Note

The Vitro Fermentation of Six Functional Oligosaccharides by Clostridium butyricum TK2 and Clostridium butyricum CB8

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In vitro fermentation of six functional oligosaccharides including fructooligosaccharides (FOS), galactooligosaccharides (GOS), xylooligosaccharides (XOS), isomaltooligosaccharides (IMOS), raffinose and stachyose was performed by using Clostridium butyricum TK2 and Clostridium butyricum CB8. Among the six oligosaccharides, IMOS revealed the strongest fermentability for both strains, which was supported by the highest viable cells (10.1 ± 1.05 and 9.8 ± 0.41 log (CFU / mL)) and the highest levels of SCFAs or butyrate (11.77 ± 0.37 and 14.47 ± 1.05 mM) from fermentation with this functional oligosaccharide. The fermentability of GOS to two strains was better than raffinose or stachyose. The last groups were FOS and XOS. However, every oligosaccharide showed no significant difference in promoting the growth of both of C. butyricum (p > 0.05), and exerted some differences in the production of SCFAs between two strains. This study provides a rational basis for establishing synbiotics with C. butyricum to improve the gut health.

Keywords: Clostridium butyricum, functional oligosaccharides, synbiotics

Introduction

In the human large intestine, the bacterial population is composed by several genera among which Clostridium butyricum is identified as a butyric acid producing beneficial bacterium and plays a crucial role in host health and maintaining gut function. They can decreases ammonia, amine and benzopyrrole from abnormal fermentation in intestinal tract and inhibit some pathogenic bacteria. The bacterium show remarkable stability but can be modulated by many endogenous and exogenous factors, especially prebiotics. Resistant starch type 3 (RS3, a potential prebiotic) is retrograded starch which is not digested by human starch degrading enzyme, and will thus undergo bacterial degradation in the colon. Current research suggests that C. butyricum have the capability to ferment RS3 with featured regarding short chain fatty acids (SCFA) released in the human colon with potential health implication (Purwani et al., 2011).

Prebiotics are defined as “non-digestible food substances that beneficially affect the host by selectively stimulating the growth and/or activity of a limited number of bacterial species already resident in the colon and thus attempt to improve the host health” (Gibson and Roberfroid, 1995). In brief, prebiotics have better effect on normal intestinal flora. Many prebiotics belong to the group of nondigestible oligosaccharides, which resist digestion and

Abbreviations

IMOS; Isomaltooligosaccharides, GOS; Galactooligosaccharides, FOS; Fructooligosaccharides, XOS; Xyloooligosaccharides

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absorption in the human small intestine and are fermented into SCFA in the large intestine. The SCFA (acetate, propionate and butyrate) have an beneficial influence on colonic health (Mortensen and Clausen, 1996; Scheppach et al., 2001; Williams et al., 2001; Wong et al., 2006; Macfarlane and Macfarlane, 2012). In particular butyrate is the prime energy substrates for the colonic mucosa and has an effect on immune function (Roediger, 1980; Fleming and Floch, 1986; Wong et al., 2006; McOrist et al., 2008; Serpa et al., 2010). Furthermore, in vitro studies as well as animal studies indicate that butyrate may reduce risk factors of gut inflammation (Roediger, 1990; Cummings and Englyst, 1991; Segain et al., 2000; Galvez et al., 2005). It also has been observed to reverse the resistance of colorectal cancer cells to apoptosis (Caderni et al., 2006; Serpa et al., 2005). Therefore, the current study aimed to evaluate and compare the fermentation characteristics of various functional oligosaccharides depending on the growth of two types of C. butyricum.

Materials and Methods

Functional oligosaccharide The functional oligosaccharides used in this study are food material and described in Table 1.

Biochemical characterization Strain TK2 and CB8 used in this study were originally isolated from the soil and human feces, respectively. Fermentation tests of carbohydrate and traditional biochemical tests for the two strains were performed according to Berger’s Manual of Systematic Bacteriology (Sneath et al., 1986).

16S rDNA gene sequencing analysis Strain TK2 and CB8 were identified through 16S ribosomal DNA (rDNA) analysis. The 16S rDNA sequences of the isolated strains were determined by Beijing Liuhe Huada gene technology Co., Ltd. A homology search using the reference strains registered in DDBJ/EMBL/GenBank was performed using NCBI BLAST.

