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In vitro assessment of the prebiotic potential of *Caulerpa lentillifera, Gracilaria arcuata*, and *Sargassum polycystum* on probiotic *Lactobacillus* species

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Abstract

Macroalgae are rich in complex polysaccharides making them a good source of potential prebiotics – non-digestible polysaccharides that promote the growth of beneficial microorganisms in the gut. This study assessed the prebiotic potential of three common Southeast Asian macroalgal species: *Caulerpa lentillifera*, *Gracilaria arcuata*, and *Sargassum polycystum*, on isolated probiotic bacterial species *Lactobacillus casei* and *Lactobacillus paracasei*. The Competitive Growth Assay (CGA) of the two *Lactobacillus* species against the hospital-isolated *Escherichia coli*, done in Luria Broth with 2% glucose supplementation, was measured using the drop plate method on selective media. The following bacterial combinations for the CGA were: (1) *L. casei* + *E. coli*, (2) *L. paracasei* + *E. coli*, and (3) both *Lactobacillus* species + *E. coli*. Prebiotic potential was assessed by comparing the ratio of *Lactobacillus* species to *E. coli* pre- and post-treatment with macroalgae. Data showed that all three macroalgae exhibited significant prebiotic potential ($p<0.05$) when compared to no prebiotic (negative control), and their prebiotic potentials were comparable to the prebiotic potential of the commercially available prebiotic inulin (positive control). Furthermore, all macroalgae exhibited a significantly stronger prebiotic potential ($p<0.05$) on *L. casei* compared to *L. paracasei*. It is recommended that these macroalgae be part of the regular diet together with the probiotic *L. casei*. Furthermore, *in vivo* studies are encouraged to confirm if these macroalgae continue to exhibit their prebiotic effect.

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Introduction

The human intestinal tract contains a wide variety of microorganisms, with a mix of potentially harmful and useful bacteria. The maintenance of the balance between these two bacterial categories is critical in homeostasis and in the regulation of digestion, inflammation and immunity (Hardy *et al.,* 2013).

The health of a host animal deteriorates due to the abnormal proliferation of harmful bacteria in the intestines. On the contrary, it is maintained in a normal condition or is improved by the growth of useful intestinal bacteria such as *Lactobacillus* and *Bifidobacterium*. These strongly suggest that the deliberate selective growth of such useful intestinal bacteria is important for the prevention and treatment of diseases (Hardy *et al.,* 2013). One way to promote gut homeostasis is the intake of prebiotics, which are ingredients that allow changes that confer benefits upon the host's health. These prebiotics resist enzymatic digestion in the human small intestine and are metabolized by the human gut microflora (Slavin, 2013).

Macroalgae are a significant contributor to the Philippine fishing industry. According to the Bureau of Fisheries and Aquatic Resources (BFAR, 2014), the Philippines is actually the world's third largest producer of aquatic plants, including macroalgae, producing a total of 1.56 million metric tons or nearly 5.78% of the world total production. It is the second top export commodity, following tuna, with an export value of US$ 264 million in 2014 or 21% of the total export earnings for the said year (BFAR, 2014). The Philippines has a diverse array of macroalgal species, of which the major commercial kinds are *Eucheuma* spp., *Kappaphycus* spp., *Gracilaria* spp. and *Caulerpa lentillifera*. Other economically important macroalgae are *Codium* spp., *Gelidielaacerosa*, *Halymenia*, *Porphyra*, and *Sargassum* spp. (BFAR, 2010).

Complex carbohydrates are known to exhibit prebiotic effects (Slavin, 2013). These carbohydrates have been noted in several macroalgae (Critchley, 1993; Kuda and Ikemori, 2009; Ortiz and Trono, 2007; Pulz and Gross, 2004).

A diverse array of these macroalgae area bundant and inexpensive, albeit underutilized, in the Philippines (Ganzon-Fortes, 2012). This study was an effort to assess the prebiotic potential of select species of indigenous macroalgae, and possibly introducing these as inexpensive, accessible, natural health food.

