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In vitro prebiotic evaluation of exopolysaccharides produced by marine isolated lactic acid bacteria

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ABSTRACT

Lactic acid bacteria (LAB) selected based on high EPSs production yields of 14, 7.6, 4.9 and 5 g/L in sucrose containing MRS broth were identified as *Weissella cibaria*, *Weissella confusa*, *Lactobacillus plantarum* and *Pediococcus pentosaceus*, respectively based on their 16S rDNA sequences. EPSs produced by these strains did not stimulate secretion of interleukin (IL)-8, and were resistant to stomach acid and human pancreatic amylase. In pure culture system, only *Bifidobacterium bifidum* DSM 20456 exhibited the ability to utilize these EPSs as carbon sources but not *L. plantarum* TISTR 875 and *Lactobacillus acidophilus* TISTR 1034. EPSs from *W. cibaria* exhibited strong bifidogenic effect in the mixed-culture of human fecal microflora using the three-stage fermentation model. In the transverse and distal colon, bifidobacteria and lactobacilli as well as acetate and propionate increased significantly. Butyrate slightly decreased in the proximal colon region after feeding EPSs, but increased in the distal region.

1. Introduction

Exopolysaccharides (EPSs) are long-chain polysaccharides which are secreted mainly by bacteria and microalgae into their surrounding during growth. For the cells, EPSs are thought to play a role in protection against desiccation, toxic compounds, bacteriophages, osmotic stress, and to permit adhesion to solid surfaces and biofilm formation (De Vuyst & Degeest, 1999). Apart from the stabilizing, emulsifying or gelling properties (Tallon, Bressollier, & Urdaci, 2003); health-promoting effect of EPSs with the antitumor, antiulcer, immunomodulating and cholesterol-lowering activities, has resulted to their valuable ingredients for application in food industry (Dal Bello, Walter, Hertel, & Hammes, 2001; De Vuyst & Degeest, 1999; Kitazawa et al., 1991; Oda, Hasegawa, Komatsu, Kambe, & Tsuchiya, 1983; Ruas-Madieido, Hugenholtz, & Zoon, 2002). In addition, bacterial EPSs exhibited high resistance to human gastrointestinal digestion, and selective enhancement of beneficial bacteria colonized in the colon function in the same way to other prebiotics, non-digestible polysaccharides such as fructooligosaccharides, galactooligosaccharides and inulin by acting as carbon source acquired by gut bacteria. Short chain fatty acids (SCFA) and lactate generating from bacterial fermentation of these resistant carbohydrates involved in suppression of pathogenic intestinal bacteria, immune system modulation, energy supply for colonic epithelium and modulation of cholesterol and lipid metabolism as well as gut microbiota composition (Cummings & Macfarlane, 1997; Topping & Clifton, 2001). Recently, the *in vitro* fermentation of EPSs from *Bifidobacterium* strains by fecal microflora was demonstrated (Salazar, Gueimonde, Hernández-Barranco, Ruas-Madiedo, & de los Reyes-Gavilán, 2008).

Lactic acid bacteria (LAB) are classified as generally regarded as safe (GRAS) by US-FDA and considered as an alternative source of EPSs production with a wide structural variety including homopolysaccharides (HoPS), which contain a single type of monosaccharide, and heteropolysaccharides (HePS), which comprise repeating units of different monosaccharides (De Vuyst & Degeest, 1999; Monsan et al., 2001).

Many non-digestible polysaccharides have been widely proven to have "prebiotic" potential, meanwhile only a few reports regarding the use of EPSs produced by LAB as prebiotics, apart from other beneficial effects mentioned above. Biodegradability of EPSs differs greatly, hence the diverse variation of their physiological function. EPSs produced by *Lactococcus lactis* subsp. *cremoris* showed 100% recovery through rat digestive passage as well as simulated digestion in humans (Looijesteijn, Trapet, de Vries, Abee, & Hugenholtz, 2001). EPSs from *L. lactis* ssp. *cremoris* B40, *Lactobacillus sakei* 0-1, *Streptococcus thermophilus* SFi20, and *Lactobacillus helveticus* Lh59 were highly resistant to degradation by both soil and fecal inocula,

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meanwhile *S. thermophilus* SFi 39 and SFi 12 produced EPSs which were readily degraded (Ruijssenaars, Stingele, & Hartmans, 2000). Levan-type EPSs from *Lactobacillus sanfranciscensis* displayed bifidogenic effect, although growth of clostridia was also enhanced (Dal Bello et al., 2001). Significant increase in bifidobacterial population was observed in fecal sample from human volunteer who had consumed ropy, oat-based product co-fermented with EPSs producing strain, *Pediococcus damnosus* 2.6 (Martensson et al., 2005).

This study focused on screening of EPS-producing lactic acid bacteria for potential prebiotic source. The prebiotic potential of EPS in term of gut enzyme resistance and selective stimulation of probiotic bacteria as well as cytokine secretion were also evaluated. *In vitro* continuous fermentation of EPS by fecal microflora was conducted and microbial population profile, prebiotic index (PI) and SCFA content were also determined and reported.

