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Effect of Novel Propionyl-fructooligosaccharides on Growth of Intestinal Bacteria

신규 프로피오닐 프락토올리고당이 장내 균 성장에 미치는 영향

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Department of Food and Nutrition

Graduate School

Seoul National University

Seungeun Lee

Abstract

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Seungeun Lee

Department of Food and Nutrition

The Graduate School

Seoul National University

Numerous studies reported that ingestion of fructooligosaccharides (FOS) can promote the growth of *Bifidobacterium* in large intestine. Therefore, FOS is currently used as prebiotics. Propionic acid (PA) has an inhibitory effect on the growth of pathogenic molds and bacteria. Propionates such as sodium propionate and calcium propionate are used as preservatives for food. In this study, the effect of novel propionyl-fructooligosaccharides (P-FOS) on the growth of various intestinal bacteria was assessed. According to the structural analyses using FT-IR, MALDI-TOF MS, LC-ESI-MS, and LC-ESI-MS/MS, the major components of P-FOS used in this study contained FOS with 1-3 propionyl groups attached. P-FOS

promoted the growth of the most experimental *Bifidobacterium* and some of the other lactic acid bacteria. In contrast to FOS, P-FOS showed no growth promotion or slight suppression against most of the non-probiotic bacteria. The novel P-FOS is expected to be useful for the improvement of human intestinal microflora.

Keywords : fructooligosaccharides, bacterial growth, structural analysis of oligosaccharides

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List of Abbreviations

P-FOS; Propionyl-fructooligosaccharides

LAB; Lactic acid bacteria

BHIB; Brain-heart infusion broth

FOS; Fructooligosaccharides

DW; Distilled water

TLC; Thin-layer chromatography

FT-IR; Fourier transform infrared spectroscopy

MALDI-TOF MS; Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry

LC-ESI-MS; Liquid chromatography-electrospray ionization-mass spectrometry

1. Introduction

The composition of microflora in human gut changes through lifespan [1]. The importance of gut microflora to human health and disease has been reported [2, 3]. *Bifidobacterium* and the lactic acid bacteria (LAB) produce antimicrobial compounds such as organic acids and bacteriocins [4]. The presence of *Bifidobacterium* and LAB can inhibit the growth of other enterobacteria and pathogenic bacteria. Therefore, *Bifidobacterium* and LAB are used as probiotic strains to improve the composition of human gut microflora [5-8]. LAB produce lactic acid as a major product of carbohydrate fermentation. Genera such as *Lactobacillus, Leuconostoc, Streptococcus, Pediococcus*, and *Aerococcus* are parts of LAB [9]. *Bifidobacterium* is often considered as a part of LAB. However, *Bifidobacterium* has a unique hexose fermentation pathway, so called fructose-6-phosphate shunt (also called as bifid shunt) [10]. *Bifidobacterium* belongs to the phylum *Actinobacteria*, class *Actinobacteria* [11, 12]. Considering the taxonomic differences of *Bifidobacterium* from LAB, *Bifidobacterium* is distinguished from LAB in this study.

Frucooligosaccharides (FOS) are polymers of β-2,1-linked fructosyl units (F) with a terminal α-D-glucose (G) by 1→2 linkage [13]. The major components of FOS are 1-kestose (GF2), nystose (GF3), and 1F-fructofuranosyl nystose (GF4). According to

many previous studies, FOS promotes the growth of *Bifidobacterium* thus being regarded to be beneficial to the host [14, 15]. However, some harmful bacteria such as *Enterobacter cloacae* and *Escherichia coli* also can utilize FOS [16].

Propionic acid (PA, C_2H_5COOH) is a naturally occuring short-chain fatty acid produced by bacterial fermentation of carbohydrates in the colon [17]. *Propionibacterium* is the main bacteria to produce PA as a product of fermentation [18]. PA, as an organic acid, reduces pH of intestine and exerts antimicrobial activity. An inhibitory effect of PA on growth of pathogenic molds and bacteria have been reported [19]. Therefore, PA is used as a preservative for food as a form of sodium propionate and calcium propionate. Also, PA is applicated in the selective media for bifidobacteria to inhibit the growth of other bacterial strains such as *Enterobacteriaceae*, *Enterococcus*, and *Staphylococcus* [20, 21]. However, high concentration of PA can inhibit the growth of lactobacilli [22].

Recently, propionyl-fructooligosaccharides (P-FOS) was newly synthesized to exploit the merit of antimicrobial activity of propionate and prebiotic effect of FOS. Additionally, it may be expected that antimicrobial activity of the PA against LAB and utilization of FOS by some of the harmful bacteria could be avoided by using P-FOS. The aim of this study was to assess the possibility of P-FOS in overcoming the defect of FOS.

Here, we present the characterization on the structure of P-FOS and the result of various bacterial growth on P-FOS.

2. Materials and methods

2.1. Materials

2.1.1. Source of P-FOS

P-FOS was synthesized and provided by BIFIDO (Hongchun, Korea).

2.1.2. The bacterial strains and culture condition

The bacterial strains used in the study are listed below (Table 1). 10 strains of *Bifidobacterium* and LAB including 6 strains of lactobacilli, *Lactococcus lactis* subsp. *lactis* KCTC 2013, and *Streptococcus salivarius* subsp. *thermophilus* KCTC 5092 were activated by two successive preculture in de-Mann-Rogosa-Sharpe broth (Difco, Detroit, USA) with 0.05% (w/v) cysteine-HCl at 37℃ for 18 h. Other non-probiotic strains were precultured in brain-heart infusion broth (BHIB, Difco) and activated by same culture condition.

Table 1. List of the experimental bacterial strains

Test strains	Abbreviation
Bifidobacteria	
Bifidobacterium adolescentis KCTC 3216	*B. adolescentis*
B. angulatum KCTC 3236	*B. angulatum*
B. animalis subsp. *animalis* KCTC 3219	*B. animalis*
B. bifidum BGN4	*B. bifidum* BGN4
B. breve KCTC 3419	*B. breve*
B. catenulatum KCTC 3221	*B. catenulatum*
B. longum BORI	*B. longum* BORI
B. longum subsp. *infantis* KCTC 3249	*B. infantis*
B. longum subsp. *longum* RD47	*B. longum* RD47
B. thermophilum KCCM 12097	*B. thermophilum*
Lactic acid bacteria	
Lactobacillus acidophilus KCTC 3154	*L. acidophilus* KCTC 3154
L. acidophilus KCTC 3168	*L. acidophilus* KCTC 3168
L. casei KFRI 699	*L. casei*
L. paracasei KF10	*L. paracasei*
L. plantarum KFRI 708	*L. plantarum*
L. rhamnosus KCTC 3237	*L. rhamnosus*
L. lactis subsp. *lactis* KCTC 2013	*L. lactis*

