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Full Length Research Paper

Characterization and fermentability of sucrose thermolysis oligosaccharides caramel by human intestinal microbiota

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Sucrose thermolysis oligosaccharides caramel (STOC) was prepared by thermal treatment (160°C) under vacuum of acidified sucrose using citric acid as caramelization catalyst. Total carbohydrate content was 59.95% in STOC, which was constituted by kestose, mono- and di-oligosaccharide. In addition to carbohydrates, STOC also contained structurally unknown melanoidins. STOC was further subjected to *in vitro* fermentation with human fecal microbiota. After 24 h of incubation with human fecal suspension, STOC was extensively degraded (84.3%). Moreover, the results of total cell counts, major bacterial groups and short-chain fatty acid analysis indicated that STOC were the preferred substrates for human fecal microbiota. Significant increases after 24 h occurred in the total bacterial cells, *Bifidobacteria* and *Bacteroides-Prevotella* group in the presence of STOC ($p<0.01$). The increase in amounts of acetic and propionic acid was consistent with the role of these bacteria in the degradation of STOC.

Key words: Caramelization, oligosaccharide, intestinal microbiota, short-chain fatty acid.

INTRODUCTION

Caramelization was one of the important reactions in producing caramel which had undergone a thermal process (Kitts et al., 2006, Tsai et al., 2009). When crystalline sucrose was heated at 160°C, or above, and in the absence of amino acids or peptides, brown pigments collectively referred to as caramel were formed (Defaye and García, 1994). These brown pigments resulted from a series of chemical reactions that included hydrolysis, dehydration and polymerization, collectively referred to as caramelization. The caramelization depended strongly on many factors including temperature, time, pH, reactant concentrations and nature of reactants (type of sugar, type of amino acid or protein). The controlled thermal treatment of sucrose, preferably with an acidic catalyst such as acetic or citric acid, was a manufacturing process used in the large-scale production of caramel. Sucrose thermal oligosaccharides caramel (STOC) was the ethanol-soluble fraction containing di-D-fructose dianhydrides, oligosaccharides and monosaccharides. Some di-D-fructose dianhydrides or a caramel with high content of difructose dianhydrides were previously identified and further raised an intense research on their prebiotics, promoting the proliferation of *Bifidobacteria* and mineral absorption in the large intestine of the animals (Manley-Harris and Richards, 1996; Al-Rawashdeh et al., 2000; Minamida et al., 2006; Arribas et al., 2010).

The human large intestine was a complex ecosystem inhabiting a vast range of microbial population (Eckburg et al., 2005; Egert et al., 2006), which was one of the most densely populated microbial ecosystem on earth. A large microbial population was present in the human colon at a level of 10^{13} to 10^{14} colony-forming units (cfu)/g

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wet weight, with more than 50 genera and over 400 species of bacteria being identified in human feces (Gill et al., 2006; Wong et al., 2006). The vast majority of these microbes (10 to 100 trillion) inhabited our gastrointestinal tract, with the greatest number residing in the distal gut, where they synthesized essential amino acids and vitamins and transformed components of otherwise indigestible contributions to our diet such as plant polysaccharides (Gill et al., 2006) and *Firmicutes*, *Bacteroidetes* and *Actinobacteria* were the most widely noticed representatives. It had been recognized that endogenous gastrointestinal microbiota played a fundamentally important role in health and disease, and associated with absorption of mineral (Andrieux et al., 1980), obesity (Ley et al., 2006) and inflammatory diseases (Marsland, 2012). However, due to the sensitivity of cultivation, the *in situ* functions of distinct groups of the intestinal microbiota were largely unknown. Fecal samples were often used in *in vitro* fermentation model to investigate the intestinal microflora because they were easily collected, and molecular fingerprinting methods and sequence analysis of 16S ribosomal RNA (rRNA) were often used in researching the functionality of intestinal microflora. Moreover, metagenomic approach was also used to reveal microbial genomic and to identify some of the distinctive functional attributes encoded in our distal gut microbiome (Gill et al., 2006).

