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Characterization of Physico-chemical of Crude Laminaran from Sargassum duplicatum and SCFA Profile with Bacterial Fermentative from Wistar Rats Feces

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ABSTRACT

Sargassum duplicatum algae was extracted using two methods, the first is extraction with 0.09 M sulfuric acid solution (LAE), and the second is extraction with 0.09 M sulfuric acid followed by water extraction of its residue (LME). Laminaran was analyzed for total fiber, water-soluble fiber, water-insoluble fiber, water holding capacity, oil holding capacity, and in vitro laminaran degradation. In vitro assay was done by fermenting the laminaran (LAE and LME) at 37 °C for 36 h using caecal bacteria from 2.5 mo. old male Wistar rat. Fermented products at 0, 9, 12, 24 and 36 h of incubation were analyzed for its organic acids content using gas chromatography. The results show that LME has higher content of dietary fiber, water-soluble fiber, water-insoluble fiber, and water-holding capacity than LAE. LME and LAE was degraded during fermentation to yield acetic, propionic, and butyric acids during 36 h of fermentation, with the acetate: propionate: butyrate molar ratio of 62: 30: 8 and 63: 29: 8, respectively. Butyric acid production from LME after 36 h fermentation was 2.5 times higher than from LAE.
Keywords: laminaran, *Sargassum duplicatum* extract, caecal bacteria, dietary fiber.

INTRODUCTION

The role of gut micro flora is very important to a person's health. Unbalanced gut micro flora population may lead to many cases of gut-related health problems. Interactions between diet, colonic bacteria and body health drive the development of a new dietary strategy which supports the growth of health-promoting colonic bacteria. The increase in society's demand for functional foods is caused by the increase in gut-related health problems like diarrhea, constipation, irritable bowel syndrome, and colorectal cancer. Colorectal cancer is caused by many factors, but diet holds a very significant role in the cancer etiology [1]. Diet containing fiber and resistant starch can prevent colorectal cancers. Both of them can be fermented in the colon to produce short chain fatty acids (SCFA) like acetate, propionate and butyrate. Butyrate was reported to be effective in reducing colorectal cancer risk [2]. Resistant starch and dietary fiber are food components that have become main interest to many researchers. Dietary fiber is edible plant polysaccharide, resistant to digestion, not absorbed in human intestines but can be partly or totally fermented in the colon [3]. Many fiber sources are being used for food supplements, like FOS (fructo-oligosaccharide), GOS (galacto-oligosaccharide), and cereals. Today, the demand for dietary fiber keeps rising. It drives the search for alternative source of dietary fiber, like algae from aquatic or marine organisms. Algae is a fiber source, rich in nutrients, low calories, has high total and water-soluble fibers, high antioxidant capacity, high degree of ferment ability, and high water holding capacity [4]. Algae is also a potential dietary fiber source which has different chemical and physic-chemical composition compared to land-based plants, thus may be having different physiological effects to human body [5][6].

Mostly, algal polysaccharides are resistant to hydrolysis by various human digestion enzymes. It can then be categorized as dietary fiber [7][8]. Brown alga is rich in dietary fiber, especially water-soluble fiber. *Sargassum* sp. is classified as brown alga which is widely distributed in Indonesia, mostly in rocky coastlines with big waves. It is currently underutilized. Some minor fraction is used for raw material for alginate. Brown alga is rich in carbohydrates (in *Laminaria* spp. it can reach 55% of algal dry weight). The carbohydrates are consisted of laminaran (β-1,3-glucan), fucoidan, cellulose, alginate, and mannitol. Laminaran is the main carbohydrate storage of brown algae (with the range from 0 to 35 % as influenced seasonally) [9]. This compound is a short polymer with 20-25 glucose molecules with β (1, 3) bonds and branches at β (1, 6) bonds [10]. In the last few years, the characterization of physiological effects becomes well developed research focus. Some mechanisms have been postulated to explain many physiological effects of dietary fiber. Among them is the ability of some kinds of fiber to form gel and slow down carbohydrate and lipid absorption in the intestines [11]. The SCFA which is produced during fiber fermentation in the colon can bring some health benefits to the body, among which is reducing cholesterol or preventing colorectal cancers [12]. Fiber is degraded in the colon by bacteria which are growing during fermentation, its relation with the increase of fecal bulkiness may be important in reducing colon cancer and IBS (Irritable Bowel Syndrome) cases [13].

