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Short-chain fructo-oligosaccharides produced by enzymatic hydrolysis enhance the growth of probiotics isolated from cultured milk drinks

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ABSTRACT

Aims: Short-chain fructo-oligosaccharides (scFOSs) are good prebiotics that enhance the growth of probiotic bacteria. The aim of this study is to determine the effect of scFOSs produced by levan hydrolysis using recombinant endo-levanase from *B. lehensis* G1 on the growth of probiotics isolated from commercially cultured milk drinks.
Methodology and results: Two probiotic bacteria, *Lactobacillus casei* and *L. rhamnosus*, were isolated from commercially cultured milk drinks. ScFOSs were produced by levan hydrolysis using recombinant endo-levanase from *B. lehensis* G1. The scFOS and levan (control) were added independently to the growth medium, and the growth rates of the probiotic bacteria were determined. Results showed that the growth rate of *L. casei* decreased in the presence of levan compared with the control medium but increased by approximately 20% when supplemented with scFOS produced by Levblg1-N28S. Similarly, the growth rate of *L. rhamnosus* increased by approximately 20% when supplemented with scFOS produced by Levblg1 and Levblg1-N28S.
Conclusion, significance and impact of study: The scFOSs produced by the enzymatic hydrolysis of levan using a recombinant endo-levanase from *B. lehensis* G1 have significant potential as prebiotics because they were able to promote the growth of the probiotic bacteria.

Keywords: Lactobacillus, prebiotic, probiotic, fructo-oligosaccharide, fructan

INTRODUCTION

Probiotic bacteria enhance the function of the human intestinal immune system by converting the microflora in the gastrointestinal tract to an improved microorganism population. In addition, mineral absorption is improved and the risk of colon cancer is reduced in the presence of probiotic bacteria (Kilian *et al*., 2002; Knol *et al*., 2005). The growth of probiotic bacteria in the gastrointestinal tract is stimulated by prebiotics, which are carbon sources that can be readily metabolised by probiotic bacteria; notably, prebiotics are not absorbed by pathogenic microbes in the human gut or by the host (Slavin, 2013). Several reported prebiotics include fructans, such as levan (β-(2→6) linkages between fructose units in the main polymer chain) and inulin (β-(2→1) linkages between fructose units) and fructo-oligosaccharide (FOS; either β-(2→1) or β-(2→6) linkages). However, most probiotic bacteria prefer simple sugars (FOS) as carbon sources over complex sugars (fructans) (Marx *et al*., 2000; Biedrzycka and Bielecka, 2004; Porras-Dominguez *et al*., 2014).

FOS intake promotes healthy intestine and has been formulated in infant formulas, in fermented milk products (cheese, yogurt and cultured milk drinks) and in supplement tablets (Gibson *et al*., 2004; Roberfroid, 2007). On the basis of several reports, most probiotic *Bifidobacteria* species, such as *B. breve*, *B. longum*, *B. adolescentis*, *B. infantis*, *B. animalis*, *B. dentium*, *B. catenulatum* and *B. pseudocatenulatum*, can use FOSs as carbon sources (Hidaka *et al*., 1986; Muramatsu *et al*., 1994; Mitsuoka, 1996; Hartemink, 1999; Kaplan and Hutkins, 2000; Marx *et al*., 2000; Biedrzycka and Bielecka, 2004; Rossi *et al*., 2005; Porras-Dominguez *et al*., 2014). Similar to *Bifidobacteria* species, *Lactobacillus* species, such as *L. casei*, *L. acidophilus*, *L. gasseri*, *L. salivarius*, *L. plantarum* and *L. paracasei*, use FOSs as carbon sources. By contrast, *L. bulgaricus*, *L. lactis* and *Lactobacillus* strain GG (Mitsuoka, 1996; Sghir *et al*., 1998; Kaplan and Hutkins, 2000; Porras-Dominguez *et*

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al., 2014) and pathogenic bacteria, such as *Escherichia coli*, *Fusobacterium*, *Streptococcus pyogenes* and *Clostridium perfringens*, cannot metabolise FOSs (Mitsuoka, 1996; Kilian *et al*., 2002). These observations indicate that the consumption of FOSs as carbon sources is dependent on bacterial species.

