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A potential synbiotic beverage from fermented red seaweed (*Gracilaria fisheri*) using *Lactobacillus plantarum* DW12

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Abstract

A novel functional beverage was produced from red seaweed (Pom-nang seaweed), by fermentation using a probiotic bacterium *Lactobacillus plantarum* DW12 as a starter culture. In addition to the normal fermentation products the bacterium produced γ-aminobutyric acid (GABA) and prebiotic compounds. A cell free culture supernatant also inhibited the growth of potential foodborne pathogens and spoilage bacteria (*Bacillus cereus* TISTR2687, *Staphylococcus aureus* PSSCMI0004, *Escherichia coli* PSSCMI0001, *Salmonella* Typhi ATCC19430 and *Vibrio parahaemolyticus* PSSCMI0004). In addition, the cell free culture supernatant had antioxidant activity as shown by its ability to scavenge 2, 2/-azino-bis (3-ethylbenzthiazoline-6-sulphonic acid (ABTS) and 2, 2-diphenyl-1-picrylhydrazyl (DPPH) at a level of 60 and 68%, respectively. Using *in vitro* tests this bacterium was resistant to 1 mM H_2O_2 for 8 h and grew at various pH values from 2 to 9 and survived for 12 h in the presence of bile salts (0.15-0.30%). Results of antibiotic susceptibility tests showed that 8 of 15 antibiotics were inhibitory to strain DW12 at susceptible and intermediate levels. The fermented red seaweed beverage (FSB) contained 5.41 log CFU/ml lactic acid bacteria, 1284 mg/L GABA, 11.79 mg/ml sugars (fructose, glucose and sucrose) and 0.31 mg/ml of fructooligosaccharides (FOS, kestore and fructosylnystose). Freeze dried FSB at the concentrations tested (0.5-2.0%), that contained 78.61-314.42 mg/100 ml of sugars and 2.10-8.20 mg FOS/100 ml acted as a prebiotic by promoting the growth of strain DW12.

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Introduction

Food products that contain either probiotic microbes or prebiotic have been considered to be one of the functional foods that can promote health and prevent diseases (Qiang *et al*., 2009). It has long been known that some lactic acid bacteria (LAB) have probiotic properties and they have been used as starter cultures in various fermented foods; particularly for fermented dairy products. Probiotics, are defined as "live microorganisms that, when administered in adequate amounts orally, confer a health benefit on the host." (Joint FAO/WHO, 2002). Probiotics have become a major topic for LAB that possess health-promoting effects and also play important roles in regulating the balance of microflora in the gastrointestinal (GI) tract. One of the beneficial properties of probiotics is their ability to inhibit the growth of foodborne pathogens in the GI tract including food spoilage organisms (Duangjitcharoen *et al*., 2009; Ratanaburee *et al*., 2013). Production of antioxidative activities is another characteristic used to select probiotic bacteria. The process of an incomplete reduction of oxygen produces toxic reactive oxygen species (ROS) that include superoxide anion, hydroxyl radicals, hydrogen peroxide and singlet oxygen. The ability to scavenge for free radicles such as the 2, 2-diphenyl-1-picrylhydrazyl radical ($DPPH^{\bullet}$) and 2, 2′-azino-biz (3-ethylbenzthiazoline-6-sulfonic acid) ($ABTS^{\bullet+}$) including lipid peroxidation by LAB have been reported to provide health benefits to hosts (Zhang *et al*., 2011).

Probiotic microbes must overcome biological barriers such as an ability to survive in strongly acidic conditions and resistance to bile salt in the GI tract, in order to survive and colonize the GI tract. The pH value in the human stomach ranges from 1.5 to 3.0, and food digestion can take up to 3 h depending of the meal type (Olejnik *et al*., 2005). Hence, after a meal, probiotics would need to survive pH values range of from 2.0 to 8.0 and bile salt concentrations

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between 0.15 and 0.30% in the GI tract for at least 6 h (Jacobsen *et al*., 1999). Most of the probiotic strains in fermented food products, particularly fermented dairy products belong to the genera of *Lactobacillus* and *Bifidobacterium.* However, lactose intolerance and the cholesterol contents are the two drawbacks concerned with these products. Hence, an alternative choice is to produce non dairy probiotic products or probiotic plant products such as non alcoholic fermented plant beverages (FPBs). However, at present there are a little commercial probiotic plant products concerned with promoting human health although there are many commercial products of (FPBs).

FPBs are a traditional household product in Thailand produced by LAB from different parts of plants such as fruits, and from cereals and vegetables. They are considered to be one of the functional food products (Duangjitcharoen *et al.*, 2009). Indeed, many functional plant beverages such as from soy bean, black raspberry, grape and red seaweed have been produced by using γ-aminobutyric acid (GABA) LAB strains. For example, a high GABA content was found in a fermented red seaweed beverage (FSB) when *Lactobacillus plantarum* DW12 was used as a starter culture because this strain is a GABA producing strain (Ratanaburee *et al.*, 2011). Hence, it would be of interest to investigate its probiotic properties for use as a starter culture for producing probiotic plant products.

