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Bifidobacterium adolescentis P2P3, a Human Gut Bacterium Having Strong Non-Gelatinized Resistant Starch-Degrading Activity

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Resistant starch (RS) is metabolized by gut microbiota and involved in the production of short-chain fatty acids, which are related to a variety of physiological and health effects. Therefore, the availability of RS as a prebiotic is a topic of interest, and research on gut bacteria that can decompose RS is also important. The objectives in this study were 1) to isolate a human gut bacterium having strong degradation activity on non-gelatinized RS, 2) to characterize its RS-degrading characteristics, and 3) to investigate its probiotic effects, including a growth stimulation effect on other gut bacteria and an immunomodulatory effect. *Bifidobacterium adolescentis* P2P3 showing very strong RS granule utilization activity was isolated. It can attach to RS granules and form them into clusters. It also utilizes high-amylose corn starch granules up to 63.3%, and efficiently decomposes other various types of commercial RS without gelatinization. In a coculture experiment, *Bacteroides thetaiotaomicron* ATCC 29148, isolated from human feces, was able to grow using carbon sources generated from RS granules by *B. adolescentis* P2P3. In addition, *B. adolescentis* P2P3 demonstrated the ability to stimulate secretion of Th1 type cytokines from mouse macrophages in vitro that was not shown in other *B. adolescentis.* These results suggested that *B. adolescentis* P2P3 is a useful probiotic candidate, having immunomodulatory activity as well as the ability to feed other gut bacteria using RS as a prebiotic.

Keywords: *Bifidobacterium adolescentis,* human intestinal bacteria, immunomodulatory effect, probiotics, resistant starch

Introduction

Starch is a carbohydrate and a major component of human and animal diets. In general, most starch is consumed as gelatinized starch and easily degraded by digestive enzymes upon ingestion, followed by utilization as an energy source for the human body [1]. However, raw (uncooked) starch is not digested well in the stomach and small intestine and reaches the large intestine relatively intact [2]. These undamaged starch molecules are utilized by gut bacteria in the large intestine, and the enriched bacterial communities transform them into short-chain fatty acids (SCFAs), which provide a positive effect on human health [3].

Starches, which are composed of amylose and amylopectin complexes, exist in most plants of various sizes [4, 5]. They are stored in an insoluble and tightly packed manner and

are rigid due to their crystalline structure, cracks, and pores [6]. Therefore, common raw granular starches are resistant to human digestive enzymes. Non-digestible starches are called resistant starch (RS) and generally classified into five types (RS1: physically inaccessible, RS2: native granular structure, RS3: retrogradation, RS4: chemically modified, and RS5: amylose-lipid complexes) [7].

The disgestibility of RS is influenced by many factors including storage conditions, processing, cooking, origin, and water content of the starches. Also, the properties of starch granules depend on how the two major components (amylose and amylopectin) are organized in the granules [8]. Thus, RS digestibility is closely affected by the ratio of amylose and amylopectin; generally, resistance to digestibility is positively correlated with the amylose level in the granules [9−11]. In other words, starch granule structure can be enforced by increasing both the branched chain length and content of amylose [12−14]. Among native granular starches (RS2), potato starch and high-amylose corn starch (HACS) have much higher resistance against digestive enzymes than other starch granules due to the relatively large granule size (potato) and the long amylose branching chains with rigid granules from high amylose content, respectively [7].

Although many granular starch-degrading microorganisms have been reported in various environments [15, 16], only a few studies have been conducted on HACS granule-degrading bacteria in the human intestinal environment. Ze *et al*. studied the utilization of different starches including high-amylopectin corn starch, HACS, RS2, and RS3 by four amylolytic bacteria that represent relatively abundant groups in the human gut [17]. Whereas *Bacteroides thetaiotaomicron* (0%) and *Eubacterium rectale* (ca 10%) barely consumed HACS granules, *Ruminococcus bromii* and *Bifidobacterium adolescentis* isolated from a healthy male infant were able to decompose HACS granules up to 54.3 and 43.8%, respectively. In conclusion, they claimed that bacteria related to *R. bromii* appeared to have a pivotal role in the degradation of dietary RS in human. Jung *et al*. found that *Bifidobacterium choerinum* FMB-1 from rumen fluids of Korean native cattle (*Bos taurus coreanae*) can effectively degrade HACS granules and form granule clusters during incubation with HACS granules [18]. They suggested that the growth of other bacteria might be promoted by utilizing small saccharides generated by a primary RS-degrader such as *B. choerinum* FMB-1. These results imply that the primary RS-degrader is an important factor for promoting other bacteria and maintaining balanced intestinal microbiota. Therefore, the discovery and isolation of bacteria that can act as primary degraders are very important for a study of intestinal microbiota and probiotics related to human intestinal health.

Previously, the formation and precipitation of unusual HACS granule clusters were observed before HACS granules were decomposed by *B. choerinum* FMB-1 [17]. This phenomenon allows us to presume that intestinal microbial cells with an ability to utilize the granular RS may be related to the formation HACS granule clusters and there is also high probability that they possess RS-degrading enzymes. In this study, we separated the HACS granule clusters formed while incubating human feces with RS-containing media from the supernatant. The intestinal microorganisms stuck to the separated HACS granule clusters were isolated and identified, and their RS-degrading abilities were determined. Among those strains, isolated *B. adolescentis* P2P3 degraded various RS including commercial products more effectively than the previously reported *Bifidobacterium* species [17, 18]. In addition, the possibility that *B. adolescentis* P2P3 could work as a primary degrader of RS to support and regulate the growth of other intestinal microorganisms was examined by a cross-feeding experiment. Finally, in vitro expressions of TNF-α, IL-6, and IL-12 in mouse macrophages by *B. adolescentis* P2P3 were investigated.

Materials and Methods

Bacterial Strains

Bacterial strains, *Bacteroides thetaiotaomicron* (ATCC 29148) and *B. adolescentis* (DSM 20083, DSM 20086, DSM 20087, and DSM 24849), were purchased from the American Type Culture Collection (ATCC, USA) and Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSMZ, Germany). *Bifidobacterium adolescentis* L2-32 was kindly provided from BEI resources (USA). All strains were cultivated according to the manual provided by the corresponding suppliers. The *B. adolescentis* P2P3 strain isolated in this study was deposited in the Korean Agricultural Culture Collection (KACC, Korea) under collection number KACC 92235P.