Recently, some investigations were performed to produce caramel with high contents of oligosaccharides or difructose dianhydrides and glycosylated derivatives. These caramels also promoted the growth performance of pigs and more favorable intestinal microbial populations (Orban et al., 1997; Arribas et al., 2010; Saminathan et al., 2011), as well as induced higher concentrations of short-chain fatty acid (SCFA), which was the primary energy sources of epithelial cell and regulated inflammation (Marsland, 2012). Some investigations also proved that the intestinal microflora was able to metabolize Maillard reaction or caramelization products, and facilitated the absorption of minerals in the colon to some extent (Andrieux and Sacquet, 1984; Delgado-Andrade et al., 2008; van Boekel et al., 2010). Besides, the vacuum treatment may produce significant benefits for future food manufacturing, such as improvement of product safety and quality, reduction of oil oxidation and toxic compound formation (Granda et al., 2004; Quarta and Anese, 2012). Therefore, STOC was prepared under vacuum conditions. Therefore, the objective of this study was to analyze the characterization and to determine the fermentability of the STOC fraction prepared using dry process under vacuum.

MATERIALS AND METHODS

Chemicals

Sucrose, citric acid and 1,1-diphenyl-2-picryl-hydrazyl (DPPH) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Sodium hydroxide, sodium acetate (NaOAC) and ethanol were chromatographic grade and purchased from Merck (Damstadt, Germany). All other chemicals were analytical grade materials.

Preparation of sucrose thermolysis oligosaccharide caramel (STOC)

Anhydrous, amorphous sucrose (11.88 g) was mixed with 0.12 g anhydrous citric acid, and then ground thoroughly according to the procedure of Manley-Harris and Richards (1991) and Suarez-Pereira et al. (2010) with slight modification. The powders were dissolved in deionized water (30 mL), which was then mostly removed (85-88%) at < 40°C under 0.1 MPa. The samples were heated in a thermostated oven connected to a vacuum pump (-0.1 MPa) at 160°C for 1 h. Upon removal from the oven, the mixture solidified almost immediately to give a walnut-colored glass, which cracked upon standing. The aqueous ammonia (28 mL, 0.1 M) was added drop wise to dissolve the glassy product and the ethanol also added drop wise with vigorous stirring to 95% concentration. The resulting solution was refrigerated overnight before centrifuging (9000 rpm for 10 min, 4°C). The supernatant was concentrated under reduced pressure and dried in a vacuum (<40°C) to a yellowish-brown STOC.

Fecal samples and culture conditions

Fecal samples were collected from three healthy volunteers (one female, two males, aged 22 to 35 years) who had no previous history of gastrointestinal disorders and had not been taking antibiotics for at least 3 months prior to the study. Freshly voided feces were immediately homogenized for 3 min with 10-fold (w/v) of phosphate-buffered saline (PBS, 8 g/L NaCl, 0.2 g/L KCl, 1.15 g/L Na_2HPO_4 and 0.2 g/L KH_2PO_4, pH 7.3) and centrifuged (1 min, 300 g) to remove large particles. The phosphate buffer solution was pre-boiled and then cooled under a stream of oxygen-free nitrogen and kept at 37°C.

Batch culture *in vitro*-fermentation was carried out in 250 mL glass vessels (100 mL liquid)/16 mL glass tubes (9 mL basal liquid medium) under anaerobic conditions (80% nitrogen, 10% carbon dioxide and 10% hydrogen at 37°C), according to the described procedures with some modification (Ames et al., 1999; Borrelli and Fogliano, 2005; Sanz et al., 2005; Gniechwitz et al., 2007). From the fecal homogenate, 20 mL aliquots of inoculums as a bacterial source were transferred to 250 mL glass bottles containing either 20 mL of phosphate buffer alone (blank) or phosphate buffer with 400 mg (80 mg) of sample. The final concentrations of sample solution and fecal suspension were 10 g/L and 10 g feces/L, separately. One sample was prepared without any carbohydrate addition as a negative control. As positive control, 400 mg (80 mg) glucose was dissolved in anaerobic phosphate buffer and then 20 mL fecal slurries prepared above were added. Each fermentation experiment was carried out in triplicate and incubated at 37°C. Samples were removed after 0, 6, 12, 18 and 24 h of fermentation, and stopped fermentation by placing the suspension on ice. Each sample was transferred to 50 mL tubes and centrifuged (4200 g, 4°C) for 30 min. The fermentation supernatants, sterilized by filtration (pore size 0.22 μm), were divided into aliquots and stored at -20°C until use. The fermentation experiments were performed in triplicate. All additions, inoculations and incubations were carried out inside an anaerobic cabinet. After fermentation, an aliquot (100 μl) of bacterial cultures was transferred to 96-well microtiter plates. Cell growth was evaluated by changes in the optical density (OD) of the bacterial cultures at λ_{630nm} by a microtiter plate reader (MK3, Ladsystems Inc., Finland).

