The Effect of *Sargassum angustifolium* Brown Seaweed Extracts on Gut Microbiota in Induced Obese Male Rats

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Abstract

Marine organisms, especially brown algae are good dietary complements with probiotic and anti-obesity activities. The objective of this study was to find the effects of Persian Gulf marine algae *Sargassum angustifolium* (SA) on the intestinal microbiota of animals exposed to a high-fat diet (HFD). Rats were fed with hot water (HW) and ethanolic (E) extracts of *Sargassum angustifolium* for two months. At the end of the experimental period, serum indices, food intake, and weight loss were measured. We also analyzed the intestinal microorganisms through 16SrRNA sequencing in all groups. The distribution ratio of intestinal microorganisms showed that *Bacteroides* and *Firmicutes* as dominant phyla in the intestinal rats, and obesity-associated bacteria were decreased and leanness-associated genera were increased in all treatment groups compared with the control obese group. *Clostridium* and *Lactobacillus* were the dominant genera in all groups with pathogenic potentials, and lactic acid genera, respectively. In addition, all the extracts could decrease the level of cholesterol, inflammatory factors including IL1 and TNF-α, and liver enzymes, and also led to reduction of the food intake and weight loss due to high fibers. Our results indicate that consumption of *Sargassum angustifolium* seaweeds can balance intestinal microbiota as well as decrease serum indices.

**Keywords:** *Sargassum angustifolium*; intestinal microbiota; obesity; 16SrRNA.

**Abbreviations:** Alkaline phosphatase (ALP), Aspartate aminotransferase (AST), Ethanolic(E), Ethanolic *Sargassum angustifolium* (E-SA), High-fat diet (HFD), Hot water (HW), Hot water *Sargassum angustifolium* (HW-SA), Interleukin 1 (IL1), One-way analysis of variance (ANOVA), Operational taxonomic units (OTU), Short-chain fatty acids (SCFAs), Tumor necrosis factor alpha (TNF-α).

INTRODUCTION

Obese people are at risk for diseases such as type 2 diabetes, dyslipidemia, high blood pressure, cardiovascular disease, non-alcoholic fatty liver disease, hypertension, hypercholesterolemia, tumors, immune dysfunction, rapid onset of infection, delayed wound healing and certain types of cancers (Wan-Loy and Siew-Moi, 2016). COVID19 pandemic changes the life style; and as a result, the obesity prevalence might be increasing. Besides, Insufficient knowledge of people about healthy nutrition and inappropriate method for weight losing will lead to more serious complications (Matsuzawa, 2007; Park et al., 2011). On the other hand, gut microbiota plays an important role in several metabolic processes and human diseases. Various dietary factors, including complex carbohydrates, such as polysaccharides, provide abundant nutrients and substrates for microbial metabolism in the gut, affecting the members and their functionality. It was demonstrated that intestine microbiota has been considered as one of the main causes of obesity (Kelly et al., 2016; Kim et al., 2018). In addition, the intestinal microbiota has a major impact on the immune system and immune responses of the host (Wiertsema et al., 2021). The gut bacterial composition is commonly affected by various factors, including age, antibiotics, diet, and disease, and it is now found that diet plays a significant role in shaping the microbiome (Walker, Ince et al., 2011). Some studies suggest that the consumption of probiotic substances and dietary fibers can beneficially alter the gut microbiome composition in a short time (Wen and Duffy, 2017; Hasan and Yang 2019). Besides, recent findings have shown that *Firmicutes* and *Bacteroidetes* are two dominant phyla in human gut microbiota and an increased *Firmicutes*/*Bacteroidetes* ratio correlated with obesity (Kim et al., 2018).

