



# Identification of a Novel Potential Probiotic *Lactobacillus plantarum* FB003 Isolated from Salted-Fermented Shrimp and its Effect on Cholesterol Absorption by Regulation of NPC1L1 and PPAR $\alpha$

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## Abstract

Cholesterol-lowering activity is an important health benefit of lactic acid bacteria (LAB). This study aimed to screen LAB strains with cholesterol-lowering activities from salted fermented shrimp and evaluate probiotic characteristics and cholesterol-lowering potentials of these LAB isolates. Among 191 lactic acid strains isolated from traditional salted-fermented shrimp food, FB003 isolate showed the highest cholesterol-lowering activity and investigated as probiotics with cholesterol-lowering ability. Biochemical analysis and 16S rRNA sequencing revealed that this LAB isolate was *Lactobacillus plantarum* FB003. To screen probiotic trait, *L. plantarum* FB003 was found to be susceptible to six antibiotics tested and broad-spectrum antimicrobial activity. It also produced various enzymes such as galactosidase, glucosidase, and mannosidase. In addition, this strain showed autoaggregation, and coaggregation capacity for various pathogens. Moreover, it could adhere to Caco-2 cells and be exerted lowering cholesterol effects in Caco-2 cells via an upregulation of PPAR $\alpha$  to inhibit NPC1L1 mRNA expression. Strain *L. plantarum* FB003 might be effective as a candidate probiotic with high cholesterol-lowering activity. The results of the present study suggest that *L. plantarum* FB003 have an impact on preventing high cholesterol level and may be used as starter culture for shrimp fermentation.

**Keywords** Cholesterol · *Lactobacillus plantarum* · NPC1L1 · PPAR $\alpha$  · Probiotic

## Introduction

It is well known that consuming a high cholesterol and fat diet is closely related to elevated serum cholesterol level. High blood cholesterol levels or hypercholesterolemia can raise the development of obesity, colon cancer, and cardiovascular disease. The effect of fermented food containing lactic acid bacteria (LAB) for decreasing cholesterolemia was first studied in Maasai [1]. LAB strains are garnering increasing scientific and commercial interest as apart of healthy diet for humans and animals due to its non-pathogenic characteristic and high acid and bile tolerance ability [2]. In addition, they can provide an effective barrier against microbial infections [3]. Most LAB

are considered as probiotics including two genera: *Lactobacillus* and *Bifidobacterium*.

Recently, LAB have attracted attention of studies because of its beneficial effect on lipid metabolism as potential cholesterol-lowering food additives in animal models [4, 5]. There are a number of mechanisms underlying the lowering cholesterol activity of probiotic bacteria, as increased deconjugation of bile resulting in increasing cholesterol synthesis, reducing cholesterol transport and serum cholesterol [6]. Indeed, *L. acidophilus* ATCC 4962 removed cholesterol and produces short-chain fatty acid (SCFA) in medium with mannitol, FOS, and inulin supplement [7]. The probiotic formula of *L. rhamnosus* BFE5264 and *L. plantarum* NR74 decreased the cholesterol levels by upregulating ABCG5/8 [8] and inhibiting of NPC1L1 expression [9] in Caco-2 colon epithelial cells. In addition, the cholesterol-lowering effect thought PPAR $\alpha$ -agonistic activity has been only reported by *L. amylovorus* CP1563 in a mouse model of HFD-induced obesity [10]. However, the application of *Lactobacillus* in hypercholesterolemia treatment represents only in vivo studies, due to the mode of action varies depending on LAB species [11].

Salted and fermented small shrimp (SFS) is one of the most important ingredients used in *kimchi*, a Korean fermented

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vegetable product made by adding 20–30% (*w/w*) salt [12]. Although LAB accounts about 1% of the microorganism community of SFS due to its salt concentration, many LAB strains have been isolated and characterized as probiotics and bacteriocin producers for future applications [3, 13, 14]. SFS has the ability to suppress cholesterol levels in mice model [15]. The capacity of LAB to degrade cholesterol in cholesterol-containing fermented foods might be beneficial for human health [3, 13]. Hence, this study proposed that traditional salted and fermented small shrimps might be a good source for screening LAB strains with high cholesterol-lowering property.

Many studies have investigated cholesterol-lowering profiles of LAB strains isolated from fermented products [16, 17]. To the best of our knowledge, little information is available regarding cholesterol-lowering characteristics of LAB strains isolates from SFS. Therefore, the aim of the study was to screen LAB strains with high cholesterol-lowering activities from SFS and evaluate probiotic characteristics and cholesterol-lowering potentials of these LAB isolates through *in vitro* assays.