Growth conditions C. butyricum TK2 and C. butyricum CB8 were stored at ~80°C in 20% sterile glycerol. Before use, the strains were propagated twice in 20 mL test tube with 10 mL liquid medium (peptone 20 g/L, glucose 5 g/L, K₂HPO₄ 5 g/L, MnSO₄ 0.2 g/L, MgSO₄ 0.2 g/L, pH 7.4) under anaerobic gas phase (H₂:CO₂:N₂, 10:10:80, v/v/v) at 37°C for 16 h. The cells were then harvested and resuspended in sterilized physiological saline and adjusted to 7.30 log (CFU / mL). The cells suspensions were ready to serve as inoculums for in vitro fermentation.

In vitro fermentation Fermentation studies were carried out in 50 mL bottom flasks with 40 mL fermentation medium (peptone 20 g/L, functional oligosaccharides 5 g/L, K₂HPO₄ 5 g/L, MnSO₄ 0.2 g/L, MgSO₄ 0.2 g/L, pH 7.4). Each flask was sterilized at 115°C for 20 min. Then the cells suspensions of C. butyricum TK2 or C. butyricum CB8 (5%, v/v) were inoculated into the flask. It was then incubated under anaerobic gas phase (H₂:CO₂:N₂, 10:10:80, v/v/v) at 37°C. Fermentation was carried out for 48 h and performed in triplicate.

Viable count method The number of viable cells was determined using the Semi-solid AGAR method. And the counting medium is consisted of the following (in g/L): peptone, 20; glucose, 5; agar, 10 g.

The viable cells were counted as follows. Serial 10-fold dilutions were performed in sterile phosphate-buffered saline. Then 1 mL of appropriate dilutions were injected into anaerobic tubes, which had been added into counting medium (9 mL), and incubated under anaerobic gas phase at 37°C for 36 h.

Preparation of samples Culture medium was centrifuged at 10000 rpm for 10 min and the supernatant remove 2.0 mL, diethyl

<table>
<thead>
<tr>
<th>Table 1.</th>
<th>Various types of functional oligosaccharides used in this study (Sako et al., 1999; Patel and Goyal, 2011).</th>
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<tr>
<td>Type</td>
<td>Nomenclature</td>
</tr>
<tr>
<td>Fructooligosaccharides</td>
<td>FOS</td>
</tr>
<tr>
<td>Galactooligosaccharides</td>
<td>GOS</td>
</tr>
<tr>
<td>Xylooligosaccharides</td>
<td>XOS</td>
</tr>
<tr>
<td>Isomaltooligosaccharides</td>
<td>IMOS</td>
</tr>
<tr>
<td>Raffinose</td>
<td>—</td>
</tr>
<tr>
<td>Stachyose</td>
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</table>
Vitro Fermentation of Functional Oligosaccharides by *Clostridium butyricum*

ether (2 mL) and about 50% H$_2$SO$_4$ (0.4 mL) were then added. The sample was centrifuged at 3000 rpm for 5 min at room temperature after mixed for 45 min with an orbital shaker, and the organic phase was filtered through a 0.22 μm filter into a 1.5 mL eppendorf tube for detecting (Schneider et al., 2006).

**Short chain fatty acid analysis** Samples of 1 µL were injected into a high-resolution gas chromatography (Agilent 7890A GC System), which is equipped with an HP-Innowax 19091 N-213 column (30 m × 0.32 mm × 0.5 µm) and a flame ionization detector. The flow rate of carrier gas was 1.8 mL/min, and the split ratio was 40:1. Injector and detector temperatures were 275°C. The column temperature was held at 90°C for 0.5 min, increased to 110°C at 10°C min$^{-1}$, and then increased to 170°C at 5°C min$^{-1}$ and maintained for 5 min. Concentration of SCFA was determined used external standards.

**Statistical analysis** Data were expressed as the mean values ± standard deviation (SD) for each measurement. The data were also analyzed by one-way analysis of variance (one-way ANOVA). Tukey’s procedure was used for significance of difference (p < 0.05). Analysis was performed with SPSS 16.0 (SPSS, Inc., Chicago, IL).