Marine organisms, especially brown algae are good dietary complements with probiotics and anti-obesity potentials for those who want to lose weight (Hu et al., 2016). Furthermore, human consumption of marine
seaweeds has been increasing because of their benefits with health-enhancing biological properties including anti-inflammatory, anticancer, and immune-enhancing functionalities (Lopez-Santamarina et al., 2020; Oh et al., 2022). The potential benefits of brown seaweeds are partly due to the high dietary fibers, sulfated polysaccharides, that are not digested a long time in the intestine. Such fibers can increase satiety feeling through bulking capacity (Draget and Taylor, 2011). Moreover, gut microbiota can degrade seaweeds polysaccharides into other bioactive compounds, such as oligosaccharides and short-chain fatty acids (SCFAs) acting as food sources for these organisms (Oh et al., 2022). It was found that other bioactive molecules such as polyunsaturated fatty acids, phytochemicals, and polyphenolic compounds in seaweeds have the prebiotic activities and potential health benefits of managing obesity(Wan-Loy and Siew-Moi, 2016). Besides, the consumption of brown seaweed can inhibit the growth of pathogenic bacteria in the gut and promote the growth of beneficial bacteria such as lactic acid bacteria. Recent evidence shows that increasing beneficial intestinal microbes following the intake of brown seaweeds can reduce weight gain in rats (Kim et al., 2016).

In the present study, we aimed to evaluate the effect of hot water and ethanol extracts of Persian Gulf brown algae Sargassum angustifolium, on the intestine microbiota, food intake, and weight loss in rats that fed with high-fat diet.

MATERIAL AND METHOD

Collection and identification of species
Sargassum angustifolium was collected from the coastal of Qeshm island in the south of Iran during March. The specie was confirmed by comparing the algae morphology with the information presented in www.Algaebase.org, different identification keys (Leliaert & Coppejans, 2003; Braune, 2011), and Iranian Marine Macroalgae Checklist (Kokabi & Yousefzadi, 2015). Fresh samples were initially washed with seawater to remove epiphytes, and then they were washed again with tap water in the laboratory. The clean seaweeds were dried in the shade for 1 week. The dried samples were pulverized using a grinder, and then stored in airtight plastic bags at −20 °C until use.

Preparing of the extracts
Ethanolic and hot water extracts were prepared by adding 10g of seaweed powder in 100 mL of either 70% ethanol or double-distilled water, respectively, on a shaker for 72 h at dark. The mixture was centrifuged two times at 4000 g for 10 min at 4 °C.

Animal treatment
A total of sixty male Wistar rats (four-week-old and 200-230 gr weight) were purchased from the animal Lab of Shiraz University of Medical Sciences. All mice were acclimated for two weeks at controlled temperature (22-24°C), humidity (55-60%), and lighting (12 h light/dark cycle) with free access to water and routine food. Then, the rats were weighed and fed with high-fat diet which contained 60% cholesterol (Merck, Germany) for one months.

Experimental design
The rats were divided into two control and two experimental groups. The experimental groups were fed with either ethanolic or HW extracts of SA. Experimental groups were further divided into two subgroups which were treated with one of the aforementioned extracts at a dose of either 100 or 200 mg/kg for HW or 250 and 500 mg/kg for ethanolic extract (n=10). Our experiments were continued for two months. At the end of the experiment, the rats were euthanized by CO₂, and blood samples were collected (three rats in each group) to measure the concentration of cholesterol, ALP, AST, TNF-α and IL1, fecal samples were taken (three rats in each group) for isolation and identification of dominant microbial strains.

Enrichment and isolation of microbial mixtures
For the initial culture of the samples, non-selective media were used to grow the majority of bacteria. Nutrient Broth, Nutrient agar, Blood agar, MacConkey agar, Eosin methylene blue 4, media were provided and samples were cultured on the aforementioned media, then all of them were incubated for 24 hours at 37°C. After incubation, other tests, including gram staining to detect Gram-positive and negative bacteria, triple sugar iron (TSI), sulfide indole motility (SIM), Simon citrate, urease, oxidase, and oxidation, and fermentation tests were performed for them.