Table 1. 16S rRNA targeting oligonucleotide probes used for FISH.

Probe	Sequence (5'-3')	Target organism
Bif 164	CATCCGGCATTACCACCC	*Bifidobacteria*
Bac 303	CCAATGTGGGGGACCTT	*Bacteroides-Prevotella* group
Erec 482	GCTTCTTAGTCARGTACCG	*Clostridium coccoides-Eubacterium rectal* group

Fluorescence *in situ* hybridization (FISH) and enumeration of bacterial cells

The oligonucleotide probes used in this study are listed in Table 1 (Reichardt et al., 2009). Fecal batch culture aliquots (1 mL) were centrifuged (5 min, 4°C, 12000 g). The remaining pellet was washed twice and resuspended in 0.5 mL of filtered (polyvinylidene difluoride filter; pore size, 0.22 μm) phosphate-buffered saline, and added to ice-cold 4% paraformaldehyde (pH 7.2) at a ratio of 1:3 (vol:vol) in a Eppendorf tube and were stored at 4°C for 4 h. Microbial populations were enumerated using fluorescently labeled 16S rRNA-targeted oligonucleotide probes and fluorescent *in situ* hybridization. Slides were enumerated using a Nikon (Tokyo, Japan) microscope (×1,000) fitted with an epifluorescence attachment; 15 randomized views were counted for each sample.

The hybridization was carried out as previously described by Connolly et al. (2012) using genus- and group-specific 16S rRNA gene-targeted oligonucleotide probes labeled with Cy3 (Sigma-Aldrich, St. Louis, MO, USA) or the nucleic acid stain 4',6-diamidino-2-phenylindole for total cell counts. A 20 μL volume of each sample was pipetted onto Teflon coated, 6-well (each 10 mm in diameter) slides. Samples were dried onto the slides at 46°C for 15 min and afterwards dehydrated in an alcohol series (50, 80 and 96%, 3 min each). The ethanol was allowed to evaporate from the slides before the probes were applied to the samples. A probe/hybridization buffer mixture (5 μL of a 50 ng/μL stock of probe plus 45 μL of hybridization buffer) was applied to the surface of each well. Hybridization was performed for 4 h at 46 or 50°C (depending on the probe used) in an ISO20 oven (Grant Boekel, Cambridge). Slides were stored in the dark at 4°C (for a maximum of 3 days) until cells were counted. Slides were enumerated using a Nikon E400 Eclipse microscope fitted with an epifluorescence attachment; the fluorescence filter set 15 (excitation: 546 nm; emission: 590 nm) and 15 randomized views were counted for each sample.

Analysis of carbohydrate

Aliquots of sample solutions were appropriately diluted with deionized water, and then passed through a 0.22 μm nylon filter, and analyzed by high performance anion-exchange chromatography with pulsed amperometric detection (HPAEC-PAD) (ICS-5000, Dionex, Sunnyvale, CA). The system was composed of an autosampler, a gradient pump with on-line degassing, and an electrochemical detector. A three-eluent system comprising deionized water, a 500 mM sodium hydroxide (NaOH) aqueous solution and a 500 mM sodium acetate (NaOAC) aqueous solution was used. Separation was accomplished on a CarboPac PA1 anion exchange column (250 × 4 mm, Dionex) and a CarboPac PA1 guard column (50 × 4 mm, Dionex) using the isocratic elution with 25 mM NaOAC for 5 min followed by a linear gradient from 25 to 400 mM NaOAC for 20 min and then isocratically with 400 mM NaOAC for 10 min (Davidek et al., 2003). The NaOH concentration was maintained at 100 mM throughout the run. The eluent flow was always kept at 1.0 ml/min throughout the program. Quantification was carried out using external monosaccharides standards: D-fructose, D-glucose, D-xylose and D-arabinose or sucrose, trimers of fructo-oligosaccharides. The injection volume was 20 μL and the system was maintained at 25°C. Residual total carbohydrate in the samples was determined by the phenol-sulfuric acid method for total sugar (Dubois et al., 1956).