Table 1. (Continued)

Streptococcus salivarius subsp. *thermophilus* KCTC 5092	*S. thermophilus*
Non-probiotic bacteria	
Bacteroides cellulosilyticus KCTC 5800	*B. cellulosilyticus*
Bac. coprocola KCTC 5443	*B. coprocola*
Bac. fragilis ATCC 25285	*B. fragilis*
Clostridium ramosum KCTC 3323	*C. ramosum*
Enterobacter cloacae subsp. *cloacae* KCTC 2361	*E. cloacae*
Enterococcus faecalis KCTC 3511	*E. faecalis*
Escherichia coli DH5 α	*E. coli* DH5 α
E. coli KCTC 1039	*E. coli* KCTC 1039
Eubacterium rectale KCTC 5835	*E. rectale*
Listeria monocytogenes ATCC 19115	*L. monocytogenes*
Prevotella intermedia KCTC 5694	*P. intermedia*
Staphylococcus aureus ATCC 6358	*S. aureus*

2.1.3. Chemicals and reagents

All chemicals and reagents were purchased from Sigma-Aldrich (Sigma, St. Louis, MO, USA), unless described specifically.

2.1.4. The media and carbohydrate sources for bacterial growth test

The basal media used for the bacterial growth test was dextrose-free BHIB (MB Cell, Los Angeles, CA, USA). α-D-glucose (Sigma) and FOS (BIFIDO) were added to the basal media and compared to the P-FOS added media.

2.2. Purification and preparation of P-FOS

To purify P-FOS, column chromatography was performed using synthetic absorbent Diaion HP20 (Mitsubishi, Tokyo, Japan). Before the purification process, a 50 × 5 cm Glass Econo-Column column (Bio-Rad, Hercules, CA, USA) was packed with a fixed quantity of Diaion HP20. The column was washed by distilled water (DW). Solution of unpurified P-FOS was diluted by same volume of DW. The diluted solution was loaded on to the column packed with Diaion HP20. Washed DW and 10-30% (v/v) ethanol was discarded. The fractions eluted by 40-100% (v/v) ethanol were collected. The purity of P-FOS was determined by thin-layer chromatography

(TLC). The purified P-FOS collections were loaded on the silica gel plate 60 F254 (Merck, Darmstadt, Germany), and developed by 1-propanol/water/ethyl acetate (7:2:1, v/v). The sulfuric acid/water (1:9, v/v) solution was sprayed on the plate followed by tarring at 120℃ for 5 min [23, 24]. The purified P-FOS was concentrated by a speed vacuum concentrator ScanSpeed 40 (Labogene, Lynge, Denmark) and freeze-dried by Freeze dryer (Ilshin Biobase, Yangju, Korea).

2.3. Structural analysis of P-FOS

2.3.1. Determination of the linkages in P-FOS by FT-IR

Purified P-FOS in powder form was analyzed by Fourier transform infrared spectroscopy (FT-IR) to identify a chemical bond between propionate and FOS. The FT-IR spectra were taken using the KBr pellet technique and TENSOR27 (Bruker Optics, Ettlingen, Germany) at NCIRF of Seoul National University.

2.3.2. Mass analysis by MALDI-TOF MS and LC-ESI-MS

Mass spectra of FOS and P-FOS were characterized by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) Voyager-DETM STR

Biospectrometry Workstation (Applied Biosystems, Foster City, CA, USA) at NCIRF of Seoul National University. 2,5-Dihydroxybenzoic acid was used as matrix substance for MALDI-TOF MS. Mass spectra for P-FOS were obtained in the m/z range 350-3000. The mass spectra for FOS were obtained in the m/z range 300-3000. Mass analyses using liquid chromatography-electrospray ionization-mass spectrometry (LC-ESI-MS) and LC-ESI-MS/MS system were performed using high resolution LC-ESI-MS spectrometer, Q-TOF 5600 (AB Sciex, Foster City, CA, USA) at NICEM of Seoul National University. Mass spectra for P-FOS and FOS were obtained in the m/z range 500-1500.

2.4. Effect of glucose, FOS, and P-FOS on growth of intestinal bacteria

The effect of glucose, FOS, and P-FOS on bacterial growth was performed as described by Louise et al. with several modifications [25]. To exclude glucose or other carbohydrates contained in growth media, the grown cells were centrifuged (16,000 × g, 5 min) and harvested by centrifugation. The supernatant was discarded, and the cell pellets were washed twice by sodium phosphate buffer (pH 6.6) [23]. P-FOS, FOS, and glucose were dissolved in sterile DW (10%, w/v), sterilized by 0.2 ㎛ membrane filter. Dextrose-free BHIB

(198 ㎕) and the glucose, FOS, P-FOS solutions (22 ㎕) were added to 96-well microtiter plates. Sterile DW (22 ㎕) was added in a group without carbohydrates. The cell suspension of bacteria (2 ㎕) was inoculated in each broth. Final concentration of carbohydrates and the cell suspension of bacteria was 0.91% w/v and 0.91% v/v in total volume of 222 ㎕. The broth without cells was used as blank control. Three replicates were performed. The cultures were incubated at 37℃ for 95 h in anaerobic conditions using Whitley jar gassing system (Don Whitley Scientific, Shipley, UK). The microtiter plates were agitated before measurements. The optical density (OD) was measured at 600 nm with a microplate reader (BioRad, Hercules, CA, USA).

3. Results and Discussion

3.1. Structural analysis of P-FOS

3.1.1. Investigation of linkages in P-FOS using FT-IR

The purified P-FOS was confirmed by TLC (Fig. 1). The structure of purified P-FOS was performed by FT-IR as follows. Fig. 5 shows FT-IR absorbance spectra of FOS and P-FOS. The absorbance spectra of both chemicals represent bands assigned to each functional group and band. The broad bands in the 4000-2500 cm^{-1} range assigned to hydroxyl groups are observed in both FOS and P-FOS [26]. The bands at the range of 2700-3000 cm^{-1}, and 1600-1630 cm^{-1} are attributed to the C-H and $(COO)^-$ stretching bands, respectively. Bending vibration of (OCH), (COH), (CCH) groups are observed in the region between 1300-1500 cm^{-1} [27]. In the 1000-1200 cm^{-1} region, stretching vibration of glycosidic bonds are also observed [28]. However, the absorption at 1730 cm^{-1} attributed to (C=O) bond of ester groups is observed only in the data of P-FOS [26]. By this result, it is assumed that P-FOS is a product of ester linkage between propionate and FOS.