DNA Extraction and PCR
DNA was extracted from stool samples using QIAamp DNA stool mini kit (Qiagen, Hilden, Germany) according to the manufacturer. Amplification and barcoding of PCR products of different samples were performed in two steps. In first step, the V3–V4 region of 16S rDNA was amplified using primers Bact-0341F 5’-CCTACGGGNGGCWGCAG-3’ and Bact-0785R 5’-GACTACHVGGGTATCTAATCC-3’. PCR conditions were performed in the first step: 5 min at 95°C (initial denaturation), 25 cycles at 95°C for 30s (denaturation), 55°C for 30s (annealing), and followed by 72°C for 1 min (extension) and finally 72°C for 7 min (final extension). The sizes of amplicons were 400-500 bp for bacteria. 1 μL of the PCR products from each sample was run on 2% agarose gel to confirm PCR.

second PCR was performed with 2 μL PCR products, and 15 cycles at 55°C for Barcoding amplified products by using Overhang adapter sequences related to Illumina Company. 1 μL of the PCR products from each sample
was run on 2% agarose gel to confirm PCR. 7 μL of purified products were sequenced by Macrogen company (Macrogen Inc. (Seoul, South Korea). The volume of materials was used in this study showed in Table 1.

**Table 1.** The volume of materials for PCR.

<table>
<thead>
<tr>
<th>Materials of PCR</th>
<th>Volume</th>
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</thead>
<tbody>
<tr>
<td>Primer F</td>
<td>1μL</td>
</tr>
<tr>
<td>Primer R</td>
<td>1μL</td>
</tr>
<tr>
<td>PCR Master Mix (2X)</td>
<td>10μL</td>
</tr>
<tr>
<td>DDW</td>
<td>12μL</td>
</tr>
<tr>
<td>DNA template</td>
<td>2μL</td>
</tr>
<tr>
<td>TOTAL VOLUME</td>
<td>25μL</td>
</tr>
</tbody>
</table>

**Measurement of serum Index**

Blood samples were taken from the heart and sera were separated by centrifugation 1500g for 15 min. Total cholesterol were performed by EverlyWell kit, serum Index of Interleukin 1(IL1) by Eliza kit (Abcam, England), Tumor necrosis factor alpha (TNF-α) by Eliza kit (Bender Medsystem, England), and some liver enzymes were determined using kits Man (Applied biosystems French).

**Statistical analysis**

Statistical analysis was done using one-way analysis of variance (ANOVA). The data were analyzed and the graphs were depicted by graph pad prism 8,3/4. A p-value less than 0,05 was accepted as the significant difference. PCR products were analyzed by BLAST. All the tests were performed in triplicate.

**RESULTS**

**Analysis of Intestinal Microbiota**

After 60 days of exposure to different extracts, the intestine bacteria were evaluated in all treated groups and compared to control groups. The distribution ratios of intestinal microorganisms revealed that *Firmicutes* and *Bacteroides* were dominant bacteria in rats' intestines in all groups so that, they altogether accounted for 96% of intestinal microorganisms. We observed *Firmicutes* decreased and *Bacteroidetes* increased in all treatment groups compared to the obese control group. Both concentrations of HW-SA sufficiently decreased *Firmicutes* to reach the level of normal control. The dominant species of bacteria we found in the fecal rats in each group are shown in Table 2.

**Table 2.** Dominant species bacteria in the fecal rats in treatment groups.

<table>
<thead>
<tr>
<th>Group</th>
<th>Species</th>
<th>Doses</th>
<th>Dominant Genus</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td><em>Sargassum angustifolium</em></td>
<td>HW-E 200mg/kg</td>
<td>Alistipes, Bacteroides, Turicibacter, Prevotellaceae,</td>
</tr>
<tr>
<td>2</td>
<td></td>
<td>HW-E 100mg/kg</td>
<td>Alistipes, Bacteroides, Turicibacter, Coprobacillus</td>
</tr>
<tr>
<td>3</td>
<td></td>
<td>E-E 500mg/kg</td>
<td>Alistipes, Bacteroides, Coprobacillus, Prevotellaceae</td>
</tr>
<tr>
<td>4</td>
<td></td>
<td>E-E 250mg/kg</td>
<td>Alistipes, Prevotellaceae, Coprobacillus</td>
</tr>
<tr>
<td>5</td>
<td>control</td>
<td></td>
<td>Allobacillus, Turicibacter, Coprobacillus, Butyricoccus, Firmicutes, Alistipes, Escherichia, Enterobacter</td>
</tr>
</tbody>
</table>

