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In vitro fermentation behaviors of fucosylated chondroitin sulfate from *Pearsonothuria graeffei* by human gut microflora

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ABSTRACT

A fucosylated chondroitin sulfate (FCS-*pg*) with highly repeated structure from *Pearsonothuria graeffei* was subjected to a *in vitro* fermentation model to investigate its fermentability and effects on human gut microflora. High performance liquid chromatography (HPLC) measurement found FCS-*pg* can be fermented to short chain fatty acids (SCFAs) by gut microflora from partial human fecal samples. 16S rRNA gene-based polymerase chain reaction-based denaturing gradient gel electrophoresis (PCR-DGGE) profiling and real-time quantitative PCR analysis showed that FCS-*pg* mainly increased the proportions of *Clostridium cluster* XI, *Bacteriodes prevotella* group, *Bifidobacterium genus*, *Clostridium cluster* I and *Clostridium cluster* XIVab, whereas the numbers of the *Enterobacteriaceae* and *Lactobacillus* decreased. These results indicated that FCS-*pg* was mainly fermented by *Bacteroides*, *Bifidobacterium* and *Clostridium*. It increased the content of probiotics bacteria in achieving health-enhancing effect, was slightly different than most sulfated polysaccharides from marine animals. The current study provides useful new information on the mechanism of absorption and functional activity on FCS-*pg* within the gastrointestinal tract of the human body.

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1. Introduction

Sea cucumber is an economically important aquacultural food and medicine resource [1]. Its role in human nutrition and health are widely appreciated. Fucosylated chondroitin sulfates (FCSs) from sea cucumbers are sulfated polysaccharides extracted from the body wall of sea cucumber that contain a chondroitin sulfate E backbone with sulfated fucose branches [2]. FCSs are unique bioactive ingredients in sea cucumber and laminaria, they are also the most widely distributed and well-studied polysaccharides in marine organisms. The unique biological activities of FCSs have attracted the attention of researchers, particularly in development of anticoagulants [3] and anti-atherosclerotics for the reduction of hyperlipoproteinemia [4].

An *in vitro* study suggested that FCS primary structure is key factor to influencing its biological activities [1,2,5], furthermore, there is a minimum active unit [6]. Animal experiments on orally administered FCS demonstrated that the anti-thrombosis and blood lipid regulation depend on integrated structure [1,5,7]. However, most non-starch polysaccharides (NSP) such as pectin, arabinoxylan oligosaccharides, as well as resistant starch demonstrate resistance to digestion in the human small intestine (this impedes the absorption of polysaccharides particles through the intestinal mucosa), undergoing fermentation by intestinal bacteria to SCFAs [8].

It is reported that intestinal flora, in a portion of the human population, can ferment fucoidan sulfate derived from seaweed to afford low molecular weight oligosaccharides [9]. Therefore, it is necessary to take investigation of the internal digestibility of FCSs from sea cucumbers and their true prebiotic effects in the intestinal tract. Since the internal environment that impacts the digestion of polysaccharides is so complicated, the influence of intestinal microbiota on fermentation of FCSs from sea cucumbers and their metabolites in people have not been studied. In recent years,

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simplified models, simulating digestion and absorption system, have demonstrated their potential for studying on the digestion and absorption of polysaccharides. Continuous culture *in vitro* gut microbe cultivation models, provide a great convenience to study changes in polysaccharides during digestion and absorption in the gastrointestinal tract [10] and have already demonstrated a large number of applications in the field of intestinal fermentation of polysaccharides and oligosaccharides [11,12].

In the present study, highly active fucosylated chondroitin sulfate from *Pearsonothuria graeffei* (FCS-*pg*) was used in fermentation study using intestinal microbiota in a simulated *in vitro* digestion model. The ferment digestibility of FCS-*pg* by human intestinal microbes and the production of SCFAs were investigated. Additionally, the effect of FCS-*pg* on human colonic bacterial communities was analyzed using denaturing gradient gel electrophoresis (DGGE) and with phylogenetic analysis relying on real time quantitative PCR (qPCR).

2. Materials and methods

2.1. Materials and chemicals

Dry sea cucumbers *Pearsonothuria graeffei* (*P. graeffei*) were purchased in a local market in Qingdao, China. Monosaccharide standards were from Sigma (St. Louis, MO, USA). The derivatization reagent 1-phenyl-3-methyl-5-pyrazolone (PMP) was from Sinopharm Chemical Reagent (Shanghai, China). All others reagents and chemicals used were of analytical grade.

2.2. Polysaccharide preparation and physicochemical properties

Polysaccharide, from the body wall of the sea cucumber *P. graeffei* (FCS-*pg*), was extracted and purified as previously described [2]. Monosaccharide composition was measured following PMP derivatization using high performance liquid chromatography (HPLC) as previously described [13]. Sulfate content was determined by ion chromatography [14] and protein content was determined according to the Bradford method [15]. Measurement of molecular weight was performed by gel filtration chromatography on a TSK 4000 column [2].