Probiotics also need to have the ability to survive in the FPB in sufficient numbers during production and storage. It is well recognized that prebiotics promote probiotic growth and thus there is a synergy between prebiotic and probiotic microbes. Synbiotics are defined as a combination of a prebiotic and a probiotic organism (Teitelbaum and Walker, 2002). Prebiotics are "non-digestible food ingredients such as oligosaccharides that beneficially affect the host by selectively stimulating their growth and/or the activity of one or a limited number of bacteria like bifidobacteria and lactobacilli in the colon, and thus improve host health" (Teitelbaum and Walker, 2002; Mussatto and Mancilha, 2007). Therefore, one of the principal benefits of synbiotics is believed to be an increased persistence of the probiotic in the GI tract, as they facilitate probiotic's survival, by selectively stimulating the growth and or metabolic activity of one or a limited number of health-promoting bacteria such as LAB. Red seaweed, *Gracilaria fisheri* or Pom-nang seaweed is its Thai name, is classified in the division rhodophyta, class rhodophyceae and it is often used for cooking as it is high in nutrients i.e. amino acids, carotenoids and iodine, and also contains bioactive compounds such as polyphenols and phenolic acids. Seaweeds are rich in polysaccharides that could potentially act as prebiotic functional ingredients for human health applications (Sullivan *et al.*, 2010).

Hence, there is a potential for synergy between prebiotics and probiotics by producing a novel functional FPB from fermented red seaweed (FSB) using *L. plantarum* DW12 as the starter culture. The objectives of this study were to evaluate the probiotic properties of *L. plantarum* DW12, on the basis of its probiotic properties as previously described, and the potential for a prebiotic FSB by determination of the oliosaccharides in FSB including an ability to support the growth of *L. plantarum* DW12.

Materials and Methods

Bacterial strains used

Lactobacillus plantarum DW12 was isolated by us from a fermented red seaweed (Rattanaburee *et al.*, 2011), and used as a starter culture for producing the FSB. This organism is a facultative bacterium (our unpublished data) and was twice subcultured in de Man Rogosa Sharpe (MRS, Merck, Germany) and incubated at 35°C for 24 h to obtain an active culture for testing. The following potential foodborne pathogens; *Bacillus cereus* TISTR 2687, *Salmonella* Typhi ATCC19430, *Staphylococcus aureus* PSSCMI0004 and *Vibrio parahaemolyticus* PSSCMI0064, and the bacterial indicator; *Escherichia coli* PSSCMI0001, were used as target organisms. They were cultured in tryptic soy broth (TSB, Merck, Germany) and all indicator strains were subcultured twice prior to use in each experiment for obtaining active cultures.

Investigating the probiotic properties of the starter culture

The antibacterial activity of *L. plantarum* DW12 was investigated with target organisms as previously described, by the agar well diffusion method (Ratanaburee *et al.*, 2013). Briefly, each bacterial indicator was adjusted to approximately 1.5 x 10^8 CFU/ml based on the 0.5 McFarland number and then it was diluted to obtain 1.5 x 10^6 before being spread onto the surface of a TSA plate. Cell free culture supernatant, obtained by centrifugation of 24 h culture broth of DW12 at 5000 rpm for 20 min and the supernatant was sterilized through a 0.45 µm pore membrane, and 140 µl was then added into a well with a diameter of 8 mm on a TSA plate. Uninoculated MRS medium served as a control. All TSA plates were incubated at 35°C for 24 h and the diameters of the clear zones were measured using a

vernier caliper.

Culture supernatants of strain DW12 were used to investigate the antioxidant activity by the following assays; ABTS scavenging activity, DPPH scavenging activity and lipid peroxidation assay based on malondialdehyde (MDA) according to the methods described by (Singhatong *et al*., 2010). Resistance to hydrogen peroxide (H_2O_2) was also determined by growing strain DW12 in MRS broth as previously described, then washed cells were adjusted to 10^7 CFU/ml in phosphate buffer saline, pH 7.4 containing 1 mM H_2O_2. The cell suspension was incubated for 8 h prior to making viable counts on MRS agar.

A disc diffusion method was used to measure the antibiotic susceptibility of *L. plantarum* DW12 grown on MRS agar with 15 antibiotics according to Duangjitcharoen *et al*. (2009): vancomycin (30 µg), bacitracin (10 units), gentamycin (10 µg), kanamycin (30 µg), streptomycin (10 µg), norfloxacin (10 µg), polymyxin (300 units), ampicillin (10 µg), cephalothin (30 µg), ceftazidime (30 µg), chloramphenical (10 µg), erythromycin (15 µg), penicillin (10 units), ceferperazone (75 µg) and tetracycline (30 µg). A cell suspension of roughly 1 x 10^6 CFU/ml, was swabbed onto an MRS agar plate and then each antibiotic disc was placed on the surface of the agar. The inhibition zone diameters were measured after incubation at 35ºC for 24 h and compared with those in the interpretation standard chart (NCCLS, 2004). Based on NCCLS in therapeutic terms, the following standard therapeutic doses that can be attained in body fluids; resistant meant that there was no inhibition of a microbe by that particular concentration of an antimicrobial agent whereas susceptible meant that a microbe was inhibited by that particular concentration of an antimicrobial agent. Intermediate meant that a susceptibility result implied clinical utility in certain body sites in which the antibiotics became physiologically concentrated i.e. quinolones and beta-lactams in urine or when a high dosage of a drug can be used i.e. beta-lactams (Stephen *et al.*, 2005). Results have been presented in terms of resistance, intermediate and susceptible.