**Results**

**Description of *C. butyricum* TK2 and *C. butyricum* CB8** Table 2 shows the results of the tests usually applied for characterization of strain TK2 and CB8. And the two strains were identified as *C. butyricum* based on an analysis of 16S rDNA sequences, which revealed 99% and 100% homology, respectively. The 16S rDNA gene sequences of strain TK2 and CB8 have been deposited in GenBank under accession number KJ558432 and KJ558433, respectively.

**Effect of different functional oligosaccharides on the growth of *C. butyricum* TK2 and *C. butyricum* CB8** The capacity for microbial degradation of the functional oligosaccharides during fermentation of *C. butyricum* TK2 and *C. butyricum* CB8 can be indicated by the number of the viable cells (Fig.1). Overall, the six functional oligosaccharides were able to be fermented by both of *C. butyricum* and every oligosaccharide exerted no significant difference on the propagation of the two cells from different source (p > 0.05). However, the cell concentrations of *C. butyricum* TK2 or *C. butyricum* CB8 were different depending on the different carbon source. Among the six oligosaccharides, the highest number of *C. butyricum* TK2 and *C. butyricum* CB8 were noticed in the group of fermenting with IMOS, 10.1 ± 1.05 log (CFU / mL) and 9.8 ± 0.41 log (CFU / mL), respectively. GOS resulted in higher populations of the two types of *C. butyricum* than raffinose or stachyose. Fig.1 showed that FOS and XOS caused a much lower concentration than the other oligosaccharides fermented by *C. butyricum* TK2 and *C. butyricum* CB8 (p < 0.05).

**The changes of pH of medium during fermentation** Fig. 2 shows the changes of pH of medium fermented by *C. butyricum* TK2 and *C. butyricum* CB8. The pH of both medium with IMOS decreased more sharply than that with other functional oligosaccharides during the initial 24 h of fermentation, and then tended towards stability. It is founded that IMOS was preferentially utilized by *C. butyricum* used in the study. In addition, the pH of the medium with IMOS, GOS, stachyose and raffinose were apparently lower than that with FOS and XOS.

**Production of short chain fatty acids during the vitro fermentation** The short chain fatty acids were produced by *C. butyricum* TK2 and *C. butyricum* CB8 during vitro fermentation of varied oligosaccharides, as shown in Table 3, and the total SCFA is considered as a symbol of functional oligosaccharides fermentability (Li et al., 2012). Although SCFA were composed of acetate, propionate and butyrate, propionate was not detected for the two strains. Acetate and butyrate were the major end products and butyrate was the dominant SCFA during the fermentation of six functional oligosaccharides by both strains. In contrast to other

<table>
<thead>
<tr>
<th>Oligosaccharides</th>
<th><em>C. butyricum</em> TK2</th>
<th><em>C. butyricum</em> CB8</th>
<th><em>C. butyricum</em> MIYAIRI 588</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gas from glucose</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>nitrate reduction</td>
<td>−</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>Gelatin liquefied</td>
<td>−</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>Milk coagulation</td>
<td>−</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>Fermentation:</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>glucose</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>lactose</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>starch</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>maltose</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>mannose</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>raffinose</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>ribose</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

Explanation of the symbols: +, Test result is positive; −, Test result is negative.
oligosaccharides, fermentation of IMOS by the two types of *C. butyricum* resulted in the highest concentration of SCFA and butyrate (11.77 ± 0.37 mmol/L and 14.47 ± 1.05 mmol/L). Moreover, the yield of SCFA and butyrate from fermentation of FOS and XOS were the least for *C. butyricum* TK2 and *C. butyricum* CB8.