2.3. In vitro *degradation by intestinal microbial*

Fresh feces were obtained from six healthy human volunteers (Subjects A-F, living in Hangzhou, China) of diverse genders and ages, who were diagnosed without bowel disorders and had not received treatment with antibiotics, probiotics- or prebiotics for at least three months prior to sample collection. Individual and mixed (it was mixed the six individual fresh fecal samples with an equal weight) fresh fecal samples were each inoculated into medium (Table S1) that selected FCS-*pg* as sole carbon source, and cultured without oxygen for days at 37 °C, to assess the degradation and utilization of FCS-*pg*.

The specific operation was devised into two steps, sampling and inoculation as follows. In the sampling step, fresh feces were mixed with anaerobic broth in a ratio of 1:25 (m/v), and then immediately transferred to miniMACS anaerobic workstation (Do Whitley Scientific, UK). Under anaerobic conditions, the mixture was ground, stirred, and filtrated. This process was repeated 3-times. The resulting filtrate was diluted with water to 25 mL, and transferred to 75 mL anaerobic broth (Table S1), and cultured for 48 h at 37 °C (defined as the 1st culturing). In the inoculating step, 25 mL of 1st culturing was transferred to a new 75 mL of anaerobic broth (Table S1) and cultivated for 72 h at 37 °C, (defined as the 2nd culturing). A 3rd culturing was performed as described above to verify the accuracy of the experiment. Changes in the intestinal microbial communities and the degradation of polysaccharide were investigated in the 2nd and 3rd culturing.

2.4. Gut bacterial collection, DNA extraction and PCR amplification

Total DNA, isolated from gut bacteria cultured *in vitro*, was used as a template for PCR amplification to determine bacterial diversity in the human intestinal tract. Fermentation broth was centrifuged at 9000 rpm/min for 5 min to obtain supernatant which was reserved for detection of short chain fatty acids (SCFAs). The pelleted bacteria were fully re-suspended in three 1 mL aliquots of 0.1 mol/L PBS for extraction.

Sample DNA extraction was conducted by the method of the manufacturer's instruction with a QIAamp DNA Stool Mini Kit (QIAGEN, Inc., Shanghai, China). PCR amplifications with V3 region of bacterial 16S rDNA were analyzed by the method of DGGE analysis, the extracted total DNA of the intestinal contents were used as template of PCR reaction. There are two universal primers in PCR reaction system, such as GC-341F (5′-CGC CCG CCG CGC GCG GCG GGC GGG GCG GGG GCA CGG GGG GCC TAC GGG AGG CAG CAG-3′) and 534R (5′-ATT ACC GCG GCT GCT GG-3′). A 50 μL volume of PCR reaction system, containing 5 μL of 10 × PBS buffer, 4 μL of dNTP mixture, 1 μL of individual primer (10 mmol/L), 2 μL of DNA template, 0.5 μL of Taq DNA polymerase (5 U/μL), and made to volume with sterile water. The 10 × PBS buffer, dNTP mixture, and Taq DNA polymerase were purchased from TransGen Biotech (Beijing, China). A negative control, without DNA template, was also similarly prepared. The PCR reaction process used the following conditions: an initial denaturation of 94 °C for 4 min, followed by 30-cycles of denaturation of 94 °C for 1 min, 56 °C for 45s, and 72 °C for 45s, and a final elongation at 72 °C for 10 min. Finally, 5 μL of amplification product was analyzed by electrophoresis on an agarose gel (1.0%) and stained with ethidium bromide and visualized under UV-light.

2.5. Identification of DGGE DNA bands and phylogenetic analysis

The procedures of isolating DNA bands from the DGGE gel, cloning of excised bands, and sequence analysis were previously described [16]. The sequences of PCR products were submitted to NCBI website (http://www.ncbi.nlm.nih.gov) of BLAST database for sequence alignment to identify their most related bacterium and species. Programs of *Mega*, *Clustalx* and *DNAstar* were used for phylogenetic analysis.

2.6. Real-time fluorescence quantitative PCR(qPCR) analysis

qPCR was performed using SYBR Green I to count targeted bacteria, and the specific primers used are listed in Table S2. The reaction system was composed of 4.2 μL of distilled water, 10 μL of SYBR Green Real time PCR Master Mix, 0.4 μL of the upstream and downstream primers (10 μmol/L), respectively, and 5 μL of DNA template (1–10 ng/μL). The amplification procedure was conducted on iCycle TM (Bio-Rad, USA) with an initial denaturation of 94 °C for 10 s, then cycled 40-times with denaturing at 95 °C for 5 s, annealing at 58 °C for 5 s and extension at 72 °C for 20 s and detection at 80 °C for 0.5 s. Melting curve analysis was carried out immediately after the PCR amplification to verify the specificity of amplification. The conditions used were as follows: denaturation at 95 °C for 10 s, incubation at 70 °C for 10 s, and then warming to 95 °C at a rate of 0.2 °C per second. Fluorescence measurements were determined continuously in the process, and the temperature was dropped to 40 °C after detection is complete.