The acid-base tolerance of *L. plantarum* DW12 was tested in MRS broth with various pH values (pH 2, 3, 4, 5, 8 and 9) as described by Conway *et al.* (1987). A bacterial suspension of approximately 10^8 CFU/ml based on the 0.5 McFarland number was used as the inoculum in this study and for further studies. To test, 0.5 ml of inoculum was added into 5 ml of each condition tested and incubated anaerobically in an anaerobic jar at 35ºC. Viable cells of strain DW12 at 0, 2 and 3 h were counted by a pour plate method using MRS agar.

The tolerance to bile salt of *L. plantarum* DW12 was tested in MRS broth containing bile salt at concentrations of 0.15 and 0.30% following the method of Erkkilä and Petäjä (2000). An inoculum of 0.5 ml was made into 5 ml of MRS broth supplemented with bile salt and incubated anaerobically in an anaerobic jar at 35˚C. Viable cells at 0, 6, and 12 h were counted by a pour plate method using MRS agar. All experiments were conducted with three replicates.

Red seaweed fermentation

Dried red seaweed after cleaning by potable water was soaked in potable water until saturated and it was then ready for use after being drained. *L. plantarum* DW12 was used as the starter culture by preparing cell suspension according to the methods as described by Ratanaburee *et al.* (2011). A fermented red seaweed beverage was produced as follows: in brief: addition of 0.5% monosodium glutamate (MSG) and a 5% starter culture of 5.2 x 10^9 CFU/ml into the following ingredients; red seaweed, cane sugar and potable water in a ratio of 3: 1: 10 with an initial pH of 6 for 60 fermentation days. Samples were taken at days 0 and 60 for determination of LAB, oligosaccharides and the GABA content. After 60 days fermentation culture was sampled because such conditions were normally used to obtain a finished beverage product. MRS agar was used to count LAB by the pour plate method, while the amount of GABA was determined by HPLC (Agilent 1100 series, HP) according to the method as described by Ratanaburee *et al*. (2011).

Determination of carbohydrate size (Degree of polymerization)

Determination of oligosaccharide sizes were based on the degree of polymerization (DP) of seaweed extracts that were investigated according to the amounts of total sugar and reducing sugar. Total sugar and reducing sugar were determined by the phenol-sulfuric method (Dubois *et al*., 1956) and the dinitrosalicylic acid method (DNS) (Miller, 1959), respectively. The following equation was used to calculate DP.

Degree of polymerization (DP) = (Amount of total sugar)/ (Amount of reducing sugar)

Analysis of sugars and oligosaccharides

To investigate the possibility that strain DW12 may utilize red seaweed to produce sugars and oligosaccharides for the FSB; thereby both the red seaweed and FSB were checked for the presence of these carbohydrates. FSB at day 60 was freeze dried to analyze for sugars and oligosaccharides by HPLC. The freeze dried FSB was then extracted by using hot

water at 80°C for 3 h then filtered through a 0.45 µm filter prior to use for HPLC analysis. In the case of red seaweed or FSB they were first dried and then extracted by adding 0.05 N HCl and incubated in a water bath at 95ºC for 3 h. Filtration was used to remove the solid and the solution was adjusted to a pH of 7.0 by 0.1 N NaOH, followed by adding 3 times the amount of 95% ethanol to remove precipitable material and the clear solution was further filtered through a 0.45 µm filter. The HPLC was equipped with an amino column (NH2P-50 4E) (Shodex co. Ltd.) and a RI detector. The mobile phase was 70% acetonitrile and the column was run at 30°C at a flow rate of 0.9 ml/min. Authentic sugars and oligosaccharides at various concentrations were used as standard solutions for making standard curves for calculating the amount of each sugar and oligosaccharide in the samples.

Effect of FSB on L. plantarum DW12 growth

The prebiotic property of the FSB was also evaluated based on the growth of *L. plantarum* DW12 by comparing its growth in a minimal medium containing FSB with positive and negative controls. The bacterium was grown in MRS broth for 18 h at 35°C and cells were harvested by centrifugation at 5000 rpm for 20 min. The cell pellet was washed twice with 0.85% NaCl prior to use as an inoculum by adjustment to 10^7 CFU/ml in normal saline solution. A 3% cell suspension was inoculated into 3 treatments and three controls; minimal medium with addition of 2% glucose (set A: positive control) and MRS medium as a standard medium for growing lactobacilli (Positive control MRS), minimal medium (set B: negative control) and minimal medium supplemented with 0.5, 1 and 2% (w/v) freeze-dried FSB obtained from day 60 of a fermentation (sets; C, D and E). Minimal medium consisted of (g/L) peptone water, 2.0; yeast extract, 2.0; NaCl, 0.1., K_2HPO_4, 0.04; KH_2PO_4, 0.04; $CaCl_2.6H_2O$, 0.01; $MgSO_4.7H_2O$, 0.01; $NaHCO_3$, 2.0; Cysteine-HCL, 0.5; Bile salt, 0.5 and 2 ml/L of tween 80 (Fooks *et al.*, 1999). Sample tubes were incubated anaerobically at 35°C and viable counts of strain DW12 were determined by a pour plate method at 0, 6, 12, 24 and 48 h using MRS agar. Three replications were made for the experiment.