Currently, the industrial production of FOSs is either performed through the transfructosylation of sucrose by fructosyltransferase enzymes or through the hydrolysis of plant inulin by endo-inulinases (Singh and Singh, 2010; Vega-Paulino and Zúniga-Hansen, 2012). However, the production of FOSs from plant inulin (β-(2→1) linkages between fructose units) is limited by seasonal conditions and low inulin content in plants (Mussatto *et al*., 2012). Therefore, the production of FOS from other sources is indispensable to meet market demand. Levan synthesised by bacteria is a suitable substrate because it can be produced in large quantities in a bioreactor (Srikanth *et al*., 2015). However, bacterial levan is a polymer composed of fructose unit with β-(2→6) linkages, and thus the hydrolysis of this polymer into short-chain fructo-oligosaccharides (scFOS) requires levanase, an enzyme that catalyse the random hydrolysis of 2,6-β-D-fructofuranosidic linkages in levan. In the present study, we evaluated the ability of bacterial levan and scFOS, produced via enzymatic hydrolysis of the levan and as support for the growth of probiotic bacteria. To render this study industrially relevant, we isolated the probiotics used in the experiment from two commercially cultured milk drinks available in the Malaysia market.

MATERIALS AND METHODS

Probiotic bacterial isolation

Probiotics used in this study were isolated from commercially cultured milk drinks (Yakult and Nutrigen). Approximately 0.1 mL of diluted cultured milk drinks (DF 10^{-1}–10^{-8}) were spread on de Man, Rogosa and Sharpe (MRS) agar plates (de Man *et al*., 1960) and incubated at 37 °C for 48 h. A single colony of bacteria was inoculated into 10 mL of MRS growth medium and was incubated at 37 °C for 48 h with 250 rpm agitation. Bacterial DNA genome was extracted by using Wizard® SV Genomic DNA Purification kit (Promega, USA). The genomic DNA was analysed by 1% agarose gel electrophoresis and stored at −20 °C for further use.

Identification of bacteria species

The primer pair 27F (5-′AGAGTTTGATCMTGGCTCAG-3′) and 1492R (5-′TACGGYTACCTTGTTACGACTT-3′) was used for the amplification of nucleotide sequence-encoded 16S ribosomal subunit (16S rDNA) (Kim *et al*., 2012). The 16S rDNA was amplified from the bacterial DNA genome by using KOD Hot Start DNA Polymerase (Merck, USA). The PCR cycling conditions were one cycle of 95 °C for 4 min, followed by 30 cycles of 95 °C for 1 min, 50 °C for 1 min and 72 °C for 2 min and final extension at 72 °C for 10 min. The PCR product was analysed by 1% agarose gel electrophoresis and purified by using QIAquick® Gel Extraction Kit (Qiagen, Germany).

PCR product analysis

The PCR product was sent to First BASE Laboratories Sdn. Bhd. (Malaysia) for sequencing. We analysed the 16S rDNA sequence via BLAST tools (https://blast.ncbi.nlm.nih.gov/Blast.cgi) to determine its relationship to other reported species (Altschul *et al*., 1990).

Preparation of scFOS

ScFOS was prepared by treating the bacterial levan (Sigma, USA) with either *Bacillus lehensis* endo-levanase (Levblg1) or modified *B. lehensis* endo-levanase (Levblg1-N28S). Both enzymes were produced in-house, and Levblg1-N28S was modified for the enhancement of FOS production with the degree of polymerisation (DP) between 3 and 4. The reaction mixture contained 1 µg of enzyme, 50 mM of potassium phosphate buffer (pH 7.0) and 1% (w/v) of levan. Hydrolysis was performed at 30 °C for 2 h and was stopped by boiling at 95 °C for 10 min. The degradation product of levan was analysed by high-performance liquid chromatography using LaChrom Elite system (HITACHI, Japan) with refractive index (RI) detection. The filtered sample was applied to an analytical TSKgel Amide-80 column (TOSOH, Japan) (4.6 × 250 mm) for carbohydrate analysis at incubation temperature of 80 °C and flow rate of acetonitrile/water (75/25) (v/v) of 1 mL/min. Retention time was used for the identification of reducing sugars, and RI units were utilised for the calculation of their concentrations.