## Materials and Methods

### Bacteria Isolation and Pathogen Strains

LAB strains were screened and isolated from salted shrimp obtained from a household in Yeosu, Korea, using MRS agar plates for 24 to 48 h in incubator at 37 °C. In this study, the pathogenic strains and the reference stains including *Lactobacillus rhamnosus* GG (LGG) and *Pediococcus pentosaceus* KACC 12311 were obtained from the Korea and USA microbial culture collections such as KACC, KCCM, KCTC, and ATCC. The pathogens were maintained and grew in brain-heart infusion (BHI) medium at same above condition.

### Screening Cholesterol Assimilation Activity

The isolated strains were grown in 5 mL MRS broth and adjust to reach the A<sub>600</sub> of 0.5 before cholesterol assimilation experiments. About 5 mL of Cho-MRS broth (0.1% cholesterol and with or without 0.3% bile oxgall) was inoculated with 1% of bacterial culture overnight at 37 °C. After incubation, the media was centrifugated at 9000×*g* for 5 min. Cholesterol assimilation activity of the bacterial supernatant and sterile Cho-MRS media was then determined using the o-phthalaldehyde method [18].

### Strain Identification and Biochemical Test

Selected FB003 strain was further identified using both biochemical methods by API 50 CHL kit, API ZYM kit (bioMérieux, France) and identification using the 16S rRNA gene sequence conducted by Bioneer Co. (South Korea). The sequence of

FB003 was analyzed and deposited at NCBI GeneBank with accession number MF436193 as *Lactobacillus plantarum*. A phylogenetic tree was constructed using MEGA7 program [19].

## Characterization of Probiotic Properties

Antibacterial activity of *L. plantarum* FB003 against foodborne pathogens was assessed using agar well diffusion method [20]. Briefly, the bacterial broth of FB003 after 18 h of culture was filtered to remove the cells and tested against *Bacillus cereus*, *Escherichia coli* K99, *L. monocytogenes*, *Staphylococcus gallinarum*, *Salmonella enterica*, *S. typhi*, *Shigella boydii*, and *Yersinia enterocolitica*.

The minimal inhibitory concentrations (MIC) for a relevant range of antibiotics for strain FB003 were estimated using broth microdilution method [21]. The standard MIC values for *Lactobacillus* the European Food Safety Authority [22] were used to define the antibiotic susceptibility of the strain.

The safety assessment of FB003 strain was evaluated to produce biogenic amine, degrade mucin, and hemolytic activity. The strain was streaked on Møller decarboxylase medium supplied 0.5% various of amino acids and incubated overnight at 37 °C to determine biogenic amine production [23]. The colony of FB003 was streaked on MRS, containing 5% (*w/v*) sheep blood to evaluate the hemolytic activity. Mucin degradation was established using protocol described by Abe et al. [24].

The digestion tolerance assay was performed to evaluate survival of the strain after passage through human gastrointestinal tract using published method [25].

The autoaggregation and coaggregation activity were estimated according to Collado et al. [26] with slight modifications. Briefly, bacteria were grown in MRS broth at 37 °C for 18 h and followed by centrifugation at 9000×*g* for 5 min at 4 °C. The cell was suspended in PBS and mixed well by vortexing for 10 s. The suspension was incubated at 37 °C for 5 h. The aliquot was taken out every hour to measure absorbance at 600 nm. The autoaggregation percentage was expressed as

$$\text{Autoaggregation (\%)} = 1 - (A_t/A_0) \times 100 \quad (1)$$

where  $A_t$  was the absorbance at a certain time point and  $A_0$  was the absorbance at 0 h.

To test the coaggregation, the FB003 suspensions were prepared as described for autoaggregation. They were then mixed with pathogen strains at equal volume followed by incubating at 37 °C without agitation. The coaggregation percentage was expressed as:

Coaggregation

$$= \left\{ \left[ (A_{\text{pathogen}} + A_{\text{FB003}}) / 2 - A_{\text{mix}} \right] / (A_{\text{pathogen}} + A_{\text{FB003}}) / 2 \right\} \times 100 \quad (2)$$

where  $A_{\text{pathogen}}$ ,  $A_{\text{FB003}}$ , and  $A_{\text{mix}}$  were absorbance values (600 nm) of pathogen, FB003 suspension in control tube, and mixed bacterial suspension at 5 h, respectively.

### Caco-2 Cell Culture

The Caco-2 epithelial cell line was maintained in DMEM with 10% (v/v) heat-inactivated FBS and 100 units/mL of penicillin-streptomycin at 37 °C in a humidified atmosphere of 95% air and 5% CO<sub>2</sub>. Caco-2 cells were then seeded at 10<sup>5</sup> cells/mL in 12-well plates containing DMEM with 10% FCS and incubated at 37 °C with 5% CO<sub>2</sub> until a confluent monolayer was obtained. Medium was refreshed daily. Caco-2 monolayer was pre-incubated with non-antibiotic-supplemented DMEM for 2 h before further assay.