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The descriptions above show that dietary fiber has potential to reduce the incidence of colorectal cancers. Many of dietary fiber sources can be found in Indonesia, but marine-based dietary fibers have not been utilized widely. *Sargassum duplicatum* (brown alga) has the potential as a dietary fiber source. The aim of this research was to determine the dietary fiber content of *S. duplicatum* extract; its fermentability, especially the crude laminaran fraction by analyzing the SCFA profile produced during fermentation; and its water and oil holding capacity (WHC and OHC, respectively). *In vitro* method was used to predict one physiological effect arising from dietary fiber consumption.

MATERIALS AND METHODS

Materials

The main material used in this research is brown algae *Sargassum duplicatum* which was obtained from Talango Island, Sumenep, and Madura. It is harvested from its wild native habitat, the age was not known, in a 50-250 cm depth tidal-affected coastal area in a rainy season, February 2009.

Dietary fiber source

Substrate used in this research is acidic extract [14] and water extract of the residue as the original substrate, and inulin (Merck) as a comparison.

Extraction Method

Crude laminaran extract was produced using modified Yvin method [14]. 50 mg of algal powder was extracted with 0.09 M sulfuric acid solution (1 : 14) in a shaker water bath (120 rpm) at 70 °C for 150 min, to yield first filtrate and residue. Then, residue was extracted again with 0.09 M sulfuric acid solution (1: 14) to yield second filtrate. Both filtrates are then mixed, centrifuged, evaporated, precipitated with 96% ethanol and centrifuged at 5,000 rpm, after that the resulting pellets was dried and powdered (LAE). The second extraction method (LME) was 50 mg algae powder extracted with 0.09 M sulfuric acid solution (1: 14) in a shaker water bath (120 rpm) at 70 °C for 150 min, to yield first filtrate and residue. Then residue was extracted again with water, then the resulting filtrate was mixed with first filtrate and the next steps were the same with first method.

Physicochemical Analysis

Total dietary fiber was analyzed using method from Prosky *et al*. [15]. Water holding capacity (WHC) and oil holding capacity (OHC) was analyzed using procedures from Chau and Cheung [16] and were calculated as gram of water or oil loss during drying, divided by residual dry weight. WHC from substrate was analyzed by hydrating substrate (250 mg) in 25 ml distilled water for 24 h.

Fermentation Procedure

Substrate was fermented *in vitro* for 36 h with caecal bacteria from 2.5 Mont old male Wistar rat. Fresh caecal material was collected and directly used for experiment. Caecal material was mixed and solubilized in 9 volume of GAM ¼ which contains ingredients shown in Table 1.

Table 1 Medium composition for *in vitro* fermentation

No.	Component	Concentration (per 1000 ml distilled water, pH 7.1)
1.	Peptone	2.5 g
2.	Soy-peptone	0.75 g
3.	Proteose–peptone	2.5 g
4.	Digested serum powder	4.35 g
5.	Yeast extract	1.25 g
6.	Meat extract	0.55 g
7.	Liver extract	0.3 g
8.	NaH_2PO_4	0.625 g
9.	NaCl	8.5 g
10.	L L-cysteine–HCl	0.075 g
11.	Sodium thioglycolate	0.075 g

Source: Kuda *et al.* [17]

Then caecal slurry (0.5 ml) was inoculated into 4 ml GAM ¼ which contain urea (1 mg/ml) and tyrosine (5 mg/ml) [17], with or without 10 mg/ml LAE or LME extract or inulin. The culture was incubated at 37 °C for 36 h (gas generating kit was used to remove CO_2 and keep the medium pH from decreasing drastically). Analysis was done at 0, 9, 12, 24, and 36 h of incubation. Organic acids content was determined using gas chromatography by the procedure of Miller and Wolin (1996). Sample for SCFA analysis (2 ml) was mixed with 0.5 ml of 250 g/l meta-phosphoric acid, precipitated at room temperature for 20 min, then centrifuged at 25,000×g for 20 min.

