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SCREENING BILE SALT HYDROLASE ACTIVITY OF *LACTOBACILLUS* ISOLATED FROM VIETNAMESE HUMAN ORIGINS

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SUMMARY

Hypercholesterolemia is a major cause of cholesterol build-up in the coronary arteries, which can subsequently lead to heart disease or atherosclerosis. Cholesterol levels can be lowered by cholesterol-lowering drugs but some of these drugs may have harmful side effects, while supplementation of *Lactobacillus* has shown the potential to reduce serum cholesterol levels by virtue of bile salt hydrolase (*bsh*) activity. In this study, *Lactobacillus plantarum* VFE-04, *L. rhamnosus* VFE-08, and *L. plantarum* VFE-14, had been isolated from Vietnamese healthy adults, were able to deconjugate glycodeoxycholate (GDC) on MRS plates and MRS broth supplemented with GDC. In addition, deconjugating activity of *L. plantarum* VFE-04, *L. rhamnosus* VFE-08, and *L. plantarum* VFE-14 were also found in cell-free extract as expressed by amount of glycine that released in the supernatant. Four *bsh* genes including *bsh*1, *bsh*2, *bsh*3, and *bsh*4 have been identified by PCR in these strains. In addition, *L. plantarum* VFE-04, *L. rhamnosus* VFE-08, and *L. plantarum* VFE-14 also showed high ability to resist bile salts and low pH. The results of 16S rRNA gene analyses showed that *L. plantarum* VFE-04, L. rhamnosus VFE-08, and *L. plantarum* VFE-14 and had high similarity scores with *L. plantarum* ZZU 23 (100%), *L. rhamnosus* JCM 1136 (99%) and *L. plantarum* S7 (98.65%), respectively. This study suggests that *L. plantarum* VFE-04, *L. rhamnosus* VFE-08, and *L. plantarum* VFE-14 have the potential to be explored as probiotics in the management of hypercholesterolaemia in near future.

Keywords: *Lactobacillus,* bile salt deconjugation, cholesterol, deconjugate glycodeoxycholate, human origins

INTRODUCTION

Hypercholesterolemia is a major cause of cholesterol build-up in the coronary arteries, which can subsequently lead to heart disease or atherosclerosis (Argyri, Zoumpopoulou *et al.*, 2013) Based on a meta-analysis, a 1% decrease in plasma cholesterol levels can lower the risk of coronary events by up to 3%. Cholesterol levels can be lowered by several independent mechanisms, including decreasing hepatic cholesterol biosynthesis, increasing plasma

cholesterol removal, and disruption of bile acid reabsorption (Duary *et al.,* 2010; Gu *et al.,* 2014). Bile acids synthesized from cholesterol in the liver are important end products of cholesterol metabolism in mammals, and are secreted mostly as conjugated with glycine or taurine by an amide bond. Conjugated and unconjugated bile acids are absorbed by passive diffusion along the entire gut and by active transport in the terminal ileum (Heunis *et al.,* 2014). In the gut, intestinal microbes are capable of hydrolyzing the glycine or taurine conjugated with bile acids using bile salt hydrolase (BSH) enzymes. After the removal of glycine or taurine, deconjugated bile salts are less water-soluble and are more easily excreted via the feces. Enhanced fecal loss of bile acids may result in an increased requirement for cholesterol as a precursor for the de novo synthesis of bile salts in order to maintain bile salt homeostasis. Therefore, this drain on the bile salt pool might be regarded as a 'biological' alternative to common therapeutic interventions to treat hypercholesterolemia (Patel *et al.,* 2010; Nie *et al.,* 2015).