Resistant Starches

S4180 was purchased from Sigma–Aldrich (USA). S4180 is an unmodified HACS classified as RS2 type and composed of 70% amylose and 30% amylopectin. Hi-Maize (HM) 260, HM 958, Novelose (NV) 330, Versafibe (VF) 1490, and VF 2470 are food-grade commercial RSs and were provided by Ingredion (USA). According to the manufacturer's technical specifications, RS2 type starches (HM 260 and 958) were made from different base HACS but using similar hydrothermal methodology. HM 260 is a hydrothermal treated starch based on Hylon VII (RS2 type starch, Ingredion), which is a commercial HACS (70% amylose). HM 958

Table 1. Resistant starches used in this study.

	S4180	Hi-maize 260*	Hi-maize 958*	Novelose 330*	Versafibe 1490*	Versafibe 2470*
Source	Unmodified HACS	Unmodified HACS (Hylon VII, commercial)	Unmodified HACS	Hydrolyzed products of HACS	Regular potato starch	Unmodified HACS
Treatment	Non-treated	Hydrothermal	Hydrothermal	Retrograded	Chemical	Chemical
RS type	RS 2	RS 2	RS 2	RS 3	RS 4	RS 4
Starch (%)	65	31	64	65	10	29
Dietary Fiber (%)	23	56	22	23	74	60
Total Carbohydrate (%)	88	87	86	88	84	89

*Food-grade commercial starches except for S4180 were kindly provided by Ingredion Incorporated.

is an RS2 type starch prepared by the hydrothermal method from an unmodified HACS. RS3 type starch (NV 330) is a retrograded starch generated from hydrolyzed high-amylose corn starch products. VF 1490 and 2470 (RS4 type starch) were derived from potato starch and HACS, respectively. VF 1490 is a distarch phosphate modified using phosphorus oxychloride, and VF 2470 is created through hydrolysis and heat treatment of high-amylose maize starch. Table 1 describes the various RSs used in this study.

Growth Media

The medium for bacteria isolation was chopped meat (CM) broth containing 15% bovine rumen fluid (CMR). The CM broth consisted of (per 100 ml) chopped meat pellets (1.02 g), pancreatic digest of casein (3.0 g), yeast extract (0.5 g), dipotassium phosphate (0.5 g), L-cysteine (0.05 g), resazurin (0.1 mg), vitamin K_1 (0.1 mg), and hemin (0.5 mg). The CM medium containing 1% (v/v) trace mineral and vitamin solutions (CMTV) was used for the growth of bifidobacteria. The trace mineral solution (per liter) consisted of nitrilotriacetic acid (1.5 g), $MgSO_4 \cdot 7H_2O$ (3 g), $MnSO_4$ (0.5 g), NaCl (1 g), $FeSO_4 \cdot 7H_2O$ (0.1 g), $CoSO_4 \cdot 7H_2O$ (0.18 g), $CaCl_2 \cdot 2H_2O$ (0.1 g), $ZnSO_4 \cdot 7H_2O$ (0.18 g), $CuSO_4 \cdot 5H_2O$ (0.01 g), $KAl(SO_4)_2 \cdot 12H_2O$ (0.02 g), H_3BO_3 (0.01 g), $Na_2MoO_4 \cdot 2H_2O$ (0.01 g), and $NiCl_2 \cdot 6H_2O$ (0.025 g). The vitamin solution (per liter) consisted of biotin (2 mg), folic acid (2 mg), pyridoxine-HCl (10 mg), thiamine-HCl·$2H_2O$ (5 mg), riboflavin (5 mg), nicotinic acid (5 mg), D-pantothenic acid (5 mg), vitamin B_{12} (0.1 mg), 4-aminobenzoic acid (5 mg), and lipoic acid (5 mg). The medium (20 ml) was dissolved in serum vials and autoclaved. And then, 0.1 g of sterilized starch substrates [17] were mixed with a sterilized medium, followed by flushing with 99.5% CO_2 gas set up with a 0.2-μm filter.

Human Feces and Culture Condition

A fecal sample was collected from a healthy male (age 29) with no history of gastrointestinal diseases and who had not taken antibiotics over one year. Feces was provided after three weeks on a regular diet. The fecal sample was packed and transported anaerobically within one hour after excretion, then diluted five times (30 g/150 ml) in an aseptic pack and homogenized for inoculation. All work was performed in an anaerobic chamber filled with CO_2 gas. To investigate RS degradation by microorganisms in human feces, 200 μl of the five times diluted human feces (w/v) was inoculated into 20 ml of CMR broth containing 0.5% S4180, and then incubated at 37°C with shaking at 150 rpm under anaerobic conditions. Samples were taken every 6 h after incubation.

Scanning Electron Microscopy (SEM)

Samples were carefully dried with nitrogen gas and then placed on a carbon tape over a microscope slide to be coated with gold under vacuum. The images were obtained with the Tabletop Microscope TM 3000 (Hitachi, Japan). The particle size was determined from the micrograph with scale bars of 30 μm (2,000 ×) and 20 μm (4,000 ×) units, respectively.

Isolation Procedures

Strain isolation proceeded in six steps as follows. 1) Subculture process: 200 μl of five times diluted human feces (w/v) was inoculated into 20 ml of CMR broth containing 0.5% S4180. After culturing at 37°C for 48 h, subculture was repeated four more times. 2) Measurement of consumed RS: The RS utilization was measured when the subculture was completed. 3) Natural precipitation of granule clusters: The subculture vial was left untouched until granule clusters settled. 4) Separation of granule clusters and supernatant: The supernatant was removed from the vial and placed in a separate tube, and the pellet was immediately dispersed with an equal volume of fresh 0.8% NaCl solution. 5) Washing process: The precipitated granule clusters were resuspended in 0.8% NaCl solution and settled again, followed by removing the supernatant. This washing step was performed twice. The original supernatant was mixed with starch granules and RS granule binding cells were confirmed by checking the formation of starch granule clusters. 6) Spreading of washed pellet and supernatant: 100 μl of washed pellet and supernatant (properly diluted) were incubated on solid CMR medium containing 0.5% maltose and 0.5% soluble starch. All work was done in an anaerobic chamber filled with CO_2 gas. The isolation procedure is shown in Fig. S1.

