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Characterization and Prebiotic Activity of Aqueous Extract and Indigestible Polysaccharide from *Anoectochilus formosanus*

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ABSTRACT: *Anoectochilus formosanus* (Orchidaceae) is a folk medicine in Asia. This study investigated the in vivo and in vitro prebiotic effects of an aqueous extract of *A. formosanus* (SAEAF) and of an indigestible polysaccharide (AFP) isolated from SAEAF. Chemical analyses showed AFP was mainly composed of arabinogalactan type II (AG-II), with an average molecular weight of 29 kDa. Following 4 weeks of oral administration to rats, SAEAF exhibited prebiotic effects including a decrease in cecum pH and increases of calcium absorption and fecal bifidobacteria. Furthermore, through a bioactivity-guided separation strategy, AFP was proven to be a bifidogenic component in vitro fecal strains fermentation and in vivo administration to mice. In RT-PCR analysis of *Bifidobacterium*, AFP increased the expression of ABC transporter related to nutrient uptake. Thus, AFP, a polysaccharide from *A. formosanus*, was demonstrated to be a prebiotic that has a positive health effect on gut microbiota.

KEYWORDS: *Anoectochilus formosanus, calcium absorption, type II arabinogalactan, prebiotic*

INTRODUCTION

Several indigestible carbohydrates are potential prebiotics, such as inulin-type fructans or pectin.[1,2] These escape digestion in the upper gastrointestinal tract and reach the large intestine virtually intact, are quantitatively fermented, and have prebiotic properties.[1] Many studies have shown the effects of inulin-type fructans on the gut microbiota both in vivo and in vitro, yielding a selective stimulation of growth of the beneficial microbiota, namely, bifidobacteria.[1] The glycoside hydrolases produced from probiotics could be induced by related carbohydrates, allowing the breakdown of indigestible carbohydrates. Bifidobacteria are a cluster of anaerobic bacteria in the large intestine that benefit humans in improving mineral absorption.[1] The short-chain fatty acids (SCFAs) produced by prebiotic fermentation could reduce pH values in the large intestine. The lower pH increases mineral solubility and may result in enhanced calcium (Ca) absorption, or SCFAs may increase transcellular Ca absorption directly.[3]

Ca is required for normal bone growth and development. To minimize bone resorption in old age, maximizing peak bone mass during adolescence may be key to postponing or even preventing bone fractures caused by osteoporosis later in life. Thus, adequate Ca intake during adolescence is critical. One approach to accomplish this is by increased dietary intake of Ca. The body typically absorbs only approximately 30% of total dietary Ca that is deposited as bone.[4] Thus, searching for various dietary components to enhance mineral absorption and bone mineralization may assist bone health, which is a well-researched topic.

Anoectochilus formosanus Hayata (Orchidaceae) is a valuable medicinal plant broadly cultivated by tissue culture technology. *A. formosanus* is used in cuisine or as an herbal tea in Taiwan and other Asian countries. Pharmacological studies have indicated several beneficial properties of this herb including antihyperglycemia and antihyperliposis, with hepatoprotective properties.[5,6] Previous studies on aqueous extracts of *A. formosanus* have shown that it possesses antiosteoporosis properties.[7] The entire plant has also been used since ancient times as a folk medicine for the treatment of stunted growth in children. Its application in folk medicine has led to the speculation on whether *A. formosanus* could enhance Ca absorption and, moreover, its possible relationship to prebiotic action. Many previous studies indicated that inulin stimulates mineral absorption; however, inulin use may result in gastrointestinal discomfort, including bloating, flatulence, distension, loose stools, and increased stool frequency.[8] This study therefore examined the prebiotic action and Ca absorption effects of aqueous extracts of *A. formosanus* and isolated the active component type II arabinogalactan (AG-II) from *A. formosanus* using bioactivity-guided fractionation to understand the effects of this prebiotic.

MATERIALS AND METHODS

Preparation of a Standardized Aqueous Extract and Polysaccharide of *A. formosanus*. Cultured *A. formosanus* plants were purchased from Yu-Jung Farm (Pu-Li, ROC). The Institute of Chinese Pharmaceutical Science, China Medical University (Taichung, ROC) identified the plant, indexed under plant specimen CMCP 1253.

Our previous bioactivity-guided fractionation studies showed that the ethyl acetate fraction of *A. formosanus* aqueous extract promotes hepatitis in mice, caused by carbon tetrachloride.[6] A standardized aqueous extract of *A. formosanus* (SAEAF), from which the ethyl acetate fraction was removed, was prepared and used for this study. Briefly, fresh whole plants of cultured *A. formosanus* were homogenized and extracted with distilled water, and the filtrate was partitioned with

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ethyl acetate. The aqueous fraction was filtered and evaporated under reduced pressure to yield a purpuric residue, SAEAF, with a yield of approximately 2.8% (w/w).

For polysaccharide preparation, the SAEAF solution (100 mg/mL) was precipitated by adding a 4-fold volume of 95% ethanol, stored at 4 °C overnight, and then centrifuged. The precipitate was collected and redissolved in distilled water, and the insoluble part was discarded. The water-soluble fraction was then filtered to obtain the crude polysaccharide (AFCP). Protein and starch were removed from AFCP according to method 991.43 of the Association of Official Analytical Chemists, using a total dietary fiber assay kit (Megazyme, Wicklow, Ireland). The AFCP was sequentially treated with α-amylase, protease, and amyloglucosidase. After digestion by the enzymes, a 4-fold volume of 95% ethanol was added and then stored at 4 °C overnight to precipitate the indigestible polysaccharide (AFP). After centrifugation, the precipitate was wash three times with 95% ethanol and then stored in 75% ethanol before use. For analysis and experimental uses, AFP was redissolved in hot water and diluted by distilled water.

Isolation and Characterization of AFP. The protein content of AFP was determined by using a modified Bradford method assay with bovine serum albumin as the standard.[9] The carbohydrate content of polysaccharides was determined by using the phenol−sulfuric acid method with glucose as the standard.[10] The content of uronic acid was determined by the *m*-hydroxydiphenyl method using galacturonic acid as the standard.[11]

AFP relative molecular mass (M_r) was determined by high-performance size exclusion chromatography using a TSKgel guard column PWH (75 × 7.5 mm i.d.; TOSOH, Tokyo, Japan), TSKgel $G4000_{PWXL}$, and $G2500_{PWXL}$ (300 × 7.8 mm i.d., TOSOH) connected in series. The column was eluted with 0.3 N sodium nitrate solution containing 0.02% sodium azide with a flow rate of 0.8 mL/min at 65 °C. Peaks were detected by an Interferometric Refractometer (Wyatt, CA, USA). The average M_r of the AFP was estimated by comparison with the retention time of pullulan standard P-82 kit (molecular weights of standards: P-800, 708 kDa; P-400, 375 kDa; P-200, 200 kDa; P-100, 107 kDa; P-50, 47.1 kDa; P-20, 21.1 kDa; P-10, 9.6 kDa; and P-5, 5.9 kDa; Shodex, Kanadawa, Japan).

The presence of AG-II was detected by the β-glucosyl-Yariv reagent (Biosupplies, Bundoora, Australia) affinity test using gum arabic (4 mg/mL) as a positive control.[12] A positive reaction was identified by a reddish circle forming around the well.

For monosaccharide composition analysis, samples were hydrolyzed at 100 °C for 4 h with 2 M trifluoroacetic acid (Sigma-Aldrich, St. Louis, MO, USA), and the resulting samples were determined by high-performance anion exchange chromatography with pulsed amperometric detection (HPAEC-PAD). The peaks were detected by a 817 Bioscan PAD detector (Metrohm, Zofingen, Switzerland) using a CarboPac PA1 column (Dionex, Sunnyvale, CA, USA). The eluent was 10 mM sodium hydroxide and 1 mM barium acetate (Sigma-Aldrich) at a flow rate of 1.0 mL/min.