Statistical analysis

All experiments were performed in triplicate and the raw data were analyzed using ANOVA. Mean values with standard deviations are presented. Statistical significance was evaluated using LSD (Least Significant Difference) and a $P < 0.05$ was considered to be a significant difference.

Results

Probiotic properties

The inhibitory effect of strain DW12 against the growth of target organisms is presented in Table 1A. A cell-free culture supernatant of *L. plantarum* DW12 strongly inhibited the growth of almost all the indicator organisms tested (both Gram positive and Gram negative bacteria). Amongst the bacteria tested, *S.* Typhi ATCC19430 was the most sensitive strain followed by *V. parahaemolyticus* PSSCMI0064 whereas *E. coli* PSSCMI0001 was the most resistant strain. The inhibitory effect of strain DW12 against the Gram positive bacterial growth (*B. cereus* TISTR2687 and *S. aureus* PSSCMI0004) was similar.

The antioxidant activities of strain DW12 are shown in Table 1B. With the use of the culture supernatant collected from a culture in MRS broth, strain DW12 demonstrated antioxidative activities to scavenge for both DPPH and ABTS free radicals at 68 and 60%, respectively. However, there was no inhibition of lipid peroxidation. On the other hand, this bacterium was able to resist 1 mM hydrogen peroxide for 8 h as cells decreased by only 0.5 log CFU/ml.

L. plantarum DW12 was susceptible to ampicillin, cephalothin, ceftazidime and chloramphenicol but was resistant to 7 antibiotics; vancomycin, bacitracin, gentamycin, kanamycin, streptomycin, norfloxacin and polymyxin. The following antibiotics; erythromycin, penicillin, ceferperazone and tetracycline gave intermediately resistance to strain DW12.

The effect of acid-base conditions (pH 2, 3, 4, 5, 8 and 9) on the viability of *L. plantarum* DW12 is shown in Table 2. No significant differences were found for the initial cell density (8.26 log CFU/ml) among the pH values tested. The numbers of viable cells at all pH values tested significantly decreased over longer incubation times; however, after 3 h incubation viable cells were still between 5.87-7.21 log CFU/ml. Strain DW12 was the most sensitive to pH 9 followed by pH 2 while pH 4 produced the least destruction of the viable cells. The results indicated that strain DW12 did survive under both acidic and basic conditions.

The initial cell density was the same (8.38 log CFU/ml) for both 0.15 and 0.30% bile salt concentrations, but after 6 h the higher concentration of 0.30% decreased the cell counts by 2.32 log CFU/ml while the lower concentration decreased the cell count by only 0.13 log CFU/ml (Table 3). The viability of this strain continued to decrease in the presence of bile salt with increasing time of incubation and at 12

Table 1. Probiotic properties of *Lactobacillus plantarum* DW12 tested *in vitro* (A), antibacterial activity and (B) antioxidant activity

(A), Antibacterial activity: Inhibition zone (mm)				
B. cereus	*E. coli*	*S.* Typhi	*S. aureus*	*V. parahaemolyticus*
TISTR2687	PSSCMI0001	ATCC19430	PSSCMI0004	PSSCMI0064
16.10 ± 0.20	9.80 ± 0.11	21.45 ± 0.29	15.35 ± 0.07	19.18 ± 0.04

(B), Antioxidant activity[a]	
Scavenging ability of DW12 on DPPH•	68 ± 0.26%
Scavenging ability of DW12 on ABTS•+	60 ± 0.45%
Lipid peroxidation	No inhibition
Resistance to 1 mM H_2O_2 for 8 h (initial cell, 7.05 ± 0.01 log CFU/ml)	6.55 ± 0.02 log CFU/ml

a% DPPH• scavenging activity = $[(A_{517\ control} - A_{517\ test\ sample}) / A_{517\ control}] \times 100$
%ABTS•+ scavenging activity = $[(A_{734\ control} - A_{734\ test\ sample}) / A_{734\ control}] \times 100$
A = Absorbance

Table 2. Survivors of *Lactobacillus plantarum* DW12 in MRS broth incubated for 3 h at various pH values

pH	Log CFU/ml		
	0 h	2 h	3 h
pH 2	8.26 ± 0.01^{a}	$6.99 \pm 0.21^{b\ A}$	$6.76 \pm 0.06^{c\ A}$
pH 3	8.26 ± 0.01^{a}	$7.05 \pm 0.02^{b\ B}$	$6.94 \pm 0.03^{c\ A}$
pH 4	8.26 ± 0.01^{a}	$7.32 \pm 0.24^{b\ C}$	$7.21 \pm 0.03^{c\ B}$
pH 5	8.26 ± 0.01^{a}	$7.17 \pm 0.02^{b\ B}$	$7.00 \pm 0.03^{c\ C}$
pH 8	8.26 ± 0.01^{a}	$7.16 \pm 0.05^{b\ D}$	$6.89 \pm 0.02^{c\ C}$
pH 9	8.26 ± 0.01^{a}	$6.89 \pm 0.05^{b\ E}$	$5.87 \pm 0.10^{c\ D}$

Data are presented as a mean value ± standard deviation (n = 3).
Different lowercase letters in each row indicate significant differences (P < 0.05). Different uppercase letters in each column indicate significant differences (P < 0.05).