Probiotic *Lactobacillus* are considered as normal components of the intestinal microflora in both humans and animals, and have been invariably associated with various health-promoting functions (Kim, Lee, 2005; Duary *et al.,* 2010, Patel *et al.,* 2010; Kumar *et al.,* 2011; Kumar *et al.,* 2012). Previous studies have demonstrated that consumption of *L. plantarum* Lp91 and *L. plantarum* Lp09 (Duary *et al.,* 2012) as probiotic dietary adjuncts are useful in reducing plasma cholesterol levels (Duary *et al.,* 2010; Duary *et al.,* 2012). Besides deconjugation of bile via *BSH* enzymes, there are several mechanisms for cholesterol removal by probiotics *Lactobacillus* viz.; assimilation of cholesterol by growing cells, binding of cholesterol to the cellular surface, incorporation of cholesterol into the cellular membrane, co-precipitation of cholesterol with deconjugated bile, binding of bile by fiber, and production of short-chain fatty acids by oligosaccharides (Ridlon *et al.,*2006; Lambert, 2008, Sedláčková *et al.,* 2015; Shehata *et al.,*2016). However, in recent years, the possibility of using bile salt deconjugation by *Lactobacillus* to lower serum cholesterol levels *in vivo* has received considerable attention. Bile salt hydrolase activity of probiotic *Lactobacillus* is more often found in strains isolated from the gastrointestinal tract, where bile salts are present, rather than in strains from the non-bile salt environment (Ramasamy *et al.,* 2010; Öner *et al.,* 2014; Tsai *et al.,* 2014; Bustos *et al.,*2018). The aims of this study were to select *Lactobacillus* strains with bile salts deconjugate activity from Vietnamese adult origins and screen probiotic potential of selected *Lactobacillus* that can be used as probiotics in near future to help hypercholesterolaemia patients.

MATERIALS AND METHODS

Chemicals and reagents

All chemicals and solvents used in this study were of the highest purity commercially available. Standard bile salts, including glycocholate (GC), glycodeoxycholate (GDC), taurocholate (TC), taurodeoxycholate (TDC) and oxgall powder were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Isolating and screening bile salts deconjugation-positive *Lactobacillus*

The *Lactobacillus* strains were isolated as described in previous study (Thu *et al.,*2020). *Lactobacillus* colonies were first screened for catalase activity and Gram staining, and only those that were catalase-negative and Gram-positive were used for further investigations.

Deconjugation ability was carried out on the MRS plate supplemented with 0.3% oxgall and 0.37 g/L $CaCl_2$ (Öner *et al.,*2014). Once agar plates were solidified, overnight MRS broth cultures were streaked on and incubated anaerobically at 37^{o}C for 48-72 h. MRS agar plates without oxgall were used as controls. The white precipitates around colonies and the clearing of the medium are indicative of deconjugating activity (McAuliffe *et al.,*2005).

Determination of acid tolerance

Acid tolerance of the Lac.VFE-04, Lac.VFE-08, and Lac.VFE-14 were studied. 10^8 CFU/mL of selected *Lactobacillus* cultures were inoculated in MRS media pre-adjusted to pH 2.0 and 3.0 using 1 M HCl and incubated at 37ºC for 3 h. Viability was determined using the plate count method. Serial tenfold dilutions of the sample were plated onto MRS agar and incubated anaerobically at 37ºC for 24-48 h to obtain the colony count (Zheng *et al.,* 2013).

Determination of bile tolerance

Bile tolerance is an important criterion in the selection of probiotic strains. Previous reports have suggested that a concentration of 0.3% oxgall closely approximates the bile levels found in human gastrointestinal tract (McAuliffe *et al.,* 2005). To evaluate the bile tolerance ability of *Lactobacillus*, isolates were inoculated (2% w/v) into MRS broth with 0.3%, 0.5%, or 1% (w/v) of oxgall. After 24 h cultivation at 37ºC, A560 nm was measured and the results were expressed by the percentage of growth (A560 nm) in the presence of oxgall compared to the control culture without oxgall (Isa, Vasavi, 2017).

Substrate specificity

To screen the substrate specificity, oxgall-hydrolyzed *Lactobacillus* strains were investigated. Agar plates containing 1.5% (w/v) agar and 0.37 g/L $CaCl_2$ or MRS broth supplemented with either 5 mM GC, 5 mM GDC, 5 mM TC, or 5 mM TDC. The centrifuged and concentrated cultures (10 µL) were spotted on the plates and incubated anaerobically at 37ºC for 48-72 h. MRS agar plates without bile salts were used as controls. Each strain was tested in triplicate. The white precipitates around colonies, the clearing of the medium or the broth cloudy are indicative of BSH activity (Begley *et al.,* 2006).

BSH activity was also further monitored by measuring glycine liberation from GDC following the protocol described by Grill. To summarize, cell-free extract was mixed with 5 mM GDC and incubated for 24 h at 37ºC. Controls were performed in the absence of GDC. Glycine concentration was measured at 416 nm using a spectrophotometer. A standard curve was established using free glycine (Grill *et al.,* 2000).