16S rRNA Gene Identification

Genomic DNA was prepared using a stool DNA extraction kit (Qiagen, USA) in accordance with the manufacturer's instructions, including bead-beating for 5 min. The 16S rRNA gene amplification was carried out using universal primers (27F and 1492R) and PCR was performed using recombinant KOD polymerase (Toyobo, Japan). The PCR conditions included an initial denaturation at 95°C for 5 min; 30 cycles consisting of 30 sec at 95°C, 30 sec at 55°C, and 1.5 min at 72°C; and 5 min of final extension at 72°C. The amplified fragments were sequenced and analyzed using the Bacteria and Archaea database of BlastN+2.8.0 [19]. The nucleotide sequences of the isolates were deposited in the GenBank database.

RS Degradation by Bifidobacteria

To examine RS degradation by bifidobacteria, the isolated bacteria were grown on 5 ml of CMTV broth containing 0.5% maltose. After reaching an optical density at 600 nm of 0.6, 200 μl of the cultures were inoculated into 20 ml of CMTV medium containing 0.5% RS (S4180, HM 260, HM 260, NV 330, VF 1490, and VF 2470, respectively). Next, the cultures were incubated for 72 h (almost no decomposition occurs from 48 h to 72 h) at 37°C with shaking at 150 rpm under anaerobic conditions. Experiments were repeated in triplicate.

Quantitation of Residual RS and Reducing Sugars

Residual RS in the cultures was measured via the phenol-sulfuric acid method [20, 21]. In brief, 5% phenol solution (250 μl) was added to 250 μl of culture. Next, 1.5 ml of 99% sulfuric acid was added and the reaction was gently mixed and incubated at 30°C for 20 min. Reducing sugars in the culture were measured using the dinitrosalicylic acid (DNS) method [22]. Briefly, 100 μl of cell-free supernatant was mixed with 300 μl of DNS solution, followed by boiling for 5 min. The absorbance of the phenol-sulfuric acid and DNS reactant was measured at 490 nm using an iMark microplate reader (BioRad, USA).

Coculture Experiments

To create a seed culture, *B. adolescentis* P2P3 and *Bac. thetaiotaomicron* ATCC 29148 were grown independently in 5 ml of CMTV broth containing 0.5% maltose until an optical density at 600 nm of 0.6 was reached. Next, 200 μl of *B. adolescentis* P2P3 and 200 μl of *Bac. thetaiotaomicron* ATCC 29148 were simultaneously inoculated into 20 ml of CMTV medium containing 0.5% S4180. As parallel controls, 200 μl of *B. adolescentis* P2P3 and 200 μl of *Bac. thetaiotaomicron* ATCC 29148 were inoculated individually into 20 ml of CMTV medium containing 0.5% S4180. The inoculated medium was incubated at 37°C with shaking at 150 rpm under anaerobic conditions. Samples were collected every 12 h until 36 h for quantitative polymerase chain reaction (qPCR). Experiments were repeated in triplicate.

Quantitative Real-Time PCR

Genomic DNA was prepared using a stool DNA extraction kit (Qiagen, USA) in accordance with the manufacturer's instructions, including bead-beating for 5 min. The *B. adolescentis* P2P3 and *Bac. thetaiotaomicron* ATCC 29148 levels were quantified by amplification of a specific region of the 16S rRNA gene. Amplification was performed using specific primers set for *B. adolescentis* P2P3 (forward: 5′-TGT CGG GAA AGA TTC ATC GGT AT and reverse: 5′-TTT AAG GGA TCC GCT CCA CC-3′) and *Bac. thetaiotaomicron* ATCC 29148 (forward: 5′-GTA TAA TCA GAC CGC ATG GTC TTG-3′ and reverse: 5′-GAT TAG CAT CCT GTC ACC AGG TA-3′). SYBR Green I (Dyne qPCR premix, DYNE BIO, Korea) was used to amplify with a CFX Connect Real-Time PCR Detection System (BioRad). The PCR thermocycling conditions were as follows: initial denaturation at 95°C for 10 min followed by 40 cycles of 30 sec at 95°C, 30 sec at 58°C, and 1 min at 72°C. Cycle threshold (Ct) values were calculated using Bio-Rad CFX 96 Manager 3.0 software set to single threshold mode.

Measurement of Nitrites

Each bacterial strain was cultivated in 300 ml of CMTV medium containing 1% glucose at 37°C for 18 h under anaerobic conditions. The cultivated cells were killed in boiling water for 6 min, followed by washing with 0.85% saline and centrifugation (7,000 ×*g*, 5 min). Cells were lyophilized and used after suspending in phosphate buffered saline. For in vitro assays, 2×10^5/0.5 ml of macrophage RAW 264.7 cells (Korea Cell Line Bank, Korea) were treated with 100 μg/ml of cells or 100 ng/ml of lipopolysaccharides (LPS) (Sigma–Aldrich) for 24 h. For analysis of nitrite level, the supernatant was reacted with an equal volume of Griess Reagent (Sigma–Aldrich) for 15 min at room temperature. The absorbance was measured at 540 nm using an iMark microplate reader (BioRad).

Analysis of Peritoneal Macrophages In Vitro Stimulated with Bacterial Strains

Two milliliters of thioglycollate medium (BD, USA) was injected intraperitoneally into healthy mice (male Balb/c, 7 weeks of age). After three days, macrophages were collected via peritoneal lavage with Dulbecco's modified Eagle's medium (DMEM) (Hyclone, USA). After removing the supernatant by centrifugation (1,000 rpm, 10 min), the cells were resuspended in DMEM with 10% fetal bovine serum (Hyclone) and 1% penicillin-streptomycin. The cells were incubated overnight in a humidified atmosphere of 5% CO_2 at 37°C. Non-adherent cells were removed and the macrophages (4×10^5 cells/ml) were treated with prepared bacterial cells (4, 20, and 100 μg/ml) or 100 ng/ml LPS (Sigma–Aldrich) for 24 h. The supernatant containing cytokines was collected and diluted for ELISA analysis. ELISA analysis was performed according to the manufacturer's protocol (BD OptEIA Set Mouse IL12p70, TNF-α, IL-6) and optical density was measured at 450 nm using an iMark microplate reader (BioRad).