Linkage analysis was determined by methylation analysis according to the method by Liane et al.[13] Methyl iodide (Sigma-Aldrich) in DMSO and sodium hydroxide were applied to methylation. After methylation, samples were hydrolyzed by 2 M trifluoroacetate, followed by silylation with *N,O*-bis(trimethylsilyl)trifluoroacetamide containing 10% trimethylchlorosilane (Sigma-Aldrich). GC-MS of samples was conducted on a 30 m, 0.25 mm, HP-5 column (film thickness = 0.25 μm) with a detector (HP5973 series mass selective detector) at the following temperature program: 100 °C for 2 min; the gradient was warmed at 4 °C/min to 220 °C (lasted 2 min); when it reached 220 °C, the temperature was increased at a rate of 15 °C/min until to 300 °C (lasted 2 min). The carrier gas was helium (1 mL/min, constant flow). The detector conditions were 70 eV and 40−600 amu. Identification of the sugar components and linkage was based on matching the mass spectra of the Wiley library of the mass spectral database and relative retention time of the TMS derivative.

Animals. Wistar male rats (4 weeks old) and BALB/C male mice were purchased from BioLASCO Co., Ltd. (Taipei, ROC). All conditions and treatments of animals were approved by the Institutional Animal Care and Use Committee of China Medical University. The experimental animals received humane care, and the study protocols complied well with the China Medical University institutional guidelines for use of laboratory animals. The animals were housed in an air-conditioned room (21−24 °C) with 12 h of light (7:00 a.m.−7:00 p.m.) and were allowed free access to food pellets and water throughout the study.

Studies on Ca Balance and Prebiotic Activity of SAEAF in Growing Rats. Forty rats weighing 140−150 g each were divided into four groups according to their body weights. The animals in each group received orally H_2O, SAEAF (200 or 400 mg/kg body weight per day), or inulin (400 mg/kg body weight per day) for 4 weeks. The purity of inulin (Alfa Aesar, Heysham, UK) used in this paper was 98% according to the manufacturer.

The method used for studying Ca balance was as described in our previous paper.[14] During the period spanning days 21−26 following SAEAF administration, all of the rats were housed in individual metabolism assessment cages for collecting the feces and urine separately.

Food consumption was monitored daily during the 4 day metabolic balance study. Portions of the urine samples were acidified with 12 M HCl (Wako, Osaka, Japan; 225 μL/dL urine) and stored at −20 °C until analysis. The fecal samples were dried at 100 °C. Feed and dried fecal samples were ash-dried at 700 °C for 12 h. The feed ash and fecal ash were each solubilized with 6 M HCl in preparation for the Ca assay. Total Ca was determined according to the *o*-cresolphthalein complex one method using a commercial kit (Randox, Crumlin, UK).

Apparent Ca absorption, apparent Ca absorption rate, and apparent Ca balance were calculated using eqs 1, 2, and 3, respectively: (eq 1) apparent Ca absorption = Ca intake − fecal Ca; (eq 2) apparent Ca absorption rate (%) = 100 × (Ca absorption/Ca intake); and (eq 3) apparent Ca balance = Ca intake − fecal Ca − urinary Ca.

For determination of bacteria in feces, fresh feces were collected directly from each rat on day 28 after SAEAF administration, weighed, and immediately placed in solution. After homogenization of the feces, serial decimal dilutions (0.1% peptone solution) were prepared, taking care to avoid aeration. The numbers of *Bifidobacterium* spp. and *Clostridium perfringens* populations were counted on bifidobacteria iodoacetate medium-25 agar and tryptose-sulfite-D-cycloserine agar (Oxoid, Hampshire, UK), respectively, after incubation at 37 °C for 72 h in an anaerobic oven with a controlled atmosphere (95% N_2, 3% CO_2, and 2% H_2).[15,16] The results were expressed as the logs of colony-forming units (CFUs) per gram of feces.

After blood was drawn from the rats between 8:00 a.m. and 10:00 a.m. on day 29, the animals were sacrificed, and the cecum and its content were immediately removed and weighed. The cecal contents were transferred into tubes; pH was measured by IQ 150 pH-meter (Spectrum Technologies, IL, USA), and the samples were then centrifuged at 1500*g* at 4 °C for 10 min. The supernatant was diluted for the subsequent Ca assay. Ca concentrations of blood plasma and cecal contents were assayed using commercial test kits (Randox).

In Vitro Fermentation by Collection Strains. We studied the growth of strains of intestinal bacteria using in vitro fermentation. The strains used in this study, *Bifidobacterium longum* (ATCC 15697), *Bifidobacterium breve* (ATCC 15698), *Lactobacillus acidophilus* (ATCC 4356), and *C. perfringens* (ATCC 27060), were purchased from the Bioresource Collection and Research Center (Hsinchu, ROC). Lactobacilli MRS broth (Difco, MD, USA) with 0.05% cysteine (Wako) was used for the culture of *B. longum* and *B. breve*. Lactobacilli MRS broth was used for *L. acidophilus*, and reinforced clostridial medium (RCM; Difco) was used for the *C. perfringens* culture.

The media were inoculated with 10^5−10^6 CFUs/mL of active strains and incubated for up to 48 h. Each fermentation test was incubated at 37 °C and conducted in triplicate. All additions, inoculations, and incubations were conducted under anaerobic condition. The optical density (OD) value of fermentation products was measured at 600 nm on a spectrophotometer (Hitachi, Tokyo, Japan) every hour, from 0 to 24 h. The relationship between the OD_{600} value and the number of bacteria was determined previously by

counting CFUs per milliliter on the MRS or RCM agar plates at 37 °C after 48 h under anaerobic conditions.

A growth curve plotting the logarithm of the number of organisms against time showed a lag phase immediately after $t = 0$, followed by an exponential growth phase. The growth curve is described by $[y = \ln(N_t/N_0)]$ against time (x), where N_t and N_0 are optical density measurements at time t and time 0, respectively. The specific growth rate, μ, is defined as the x-axis intercept of the tangent at any given point.[17]

SAEAF was dissolved into MRS or RCM to provide final serial concentrations. The fermentation broth OD_{600} was measured for *B. breve* and *B. longum* after 18 h of incubation. Fermentation broth OD_{600} was measured for *L. acidophilus* and *C. perfringens* after 5 h of incubation. The strain *B. breve* was used to study the effects of AFP and inulin on the growth of bifidobacteria. AFP and inulin were dissolved in MRS to yield final serial concentrations. The effects of AFP and inulin on *B. breve* growth kinetic curves were determined. To confirm the prebiotic activity of AG-II, the AG-II portion in AFP was removed by precipitation with β-glucosyl-Yariv reagent according to the method of Classen and Blaschek.[18] The non-AG-II polysaccharides in AFP remained in the supernatant. The supernatant was added to the broth for *B. breve* fermentation test.

The pH values and SCFA contents were determined by a pH-meter and HPLC. The peaks were detected by a Shodex RI-71 refractive index detector (Showa Denko, Tokyo, Japan) using a Transgenomic ICSep Transgenomic (300 × 7.8 mm, Omaha, NE, USA) at 65 °C and eluted with 0.0085 N sulfuric acid at a flow rate of 0.4 mL min^{-1}.

Gene expression analysis by RT-PCR of *B. breve* was conducted at various concentrations of AFP in the MRS broth at 9 and 18 h of cultivation. The RT-PCR process used an RNA Purification Kit (Epicenter, WI, USA) in accordance with the method of Yuan et al.[19] Synthetic cDNA (1 μg) was used as a template for PCR amplification in a PCR system 9700 machine (Applied Biosystem, CA, USA) with primers as shown in Table 1. The amplicons were electrophoresed in 2% agarose gels containing ethidium bromide staining.

In Vitro Fermentation by Mouse Fecal Microbiota. The broth used in the fermentation experiments was modified from that described by Sanz et al.[20] to determine the growth of fecal bacteria in response to different concentrations of SAEAF, AFP, and inulin as a positive control. The broth was inoculated with mouse fecal slurry to yield a final concentration of 0.1% v/v. All incubations were conducted under anaerobic condition. Samples of the fermentation solution were taken at the start and after 24 h of fermentation for (1) amplification of bacteria by the PCR and (2) determination of the pH.