Bsh gene investigation

Genomic DNA of selected *Lactobacillus* was extracted using a Genomic DNA Isolation Kit (Invitrogen Inc.) according to the manufacturer's instruction. *bsh* genes were PCR-amplified from the genomic DNA of Lac.VFE-04, Lac.VFE-08, and Lac.VFE-14 using the primers (Table 1), which were designed based on the published genome sequence of *L. plantarum* WCFS1 (NC_004567.2) PCR products were analyzed and compared with the reference sequences available in GenBank (EMBL) The *bsh*1 of 3 reference strains were submitted to the GenBank database under accession numbers FJ179487, FJ179486, and EU822811, respectively.

Table 1. Nucleotide sequences of 4 pairs of primers.

Primer	Nucleotide Sequences
*bsh*1-F	GGAATTCCATATGATGTGTACTGCCATAACTTATC (35)
*bsh*1-R	CCGCTCGAGTTAGTTAACTGCATAGTATTGTG (32)
*bsh*2-F	GGAATTCCATATGATGTGCACTAGTCTAACTTATACAAA (39)
*bsh*2-R	CCGCTCGAGTTAATGGGCCGCTGGCAA (27)
*bsh*3-F	GGAATTCCATATGATGTGTACTAGTTTAACGATTCA (36)
*bsh*3-R	CCGCTCGAGTTAGTTTGCTAACCGGAAC (28)
*bsh*4-F	GGAATTCCATATGATGTGTACCAGCTTAACTTATCTTG (38)
*bsh*4-R	CCGCTCGAGTCAATCGGCAGGAAAGAGGT (29)

Note: the number of nucleotides in parenthese

RESULTS AND DISCUSSION

Screening BSH-positive lactic acid bacteria

In recent years, the interest in lactic acid bacteria that can deconjugate bile salts to prevent hypercholesterolaemia in obese people, or can lower serum cholesterol levels in hypercholesterolaemic people, has increased. *BSH* activity in the intestinal microflora has been screened in a number of studies and for specific groups of bacteria like *Lactobacillus*, *Bifidobacterium*, and *Enterococcus* (Begley *et al.*, 2006). To understand the distribution of *BSH* activity in lactic acid bacteria, we have screened *BSH* and bile salt tolerance of *Lactobacillus* isolated from Vietnamese adult origins.

Oxgall powder from Sigma-Aldrich was chosen because this mixture was closer to human bile in terms of its glycine-conjugated bile acids to taurine-conjugated bile acids ratio. In previous studies, 0.3% bile has been considered to be the crucial concentration at which to evaluate bile-tolerant probiotic *Lactobacillus*. In this regard, 11 strains of *Lactobacillus* showed good resistance to bile (data not shown). These findings corresponded with those of studies where *L. plantarum* strains from cheeses displayed good resistance to bile salts (Georgieva *et al.*, 2008; Pisano *et al.*, 2008; Zago *et al.*, 2011).

Acid tolerance

The ability of probiotic organisms to survive in acidic environments is an important characteristic of probiotics. In this study, *Lactobacillus* strains were incubated under conditions of pH 2 and pH 3 to evaluate their tolerance to acidic pH. As shown in Table 2, of the five tested *Lactobacillus* strains, Lac.VFE-14 had the highest acidic tolerance ability while this activity of Lac.VFE-09 was lowest as expressed by cell survival percentage.

At pH 3, the viability of Lac.VFE-14 was 98.54% ± 6.68, followed by Lac.VFE-04, Lac.VFE-08, Lac.VFE-11, and Lac.VFE-09. At pH 2, the cell viability was reduced in all of them. The survival of Lac.VFE-14 was 88.22 ± 5.14, where as in Lac.VFE-09, this percentage was 69.06 ± 3.89 (Table 2). Similar results were reported by Mirelahi, where the *L. plantarum* A7, *L. rhamnosus* GG, and *L. acidophilus.* Lac. strains showed tolerance to pH 3 (Pisano *et al.*, 2008). Another study was conducted by Deshpande demonstrated that *L. casei* can survive at pH 2, 3, and 4 for at least 90 min (Mirelahi *et al*., 2009, Deshpande *et al*., 2014, Zago *et al.*, 2011). However, *Lactobacillus* strains isolated from Koozeh cheese decreased significantly after incubation at pH 3 and at pH ≤ 2, and there were no viable bacterial cells detected after the first hour of incubation (Hassanzadazar *et al.* ,2012).

Bile salt tolerance

Bile tolerance is considered as another important characteristic of *Lactobacillus* strains, which enables them to survive, grow, and exert their action during gastrointestinal transit. Five selected *Lactobacillus* strains isolated from Vietnamese healthy adult feces were screened for their bile salt tolerance by exposure to oxgall up to 1%.