Statistical Analysis

Significance between groups was analyzed using one-way

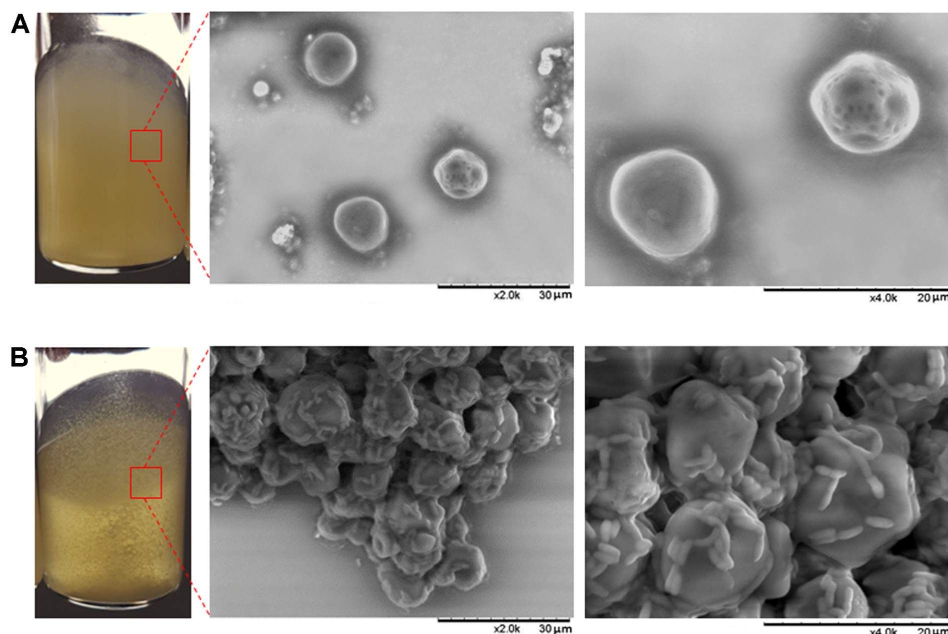

Fig. 1. Formation of RS granule clusters in liquid culture of human feces.
The scanning electron microscopic (SEM) images display (**A**) initial dispersion of RS granules at 0 h of incubation and (**B**) the formation of RS granule clusters by human gut microorganisms at 18 h of incubation.

ANOVA and expressed as a mean ± standard deviation. The software used was IBM SPSS Statistics ver. 23. The *p*-values less than 0.05 were considered significant.

Results

Cultivation of Human Feces with RS

Human feces from one healthy male was anaerobically cultured with non-gelatinized raw S4180 starch and the total amounts of RS and reducing sugars in the culture medium were determined during the cultivation. Until 18 h after inoculation, the amount of RS in the medium remained about 90% (Fig. S2). After 18 h, the RS was continuously degraded until 48 h. The level of reducing sugars gradually increased from the point of starch decomposition to the early stage of RS degradation (12 to 24 h). As the starch continuously degraded, the amount of reducing sugars in the medium gradually decreased (24 to 48 h). Finally, about 60% of the total RS was decomposed overall.

At the initial stage of the cultivation with RS granules, each granule individually dispersed in the liquid medium (Fig. 1A). Interestingly, however, after 18 h of incubation, the relatively large white clusters formed by aggregation of RS granules were observed and the presence of bacterial strains adhered to the clustered granules was monitored (Fig. 1B). The granule clusters maintained even if they were separated from supernatant and washed several times. When the shape of the granule clusters was observed by SEM, many cells were still attached to the surface of the RS granule clusters. It was assumed that the cells attached to the granules were strongly related to the formation of granule clusters. These RS granule clusters were previously observed in RS-degrading *B. choerinum* FMB-1 isolated from bovine rumen fluids [17].

Isolation of Bacterial Strains from Clusters of RS Granules

Since the solid relationship between the formation of RS granule clusters and RS degradation was discerned, the gut bacterial strains that might degrade the RS granules were isolated as described in Methods (Fig. S1). The supernatant was separated from the RS granule clusters. And Next, both supernatant and RS granule clusters were transferred to fresh CMTV medium containing 0.5% S4180. The main observation was that the supernatant, from which the RS granule clusters were separated, did not show an ability either to form RS granule clusters or to degrade RS granules, while the transfer of small amounts of RS granule clusters always showed an adhesion ability to form RS granule clusters as well as RS-degrading activity in fresh medium. This indicated that the bacterial strains attached to the RS granules forming clusters were responsible for the RS degradation.

Table 2. Isolated organisms from granule clusters and supernatant in this study.

	Collection no.	Matched organisms	Top hit strain	Accession No.
Clusters	P2P3	*Bifidobacterium adolescentis*	Eq1	MH828351
Supernatant	C11	*Collinsella aerofaciens*	JCM 10188	MH828352
	S10	*Bacteroides vulgatus*	ATCC 5826	MH828353
	R3-8	*Paraclostridium benzoelyticum*	JC272	MH828354
	P8-2	*Clostridium perfringens*	JCM 1290	MH828355
	AR5-10	*Shigella sonnei*	CECT 4887	MH828356
	SS1-3	*Pediococcus pentosaceus*	DSM 20336	MH828357
	EC8	*Enterococcus faecium*	DSM 20477	MH828358
	ST3	*Streptococcus lutetiensis*	CIP 106849	MH828359
	R12	*Escherichia coli*	U5/41	MH828360
	SS1-1	*Escherichia fergusonii*	ATCC 35469	MH828361
	R7	*Bacillus paralicheniformis*	KJ-16	MH828362
	R5	*Bacillus licheniformis*	DSM 13	MH828363
	R8	*Lactobacillus rhamnosus*	NBRC 3425	MH828364

More than 100 colonies were picked from the solid plates cultured with granule clusters and supernatant parts. The isolated strains are listed in Table 2. Among the isolated strains, only *B. adolescentis* P2P3, isolated from granule clusters, was able to form clusters of RS granules and to utilize RS (S4180) at almost 63.3%. The strain was also able to adhere to raw starch granules at the initial culture stage and remain on the raw granules as they began to be destroyed (Fig. S3).

Related to the clustered RS granules, several interesting findings were detected (data not shown). First, separately grown cells of *B. adolescentis* P2P3 without RS also had an ability to immediately make clumps of RS granules as soon as they were mixed with RS granules. Second, the cells treated with proteinase lost their ability to form the clusters of RS granules and were unable to attach to RS granules. Third, this granule clustering was observed only in granules with α-1,4-glucose linkages such as starch and amylopectin. This phenomenon was not observed with other substrates such as pullulan, cellulose, and xylose. As a result, it was presumed that this aspect was due to the proteins possessing starch, binding domains (SBDs) existing in the cell envelop. Generally, SBDs exist only in α-1,4-glucan-related enzymes as an auxiliary module of amylolytic enzymes.