Quantification of SCFA

Samples were centrifuged at 12000 rpm for 15 min, and 20 μL was injected onto the 1100 HPLC system (Agilent, Waldbronn, Germany) equipped with UV detector. The column was an ion-exclusion Aminex HPX-87H (300×7.8 mm, Bio-Rad) maintained at 50°C. The eluent was 0.005 mM sulfuric acid in HPLC grade water, and the flow rate was 0.6 ml/min. Detection was performed at 210 nm, and data were acquired using Chem Station for LC3D software (Agilent Technologies). Quantification of the samples was carried out using calibration curves of acetic, propionic, butyric and lactic acids in concentrations between 0.5 and 100 mM.

Spectroscopic analysis and color intensity EBC units determination

The absorption spectra of sample solutions were recorded in the range of 200 to 800 nm, after a suitable dilution, in 10 mm pathlength cells, using a TU-1901 spectrophotometer (Beijing Purkinje General Instrument Co. Ltd., China). Color intensity was calculated by the Chinese National Procedure GB 8817-2001.

Statistical analysis

Data were presented as means ± SD for three replicate measurements. The statistic significance of differences among groups was evaluated by one-way ANOVA using SPSS 17.0 program (SPSS 17.0 for windows, SPSS Inc, Chicago, IL). The differences between the means were assessed using Duncan's multiple-range test and significance was identified with a value of $p < 0.05$.

RESULTS AND DISCUSSION

Adsorption spectra and carbohydrate composition of STOC sample

Adsorption spectra of STOC solution (1‰) were recorded (Figure 1a) and there were two absorption maxima at 282 and 225 nm, respectively. The wavelength selected for measuring caramel was often chosen at 510 and 610 nm marked as red index (8.158) and yellow index (9.116). The color intensity was also calculated by the absorption at 610 nm, which was 15000 EBC. The STOC sample

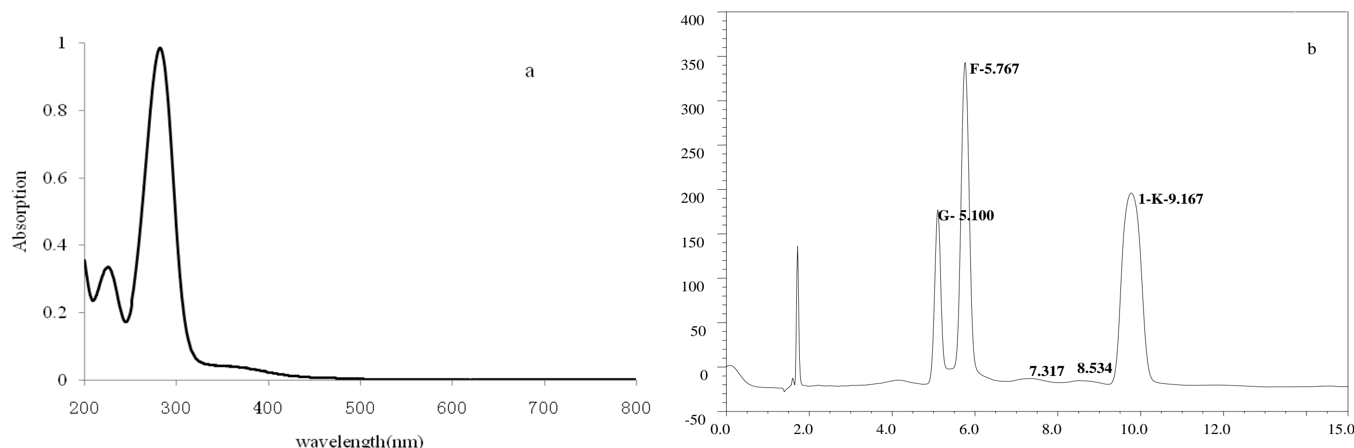

Figure 1. Absorption spectra (a) and carbohydrate analysis chromatogram (b) of STOC, G-glucose, F-frucose and 1-K-kestose.