Statistical Analysis

Data was analyzed using Microsoft Excel 2007. Statistical analysis was done with ANOVA and continued with Duncan's Multiple Range Test at 5 % significance level.

RESULT AND DISCUSSION

Dietary Fiber

Algal dietary fiber is different in its composition, chemical structure, physicochemical properties, and biological effects compared to terrestrial plant. The dietary fiber content of laminaran for total fiber (TDF), soluble fiber (SDF), and insoluble fiber (IDF) for both LAE and LME are 31-53.6 %; 26.6-45.9 %, and 4.4-7.7 % (db), respectively, as shown in Figure 1.

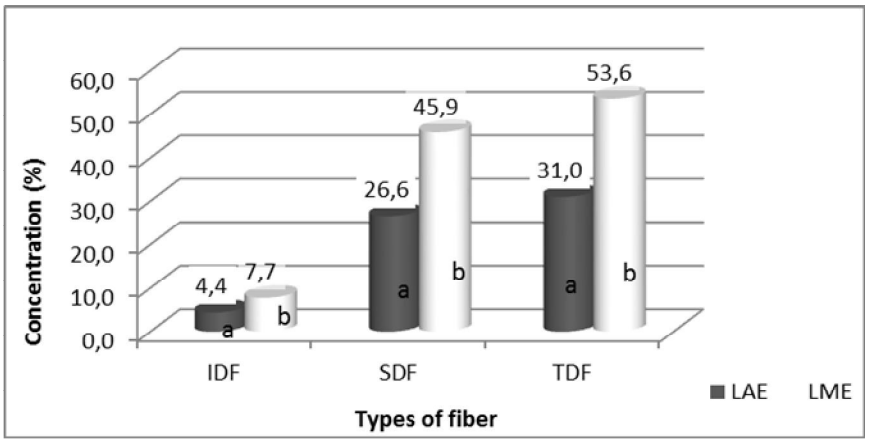

Figure 1 Dietary fiber content of crude laminaran

From Figure 1, it is known that dietary fiber content of LME was higher than LAE for each categories of fiber (TDF>SDF>IDF). TDF, SDF, and IDF of LME was higher (53.6 %, 45.9 %, and 7.7 %) than LAE (31.0 %, 26.6 %, and 4.4 %), respectively.

The higher dietary fiber content of LME compared to LAE was probably caused by the difference of extracting. Acidic solution produced lower total fiber since laminaran is more soluble in water, so that water extraction can maximize laminaran yield. It is in agreement with Fleming and Manners (1965) in Michael and Macfarlane [9] that laminaran polymer is neutral and soluble in hot water. In acid extraction, non-fiber components were more easily extracted so the fiber content was lower. Soluble and insoluble dietary fiber of LME was higher than LAE, probably it was because LME extraction was using water. LAE used acid extraction, so it may account for the formation of more rigid and more insoluble sulfate bonds. High content of insoluble dietary fiber in LME is the residue of laminaran extract, so it probably consists of many algal materials which are water-insoluble. It can be said that TDF = SDF + IDF. Total dietary fiber content in this experiment is in agreement with other publications, TDF of brown algae species ranges from 34.7 % to 74.6 % [18][19].