2. Experimental

2.1. Marine fish, bacterial medium and chemicals

Various marine fish, shellfish and shrimp were purchased from fresh markets in Songkhla and directly obtained from fishermen docking along Songkhla shoreline. Ruthenium red was obtained from Fluka (Buchs, Germany). Glucose, fructose, lactose and sucrose were supplied by Ajex Finechem (NSW, Australia). MRS medium was purchased from Himedia Laboratories (Mumbai, India)

2.2. Isolation and screening of EPSs producing lactic acid bacteria

Exopolysaccharides-producing LAB was isolated from gastrointestinal tracts of fish, shellfish and shrimp on MRS agar contained 0.8% ruthenium red (RR). Gastrointestinal tracts were aseptically removed and weighed. Twenty-five gram of each sample were ground and diluted in sterilized sea water to obtain the dilutions of 10^{-1}, 10^{-2}, 10^{-3} and 10^{-4}. One hundred microliter of each dilution was then spread on ruthenium red containing MRS (RR-MRS) agar. Bacterial isolation was performed on the RR-MRS with 4 different carbon sources including glucose, lactose, fructose and sucrose. After incubation at 37 °C for 24–48 h, EPSs-producing colonies were selected based on their ropy appearance by touching them with a sterile inoculation loop and their ability to resist the penetration of ruthenium red, which appeared as white colonies on pink background of the MRS-RR agar plate (Stingle, Neeser, & Mollet, 1996).

2.3. EPS preparation

EPS producing strains were cultivated in MRS broth supplemented with 2% of specific sugar used for the isolation. After 48 h of incubation at 37 °C, the culture broth was centrifuged at 8000 rpm for 15 min at 4 °C to remove bacterial cell. Addition of 2 vol of 95% chilled ethanol to the supernatant was performed at 4 °C and the mixture was left overnight to precipitate EPSs, which were later collected by centrifugation at 8000 rpm for 15 min at 4 °C and dried at 60 °C until constant weight was obtained (Smitinont, Tansakul, Tanasupawat, Keeratibul, & Navarini, 1999).

2.4. Effect of EPSs on cytokine stimulation

HT-29 epithelial cells were grown as monolayers in RPMI 1640 medium supplemented with 10% heat inactivated fetal calf serum, 2 mmol/L glutamine, 1 mmol/L sodium pyruvate, 2% sodium bicarbonate and 10 mmol/L HEPES. Cell were seeded at a density of 1×10^6 cell/well in microtiterplate and incubated at 37 °C under 5% CO_2 atmosphere. Cell cultures were regenerated daily with fresh media until confluent monolayer was obtained. The old media were decanted and the monolayer was incubated in 150 μL of fresh serum-free RPMI 1640 at 37 °C under 5% CO_2 atmosphere for 2 h, then discarded the supernatant thereafter. The confluent monolayer was later incubated in 200 μL of EPS (0.001, 0.01, 0.1 and 1% (w/v) in serum-free RPMI 1640) for 1 h, whereas human recombinant α-TNF (10 ng/mL (w/v) in serum-free RPMI 1640) was added to the monolayers in each positive control well. Each treatment was conducted in three separate runs.

Cytokine secretion was (IL-8) quantitatively measured by cytokine-specific sandwich enzyme linked immunosorbant assays (ELISA) on 96-well (Costar#3369) as previously described by (Jijon et al., 2004). Recombinant mouse IL-8, antimouse IL-8, biotinylated anti-mouse IL-8 from R&D System were used as standard, capture antibody, and detection antibody, respectively.

2.5. EPS digestibility under simulated gastrointestinal conditions

EPSs were tested for the digestibility under acidic condition (0.14 M HCl buffer pH 1 + pepsin) at 37 °C for 4 h according to Korakli, Gänzle, and Vogel (2002). Thereafter, the EPSs solution adjusted to pH 6.9 using 1 M NaOH followed with addition of 1 unit/mL of human pancreatic α-amylase (Sigma) was further incubated for another 6 h. Total carbohydrate (expressed in glucose equivalents) and reducing sugar were determined before and after the digestion by phenol sulfuric acid method and copper–bicinchoninate, respectively (Fox & Robyt, 1991). Percentage of hydrolysis was calculated from reducing sugar released from the digestion divided by total carbohydrate.

2.6. Effect of EPSs on growth of probiotic bacteria and co-cultivation with food-borne pathogens

Growth and EPSs utilization by many probiotic bacteria (*Bifidobacterium longum* ATCC 2014, *Bifidobacterium aldolescentis* ATCC 2018 *Bifidobacterium bifidum* DSM 20456, *Lactobacillus reuteri* ATCC 3042, *Lactobacillus animalis* ATCC 3045, *Lactobacillus acidophilus* ATCC 3066 and *L. acidophilus* TISTR 3180) were evaluated in minimal medium containing 2.0 g peptone water, 2.0 g yeast extract, 0.1 g NaCl, 0.04 g K_2HPO_4, 0.04 g KH_2PO_4, 0.01 g $CaCl_2{\cdot}6H_2O$, 2 g $NaHCO_3$, 0.01 g $MgSO_4{\cdot}7H_2O$, 0.5 g bile salt, 2 mL Tween-80, 0.05 g hemin, 0.5 g L-cysteine and 1% EPSs (glucose as positive control) at 37 °C for 72 h (Salazar et al., 2008). Bacterial growth was determined by cultural plating on MRS agar for lactic acid bacteria, and direct microscopic count of stained cells (LIVE/DEAD BacLight™ Bacterial Viability Kits, Invitrogen) under fluorescent microscope was applied for bifidobacterial count. Utilized EPS was reported as total carbohydrate analyzed by phenol sulfuric acid method according to Fox and Robyt (1991).