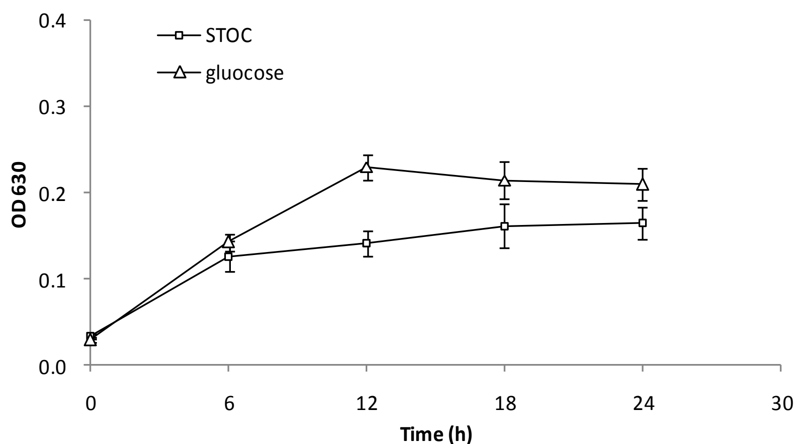

Figure 2. Growth curve of human fecal microbiota in basal medium supplemented with glucose or STOC assessed by optical density.

possessed some caramel characteristics due to the presence of melanoidins. The carbohydrate content of STOC was analyzed by using phenol-sulfuric acid method, and the content was 59.95 ± 1.82%. On the other hand, the carbohydrate composition of STOC was investigated using HPAEC-PAD, and the results are shown in Figure 1b. The carbohydrate constitute of STOC was 47.37% kestose, 14.74% glucose and 35.67% fructose. The constituent of STOC was consistent with that of di-D-fructose dianhydride-enriched caramel containing some D-fructose and higher fructooligosaccharides (Suarez-Pereira et al., 2010).

Fermentability of the STOC fraction by human fecal microbiota

The fermentability of STOC fraction was investigated by *in vitro* fermentation experiments with human fecal microbiota. The increment of OD during fermentation indicated that STOC supported the growth of fecal microbiota *in vitro* as the only carbon sources (Figure 2). There was no significant difference in the growth rate of human fecal microbiota at initiatial 6 h ($p > 0.05$), which was attributed to the presence of glucose in STOC. After disappearance of available glucose in STOC, oligosaccharides disappearance was observed during secondary logarithmic growth with the reduced growth rate. The OD of the fecal bacteria suspensions supplemented with glucose was maximal at 12 h, however, the OD of the fecal bacteria supplemented with STOC increased more slowly during 24 h without reaching a maximum. Additionally, the degradation of total carbohydrate was consistent with the OD of fecal microbiota (Table 2), and the STOC was slowly utilized by fecal microbiota.

Table 2. Degradation of carbohydrates during fermentation of the STOC and glucose as control.

Substrate	Fermentation time (hours)	Total carbohydrate degradation (%)
Glucose	0	0
	6	42.6 ± 0.2
	12	64.4 ± 0.9
	18	69.4 ± 0.8
	24	71.5 ± 0.4
STOC	0	0
	6	18.9 ± 2.0
	12	30.0 ± 3.5
	18	43.1 ± 3.2
	24	49.3 ± 1.5

Values given as mean ± SD.