Dietary fiber can be classified into soluble and insoluble forms based on its dispersion profile when mixed with water. Soluble dietary fiber of some algal species ranges from 17.2 % to 59.7 % [18]; while Nishimune *et al*. [20] found that algal soluble fiber ranges from 51 % to 85 %. IDF content according to Ruperez and Saura-Calixto [21] is between 16.3 – 19.2 %. The ratio between soluble and insoluble dietary fiber of crude laminaran (LME and LAE) was 6: 1, while Deville *et al*. (2004) found it was 2.5: 1. Phorpira algae has soluble and insoluble fiber ratio of 6.9: 1 [22].

This high soluble dietary fiber content of crude laminaran makes it a potential source of dietary fiber, and it may also give health benefits. Lahaye [5] stated that marine algae are rich in enzymatically-indigestible polysaccharide, which is very good as a source of soluble dietary fiber. Clinical studies for the intake of soluble fiber shows its ability to reduce absorption rate of metabolizable nutrient in the intestines, and that in turn will reduce glycemic load of the body [23]. Laminaran fiber reduces insulin response level as a consequence of absorption rate reduction. It in effect may minimize the risk of cardiovascular disease, diabetes mellitus type II, and obesity [24][25]. Types of soluble dietary fibers are β-glucan, xyloglucan, galactomannan, hemicellulose, pectic substance, gum, and mucilage. However, cellulose, lignin, arabinoxylan, hemicellulose, and resistant starch are components of insoluble fiber [26].

Water Holding Capacity (WHC)

WHC is an important function of dietary fiber from physiological and technological perspective [27]. WHC data from this experiment is shown on Figure 2.

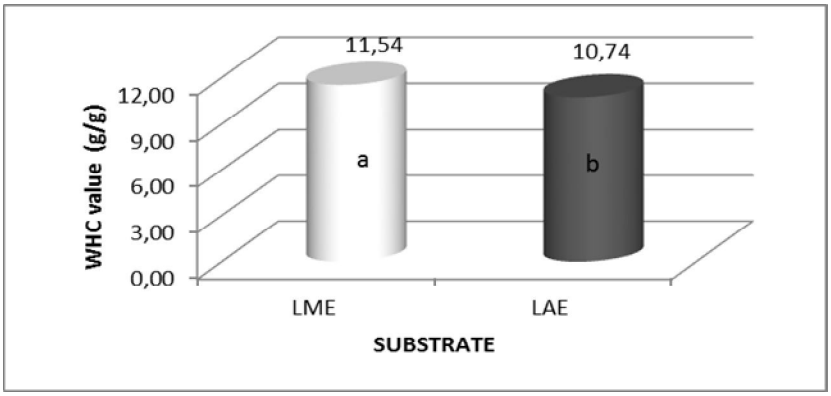

Figure 2 WHC of crude laminaran

From Figure 2, it is clear that WHC value of LME (11.535 g water/g fiber) is significantly higher than LAE (10.739 g water/g fiber) ($P \leq 0.05$). This difference is probably due to lower active sides and higher soluble fiber concentration of LME because of water extraction, so that it water holding capacity gets higher too. Besides that, laminaran is classified as water soluble polysaccharide, where water is held by the fiber matrix from hydrophilic polysaccharide or by the entrapment of intestinal mucosal cell wall [28]. Since LAE was extracted with acid, it just has sulfate active sides. According to Robertson and Eastwood [29], fiber structure affects the amount active sites that hold water. Fiber hydration happens because of surface adsorption of water and water entrapment in gel pores or fiber matrices.

WHC of *Enteromorpha* algae (10.44 ± 0.17 g/g, db) is comparable with the WHC of crude laminaran (LME and LAE), but *Wakame* algae has much higher WHC value (38.6 g/g, db). Algal species, harvesting time, and habitat may account for this difference. These WHC value is in agreement with its species according to Grigelmo-Miguel *et al*. [30]; Wachirasiri *et al*. [27]. Quite high value of crude laminaran WHC may be because laminaran has similar structure with amylose. Rodríguez-Ambriz *et al*. [31] stated that amylose as the main component of starch has the properties in affecting water molecule binding to yield high WHC.