RS Degradation of Isolated Strains and Comparative Study among *Bifidobacterium* sp.

Since the isolated RS-degrading strain was identified as *B. adolescentis*, we compared six *B. adolescentis* strains by RS cluster formation and RS-degrading ability. Among *B. adolescentis* strains, in particular, *B. adolescentis* P2P3 was considered the most efficient RS-utilizing strain. *B. adolescentis* P2P3, DSM 20087, DSM 24849, and L2-32 (reported as RS-degrading strain [18]) could utilize RS almost 63.3%, 9.8%, 47.3%, and 43.8%, respectively, while DSM 20083 and DSM 20086 did not degrade RS at all (Fig. 2A). Interestingly, *B. adolescentis* P2P3, DSM 20087, DSM 24849, and L2-32 were found to form granule clusters while also being able to degrade RS (Fig. S4). Non-RS-degrading strains, *B. adolescentis* DSM 20083 and DSM 20086, did not form granule clusters as expected (Fig S4). In addition, *B. longum* S3, *B. bifidum* S6, *B. animalis* S7, and *B. pseudocatenulatum* 12 (Table S1, additional strains isolated using *Bifidobacterium* selective media in this study) were unable to degrade RS and did not form granule clusters (data not shown). Also, *B. choerinum* FMB-1 and *B. pseudolongum* FMB-2, known to degrade RS in a previous study, also formed granule clusters [17]. As a result, the attachment to RS granules and the formation of RS granule clusters by *Bifidobacterium* sp. might be strongly related to the degradation of the RS granule structure. The proof of this assumption requires further study on other bacterial genera with the ability to degrade RS granules.

The utilization rates of commercially available RS (HM 260, HM 958, NV 330, VF 1490, and VF 2470) by *B. adolescentis* P2P3 (highest RS utilization strain) were measured without gelatinization and compared with *B. choerinum* FMB-1 [RS-

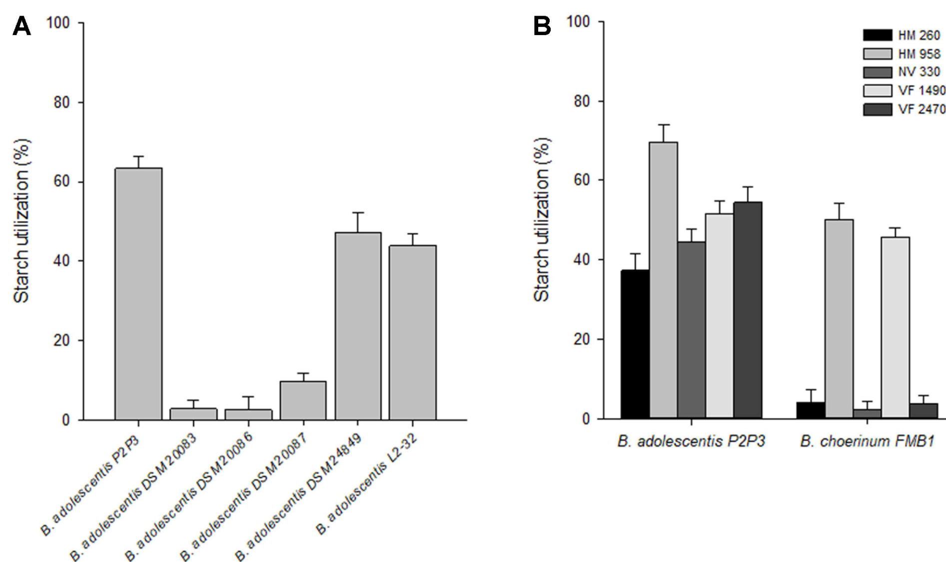

Fig. 2. Quantification of residual starch during fermentation with bifidobacterial strains.

Fermentation was carried out in CMTV-RS medium for 72 h. (**A**) Fermentation of various *B. adolescentis* strains with 0.3% S4180. (**B**) Fermentation of *B. adolescentis* P2P3 or *B. choerinum* FMB-1 with 0.3% commercial RS.

degrading strain reported in Jung *et al.* (2018)]. *Bifidobacterium choerinum* FMB-1 showed RS utilization similar to P2P3 in the case of S4180 (unmodified HACS). However, the two species showed different utilization rates with other commercial RS. Fig. 2B shows the utilization of commercial RS when cultured with *B. adolescentis* P2P3 and *B. choerinum* FMB-1 for 72 h at 37°C. In the case of *B. adolescentis* P2P3, it utilized HM 260, HM 958, NV 330, VF 1490, and VF 2470 at 37.3%, 69.6%. 44.5%, 51,6%, and 54,4%, respectively, while *B. choerinum* FMB-1 utilized at 4.0%, 50.3%, 2.4, 45.7%, and 3.8%, respectively. The difference in utilization between the two strains may be due to their enzymes involved in the degradation of RS or dietary fiber contained in the RS. However, it is currently unclear what differences between *B. adolescentis* P2P3 and *B. choerinum* FMB-1 may cause this result. Therefore, further research on their RS-degrading enzymes and mechanisms, in addition to their genomes, is underway.

RS Degradation and Growth Proportion in Co-Culture

When non-gelatinized S4180 was cultured with *B. adolescentis* P2P3, reducing sugars such as glucose, maltose, and maltotriose were detected in TLC analysis (data not shown). To determine whether other microorganisms could utilize reducing sugars generated by *B. adolescentis* P2P3, *Bac. thetaiotaomicron* ATCC 29148 (well-studied gut bacterium with starch-degrading enzymes) [23] was simultaneously grown with S4180 as the only carbon source. Fig. 3 shows the amount of residual RS and 16S rRNA genes changed in individual and simultaneous cultures of *B. adolescentis* P2P3 and *Bac. thetaiotaomicron* ATCC 29148. When *Bac. thetaiotaomicron* ATCC 29148 was cultured alone in CMTV medium containing 0.5% S4180, S4180 was not degraded and therefore it did not grow well, but it could grow well in CMTV medium containing 0.5% soluble starch (data not shown). However, *B. adolescentis* P2P3 grew well by degrading RS about 22% and 55% during 24 h and 36 h incubation, respectively (Fig. 3), whereas when *B. adolescentis* P2P3 and *Bac. thetaiotaomicron* ATCC 29148 were incubated in one pot, RS was utilized about 9% and 42% for 24 h and 36 h, respectively. When both strains were co-cultured at the same time, the RS degradation rate decreased, even if only a little, compared to *B. adolescentis* P2P3 cultured alone. These results suggested that the growth of *Bac. thetaiotaomicron* ATCC 29148 demanded small saccharides released from RS by *B. adolescentis* P2P3. On the other hand, as *Bac. thetaiotaomicron* ATCC 29148 grew, the relative proportion of RS-degrader *B. adolescentis* P2P3 decreased, resulting in decreased RS degradation. The quantitative PCR results supported this assumption. The ratios of *B. adolescentis* P2P3 to *Bac. thetaiotaomicron* ATCC 29148 in individual and simultaneous cultures were examined through quantitative analysis of 16S rRNA genes. When *B. adolescentis* P2P3 and *Bac. thetaiotaomicron* ATCC 29148 were grown independently in CMTV medium containing 0.5% S4180 as the only carbon source, *Bac. thetaiotaomicron* ATCC 29148 had a