The study determined the optimum dosage of *Caulerpa lentillifera, Gracilaria arcuata,* and *Sargassum polycystum* for the growth of *Lactobacillus paracasei,* and *Lactobacillus casei* strain Shirota. It also attempted to determine the selectivity of macroalgae towards the growth of probiotic bacteria as opposed to *E. coli,* and it assessed the effect of the selected macroalgae on mixtures of bacteria.

Materials and methods

Materials and Media Preparation

Lactobacillus MRS Agar (HIMedia, India) was used in the isolation and selective enumeration of *Lactobacillus* species. MacConkey Agar (HI Media, India) was used in the isolation and selective enumeration of *Escherichia coli*. Luria Broth (HI Media, India) supplemented with 2% Glucose (Univar, USA) (LGB) was used in the maintenance of cultures and competitive growth assays.

Macroalgae Procurement and Processing

Dried specimens of *Sargassum polycystum* were provided by University of the Philippines - Marine Science Institute through Dr. Teresita Ramos-Perez. Fresh specimens of *Caulerpa lentillifera* and *Gracilaria arcuata* were collected from local wet markets. Proper identification of the macroalgae was done by the National Museum of the Philippines, Botany Division.

The macroalgae were air dried and grounded. To simulate gastrointestinal digestion of food substances, the powdered macroalgae underwent *in vitro* digestion. Powdered macroalgae (5 g per sample) were reconstituted with 90 mL distilled water. It was acidified to pH 2.0 with 6 M HCl and was left to stand for 15 minutes at 37°C.

Then, pH was checked and adjusted. Pepsin (HI Media, India) (0.01 g per 5 g sample) was added and incubated at 37°C for 2 hours. Mixture was neutralized to pH 5.0 using 1 M $NaHCO_3$. Pancreatin (HIMedia, India) and bile salts (Hardy Diagnostics, USA) (0.0025g pancreatin and 0.015g bile salts per 5 g sample) were added and incubated at 37°C for 2 hours. After incubation, pH was adjusted to pH 7.2 with 0.5 M NaOH (Laparra *et al.*, 2003). Inulin (Sigma-Aldrich, USA) was also digested *in vitro* and served as positive control while distilled water served as negative control.

Isolation of Bacteria

Probiotic bacteria were isolated from commercially available food products using Lactobacillus MRS Agar (HI Media, India). Isolated colonies were screened through Gram staining and Catalase test. Presumptive *Lactobacillus* isolates were preserved in Lactobacillus MRS broth (HI Media, India) supplemented with 20% glycerol for further identification.

E. coli was obtained via hospital isolate. The *E. coli* sample was preserved in MacConkey broth (HI Media, India) supplemented with 20% glycerol for further identification. All bacterial isolates were sent to Macrogen, Inc., Korea for 16S rRNA genome sequencing and identification of was done through BLAST score analysis.

Determination of Macroalgal Dosage

Varying concentrations of each processed macroalgae ranging from 0.5 – 2.5% w/v at 0.5% increments was tested against each bacteria. Bacteria were inoculated in LGB supplemented with macroalgae and was incubated at 37°C for 48 hours. The same treatment was done with inulin (1% w/v) and distilled water, which served as the positive control and negative control, respectively. The dosage that yielded the maximum growth for *E. coli*, as determined by the CFU count, was used in the subsequent experiments. This was to ensure that any relative increase in the ratio between *Lactobacillus* and *E. coli*, in the succeeding assay, was due to the increase in *Lactobacillus* growth and not due to the decrease in growth of *E. coli*.

Competitive Growth Assay

Prebiotic potential was assessed by comparing the CFU/mL ratio of *Lactobacillus* species to *E. coli* pre- and post-treatment with macroalgae using a Competitive Growth Assay (CGA) done in LGB. The following bacterial combinations for the CGA were: (1) *L. casei* + *E. coli*, (2) *L. paracasei* + *E. coli*, and (3) both *Lactobacillus* species + *E. coli*. To ensure that the pre-treatment ratio of probiotic *Lactobacillus* and *E. coli* is 1:1 and the same number of bacteria will be inoculated in the experimental set-ups, direct cell counting using a hemocytometer (Neubauer) was employed. Each species of probiotic *Lactobacillus* was co-cultured with *E. coli* in LGB supplemented with the optimum dose of macroalgae as determined in the previous experiment. LGB supplemented with inulin (1% w/v) and distilled water served as positive and negative controls, respectively. All assays were incubated at 37°C for 48 hours. Quantification of growth rate was done through the drop plate method on selective media and computing for the CFU/mL. Data was reported as the ratios of the log CFU/mL counts of the bacteria.