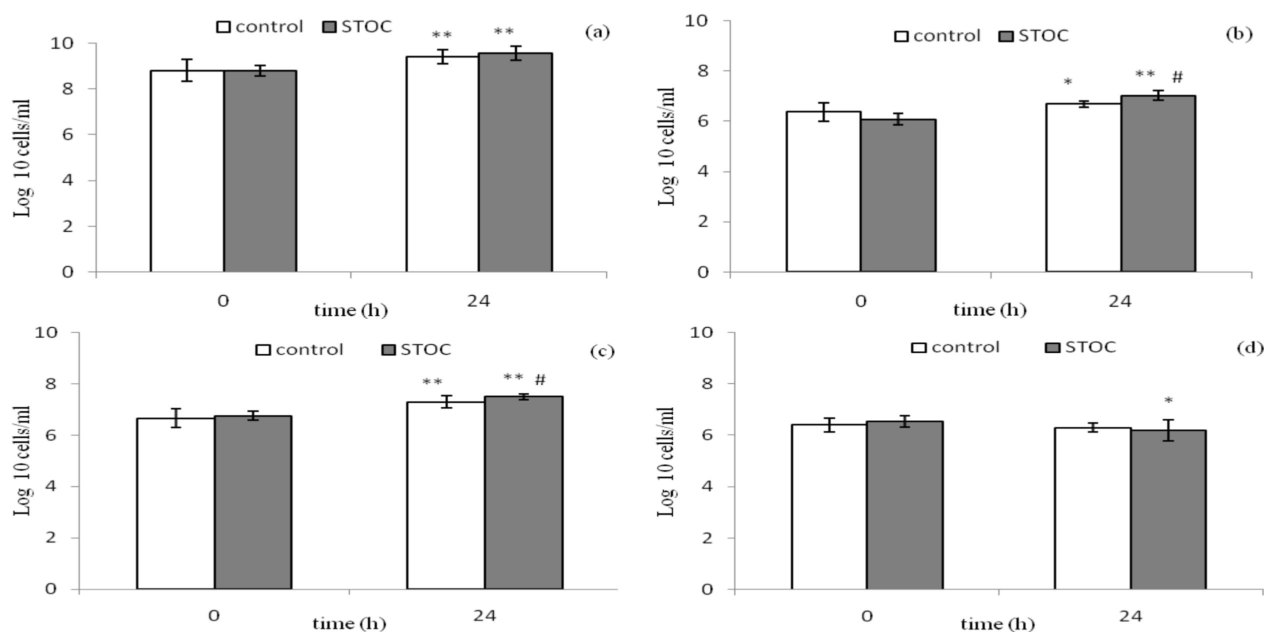

Figure 3. Changes of different bacteria after 24 h fermentation of the STOC and glucose as control. Asterisks indicate a significant difference between bacterial counts in the original community and in the fermented samples: *, $p < 0.05$; **, $p < 0.01$. Pound signs indicate a significant difference between bacterial counts after fermentation of STOC and after fermentation of glucose: #, $p<0.05$. (a) total anaerobes; (b) *Bifidobacteria*; (c) *Bacteroides-Prevotella* group; (d) *Eubacterium rectale-Clostridium coccoides* group.

These results indicated that oligosaccharides and melanoidins or Amadori compounds in STOC were able to serve as a carbon source for human fecal microbiota, which were demonstrated by *in vitro* or *in vivo* model that melanoidins and Amadori compounds were metabolized and played an important role in digestive physiology (Ames et al., 1999; Erbersdobler and Faist, 2001; Tuohy et al., 2006; Deppe et al., 2011). The slower increase in OD of the STOC reflected the complex characterizations of STOC fractions which were more difficult to break down and similar to those of lactose or other poorly digestible carbohydrates.

Growth of human fecal microbiota and SCFA production

FISH approach was precise and stable than the more frequently used plate culture technique, and carried out to monitor changes in the proportion of dominant bacterial groups during *in vitro* fermentation. Changes in bacterial populations after 0 and 24 h of *in vitro* fermentation with the different test substrates are shown in Figure 3. It could be seen that the numbers of total bacteria significantly increased in all fermentation experiments ($p<0.01$), with numbers increasing from 8.81 to 9.41

Table 3. SCFA production in *in vitro* fermentation at 0, 12 and 24 h.

Substrate, time (h)	Concentration (mmol/L)			
	Total SCFA	Acetic acid	Propionic acid	Butyric acid
Glucose				
0	1.1±0.1[a]	0.8±0.1[a]	0.2±0.0[a]	0.1±0.0[a]
12	24.7±1.1[b]	15.7±0.9[b]	5.7±0.3[b]	3.3±0.4[b]
24	31.0±2.0[c]	21.3±1.7[c]	6.0±0.7[b]	3.7±0.6[b]
STOC				
0	1.2±0.1[a]	0.9±0.1[a]	0.2±0.0[a]	0.1±0.0[a]
12	30.1±0.9[b]*	24.6±0.8[b]*	1.2±0.1[b]*	4.3±0.1[b]
24	43.6±3.6[c]	31.2±4.5[c]*	7.1±0.4[c]	5.3±0.7[b]

Values given as mean ± SD; Different letters indicate significant difference ($p<0.05$) for each substrate and different fermentation time; Paired t test was used to determine a significant increase in SCFA concentration of different substrates, *, $p<0.05$.

