 (13.414 min) belong to glucose. It could be seen that peak of glucose was substantially greater than that of maltose, but other peaks could not be determined because of the absence of other neutral sugar standards. It is possible that the rest peaks are other saccharides constituting polysaccharides of brown algae, such as fucoidan, alginate, funoran and manuronan considering that the test sample was crude laminaran. But the existence of these other components did not interfere with the identification of the main components.

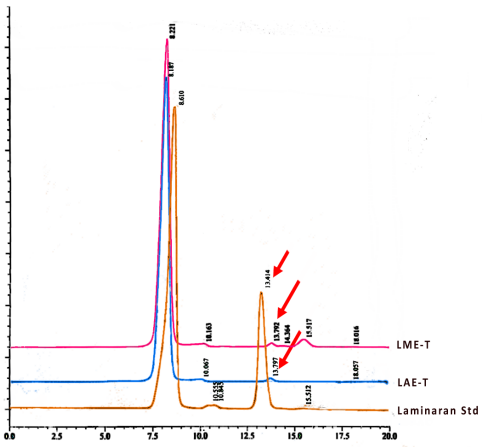

Figure 1. Chromatogram of the total hydrolysis product of laminaran standard, LAE and LME.

Analysis of neutral sugar content of LAE and total LME by total hydrolysis resulted in simple sugars monosaccharides, which were more heterogeneous than from that when only partially hydrolyzed. Investigation result on the composition of LME and LAE extracts by using HPLC showed that samples from all treatments contained glucose. This means that both extracts are laminaran compound. The content of glucose in the hydrolysate is associated with the presence of a laminaran substance in biomass [19]. This was also supported by previous study [20] showing that based on the composition of carbohydrate; glucose was the dominant component in laminaran with smaller amounts of mannit. Thus, it could be inferred that *S. crassifolium* contains laminaran.

3.4. Prebiotic index

Prebiotic index describes the relationship between changes in beneficial microflora with those in unwanted microbes associated with their initial levels. Based on Palframan equation, the increase in the population of *Bifidobacteria* or *Lactobacilli* is assumed as a positive effect, while the increase in Bacteroides and Clostridia (sub grouphistolyticum) is considered as negative effects. The number of bacterial colonies grown in the 0 h and 12 h of each treatment is shown in table 2.

As described in table 2, the number of *Bifidobacterium* colonies grown during 0 h to 12 h on a medium with a carbon source and without the addition of a carbon source showed an increase in average by 4 log cycle, whereas the *Lactobacillus* bacteria with the same conditions could only produce an increase in average by 3 log cycle. This indicates that both *Bifidobacterium* and *Lactobacillus* are equally capable of using crude laminaran (LAE and LME) and inulin as a carbon source for growth, but the ability to utilize crude laminaran of *Bifidobacterium* is better than that of Lactobacillus. Lactobacillus to take advantage of β-glucan (laminaran) must be able to break down the glycosidic bond between glucose, so glucose loses and can be used to stimulate growth. On the other hand, though *Bifidobacterium* could not utilize laminaran directly, cross-feeding possibly occurred. *Bifidobacterium* is not able to directly degrade laminaran, but the result of degradation laminaran by *Clostridium* and *Bacteroides* can be used as a carbon source for growth [8, 21]. This is the reason why the total growth of *Bifidobacterium* higher than *Lactobacillus* for all substrates.

Table 2. The growth of bacteria on various substrates at 0 and 12 hours incubation.

Bacterial	Substrate	Number of Bacteria (log 10 cells·g^{-1} feces)	
		0 h	12 h
Bifidobacterium	LAE	5.98	9.23
	LME	5.86	9.32
	Inulin	5.96	9.26
	Control	5.91	9.00
Lactobacillus	LAE	6.04	9.15
	LME	5.34	9.15
	Inulin	5.83	9.18
	Control	5.80	9.23
Bacteriodes	LAE	5.84	8.70
	LME	6.24	8.00
	Inulin	5.87	8.60
	Control	5.91	8.93
Clostridium	LAE	6.26	8.30
	LME	6.02	9.13
	Inulin	6.18	7.28
	Control	6.18	8.32

Table 2 shows that the substrate of crude laminaran (LAE and LME) was capable of supporting the growth of pathogenic bacteria, although its number is still lower than that of beneficial bacteria. This may be related to the factor of cross-feeding, where nature has set the balance between the microflora present in the colonic ecosystem. *Bifidobacteria* could not directly degrade laminaran. Only after it is

degraded by Clostridia or Bacteroides, then the resulted compounds could be utilized by *Bifidobacteria*. Thus, the presence of both pathogens was still needed if their number could not exceed the balance.