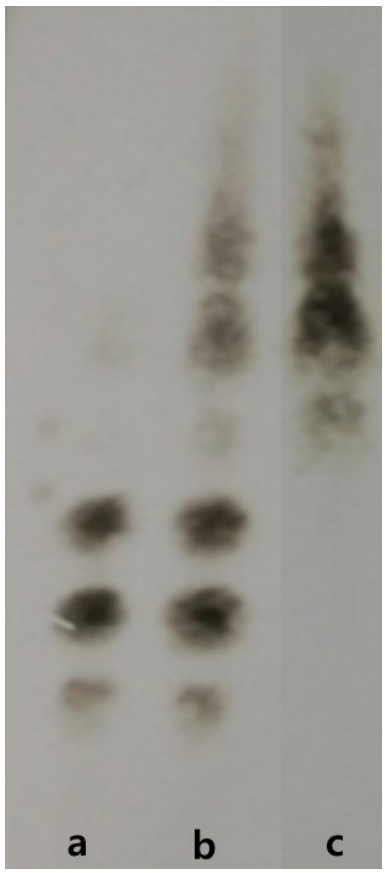

Fig. 1 Determination of purified propionyl-fructooligosaccharides by TLC. a : FOS, b : unpurified P-FOS, c : purified P-FOS.

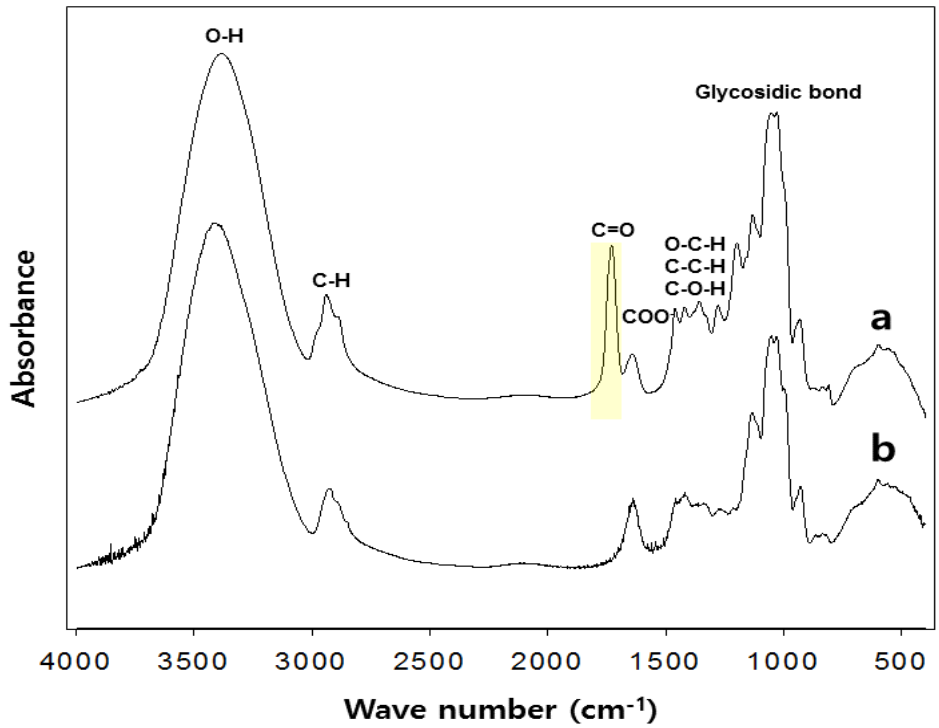

Fig. 2 Absorbance spectra of P-FOS and FOS using FT-IR. a : P-FOS, b : FOS.

3.1.2. Mass spectra analysis of FOS by MALDI-TOF MS and LC-ESI-MS

MALDI-TOF MS (Fig. 6A) and LC-ESI-MS (Fig. 6B) analyses were performed to analyze mass spectra of FOS. The peaks of m/z 527, 689, 851, 1013 and 1175 corresponded to $[M+Na]^+$ ions of FOS from GF2 to 6 by using MALDI-TOF MS. The pattern of FOS by LC-ESI-MS analysis was similar to the patterns from MALDI-TOF MS analysis. However, an ionized mass of GF7 was detected by LC-ESI-MS analysis (data not shown). The data of MALDI-TOF and LC-ESI-MS analyses suggested that FOS used as a substrate of synthesis was composed of GF2-GF7.