Effect of scFOS and levan on the growth of probiotic bacteria

A single colony of probiotic bacteria was inoculated into 5 mL of MRS growth medium and incubated at 37 °C with 250 rpm agitation. An overnight culture was subsequently subcultured into a new MRS growth medium with a ratio of 1:100 (v/v) and was used for final scFOS or levan fermentation. Filtered sterile solutions of levan (1% final concentration (v/v) of levan solution), scFOS produced by Levblg1 (16% final concentration (v/v) of scFOS solution dominated with DP 3 and DP 4) and scFOS produced by Levblg1-N28S (23% final concentration (v/v) of scFOS solution dominated with DP 3 and DP 4) were then added to the growth medium at a ratio of 1:100 (v/v). Meanwhile, a culture solution that did not contain levan or scFOS was used as a control. The culture was then distributed into a 50 mL falcon tube with each tube containing 5 mL of culture and incubated at 37 °C with 250 rpm agitation. Samples were withdrawn every 4 h and growth was measured by monitoring the increase of the biomass via optical density at 600 nm (OD_{600}). The experiments were performed in duplicate, and three replicate readings were recorded for each time point for both *L. casei* and *L.*

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Table 1: Growth rates (means ± standard deviation) of *L. casei* and *L. rhamnosus* with the presence of levan and scFOS as carbon sources.

Probiotic bacteria	Growth rate (1/h)			
	Control	ScFOS+Levblg1	ScFOS+Levblg1-N28S	Levan
L. casei	0.091 ± 0.009	0.101 ± 0.004	0.109 ± 0.004*	0.076 ± 0.008*
L. rhamnosus	0.113 ± 0.015	0.136 ± 0.001*	0.135 ± 0.006*	0.125 ± 0.008

* $p < 0.05$; one-way ANOVA with Bonferroni *Post Hoc*

rhamnosus. The bacterial growth curve was then plotted on the basis of OD_{600} reading against incubation time. The growth rates of the bacteria were calculated in accordance with the mathematical equation described by Stensjøen *et al.* (2015).

Exponential phase equation:
$y = Ae^{Bx}$, where A and B are numbers

Doubling time equation (T_d):
$y = Ae^{Bx} \rightarrow x = ln\ (y/A)/B$
When $y = 1$, $x1 = ln\ (1/A)/B$
When $y = 2$ (i.e. when y is doubled) $x2 = ln\ (2/A)/B$
$T_d = x2 - x1 = [(ln2 - lnA) - (ln1 - lnA)]/B$
$ln1 = 0$
$T_d = ln2/B = 0.693/B$

Growth rate (μ):
$\mu = ln2/T_d$

Data were provided as mean values ± standard deviation.

Statistical analysis

The one-way analysis of variance (ANOVA) was used to determine whether differences among four groups of growth medium (MRS medium only, MRS medium supplemented with scFOS produced by Levblg1, MRS medium supplemented with scFOS produced by Levblg1-N28S and MRS medium supplemented with levan) with respect to the growth rates of *L. casei* and *L. rhamnosus* are statistically significant. Then, Bonferroni was applied as a post hoc test for multiple comparisons among groups. The results were considered statistically significant at p-value < 0.05 at 95% confidence interval. The IBM SPSS software version 21 was used for all statistical analyses.