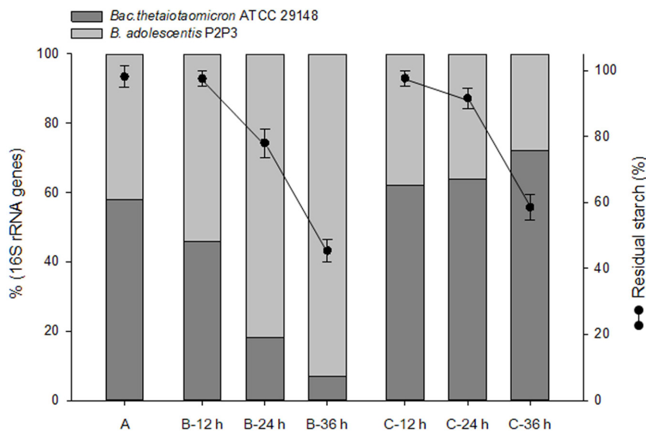

Fig. 3. Residual starch and quantitative PCR results during individual and cocoulture of *B. adolescentis* P2P3 and *Bac. thetaiotaiomicron* ATCC 29148.

Fermentation was carried out in CMTV medium containing 0.5% S4180 for 72 h. Black circles represent residual starch. Quantitative PCR results were expressed by the ratio of amplified 16S rRNA genes. Grey represents *Bac. thetaiotaiomicron* ATCC 29148 and light grey represents *B. adolescentis* P2P3. Equal volumes of *B. adolescentis P2P3* and *Bac. thetaiotaiomicron* ATCC 29148 (individual cultures) were mixed immediately (**A**) after inoculation or (**B**) after growth. (**C**) *B. adolescentis* P2P3 and *Bac. thetaiotaiomicron* ATCC 29148 were cocultured.

much slower growth rate than *B. adolescentis* P2P3, with relative ratios of 46% to 54%, 18% to 82%, and 7% to 93% for 12 h, 24 h, and 36 h incubation, respectively. However, co-culture of both strains increased the ratio of *Bac. thetaiotaomicron* ATCC 29148 by up to 62%, 64%, and 72% for 12 h, 24 h, and 36 h incubation, respectively. Clearly, when the two strains were cultured simultaneously, the ratio of *Bac. thetaiotaomicron* ATCC 29148 was significantly increased compared to its single culture. This was an undoubted phenomenon caused by co-culture with *B. adolescentis* P2P3. These observations indirectly suggested that *B. adolescentis* P2P3 might be a strong RS-metabolizing human gut microorganism and a potential probiotic-stimulating primary degrader, which possibly generates the small maltooligosaccharides needed for the growth of neighboring human microbiota from RS.

Immunomodulatory Effect and β-Glucuronidase Activity of *B. adolescentis* P2P3

The immunomodulatory effects of isolated bacteria were examined to check the possible probiotic activity of the selected microorganism. Activated macrophages release nitric oxide (NO), tumor necrosis factor-α (TNF-α), reactive oxygen intermediates, and other substances to protect the host [24]. As NO modulates cytokine release and induces T helper (Th) cell differentiation, it plays an important role in antimicrobial and antitumor activities in the host immune system [25]. RAW 264.7 cells were stimulated by fifteen bacterial strains, including *Bifidobacterium* and other human gut bacteria, and their nitrite production levels were observed. In Fig. 4A, *B. choerinum* FMB-1, *B. adolescentis* P2P3, *B. bifidum* S6, and *E. fergusonii* SS1-1 showed nitrite production of 16.9, 19.8, 16.1, and 22.5 μM, respectively, higher than the positive control, LPS (14.1 μM). Next, mouse peritoneal macrophages were stimulated by *B. choerinum* FMB-1, *B. adolescentis* P2P3, and *B. bifidum* S6 (except for *E. fergusonii* SS1-1, considered as a pathogen) with increasing doses of bacterial cells for 24 h, followed by measurement of TNF-α, IL-6, and IL-12 production. TNF-α showed almost similar production for the three strains, except for low production at the 4 μg/ml dose of *B. bifidum* S6 (Fig. 4B). For IL-6, all doses of *B. choerinum* FMB-1 and 100 μg/ml doses of *B. adolescentis* P2P3 showed lower production than the others did, but the other cells showed almost similar production to each other (Fig. 4C). Overall, TNF-α and IL-6 productions were higher than LPS-treated cells. In the case of IL-12, known to induce Th1 type immune responses, only *B. adolescentis* P2P3 showed higher production than LPS-treated cells. IL-12 stimulated by *B. adolescentis* P2P3 was two to four times higher than the IL-12 level produced by LPS-treated cells (Fig. 4D). Based on these results, the RS-degrading bacterium *B. adolescentis* P2P3, isolated from human feces, was considered to induce Th1 type cytokines in vitro. In a previous report, *B. adolescentis* DSM20083, 20086, and 20087 did not induce Th1 type cytokines in vitro [26].

Since several studies suggest that β-glucuronidase activity is involved in the mutagenic activation of metabolites and correlated with colon cancer, the presence of β-glucuronidase activity in *Bifidobacterium* is not a desirable characteristic as a possible probiotic. Enzyme activity assays of *B. adolescentis* P2P3 with API ZYM system revealed that this strain does not show β-glucuronidase activity (data not shown), which fulfills a basic safety characteristic for probiotics in the food industry.