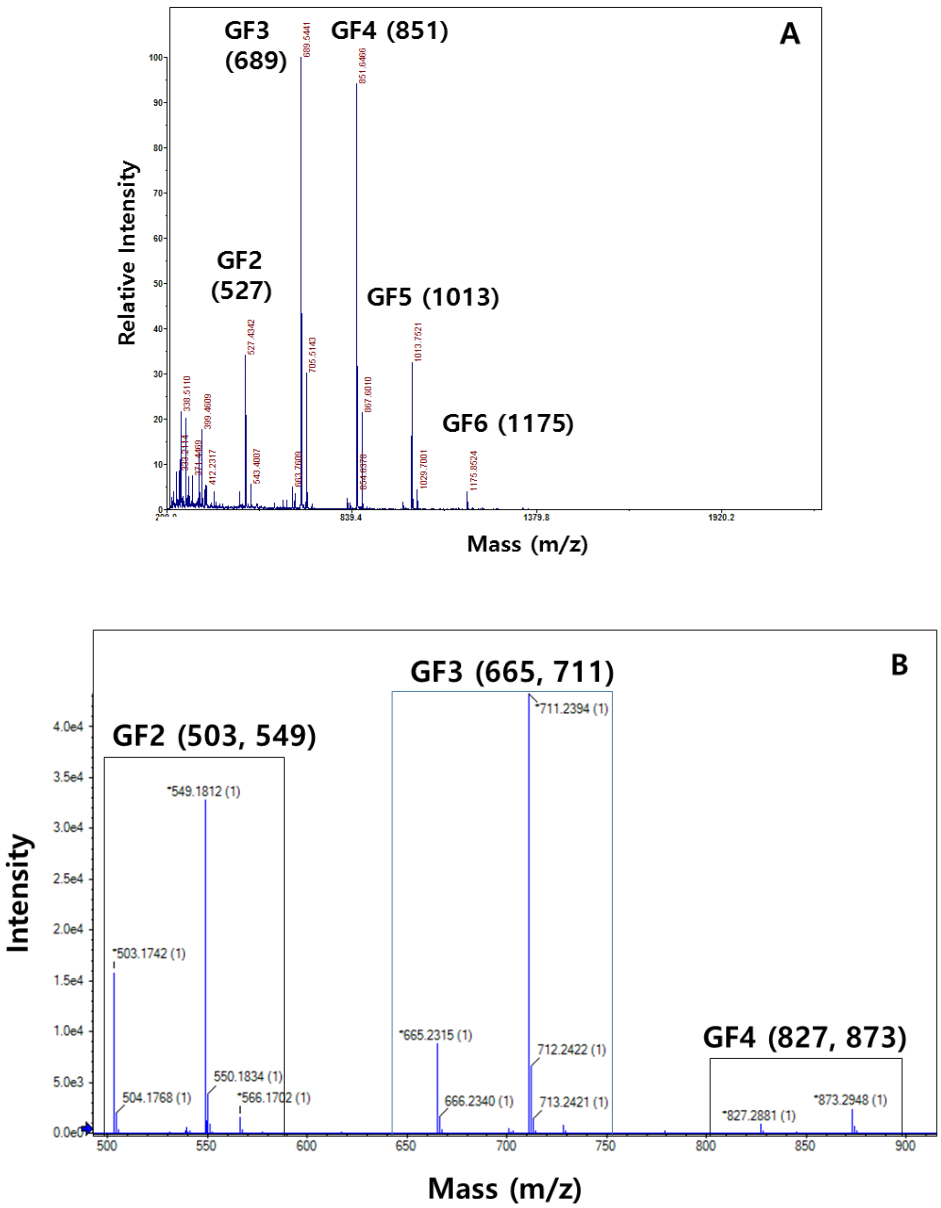

Fig. 3 Mass spectra of fructooligosaccharides by MALDI-TOF MS (A) and LC-ESI-MS (B) analysis.

3.1.3. Mass spectra analysis of P-FOS by MALDI-TOF MS, LC-ESI-MS, and LC-ESI-MS/MS

Mass spectra of P-FOS was analyzed by MALDI-TOF MS (Fig. 5A) and LC-ESI-MS (Fig. 5B). The peaks at m/z 583, 639, 695 by MALDI-TOF MS analysis represented the $[M+Na]^+$ ions of GF2 with 1-3 propionyl groups. The peaks at m/z 745, 801, 857, 907, 963, 1019 represented ionized GF3 and GF4 with 1-3 propionyl groups. The mass peaks of ionized GF5 with 1-4 propionyl groups were detected at m/z 1069, 1125, 1181, and 1237.

The LC-ESI-MS analysis of P-FOS had a similar pattern to that of MALDI-TOF MS analysis. The $[M-H]^-$, $[M-H+CH_2O_2]^-$ ions of GF2-GF5 with propionyl groups were detected. Ionized GF5 with 4 propionyl groups was not detected by LC-ESI-MS analysis. However, P-FOS was not well separated by liquid chromatography, and a future research about the separation of P-FOS compounds on liquid chromatography is needed. The presumed mass peaks of ionized propionyl GF6 and GF7 were additionally detected by LC-ESI-MS (data not shown). The mass peaks of various P-FOS compounds were detected by MALDI-TOF MS and LC-ESI-MS, but the mass peaks of FOS compounds were not detected. It is assumed that FOS was removed by purification.

Propionyl-1-kestoses (GF2) and propionyl-nystoses (GF3) were analyzed by LC-ESI-MS/MS. The major product ions of 1

propionyl-1-kestose is shown in Fig. 8A. The peak of product ions at m/z 503 and 485 were related to the loss of $C_3H_5O^+$ and $C_3H_5O_2^+$. As shown in Fig. 8B, the loss of $C_3H_5O^+$ and $C_3H_5O_2^+$ of 1 propionyl-nystose was also detected. The loss of $C_3H_5O^+$ and $C_3H_5O_2^+$ were also detected in the other propionyl-1-kestoses and propionyl-nystoses (Data not shown). By this result, ester linkage of P-FOS was reconfirmed.

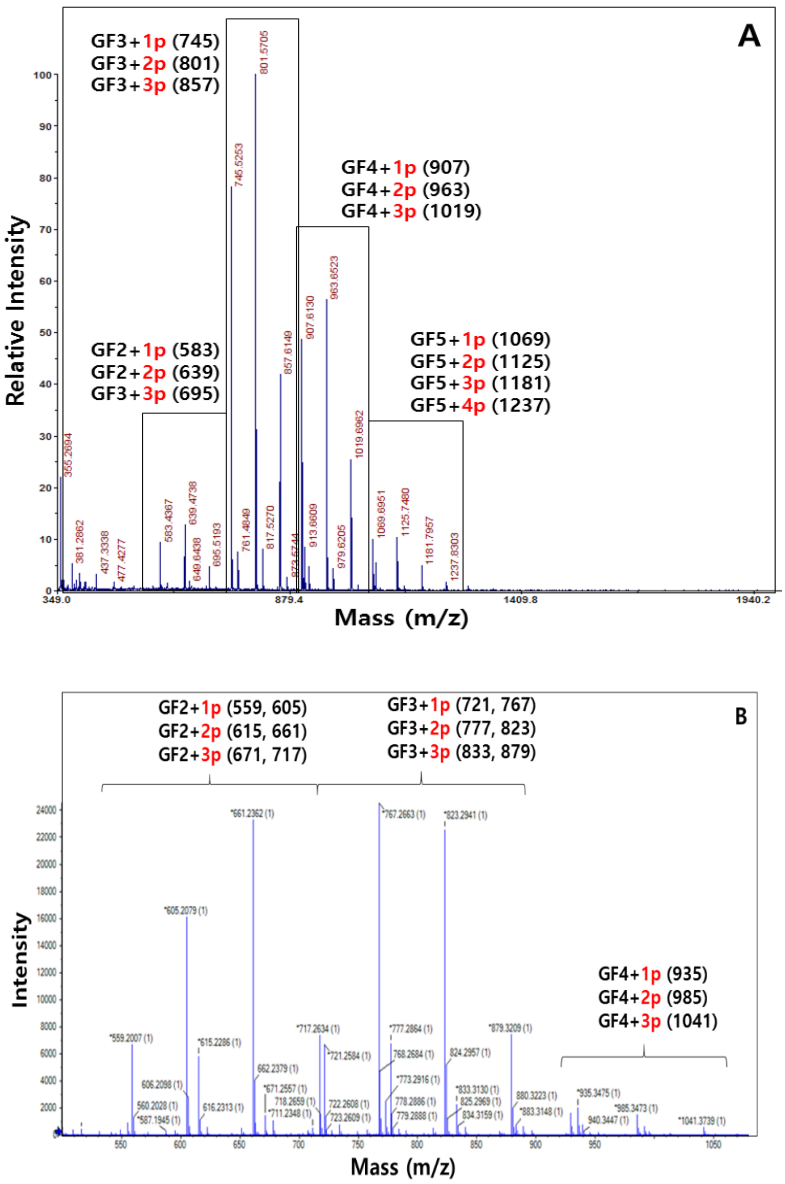

Fig. 4 Mass spectra of propionyl-fructooligosaccharides by MALDI-TOF MS (A) and LC-ESI-MS (B) analysis. +1p : with 1 propionyl group, +2p : with 2 propionyl groups, +3p : with 3 propionyl groups, +4p : with 4 propionyl groups.