Table 3. Survivors (log CFU/ml) of *Lactobacillus plantarum* DW12 in 0.15% and 0.30% bile salt for varying incubation times

Bile salt	Log CFU/ml		
	0 h	6 h	12 h
0.15%	8.38 ± 0.06^{a}	$8.25 \pm 0.33^{b\ A}$	$7.36 \pm 0.06^{c\ A}$
0.30%	8.38 ± 0.06^{a}	$6.06 \pm 0.02^{b\ B}$	$4.81 \pm 0.55^{c\ B}$

Data are presented as a mean value ± standard deviation (n = 3).
Different lowercase letters in each row indicate significant differences (P < 0.05). Different uppercase letters in each column indicate significant differences (P < 0.05).

Table 4. Amount of sugars and fructooligosaccharides found in red seaweed (*Gracilaria fisheri*) and the finished product of fermented red seaweed beverage (FSB)

Sugar and Fructooligosaccharide (mg/g dry weight)	Sample[a]		
	Red seaweed	Fermented red seaweed	FSB in a freeze dried form[b]
Fructose	86.77 ± 0.12	159.89 ± 0.34	68.08 ± 0.09
Glucose	15.04 ± 0.07	119.02 ± 0.23	73.35 ± 0.15
Galactose	25.71 ± 0.08	28.83 ± 0.07	-
Sucrose	-	-	15.78 ± 0.04
Maltose	3.98 ± 0.01	10.83 ± 0.02	-
Kestose (GF2)	-	-	2.88 ± 0.00
Nystose (GF3)	-	-	-
Fructosylnystose (GF4)	-	-	1.22 ± 0.01

[a]Red seaweed is a raw plant material used for producing the fermented beverage, which is called fermented red seaweed (FSB), and FSB was dried by a freeze drier to obtain a freeze dried form for measuring the concentrated substances.
[b]1 g freeze dried form obtained from 13.33 ml of FSB
- = not detected with the limit of detection at 0.30 mg/ml

h exposure to bile salt, the survivors were in a range of 4.81 and 7.36 log CFU/ml for the 0.30 and 0.15% bile salt, respectively.

Prebiotics in FSB

The DP value of the dried red seaweed before and after 60 days fermentation had the same value of 3.5. Whilst in the FSB, at the start of fermentation the amount of total sugar was 10% and at day 60 the total sugar was 0.56%. In addition, extraction of samples of red seaweed both before and at 60 days fermentation for determination of sugar and oligosaccharides detected the same types of sugar but no oligosaccharides were detected (Table 4). In contrast, a freeze dried FSB collected from day 60 found sugars and fructooligosaccharides (FOS) in

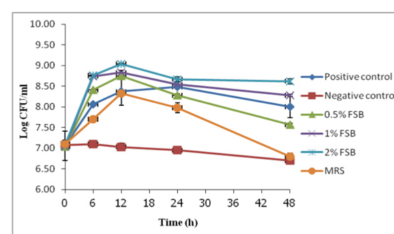

Figure 1. Effect of various concentrations of freeze dried FSB as prebiotic on the growth of *Lactobacillus plantarum* DW12

mg/g: fructose, 68.08; glucose, 73.35; sucrose, 15.78; kestose (GF2), 2.88 and fructosylnystose (GF4), 1.22 (Table 4). As 1 g of the freeze dried form was obtained from 13.33 ml of FSB, therefore It means that FSB consisted of 11.79 mg/ml of all detected sugars and 0.31 mg/ml FOS. In addition, a finished FSB product had a GABA content of 1284 mg/L and the LAB number was 5.41 log CFU/ml.

Effect of FSB on Lactobacillus plantarum DW12 growth

The growth of *L. plantarum* DW12 in minimal medium containing FSB extracted from a freeze dried form and control sets is presented in Figure 1. The initial cell density of strain DW12 in all sets was approximately 7.0 log CFU/ml. There was no growth of strain DW12 in the negative control as viable cells slightly decreased throughout the 48 h of incubation. An increase of cell numbers was found in all other treatments with 0.5-2.0% freeze dried FDS including positive controls and MRS broth after 6 h of incubation with another small increase at 12 h of incubation after which the cell numbers slightly decreased, except for the MRS broth in which there was a marked reduction in cell numbers. Cell density increased along with the increase of the freeze dried FSB from 0.5 to 2.0% and they were significantly higher than both the positive controls. The maximum population at 9.5 log CFU/ml was found in set E with 2% of the freeze dried FSB while the maximum cell numbers in both positive controls was 8.8 log CFU/ml.

Discussion

One of the most beneficial probiotic properties is their antibacterial activity, and in our study, *L. plantarum* DW12 strongly inhibited the growth of all potential pathogens tested; however, its ability to inhibit the bacterial indicator, *E. coli* ATCC25922 was quite low (Table 1A). It is well recognized that *L. plantarum* varieties produce various antimicrobial compounds such as organic acids, bacteriocins, hydrogen peroxide, reuterin and phenyllactic acid that can inhibit spoilage and pathogenic bacteria such

as *Salmonella* Enteritidis, *Clostridium perfringens*, and *Staphylococcus aureus* (Duangjitcharoen *et al*., 2009). Our results indicated that *L. plantarum* DW12 could control both Gram positive and negative pathogenic bacteria although a better result was found against Gram negative bacteria (Table 1A). This is because some of the Gram negative bacteria that are enteropathogens are sensitive to organic acids produced by lactobacilli (Gonzales *et al*., 1993). These results are also supported by Makras *et al*. (2005) who reported that probiotic lactobacilli strains displayed strong antibacterial activity towards the *Salmonella enterica* serovar Typhimurium by production of organic acids, and in particular lactic acid.