Statistical Analysis

Data was reported as the average of the ratios of the log CFU/mL counts of the MRS and MacConkey plates. Statistical analysis was done using the IBM© SPSS© Statistics Version 20.0.0 computer software. Significance was set at $p<0.05$. Test for homogeneity of variances (Levene) was done, followed by nonparametric Kruskal-Wallis Ranks with post-hoc Mann-Whitney U test.

Results and discussion

Isolation of Probiotic Bacteria

Probiotic bacteria were isolated from locally available commercial probiotic food products because these bacteria are optimized strains of those found in the human gastrointestinal tract. At the same time, hospital isolates of *E. coli* were used to ensure the potential pathogenicity of the bacteria. All isolates were sent to Macrogen Inc., Korea for 16s rRNA sequencing using the 27F and 1492R universal primers.

Isolates were identified as *Lactobacillus casei* and *Lactobacillus paracasei* through NCBI BLAST score analysis. The identity of the *E. coli* hospital isolate was also confirmed by sequencing done by Macro Gen Korea, Inc. and BLAST score analysis.

Table 1. NCBI BLAST score analysis of isolated probiotic bacteria from commercially available food products and *E. coli* hospital isolate.

Isolate	Accession Number	% Identity	Nearest Phylogenetic Affiliation
A	JQ398845.1	98%	*Escherichia coli* strain RRL-36
B	KF673509.1	98%	*Lactobacillus casei* strain SWU91231
C	KU315085.1	99%	*Lactobacillus paracasei* strain FC4

Macroalgal Dosage

The dosage used in the CGA was determined by inoculating *E. coli* in LGB supplemented with varying concentrations of macroalgae. The dose of macroalgae exhibiting the highest concentration of *E. coli* was used in the succeeding CGAs. This was to ascertain that any prebiotic effect, indicated by the *Lactobacillus* to *E. coli* ratio, is due to an increase in *Lactobacillus* concentration and not due to *E. coli* suppression. In addition, LGB is known to support the growth of *E. coli*; hence, an increase in this ratio should definitely demonstrate the efficacy of the prebiotic, which is to promote the growth of beneficial microorganisms. Fig. 1 shows the average log CFU/mL counts of the bacteria grown in 2% w/v macroalgae, which was the concentration that exhibited the highest growth of *E. coli* (data not shown). Hence, the dose that was used for the CGAs was 2%.

Prebiotic Potential

The presence of prebiotic potential was assessed by comparing CFU/mL ratio of *Lactobacillus* species to *E. coli* pre- and post-treatment with the macroalgae. All pre-treatment ratios were set to 1:1 by inoculating the same amount of *Lactobacillus* species and *E. coli*. Post-treatment ratios for each macroalgal species are shown in Figs. 2, 3, and 4.

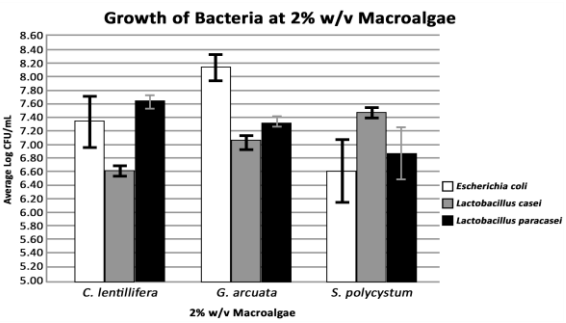

Fig. 1. *Escherichia coli* (white bar), *Lactobacillus casei* (gray bar), and *Lactobacillus paracasei* (black bar) treated with 2%w/v *C. lentillifera, G. arcuata,* and *S. polycystum*. Data reported as the average log CFU/mL ± SEM.