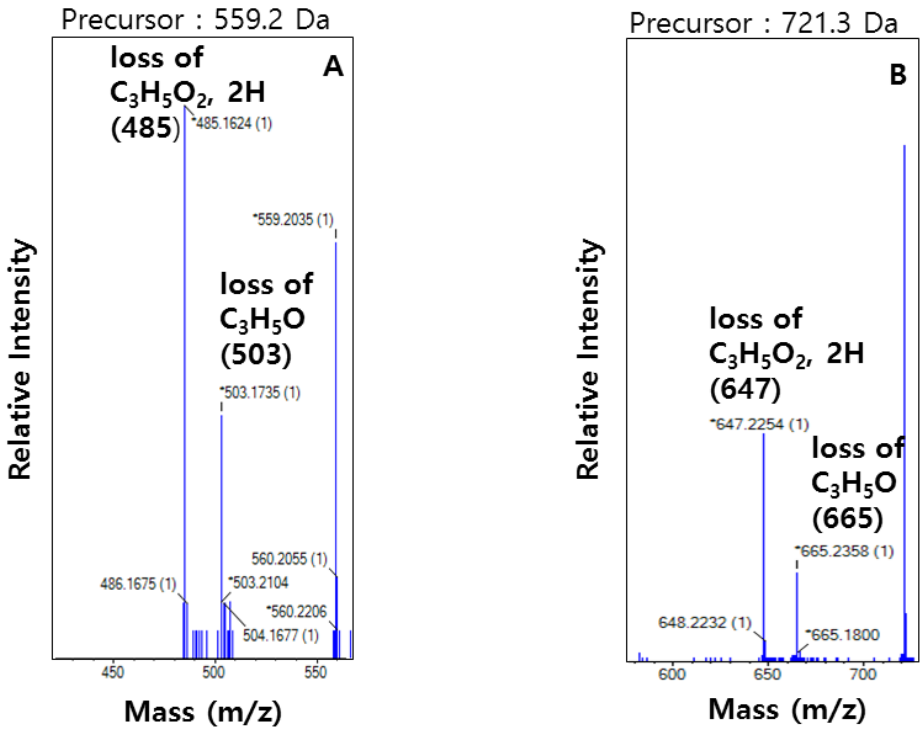

Fig. 5 LC-ESI-MS/MS data of 1 propionyl-1-kestose (A) and 1 propionyl-nystose (B).

3.2. Growth of various bacteria in the presence of P-FOS, FOS, and glucose

The growth of various bacterial strains on P-FOS, FOS, and glucose is shown in Fig. 6-8. Glucose was used well by all of the experimental bacteria. In the basal media, some of the bacteria grew very poorly and some of the other bacteria grew to some degree but evidently to a considerably lower level than in glucose-media.

The growth patterns of bifidobacteria are shown in Fig. 6. The growth of all bifidobacterial strains were promoted by FOS compared to the control. The growth of bifidobacteria was enhanced by P-FOS, except for *B. longum* RD47. *B. longum* BORI grew better on P-FOS than FOS. The growth patterns of LAB are shown in Fig. 7. Among LAB, *L. casei* did not grow on both FOS and P-FOS. FOS promoted considerable growth of *L. acidophilus* KCTC 3154, *L. acidophilus* KCTC 3168, *L. paracasei*, *L. plantarum*, and *S. thermophilus*. P-FOS promoted considerable growth of *L. acidophilus* KCTC 3168, *L. paracasei*, *L. plantarum*, *L. lactis*, and *S. thermophilus*. *L. paracasei*, *L. lactis*, and *S. thermophilus* grew better on P-FOS than FOS. P-FOS did not suppress the growth of all bifidobacteria and LAB. The growth of bifidobacteria and LAB was promoted by P-FOS in general.

The growth patterns of the non-probiotic bacterial strains are shown in Fig. 8. P-FOS showed no growth promotion or slight suppress against most of the non-probiotic bacteria except *B. cellulosilyticus*, *B. coprocola*, and *E. rectale*. Especially, the growth of S. aureus was significantly inhibited by P-FOS. As reported in previous study [16], FOS was well used by harmful bacteria such as *B. cellulosilyticus, B. coprocola*, *C. ramosum*, *E. cloacae*, *E. coli* KCTC 1039, and *E. rectale*. Especially, *B. cellulosilyticus* and *B. coprocola* grew better on FOS than glucose.

In this experiment, both FOS and P-FOS functioned as prebiotics by promoting the growth of bifidobacteria and LAB generally. However, the non-probiotic bacteria shows a tendency to use FOS more than P-FOS. With this point view, P-FOS has a greater merit than FOS as a prebiotic.