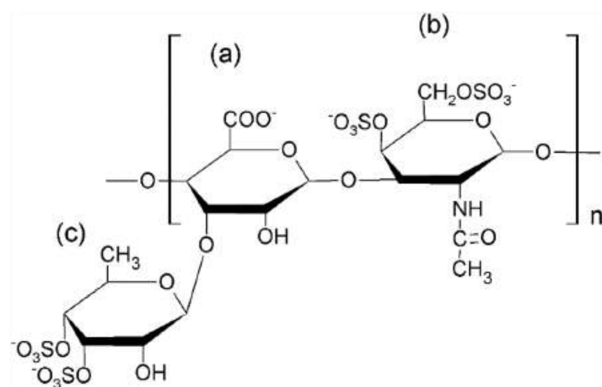

Fig. 1. The structure of FCS-*pg* isolated from the body wall of the sea cucumber *P. graeffei*. (a) 1,4-linked β-D-glucuronic acid residue. (b) 1,3-linked 4,6-*O*-disulfo *N*-acetyl-β-D-glucosamine residue. (c) 3,4-*O*-disulfofucose attached at the *O*-3 position of β-D-glucuronic acid residue.

2.7. Measurement of polysaccharides and SCFAs

The culture supernatant was employed to determine changes of the metabolites, such as polysaccharides and short chain fatty acids. Polysaccharides in supernatant were analyzed on a Waters 1525-2414 system to determine their molecular weight distribution. The chromatographic system consisted of a Waters 1525 binary HPLC pump (Waters Corp., USA) equipped with a TSK-gel G4000PW$_{XL}$ column (7.8 mm × 300 mm, 10 μm, TOSOH, Japan) and a Waters 2414 differential refractive index detector (Waters Corp., USA). NaCl solution (0.2 mol/L) was used as eluent at a flow rate of 0.5 mL/min and the measurement was performed at a temperature of 25 °C. Samples were prepared by filtering through a 0.22 μm filter before a 20 μL aliquot was injected into the column.

SCFAs were obtained from the supernatant, which had been acidified with an equal volume of 0.1 mol/L H_2SO_4 and then extracted into an equal volume of ether. A 10 μL aliquot of the resulting extract was analyzed as previously reported with minor modifications [17]. In brief, a 7890A gas chromatograph (Agilent Technologies, Stockport, UK) with flame ionization detector and equipped with a 30 m × 0.25 mm × 0.25 μm HP-INNOWax column (No. 19091N-133; Agilent Technologies) was utilized to analyze the composition of SCFAs. The determination program for SCFAs was as follows: the temperatures of injector and detector were 225 °C and the column temperature was 200 °C. Split injection (40:1). The initial column temperature was 110 °C and maintained for 1 min, thereafter increasing at a rate of 10 °C/min until reaching 200 °C, which was held there for 2 min. The flow rates of N_2 (carrier gas), H_2 (make-up gas), and air were 20, 15 and 150 mL min^{-1}, respectively. All samples were quantitated by comparison of peak heights with those of authentic standards.

3. Results

3.1. Physicochemical properties analysis of FCS-pg

Polysaccharides (FCS-*pg*) were prepared from the body wall of the sea cucumber *P. graeffei* as previously described [2]. Chemical composition analysis indicated that FCS-*pg* was an acidic polysaccharide with molecular weight of 73 KDa, composed of GlcA, GalNAC and Fuc in a ratio of 1.0:0.8:1.5, consistent with the report of Chen et al. [2]. The sulfate and protein contents of the FCS-*pg* were 31.00% and 0.12%, respectively. The structure of FCS-*pg* has been characterized in our previous work [2] and corresponds to the structure shown in Fig. 1, a repeating disaccharide unit of alternating 1,3-linked 4,6-*O*-disulfo *N*-acetyl-β-D-glucosamine (b) and 1,4-linked β-D-glucuronic acid (a) residues, containing a 3,4-*O*-disulfofucose branch (c) at the β-D-glucuronic acid residue.

Table 1
Experimental schedule.

Fecal sample from	Items investigated in medium (24 h–72 h)			pH	Areas
	Microbiota	SCFAs	Retention time (min)		
Subject A	O[a]	O	O	O	O
Subject B	O	O	O	O	O
Subject C	O	O	O	O	O
Subject D	O	O	O	O	O
Subject E	O	O	O	O	O
Subject F	O	O	O	O	O
Subject MIX	O	O	O	O	O

[a] O: Investigation was performed.

Table 2
Bacterial degradation of FCS-*pg* assay by HPLC.