C. lentillifera significantly ($p<0.05$) increased the ratio of both *Lactobacillus* species to *E. coli* to 1.17 when compared to the negative control and a comparable prebiotic effect to inulin. Furthermore, the *L. casei* to *E. coli* ratio (1.40) was higher than the *L. paracasei* to *E. coli* ratio (0.83) in the presence of *C. lentillifera* (Fig. 2).

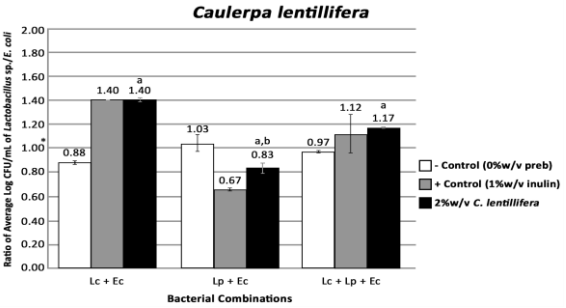

Fig. 2. *Lactobacillus casei* (Lc) and/or *Lactobacillus paracasei* (Lp) in combination with *Escherichia coli* (Ec) treated with 2%w/v *C. lentillifera* (black bar). Negative control is distilled water (white bar), positive control is 1%w/v inulin (gray bar). Data reported as the ratio of average log CFU/mL ± SEM between *Lactobacillus sp.* and *E. coli*. Significance set at $p<0.05$. *Starting Ratio of *Lactobacillus sp./Escherichia coli*. (a) Significantly different from the negative control. (b) Significantly different from the positive control.

Algae belonging to Division Chlorophyta, like *Caulerpa,* are rich in glucomannans, mannans, xylans, sulfated polysaccharides and pectins. Sulfated polysaccharides found in a wide range of algae exhibit their prebiotic effect by inhibiting the adhesion of pathogenic bacteria such as *Helicobacter pylori* to the mucous membrane.

In addition, they also exhibit anti-inflammatory effects on colitides, peptic-ulcer disease and other disorders of the gastrointestinal tract (Zaporozhets *et al.*, 2014).

G. arcuata also significantly ($p<0.05$) increased the ratio of both *Lactobacillus* species to *E. coli* to 1.32 when compared to the negative control and a comparable prebiotic effect to inulin. In addition, the presence of *G. arcuata* also seemed to favor more the growth of *L. casei* (1.35) than that of *L. paracasei* (0.94) when comparing *L. casei* and *L. paracasei* each in combination with *E. coli* (Fig. 3).

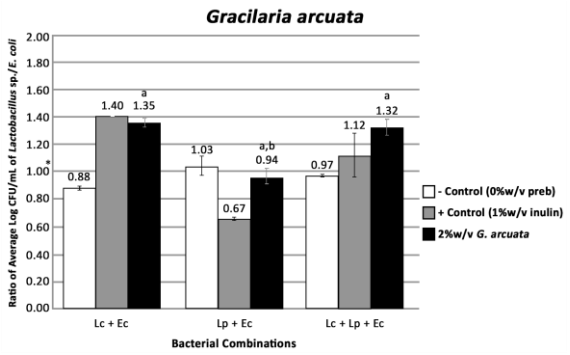

Fig. 3. *Lactobacillus casei* (Lc) and/or *Lactobacillus paracasei* (Lp) in combination with *Escherichia coli* (Ec) treated with 2%w/v *G. arcuata* (black bar). Negative control is distilled water (white bar), positive control is 1%w/v inulin (gray bar). Data reported as the ratio of average log CFU/mL±SEM between *Lactobacillus sp.* and *E. coli*. Significance set at $p<0.05$. *Starting Ratio of *Lactobacillus sp./Escherichia coli*. (a) Significantly different from the negative control. (b) Significantly different from the positive control.

Algae belonging to Division Rhodophyta, like *Gracilaria*, are rich in floridean starch, sulfated galactans, agarose, carageenan and to lesser degree xylans and mannans. *In vitro* studies on the prebiotic effects of neoagaro-oligosaccharides from the enzymatic hydrolysis of agarose have shown that these polysaccharides significantly increased the population of Bifidobacteria and Lactobacilli. In addition, when tested *in vivo* these polysaccharides not only increased the population of beneficial bacteria but also decreased the population of putrefactive microorganisms (Hu *et al.*, 2006).