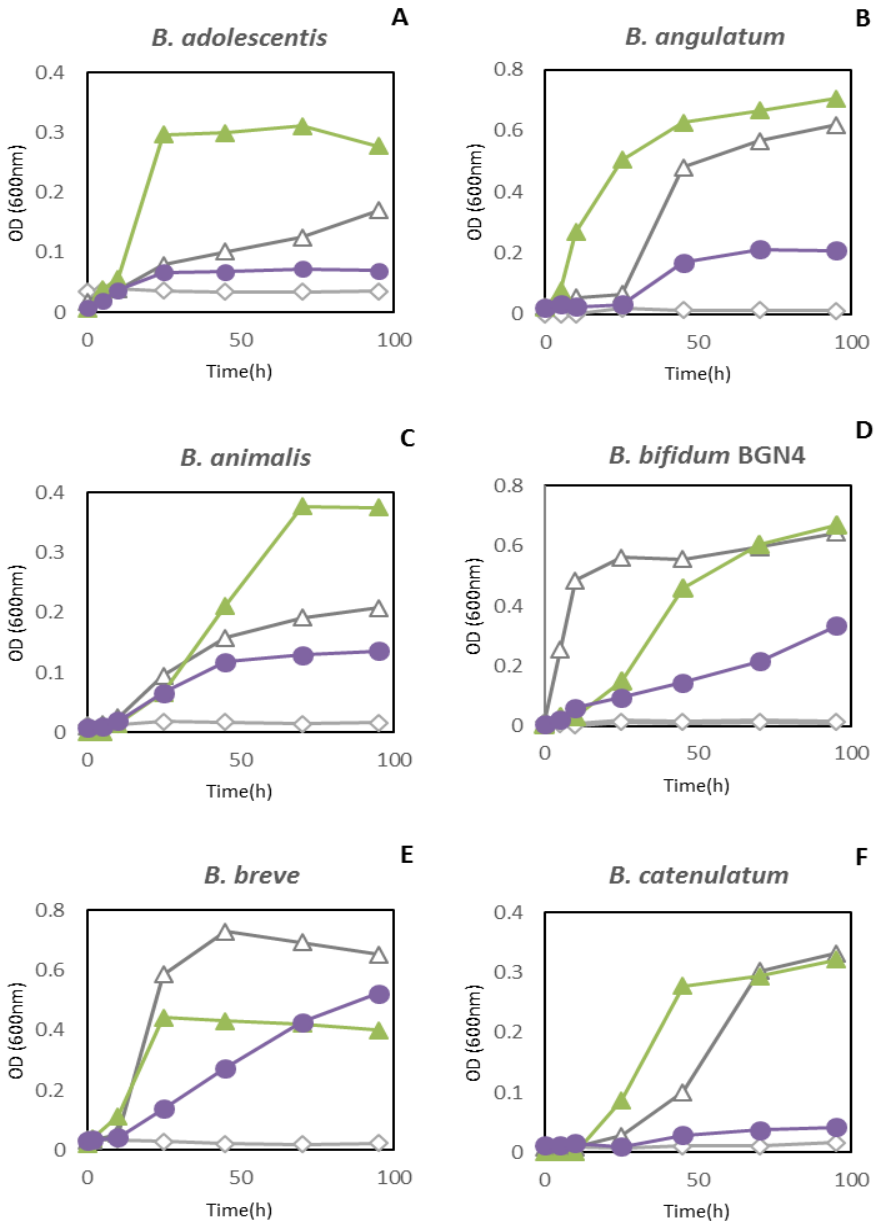

Fig. 6 Growth curve of bifidobacterial strains grown in media with different carbohydrate sources. Each medium with glucose (△), FOS (▲), P-FOS (●) and sterile DW (◇).

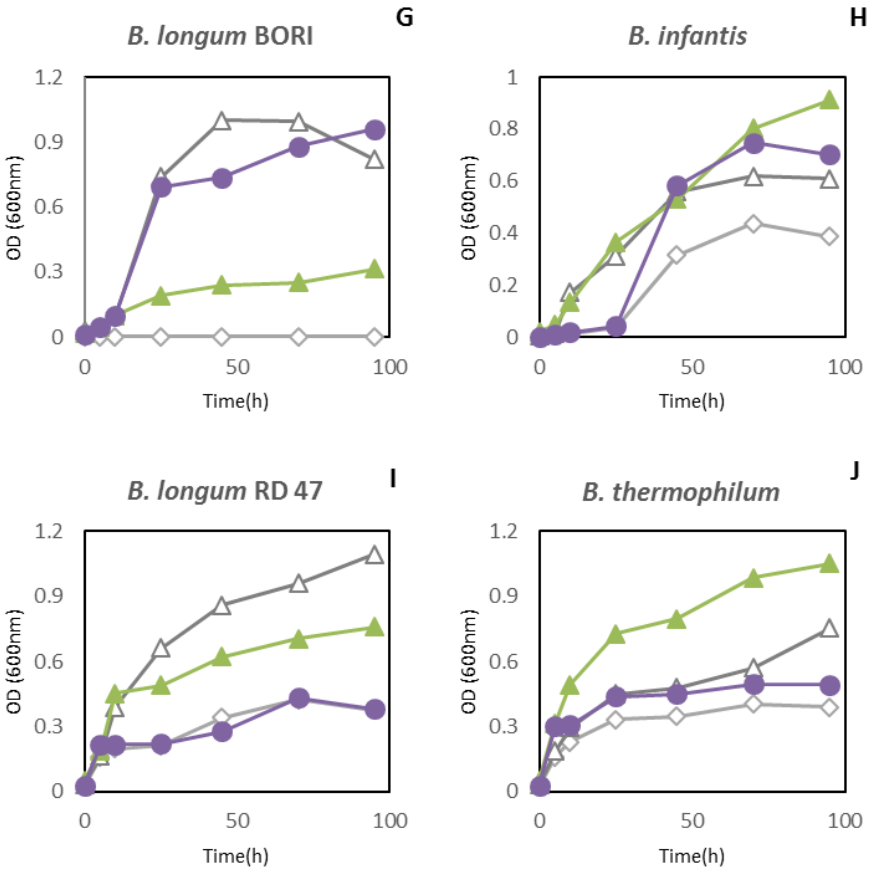

Fig. 6 (Continued) Each medium with glucose (△), FOS (▲), P-FOS (●) and sterile DW (◇).