Table 2. Survival of *Lactobacillus* isolates in the presence of Oxgall and low pH.

Strain	Growth (%) in the presence of oxgall			Growth (%) at low pH	
	0.3%	0.5%	1.0%	pH2	pH3
Lac.VFE-04	+++	++	++	85.12 ± 5.44	95.27 ± 3.21
Lac.VFE-08	+++	+++	+	84.51 ± 8.01	94.15 ± 5.69
Lac.VFE-09	++	++	+	69.06 ± 3.89	75.46 ± 6.62
Lac.VFE-11	+++	++	++	80.71 ± 4.22	86.47 ± 9.11
Lac.VFE-14	+++	+++	++	88.22 ± 5.14	98.54 ± 6.68

As described in Table 2, all five of the *Lactobacillus* strains could grow well at 0.3% and 0.5% oxgall. At 1% oxgall, except Lac.VFE-09, all remaining strains were able to grow and survive. In some reports, *Lactobacillus* spp. does not grow at levels greater than 0.3% of oxgall (Zheng *et al.*, 2013). In one study, researchers showed that survival rates of *L. plantarum* CGMCC 8198 was 48%, while *L. casei* 1.539 had a survival rate of only 8% when cultured in MRS broth containing 0.3% bile salts for 4 h (Gu *et al.*, 2014). McAuliffe *et al.*, 2005, indicated that *L. acidophilus* NCFM did not grow in MRS broth, nor on MRS plates supplemented with 0.3% and 0.5% oxgall (McAuliffe*et al.*,2005). In line with the findings of our investigation, Nawaz *et al.*, 2017, revealed that among the 25 tested lactic acid bacterial strains, there were 10 strains that could grow well in an MRS medium supplemented with bile salt up to 1% oxgall (Nawaz *et al.*, 2017). As such, isolated *Lactobacillus* strains from Vietnamese healthy people are able to low pH tolerance, especially these *Lactobacillus* strains showed the highly tolerance to oxgall.

The fact that some *Lactobacillus* strains were able to grow in the presence of conjugated bile salts, while they were not able to deconjugate them, means that the capacity to express bile salt hydrolase is not related to the capacity to resist the toxicity of conjugated bile salts (Belicová *et al.*, 2013). That explains why all *Lactobacillus* strains that were able to survive in the presence of oxgall were selected to examine for bile salt deconjugation.

Bile salt deconjugation

In our study, bile salt deconjugating activity of all *Lactobacillus* strains was tested with 5 mM of GC, 5 mM of GDC, 5 mM of TC, and 5 mM of TDC. Five strains (Lac.VFE-04, Lac.VFE-08, Lac.VFE-09, Lac.VFE-11, and Lac.VFE-14) were concentrated and spotted on the same medium for the direct plate assay. The BSH-positive strains could be identified by halos of precipitation and/or clearing of the medium around active colonies (Fig. 1). The obtained results indicated that the Lac.VFE-04, Lac.VFE-08, and Lac.VFE-14 were able to deconjugate GDC.

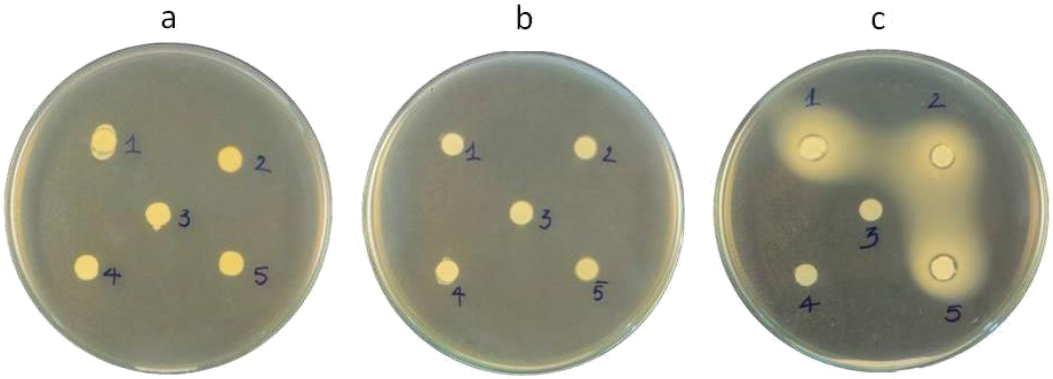

Figure 1. BSH plate activity by *Lactobacillus* on solid MRS medium. Plates were incubated anaerobically for 48 h at 37ºC. (a) MRS; (b) MRS containing 5mM GC; (c) 5 mM GDC; (1) Lac.VFE-04; (2) Lac.VFE-08; (3) Lac.VFE-09; (4) Lac.VFE-11; (5) Lac.VFE-14.