**It was found that Firmicutes was decreased from 89% in the obese control group (group one) to 32% in 200mg/kg HW-SA group (group two), 33% in 100mg/kg HW-SA group (group three), 38% in 500mg/kg E-SA group (group four) and 40% in 250mg/kg E-SA group (group five). Bacteroides was increased in all treatment groups when compared to the obese control group. The distribution of Bacteroides was 7% in the obese control group and in all treatment groups respectively were increased to 64% in group two, 63% in group three, 57% in group four, and 56% in group five (Fig. 1). Based on the above results, it can be concluded that consumption of brown seaweeds daily can increase Bacteroides.**
Moreover, the microbiome data were analyzed to determine bacterial groups at the genus level according to the characteristics of different species. The analysis showed 4 functional groups including the obesity-associated genera, the leanness-associated genera, the genera with pathogenic potentials, and the genera belonging to lactic acid genera.

The obesity-associated genera included Allobaculum, Turicibacter, and Oscillibacter were observed in all groups. However, the frequency of these genera in treatment groups was significantly reduced compared to the obese control group. Both concentrations of HW-SA groups showed the highest decrease in the obesity-associated genera (Fig 2).

The current study indicated that Bacteroides, Prevotella, and Alistipes were the leanness-associated genera in all groups. All treatment groups showed a significant increase in the frequency of these genera compared to the control group. Bacteroides and Alistipes were the dominant genera in the stool samples of treated animals so that, Bacteroides and Alistipes revealed approximately seven (P=0.003 compared to control) and four (P=0.002 compared to control) folds’ increase (Fig. 2).

As shown in Fig 3, both concentrations of HW-SA extracts led to a significant decrease in pathogenic genera compared to both control group (P=0.02 for 200mg/kg SA vs both control and P=0.003 for 100mg/kg SA vs both controls). The genera with pathogenic potentials including Bacteroides, Clostridium, Escherichia, Mollicute, and Prevotella were observed in all groups. The frequency of pathogenic genera in all treated groups was significantly reduced compared to the obese control group. Escherichia was not present in the treatment groups and Clostridium, the dominant genera, was accounted for in all groups (P < 0.05). (Fig. 3).

According to Fig 4, all extracts could significantly elevate the lactic acid microorganisms level compared to control obese groups. Lactic acid genera have received much attention for their probiotic properties and properties such as inhibiting pathogenic genera and increasing the immune system. Enterococcus, Lactobacillus, and Streptococcus were lactic acid genera that were observed in all groups. Our results showed that Lactobacillus was the dominant species in the treated groups. The aforementioned extracts led to a 98% increase in Lactobacillus frequency (P < 0.05) (Fig. 4).

Liver enzymes
As compared to the obese control group, both concentrations of HW-SA and E-SA extracts led to a significant decrease in ALP (P=0.01 for 200mg/kg HW-SA, P=0.04 for 100mg/kg HW-SA and P=0.005 for E-SA) so that, it reached to the normal level. Both concentrations of HW-SA extracts could significantly reduce the serum level of AST (P=0.01 for all groups, except for 250 mg/kg ethanolic extract) compared to obese control. Although E-SA extracts were also effective in reducing the level of AST, 250mg/kg E-SA could not decrease as well as other extracts. 250 mg/mL.
E-SA extract had no impact on serum AST compared to the normal controls (Fig. 5).

**Figure 5.** A: Alkaline phosphatase (ALP), B: Aspartate Aminotransferase (AST) Data are represented as mean ± SD (n = 3). *significant difference between the groups (*P<0.05, **P<0.01, ***P<0.001, **** P<0.0001).

**Cholesterol and Inflammatory factors**

The data of the present study indicated that 200mg/kg HW-SA, and 500mg/kg E-SA led to a significant decrease in the serum level of cholesterol (P=0.004 for 200mg/kg HW, P=0.0005 for 500mg/kg ethanolic extracts) compared to the obese control group, so that they could reach the normal level. 100mg/kg HW and 250mg/kg ethanolic extracts of SA were also efficient in diminishing the level of cholesterol (P=0.0002 for 100mg/kg HW, and P=0.00004 for 250mg/kg ethanolic extracts), but not as efficient as 200mg/kg HW-SA and 500mg/kg E-SA extracts. All extracts prepared from SA extracts led to a significant decrease in the level of IL1 (P=0.001 for all extracts) and TNF-α (P= 0.002 for 100mg/kg HW and 500mg/kg ethanolic, P=0.03 for 200mg/kg HW, and P=0.0008 for 250mg/kg ethanolic extracts) as low as the normal level.