Samples	Culture	Retention time (min)	Area (μv*sec)	pH
SA[a]	48 h	15.176	27698963	6.6
	72 h	15.481	27634506	6.4
SB	48 h	16.188	19158526	6.1
	72 h	17.095	12498035	5.2
SC	48 h	16.782	12487398	5.9
	72 h	17.362	10520398	4.9
SD	48 h	15.167	22981462	6.5
	72 h	15.388	22416203	6.4
SE	48 h	15.091	24527611	6.5
	72 h	15.152	22243530	6.3
SF	48 h	16.155	20447534	6.1
	72 h	17.538	11537563	5.4
MIX	48 h	16.075	19204541	5.8
	72 h	18.253	11326794	4.5

[a] SA–MIX were the bacterial derived from fecal samples of Subjects A-MIX, respectively.

3.2. Degradation of FCS-pg by human intestinal flora

There are significant differences in the composition of intestinal flora among various individuals, therefore, six individuals of different genders and ages were recruited for the current research, to study the impacts of the polysaccharide FCS-*pg*, on human intestinal flora and its metabolism during simulated intestinal digestion *in vitro*. In this process, changes of intestinal microflora, degradation of polysaccharide and its microbial metabolites in the 2nd and 3rd culture medium were investigated at different times (Table 1).

The media, after fermentation for 48 h and 72 h in the 3rd culturing, were measured by HPLC, for detection changes in the degradation of polysaccharide, and the pH was also determined (Table 2). The results for samples SB, SC, SF and MIX revealed delayed retention times and reduced peak areas and pH values. These changes in the MIX sample were the most significant, perhaps due to the diversity of microflora coming from all six individuals. No remarkable changes were observed for samples of SA, SD and SE. Intestinal bacteria with the capacity of degradation of FCS-*pg* was present in samples SB, SC, SF and MIX, and these bacteria could utilize FCS-*pg* as carbon source for their growth, meanwhile may produce acidic products that decreased the pH of the media (Table 2).

3.3. SCFAs production from FCS-pg fermentation

SCFAs are organic aliphatic acids with a 2–6 carbon atoms, largely generated by glycolysis in anaerobes in the intraluminal colon from indigestible starches, fibers, polysaccharides, in the human diet [18]. The main SFCA components are acetic acid, propi-

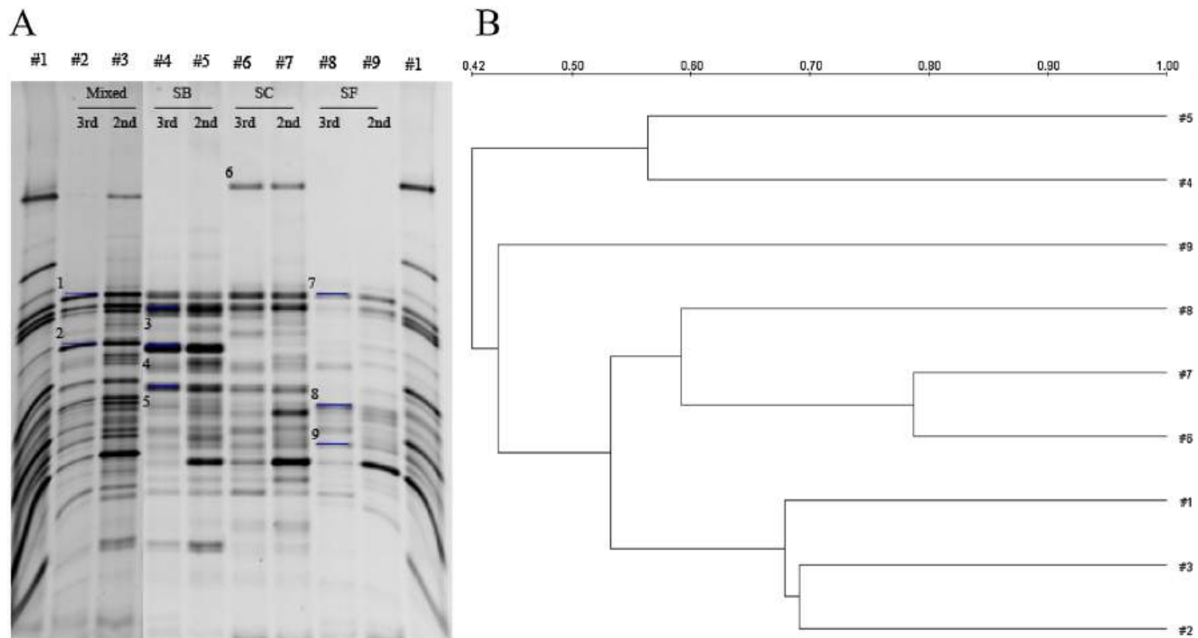

Fig. 2. DGGE profiling of the bacterial community in the 2nd and 3rd subcultures. #1 stands for DNA markers for the gradient, which consist of 16 clones of different bacteria. (A) Numbered 9 DGGE DNA bands were excised for cloning and sequencing. (B) UPGMA cluster analysis of DGGE bacterial profiles. Bacteria samples came from Subject B (SB), Subject C (SC), Subject F (SF), and Mixed fermentation (MIX).

onic acid, and butyric acid, and these are absorbed by the intestinal epithelial cells in their ionic and non-ionic forms and promote the absorption of aqueous electrolytes [19]. SCFAs, a preferred energy substrate for colonic mucosa, enhance sodium absorption, promoting proliferation and growth of colonic mucosal epithelial cells, providing metabolic energy, increasing intestinal blood flow, stimulating production of gastrointestinal hormones, and are important nutrients for the colonic mucosa [20,21].