Figure 1 shows the results of positive and negative hydrolysis on solid plates of MRS medium supplemented with bile salts by five strains of *Lactobacillus*. As we can see on the Table 3, three strains Lac.VFE-04, Lac.VFE-08, and Lac.VFE-14 were able to hydrolyze GDC, whereas this activity of Lac.VFE-09 and Lac.VFE-11 was not stable.

Lim *et al.*, observed that only 12.8% of *Lactobacillus* strains isolated from faeces of healthy humans had BSH activity (Lim *et al.*, 2004). Similar to our findings, in a study

conducted by Rani *et al.*, 2017, the researchers tested the ability of BSH from *L. gasseri* FR4 to GC, GDC, TC, and TDC. BSH of *L. gasseri* FR4 showed greater hydrolysis capability towards glycine-conjugated bile salts (GC and GDC) than to taurine-conjugated bile salts (TC and TDC) (Rani, Anandharaj *et al.*, 2017). The highest BSH activity was observed with the GDC substrate.

In contrast with our findings, the investigation conducted by Belicová in 2013 indicated that 11 *Lactobacillus* strains that were isolated from Slovak Bryndza cheese could grow in the presence of TC, TDC, GC, and GDC, but not all of them were able to deconjugate bile salts. Among them, *L. plantarum* CK06, B01, K21, and ZS15 strains showed deconjugation activity on GC, TC, and TDC, but there were no strains that demonstrated deconjugation activity on GDC (Hassanzadazar *et al.*, 2012).

In another study, González-Vázquez *et al.* investigated the hydrolysis of GC, GDC, TC, and TDC by *L. casei* J57 isolated from Pulque and *L. casei* Shirota. They reported that *L. casei* J57 showed bile salt hydrolase activity with all bile salts at different concentrations expressed as unit/mg of protein. With GC, the value was 44.91, whereas with GDC, TC, and TDC, the values were 45.27, 671.72, and 61.57, respectively. However, *L. casei* Shirota did not show any activity in glycine conjugates, while its activity in the TC was 1046.15 and TDC was 264.69 unit/mg of protein (Zhang *et al.*, 2009).

Table 3. The bile salt hydrolase activity on different substrates of selected Lactobacillus strains.

Strain	Bile salt deconjugating assays			
	GC (5mM)	GDC (5mM)	TC (5mM)	TDC (5mM)
Lac.VFE-04	-	+	-	-
Lac.VFE-08	-	+	-	-
Lac.VFE-09	-	+/-	-	-
Lac.VFE-11	-	+/-	-	-
Lac.VFE-14	-	+	-	-

Hydrolase activity and glycine

Deconjugation is catalyzed by bile salt hydrolases, which hydrolyze the amide bond and liberate the glycine/taurine moiety from the steroid core of the bile salts. The resulting free bile acids or deconjugated bile acids are excreted via the feces.

Table 4. The bile salt hydrolase activity and glycine.

Strain	GDC (5 mM)	Glycine (µg/mL)
Lac.VFE-04	+	1.1
Lac.VFE-08	+	0.6
Lac.VFE-14	+	0.9

In MRS broth, BSH catalyzes the hydrolysis of glycine-conjugated bile acids into the glycine amino acid residue and the free bile acid. BSH activity in a cell-free extract of each strain Lac.VFE-04, Lac.VFE-08, and Lac.VFE-14 was determined by measuring the amount of amino acids resulting from the hydrolysis of the amide bond of bile salts. Using HPLC assay, most results were consistent with those of the plate assay (Table 4). The plate assay showed the BSH-positive activity in the presence of 5 mM GDC, and as the culture spotted on the plates was more concentrated, the precipitated halo zones were observed. Moreover, BSH activity in the supernatant could be further investigated.

Bile salt hydrolase genes

The presence and genetic organization of *bsh* genes in *Lactobacillus* are highly variable. In some species, only a single *bsh* gene has been reported (Zhang *et al.*, 2009; Kumar *et al.*, 2013),

while in *L. plantarum* WCFS1, *L. acidophilus* NCFM, *L. johnsonii* NCC533, and *L. gasseri* ATCC 33323, multiple *bsh* genes have been recorded and characterized (Lambert *et al.*, 2008; Zhang *et al.*, 2009, Kumar *et al.*, 2013). In our study, four predicted bile salt hydrolase genes (*bsh*1, *bsh*2, *bsh*3, and *bsh*4) were found in Lac.VFE-04, Lac.VFE-08, and Lac.VFE-14 but they could only deconjugate GDC (Fig. 2).