$\log_{10}$ cells/mL for glucose, and for STOC from 8.80 to 9.56 $\log_{10}$ cells/mL, while the numbers of total bacteria were similar in different substrate ($p>0.05$). Significant increases after 24 h occurred in the *Bifidobacteria* in the presence of STOC ($p<0.01$), with numbers increasing from 6.1 to 7.0 $\log_{10}$ cells/mL, while there was slight significant increase of *Bifidobacteria* in the glucose fermentation ($p<0.05$). The cause of difference possibly lies in the slower degradation of STOC. In contrast, STOC and glucose fermentations resulted in no significant increases in the *Eubacterium rectale*-*Clostridium coccoides* group after 24 h, and the proportion of members of the *E. rectale*-*C. coccoides* group was fairly stable over the incubation (Figure 3d). Nevertheless, *Bacteroides*-*Prevotella* group increased significantly ($p<0.01$) for all tested substrates, with numbers increasing from 6.66 to 7.3 $\log_{10}$ cells/mL for glucose, and for STOC from 6.76 to 7.5 $\log_{10}$ cells/mL. The *Bacteroides*-*Prevotella* group was possibly capable of partly utilizing STOC as a substrate. *Bacteroides* species are well known for their ability to degrade various carbohydrates because they possess a wide range of carbohydrate-depolymerizing enzymes (Gniechwitz et al., 2008; Reichardt et al., 2009).

Change in SCFA concentrations after 0, 12 and 24 h of *in vitro* fermentation with the different tested substrates are shown in Table 3. All substrates had a significant increase in total (acetic acid, pronionic acid and butyric acid combined) SCFA concentration over the fermentation period by the fecal microbiota. Acetic acid was the dominant SCFA produced in all fermentation experiments, and a significant difference was observed when STOC was used as substrate when compared with the glucose group ($p<0.05$). Propionic acid and butyric acid increased significantly after *in vitro* fermentation for both STOC and glucose, whereas only propionic acid resulted in a significant difference after 12 h in different substrate. Also, a better indicator was given by the ratio of the individual SCFA to total acid (Figure 4). There were no significant differences in the proportion of butyratic acid in different substrates. A significant difference was observed when the larger proportion of acetic acid after 12 h of STOC fermentation was compared with lower values after glucose fermentation ($p<0.05$). The ratio of propionic acid after 12 h glucose fermentation was significantly greater than that after 12 h STOC fermentation ($p<0.01$). Acetate was the most abundant SCFA produced by our gut microbiota, and propionate was produced as a metabolite of a complex mixed microbial community, such as *Bacteroides*, *Prevotella*,

Propionibacterium, etc (Hosseini et al., 2011). The decrease of propionic acid was due to an increase in other types of SCFA after 12-h fermentation; moreover, the OD of the fecal bacteria suspensions supplemented with glucose was maximal at 12 h, while the STOC group slowly increased. And the relatively more acetate was produced, so the ratio of propionic acid to total SCFA was much lower in STOC substrate fermentation.

SCFA are fermentation end-products of bacterial carbohydrate fermentation in the colon, in particular, n-butyrate is generally considered to be a desirable metabolite of gut bacterial function, and some *in vitro* fermentation experiments have demonstrated that butyrate is metabolized by the colonic epithelium and modulates inflammation or intestinal immune function of host (Scheppach and Weiler, 2004; Tuohy et al., 2006; Vinolo et al., 2011). Furthermore, propionate and butyrate were often produced in equal concentrations, the concentration of propionate increased or decreased, the butyrate concentration reflected the variation, either increasing or decreasing. The fructo-oligosaccharides, the dimerization reaction product of D-fructose moiety, was not neglected or discarded in caramel industry. All results showed that the STOC seem to selectively