**Figure 6.** Effects of alcoholic and hot water extracts on seaweed on rat blood serum biochemistry in HFD-induced obese rat. A: cholesterol, B: Interleukin 1(IL1), and C: Tumor necrosis factor alpha (TNF-α). Data are represented as mean ± SD (n = 3). *significant difference between the groups (*P<0.05, **P<0.01, ***P<0.001, **** P<0.0001).

**Bodyweight and Food intake**

We measured body weight loss and food intake in all animals, weekly. Treating the animals with all the SA extracts for 60 days significantly decreased weight and food intake. The food intake and weight reached normal for all treated animals after 30-37 days. The animals
were fed with 250mg/kg E-SA extract lost weight faster than the other groups. These animals could reach the normal weight on day 30. Feeding the animals with 500mg/kg E-SA extract caused slowly lost weight compared to other groups so that, bodyweight took the normal level after 37 days. The same way is repeated for food intake as well. After this period, the weight loss and decrease in food intake continued so that, the weight reached 179-174 gr, and the food intake reached 17-14 gr at the end of the 60 days (Fig. 7).

**DISCUSSION**

Seaweeds have beneficial components including polysaccharides, polyphenols, and peptides. They are a source of abundant dietary fibers that may act on gut health by prebiotics activities and could be digested by gut microbiota. It is important to note that bioactive compounds of marine algae with anti-obesity activity modulate the distribution of intestinal microorganisms (O'Sullivan & Murphy et al., 2010). Previous studies revealed that *Proteobacteria, Firmicutes, Actinobacteria,* and *Bacteroidetes* are the main phyla in the gut microbiome (Mikelsaar et al., 2011; Hugon et al., 2015; Hugon et al., 2015). Recent findings have shown that seaweeds are a source of abundant dietary fibers that change intestinal microorganisms in a short time, thereby improving obesity and weight gain (De Filippo et al., 2010; De Filippo et al., 2010; Neyrinck et al., 2011). Our studies also confirmed that *Firmicutes* and *Bacteroidetes* are the two main genera of intestinal microorganisms in all groups and the extracts prepared from SA were effective in changing intestinal microorganisms in obese rats; however, HW extracts were more efficient in reduction of the relative abundance of obesity-associated genera and elevation of the leanness-associated genera, respectively. Some studies showed that intestinal microorganisms play an important role in obesity and metabolic disorders (Turnbaugh et al., 2008), and it causes an increase in the level of *Firmicutes* and a decrease in the level of *Bacteroidetes* in the gut (Schwertz et al., 2010; Kim et al., 2016; Kim et al., 2018). Our data is in the same line with the other reports that indicated brown algae meals can reduce *Firmicutes/Bacteroidetes* ratio in the gut of all treatment animals.

The current study indicated that *Prevotella, Bacteroides,* and *Alistipese* were dominant genera when the animals were treated with HW and ethanolic extracts of SA. We also found that all extracts of SA administration can change the distributions of intestinal microorganisms in favor of those that prevent or improve overweight and obesity in rats. Consistent with our results, De et al (2010) reported that *Prevotella* and *Bacteroides* genera were extremely observed in African children who eat vegetables and diet with high fibers compared to European children who eat high-fat foods (De Filippo et al., 2010). *Prevotella, Bacteroides,* and *Alistipese* belong to the phylum *Bacteroidetes* can hydrolyze cellulose, and resulted in a higher decrease of fat accumulations (Neyrinck et al., 2011; Kelly et al., 2016; Kim et al., 2018).