The quite high WHC values of crude laminaran (LME and LAE) are advantageous. According to Brownlee *et al*. [32] the physiological effect of dietary fiber (like bulking luminal content, reducing transit time and increasing mutagen binding) may reduce the exposure of potential destructive agents that come from bacteria, food, and native residing endogenous bacteria to colonic mucosa. In the upper part of the gut, WHC may affect nutrient absorption pattern, postprandial satiety, and gut motility, it may also affect the participation of water–soluble nutrients into a gel structure. In turn, it may reduce diffusion rate of absorption at mucosal lining. It was found that fresh and dried stool weight increase in livestock feeding trial with seaweed addition [33], which shows that the high WHC value of undigested diet mostly comes from seaweed [21].

Oil Holding Capacity (OHC)

Oil holding capacity is another important functional property that affects sensory profile from food formula [34]. OHC values from this experiment are shown on Figure 3.

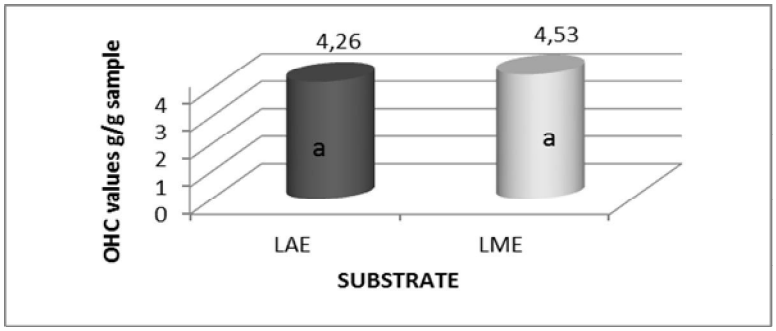

Figure 3 OHC value of crude laminaran

In Figure 3 it is shown that oil holding capacity between LAE and LME is not significantly different. This is probably caused by the same particle size, total charge density, and natural hydrophobic from individual particle [35].

OHC values in this experiment is in agreement with other results like *Enteromorpha* algae (4.29 ± 0.05 g oil/g sample) (Mamatha *et al*., 2006); *U. intestinalis* (4.62 g oil/g) (Benjama and Masniyom, 2011); *U. fasciata* meal (4.52 g oil/g DW) (Carvalho *et al*., 2009), but higher than *L. digitata* (0.16-0.41 g/g) [35]; *U. pertusa* (1.53 g oil/g DW); and *U. lactuca* (1.46-1.68 g oil/g DW) [36]. OHC property of food materials is associated with surface property, total charge density and lipophilic and hydrophilic constituents [35]. High OHC materials are the best choice for stabilizers in formulated foods. In addition, this fiber can reduce blood lipid level, obesity, and risk of coronary heart disease [36].

SCFA Content of Laminaran

Short chain fatty acids (SCFA) are organics acid with 1-6 carbon atoms in anionic form, which comes from bacterial fermentation with polysaccharides, oligosaccharides, proteins, peptides, and glycoproteins as precursors inside the colon [37]. Result of analysis of SCFA production using gas chromatography from *in vitro* fermentation of crude laminaran is shown on Figure 4 and Figure 5.