Since samples SB, SC, SF and MIX contained intestinal bacteria which could degrade FCS-*pg* (Table 2), we measured the production of SFCAs in media of samples SB, SC, SF and MIX cultured for 48 h and 72 h in the 3rd stage of culturing. This allowed us to further study the impact of intestinal microbial on the metabolites of FCS-*pg*. A higher content of acetate, propionate and butyrate was observed (Table 3), indicating that they were the major products of microbial metabolism. The production of SFCAs were significantly increased as a function of growth time and the total production of SCFAs in the samples were SC > MIX > SB > SF. The effect of FCS-*pg* on the bacterial community of SB, SC, SF and MIX requires further study.

3.4. Effect of FCS-pg on bacterial communities

Bacteria samples from the 2nd and 3rd subcultures, were cultured for 72 h and analyzed using DGGE method to further understand the influence of polysaccharide FCS-*pg* on the SB, SC, SF and Mix communities. Each band shown in Fig. 2A represents a single microbe and the number of bands reveals the number of different species of bacteria in sample and the cluster analysis and abundance of each band and the similarity of different microflora *via*. Moreover, the shade of the band indicates content of bacteria and changes in microflora are preliminary obtained according to a semi-quantitative analysis of band shade derived from the same microflora undergoing different treatment.

The abundance of band in MIX was the highest, which corresponds to the greatest diversity of microflora in the MIX sample. However, along with increase in the number of transformations, the growth of many microbes was inhibited because of their lack of ability to utilize FCS-*pg*. Therefore, only bands of C1 and C2 maintained a higher content, indicating microflora in which bands C1 and C2 represents involvement in the metabolism of the polysaccharide, FCS-*pg*. In addition, some microflora might be involved in metabolizing FCS-*pg* in sample SB (C3, C4, C5), SC (C1, C3, C6) and SF (C7, C8, C9). UPGMA cluster analysis of DGGE bacterial profiles (Fig. 2B) shows that the bacterial community of SB and SF were of low similarity, nevertheless, those of MIX and SC reached steady state, which suggests that the influence of FCS-*pg* intake is a major factor affecting the microbial community.

Based on sequencing selected bands and the use of sequence alignment, the genetic relationship among the members of the microbial community were constructed as a phylogenetic tree. The 9 bands shown in Fig. 3 were distributed in the genuses of *Firmicutes*, *Proteobacteria* and *Bacterioidetes*. The diversity of microflora assigned to the genus *Firmicutes* were most abundant, including bands of C2, C4, C5 and C8. These are the closest phylogenetic relationship with *Clostridium* and butyric acid-producing bacteria, and typically play a role in degrading polysaccharides and producing short-chain fatty acids [22]. Bands of C1 and C3 were associated with *Actinobacteria.* These show a close relationship to *Bifidobacterium*, which is a class of intestinal microorganisms that possess beneficial effect on human health. Band C6 was attributed to *Bacteroidetes*. Reportedly, during the metabolism of polysaccharides, *Bacteroides* can simultaneously produce SCFAs such as acetic acid, propionic acid and butyric acid [23]. Meanwhile, some genes involved in the degradation of polysaccharides which human beings do not possess, were discovered in *Bacteroides* [24]. Thus, *Bacteroides* plays a very important role in the metabolism of polysaccharides in the gut. Bands C7 and C9 belong to *Proteobacteria*. They might be responsible for the partial utilization of sulfate present in the sample, as they are closely related to sulfide-degrading bacteria [25–27].

Table 3
Amount of individual SCFA production out of the total production of SCFAs in 3rd subcultures initially inoculated with adult human fecal microbiota.

Samples	Amounts (mM) of SCFAs present at time (h)											
	Actate		Propionate		Isobutyrate		Butyrate		Isovalerate		Valerate	
	48	72	48	72	48	72	48	72	48	72	48	72
SB[a]	9.35 ± 1.23[b]	19.62 ± 2.24	5.65 ± 1.06	9.03 ± 1.47	0.25 ± 0.04	1.67 ± 0.30	11.62 ± 1.32	13.82 ± 2.13	1.61 ± 0.35	4.34 ± 0.36	5.12 ± 1.12	6.76 ± 1.33
SC	21.52 ± 3.26	38.13 ± 3.68	21.54 ± 4.29	30.03 ± 3.46	4.52 ± 0.43	8.57 ± 0.33	21.31 ± 3.35	30.45 ± 4.36	9.36 ± 1.38	17.75 ± 2.35	9.77 ± 1.41	28.25 ± 4.71
SF	9.57 ± 1.35	11.00 ± 1.75	5.32 ± 0.27	11.43 ± 2.18	0.15 ± 0.02	1.85 ± 0.06	11.95 ± 2.81	14.20 ± 3.14	0.55 ± 0.06	3.83 ± 0.47	3.24 ± 0.39	2.84 ± 0.26
MIX	12.25 ± 2.79	23.50 ± 2.26	12.77 ± 1.44	26.79 ± 3.29	0.70 ± 0.31	3.63 ± 0.72	14.42 ± 2.35	30.09 ± 4.15	2.73 ± 0.31	7.70 ± 1.08	5.71 ± 0.40	11.56 ± 2.41

