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Bifidobacterial Growth Stimulation by *Lactobacillus casei* via Whey Fermentation

– Research Note –

Gi-Seong Moon

Department of Biotechnology, Chungju National University, Chungbuk 368-701, Korea

Abstract

Three-hundred bacterial isolates from a natural cheese were screened for the production of bifidobacterial growth factor by whey fermentation. Based on this screen, two whey samples fermented by strains designated as CJNU 0421 and CJNU 0588 were found to effectively stimulate the growth of a bifidobacterial strain, *Bifidobacterium longum* FI10564, by 1.6～1.7 fold compared to a control, in which non-fermented whey medium was added. The two isolates were identified to be *Lactobacillus casei* (99% identity) by 16S rRNA gene sequencing and named *Lactobacillus casei* CJNU 0421 and CJNU 0588, respectively. The whey sample fermented by CJNU 0588 did not enhance the growth of other bacteria such as *Escherichia coli* and *Listeria monocytogenes*, suggesting that the whey fermentation metabolites from the isolate could be used for the selective stimulation of bifidobacteria.

Key words: *Lactobacillus casei*, bifidobacterial growth factor, whey fermentation

INTRODUCTION

One method to identify novel materials that can selectively stimulate the growth of bacteria beneficial to human health, such as bifidobacteria and lactobacilli, which belong to the normal microflora in human intestines, is to screen materials from natural sources. Bifidobacteria are one of the most beneficial bacteria and their functional properties have been extensively investigated by many researchers (1,2). Recent studies examining the relationship between intestinal microbiota and diseases have shown that the balance of intestinal microbiota is very important to maintain a healthy state, and the bifidobacterial population is recognized as playing a key role in sustaining this balance (3,4). Bifidobacteria were previously found to be the most predominant bacteria in human intestines during the early stage of life and the bifidobacterial population was shown to gradually decrease with age (5), which is not desirable for human health. Thus, therapeutic strategies to recover this population to levels seen in the early stage of life could be very important for improving an individual's health status. Regular intake of compounds including prebiotics (6,7), which selectively stimulate the growth of bifidobacteria, might be a solution to this problem.

Whey is a byproduct from the manufacturing of natural cheese and has been historically recognized as wastewater (8). However, whey containing organic compounds such as proteins, lactose, minerals, etc. has recently been reprocessed and used mainly for dairy products and pharmaceuticals (9,10). Nevertheless, whey is still a cost-effective product and its production yield has increased due to the increasing demand for natural cheese (11). Therefore, whey can be a good medium for the production of valuable metabolites via microbial fermentation (12,13). In this study, we assessed the potential use of whey as media and screened 300 bacterial isolates from a natural cheese for the production of bifidobacterial growth factors via whey fermentation. From this initial screen, two isolates were selected for this purpose.

MATERIALS AND METHODS

Bacterial strains and culture conditions

Bacterial isolates from a natural cheese (Cheddar cheese, 3 year ripening period), which was obtained from a local dairy company (Namyang Dairy Products Co., Gongju, Korea), were cultured in MRS (de Man, Rogosa, and Sharpe) broth (Difco, Sparks, MD, USA) or RCM (Reinforced Clostridial Medium) broth (Difco) at 37°C without shaking. *Bifidobacterium longum* FI10564, which was used as the target cells when testing for the production of bifidobacterial growth factors, was anaerobically cultured in RCM broth (Difco) at 37°C in an anaerobic jar (Oxoid, Cambridge, UK).

Isolation of bacteria from a natural cheese sample

The cheese sample (10 g) was placed into a sterilized

E-mail: gsmoon@cjnu.ac.kr
Phone: +82-43-820-5251, Fax: +82-43-820-5272

filter bag and peptone water (90 mL) was added. The sample was homogenized in a stomacher (Seward, West Sussex, UK) and the filtrate was serially diluted by 10-fold with peptone water (10%, w/v). Appropriate diluents were spread on MRS or RCM agar plates and colonies on the plates were chosen and incubated in 5 mL of MRS or RCM broth at 37°C for 48 hr. The cultures were streaked on MRS or RCM agar plates 3 times. Finally, the single colonies were cultured and mixed with an equal volume of 80% (v/v) glycerol and stored at -76°C in a deep freezer (Ilshin Lab Co., Yangju, Korea) until use.

Selection of isolates producing bifidobacterial growth factors via whey fermentation

Three-hundred isolates from the natural cheese were screened for the production of bifidobacterial growth factors. The isolates were incubated in 5 mL of MRS or RCM broth at 37°C for 24 hr and subcultured in 5 mL of whey (Demineralized Whey Powder 90; Irish Dairy Board, Dublin, Ireland) medium (10%, w/v) at 37°C for 48 hr in an anaerobic jar (Oxoid). Ten culture samples were mixed and 1 mL of each mixed culture was removed, centrifuged at 7,000×*g* for 10 min, and then the culture supernatant was filtered with a 0.2 μm syringe filter (Millipore, Billerica, MA, USA). Fifty μL of the filtrate was added to RCM broth containing *B. longum* FI10564. The inoculated samples were incubated at 37°C for 12 hr under anaerobic conditions. After incubation, optical densities were measured at 600 nm using a BioPhotometer (Eppendorf, Hamburg, Germany). The mixed samples that produced bifidobacterial growth factors were separated into one unit and the individual samples were tested again as described above.