2.7. Fermentation of EPS by fecal microflora in continuous culture system

The continuous culture system consisted of three vessels (V1, V2 and V3) with working volumes of 220, 320 and 320 mL, respectively. The fermentation condition was conducted under closed anaerobic (continuous N_2 flushing) system and respective pH of 5.5, 6.2 and 6.8 were automatically controlled by addition of 0.1 N HCl–NaOH as appropriate. Each vessel was continuously stirred and maintained at 37 °C using circulating water jacket. The culture medium was continuously introduced to V1 by peristaltic pump and sequentially overflowed through V2 and V3 with operating rate of 25–27 mL/h. The culture medium consisted of the following composition in 1 L of distilled water: 5.0 g soluble starch, 2.0 g pectin (citrus), 1.0 g guar gum, 4.0 g mucin (porcine gastric type III), 2.0 g xylan (oat spelt), 2.0 g arabinogalactan, 1.0 g inulin, 3.0 g casein, 5.0 g

peptone water, 5.0 g tryptone, 0.4 g bile salt, 4.5 g yeast extract, 0.005 g $FeSO_4{\cdot}7H_2O$, 4.5 g NaCl, 4.5 g KCl, 0.5 g KH_2PO_4, 1.25 g $MgSO_4{\cdot}7H_2O$, 0.15 g $CaCl_2{\cdot}6H_2O$, 1.5 g $NaHCO_3$, 0.8 g cysteine–HCl, 0.05 g hemin, 1.0 mL Tween-80, 0.01 mL vitamin K and 4 mL of 0.025% (w/v) rezasurin solution (Macfarlane, Macfarlane, & Gibson, 1998).

The 10% fecal slurry from a healthy human volunteer was freshly prepared with pre-warmed phosphate-buffered saline (PBS pH 7.2) and used as an inoculum. After fecal inoculation, the system was left overnight as batch cultivation and later switched to continuous feeding until flow rate of 25–27 mL/h was stabilized (within approximately 48 h). The fermentation continued to reach steady state (about 10 days), at which EPS was added into V1 at the amount of 7 g/day for 10 days or until a further steady state was reached. Samples of culture fluid was removed from each vessel at time of inoculation, flow initiation, 10 days after, before and after EPS addition for subsequent bacterial enumeration and short-chain fatty acid analysis.

2.8. Enumeration of bacterial population in fecal sample by fluorescent in situ hybridization (FISH) technique

Differences in bacterial populations were assessed through FISH technique (Probert, Apajalahti, Rautonen, Stowell, & Gibson, 2004) with oligonucleotide probes designed to target specific region of 16S rRNA and labeled with the fluorescent dye Cy3. The probes used were Bif164 (5′-CATCCGGC ATTACCACCC-3′), Bac303 (5′-CCAATGTGGGGGACCTT-3′), Lab158 (5′-GGTATTAGCA(T/C)CTGTTTCCA-3′), Chis 150 (5′-TTATGCGGTATTAATCT (C/T)CCTTT-3′) and Eub338 (5′-GCTGCCTCCCGTAGGAGT-3′) specific for bifidobacteria, bacteroides, *Lactobacillus*/*Enterococcus* spp., *Clostridium histolyticum* group, and *Eubacterium* group, respectively. The nucleic acid stain 4, 6-diamidino-2-phenylindole (DAPI) was used for total bacterial count. Samples (375 μL) obtained from the fermentation vessels were diluted in 4% (w/v) paraformaldehyde and fixed overnight at 4 °C. The cells were then centrifuged at 10,000 × *g* for 5 min, washed twice with phosphate-buffered saline (PBS; 0.1 M, pH 7.2), resuspended in 300 μL of PBS/99% ethanol (1:1 (w/v)) mixture and stored at −20 °C for at least 1 h. The 20 μL of cell suspension appropriately diluted with PBS were dried on TEFLON poly-L-lysine coated slide at 45 °C for 10–12 min. The slide was then sequentially dipped into 50, 80 and 96% ethanol and finally dried on slide dryer to expose bacterial DNA. Prewarm hybridization buffer (45 μL) and 5 μL DNA probe were mixed and flushed on each slide well and probe hybridization was allowed for 4 h at the appropriate temperature for each probe in hybridization oven. The slide was later dipped into washing buffer contained 50 ng/μL DAPI and incubated at the appropriate temperature for 15 min, followed by dipping into cold distilled water before air blow-drying. SlowFade (Molecular Probes, Eugan, OR, USA) was added onto slide well and the cover slide was placed over. The bacterial cells were examined and counted under a fluorescent microscope (Nikon Eclipse, E400). The DAPI stained cells were examined under UV light and hybridized cells viewed using a DM510 filter. For each slide well, at least 15 different fields were counted.