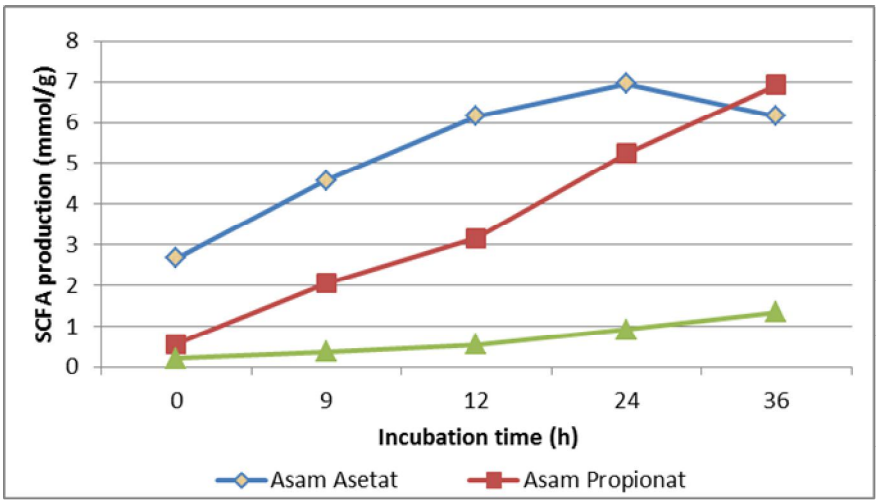

Figure 4 SCFA production during *in vitro* fermentation of LME

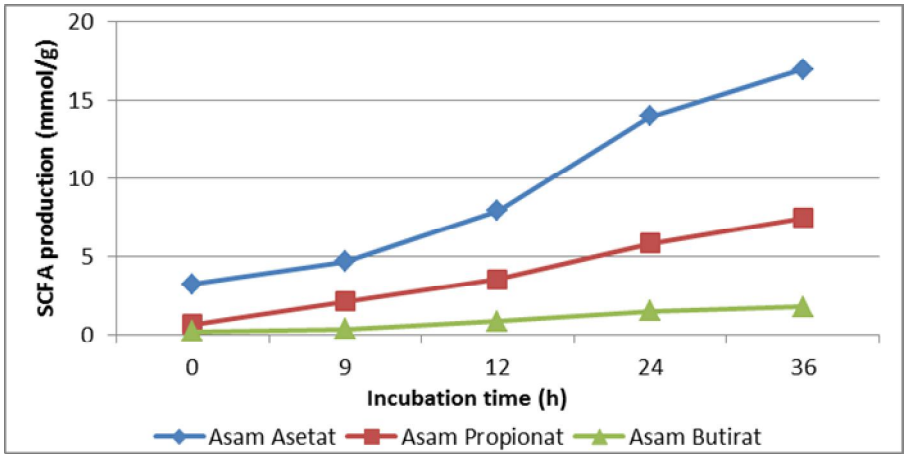

Figure 5 SCFA productions during i*n vitro* fermentation of LAE

It is known from Figure 4 and 5 that in the start of fermentation (0 h incubation), both LME and LAE had already had acetic, propionic and butyric acid of 3.23; 0.65; 0.22 mmol/g and 2.68; 0.56; 0.20 mmol/g, respectively. The presence of short chain fatty acids at the start of incubation probably come from the inoculums itself (the rat feed). Up to 9 h of incubation, total SCFA of LME and LAE increased slowly, this is probably

because laminaran started to be degraded after 6 h of incubation. According to Michel *et al*. [9], SCFA formation in brown alga happens after 6 h of incubation, as much as 50 % of total production. The lag of fermentation is probably due to the time needed for induction and synthesis of laminarinase. Also, the fermentation substrate is crude laminaran which probably is still containing other polysaccharides like alginate that can only partially fermented, or fucoidan that cannot be fermented [38].

At the start of LME incubation, butyric acid content was 0.22 mmol/g and it became 1.81 mmol/g after 36 h, an increase of 7.23 fold. In LAE it was initially 0.20 mmol/g and increased to 1.34 mmol/g after 36 h, an increase of 5.7 fold. The increase of butyric acid concentration is probably due to the activity of extracellular enzymes in the supernatant from microbes of rat's digest. This is supported by Michel *et al*. [9] that laminaran is degraded by gut micro flora to yield high level of butyrate. The difference of 2.5 fold of butyrate concentration may be because LME is easier to be degraded by gut micro flora than LAE. This is also supported by the higher concentration of soluble dietary fiber in LME. Butyric acid concentration rose sharply during incubation, both in LME and LAE. The same thing is also found in Michel *et al*. [9] that after 24 h of incubation there is oligomers accumulation in the culture, degraded perfectly and converted about 85 % to SCFA. Butyrate production indicates the general process of fermentation of glucose-containing polymer [9].