To test the tolerance to ROS in the presence of 1 mM hydrogen peroxide, *L. plantarum* DW12 showed a remarkable resistance to this compound with over 94% survivors after 8 h of exposure (Table 1B) when compared to previous studies i.e. *L. fermentum* E3 had resisted this compound at the same concentration for only 3 h (Kullisaar *et al*., 2002). Based on the scavenging ABTS assay, the antioxidative activity of whey fractions obtained after milk fermentation by 25 LAB strains was in a range of 3-53% with the highest activity found in *Leuconostoc mesenteroides* ssp. *cremoris B* (Virtanen et al., 2007). It should be noted that strain DW12 showed a higher antioxidative activity against the free radical ABTS (60%) than those strains. Strain DW12 also produced a higher scavenging activity on the DPPH radical (68%) than that found for *L. casei* subsp. *casei* SY13 and *L. delbrueckii* subsp. *bulgaricus* LJJ (27.50 and 23.99%) (Zhang *et al*., 2011). However, both LAB strains SY13 and LJJ showed a high inhibition of linoleic acid peroxidation (62.95 and 59.63%) while strain DW12 showed no inhibition of lipid peroxidation. These results indicate that the radical scavenging activity of strain DW12 was stronger than for inhibition of lipid peroxidation activity.

The antibiotic susceptibility test indicated that the strain DW12 was sensitive to 4 antibiotics and resistant to 7 antibiotics. Antibiotic resistance of some probiotic strains may be benefit for patients who have an intestinal unbalance due to the antibiotics used (Hickson *et al*., 2007). Our results are in agreement with previous work that has claimed that LAB isolates from probiotic products are usually resisted to antibiotics. Most of the kanamycin, resistant isolates belonged to the genera *Lactobacillus* and *Enterococcus* (Temmerman *et al*., 2003). It is generally known that lactobacilli resistant to vancomycin due to the majority of the lactobacilli are intricsically resistant to glycopeptide (Klein *et al*., 2000).

The ability to remain viable in the presence of acid and bile salt in the GI tract ecosystem is a requirement to select for probiotic strains because it is hoped that they will colonize the intestinal surface to prevent attachment of pathogens. Results from this study showed that *L. plantarum* DW12 could survive at various pH values, particularly at pH 2 and 9 for 3 h; *L. acidophilus* NCFM survived in condition of pH 3 for 3 h with a four-log cycle drop of cell numbers (Conway *et al*., 1987). It is of interest, that our strain DW12 was superior as this strain with the same conditions as the cells decreased roughly by only 1 log cycle (Table 2). Moreover, the strain DW12 also survived (5.87 log CFU/ml) at pH 9 after 3 h exposure. This indicated DW12 had a higher tolerance to alkaline condition when compared with previous studies. Grimoud *et al*. (2010) reported that *Lactobacillus rhamnosus* R1102, *Lactococcus lactis* R1058, *Pediococcus acidilactici* R1001 and *Streptococcus thermophiles* R0083 were resistant to the artificial gastric juice (pH 2.5) but were inhibited by simulated intestinal fluid (pH 8).

Since bile salts disorganize the structure of the cell membrane, it is toxic for living cells (Bao *et al*., 2010). Hence, bile tolerance is considered to be an important characteristic of a probiotic bacterium that enables it to survive, grow, and exert its action in gastrointestinal transit. *L. plantarum* DW12 was able to survive with both 0.15 and 0.30% bile salt for the 12 h test period and at 6 h incubation viable cells of this strain were 8.25-6.06 log CFU/ml. Hence, this strain could survive under slightly alkaline condition in the gut which bile salts in the small intestine are between 0.15 - 0.30% and probiotics will be normally exposed to this condition for an average of 6 h (Jacobsen *et al*., 1999). The resistance to bile salt of some strains has been related to the activity of bile salt hydrolase enzymes (BSHs) that will hydrolyze combined bile salt and thus reduce its toxicity and side effects of bile salt (Erkkilä and Petäjä, 2000). This means that strain DW12 may release BSHs as well and it will be further investigated. Overall, results in our study demonstrated that the strain DW12 performed most important of the probiotic properties in *in vitro* tests, thereby it should be considered as a potential probiotic isolate.