### Adhesion Assay

One milliliter of bacterial suspension (10<sup>8</sup> cfu/mL of final concentration) was added to each well and incubated at 37 °C with 5% CO<sub>2</sub> for 1 h. After washing with PBS (pH 7.2), the cell was treated with 1 mL of 1% Triton X-100. The resulting suspension was plated onto MRS and incubated at 37 °C overnight to determine the number of adhered bacterial cells.

### Bacterial Suspension and Co-Culture

*L. plantarum* FB003 and LGG were grown in 20 mL MRS broth for 24 h at 37 °C before experimental use. The viable cell was collected and adjusted to cell density of 10<sup>7</sup> cfu/mL. The monolayer of Caco-2 cells was washed with PBS buffer and followed by incubating with 2 mL of bacterial suspension or with medium for 6 h. The heat killed bacterial cells (HK, 80 °C, 30 min) were used as control. The bacterial metabolites (BM) were prepared from the filtered culture broth of the probiotics FB-003 and Caco-2 cells incubated for 6 h.

### Cholesterol Uptake Assay

Cholesterol uptake assay was analyzed by comparing better Caco-2 cell treated with the viable bacterial cells, the HK, the BM, and the non-treated Caco-2 cells. The monolayer was primarily treated with 0.15 ml micellar solution consisted of 300 μM taurocholate, 2 mCi [<sup>3</sup>H] cholesterol, and 150 μM phosphatidylcholine before 1 h collecting cells. The cells were washed twice with cold cell free DMEM medium. The cells were homogenized with 100 μl of the lysis buffer. The intracellular radioactivity was measured in a liquid scintillation counter (Winspectral 1414, Wallac, Turku, Finland).

### Real Time PCR Analysis

The Caco-2 cells were immersed in Trizol reagent (Invitrogen, Carlsbad, CA, USA) and total RNA was isolated according to manufacturer's instructions. For mRNA expression analysis, 2 μg of total RNA was reverse transcribed with PrimeScript RT Reagent Kit (TaKaRa Bio Inc., Shiga, Japan) and mRNA level of genes of interest was measured by real-time quantitative PCR (qPCR) using the appropriate primers (for NPC1L1 gene, NPC1L1F: 5'-TATGGTCGCCCCGAAGCA-3' and NPC1L1R: 5'-TGCGGTTGTTCTGGAAATACTG-3'; for PPARα gene, PPARαF: 5'-GCCTGTCTGTCGGGATGT-3' and PPARαR: 5'-GGCTTCGTGGATTCTCTTG-3'; and for β-actin gene, ActF: 5'-CCTGGCACCCAGCACAAAT-3' and ActR: 5'-GCCGATCCACACGGAGTACT-3') with SYBR Premix Ex Taq system (TaKaRa, Shiga, Japan).

### Statistical Analysis

SPSS 20 programs (IBM, USA) was used to perform one-way analysis of variance (ANOVA) with Tukey's post hoc test. Statistical significance was considered at  $p < 0.05$ . All results are presented as mean ± standard deviation.