However, the two types of *C. butyricum* exerted some differences in the production of SCFA and butyrate. In terms of the production of SCFA, IMOS, FOS and XOS resulted in significant differences between *C. butyricum* TK2 and *C. butyricum* CB8 (p < 0.05), while statistically distinctions of the output of butyrate between the two strains were observed from fermentation with IMOS, GOS and raffinose (p < 0.05).
Discussion

At the present study, the six functional oligosaccharides were utilized by *C. butyricum* TK2 and *C. butyricum* CB8 at different levels. However, there were some differences in the fermentation characteristics of the six oligosaccharides between the two types of *C. butyricum*, which may be in relation with the specificity of two strains. It has been reported that *Clostridium* species can degrade polysaccharide (Rockova et al., 2011; Nakajima et al., 2002). Montoya et al. (2001) have demonstrated that new solvent-producing *Clostridium* sp. strains, which are closely related to *C. butyricum*, can also hydrolyze a wide range of polysaccharides. It has been found that *C. butyricum* utilizes both soybean oligosaccharides and FOS and can not utilize GOS (Hayakawa, 1990; Sako et al., 2011). The production of SCFA results in decrease of pH in the colon to inhibit the growth of certain pathogenic bacterium and stimulate the growth of the beneficial bacteria (Cherrington et al., 1991; Van Immerseel et al., 2002; Van Immerseel et al., 2004; Defoirdt et al., 2006; Woo et al., 2011). In particular butyrate promotes apoptosis and inhibits growth of cancer cells in vitro (Hague et al., 1995) and plays a protective role against colorectal cancer in vivo (Avivi-Green et al., 1999). The production of SCFA results in decrease of pH in the colon to inhibit the growth of certain pathogenic bacterium and stimulate the growth of the beneficial bacteria (Cherrington et al., 1991; Van Immerseel et al., 2002; Van Immerseel et al., 2004; Defoirdt et al., 2006; Woo et al., 2011).

In fact, there has been little research about the structure–function relationships of functional oligosaccharides recently.

Most of functional oligosaccharides are not digested by humans for lack of relevant enzymes in the human body, which reaching colon as they have been eaten and are further fermented by anaerobic bacteria (Crittenden and Playne, 1996). Such metabolic process, it produces short chain fatty acids. The amounts and types of SCFA produced depend on the type of non-digestible oligosaccharides as well as on the composition of the intestinal flora (Sako et al., 1999). The production of SCFA results in decrease of pH in the colon to inhibit the growth of certain pathogenic bacterium and stimulate the growth of the beneficial bacteria (Cherrington et al., 1991; Van Immerseel et al., 2002; Van Immerseel et al., 2004; Defoirdt et al., 2006; Woo et al., 2011). In particular butyrate promotes apoptosis and inhibits growth of cancer cells in vitro (Hague et al., 1995) and plays a protective role against colorectal cancer in vivo (Avivi-Green et al., 2001; Perrin et al., 2001). Based on these experimental observations, it is hypothesized that functional oligosaccharides as an indirect source of butyrate to the gut may be beneficial to reduce risk factors for colorectal cancer and other diseases.

*Bifidobacteria* and *Lactobacilli*, which considered as the target species for prebiotic stimulation in the colon with the aim of improving host health (Durieux et al., 2001), but do not produce butyrate. However, nowadays, there is an increasing interest in the use of prebiotics that are capable of improving the yield of butyrate or the number of butyrate-producing bacterium because of the beneficial effects of butyrate on the gut. In our study, the effect of IMOS and GOS was found on the number and the butyrate