S. polycystum also significantly ($p<0.05$) increased the ratio of both *Lactobacillus* species to *E. coli* to 1.29 when compared to the negative control and a comparable prebiotic effect to inulin. In addition, the presence of *S. polycystum* also seemed to favor more the growth of *L. casei* (1.57) than that of *L. paracasei* (0.94) when comparing *L. casei*and *L. paracasei* each in combination with *E. coli*. Moreover, *S. polycystum* was able to significantly ($p<0.05$) increase the ratio of *L. casei* to *E. coli* to 1.57 when compared to both the negative control and inulin (Fig. 4).

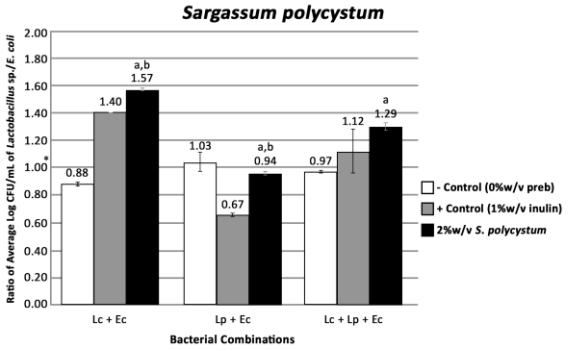

Fig. 4. *Lactobacillus casei* (Lc) and/or *Lactobacillus paracasei* (Lp) in combination with *Escherichia coli* (Ec) treated with 2%w/v *S. polycystum* (black bar). Negative control is distilled water (white bar), positive control is 1%w/v inulin (gray bar). Data reported as the ratio of average log CFU/mL ± SEM between *Lactobacillus sp.* and *E. coli*. Significance set at $p<0.05$. *Starting Ratio of *Lactobacillus sp./Escherichia coli*. (a) Significantly different from the negative control. (b) Significantly different from the positive control.

Phaeophyta, the division from which *Sargassum* belongs, are a rich source of aliginic acid and alginates, laminarans and fucoidans, which have a wide range of biological effects (Zaporozhets *et al.*, 2014). Studies on the prebiotic effect of alginates (Wang *et al.*, 2006), laminarans and fucoidans (Lynch *et al.*, 2010; Murphya *et al.*, 2013; Sweeney *et al.*, 2011) have shown that these polysaccharides can increase the populations of *Lactobacillus sp.* in rats and pigs, respectively.

All three macroalgae and inulin apparently favored the growth of *L. casei* over *L. paracasei*. This is in contrast to a study by Capra *et al.* (2014), which reported the growth rates of *L. casei* and *L. paracasei* to be 0.1±0.2% and 0.4±0.2%, respectively, in 2% (w/v) inulin. However, the said study also noted that both species were not able to ferment the prebiotic efficiently (<3.1%). The difference in the results might be due to the difference in the culture medium used and strain of *Lactobacillus*. Furthermore, according to a study done by Nagpal and Kaur (2011), prebiotics have a strain specific effect. The growth and viability of different Lactobacillus species and strains in the presence of prebiotics are strain specific.

There has been an increasing worldwide demand for prebiotics due to this generation's desire for health and wellness (Espitia *et al.,* 2016). This study was able to prove the prebiotic effect of selected species of locally abundant macroalgae. Specifically, *G. arcuata* had the greatest prebiotic effect with a ratio (1.32) of *Lactobacillus* to *E. coli* growth, followed by *S. polycystum* (1.29) and *C. lentillifera* (1.17). In addition, all three macroalgae also exhibited a strain-specific effect, particularly favoring the growth of *Lactobacillus casei* more over that of *Lactobacillus paracasei.*

A readily-ingested, affordable, and alternative natural resource such as macroalgae can therefore be beneficial not only to the overall health of consumers, but also to help boost the local economy as well.

It should be noted that this study was done *in vitro*, thus *in vivo* studies using a mammalian model are recommended to confirm their prebiotic effect. Furthermore, maximizing the prebiotic potential of the macroalgae through combination studies may be explored.

Conflict of interest

The authors declare that they have no conflict of interest.

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