If compared to arrowroot starch that is de-esterified with butyrate, butyrate concentration of crude laminaran (LME and LAE) is very small, but the production rate during incubation is much higher than butyrate de-esterified arrowroot, which is only 0.73 fold [39]. Kuda *et al* [17] showed that laminaran from *E. bicyclis* only increased acetic acid and propionic acid in rat caecal by 0.29 fold and 1.07 fold, respectively. Michel *et al*. [9] reported SCFA production from laminaran increased from 8.4 fold to 230 fold after 6-24 h incubation. It can be concluded from data above that the majority of gut micro flora of rat are able to degrade crude laminaran from *S. duplicatum*.

SCFA molar proportions of the result of dietary fiber fermentation are shown in Table 2. The analysis of SCFA from rat digesta is shown in the form of molar ratio to describe the ratio of acetate, propionate, and butyrate in one experiment.

Table 2 SCFA molar ratio from fermentation of crude laminaran by rat caecal micro biota

Substrate	Molar ratio (%)		
	Acetic acid	Propionic acid	Butyric acid
Control	69	25	6
AE Laminaran	63	29	8
ME Laminaran	62	31	8
Inulin	65	28	7

Acetate molar ratio in control sample was higher than other samples (LAE, LME and inulin). This is due to the main fiber fermented was cellulose (CMC) that will be fermented to mostly acetate (69 %). According to Miller and Wolin [39], acetate is the largest component, ranges from 60 to 75 % of SCFA total pool. Some part of acetate will be used during butyrate synthesis [39]. Most of bacteria can produce acetate, but most of them are unable to produce propionate and butyrate in the colon.

Substrate containing LAE and LME had lower acetate molar ratio. It is due to the high β-glucan content of LAE and LME that is difficult to be directly hydrolyzed by Bifidus or Lactobacillus, but can be degraded by Bacteroides and then by another bacteria (cross feeding). Salyers *et al*. [8] stated that laminaran is degraded by most of Bacteroides species (*B. thetaiotaomicron, B. distasonis*, Bacteroid 0061-1, Bacteroid T4-1). Laminaran is degraded into either glucose monomers or glucose-containing oligomers with DP from 2-6. The difference in oligomer accumulation reflects the difference in ability of different bacterial species to produce specific active enzymes against β-glucosylmannitol, laminariobiosylmannitol and gentibiosylglucose (Michel and Macfarlane, 1996). Acetate molar ratio of brown algal species *Himanthalia elongata*, *Laminaria digitata* and *Undaria pinnatifida* are 73.3; 68.5 and 74.9 %, respectively.

Butyrate molar ratio from substrate containing LAE and LME was higher than other samples. This is due to LAE and LME components were probably have been degraded by bactericides so that it will easily fermented into butyrate. Laminaran is degraded by gut micro flora to form high level of butyrate [9]. According to Macfarlane and Englyst [9], butyrate production indicates fermentation of glucose-containing polymers. Butyrate molar ratio of *Himanthalia elongata*, *Laminaria digitata* and *Undaria pinnatifida* are 9.6; 8.9 and 8.6 %, respectively. Therefore, the molar ratios between algae are similar.

CONCLUSION

LME extract is better than LAE based on its dietary fiber content of TDF, SDF and IDF: 53.6 %; 45.9 %; 7.7 % and 31.0 %; 26.6 %; 4.4 %. WHC values of LME and LAE are 11.535 g water/ g fiber and 10.739 g water/g fiber, respectively. OHC values are not significantly different between two fractions. There is an

increase in butyrate concentration in LME compared to LAE after 36 h incubation. The molar ratio of acetate: propionate: butyrate are 62: 30: 8 and 63: 29: 8 in LME and LAE, respectively.

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