**Table 3.** Production of short chain fatty acids during the vitro fermentation of *C. butyricum* TK2 and *C. butyricum* CB8.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Type of <em>Clostridium butyricum</em></th>
<th>Total SCFA (mM)</th>
<th>Acetate (mM)</th>
<th>Butyrate (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>IMOS</td>
<td>TK2</td>
<td>13.99 ± 0.44c</td>
<td>2.22 ± 0.13d</td>
<td>11.77 ± 0.37b</td>
</tr>
<tr>
<td></td>
<td>CB8</td>
<td>19.23 ± 1.03d</td>
<td>4.77 ± 0.19d</td>
<td>14.47 ± 1.05d</td>
</tr>
<tr>
<td>Stachyose</td>
<td>TK2</td>
<td>10.18 ± 1.83b</td>
<td>2.11 ± 0.59b</td>
<td>8.07 ± 1.24b</td>
</tr>
<tr>
<td></td>
<td>CB8</td>
<td>10.27 ± 0.42b</td>
<td>2.88 ± 0.18c</td>
<td>7.39 ± 0.30b</td>
</tr>
<tr>
<td>Raffinose</td>
<td>TK2</td>
<td>12.33 ± 1.18c</td>
<td>2.59 ± 0.96c</td>
<td>9.74 ± 0.71b</td>
</tr>
<tr>
<td></td>
<td>CB8</td>
<td>10.93 ± 0.07bc</td>
<td>2.80 ± 0.12bc</td>
<td>8.13 ± 0.11bc</td>
</tr>
<tr>
<td>GOS</td>
<td>TK2</td>
<td>13.48 ± 1.22c</td>
<td>2.16 ± 0.61d</td>
<td>11.32 ± 0.50b</td>
</tr>
<tr>
<td></td>
<td>CB8</td>
<td>11.45 ± 0.96c</td>
<td>2.94 ± 0.16c</td>
<td>8.51 ± 0.79c</td>
</tr>
<tr>
<td>FOS</td>
<td>TK2</td>
<td>2.81 ± 0.17a</td>
<td>0.86 ± 0.21a</td>
<td>1.95 ± 0.19a</td>
</tr>
<tr>
<td></td>
<td>CB8</td>
<td>8.60 ± 0.17a</td>
<td>2.60 ± 0.10ab</td>
<td>6.00 ± 0.15a</td>
</tr>
<tr>
<td>XOS</td>
<td>TK2</td>
<td>3.54 ± 0.46a</td>
<td>1.12 ± 0.08a</td>
<td>2.42 ± 0.39a</td>
</tr>
<tr>
<td></td>
<td>CB8</td>
<td>7.86 ± 0.18a</td>
<td>2.41 ± 0.09a</td>
<td>5.44 ± 0.11a</td>
</tr>
</tbody>
</table>

Concentration of SCFA was determined by high-resolution gas chromatography used external standards. Data were presented as the mean of triplicate measurement ± SD. IMOS, Isomaltooligosaccharides; GOS, Galactooligosaccharides; FOS, Fructooligosaccharides; XOS, Xylooligosaccharides. Indices above data bars represent significant differences within effects of six functional oligosaccharides on the levels of SCFA produced by *C. butyricum* TK2 or *C. butyricum* CB8. Different letters indicate significantly different results (P <0.05).
production of *C. butyricum*, moreover, it has been confirmed that IMOS and GOS are selectively increase *Bifidobacteria* and *Lactobacilli* in the gut (Panesar et al., 2011). *Bifidobacterium* spp hydrolysis polymeric α-(1,6) and α-(1,4) with extracellular enzymes (Ryan et al., 2006). In addition, α- or β-glucanase can also been secreted to degrade polysaccharides by some *Clostridium* strains (Montoya et al., 2001; Nakajima et al., 2002). In terms of *C. butyricum*, the enzymes for IMOS and GOS metabolism are not clear. It can be hypothesized *C. butyricum* have some kinds of extracellular or intracellular glycosyl hydrolases which metabolism oligosaccharides with hexanose units preferentially. In future, more study is needed to fully elucidate the roles of cell-associated glycosidases. Nevertheless, the present study showed that there was no significant effect of FOS and XOS on the fermentation characteristics of two types of strains.

It is a very promising direction that the development of symbiotics in the area of functional food ingredients. Symbiotic describes a combination of a prebiotic and a probiotic. The fermentation characteristics of the bacterium with various prebiotics in this study reflect the extent to which functional oligosaccharides would promote the growth and butyrate production of *C. butyricum*. Therefore, this study provides a rational basis for establishing synbiotics with *C. butyricum* to maintain intestinal health towards healthier community.

**Conclusion**

The present study determined which functional oligosaccharides could show the better fermentability by promoting the growth and SCFA or butyrate production of *C. butyricum* TK2 and *C. butyricum* CB8. The largest increase in the two types of *C. butyricum* and the highest butyrate level were seen on IMOS. While the viable cell concentration and the butyrate level from the fermentation of FOS and XOS were much lower than the other treatments. On the other hand, the different fermentation properties between the two strains were also studied. For the same functional oligosaccharide, the numbers of both bacterium and the production of SCFA were different. This study provides a rational basis for establishing synbiotics with *C. butyricum* to ensure the human health.

**Acknowledgement**

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**References**


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