Identification of isolates producing bifidobacterial growth factors

16S rRNA gene sequencing was performed to identify the isolates that produced bifidobacterial growth factors. Two isolates (CJNU 0421 and CJNU 0588) were cultured in 5 mL of MRS broth and sent to a biotech company (Macrogen Co., Seoul, Korea) where the sequences were analyzed. The BLAST (Basic Local Alignment Search Tool) program (blastn), provided by the National Center for Biotechnology Information (NCBI; http://www.ncbi.nlm.nih.gov), was used in the homology searches of the 16S rRNA gene sequences.

Growth stimulation activity for other bacteria using a whey sample fermented by CJNU 0588

Because the two isolates were identified as the same species, only isolate CJNU 0588 was used for further study. To determine if the whey sample fermented by CJNU 0588 could stimulate the growth of other bacteria such as *Escherichia coli* DH5α and *Listeria monocytogenes* ATCC 19111, the growths of these bacteria were tested in the sample. Fifty μL of the culture supernatant of the CJNU 0588-fermented whey, which was filtered with a 0.2 μm syringe filter, was added to LB (Luria-Bertani) broth (10 g/L tryptone, 10 g/L NaCl, 5 g/L yeast extract, pH 7.0) containing *E. coli* and MRS broth containing *Lis. monocytogenes,* respectively, and incubated for appropriate times. After incubation, optical densities were measured at 600 nm and compared with the controls.

RESULTS AND DISCUSSION

In the screening for the production of bifidobacterial growth factors via whey fermentation, the optical densities of two samples fermented by CJNU 0421 and CJNU 0588 were higher than those of other samples (data not shown). Therefore, the CJNU 0421- and CJNU 0588-fermented samples were selected for further studies. It has been well established that bifidobacteria, which are strictly anaerobic bacteria, are quite sensitive to oxygen. For this reason, the data acquired up to this point may have been influenced by the procedure used in the test sample preparation. To exclude the possibilities of technical error, the experimental results were further confirmed by conducting several tests in triplicate. In these multiple experiments, the two whey samples fermented by CJNU 0421 and CJNU 0588 were found to consistently enhance the growth of the employed bifidobacterial strain. The CJNU 0421 sample stimulated the growth of the bifidobacterial strain by 2.1 and 1.7 fold, respectively, compared to two controls, one that was inoculated with only the bifidobacterial strain and the other that was inoculated with the bacterium plus non-fermented whey medium. The CJNU 0588 sample similarly

Table 1. Identification of isolates that produced bifidobacterial growth factors

Strain	Best matched with	Query coverage (%)	Identity (%)	GenBank accession No.
CJNU 0421	*Lactobacillus casei* ATCC 334 gene for 16S rRNA	99	99	CP000423.1
CJNU 0588	*Lactobacillus casei* ATCC 334 gene for 16S rRNA	99	99	CP000423.1

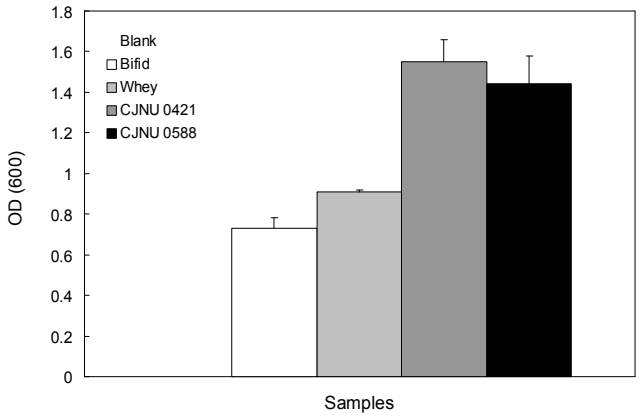

Fig. 1. Production of bifidobacterial growth stimulators from CJNU 0421 and CJNU 0588 (triplicate test). Blank, negative control; Bifid, *Bifidobacterium longum* FI10564; Whey, *B. longum* plus whey medium; CJNU 0421, *B. longum* plus whey sample fermented by CJNU 0421; CJNU 0588, *B. longum* plus whey sample fermented by CJNU 0588. The data represent the mean±standard deviation of triplicate experiments.

stimulated the growth of the bifidobacterial strain by 2.0 and 1.6 fold, respectively (Fig. 1), compared to the controls. These results indicate that the two strains, CJNU 0421 and CJNU 0588, could produce bifidobacterial growth factors via whey fermentation, in which the fermented media enhanced the growth of *B. longum* FI10564.