## Results

### Lactic Acid Bacteria in Salted Shrimp

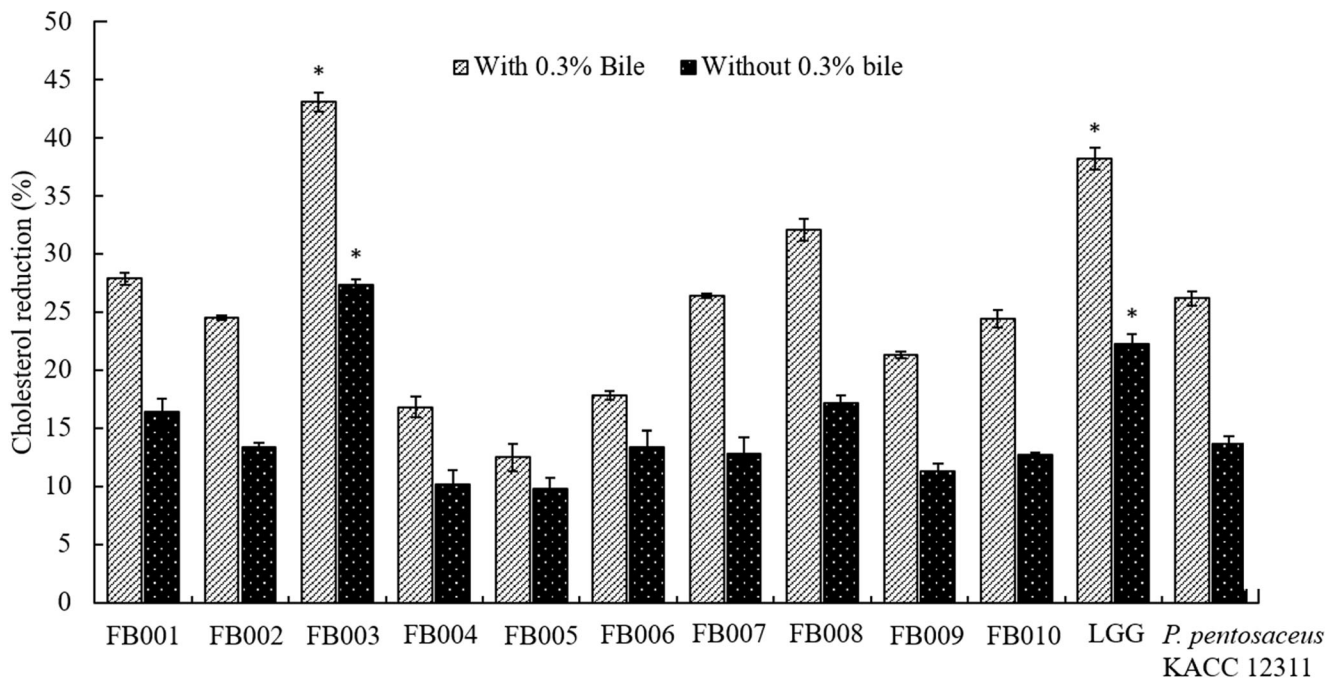
Among 191 bacteria isolated from local salted and fermented shrimp, ten isolates showing typical appearance of LAB on MRS medium were selected. These isolates were catalase negative. They had coccus and rod shape. They were found to be Gram-positive bacteria.

### In Vitro Test for Cholesterol-Lowering Activity

Results showed that these isolates had various cholesterol-lowering activities, ranging from 12.5 to 43.1 μg/mL. FB003 isolate assimilated higher amounts of cholesterol compared to *L. rhamnosus* GG (LGG) and *P. pentosaceus* KACC 12311, which assimilated 38.2% and 26.1%, respectively. The lowest cholesterol reduction rate was found for isolate FB005, which assimilated 12.5% compared to control in cholesterol-supplied MRS broth after 24 h (Fig. 1). Cholesterol reduction activity was significantly increased in the presence of 0.3% bile compared to that in bile free MRS medium supplied cholesterol.

### Identification of Selected LAB with the Highest Cholesterol-Lowering Activity

FB003 isolate with the highest cholesterol-lowering activity among all LAB strains tested was subjected to biochemical



**Fig. 1** Assimilation of cholesterol by LAB-isolated strains, *L. rhamnosus* GG (LGG), and *P. pentosaceus* KACC 12311. \* $p < 0.05$

identification using API CHL kit (Table S1) and API ZYM kit (Table S2). The 1432 bp sequence of the amplified 16S rRNA of the isolate was found to share 98.6% of identities (difference in 20 of 1432 bp) with *Lactobacillus plantarum* ATCC 14917 deposited at GenBank. Phylogenetic analysis showed that FB003 isolate was constructed in the same cluster as those of *L. plantarum* DSM 10667, *L. plantarum* NBRC 15891, and *L. plantarum* JCM 1149 (Fig. 2).

### Safety Assessment, Antimicrobial, and Antibiotic Activity

The FB003 isolate did not possess bioamine production, hemolytic activity, or mucinolytic activity. However, it exhibited various inhibitory activities towards pathogenic bacteria used in this study (Table 1). For *L. monocytogenes*, *S. aureus*, and *S. enterica*, the clear zone of inhibition was  $\geq 8$  mm. However, FB003 isolate had weak antimicrobial activities against *E. coli* and *Y. enterocolitica*. *L. plantarum* FB003 was found to be susceptible to all tested antibiotics according to cutoff values recommended by EFSA [22]. Other important characteristics when screening for potential probiotics are the absence of undesirable traits and safety aspect as those analyzed in this work.

### Survival During GI Transit

*Lactobacillus plantarum* FB003 strain has strong bile salt tolerance and resistance to lysozyme (remained 98% of viability) (Table 2). In in vitro gastric condition, the survival rate of this isolate was  $\geq 94\%$  at pH 3, 87% at pH 2, and 85% under

intestinal stress. *L. plantarum* FB003 had higher tolerance to all stresses than reference strain *L. rhamnosus* GG.