The molecular weight of the oligosaccharides extracted from the red seaweed was based on their DP values (the number of monomers combined). In our work we found that fructooligosaccharides (FOS) derived from FSB were observed as GF2 and GF4 (Table 4). Due to the raw materials that were used for FSB fermentation being red seaweed, water

and sucrose, but FOS was detected from freeze dried FSB although only in small amounts (Table 4). As red seaweed contains agaro-polysaccharide, natural oligosaccharides of agarose should be observed in the FSB. However, agaro-oligosaccharides were not detected and this corresponded to the DP value of the red seaweed before and after 60 days of fermentation that showed no change (3.5). This indicated that there was no hydrolysis of red seaweed agaro-poysaccharide during the fermentation. Hence, it means that the FOS molecules found in the freeze dried FSB were produced by the LAB, particularly strain DW12 as a starter culture and this was supported by Wiele *et al.* (2007) who reported that FOS molecules are produced by transfructosylation of sucrose using ß-fructofuranosidase and usually contain between 2 and 4 ß(2-1)-linked fructosyl units. A similar result was also reported by Vergara *et al.* (2010) that *L. mesenteroides* produced oligosaccharides in cashew apple juice.

There are many oligosaccharides including FOS have been reported to significantly enhance the growth of lactobacilli (Saulnierl *et al*., 2007). The effect of oligodextran (OD) with different degrees of polymerization (DP3-DP8) was determined for their ability to support probiotics growth as all the strains tested consumed most of the DP3 and DP4 (≥ 80%) with the exception of *B. bifidum* 02, *L. helveticum* and *L. rhamnosus* for DP4, DP7 and DP8 (Grimoud *et al.*, 2010). The results indicated that probiotic bacteria prefer to use oligosaccharides to a lesser degree than the polymers with a longer degree of polymerization. This in accordance with our results that strain DW12 grew better than both the positive controls (Figure 1) with a little sugar and FOS found in the 0.5-2.0% freeze dried FSB (calculated from Table 4, sugars 78.61-314.42 mg/100 ml and FOS 2.10-8.20 mg/100 ml) when compared with 2 g/100 ml of glucose in both the positive controls. This behavior can be attributed to the property of the prebiotic FOS that supported the growth of the potential probiotic DW12 and this is the main reason that the strain DW12 survived (5.41 log CFU/ml) in the finished FSB product from the initial number of 8.74 log CFU/ml at starting of fermentation.

According to the above results, a finished FSB could be considered to be a functional fermented beverage and also a synbiotic beverage product because it had a high GABA content, and a potential probiotic *L. plantarum* DW12 and prebiotic FOS. Hence, this fermented beverage is a unique beverage with possible health benefits that might help consumers who are lactose intolerant and prefer not to use fermented dairy products because of the effects on cholesterol levels.

Conclusions

This study proved that a functional fermented red seaweed beverage was developed with the use of GABA producing *L. plantarum* DW12 that was a potential probiotic and also it produced prebiotics (fructooligosaccharides) from sucrose. Hence, FSB enriched with GABA could be considered to be a synbiotic beverage product.

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References

Bao, Y., Zhang, Y., Zhang, Y., Liu, Y., Wang, S., Dong, X., Wang, Y. and Zhang, H. 2010. Screening of potential probiotic properties of *Lactobacillus fermentum* isolated from traditional dairy products. Food Control 21: 695-701.

Conway, P. L., Gorbach, S. L. and Goldin, B.,R. 1987. Survival of lactic acid bacteria in the human stomach and adhesion to intestinal cells. Journal of Dairy Science 70(1): 1–12.

Duangjitcharoen, Y., Kantachote, D., Ongsakul, M., Poosaran, N. and Chaiyasut, C. 2009. Potential use of probiotic *Lactobacillus plantarum* SS2 isolated from a fermented plant beverage: safety assessment and persistence in the murine gastrointestinal tract. World Journal of Microbiology and Biotechnology 25: 315-321.

Dubois, M., Gilles, K. A., Hamilton, J. K., Rebers, P. A. and Smith, F. 1956. Phenol-sulfuric total sugar. Analytical Chemistry 28: 350-356.

Erkkilä, S. and Petäjä, E. 2000. Screening of commercial meat starter cultures at low pH and in the presence of bile salts for potential probiotic use. Journal of Meat Science 55: 297–300.

Fooks, L. J., Fuller, R. and Gibson, G. R. 1999. Prebiotics, probiotics and human gut microbiology. International Dairy Journal 9: 53-61.

Gonzales, S. N., Apello, M. C., Romero, N.C., DeMacias, M. E. N. and Oliver, G. 1993. Inhibition of enteropathogens by lactobacilli strains used in fermented milk. Journal of Food Protection 56(9): 773-776.

Grimoud, J., Durand, H., Courtin, C., Monsa, P., Ouarné, F., Theodorou, V. and Roques, C. 2010. *In vitro* screening of probiotic lactic acid bacteria and prebiotic

glucooligosaccharides to select effective synbiotics. Anaerobe 16: 493-500.

Hickson, M., Souza, A.L.D., Muthu, N., Rogers, T.R., Want, S., Rajkumar, C. and Builpitt, C. J. 2007. Use of probiotic *Lactobacillus* preparation to prevent diarrhea associated with antibiotics: randomized double blind placebo controlled trial. British Medical Journal 335(7610): 80-83.

Jacobsen, C. N., Rosenfeldt, N. V., Hayford, A. E., Mølle,r P. L., Michaelsen, K. F., Paerregaard, A., Sandström, B., Tvede, M. and Jakobsen, M. 1999. Screening of probiotic activities of forty seven strains of *Lactobacillus* spp. by *in vitro* techniques and evaluation of colonization ability of five selected strains in human. Applied and Environmental Microbiology 65(11): 4949–4956.