2.9. Determination of short-chain fatty acids

Fermentation fluid was centrifuged at 13,000 rpm for 15 min to obtain culture supernatant for analysis of acetic, propionic and butyric acids using HPLC with an integrated oven compartment (50 °C). BIO-RAD Aminex HPX -87 H Ion Exclusion column with 7.8 mm diameter and 300 mm in length was used with 0.005 M sulfuric acid as a mobile phase at flow rate of 0.6 mL/h, and UV detector was set at 215 nm. The amount of each acid was calculated based on the peak area with standard curve (Olano-Martın, Mountzouris, Gibson, & Rastall, 2000).

3. Results

3.1. Isolation and screening of EPS producing lactic acid bacteria

Sixty-seven EPSs producing isolates were isolated from 4 different carbon sources of glucose, fructose, sucrose and lactose and from two conditions, aerobic and anaerobic condition. The highest numbers (14 strains) of bacterial isolates were obtained from GI tracts of Damsel fish (*Dascyllus aruanus*) whereas mussel, cockle, white shrimp and chacunda gizzard-shad provided only 2 isolates. However, the bacterial isolates obtained from green mussel and cockle produced EPSs at high levels of 14.2–14.5 g/L and 10.2, respectively.

Apart from varieties of fish, numbers of bacterial isolates obtained depended on types of sugars added to isolating medium, oxygen availability and screening methods applied. Twenty-seven isolates (40.29%) of total 67 isolates were obtained from sucrose-containing MRS agar whereas fructose, glucose and lactose containing MRS agars yielded 14, 14 and 12 bacterial isolates, respectively. Thirty-five strains were obtained from the isolation performed at aerobic condition and incubation at anaerobic condition provided thirty-two isolates. Regarding screening procedures on agar plates, slimy appearance and white colony on MRS contained ruthenium (RR) were used to detect EPSs production. Ruthenium red was a polycationic dye specific to acidic polysaccharides on bacterial cell wall, so EPSs producing bacteria could defend the penetration of this dye to its cell wall; it appeared white colony. A large number of EPSs producing LAB (62 isolates or 92.5% of total strain numbers) were obtained by using ruthenium red containing MRS (RR-MRS) whereas only 5 isolates (7.5%) were obtained by detection of slimy appearance indicating higher sensitivity in detection of EPSs production by using RR (Table 1). EPSs production in culture broth supported this conclusion as the strain produced EPSs as low as 0.8 g/L could be detected and selected on the RR-MRS agar whereas the observation of slimy appearance detected the strains, which produced EPSs at the level higher than 10 g/L as shown in Table 1. Therefore, numbers of EPSs-producing isolates obtained can be influenced by sources of isolation, types of sugars added to isolation media and sensitivity of screening or selection methods.

Amount of EPSs determined from culture broth of each isolate supported both of the agar screening methods. High EPSs producing isolates were obtained by the selection based on their ropy appearance, which confirmed by the EPSs production yield of 10–14 g/L. Five strains with high EPSs production were isolated only from sucrose containing MRS and the highest EPSs production yield of 14 g/L was obtained from the strain isolated from *Perna viridis* (green mussel) on sucrose-containing MRS agar.

The selected strains of A2, A9, A3 and 5S4 with high EPSs production yield at 14, 7.6, 4.9 and 5 g/L, respectively, were selected and identified based on the nucleotide sequence of their 16S rDNA. Alignment by the nr database using the BLAST program showed that the 16S rDNA sequences of strains A2, A9, A3 and 5S4 are significantly similar to those of 16S rDNA from *Weissella cibaria* Uga49 (99% identical over 1500 bp; GenBank accession number DQ294961), *Weissella confusa* Inje LM S-338 (99% identical over 1500 bp; GenBank accession number DQ 321751), *Lactobacillus plantarum* L5 (96% identical over 500 bp; GenBank accession number DQ 239698) and *Pediococcus pentosaceus* SL4 (98% identical over 500 bp; GenBank accession number AY 675243), respectively.

Table 1
Comparison of isolate numbers and EPs yield obtained from two screening methods for EPSs-producing lactic acid bacteria.

Screening methods	Isolates obtained		EPSs yield (g/L)	Number of isolates			
	No. of isolated	Percentage %		Sucrose	Fructose	Lactose	Glucose
Ruthenium Red (RR) detection	62	92.54	0.8–9	22	14	12	14
Slimy appearance detection	5	7.46	10–14	5	–	–	–

3.2. Resistance of EPS to degradation under simulated gastrointestinal conditions

EPSs produced from *W. cibaria* A2, *W. confusa* A9, *L. plantarum* A3 and *P. pentosaceus* 5S4 to simulated gastric and intestinal juices showed high resistance to both digestions caused by simulated gastric juice and pancreatic amylase. Under high acidic condition (pH 1) for 4 h, EPSs from those isolates were hydrolyzed at the respective rates of 0.35, 2.51, 0.55 and 1.59% (Fig. 1), whereas 0.17, 0.00, 0.14 and 0.03% hydrolysis were observed under simulated intestinal condition, respectively. Although EPSs from *W. confusa* A9 was most susceptible to acid hydrolysis, it was still intact during exposure to human α-amylase under intestinal condition for 6 h.