*Allobaculum* and *Turicibacter* are a part of the class *Erysipelotrichi* with obesity activity which increases in the gut of obese individuals (Greiner & Bäckhed, 2011). These microorganisms cause accumulation of fat in cells and mild inflammation (Kim et al., 2018). Many studies have reported that marine algae components, such as alginate, ameliorated the relative abundance of obesity-associated genera in the human or animal intestine (Chater et al., 2015). Our data also indicated that all extracts prepared from SA led to a decrease in obesity-associated genera in rat intestines. Although HW extracts were more effective than ethanolic extracts. *Oscillibacter* is one of the intestinal bacteria that increases with the consumption of high-fat diet in the intestine of humans or animals. This microorganism is also associated with mild inflammation and the accumulation of fat in cells (Singh et al., 2013). The immune system is closely related to obesity so that numerous cytokines and chemokines, including IL1, IL6, Tumor necrosis factor-α (TNF-α), and C-reactive protein secreted from adipose tissue (Zatterale et al., 2019), they are important pro-inflammatory factor in obesity. On the other hand, intestinal pathogenic bacteria.
increase in the gut due to high-fat diets that can produce endotoxins, which lead to expression of inflammatory markers. These inflammations increase the expression of TNF-α, leptin, and adiponectin effect by significantly promoting obesity (Ding et al., 2010; Lam et al., 2012). The present study identified Clostridium, Escherichia, Mollicute, and Prevotella genera in the intestinal of obese control animals, and the level of these microbes were decreased by feeding the obese rats with SA extracts. We also showed that Persian algae extracts can normalize the level of IL1 and TNF-α. Our results are in the same line with previous studies which reported that the consumption of Undaria Pinnatifida and Laminaria Japonica for 30 days led to a slight decrease in distributions of pathogenic bacteria in the gut rats (Jy & Dy, 2016).

The treatments of the diet-induced obese rats with algae promote the proliferation of intestinal lactic acid bacteria especially, Lactobacillus. In addition, our data also confirms that both HW and ethanolic extracts of Persian Gulf algae have probiotic potency. It is well known that lactic acid bacteria can inhibit the growth of pathogenic microbes by reducing PH. Consistent with our results, Kim et al (2018) showed that Laminaria japonica had a probiotic and anti-obesity effect by significantly lowering the pathogenic bacteria levels and increasing lactic acid bacteria levels in gut-obese rats (Kim et al. 2018). Intestinal lactic acid genera have a variety of physiological functions, including modulation immune system and reducing blood cholesterol and lipid components (Park et al., 2008; Settanni & Corsetti, 2008).

In addition, obesity plays a crucial role in the formation of fatty liver and develops the risk factors of metabolic diseases, such as hyperglycemia, dyslipidemia, hypertension and increased levels of some hepatic enzymes. A large number of studies showed that liver enzymes including ALP and AST were as the most sensitive biochemical indicators and associated with obesity (Xie et al., 2018). A previous study indicated that ALT decreased by feeding the obese rats with water extract of S. binderi compared to the normal control rats (Hira et al., 2017). We showed that all the extracts prepared from SA reduced the level of cholesterol as well as liver enzymes, AST and ALP in HFD rats. Furthermore, we provided evidence that the administration of HW-SA and E-SA extracts had beneficial effects on weight and food intake in HFD rats. Several hypotheses have been proposed for the mechanism of the effect of seaweeds on weight loss and food intake is that the presence of high fiber and low lipid content in the brown seaweed (Matanjun et al., 2009; Matanjun et al., 2010).

**CONCLUSIONS**

The present study demonstrated that all the extracts led to weight loss by decreasing the obesity-associated bacteria and increasing the leaness-associated bacteria. In addition, we indicated that weight loss happened due to a decrease in food intake. Besides weight loss, the extracts normalized the cholesterol and the level of liver enzymes. The best extract that normalized all criteria was 200mg/kg HW-SA. It seems that in vivo animal studies can be extended to humans as well; however, it needs more investigations.

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**Author’s contributions:** Zarrin, and Taherizadeh have a role in conceptualization; Zarrin carried out the laboratory work. Talaei-khozani Zarrin and Tanideh have a role in Data collection; Zarrin and Talaei-khozani have a role in data analysis; Zarrin and Taherizadeh have a role in Funding acquisition. Talaei-khozani and Zarrin have roles in writing - original draft; writing - review & editing.

**Ethical statement:** All experiments were approved by the ethics committee of Hormozgan University (IR.HUMS.REC.1400.280).

**Competing interests:** The authors declare no competing interest.

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