RESULTS

Probiotic bacteria were isolated from commercial cultured milk drinks available in the market (Yakult and Nutrigen) and identified according to their 16S rDNA sequences. Subsequent nucleotide sequencing of the PCR products yielded good sequence that is suitable for subsequent analysis. We analysed the 16S rDNA sequence via the BLAST software available at the National Center for Biotechnology Information (USA) to identify bacteria. The results showed that the 16S rDNA sequence from the bacteria isolated from Yakult's cultured milk drink had 100% match with the 16S rDNA sequence of *Lactobacillus casei* strain NWAFU1575, whereas the sequence generated from bacteria isolated from Nutrigen's cultured milk drink had the highest match of 99% with the 16S rDNA sequence of *Lactobacillus rhamnosus* strain CNCM I-4036. Therefore, both probiotic bacterial species were renamed as *L. casei* and *L. rhamnosus*.

Figures 1 and 2 show the growth curve of *L. casei* and *L. rhamnosus*, respectively, in the presence of levan and scFOS. The growth of *L. casei* was rapid and had no distinct lag phase (Figure 1). The log phase lasted for 24 h before the cells entered the stationary phase. The maximum number of cells was detected from growth medium containing scFOS prepared via enzymatic hydrolysis using Levblg1 and Levblg1-N28S. Meanwhile, the lowest number of cells was produced from the medium supplemented with levan. The value was considerably lower than the cells that were grown in the MRS medium (control). The initial growth of *L. rhamnosus* was considerably slower than that of *L. casei* in the MRS medium or MRS supplemented with either levan or scFOS (Figure 2). The lag phase of the cell growth lasted for 20 h before the cells entered the log phase. The log phase lasted for 36 h. The highest number of cells was detected from the culture grown in the medium supplemented with levan and scFOS produced by Levblg1-N28S, followed by the culture grown in the medium supplemented with scFOS produced by Levblg1. Given that the growth curve only showed the number of cells, we calculated bacterial growth rate to determine the effect of scFOS and levan on bacterial growth. The growth rate of *L. casei* in the MRS medium (control) was 0.091 ± 0.009/h (Table 1). In the MRS medium supplemented with scFOS produced by Levblg1 and Levblg1-N28S, the growth rates of *L. casei* were 0.101 ± 0.004/h and 0.109 ± 0.004/h, respectively. When the MRS medium was supplemented with levan, the growth rate of *L. casei* was reduced to 0.076 ± 0.008/h and considerably slower than that of the control medium (MRS alone). Meanwhile, the growth rate of *L. rhamnosus* in the MRS medium was 0.113 ± 0.015/h but increased to

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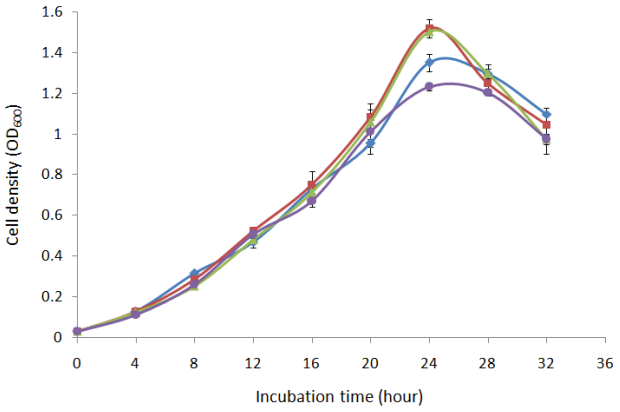

Figure 1: Growth of *L. casei* in the presence or absence of levan or scFOS. *L. casei* in the MRS medium only (close diamond), *L. casei* in the MRS medium supplemented with scFOS produced by Levblg1 (close square), *L. casei* in the MRS medium supplemented with scFOS produced by Levblg1-N28S (close triangle) and *L. casei* in the MRS medium supplemented with levan (close circle).