After fermentation, the samples were centrifuged to collect the bacterial pellets. Total DNA isolation and PCR were based on those proposed by Moura et al.[21] The DNA extracts were used as PCR templates and stored at −80 °C until the PCR assay. All primer sets were designed from 16S rRNA gene sequences, as shown in Table 1. We used the GeneBank program BLAST to confirm that the candidate primers were complementary only with the target species and not with other species. PCR tests were conducted using the isolated DNAs. Samples (2 μg) were directly added to a mixture of Mastermix (Fermentas, MD, USA) at the volume of 50 μL and 0.25 mM of each primer.

Studies on Prebiotic Activity of AFP in Mice. Twenty-eight male BALB/c mice of approximately 22 g body weight were used. Mice were randomly divided into four groups and orally treated with H_2O, inulin (400 mg/kg), or AFP (10, 20 mg/kg) daily. On days 3 and 7, feces were collected for bifidobacteria analysis. The methods were as previously described. After incubation, single colonies were counted, and the results were expressed as the log values of the CFUs per gram of feces.

Statistical Analysis. Results were expressed as the mean ± SD. All experimental data were analyzed using one-way analysis of variance with the Dunnett test. Values of $p < 0.05$ were considered to be statistically significant.

Table 1. Primers Used in This Study

gene	primer sequence[a]
ATP-binding cassette (ABC) transporter	F: CAACCTCCCTCTGACGAG R: GATGGACTCCTCCAGCTC
ATPase	F: TATGCTGCGACCAGCAGAAC R: CGCCATTAGTACCCGGCAAA
α-L-arabinofuranosidase	F: CGTCTGTTCGGCTCATTCGTA R: AGCTCCTTGACCAGGTCAAGCA
β-galactosidase	F: CCTACTCAGGAGGTACGC R: CCAGACCGGTGTAGTAGG
16S rRNA	F: GTCAGCTCGTGTCGTGAG R: GTCGCATCCCGTTGTACC
Escherichia coli	F: CGACGATCCCTAGCTGGTCT R: GAGTTAGCCGGTGCTTCTT
Bifidobacterium sp.	F: GTCAGCTCGTGTCGTGAG R: GTCGCATCCCGTTGTACC
Clostridium sp.	F: GACGGGTGAGTAACACGTGG R: TTGCTGCATCAGGGTTTCCC
Lactobacillus sp.	F: GACGGGTGAGTAACACGTGG R: CGCAGGTCCATCCAAGAGTG

[a]F, forward primer; R, reverse primer.

RESULTS

Characterization of AFP. The yield percentages of crude polysaccharide (AFCP) and indigestible polysaccharide (AFP) from *A. formosanus* were 0.75 and 0.15%, respectively, on a fresh plant basis (Table 2). Thus, AFCP contained 20% AFP that consisted of 95.5% carbohydrate and 1.0% protein. The content of uronic acid was 10.4% on a carbohydrate basis (Table 2). The average molecular weight of AFP was 29 kDa. Analysis of sugar composition revealed that AFP comprised monosaccharide units such as arabinose, galactose, glucose, and mannose (Table 3). The ratio of arabinose, galactose, glucose, and mannose is shown in Table 3. AFP contained mainly galactose units, with arabinose as a minor component, whereas glucose and mannose were present in lower proportions.

Table 2. Chemical Composition of Polysaccharide Fractions from *A. formosanus*

		protein[b]/carbohydrate[c]			
fraction	yield[a] (%)	ratio	%	uronic acid[d] (%)	M_r[e] (kDa)
AFCP	0.75	8.2	91.6	8.1	55
AFP	0.15	1.0	95.5	10.4	29

[a]The yield rate was on fresh plant basis. The moisture content of fresh plant was 89%. [b]Protein values by Coomassie blue method using bovine serum albumin as standard. [c]Carbohydrate values by colorimetric phenol−sulfuric acid method with glucose as standard. [d]Used galacturonic acid as standard. [e]Used pullulans standard P-82 as standard.

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Table 3. Linkage Analysis of the Indigestible Polysaccharide AFP from *A. formosanus*

carbohydrate	monosaccharide composition (%)	linkage	linkage composition[a] (%)
arabinose	22.38	terminal	22.72
galactose	56.54	1,3,4-Gal*p*	5.18
		1,3,6-Gal*p*	50.76
glucose	15.35	terminal	2.76
		1,4-Glc*p*	9.97
		1,6-Glc*p*	2.53
mannose	5.37	terminal	0.55
		1,4-Man*p*	4.37

[a]The percentage of the linkage composition is based on the total carbohydrate amount.

The Yariv test showed that AFP could make red precipitation diffusion circles, suggesting that it possessed AG-II characteristics.[12] The types of linkages present in AFP are listed in Table 3. The presence of a high ratio of 2, 4-di-*O*-methylgalactosyl residue in methylation analysis indicated that 3,6-linked galactosyl was the major component of AFP. On the basis of the appearance of 2,3,5-tri-*O*-methylarabinosyl during methylation analysis, terminally linked arabinofuranosyl was another major component in AFP. Thus, the AFP contained highly branched arabinogalactan with a 3,6-linked galactosyl main core and arabinosyl termini and polysaccharides with glucosyl and mannosyl residues in much lower amounts.[12]

Studies on Ca Balance and Prebiotic Activity of SAEAF in Growing Rats. Table 4 shows a summary of the study results. The body weights among the control and SAEAF- and inulin-treated groups showed no significant differences. Ca intake and fecal Ca content were measured during the Ca balance studies and represented as 4 day mean averages. The Ca intake and the fecal Ca content showed no significant differences. The apparent Ca absorption and apparent Ca absorption rates for the SAEAF (400 mg/kg) and inulin groups were greater than those of the control group. Administration of SAEAF and inulin did not affect the amount of Ca excreted in the urine. Finally, the apparent Ca retentions in the SAEAF (400 mg/kg) and inulin groups were significantly greater than that for the control group.

Table 5 shows that the numbers of fecal *C. perfringens* were unaffected by SAEAF and inulin treatments. By contrast, rats administered SAEAF (400 mg/kg) and inulin displayed greater numbers of bifidobacteria in feces compared with the control rats.

Table 5. Effect of Aqueous Extract of *A. formosanus* (SAEAF) and Inulin on Fecal *Bifidobacterium* and *C. pergringens* Growth in Young Rats[a]

group	dose (mg/kg)	*Bifidobacterium* (log CFUs/g feces)	*C. perfringens* (log CFUs/g feces)
control		6.75 ± 0.19	5.90 ± 0.19
SAEAF	200	7.17 ± 0.20	5.92 ± 0.19
SAEAF	400	7.46 ± 0.16**	5.89 ± 0.18
inulin	400	7.26 ± 0.17*	5.81 ± 0.17

[a]All values are the mean ± SD (n = 8). *, $p < 0.05$; and **, $p < 0.01$, as compared with the control group.

When animals were sacrificed, the serum Ca concentrations in the SAEAF-treated (200 and 400 mg/kg) and inulin-treated groups were significantly higher than those in the control group (Table 6). The cecal content pH was lower in the SAEAF (400 mg/kg) and inulin groups than it was in the control rats (Table 6). SAEAF (400 mg/kg) and inulin treatments significantly increased the soluble Ca concentrations to 133 and 122%, respectively.

Table 6. Effect of the Aqueous Extract of *A. formosanus* (SAEAF) and Inulin on the Calcium Concentration of Serum and Cecum (Soluble) and Cecal pH in Rats[a]

treatment	dose (mg/kg)	serum Ca (mg/dL)	cecum soluble Ca (mg/dL)	cecal pH
control		9.0 ± 0.4	2.7 ± 0.6	6.6 ± 0.1
SAEAF	200	9.7 ± 0.3*	3.0 ± 0.8	6.5 ± 0.2
SAEAF	400	10.2 ± 0.8**	3.6 ± 1.5***	6.3 ± 0.1**
inulin	400	9.7 ± 0.4*	3.3 ± 0.5*	6.2 ± 0.1**

[a]All values are the mean ± SD (n = 8). *, $p < 0.05$; and **, $p < 0.01$, as compared with the control group.