[a] SB – Subject B, SC – Subject C, SF – Subject F, MIX – Mixed fermentation.
[b] Data are means and SD, n = 6.

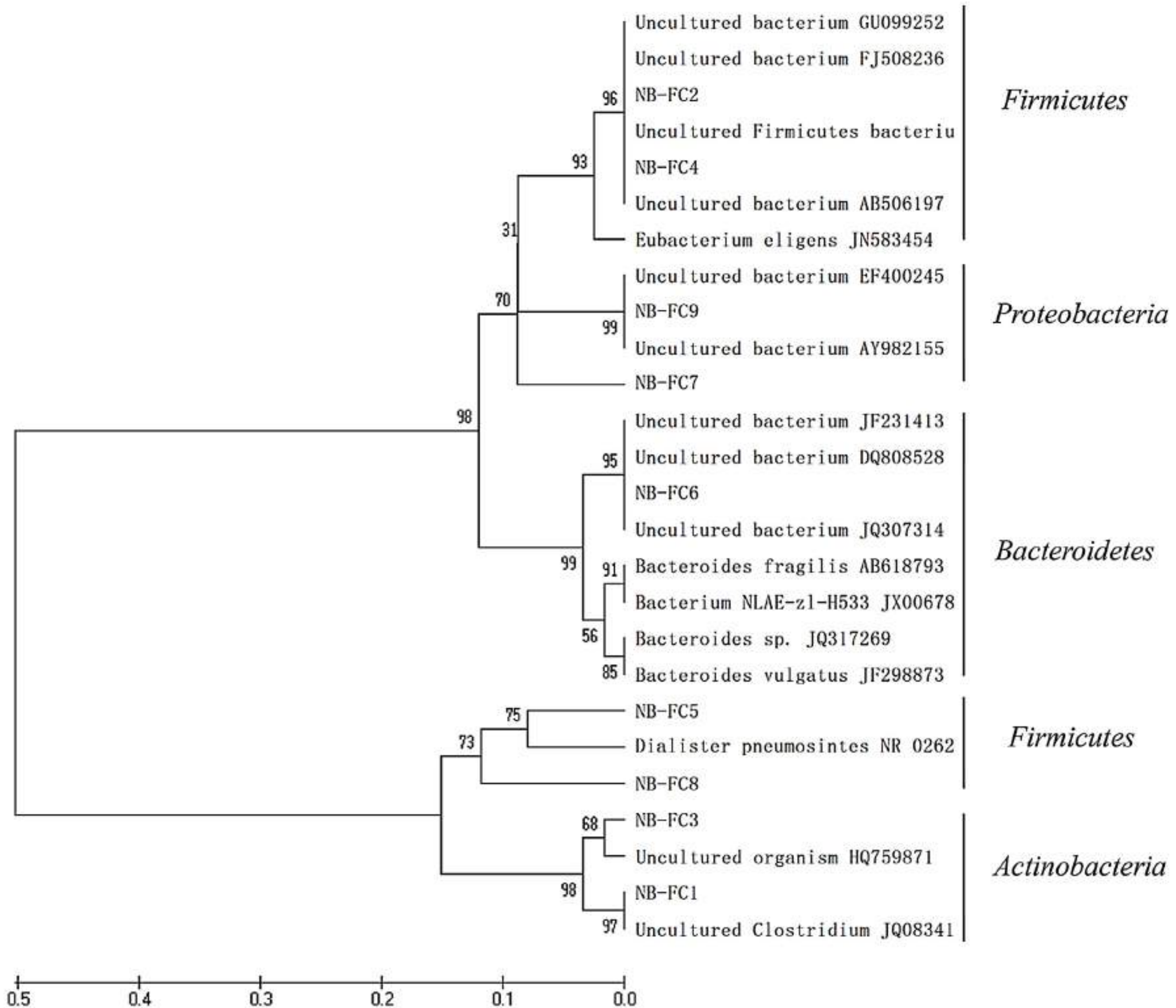

Fig. 3. Rooted phylogenetic tree of the clones from selected DGGE DNA bands. A neighbor joining analysis with the correction of Jukes and Cantor was performed on unambiguous base positions. 160 base pairs were taken into account for phylogenetic tree construction. Only Bootstrap values above 75% are shown.