3.3. Effect of EPSs on cytokine stimulation

All EPSs did not induce the production of chemokine IL-8 (inflammatory mediator) of human epithelial cell-line despite high concentration of 1% (w/v) application. Similar results were obtained in the presences of fructooligosaccharides (Raftilose), cellulose and dextran (data not shown).

3.4. Growth of probiotic bacteria and inhibition of food-borne pathogens in the presence of EPSs as sole carbon source

Only *B. bifidum* DSM 20456 exhibited the ability to utilize EPSs but not *L. plantarum* TISTR 875 and *L. acidophilus* TISTR 1034, *L. reuteri* ATCC 3042, *L. animalis* ATCC 3045. Slightly growths of *B. longum* ATCC 2014, *B. aldolescentis* ATCC 2018, *L. acidophilus* ATCC 3066 were observed at 72 h of incubation. EPSs from *W. cibaria* A2 was shown to be the best substrate for growth of *B. bifidum* with the increase cell number from 6.17 to 7.54 log CFU/mL (Fig. 2). In co-culture study between *B. bifidum* and pathogens (*Staphylococcus aureus*, *Listeria monocytogenes*, *Salmonella* Typhi and *Escherichia coli*), the pathogen inhibition was not observed in the presence of EPSs as carbon source (data not shown).

3.5. Changes in fecal bacterial population analyzed by FISH technique

EPSs from *W. cibaria* A2 selectively enhanced growth of both *Bifidobacterium* and *Lactobacillus*/*Enterococcus* groups in V1, V2 and V3 representing proximal, transverse and distal regions of human colon after feeding trial for 10 days. Bacterial population analyzed on day 10th and 11th were indifferent confirming that the system had been stabilized before EPSs feeding began. Clostridia were slightly reduced in proximal region, whereas growth of eubacteria and bacteriodes groups remained the same as they were before EPSs feeding (day 11th), when the system stabilized (Fig. 3). The result indicated that EPSs feeding could alter bacterial population to shift toward beneficial *Bifidobacterium* and *Lactobacillus*/*Enterococcus* groups and lowered detrimental bacteria such as *Clostridium* group. The residual EPSs lasted through distal colon (Fig. 4), hence the enhancement of high beneficial bacterial groups in all regions of human colon model.

3.6. Vitamins and SCFA production from in vitro continuous fermentation system by fecal microbiota

After 10 days feeding of EPSs from *W. cibaria* A2, total SCFA in V2 (transverse colon) and V3 (distal colon) increased significantly, but remained constant in V1 (Table 2). Significant increases of acetic and propionic acids were shown after 10 day-EPSs feeding (day 21st) compared with the day 11th (before EPSs feeding) when the system stabilized. However, butyric acid was slightly decreased in all vessels. Increases of SCFA in V2 and V3 indicated EPSs availability for colon bacterial fermentation in transverse and distal colons. Acetic acid was the most abundant, followed by moderate level of propionic acid and low concentration of butyric acid.

On the other hand, vitamins B1 and B2 reduced significantly in all regions after EPSs feeding was continued for 10 days (Table 3). Folic acid increased only in the region representing transverse colon. Productions of vitamins B1, B2 and folic acid correlated to

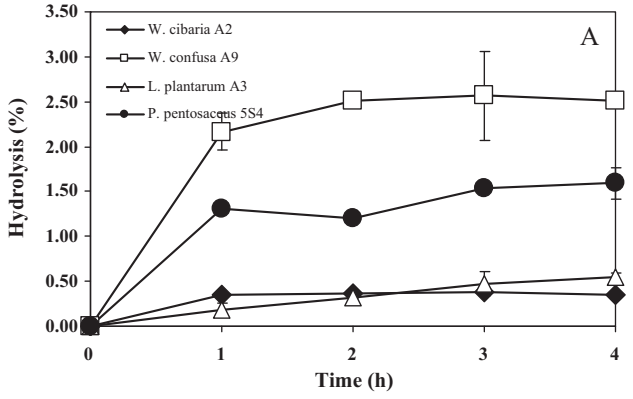

Fig. 1. Simultaneous acidic and enzymatic hydrolysis of EPSs from *W. cibaria* A2, *W. confuse A9*, *L. plantarum* A3 and *P. pentosaceus* 5S4 at pH 1 under simulated gastric and intestinal conditions. Data are obtained from triplicate analysis performed on triplicate runs and error bars are ±SD.