Discussion

Polysaccharide-specific binding domains are non-catalytic but functional domains mainly found in enzymes involved in polysaccharide metabolism. There are diverse groups of domains that specifically bind to certain polysaccharides

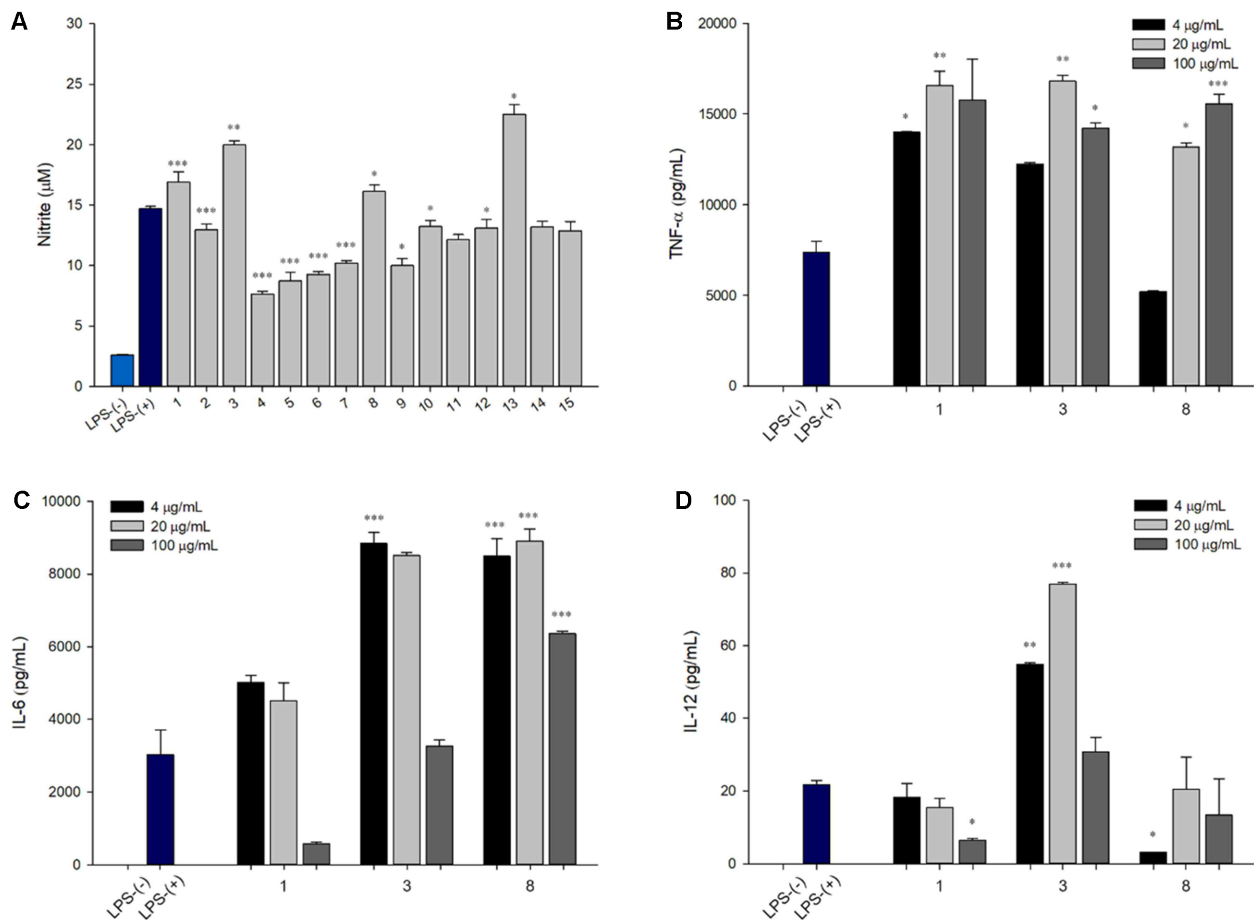

Fig. 4. The production of (**A**) nitrite, (**B**) tumor necrosis factor (TNF)-α, (**C**) interleukin (IL)-6, and (**D**) IL-12 by various bacterial strains.

Mouse peritoneal macrophages were stimulated with 4, 20, or 100 μg/ml of bacterial cells or 100 ng/ml of lipopolysaccharides for 24 h. $*p < 0.05$, $**p < 0.01$, $***p < 0.005$ versus LPS (+). 1. *Bifidobacterium choerinum* FMB-1, 2. *B. choerinum* DSM20434, 3. *B. adolescentis* P2P3, 4. *B. adolescentis* DSM24849, 5. *B. adolescentis* DSM20087, 6. *B. adolescentis* DSM20083, 7. *B. animalis* S7, 8. *B. bifidum* S6, 9. *B. pseudocatenulatum* 12, 10. *B. longum* S3, 11. *C. aerofaciens* C11, 12. *E. faecium* EC8, 13. *E. fergusonii* SS1-1, 14. *P. pentosaceus* SS1-3, 15. *Pc. benzoelyticum* R3-8.

such as cellulose, chitin, hemicellulose, inulin, lactose, mannan, starch, and xylan [27]. For example, SBDs are important components present in many enzymes involved in starch metabolism. It has been suggested that 10% of amylases and their related enzymes have SBDs with approximately 100 amino acid residues at their C- or N-terminus [28]. The main function of SBDs is assumed to increase the local concentration of substrate at the catalytic site of the enzyme by binding to starch granules, thereby facilitating easy disruption of recalcitrant raw starch granules [29]. In other cases, SBD-containing proteins are involved in glycogen metabolism by affecting its cellular localization and intracellular trafficking to lysosomes [30].

In this experiment, *B. adolescentis* P2P3 was isolated from human feces by using its remarkable RS granule clustering characteristics and confirmed to have a strong ability to degrade various types of commercial RS. In addition, these clustering characteristics as well as RS-degrading activity were lost by the treatment of protease, indicating the presence of amylase or related enzymes anchored on the cell surface of *B. adolescentis* P2P3. The draft whole-genome sequencing of *B. adolescentis* P2P3 disclosed several possible amylase-like proteins possessing SBDs in their amino acid sequences. Therefore, further research on their involvement in the formation of RS granule clusters and RS-degradation is underway.