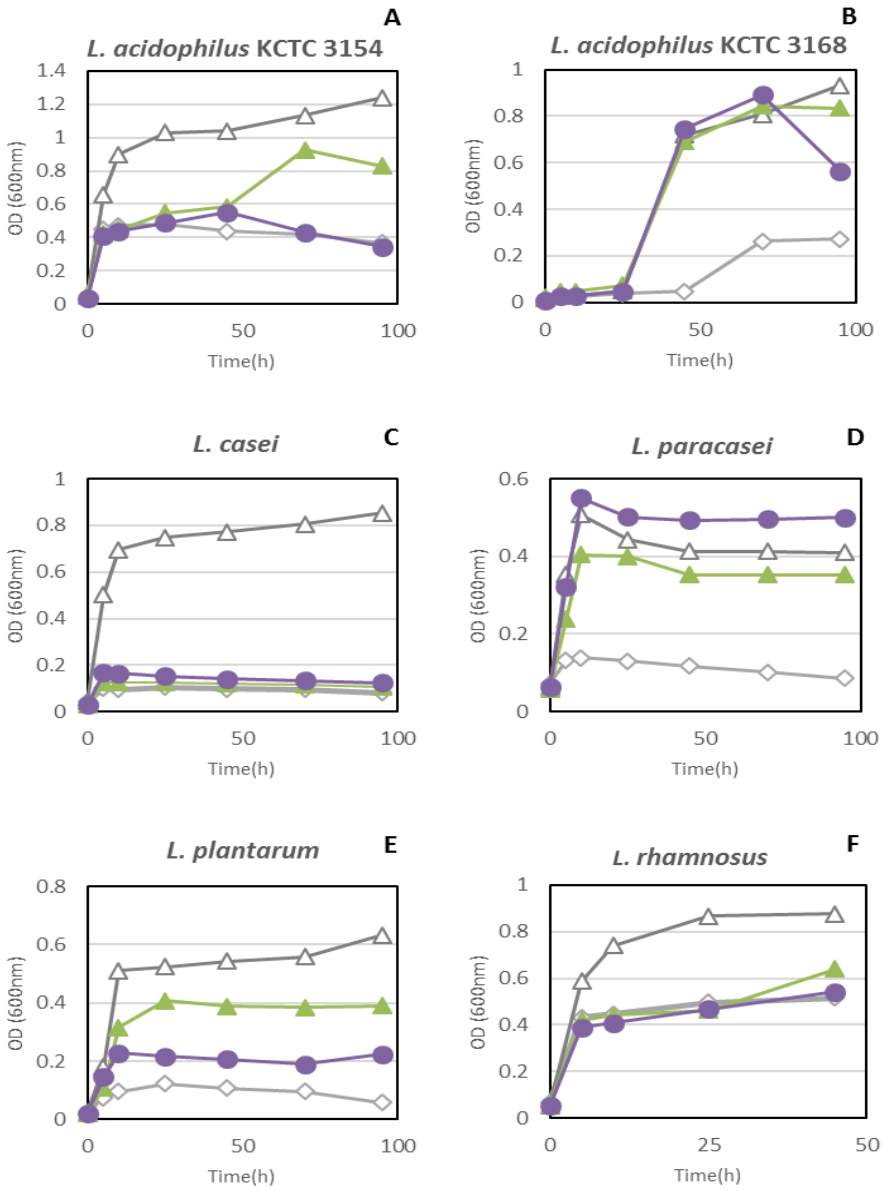

Fig. 7 Growth curve of lactic acid bacterial strains grown in media with different carbohydrate sources. Each medium with glucose (△), FOS (▲), P-FOS (●) and sterile DW (◇).

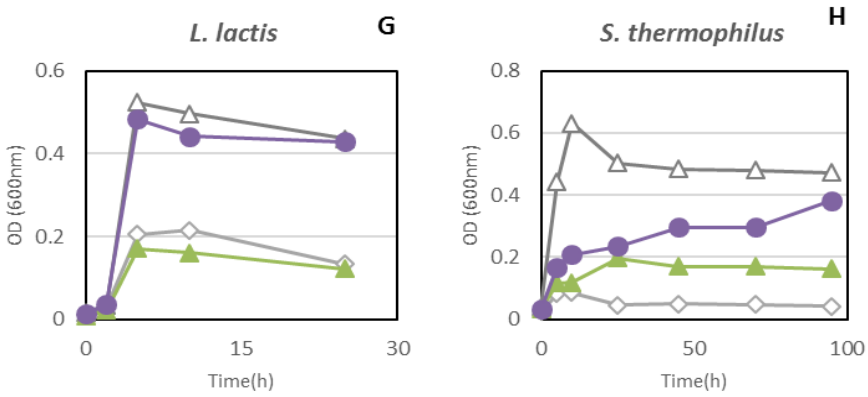

Fig. 7 (Continued) Each medium with glucose (△), FOS (▲), P-FOS (●) and sterile DW (◇).

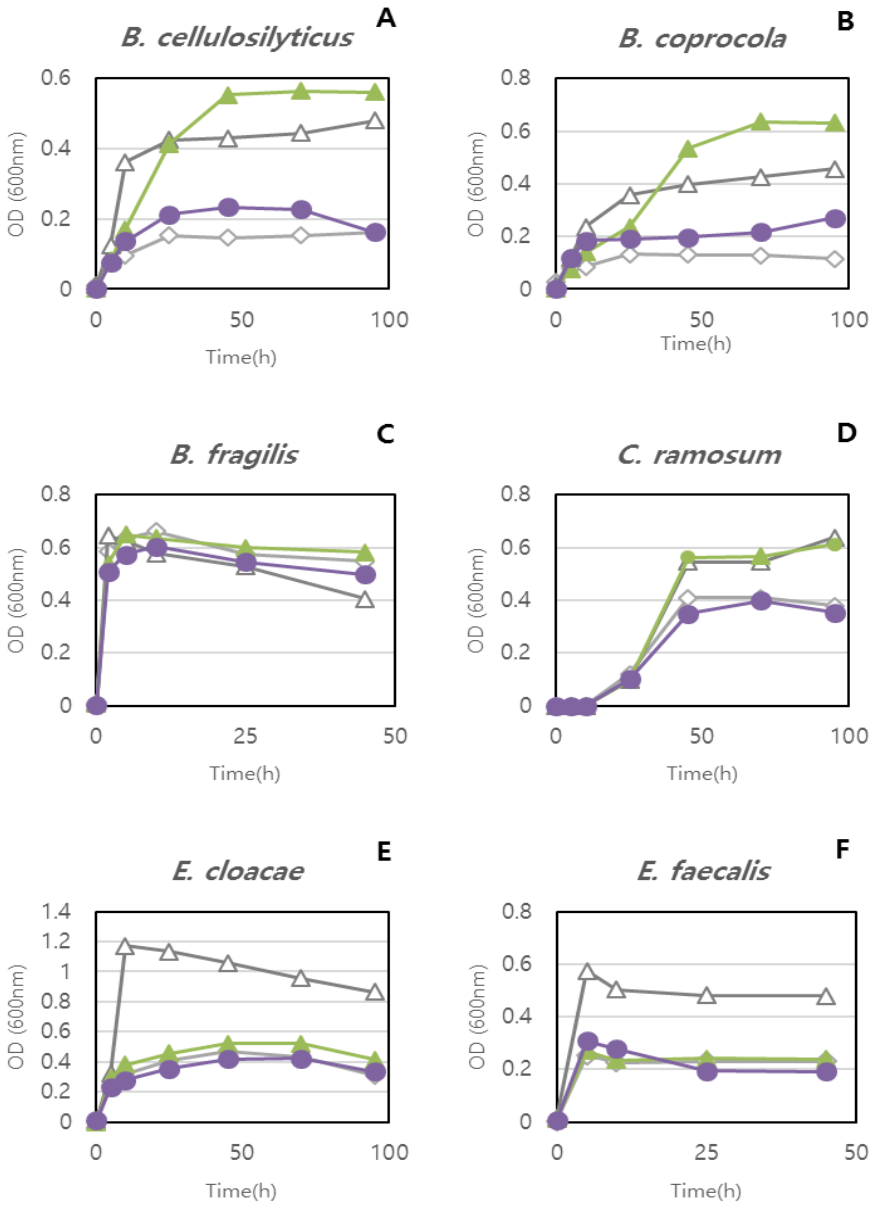

Fig. 8 Growth curve of non-probiotic bacterial strains grown in media with different carbohydrate sources. Each medium with glucose (△), FOS (▲), P-FOS (●) and sterile DW (◇).