In Vitro Fermentation by Collection Strains. Turbidity is linearly proportional to the number of colonies counted after incubation; formulas and r values describing the relationship between CFU concentration and turbidity are as follows: Y (*B. breve*) = $(1361X - 11.74) \times 10^7$, $r^2 = 0.9871$; Y (*B. longum*) = $(1094X - 0.557) \times 10^7$, $r^2 = 0.996$; Y (*L. acidophilus*) = $(4.14X - 2.84) \times 10^4$, $r^2 = 1$; and Y (*C. perfringens*) = $(40X - 2) \times 10^8$, $r^2 = 0.970$, where Y represents CFUs per milliliter and X represents OD_{600} values. This formula indicates that turbidity correlates with the number of colonies and, thus, turbidity was

Table 4. Effect of Aqueous Extract of *A. formosanus* (SAEAF) and Inulin on Intestinal Absorption and Retention of Calcium in Rats[a]

parameter	control	SAEAF 200 mg/kg	SAEAF 400 mg/kg	inulin 400 mg/kg
initial body wt (g)	145.5 ± 14.5	143.6 ± 11.4	147.8 ± 15.0	145.3 ± 15.8
final body wt (g)	237.9 ± 15.8	239.0 ± 15.5	243.7 ± 12.0	238.4 ± 16.5
Ca intake (mg)	115.2 ± 10.4	115.4 ± 8.5	118.5 ± 23.5	111.6 ± 21.5
fecal Ca (mg)	102.6 ± 9.3	95.7 ± 10.7	91.6 ± 17.5	91.2 ± 16.0
urinary Ca (mg)	1.7 ± 0.9	1.8 ± 0.9	2.5 ± 2.0	1.4 ± 0.5
absorption (mg)	12.6 ± 5.3	19.7 ± 8.1	26.9 ± 8.5**	20.4 ± 8.1
absorption (%)	10.8 ± 4.1	17.1 ± 7.0	22.4 ± 4.4**	18.3 ± 4.4*
Ca retention (mg)	10.8 ± 13.5	17.9 ± 8.1	24.4 ± 7.8**	19.0 ± 8.1**

[a]All values are the mean ± SD (n = 8). *, $p < 0.05$; **, $p < 0.01$; and ***,$p < 0.001$, as compared with the control group.

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Table 7. Effect of Aqueous Extract of *A. formosanus* (SAEAF) on the Growth of *B. breve*, *B. longum*, *L. acidophilus*, and *C. perfrigens* in Vitro Fermentation[a]

		OD_{600}			
	concn (mg/mL)	*B. breve*	*B. longum*	*L. acidophilus*	*C. perfrigens*
control		0.015 ± 0.01	0.18 ± 0.00	0.41 ± 0.04	0.24 ± 0.03
SAEAF	5	0.056 ± 0.01	0.140 ± 0.02	0.40 ± 0.03	0.21 ± 0.12
SAEAF	10	0.100 ± 0.02***	0.178 ± 0.03	0.42 ± 0.02	0.15 ± 0.08
SAEAF	50	0.200 ± 0.03***	0.271 ± 0.02**	0.45 ± 0.06	0.09 ± 0.02**

[a]All values are the mean ± SD (n = 4). *, $p < 0.05$; **, $p < 0.01$; and ***, $p < 0.001$, as compared with the control group.

Table 8. Effects of Indigestible Polysaccharide AFP and Inulin on the Growth of *B. breve* in Vitro Fermentation[a]

treatment	concn (mg/mL)	μ_m (h^{-1})	OD_{600}	pH	total SCFA (mmol/L)
control		0.20 ± 0.10	0.198 ± 0.004	5.1 ± 0.1	43.4 ± 8.4
AFP	0.5	0.41 ± 0.10*	0.297 ± 0.004**	5.0 ± 0.1	49.6 ± 8.5
	1.0	0.43 ± 0.07*	0.303 ± 0.004**	4.8 ± 0.0*	61.1 ± 2.9*
	2.0	0.49 ± 0.09**	0.319 ± 0.004**	4.7 ± 0.1**	82.1 ± 5.3**
inulin	1.0	0.44 ± 0.10*	0.221 ± 0.004	4.9 ± 0.1	51.3 ± 4.9
	2.0	0.47 ± 0.09**	0.281 ± 0.005*	4.9 ± 0.1	64.2 ± 3.8*
	5.0	0.52 ± 0.06**	0.315 ± 0.005**	4.5 ± 0.1***	95.5 ± 5.4***

[a]All values are the mean ± SD (n = 6). *, $p < 0.05$; **, $p < 0.01$; and ***, $p < 0.001$, compared with control group.

used to represent the number of colonies in the following discussion.

In the *B. breve* and *B. longum* growth kinetic curves, the maximum specific growth rates, μ_m, occurring 18 h after inoculation, were 0.201 h^{-1} for *B. breve* and 0.173 h^{-1} for *B. longum*. The maximum μ_m values for *L. acidophilus* and *C. perfringens*, both appearing at 5 h postinoculation, were 0.223 and 0.353 h^{-1}, respectively. As shown in Table 7, SAEAF administration produced a significant increase in the growth of *B. breve* and slightly up-regulated *B. longum* growth, although SAEAF had no observable effect on *L. acidophilus* and even decreased the number of *C. perfringens* present. SAEAF has a remarkable bifidogenic effect in vitro, raising the question as to which SAEAF component was the major bifidogenic fraction.

Ethanol was added to precipitate AFCP from SAEAF. AFCP was found to enhance the growth of *B. breve* (data not shown). AFP markedly increased the proliferation of *B. breve*. The Yariv precipitation showed the content of AG-II was >80% of AFP, with non-AG-II polysaccharides of <20%. The non-AG-II polysaccharide remaining in the supernatant was used for the fermentation by *B. breve*. The results showed that the non-AG-II polysaccharides in supernatant exhibited no effect on the growth of *B. breve* after 18 h of fermentation (control, OD_{600} = 0.199 ± 0.003, pH 5.2 ± 0.1; supernatant 2 mg/mL, OD_{600} = 0.200 ± 0.004, pH 5.1 ± 0.2).

A plot of *B. breve* incubation time versus turbidity shows that μ_m = 0.201 and occurs 18 h postincubation (Table 8). When AFP was added at various concentrations to a medium containing *B. breve*, μ_m shifted from 18 to 16 h (Figure 1). At 16 h, AFP concentrations 0.5, 1, and 2 mg/mL produced μ_m values of 0.409, 0.431, and 0.493, respectively. When inulin was added at various concentrations to a medium containing *B. breve*, μ_m occurred at 18 h. Inulin at concentrations of 1, 2, and 5 mg/mL produced μ_m values of 0.443, 0.470, and 0.517, respectively.

Broth pH values after 18 h of incubation of *B. breve* were markedly lower in both AFP- and inulin-treated groups (Table 8). The results of SCFAs analysis showed that both AFP and

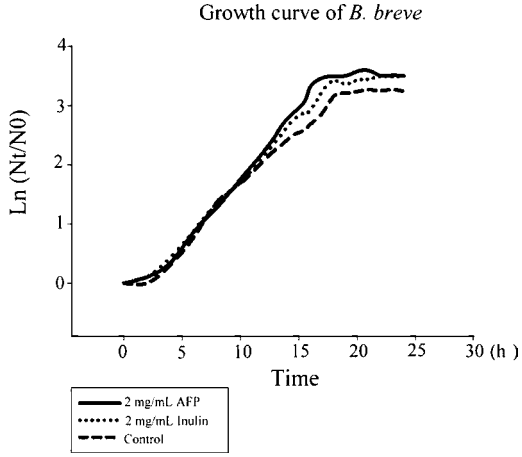

Figure 1. Growth curve of *B. breve* in MRS or in MRS supplemented with indigestible polysaccharide AFP (2 mg/mL) or inulin (2 mg/mL) in vitro. N_t and N_0 are OD_{600} measurements at time t and time 0, respectively.

inulin produced a concentration-dependent increase in total SCFA concentration (Table 8).