Real time quantitative PCR was performed to analyze the proportion of various bacteria and changes in each sample to further verify our results. Strains in sample MIX–48 h (cultured for 48 h) included *Clostridium cluster* XI, *Bacteriodes prevotella* group, *Bifidobacterium genus*, *Clostridium cluster* I, *Clostridium cluster* XIVab, *Enterobacteriaceae* and *Lactobacillus* group (Fig. 4). The proportions of *Clostridium cluster* XI, *Bifidobacterium genus*, *Clostridium cluster* I and *Clostridium cluster* XIVab were increased significantly in MIX–72 h (cultured for 72 h), while the proportions of *Bacteriodes prevotella* group had a little raise, and the remaining strains decreased. The proportions of microflora in sample SB, SC, SF and MIX (cultured for 72 h) showed similar features in comparison to those cultured for 48 h, that is *Bifidobacterium*, *Bacteriodes prevotella* group (except for sample SB) and *Clostridium* exhibited a varied extent of increase, and the *Enterobacteriaceae* and *Lactobacillus* group decreased. Therefore, the *Bifidobacterium*, *Bacteriodes prevotella* and the three species of *Clostridium* appear to involve in the metabolism of FCS-*pg*.

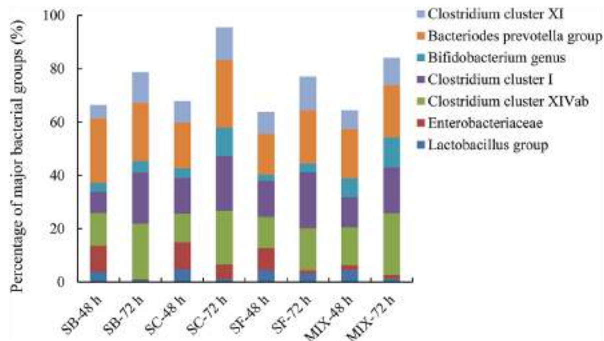

Fig. 4. The major bacterial community in fermentation samples with FCS-*pg* by real time qPCR. Bacteria samples came from Subject B (SB), Subject C (SC), Subject F (SF), and Mixed fermentation (MIX).

4. Discussion

It has been reported that FCS-*pg* possesses anticoagulant, antithrombotic and anti-hyperlipidemic activities after oral administration in animals [1,5,7]. Thus, an investigation of the fermentation of FCS-*pg* was undertaken using human intestinal microbiota in a simulated *in vitro* digestion model to understand fermentation behavior and biological role of FCS-*pg* in the gastrointestinal tract. Human intestinal microflora consists of a highly complicated and diversified microbial community and many non-digestible carbohydrates are fermented by the bacteria in our gastrointestinal tract [8]. The human intestinal microbiome is involved in various physiological functions and is essential for human health [20,28].

The present data indicates that FCS-*pg* is partially degraded by the intestinal flora of individuals, including SB, SC, SF and MIX (Table 2). These results are similar to the finding of Hehemann et al. that demonstrated fucoidan sulfate from seaweed can be fermented by the intestinal flora found in a portion of the population [9]. According to the analysis of bacterial community (Figs. 2–4), The proportions of *Clostridium cluster* XI, *Clostridium cluster* I, *Clostridium cluster* XIVab, *Bifidobacterium genus* and *Bacteriodes prevotella* group were significantly increased, indicating that *Bifidobacterium*, *Bacteriodes prevotella* and the three species of *Clostridium* may be involved in the metabolism of FCS-*pg*.

However, the utilization of FCS-*pg* by gut flora occurred at various rates among individuals. The causes of different findings, among SB, SC, SF and MIX, might be the result of the percentage of bacterial groups that can degrade FCS-*pg* (called targeted bacterial groups). While the composition of the human gut microbiota is generally similar between healthy individuals on the broad scale, there are individual differences at a species and strain level, and these result in metabolic capabilities of the microbiota can vary among people [29]. As shown in Fig. 4, the percentages of major bacterial groups among SB, SC, SF and MIX were roughly 65% at 48 h culturing, while these were diversified at 72 h culturing. Thereafter, the percentages of targeted bacterial groups, such as *Bifidobacterium*, *Bacteriodes prevotella* and the three species of *Clostridium* were absolutely dominant. Higher amounts of targeted bacterial groups resulted in better efficiency in the fermentation and SCFA production (Tables 2 and 3).

Probiotics are important live microorganisms in the intestinal microflora, which when colonized in adequate amounts confer a health benefit to the host. Most potential applications of probiotic species are attributable to *Lactobacillus* and *Bifidobacterium* [30]. Recently, it was shown that dietary fiber (inulin, resistant starch, galactooligosaccharides, *etc.*) and polysaccharides (β-glucans, arabinans, *etc.*) with prebiotic potential are capable of stimulating the growth of *Bifidobacteria* or *Lactobacillus*, balancing the colonic bacteria in the gut and having a positive effect on gut health by exhibiting antimicrobial activities that contribute to the gastrointestinal tract defense system of the host [31–33]. Analysis of intestinal microflora grown on FCS-*pg* has shown that the total count for some species of non-probiotics (*Clostridium* and *Bacteroidetes*) increased significantly (Fig. 4). In addition, the ratio of *Bifidobacterium genus* in 72 h sample was clearly increased when compared with the 48 h sample (Fig. 4). These results were slightly different those of certain reports that suggest that most sulfated marine polysaccharides were primarily fermented by non-probiotic intestinal bacteria (*Bacteroides* and *Clostridium*) in the intestinal tract without increasing probiotic bacteria [25–27,34].