Joint FAO/WHO Working Group. 2002. Guidelines for the Evaluation of Probiotics in Food, London, Ontario, Canada.

Klein, G., Hallmann, C., Casas. I. A., Abad, J., Louwers, J. and Reuter, G. 2000. Exclusion of vanA, vanB and vanC type glycopeptide resistance in strains of *Lactobacillus reuteri* and *Lactobacillus rhamnosus* used as probiotics by polymerase chain reaction and hybridization methods. Journal of Applied. Microbiology 89:815–824.

Kullisaar, T., Zilmer, M., Mikelsaar, M., Vihalemm, T., Annuk, H., Kairane, C. and Kilk, A. 2002. Two antioxidative lactobacilli strains as promising probiotics. International Journal of Food Microbiology 72: 215-224.

Makras, L., Triantafyllou, V., Fayal-Messaoudi, D., Adriany, T., Zoumpopoulou, G., Tsakalidou, E., Servin, A. and De Vuyst, L. 2005. Kinetic analysis of the antibacterial activity of probiotic lactobacilli towards *Salmonella enterica* serovar Typhimurium reveals a role for lactic acid and other inhibitory compounds. Research in Microbiology 157: 241-247.

Miller, G. L. 1959. Use of dinitrosalicylic acid reagent for determination of reducing sugar. Analytical Chemistry 31: 426-428.

Mussatto, S. I. and Mancilha, I. M. 2007. Non-digestible oligosaccharides: A review. Carbohydrate Polymers 68: 587-597

NCCLS (National Committee for Clinical Laboratory Standards). (2004). Performance standards for antimicrobial disk susceptibility testing. Fourteenth informational supplement. NCCLS document M100-514. NCCLS, Wayne, Pa.

Olejnik, A., Lewandowska, M., Obarska, M. and Grajek, W. 2005. Tolerance of *Lactobacillus* and *Bifidobacterium* to low pH, bile salts and digestive enzyme. Electronic Journal of Polish Agricultural Universities 8(1): 1-5.

Qiang, X., YongLie, C. and QianBing, W. 2009. Health benefit application of functional oligosaccharides. Carbohydrate Polymers 77: 435-441.

Ratanaburee, A., Kantachote, D., Charernjiratrakul, W., Penjamras, P. and Chaiyasut, C. 2011. Enhancement of γ-aminobutyric acid in a fermented red seaweed beverage by starter culture *Lactobacillus plantarum* DW12. Electronic Journal of Biotechnology 14(3): 0717-3458.

Ratanaburee, A., Kantachote, D., Charernjiratrakul, W. and Sukhoom, A. 2013. Selection of γ-aminobutyric acid-producing lactic acid bacteria and their potential as probiotics for use as starter cultures in Thai fermented sausages (Nham). International Journal of Food Science and Technology 48: 1371-1382.

Saulnier, D. M. A., Molenaar, D., de Vos, W. M., Gibson, G. R. and Kolida, S. 2007. Identifica tion of prebiotic fructooligosaccharides metabolism in *Lactobacillus plantarum* WCFS1 through microarrays. Applied and Enviromental Microbiology 73: 1753-1765.

Singhatong, S., Leelarungrayab, D. and Chaiyasut, C. 2010. Antioxidant and toxicity activities of *Artocarpus lakoocha* Roxb. heartwood extract. Journal of Medicinal Plants Research 4(10): 947-953.

Stephen, J.C., Ivonne, D.R., Ronald, J.H., Robert, L.S., Yvette, S.M., Susan, E.S., José, H.O. & Carol A.S. (2005). Manual of antimicrobial susceptibility testing. American Society for Microbiology. ISBN 1-55581-349-6, 39-52.

Sullivan, O. L., Murphy, B., McLoughlin, P., Duggan, P., Lawlor, G. P., Hughes, H. and Gardiner, E. G. 2010. Prebiotics from Marine Macroalgae for Human and Animal Health Applications. Marine Drugs 1660-3397.

Teitelbaum, J. and Walker, W. 2002. Nutritional impact of pre-and probiotics as protective gastrointestinal organisms. Annual Review of Nutritional 22: 107-138.

Temmerman, R., Pot, B., Huys, G. and Swings, J. 2003. Identification and antibiotic susceptibility of bacterial isolates from probiotic products. International Journal of Food Microbiology 81: 1-10.

Vergara, C. M. de A. C., Honorato, T. L. and Maia, G. A. 2010. Prebiotic effect of fermented cashew apple (*Anacardium occidentale* L) juice. LWT-Food Science and Technology 43: 141-145.

Virtanen, T., Pihlanto, A., Akkanen, S. and Korhonen, H. 2007. Development of antioxidant activity in milk whey during fermentation with lactic acid bacteria. Journal of Applied Microbiology 102: 106-115.

Wiele, T. van de., Boon, N., Possemiers, S., Jacobs, H. and Verstraaete, W. 2007. Inulin-type fructans of longer degree of polymerization exert more pronounced *in vitro* prebiotic effects. Journal of Applied Microbiology 102 (2): 452-460.

Zhang, S., Liu, L., Su,Y., Li, H., Sun, Q., Liang, X. and Lv, J. 2011. Antioxidative activity of lactic acid bacteria in yogurt. African Journal of Microbiology Research 5(29): 5194-5201.