### Autoaggregation, Coaggregation, and Adhesion to Caco-2 Cells

Autoaggregation and coaggregation were investigated on the basis of sedimentation characteristics of probiotic strain. Results showed that *L. plantarum* FB003 exhibited strong autoaggregating ability (44%) after 5 h of incubation (Fig. 3a). The ability of *L. plantarum* FB003 to coagregate with *L. monocytogenes* (43%) was significantly better than that with other pathogens such as *S. Choleraesuis* (32%), *Y. enterocolitica* (18%), and *B. cereus* (15%) after 5 h of incubation (Fig. 3b). As shown in Fig. 4, the adhesion ability of FB003 to Caco-2 cells was higher than that of *L. rhamnosus* GG.

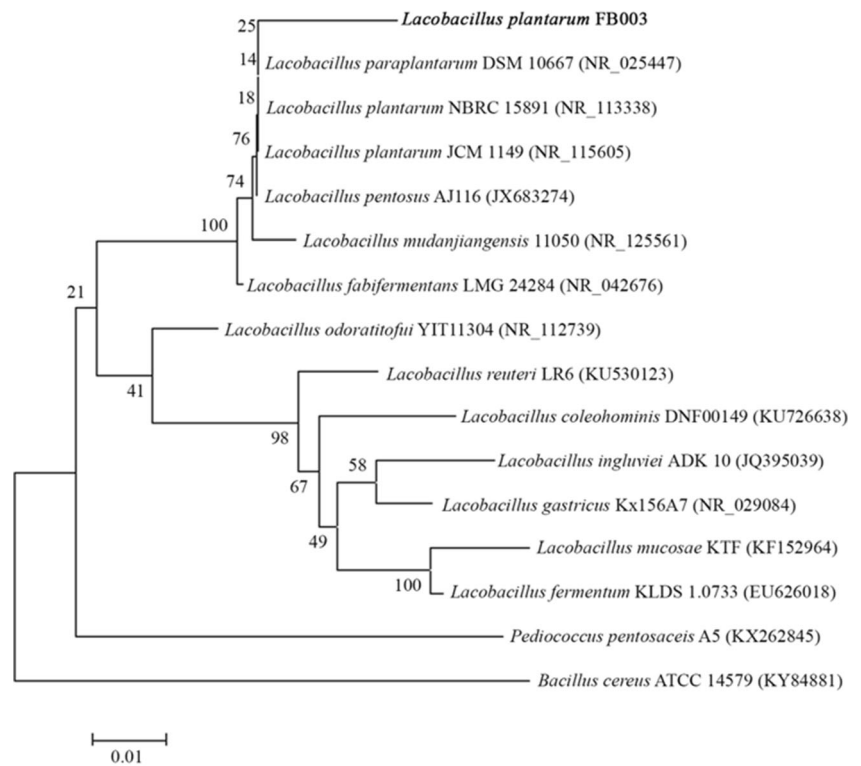
### LAB Effect on NPC1L1 and PPAR $\alpha$ Expression

To determine the mechanism of LAB affect cholesterol uptake, the mRNA expression of genes encoding protein related to cholesterol metabolism, NPC1L1, and PPAR $\alpha$  was measured (Fig. 5b, c). *L. plantarum* FB003 and LGG exhibited significant downregulation of NPC1L1 compared to non-treated control.

### Suppression of Cholesterol Uptake by Live Bacteria

To confirm effect of LAB on cholesterol uptake on Caco-2 cells, the treatment of Caco-2 monolayers with the viable bacteria, heat-killed bacteria, and bacterial metabolites was

**Fig. 2** Phylogenetic tree constructed based on 16S rRNA gene sequence of strains FB003 isolated from shrimp jeotgal. The 16S rRNA gene sequence of this strain showed 96% similarity with *Lacobacillus plantarum* JCM 1149. GenBank accession number is indicated in parenthesis



compared to non-treated control. Based on these observations, the cells treated with both viable *L. plantarum* FB003 and LGG showed the significant reduction in cholesterol uptake by fivefold (Fig. 5a). Moreover, the bacterial metabolites of LAB also decreased uptake of cholesterol, while there is not significant change in heat-killed bacteria treatment.

## Discussion

In this paper, LAB strains were isolated and selected basing on the probiotic criteria and health-promoting effects such as reducing cholesterol. Microorganisms to be applied as clinical

probiotic must overcome low-acid conditions of human gastrointestinal tract. They must exert health-promoting effects such as reducing cholesterol. The bacterial community in traditional salted and fermented seafood can vary greatly due to different types of seafood (such as shrimp, oysters, clams, and fish) and their natural environment [27]. Based on previous study, LAB detected in traditional salted and fermented shrimp accounts for 1 to 10% of the entire population of bacteria [12].