Fragments specific to ATP-binding protein cassette (ABC) transporters, adenosine triphosphatase (ATPase), α-L-arabinofuranosidase, and β-galactosidase were amplified by RT-PCR (Figure 2A). Specific target RT-PCR products were compared with 16 S rRNA as a semiquantitative measurement of the level of these genes' expression. After 9 h of incubation, expression of the ABC transporter was 1.9 and 2.1 times more than the control group significantly under AFP (2 mg/mL) and inulin treatment (Figure 2B). Expression of the ATPase was 1.7 and 2.1 times more than control group under AFP and inulin treatment after 9 h of culture and showed levels similar to the ABC transporter in AFP and inulin groups (Figure 2C). AFP groups led the expression of α-L-arabinofuranosidase, which was significantly higher than the control group, but not the inulin group (Figure 2D). β-Galactosidase expression was

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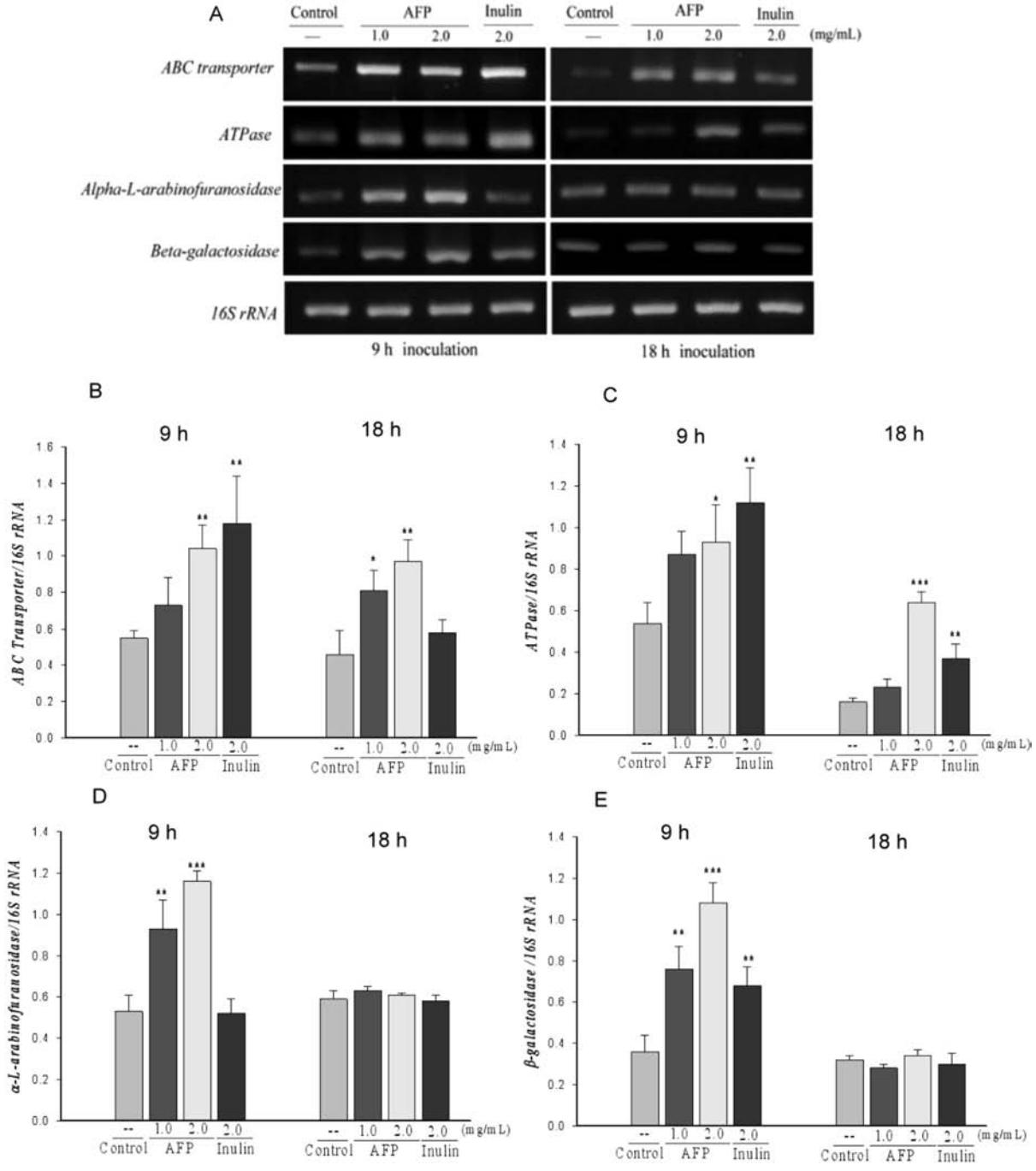

Figure 2. Effects of indigestible polysaccharide AFP and inulin on the mRNA expression of *ABC transporter*, *adenosine triphosphatase*, *α*-L-*arabinofuranosidase*, and *β*-*galactosidase* of *B. breve* for 9 and 18 h of incubation in vitro.*16S rRNA* was used as an internal control. The expression levels were measured and quantified densitometrically. Values were normalized to *16S rRNA* expression. All values were the mean ± SD ($n = 3$). *, $p < 0.05$; **, $p < 0.01$; and *** $p < 0.001$, as compared with the control group.

induced simultaneously under AFP and inulin treatments (Figure 2E). After 18 h of incubation, expression of the ABC transporter was significantly higher in AFP groups than in the control group (Figure 2B). The expression of ATPase showed 4.0 and 2.3 times increases for AFP (2.0 mg/mL) and inulin, respectively (Figure 2C). However, there were no differences between groups in the expression of *α*-L-arabinofuranosidase and *β*-galactosidase mRNA (Figure 2D,E).

In Vitro Fermentation by Mouse Fecal Microbiota. Previous papers have indicated the relationship between the number of CFUs and PCR amplicons. The relationship provides a sensitive method for detecting and quantifying bacteria.[21] Four strains were performed for testing the accuracy of PCR products using the CFU count. The test indicated that the designed *16S rRNA* primers were specific to the target microorganism; the PCR products showed a strong linear relationship to the CFU count. Equations describing the relationships are as follows: Y (*Bifidobacterium*) $= 62345X - 6 \times 10^9$, $r^2 = 1$; Y (*Lactobacillus*) $= 4929.7X - 3 \times 10^8$, $r^2 = 0.9594$; Y (*Escherichia coli*) $= 6 \times 10^7 X - 2 \times 10^{13}$, $r^2 = 0.9693$; and Y (*C. perfringens*) $= 3387.4X \times 10^8$, $r^2 = 0.9999$, where X represents the intensities (integrated density values) of the