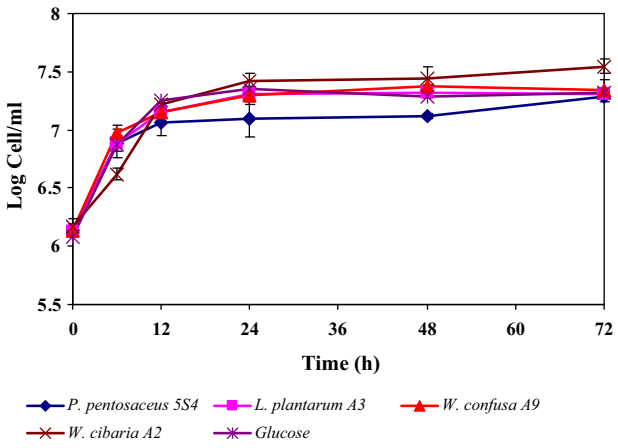

Fig. 2. Growth of *B. bifidum* DSM 20456 in minimal medium contained EPSs from *W. cibaria* A2, *W. confuse A9*, *L. plantarum* A3, *P. pentosaceus* 5S4 and glucose (positive control) as sole carbon sources at 37 °C. Data are obtained from triplicate analysis performed on triplicate runs and error bars are ±SD.

Table 2
Short chain fatty acids (from triplicate analysis) produced by fecal microflora in the three-stage continuous fermentation system using EPSs from *W. cibaria* A2 as a carbon source.

Days	Acetic acid			Propionic acid			Butyric acid		
	Vessel 1	Vessel 2	Vessel 3	Vessel 1	Vessel 2	Vessel 3	Vessel 1	Vessel 2	Vessel 3
Ti[a]	3.96 ± 0.19a	7.15 ± 0.21a	10.8 ± 3.80a	0	1.84 ± 0.16a	1.58 ± 1.00a	1.40 ± 0.25a	1.78 ± 0.09a	2.02 ± 0.90a
0[b]	57.9 ± 7.42e	73.4 ± 8.23d	80.5 ± 7.78d	23.4 ± 0.09a	24.4 ± 2.24b	27.5 ± 3.94b	22.1 ± 0.51b	22.2 ± 1.85b	18.6 ± 1.42b
10	39.8 ± 0.38b	61.0 ± 3.33c	61.0 ± 3.15c	45.5 ± 4.89c	63.2 ± 1.78e	42.3 ± 0.83d	28.5 ± 5.80c	46.2 ± 0.86d	32.4 ± 2.99d
11	54.3 ± 1.89d	52.6 ± 2.66b	57.7 ± 3.56b	41.2 ± 3.89bc	37.2 ± 6.10c	39.0 ± 4.82c	36.2 ± 1.24d	31.1 ± 6.30c	30.8 ± 3.41d
21	52.1 ± 1.27c	62.6 ± 1.20c	78.6 ± 5.43d	38.5 ± 3.70b	54.8 ± 11.03d	60.2 ± 4.09e	27.2 ± 3.05c	24.3 ± 6.11b	27.9 ± 2.74c

Note: Data are mean ± SD of three values from triplicate analysis.
Means with different online letters (a–e) within a column indicating significant difference ($P < 0.05$).
[a] Time at the inoculation for 24 h batch fermentation.
[b] Time at the continuous fermentation began. The system was operated to reach stable stage for 10 days, the culture was taken on day 10th and 11th before the EPSs feeding began.

growths of bifidobacteria and lactobacilli/enterococci rather than clostridia and bacteriodes.

4. Discussion

According to the results, sucrose appeared to be the best carbon source for screening of EPSs producing lactic acid bacteria. This phenomenon was in good agreement with Smitinont et al. (1999), who reported that seven EPSs-producing strains were selected only from sucrose containing medium, and two of them were identified as *P. pentosaceus* (named AP-1 and AP-3), which produced 6.0 and 2.5 g/L of EPS, respectively. In the same way, Van Geel-Schutten, Flesch, Ten Brink, Smith, and Dijkhuizen (1998) screened several *Lactobacillus* strains from different origins (fermented food, gastrointestinal tract of LAB animals, and human dental plaque) for EPSs production in de Man, Rogosa, and Sharpe medium supplemented with high concentrations (100 g/L) of different sugars including glucose, fructose, maltose, raffinose, sucrose, galactose, or lactose. Sixty out of one hundred eighty-two strains produced EPSs and seventeen of them rendered more than 100 mg/L, with the sucrose medium being the best for detecting the EPS phenotype. The authors attributed the higher percentage of the positive isolates (33%) to the high content of sugar used in the media. In addition, Van Geel-Schutten et al. (1998) indicated that sucrose was an excellent substrate for abundant EPS synthesis and suggested addition of high sugar concentration in liquid medium for screening LAB producing large amount of EPSs. The studies noted above indicated that the carbon source added to the screening media plays an important role in the detection of the EPSs phenotype in LAB. The total amount of polysaccharides produced seems to be strongly influenced by the sugar available in the medium. Other differences in EPS production related to the carbon source of the medium have been attributed to the presence of different sugar transport systems in the LAB strains (Chervaux, Ehrich, & Magain, 2000), if the entry of mono- and disaccharides into the cell is the initial step of EPS synthesis. Both sugar transport and the synthesis of sugar-nucleotide precursors are regulated processes that can be under catabolite repression (Boels, van Kranenburg, Hugenholtz, Kleerebezem, & de Vos, 2001; Laws, Gu, & Marshall, 2001).