Hence, LAB FB003 was subjected to strain identification and probiotic property characterization. The cholesterol-lowering activity of LAB is considered an important parameter when selecting probiotic strains with diverse health promoting benefits. A number of in vitro studies have confirmed that some

**Table 1** Antimicrobial activity of cell free supernatant broth of isolates against common pathogens, antibiotic resistance (MIC), and safety assessment

Pathogenic strains <sup>a</sup>	<i>B. cereus</i>	<i>E. coli</i> K99	<i>L. monocytogenes</i>	<i>S. gallinarum</i>	<i>S. aureus</i>	<i>S. enterica</i>	<i>S. typhi</i>	<i>S. boydii</i>	<i>Y. enterocolitica</i>	MIC (µg/mL)	Antibiotic	Activity
											Penicillin	4(S) <sup>b</sup>
											Ampicillin	1(S)
											Vancomycin	64(n.r) <sup>c</sup>
											Streptomycin	4(S)
											Kanamycin	8(S)
											Tetracycline	2(S)
										Bioamine production		ND <sup>d</sup>
										Hemolytic activity		ND
										Mucin degradation		ND

<sup>a</sup> +: ≤ 4 mm; ++: ≤ 8 mm; +++: zone > 8 mm

<sup>d</sup> NR not required

<sup>c</sup> S susceptible

<sup>d</sup> ND non-detected

**Table 2** Oro-gastrointestinal transit tolerance of LAB strains

Candidate strains	Oro-gastrointestinal transit tolerance (Log <sub>10</sub> (CFU mL <sup>-1</sup> ))					Viability after OGT assay (Log reduction)
	Initial	Oral stress (10 min)	Gastric stress pH 3 (30 min)	Gastric stress pH 2 (30 min)	Intestinal stress (120 min)	
<i>Lactobacillus plantarum</i> FB003	9.10 ± 0.04	8.94 ± 0.03	8.59 ± 0.07	7.88 ± 0.02	7.69 ± 0.04	1.57 ± 0.06*
<i>Lactobacillus rhamnosus</i> GG	9.01 ± 0.02	8.44 ± 0.02	8.39 ± 0.02	7.28 ± 0.04	7.29 ± 0.01	1.72 ± 0.02

\* $p < 0.05$

strains of *Lactobacillus* isolated from fermented beverage have various cholesterol reduction rates, ranging from 40 up to 76% [28]. The lower cholesterol reduction activity of LAB isolated from fermented shrimp might be attributed to natural selection of LAB strains with high fat concentration.

To the best of our knowledge, this is the first study reporting that *L. plantarum* FB003 strain isolated from salted and fermented shrimp could lower cholesterol. In the literature, *L. plantarum* has been recognized to be able to remove cholesterol from growth media in many studies. However, most of them were isolated from human, fermented beverages, or vegetables [29, 30].

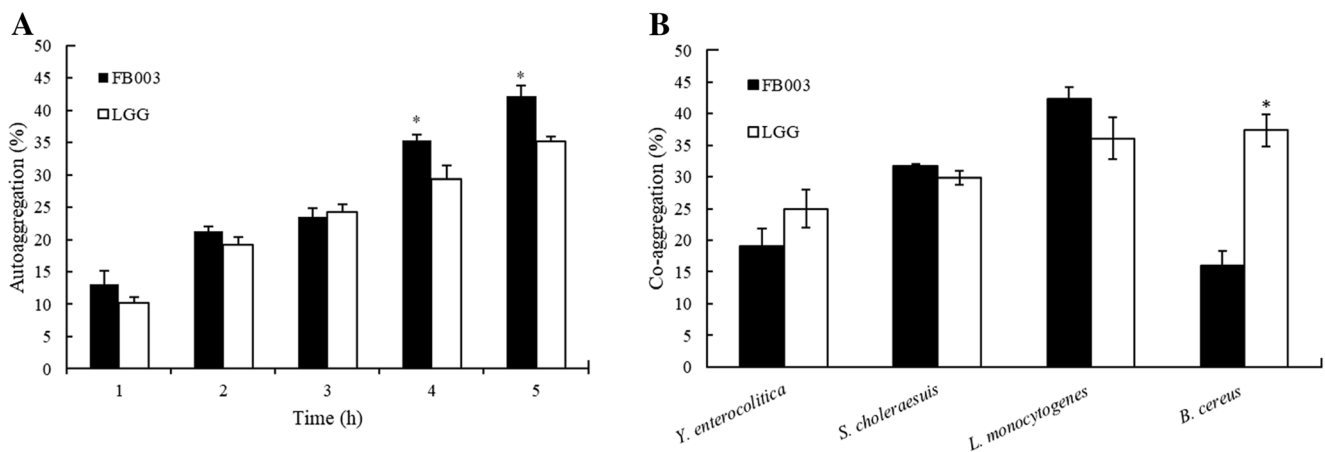
Other important characteristics when screening for potential probiotics are the absence of undesirable traits and safety aspect as those analyzed in this work. Antimicrobial properties are important selection criteria so that probiotics can be useful for clinical treatment [31]. Many previous studies have provided evidence that *Lactobacillus* has a wide inhibitory spectrum against many harmful organisms such as *Salmonella*, *E. coli*, *Listeria*, and *Streptococcus*. *L. plantarum* FB003 might have intrinsic or natural non-transmissible resistance [32]. It might be useful as feed-additive.