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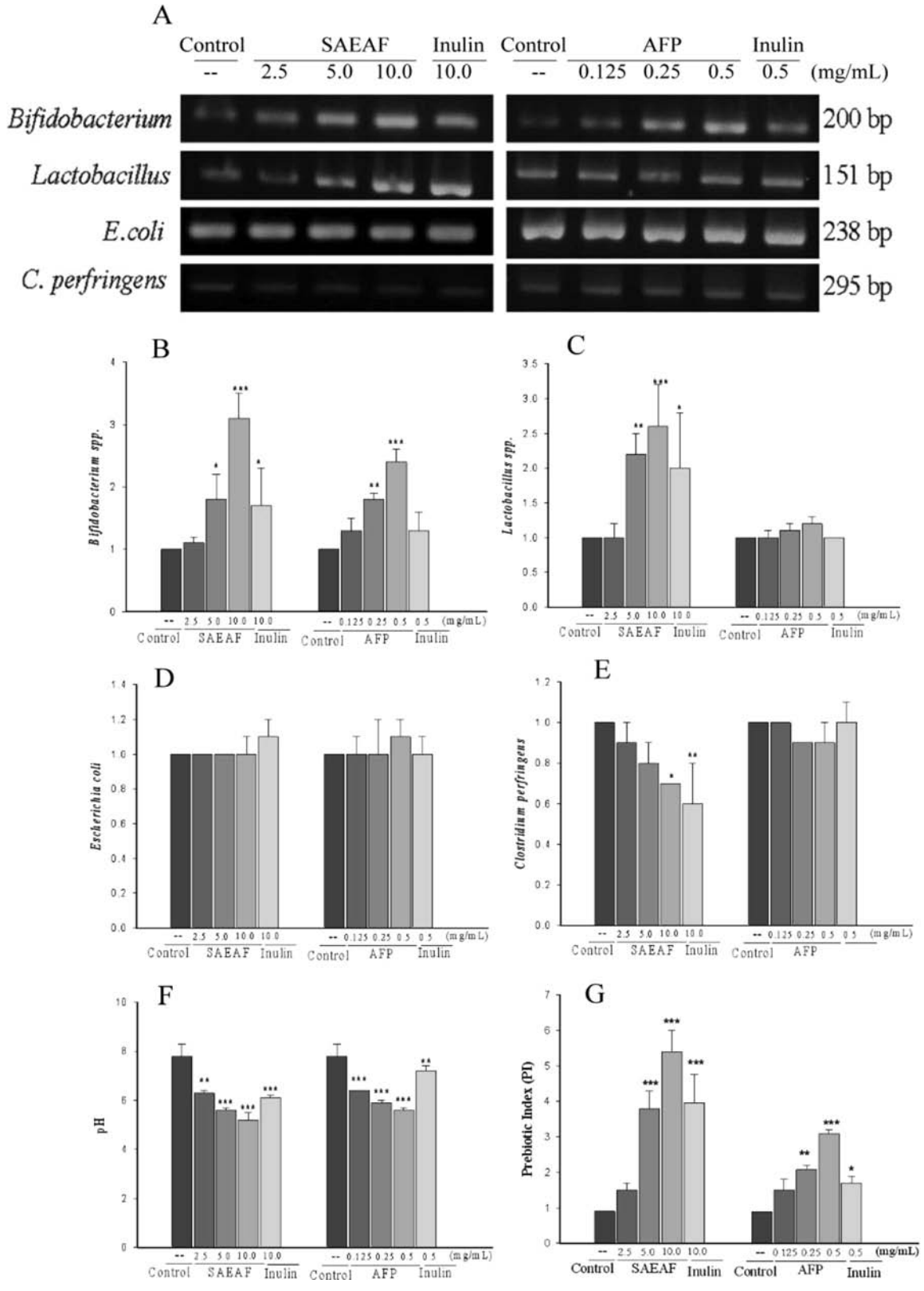

Figure 3. Effects of SAEAF, indigestible polysaccharide AFP, and inulin on 16S rRNA gene expression of *Bifidobacterium* spp., *Lactobacillus* spp., *Escherichia coli*, and *Clostridium perfringens* obtained by specific primers and pH values in vitro fecal fermentation. The expression levels were measured and quantified densitometrically. All values are the mean ± SD ($n = 3$). *, $p < 0.05$; **, $p < 0.01$; and ***, $p < 0.001$, as compared with the control group.

amplicon measured using Alpha DigiDoc 1201 software and *Y* represents the mean average of the number of CFUs per milliliter in the inoculation. The PCR results for samples prior to fermentation showed no differences between samples in the target microorganism. This test was checked to ensure that initial concentrations of target DNA between samples were the

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same. The pH values of each inoculation prior to fermentation were all close to pH 7.4 with no significant differences between samples.

The fragments in Figure 3A reflect the pooled data for three samples. Figure 3B shows that the addition of SAEAF (5 and 10 mg/mL), AFP (0.25 and 0.5 mg/mL), and inulin (0.5 and 10 mg/mL) resulted in significant increases in the *16S rRNA* expression of *Bifidobacterium* after 24 h of fermentation compared to the control group. Similarly, the *16S rRNA* expression of *Lactobacillus* showed significant increases with the addition of SAEAF (5 and 10 mg/mL) and inulin (10 mg/mL) (Figure 3C). No effect was observed by SAEAF, AFP, and inulin on the 16S rRNA expression of *E. coli* (Figure 3D); however, SAEAF (10 mg/mL) and inulin (10 mg/mL) had an inhibiting effect on the 16S rRNA expression of *C. perfringens* after 24 h of incubation (Figure 3E). Compared to the control group, pH values of the fermentation broths in SAEAF-treated (2.5−10 mg/mL), AFP-treated (0.125−0.5 mg/mL), and inulin-treated (0.5 and 10 mg/mL) groups decreased in a concentration-dependent manner (Figure 3F).

To aid the analysis of prebiotic fermentation in vitro, a quantitative equation, the prebiotic index (PI), was used for SAEAF, AFP and inulin.[22] The PI could be calculated as follows: PI = (Bif/total) + (Lac/total) − (Eco/total) − (Clos/total), where Bif is bifidobacterial numbers after 24 h of inocubation/numbers at inoculation and similarly Lac is lactobacilli numbers, Eco is *E. coli* number, and Clos is *Clostridia* number. In the PI equation, the number of probiotics, such as bifidobacteria and lactobacilli is a positive effect; however, an increase of *E. coli* and *Clostridia* is negative. The PI scores calculated from data are shown in Figure 3G.

Prebiotic Effects of AFP in Mice. As shown in Table 9, mice receiving an AFP dosage of 20 mg/kg produced an increased number of *Bifidobacterium* colonies in the stool on day 3; AFP (10 and 20 mg/kg) and inulin treatments produced increases in the number of *Bifidobacterium* colonies in the stool on day 7.

Table 9. Effects of Administration of Indigestible Polysaccharide AFP on the Number of Fecal *Bifidobacterium* from Mice[a]

		log10 CFUs/g feces	
group	dose (mg/kg)	day 3	day 7
control		6.2 ± 0.5	6.2 ± 0.5
AFP	10	6.8 ± 0.7	7.2 ± 0.1***
AFP	20	7.5 ± 0.2***	7.5 ± 0.2***
inulin	400	6.9 ± 0.7	7.5 ± 0.1***

[a]All values are the mean ± SD (n = 7). ***, $p < 0.001$, as compared with the control group.

DISCUSSION

This research demonstrates that administration of SAEAF increases Ca absorption and increases fecal numbers of bifidobacteria in growing rats. Thus, it is possible that SAEAF contains prebiotics. According to the bioactive guide, we isolated AFP from SAEAF. AFP markedly stimulates the proliferation of bifidobacteria both in vitro and in vivo, but the ethanol supernatant fraction of SAEAF exhibited no effects in bifidobacteria growth (data not shown). Chemical analysis of AFP showed that it exhibits AG-II characteristics.

The small intestine is a major Ca absorption site. However, increasing evidence supports that the large intestine can also absorb significant amounts of Ca.[23] Rat experiments have shown that fermentable indigestible carbohydrates improve Ca retention by enhancing absorption of the element.[24] Presumably, indigestible carbohydrates are not absorbed in the small intestine but are instead metabolized by microbiota in the large intestine. The end-products of carbohydrate fermentation are SCFAs, such as acetate, propionate, and butyrate. High concentrations of SCFAs in the cecum result in a decrease of cecal pH and in increased concentrations of soluble Ca.[25]

In Ca balance experiments, SAEAF treatments increased Ca absorption and retention in growing rats. The rats were sacrificed between 8:00 a.m. and 10:00 a.m., at which time cecal fermentation was still extremely active. Therefore, increases in free Ca concentration in the cecum and blood serum and decreases in cecal pH were also observed in SAEAF-treated rats. These results demonstrate that SAEAF contains indigestible carbohydrates that reach the large intestine intact, where they are fermented by intestinal bacteria.

A prebiotic is a selectively fermented component that causes specific changes, in both concentration and activity of gastrointestinal microbiota. These changes confer well-being and health benefits on the host.[26] Many studies have reported that prebiotics selectively stimulate the growth of *Bifidobacterium* spp. and, to a lesser extent, *Lactobacillus* and *Clostridium*.[1] In an in vivo study, SAEAF treatments resulted in stimulating the proliferation of bifidobacteria with no apparent effect on potentially pathogenic *Clostridium* in rat feces. A bifidogenic effect was also observed in the SAEAF in an in vitro study, where SAEAF treatment did not affect the growth curve of *L. acidophilus* and *C. perfringens*. These results strongly indicate that indigestible carbohydrates in SAEAF exhibit prebiotic characteristics.