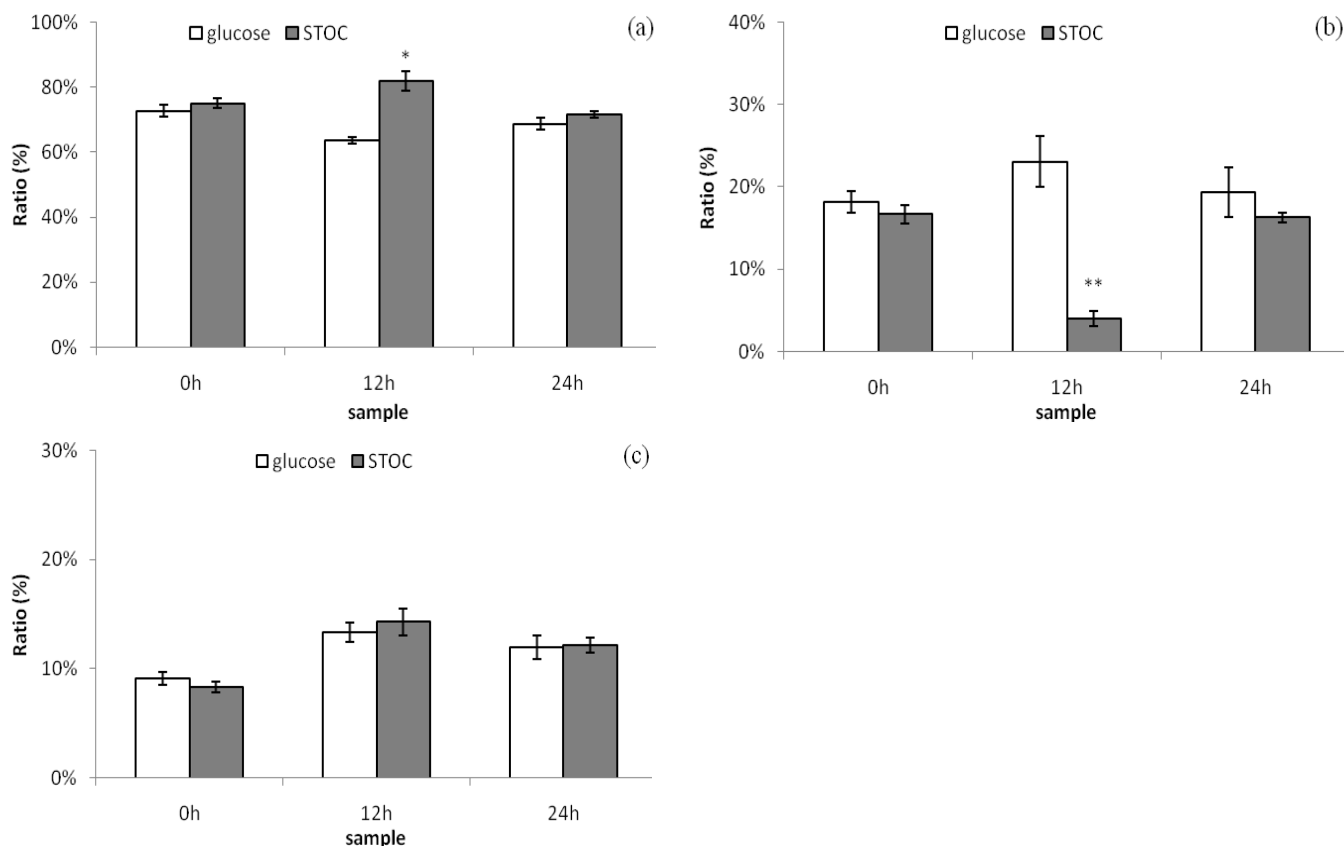

Figure 4. Ratio of SCFA in *in vitro* fermentation at 0, 12 and 24 h using the STOC and glucose as control. Asterisks indicated a significant difference between the different substrates and the time points: *, $p < 0.05$; **, $p < 0.01$. (a) acetic acid; (b) propionic acid; (c) butyric acid.

enhance the growth of beneficial bacteria. This was consistent with the study where STOC was shown to increase the populations of bifidobacteria in poultry (Arribas et al., 2010; Orban et al., 1997), which was involved in fructo-oligosaccharides, a well-characterized prebiotic. Nevertheless, further studies were needed to confirm the prebiotic activity.

Conclusion

On the basis of these data obtained from this study, STOC was composed of monosaccharidies, dimmers and oligosaccharides, *in vitro* fermentation confirmed that STOC was readily utilized by fecal microtiota, increasing the populations of beneficial microbiota in the colon. However, it is still uncertain whether the observed biological activities are attributed to a particular component or to the synergistic effect. The potential role of oligosaccharide, anhydride or melanoidins also deserves to be investigated. Our laboratory is now employing nanofiltration and activated charcoal adsorption treatment to fraction these components for further studies.

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