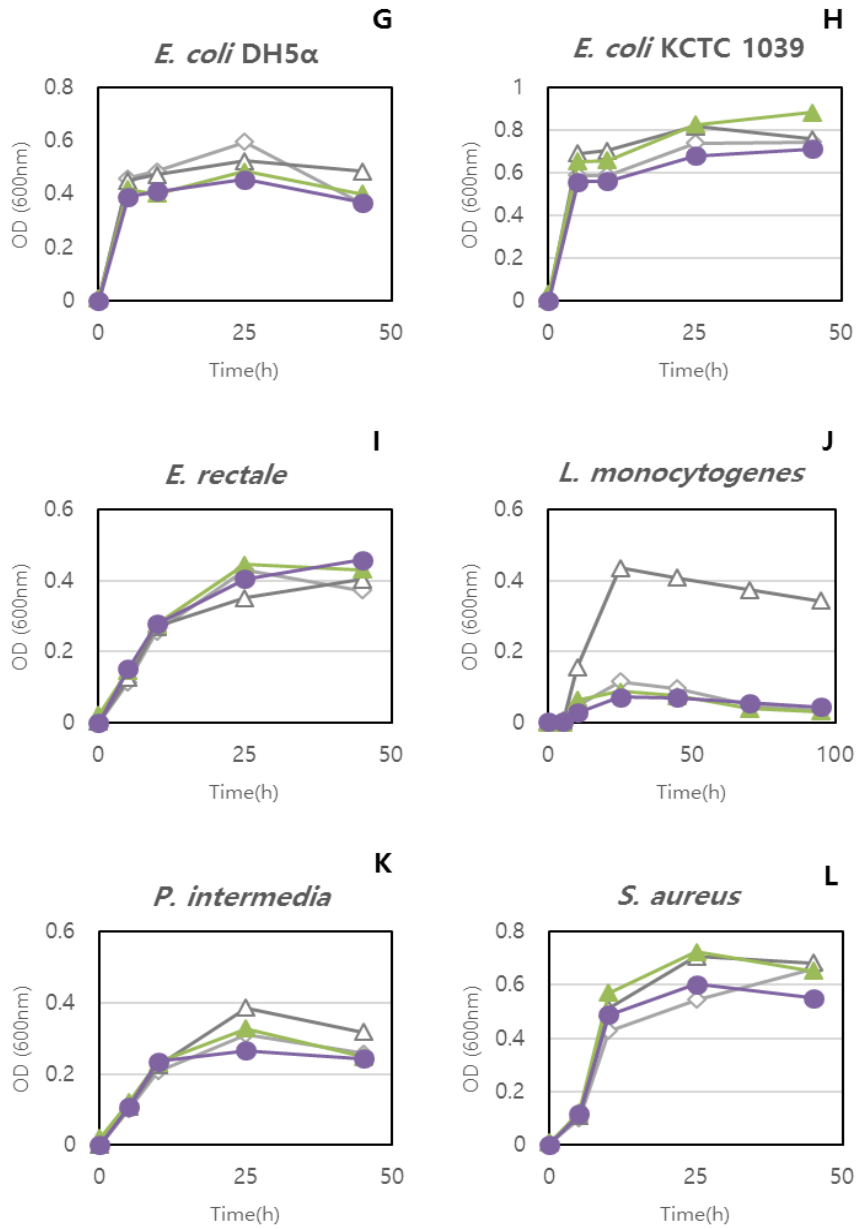

Fig. 8 (Continued) Each medium with glucose (△), FOS (▲), P-FOS (●) and sterile DW (◇).

4. Conclusion

Through the structure analyses of P-FOS by FT-IR, MALDI-TOF MS, LC-ESI-MS, and LC-ESI-MS/MS, it was found that propionyl groups were attached to FOS by ester linkage. Usually, 1-3 propionyl groups were attached.

The effect of purified P-FOS on bacterial growth was also tested in this study. The experimental bacterial strains included *Bifidobacterium*, *Lactobacillus*, *Lactococcus*, and *Streptococcus* which are known as beneficial bacteria to the host. P-FOS promoted their growth. Especially, *B. longum* BORI, *L. paracasei*, *L. lactis*, and *S. thermophilus* grew better on P-FOS than FOS. Moreover, the growth of other non-probiotic bacterial strains were further promoted by P-FOS media which were not shown in FOS media. Consequently, P-FOS may have a greater merit than FOS to improve gut microflora.

This is the first study about the effect on bacterial growth and structure of P-FOS.

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국문초록

선행 연구들에 의해 프락토올리고당 (Fructooligosaccharides)이 인간의 장내에 서식하는 유익균인 *Bifidobacterium* 의 성장을 촉진시킬 수 있음이 밝혀졌다. 따라서 프락토올리고당은 현재 프리바이오틱스로 이용되고 있다. 프로피온산 (Propionic acid)은 유해 곰팡이 및 균들의 성장을 저해하는 기능을 가지고 있다. 따라서 프로피온산나트륨, 프로피온산칼슘의 형태로써 식품 보존제로 이용되고 있다. 본 연구에서는 프로피오닐기가 결합된 새로운 형태의 프락토올리고당 (Propionyl-fructooligosaccharides, P-FOS)이 장내 균 성장에 미치는 영향을 평가하였다. FT-IR, MALDI-TOF MS, LC-ESI-MS, LC-ESI-MS/MS 를 이용하여 구조분석한 결과, P-FOS 는 FOS 에 주로 1-3 개의 프로피오닐기가 결합한 물질임이 밝혀졌다. 균 성장에 미치는 영향을 분석한 결과, P-FOS 는 실험에 사용된 대다수의 *Bifidobacterium* 과 일부 젖산균들의 성장을 촉진시킬 수 있음을 확인하였다. 프락토올리고당과 달리 P-FOS 는 대다수의 non-probiotics 균들의 성장을 촉진시키지 않거나 오히려 성장을 저해하였다. 본 연구에서는 신규 합성 물질인 P-FOS 가 추후 장내 균총 개선에 이용될 수 있는 가능성을 제시하였다.

주요어 : 프락토올리고당, 균 성장, 올리고당 구조분석

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