This study used *B. breve* for bioassay-guided separation of bifidogenic constituents of SAEAF. AFCP showed effective promotion of *B. breve* growth. AFCP treated with enzymes to remove starch content provided AFP, which markedly enhanced *B. breve* proliferation. When the Yariv reagent was added to precipitate the AG-II, the polysaccharide in the supernatant displayed no effect on *B. breve*, implying that AG-II is the prebiotic component of AFP. The efficacies of AFP (2 mg/mL) and inulin (5 mg/mL) in the OD value of *B. breve* were approximately equal (Table 8). Therefore, AFP potency for enhancing *B. breve* proliferation was approximately 2.5 times that of inulin. AFP also markedly decreased pH values and increased SCFAs content.

AFP treatments also resulted in enhanced proliferation of bifidobacteria in mouse feces, further confirming the in vivo prebiotic activity of AFP. The in vivo experiment of AFP discovered the onset time of prebiotic activity. A time-dependent trial demonstrated that high-dose treatment of AFP could significantly raise the number of fecal bifidobacteria on day 3. Low- and high-dose treatment of AFP could increase the growth of bifidobacteria within 7 days.

The mixed fecal batch culture showed that SAEAF enhanced the growth of bifidobacteria and lactobacilli in a dose-dependent manner and simultaneously suppressed the growth of the *C. perfringens* group without affecting the growth of *Escherichia*. This work confirmed the effect of AFP to markedly enhance the growth of bifidobacteria. The pH of the culturing system also lowered. These results indicate that AFP possesses a prebiotic character. The PI for in vitro fecal fermentation

scored the prebiotic effect of SAEAF, AFP, and inulin. SAEAF exhibited a more potent prebiotic effect than the relative dose of AFP. It was suggested that several carbohydrates existed in SAEAF that could enhance the number of probiotics. Even though the PI of AFP was lower than that of SAEAF, the doses of AFP used in this study were in a small amount. Compared to the same dose of inulin, AFP presented a higher PI. This indicated that AFP was a more potent prebiotic component than inulin.

To investigate the prebiotic mechanism of AFP in bifidobacteria, gene expression analysis was used. Bifidobacteria play an important role in carbohydrate fermentation in the colon. They degrade polysaccharides to lower the degree of polymerization of oligosaccharides or monosaccharides using a wide range of glycosidases.[27] Prebiotics can change glycosidase activity. For example, *B. longum* growing on larch wood AG increases β-galactosidase and α-arabinopyanosidase activities.[28] Arabinan, AG, and arabinoxylan are sources of arabinofuranosyl-containing oligosaccharides, which could be fermented by bifidobacteria and may be prebiotics.[29] α-L-Arabinofuranosidases can hydrolyze arabinosyl linkages from various hemicelluloses such as arabinans and AG.[30] β-Galactosidases are essential enzymes for bifidobacteria; their functions include catalyzing the hydrolysis of β-D-galactoside linkage,[27] and some may transfer one or more galactosyl units onto lactose.[31]

This study found that AFP and inulin enhanced mRNA expression of *β-galactosidase* after 9 h treatments (midexponential phase), but not after 18 h treatments (end of exponential phase). AFP increased *α-L-arabinopyanosidase* expression significantly after only 9 h of treatment. These results indicate that AFP could enhance mRNA expression on the AG-related carbohydrate hydrolase in bifidobacteria at the exponential phase. Inulin does not possess the β-galactoside linkage, but could still induce mRNA expression of *β-galactosidase* after 9 h of treatment. Another study demonstrated that *β-galactosidase* production could be influenced by different monosaccharides as a carbon source and the production rate could correspond to the bacteria population.[32] This study hypothesized that *β-galactosidase* expression is related to bifidobacteria growth because it is an essential enzyme to bifidobacteria.[27]

Although many *ABC transporters* are exporters and are associated with antibiotic and antifungal resistance, the first characterized ABC transporters were part of the nutrient uptake system.[33] Yuan et al.[19] showed that a solute binding protein of the *ABC transporter* system, possibly for sugars, was up-regulated in *B. longum* grown on fructose.

The present study compared mRNA expression of the *ABC transporter* on *B. breve* grown on AFP and inulin after 9 and 18 h treatments. AFP enhanced *ABC transporter* expression for both treatments; however, inulin increased these genes' expression only after 9 h of treatment. These results indicate that AFP could up-regulate bifidobacteria growth through activating the *ABC transporter* system, which influences carbohydrate uptake. Increased expression of the *ABC transporter* that occurs at the end of the exponential growth phase in *B.breve* for AFP, but does not occur on inulin, may explain the greater AFP bifidogenic activity compared to that of inulin treatments.

Previous research[34] considers that the ATPase is mainly localized in the plasma membrane, and the ATPase membrane may influence membrane transport and ATP synthesis of *Lactobacillus* and *Bifidobacterium* strains. The other important role of the ATPase membrane is that it removes protons from interior cells and maintains cytoplasmic pH in a low-pH environment with ATP hydrolysis.[35] Therefore, ATPase is related to bacteria resistance during a low-pH condition. *B. breve* fermentation produces SCFAs and reduces broth pH. This study found that AFP and inulin enhanced *ATPase* expression on *B. breve* after a 9 and 18 h of culture. RT-PCR investigations showed that AFP not only acts as a prebiotic carbon source but also stimulates the sugar intake process in bifidobacteria. This may be why AFP is so effective, even at small dosages.

To investigate the chemical properties of AFP, this study examined molecular weight, total carbohydrate, uronic acid, protein, and sugar content. The molecular weight of AFP estimated by size exclusion was approximately 29 kDa. AFP contained a high amount carbohydrate and low protein. The monosaccharide composition suggested the presence of AG. Reactivity of AFP to the β-glucosyl-Yariv reagent showed the possible presence of AG-II,[36] which is heavily branched and mainly composed of a (1→3)-β-D-galactan backbone having a (1→6)-β-D-galactan side chain.[36] In plants, AG is a major component of plant cell walls,[36] where it is thought to play important roles in plant growth and development.[36] It has also been indicated that AGs can be fermented by bifidobacteria and may be prebiotic compounds.[27] Larch AG is commercially used as a dietary fiber and prebiotic and in treating intestinal disorders.[37] This study clearly shows that *A. formosanus* AG promotes bifidobacterial growth in vivo and in vitro. The therapeutic efficacy of *A. formosanus* AG from *A. formosanus* for human welfare requires further investigation.

In conclusion, we expect that the use of *A. formosanus* AG will have a positive effect on gut microbiota.

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Notes

The authors declare no competing financial interest.

ABBREVIATIONS USED

ABC, ATP-binding protein cassette; AFCP, crude polysaccharide of *A. formosanus*; AFP, indigestible polysaccharide of *A. formosanus*; AG, arabinogalactans; ATCC, American Type Culture Collection; *ATPase*, *adenosine triphosphatase*; Ca, calcium; *Mr*, molecular mass; OD, optical density; RCM, reinforced clostridial medium; SAEAF, standardized aqueous extract of *A. formosanus*; SCFAs, short-chain fatty acids.

REFERENCES

(1) Roberfroid, M. B. Introducing inulin-type fructans. *Br. J. Nutr.* **2005**, *93* (Suppl. 1), S13−S25.

(2) Olano-Martin, E.; Gibson, G. R.; Rastall, R. A. Comparison of the *in vitro* bifidogenic properties of pectins and pectic-oligosaccharides. *J. Appl. Microbiol.* **2002**, *93*, 505−511.

(3) Weaver, C. M. Inulin, oligofructose and bone health: experimental approaches and mechanisms. *Br. J. Nutr.* **2005**, *93* (Suppl. 1), S99−S103.