Studies of microorganisms currently known to degrade raw starch granules are mainly focused on fungi such as

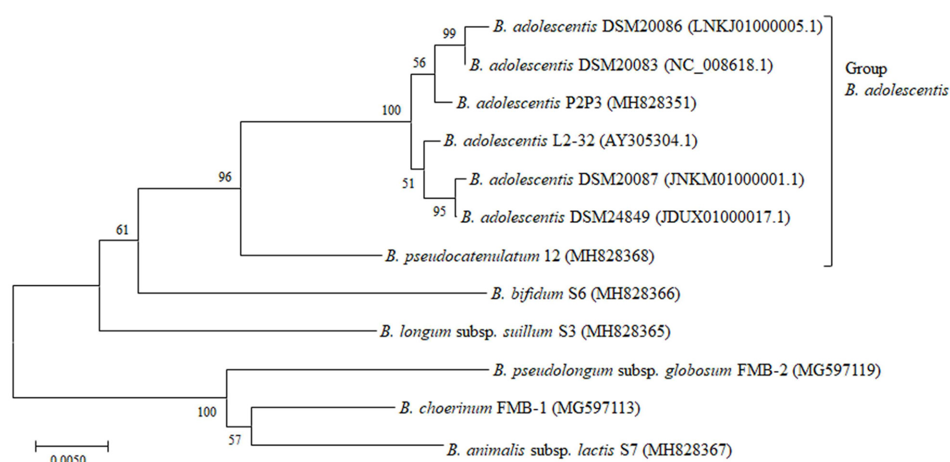

Fig. 5. Phylogenetic tree of various bifidobacterial strains based on 16S rRNA genes sequences (mean length 1,436 nt).
The tree was constructed using the neighbor-joining statistical method. Phylogeny test was performed with bootstrap replications of 1,000 dataset using MEGA7 software (https://www.megasoftware.net). GeneBank accession data are written in parentheses.

Aspergillus sp., *Penicillium* sp., and *Rhizopus* sp. Some bacteria including *Bacillus* sp. have also been mentioned previously [15, 16]. New advances in various tools in biological science including next-generation sequencing technologies have uncovered the presence of diverse microorganisms in the human gut and provided their enormous genetic information, the so-called microbiome [31]. Regarding RS-degrading gut microorganisms, however, only a few human gut bacteria have been described until now. Among the human gut bacteria, *B. adolescentis* together with *R. bromii* have been reported as major bacteria metabolizing RS as a prebiotic [18]. In this study, we isolated a non-gelatinized RS-degrading gut microorganism, which can degrade various non-gelatinized RS substrates including HM 260, HM 958, NV 330, VF 1490, and VF 2470. Compared to other strains, its degradation capacity is extraordinary. Therefore, it is worthwhile to study the RS-degradation mechanism by *B. adolescentis* P2P3 further.

Recently, studies have shown that there are special cell envelope-associated multiprotein systems for starch digestion in human gut starch-degrading bacteria. It was reported that cell wall anchored proteins (SusD and EUR_21100) are involved when *Bac. thetaiotaomicron* and *E. rectale* decompose starch substrates, respectively. In fact, these microorganisms cannot degrade RS. These proteins capture α-1,4 and 1,6 glucan near the cell envelope and help other cell surface enzymes degrade them [23, 32]. In the case of *R. bromii* (RS-degrading bacterium), the cell surface protein complex, which is called “amylosomes” and linked by dockerin and cohesin, was involved in digesting RS. This cell surface protein complex includes several amylolytic enzymes in which SBD-like modules are observed [33]. Similar to these examples, some amylases holding SBDs in *B. adolescentis* P2P3 may be involved in the cell attachment to RS granules and the formation of granule clusters in addition to RS degradation. A further disruption and complementation study, as well as the genomic information of those genes will confirm the role of an RS-degrading enzyme(s) in *B. adolescentis* P2P3.

In this study, among the *B. adolescentis* strains examined (P2P3, DSM20083, DSM20086, DSM20087, DSM24849, and L2-32), DSM20083 and DSM 20086 did not use RS. Fig. 5 shows the phylogenetic analysis result of the various bifidobacterial strains used in this study. Strains of the *B. adolescentis* group were closely related to each other. Strain P2P3 showed the highest RS utilization (about 63.3%) even though it is rather close to DSM20086 and DSM20083, which cannot utilize RS. Whereas, strain DSM20087, close to DSM24849 and L2-32 that utilized RS about 47.3% and 43.8%, respectively, exhibited only 9.8% RS utilization. In addition, there were no significant differences in growth rates using glucose, maltose, or soluble starch, but the growth rate using RS was obviously different. The growth rate of P2P3 was the fastest followed by DSM24849, L2-32, and DSM20087 (data not shown). Also, *B. choerinum* FMB-1 and *B. pseudolongum* FMB-2 utilized RS [17], but *B. pseudocatenulatum* 12, *B. bifidum* S6, *B. longum* S3, and closely related *B. animalis S7* did not utilize RS at all. In conclusion, there is no apparent relationship between genetic distance and RS utilization so far, even within the same or adjacent groups.

The immunomodulatory effect is one of the functional

effects that probiotic strains can have [34, 35]. Bifidobacteria have been shown to exhibit polarized Th1/Th2 responses in a specific manner to regulate Th1 (Crohn's/celiac disease) and Th2 (allergy inflammation) type diseases, overall inflammation, and imbalanced cytokine production characteristics [36, 37]. Young *et al*. reported that adult type *Bifidobacterium* species such as *B. adolescentis* and *B. catenumlatum* group could favor Th2-biased immune responses characteristic of allergy inflammation and demonstrated that IL-12 production was not affected by exposure to any of the bifidobacteria including *B. adolescentis* DSM20083, 20086, and 20087 [26]. In contrast, *B. adolescentis* P2P3, isolated in this study, could prefer Th1-biased immune response characteristics. In addition, *B. adolescentis* P2P3 showed ability to degrade various types of commercial RS without gelatinization and fed other bacteria using reducing sugars generated from RS. These results indicated that *B. adolescentis* P2P3 could be a useful, applicable probiotic candidate for the food industry.

Acknowledgments

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Compliance with Ethical Standards

Ethical Approval

All applicable international, national, and/or institutional guidelines for the care and use of animal and human derivatives were followed. The animal study was approved by Kyung Hee University Medical Center Institutional Animal Care and Use Committee [KHUASP(SE)-15-012]. The study of human derivatives was approved by the Institutional Review Board of Kyung Hee University (KHSIRB-17-004).

Conflict of Interest

The authors have no financial conflicts of interest to declare.

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