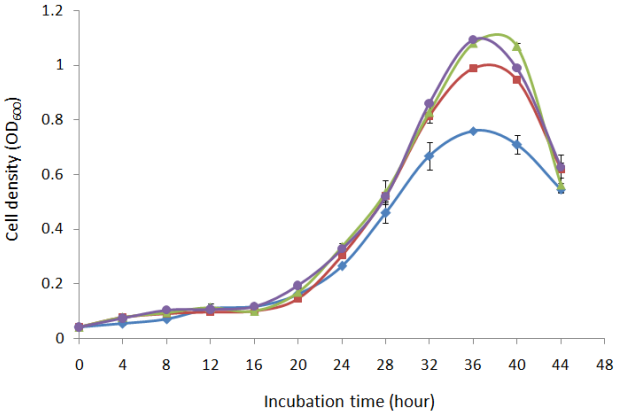

Figure 2: Growth of *L. rhamnosus* in the presence or absence of levan or scFOS. *L. rhamnosus* in the MRS medium only (close diamond), *L. rhamnosus* in the MRS medium supplemented with scFOS produced by Levblg1 (close square), *L. rhamnosus* in the MRS medium supplemented with scFOS produced by Levblg1-N28S (close triangle), and *L. rhamnosus* in the MRS medium supplemented with levan (close circle).

0.125 ± 0.008/h, 0.136 ± 0.001/h and 0.135 ± 0.006/h when supplemented with levan, scFOS produced by Levblg1 and scFOS produced by Levblg1-N28S, respectively.

The one-way ANOVA results showed that statistically significant differences were found among the growth rates of *L. casei* ($F_{(3,20)}$ = 28.783, p < 0.001) and *L. rhamnosus* ($F_{(3,20)}$ = 9.015, p = 0.001) of the four groups. The result of the post hoc Bonferroni test revealed that the growth rate of *L. casei* in the MRS medium supplemented with scFOS produced by Levblg1-N28S (0.109 ± 0.004/h, p = 0.001) was statistically higher than that in the MRS medium (0.091 ± 0.009/h). Moreover, the growth rate of *L. casei* in the MRS medium supplemented with levan was statistically lower (0.076 ± 0.008/h, p = 0.006) than that in the MRS medium (0.091 ± 0.009/h). No statistical difference was observed between the growth rates of *L. casei* in the MRS medium and in the MRS medium supplemented with scFOS produced by Levblg1 (p = 0.090). The *post hoc* Bonferroni test result also revealed that the growth rates of *L. rhamnosus* in the MRS medium supplemented with scFOS produced by Levblg1 (0.136 ± 0.001/h, p = 0.001) and Levblg1-N28S (0.135 ± 0.006/h, p = 0.002) were statistically higher than that in the MRS medium (0.113 ± 0.015/h). No statistical difference was observed between the growth rates of *L. rhamnosus* in the MRS medium and MRS medium supplemented with levan (p = 0.175).

DISCUSION

Probiotic bacteria require prebiotics as a carbon source. Prebiotic is a special food for probiotic bacteria and cannot be metabolised by other pathogenic bacteria. Previous studies showed that most probiotic bacteria, such as *Bifidobacteria* and *Lactobacillus* species, utilise FOSs as carbon sources (Hidaka *et al*., 1986; Muramatsu *et al*., 1994; Mitsuoka, 1996; Sghir *et al*., 1998; Hartemink, 1999; Kaplan and Hutkins, 2000; Marx *et al*., 2000; Biedrzycka and Bielecka, 2004; Rossi *et al*., 2005; Porras-Dominguez *et al*., 2014), although they have distinct modes of using FOSs. Some bacteria prefer to use FOSs with short sugar chains, especially those with three or four units of sugar polymers.