Probiotic strains need to survive during digestion from the stomach to the upper part of the intestinal tract to exert their effect [33]. LAB must be able to resist the strong acidic gastric

fluid in the stomach with pH of 2–3 and weakly basic in the small intestine with pH of 8 containing 0.3–3% (*w/v*) concentration of bile salts for 1–2 h [28]. Many studies have shown that *L. plantarum* has high survival rate in low pH, with survival rate ranging from 40 to 90% [34]. Therefore, the high tolerance of FB003 to GI tract conditions observed in this study complies with the prerequisites of a probiotic.

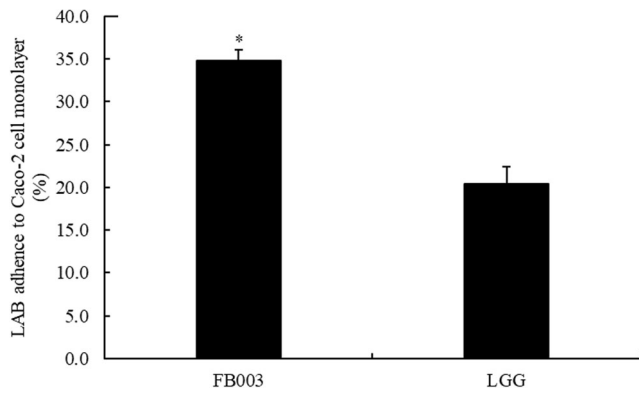
The adhesive capacity of LAB strain is related to cell surface proteins which are major factors for binding of bacteria to the human intestinal epithelium to degrade substrates and remove pathogenic bacteria [23, 35]. When pathogenic bacteria and probiotics are mixed together, the probiotics can efficiently inhibit the growth of pathogenic bacteria because antimicrobial compounds can directly target pathogens. Based on the classification of probiotics [36] as non-adhesive (<5%), adhesive (5–40%), and strongly adhesive (40%), our results demonstrated that the FB003 strain had a strong adhesive capacity to the intestinal tract.

The inhibition of NPC1L1 was reported in different *Lactobacillus* genus such as *L. acidophilus* ATCC 4356, *L. rhamnosus* BFE5264, and *L. plantarum* [9, 37, 38]. However, in addition to upregulation of NPC1L1 gene, we also observed overexpression of PPAR $\alpha$  in Caco-2 cells despite the observed cholesterol-lowering effect. A recent study showed that the administration of *L. kefir* DH5 significantly upregulated the



**Fig. 3** Aggregation, coaggregation property of *L. plantarum* FB003 from salted fermented shrimp. **a** Autoaggregation percentage of *L. plantarum* FB003 strain and *L. rhamnosus* GG (LGG) strains. **b** Coaggregation percentage recorded for *L. plantarum* FB003 and *L. rhamnosus* GG (LGG) with

*Yersinia enterocolitica* ssp. *enterocolitica* KACC 15320, *Salmonella* Choleraesuis KCTC 2932, *Listeria monocytogenes* KACC 10764, and *Bacillus cereus* KACC 11240 after 5 h of incubation at 37 °C in PBS. Error bars represent standard deviations of mean values ( $n = 3$ ). \* $p < 0.05$



**Fig. 4** Adherence ability of *L. plantarum* FB003 strain to Caco-2 cell monolayer. Error bars represent standard deviations of mean values ( $n = 3$ ). \* $p < 0.05$