EPSs from *W. cibaria* A2, *W. confusa* A9, *L. plantarum* A3 and *P. pentosaceus* 5S4 exhibited high potential prebiotic property of high resistance to gastric and intestinal digestions, selective enhancement of beneficial gut bacteria particularly bifidobacteria group indicating their prebiotic potentials. Among bifidobacteria tested, *B. bifidum* was specifically enhanced in the presence of EPSs as sole carbon source at the same level its growth in glucose. In fact EPSs could only act as carbon source of colonic bacteria if they reached the colon intact, whereas glucose was not available in the colon. The four types of EPSs tested survived *in vitro* digestion using simulated gastric and intestinal condition. The results were in agreement with previous observations that EPSs from lactic acid bacteria were either partially broken down (Mozzi, Gerbino, Font de Valdez, & Torino, 2009) or be resistant to the harsh conditions of gastrointestinal transit (Looijesteijn et al., 2001).

In mixed culture continuous colon model system, EPSs from *W. cibaria* A2 clearly enhanced growth of beneficial *Bifidobacterium* and *Lactobacillus*/*Enterococcus* groups, which were generally considered as the main bacterial target of prebiotic activity, but reduction of clostridia was resulted in V1 (Gibson & Roberfroid, 1995; Reid, 2008). In the meantime, total gut bacteria remained constant supporting specific stimulation of *Bifidobacterium* groups which was observed in pure culture experiment. Such bacterial population shift to bifidobacterial direction was correlative to addition of levan-type EPSs produced by *Lactobacillus sanfranciscensis* LTH 1729 and *L. sanfranciscensis* LTH 2590 observed by Dal Bello et al. (2001). However, *Eubacterium biforme* and *Clostridium perfringens* were also stimulated, which differed from this study. Such

Table 3
Vitamins B1, B2 and folic acid produced by fecal microflora in the three-stage continuous fermentation system using EPSs from *W. cibaria* A2 as a carbon source.

Days	Vitamin B1 (μg/mL)			Vitamin B2 (μg/mL)			Folic acid (μg/mL)		
	Vessel 1	Vessel 2	Vessel 3	Vessel 1	Vessel 2	Vessel 3	Vessel 1	Vessel 2	Vessel 3
Ti[a]	36.03 ± 1.45	29.13 ± 0.86e	37.27 ± 1.26d	27.68 ± 3.13c	32.82 ± 5.85c	44.75 ± 1.79c	95.99 ± 3.51c	85.41 ± 2.44c	138.39 ± 0.89d
0[b]	36.29 ± 1.05c	16.65 ± 0.59d	13.23 ± 0.23c	25.33 ± 1.41bc	17.34 ± 2.24b	14.03 ± 1.66a	72.13 ± 1.49b	20.73 ± 0.01b	20.15 ± 0.82b
10	14.36 ± 1.21b	11.13 ± 1.01c	8.16 ± 0.10b	22.01 ± 0.49ab	10.05 ± 0.19a	12.27 ± 1.14a	9.50 ± 1.79a	3.71 ± 0.60a	1.95 ± 0.86a
11	15.47 ± 0.15b	9.72 ± 0.51b	7.34 ± 1.13b	24.60 ± 0.70bc	15.53 ± 0.04b	41.23 ± 3.34b	ND	ND	97.35 ± 0.75c
21	12.29 ± 1.27a	8.03 ± 0.78a	5.01 ± 0.49a	18.41 ± 3.94a	16.32 ± 0.34b	ND	ND	4.08 ± 0.39a	ND

Note: Data are mean ± SD of three values from triplicate analysis.
Means with different online letters (a–c) within a column indicating significant difference ($P < 0.05$).
[a] Time at the inoculation for 24 h batch fermentation.
[b] Time at the continuous fermentation began. The system was operated to reach stable stage for 10 days, the culture was taken on day 10th and 11th before the EPSs feeding began.