Interestingly, the rising percentages of *Bifidobacterium* among SB (32.14%), SC (200.28%), SF (36.20%) and MIX (60.88%) were diverse over 48–72 h of culturing (Fig. 4). Different *Bifidobacterium* strains have varying ability to grow on fructo-oligosaccharides and inulin, for example, while all of the fifty-five *Bifidobacterium* strains tested could grow on fructo-oligosaccharides only eight strains grew on inulin [35]. This suggests that oligosaccharides are better utilized by *Bifidobacterium* strains. Therefore, a cross-feeding effect [36] from primary FCS-*pg* degraders (targeted bacterial groups) perhaps affords a certain contribution to different rising percentage of *Bifidobacterium* among individuals. FCS-*pg* can increase the content of probiotics to a certain extent and exert a probiotic-enhancing function. This suggests that the FCS-*pg* contributes to human health after oral administration.

SCFAs are fermentation products of carbohydrates, absorbed by the colonic epithelium or metabolized by other colonic bacteria, account for 90–95% of the products [37], play an important role in gastrointestinal tract and are potentially an important component of metabolic health [21]. Acetate, propionate and butyrate are the major products of microbial metabolism (Table 3). Acetic acid is the major metabolite of most bacteria in the body, most of it is absorbed into the blood, transferred to the liver for metabolism, and serve to provide energy for surrounding tissues [38]. Butyric acid is the main product of the *Firmicutes* genera, and can be absorbed by the colonic epithelial cells, and is a preferred energy source for the colon and cecum [39]. Propionic acid is metabolic product of *Bacteroidetes* genera is primarily metabolized in the liver and serves as an energy source after it is absorbed by the colon, Propionic acid can inhibit cholesterol synthesis in the liver and promote redistribution of cholesterol in plasma and liver, reducing the concentration of plasma cholesterol [40]. Moreover, propionate inhibits lipogenesis enzymes, which impact lipid levels [41]. This activity may be responsible, in part, for the functional benefits of FCS-*pg* after oral administration.

Moreover, numerous studies have suggested that some genera of bacteria in gut have the ability to produce harmful enzymes (eg, urease, β-glucuronidase and azoreductase), and potentially toxic compounds (*e.g.*, ammonia) in the intestinal tract [42–44]. In the present study, the SCFA fermentation products of FCS-*pg* result in a lower pH environment (Table 2), which plays a role in inhibiting the growth of pathogenic bacteria and influencing harmful enzyme activities in the human intestine, reducing the intestinal absorption of potentially toxic compounds. Furthermore, this low pH may also contribute to SCFA absorption [45].

Intestinal glycolysis research on polyphenols [46] demonstrate that after degradation of intestinal microflora, polyphenols that original had been considered to have a low bioavailability are metabolized and absorbed through the human gastrointestinal tract into the body to exert anti-oxidant activity in blood lipid regulation. It is noteworthy that polyphenolic bioactivities are not just dependent on changes in the intestinal microflora community, but also on their metabolic reabsorption. Research on intestinal absorption using *in vitro* models show that seaweed-derived

fucoidan sulfates can enter into the bodies' metabolic system after their intestinal fermentation [47]. Therefore, we suggest that after oral administration, the fermentation products of FCS-*pg* may be absorbed and enter the metabolic circulation, exerting their biological effect.

5. Conclusion

We investigated the internal digestibility of FCS-*pg* and its true prebiotic effects in the intestinal tract *in vitro* digestion model using human fecal samples. Results revealed that FCS-*pg* was partially degraded by the intestinal flora from human fecal samples with quite different utilization efficiency. The production of SCFAs was significantly affected by FCS-*pg*. Furthermore, analysis of bacterial community revealed that the proportions of *Bacteroides, Bifidobacterium* and *Clostridium* increased significantly during the period of culturing with FCS-*pg* as sole carbon source. These results indicate that FCS-*pg* is mainly fermented by *Bacteroides*, *Bifidobacterium* and *Clostridium*, resulting in significant increases of their proportions and the content of their metabolites in the intestinal tract. Since there was observed increase in content of probiotic bacteria, associated with health enhancing effects, the probiotic theory of intestinal microflora was suited to explain the health impact of orally administered FCS-*pg*. However, the mechanism of absorption and functional activity on FCS-*pg* within the gastrointestinal tract of the human body requires further study.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.ijbiomac.2017.04.036.

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