(4) Kruger, M. C.; Brown, K. E.; Collett, G.; Layton, L.; Schollum, L. M. The effect of fructooligosaccharides with various degrees of

polymerization on calcium bioavailability in the growing rat. *Exp. Biol. Med. (Maywood)* **2003**, *228*, 683−688.
(5) Shih, C. C.; Wu, Y. W.; Lin, W. C. Antihyperglycaemic and antioxidant properties of *Anoectochilus formosanus* in diabetic rats. *Clin. Exp. Pharmacol. Physiol.* **2002**, *29*, 684−688.
(6) Wu, J. B.; Lin, W. L.; Hsieh, C. C.; Ho, H. Y.; Tsay, H. S.; Lin, W. C. The hepatoprotective activity of kinsenoside from *Anoectochilus formosanus*. *Phytother. Res.* **2007**, *21*, 58−61.
(7) Shih, C. C.; Wu, Y. W.; Lin, W. C. Ameliorative effects of *Anoectochilus formosanus* extract on osteopenia in ovariectomized rats. *J. Ethnopharmacol.* **2001**, *77*, 233−238.
(8) Grabitske, H. A.; Slavin, J. L. Gastrointestinal effects of low-digestible carbohydrates. *Crit. Rev. Food Sci. Nutr.* **2009**, *49*, 327−360.
(9) Compton, S. J.; Jones, C. G. Mechanism of dye response and interference in the Bradford protein assay. *Anal. Biochem.* **1985**, *151*, 369−374.
(10) Dubois, M.; Gilles, K. A.; Hamilton, J. K.; Rebers, P. A.; Smith, F. Colorimetric method for determination of sugars and related substances. *Anal. Chem.* **1956**, *28*, 350−356.
(11) Blumenkrantz, N.; Asboe-Hansen, G. New method for quantitative determination of uronic acids. *Anal. Biochem.* **1973**, *54*, 484−489.
(12) van Holst, G. J.; Clarke, A. E. Quantification of arabinogalactan-protein in plant extracts by single radial gel diffusion. *Anal. Biochem.* **1985**, *148*, 446−450.
(13) Laine, C.; Tamminen, T.; Vikkula, A.; Vuorinen, T. Methylation analysis as a tool for structural analysis of wood polysaccharides. *Holzforschung* **2002**, *56*, 607−614.
(14) Yang, L. C.; Wu, J. B.; Ho, G. H.; Yang, S. C.; Huang, Y. P.; Lin, W. C. Effects of poly-γ-glutamic acid on calcium absorption in rats. *Biosci., Biotechnol., Biochem.* **2008**, *72*, 3084−3090.
(15) Munoa, F. J.; Pares, R. Selective medium for isolation and enumeration of *Bifidobacterium* spp. *Appl. Environ. Microbiol.* **1988**, *54*, 1715−1718.
(16) Harmon, S. M.; Kautter, D. A.; Peeler, J. T. Improved medium for enumeration of *Clostridium perfringens*. *Appl. Microbiol.* **1971**, *22*, 688−692.
(17) Zwietering, M. H.; Jongenburger, I.; Rombouts, F. M.; van't Riet, K. Modeling of the bacterial growth curve. *Appl. Environ. Microbiol.* **1990**, *56*, 1875−1881.
(18) Classen, B.; Blaschek, W. An arabinogalactan-protein from cell culture of *Malva sylvestris*. *Planta Med.* **2002**, *68*, 232−236.
(19) Yuan, J.; Zhu, L.; Liu, X.; Li, T.; Zhang, Y.; Ying, T.; Wang, B.; Wang, J.; Dong, H.; Feng, E.; Li, Q.; Wang, H.; Wei, K.; Zhang, X.; Huang, C.; Huang, P.; Huang, L.; Zeng, M. A proteome reference map and proteomic analysis of *Bifidobacterium longum* NCC2705. *Mol. Cell Proteomics* **2006**, *5*, 1105−1118.
(20) Sanz, M. L.; Polemis, N.; Morales, V.; Corzo, N.; Drakoularakou, A.; Gibson, G. R.; Rastall, R. A. In vitro investigation into the potential prebiotic activity of honey oligosaccharides. *J. Agric. Food Chem.* **2005**, *53*, 2914−2921.
(21) Williams, J. M.; Trope, M.; Caplan, D. J.; Shugars, D. C. Detection and quantitation of *E. faecalis* by real-time PCR (qPCR), reverse transcription-PCR (RT-PCR), and cultivation during endodontic treatment. *J. Endod.* **2006**, *32*, 715−721.
(22) Palframan, R.; Gibson, G. R.; Rastall, R. A. Development of a quantitative tool for the comparison of the prebiotic effect of dietary oligosaccharides. *Lett. Appl. Microbiol.* **2003**, *37*, 281−284.
(23) Raschka, L.; Daniel, H. Mechanisms underlying the effects of inulin-type fructans on calcium absorption in the large intestine of rats. *Bone* **2005**, *37*, 728−735.
(24) Roberfroid, M. B.; Cumps, J.; Devogelaer, J. P. Dietary chicory inulin increases whole-body bone mineral density in growing male rats. *J. Nutr.* **2002**, *132*, 3599−3602.
(25) Coxam, V. Current data with inulin-type fructans and calcium, targeting bone health in adults. *J. Nutr.* **2007**, *137*, 2527S−2533S.
(26) Gibson, G. R.; Probert, H. M.; Loo, J. V.; Rastall, R. A.; Roberfroid, M. B. Dietary modulation of the human colonic microbiota: updating the concept of prebiotics. *Nutr. Res. Rev.* **2004**, *17*, 259−275.
(27) van den Broek, L. A.; Hinz, S. W.; Beldman, G.; Vincken, J. P.; Voragen, A. G. *Bifidobacterium* carbohydrases-their role in breakdown and synthesis of (potential) prebiotics. *Mol. Nutr. Food Res.* **2008**, *52*, 146−163.
(28) Degnan, B. A.; Macfarlane, G. T. Arabinogalactan utilization in continuous cultures of *Bifidobacterium longum*: effect of co-culture with Bacteroides thetaiotaomicron. *Anaerobe* **1995**, *1*, 103−112.
(29) Van Laere, K. M.; Hartemink, R.; Bosveld, M.; Schols, H. A.; Voragen, A. G. Fermentation of plant cell wall derived polysaccharides and their corresponding oligosaccharides by intestinal bacteria. *J. Agric. Food Chem.* **2000**, *48*, 1644−1652.
(30) Van Laere, K. M.; Beldman, G.; Voragen, A. G. A new arabinofuranohydrolase from *Bifidobacterium adolescentis* able to remove arabinosyl residues from double-substituted xylose units in arabinoxylan. *Appl. Microbiol. Biotechnol.* **1997**, *47*, 231−235.
(31) Rabiu, B. A.; Jay, A. J.; Gibson, G. R.; Rastall, R. A. Synthesis and fermentation properties of novel galacto-oligosaccharides by β-galactosidases from *Bifidobacterium* species. *Appl. Environ. Microbiol.* **2001**, *67*, 2526−2530.
(32) Laxmi, N. P.; Mutamed, M. A.; Nagendra, P. S. Effect of carbon and nitrogen sources on growth of *Bifidobacterium animalis* Bb12 and *Lactobacillus delbrueckii* ssp. *bulgaricus* ATCC 11842 and production of β-galactosidase under different culture conditions. *Int. Food Res.* **2011**, *18*, 373−380.
(33) Higgins, C. F. ABC transporters: physiology, structure and mechanism. *Res. Microbiol.* **2001**, *152*, 205−210.
(34) Schneider, E. ABC transporters catalyzing carbohydrate uptake. *Res. Microbiol.* **2001**, *152*, 303−310.
(35) Krzewinski, F.; Brassart, C.; Gavini, F.; Bouquelet, S. Characterization of the lactose transport system in the strain *Bifidobacterium bifidum* DSM 20082. *Curr. Microbiol.* **1996**, *32*, 301−307.
(36) Showalter, A. M. Arabinogalactan-proteins: structure, expression and function. *Cell. Mol. Life Sci.* **2001**, *58*, 1399−1417.
(37) Pal, A. Arabinogalactan protein and arabinogalactan: biomolecules with biotechnological and therapeutic potential. In *Bioactive Molecules and Medicinal Plants*; Ramawat, K. G., Merillon, J. M., Eds.; Springer: Berlin, Germany, 2008; pp 255−270.