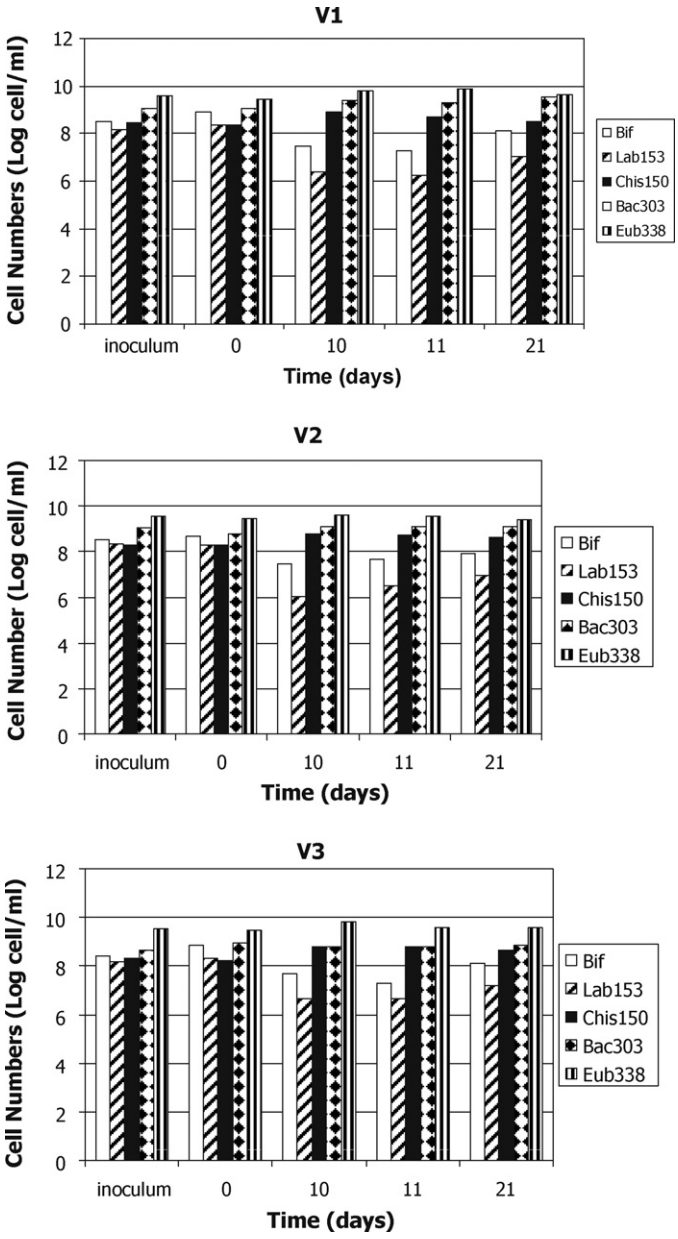

Fig. 3. Fecal bacterial population (log cell/mL) in the three-stage continuous fermentation system using EPSs from *W. cibaria* A2 as a carbon source (EPSs feeding began on the day 11th after samples were taken).

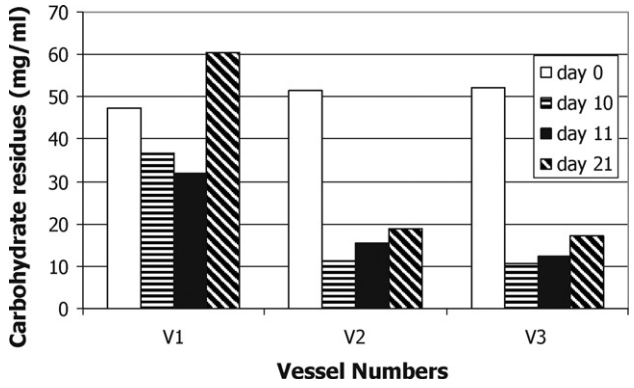

Fig. 4. Carbohydrate residues (mg/mL) in three-stage continuous fermentation system using EPSs from *W. cibaria* A2 as a carbon source.

result indicated structural differences between EPSs types from this study.

Increase of acetic acid in V2 and V3 was corresponded to the increase of bifidobacterial number, which was well-known as acetic acid producer. The result was contradicted to addition of chitosan oligosaccharides, which acetate decreased in all three vessels with increase of propionate, meanwhile butyrate elevated only in V1 and V2. However, similar correlation to bacterial groups stimulated was noted. Enhancement of bifidobacteria and lactobacilli was obvious in V1, but clostridia and *Bacteriodes* which contributed to detrimental effect became dominant in V2 and V3 (Vernazzaa, Gibsona, & Rastall, 2005). SCFA produced by certain colon bacteria are important energy source for human gut and can be transported to many parts of the body. Acetate is primarily used in muscle tissue (Topping & Clifton, 2001) and propionate is mainly utilized in liver cells (Wolever, Spadafora, & Eshuis, 1991). Butyrate is favorable energy source for colonocytes which is supposed to be constantly renewed to maintain healthy condition of human colon (Cummings, 1984). Stimulation of SCFA production by addition of external carbon source was also observed in other study, in which glucose, inulin and EPS from human *Bifidobacterium* strains were added to the batch system of fecal bacterial fermentation (Salazar et al., 2008). This phenomenon supported the role of prebiotic functionality.

5. Conclusion

The presence of EPSs produced by *W. cibaria* A2 exhibited highly bifidogenic effect with long lasting in the colon model, whereas most prebiotics commercially available were rapidly consumed by gut bacteria in proximal colon. Growths of bifidobacteria and lactobacilli were dominantly promoted under highly competitive condition of the human colon, when EPSs was added indicating its high selectivity. *W. cibaria* A2 is a potential source of prebiotic EPSs with high production yield. This study strongly proved that EPSs from lactic acid bacteria, particularly *W. cibaria* A2 can be used as a prebiotic ingredient in food industry to modulate intestinal microbiota into health beneficial direction.

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