Bifidobacteria and *lactobacilli* are known as organisms directly inhibit the growth of pathogenic bacteria, such as certain species of Clostridia [22]. Moreover, laminaran encourage the growth of cecal Bifidobacteria in rats [23]. Laminaran could suppress the growth of spoilage bacteria producing components in vitro [24]. Prebiotic effect of laminaran could be seen from an index value of its prebiotic. The quantitative calculation was used to facilitate fermentative analysis of prebiotics [9]. Compare of prebiotic index values of crude laminaran with that of other carbon sources and without the carbon source is showed in table 3.

Table 3. Average value of the prebiotic index of crude laminaran.

Substrate	Prebiotic index
LAE	1.29 ± 0.02 a
LME	2.10 ± 0.01 ab
INULIN	3.26 ± 0.02 b
CONTROL	1.06 ± 0.03 a

As shown by table 3, the PI value of LAE (1.29) and LME (2.10) indicated that both laminaran have the ability as a prebiotic, even if lower than inulin does (3.26). This is because inulin (β-(2,1) fructans linear) has selective colonic fermentation nature leading to real change in the microflora composition of the intestinal tract by increasing the number of bacteria which have potential to promote health and reduce the number and type of potentially harmful bacteria [25].

PI value of LME was higher than that of LAE. This is likely because the components contained in the LME are large compounds that are easily broken down by the intestinal microflora. The opposite occurred in LAE which contains mostly components that are not degraded by the intestinal microflora. This is supported by data of laminaran levels that were also higher in LME. In accordance with the mentioned formula, the provided treatment on both LAE and LME show their ability as a prebiotic although lower than the index of commercial inulin prebiotic.

The PI value of Wu-AX (water-insoluble wheat arabinoxylan unextractable) in 8 h fermentation by 2.03 ± 0.60 [26], when compared to the obtained laminaran PI value is not too different. Laminaribiose (G2) at 12-h of incubation could produce PI of 5.60 [10]. This much higher result is likely due to differences in raw materials, where the raw material used was not derived from algae but terrestrial plants, and due to different substrates used. POS (pectic polysaccharides), FOS (fructooligosaccharides) and OA (orange-albedo) at 10-h fermentation gave the higher value of PI (5.37; 6.32 and 4.9, respectively) [22]. Hence, we could conclude that the prebiotic index value of *S. crassifolium* algae is lower than terrestrial plants. However, there had been no publications found about PI values of other algae making it difficult to directly compare with the results obtained in this study.

The PI equation assumes that an increase in the populations of *Bifidobacteria* and/or *Lactobacilli* is a positive effect while an increase in bacteroides and clostridia (histolyticum subgroup) are negative [9]. If a bacterial group showed a relative increase more than the total population of bacteria, then such increase is considered > 1, but if the increase is relatively lower than total bacteria is considered < 1. This value was then added to the PI calculation to result in the overall scores. To determine the effect of laminaran prebiotics, then the PI score formula was used as displayed in table 4.

Table 4. Prebiotic index scores laminaran crude.

Medium	Bacterial	Calculation	PI Score
Control	*Bifidobacterium*	0.90 ± 0.05	< 1
	Lactobacillus	1.00 ± 0.02	> 1
LAE	*Bifidobacterium*	0.98 ± 0.01	< 1
	Lactobacillus	0.94 ± 0.04	< 1
LME	*Bifidobacterium*	0.97 ± 0.01	< 1
	Lactobacillus	1.15 ± 0.025	> 1
Inulin	*Bifidobacterium*	1.07 ± 0.035	> 1
	Lactobacillus	1.08 ± 0.031	> 1

From table 4, it could be inferred that the PI score of *Bifidobacterium* for crude laminaran (both LAE and LME) < 1, whereas the PI score of *Lactobacillus* > 1 only on the LME. In addition, the PI scores of Inulin > 1, which was simply because inulin is known as a prebiotic which has been tested. When examining PI scores from stirred pH-controlled batch cultures fermentation at pH 6.8 for 8 h of fermentation yielded HMP (high methylated pectin) and POS2 (pectic-oligosaccharide mixture) of 0.11 and 0.00 (PI score < 1), but when using LMP (low methylated pectin) and POS1 (pectic-oligosaccharide) yields PI values of 1.29 and 1.79 (PI > 1) [27]. When researching inulin from static batch culture fermentation of 1% for 5 h yields a score of 0.36 (< 1) and 24 h yields of 1.82 (> 1) score [28]. Thus, laminaran especially LME extract deserves to be regarded as a prebiotic candidate.

4. Conclusion

The resulted prebiotic index values obtained using Laminaran Acid extract (LAE) and Laminaran Modified Extract (LME) were 1.29 and 2.10, with a negative index score for LAE prebiotic and a positive score for LME prebiotic of *Lactobacillus* group. Laminaran especially LME extract deserves to be regarded as a prebiotic candidate.

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