Because the final goal of this study was to select GRAS (Generally Recognized as Safe) strains that could be easily commercialized and used in the food or pharmaceutical industries, the cell category identifications of CJNU 0421 and CJNU 0588 were considered very important. Since the two strains were isolated from a natural cheese, we expected that the isolates belonged to a GRAS level bacterial category. Subsequently, CJNU 0421 and CJNU 0588 were identified as *Lactobacillus casei* by homology searches of their 16S rRNA gene sequences as described in the MATERIALS AND METHODS (Table 1). *L. casei* is a representative lactic acid bacterium that is commercially used as a probiotic in dairy products (14,15). Since *L. casei* can efficiently metabolize lactose as a carbon source (14), whey, which contains high level of lactose, may be a good medium for this bacterium (16). In addition, the *L. casei* strains screened in this study produced bifidobacterial growth stimulators, which could be used as value-added ingredients, through whey fermentation. For these reasons, it would be worthwhile to extensively characterize the *L. casei* CJNU 0421 and CJNU 0588 strains for potential commercialization in the future.

To be categorized as bifidogenic growth stimulators (17), the compounds in the whey samples fermented by these strains should selectively stimulate only bifidobacteria. Because the two isolates were identified as the same species, only CJNU 0588 was used in further experiments. To determine if the fermented whey sample could stimulate other bacteria, the growth effects of the CJNU 0588 sample on other bacteria such as *E. coli* and *Lis. monocytogenes* were tested. In these experiments, no significant differences in bacterial growth were observed between the samples and controls (Fig. 2). These results support the notion that the fermented whey samples selectively stimulated the growth of bifidobacteria.

To date, the production of bifidobacterial growth stimulators via whey fermentation has rarely been studied. Nevertheless, a previous study reported that a milk whey fraction contained growth-promotional factors for *Bifidobacterium* species (18), which agrees with the results of this study (Fig. 1). Furthermore, in a recent paper (19), bovine lactoferrin, a component of milk whey, showed growth promotion capabilities for several *B. longum* strains. These results indicate that milk whey has a basic ability to promote the growth of bifidobacteria. Therefore, it would be advantageous if this ability were enhanced

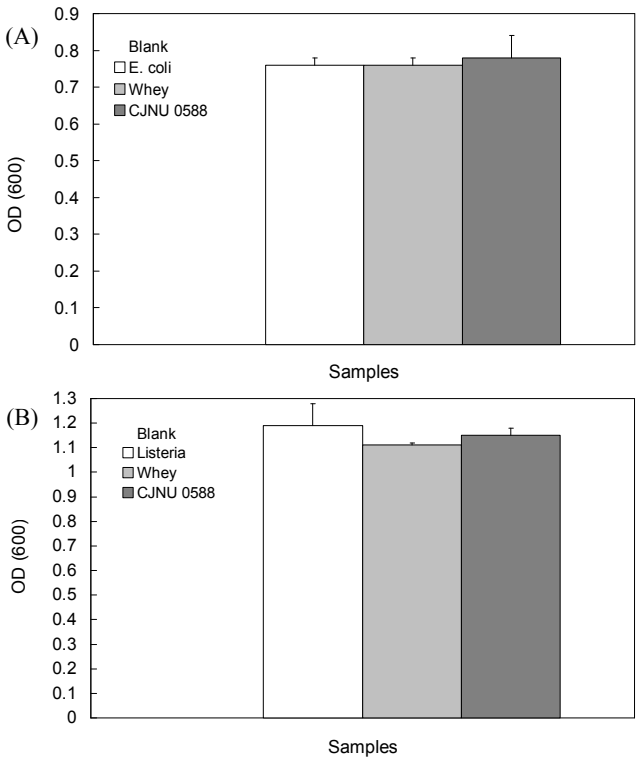

Fig. 2. Growth stimulation activity of the whey sample fermented by CJNU 0588 on *Escherichia coli* DH5α (A) and *Listeria monocytogenes* ATCC 19111 (B). Blank, negative control; Whey, bacteria plus whey medium; CJNU 0588, bacteria plus whey sample fermented by CJNU 0588. The data represent the mean±standard deviation of triplicate experiments.

by whey fermentation, as in this study. On the other hand, a metabolite, 1,4-dihydroxy-2-naphthoic acid (DHNA), produced by *Propionibacterium freudenreichii* ET-3, showed bifidogenic growth stimulation activity and has been intensively studied by a Japanese research group (20-22).

As mentioned above, whey is a good medium source for the growth of industrial bacterial strains that can metabolize lactose as a carbon source. If GRAS bacterial strains can produce beneficial metabolites via whey fermentation, the products of this type of fermentation could be commercialized and used as value-added foods of various types with higher prices. Because whey itself also contains functional ingredients (8,11), the combined functional metabolites would significantly improve the value of fermented whey. In this respect, the results of this study are highly relevant and the growth conditions of the *L. casei* strains screened in this study should be optimized to maximize the production of bifidobacterial growth factors. In addition, these occurring growth factors should be identified and characterized. This information should prove to be highly valuable in the eventual industrialization of this method and the bifidobacterial growth factors.

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