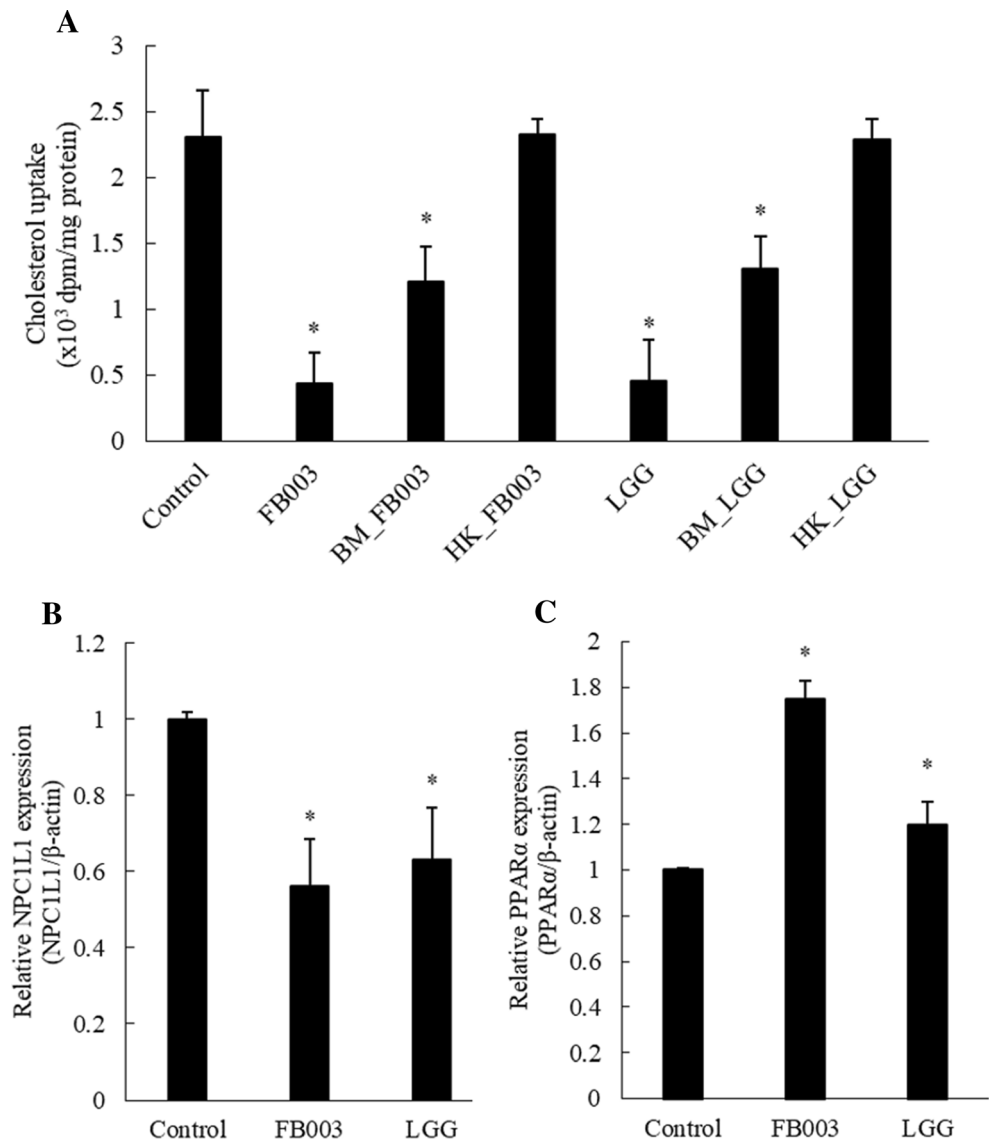
expression of PPAR $\alpha$  in the epididymal adipose tissue [39]. Moreover, Valasek et al. [40] suggested that intestinal expression of NPC1L1 may be regulated through PPAR $\alpha$ . Thus, the first

suggests the *L. plantarum* FB003 inhibits cholesterol uptake in human Caco-2 cell through a nuclear receptor PPAR $\alpha$  to inhibit NPC1L1 mRNA expression.

In summary, lactic acid bacteria isolated from salted and fermented shrimp were evaluated as probiotics for food according to guidelines of the Joint FAO/WHO working group. *L. plantarum* FB003 showed great potential for clinical treatment to reduce cholesterol level. *L. plantarum* exerted lowering cholesterol effects in Caco-2 cells via an upregulation of PPAR $\alpha$  to inhibit NPC1L1 mRNA expression. It also showed potential as a probiotic for the food industry. Further studies are needed to explore the health benefit of *L. plantarum* FB003 in vivo using foods made by this isolate.

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**Fig. 5** Cholesterol-lowering effect and expression of cholesterol-related genes in Caco-2 cells treated with lactic acid bacteria (LAB) for 6 h exposure to cholesterol. **a** Cholesterol uptake in Caco-2 cells, **b** NPC1L1, and **c** PPAR $\alpha$  expression. Expression of all genes was normalized to  $\beta$ -actin (internal control). Error bars represent standard deviations of mean values ( $n = 3$ ). \* $p < 0.05$



## Compliance with Ethical Standards

**Conflict of Interest** The authors declare that they have no conflict of interest.

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