In this study, the growth of probiotic bacteria, namely, *L. casei* and *L. rhamnosus*, isolated from two commercial cultured milk drinks, namely, Yakult and Nutrigen, in growth medium supplemented with levan and scFOS were investigated. The scFOS used in this work was prepared by the hydrolysis of the bacterial levan using an in-house recombinant *B. lehensis* endo-levanase produced in *E. coli*. Two types of endo-levanase were used in this study, namely, Levblg1 and Levblg1-N28S. The first enzyme generated scFOS containing 16% of DP 3 and DP 4 of fructose unit (β-(2→6) linkages between each unit), whereas the second enzyme generated scFOS containing up to 23% of DP 3 and DP 4 of fructose unit. The findings showed that the growth rate of *L. casei* increased significantly in the presence of scFOS produced by Levblg1-N28S and decreased in the presence of levan. Thus, *L. casei* preferred to metabolise scFOS with higher concentrations of DP 3 and DP 4 than levan. Likewise, the growth rate of *L. rhamnosus* increased in the presence of scFOS produced by Levblg1 and Levblg1-N28S. These observations suggested that *L. casei* and *L. rhamnosus* both prefer scFOS as a carbon source for growth, especially those with high DP 3 and DP 4, over levan possibly because scFOSs have simpler structures and more soluble in water than levan (Porras-

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Dominguez *et al*., 2014). According to Van Laere *et al*. (1997), the major factors affecting the preferences of probiotic bacteria with regard to oligosaccharides are chemical structure, composition of monomer units, DP, structure (whether straight or branched) and the solubility in water. Typically, short, non-branched and water-soluble FOSs are easily used by probiotic bacteria. The results obtained in this study were consistent with the studies of Biedrzycka and Bielecka (2004), who showed that *B. longum* and *B. animalis* prefer to use scFOS with $2 \leq DP \leq 4$ and oligofructose with $2 \leq DP \leq 8$ as carbon sources over inulin. Rossi *et al*. (2005) conducted studies on 55 strains of *Bifidobacteria*. Their results showed that most of these strains can use FOSs as carbon sources, whereas only eight strains can use inulin (Rossi *et al*., 2005). They observed that the *Bifidobacteria* strains that uses inulin produce β-fructofuranosidase (inulinase) that can break down fructan to produce a simple sugar of FOS. Meanwhile, Kaplan *et al*. (2000) conducted studies on the growth of *L. paracasei* 1195 in the presence of FOS with different DPs, that is, DP 3, DP 4 and DP 5, as carbon sources. Results showed that *L. paracasei* 1195 only utilised DP 3 and DP 4 as carbon sources and could not use DP 5. Additionally, they showed the importance of DP 3 and DP 4 as carbon sources that increase the growth of probiotic bacteria and compared them with long-chain FOSs. In the present study, the produced scFOS with $2 \leq DP \leq 4$ showed great potential as a prebiotic because it promoted the growth of probiotic bacteria, especially the *Lactobacillus* species.

A study was conducted on β-(2→6)-FOS, in addition to β-(2→1)-FOS, to increase the growth of *Bifidobacteria* and *Lactobacillus* species (Marx *et al*., 2000; Porras-Dominguez *et al*., 2014). The use of β-(2→6)-FOS, similar to β-(2→1)-FOS, as a carbon source is species dependent. Marx *et al*. (2000) reported that *B. adolescentis*, *B. breve*, *B. longum* and *B. pseudocatenulatum* can use FOSs as carbon sources. *B. adolescentis* can also use levan as a carbon source. Interestingly, we also observed that levan can be metabolised by *L. rhamnosus* but not by *L. casei*. Porras-Dominguez *et al.* (2014) reported that *B. longum* var. Infant ATCC 17930 and *B. longum* var. Infant NRRL 41661 have higher growth rates when they used FOSs with $2 \leq DP \leq 8$ as a carbon source than when they used levan. Moreover, the growth rates of *L. paracasei* UAM, *B. bifidum* ATCC 29521, *B. longum* ATCC 15707 and *B. breve* NRRL 41408 increased when levan was used as a carbon source than when FOS was used. The results of Porras-Dominguez *et al*. (2014) is consistent with the results obtained in this study where the *Lactobacillus* species were able to use β-(2→6)-FOS as a carbon source. The overall results showed that fructan and FOS consumption is dependent on bacterial species.

CONCLUSION

ScFOSs produced with the use of a recombinant endo-levanase from *B. lehensis* has great potential as a prebiotic because it promotes the growth of probiotic bacteria, especially the *Lactobacillus* species.

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