In some *Lactobacillus*, function of each *bsh*1, *bsh*2, *bsh*3, and *bsh*4 in deconjugation activity have been investigated by overexpression. For example, Ren *et al*. conducted a study in 2011 researching 4 *bsh* genes in *L. plantarum* ST-III. Their results showed that the proteins expressed from the *bsh*1, *bsh*2, *bsh*3, and *bsh*4 genes all reacted with the substrates tested, which included TC, GC, TDC, GDC, taurochenodeoxycholic acid (TCDC), and glycochenodeoxycholic acid (GCDC). Specifically, they found that BSH1 showed much greater hydrolysis activity than the other three proteins on GDC (Ren *et al.*, 2011). The author group suggests that all 4 bile salt hydrolases may be responsible for the bile salt hydrolysis activity in *L. plantarum* ST-III (Lambert *et al.*, 2008).

In *L. plantarum* WCFS1, the functionality of four predicted *bsh*1, *bsh*2, *bsh*3, and *bsh*4 genes was explored by overexpression. The obtained results indicated that, BSH1 was shown to be responsible for the majority of BSH activity of *L. plantarum* WCFS1. In addition, *bsh*1 of *L. plantarum* WCFS1 was shown to be involved in conferring tolerance specifically GDC, but not with TDC (Lambert*et al.*,2008).

In another study, genes coding for bile salt hydrolase of *L. plantarum* CGMCC 8198 were identified, analyzed and cloned. Using RT-PCR analysis, only *bsh*2, *bsh*3 and *bsh*4 genes could be cloned from *L. plantarum* CGMCC 8198, and they all had high similarity with *L. plantarum* WCFS1 and ST-III. The three recombinant BSHs had similar activities for the hydrolysis of TDCA, while the hydrolyzing activity of BSH2 against GDC was 2.3-fold and 5.5-fold greater than those of BSH3 and BSH4, respectively. Therefore, BSH2 might play a major role in the bile salt hydrolysis activity in *L. plantarum* CGMCC 8198 (Gu *et al.*, 2014).

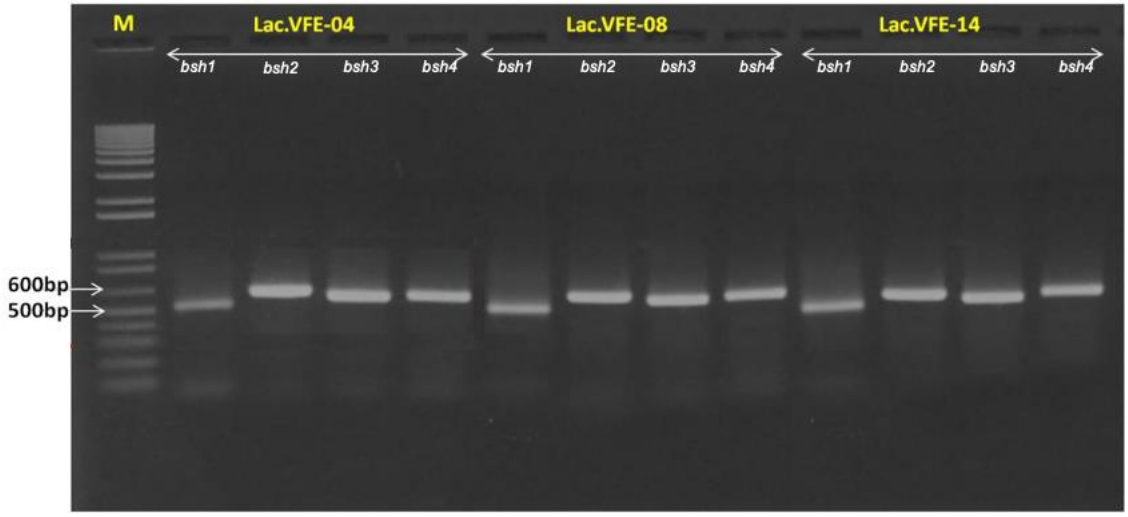

Figure 2. PCR products of *bsh*1, *bsh*2, *bsh*3, and *bsh*4 from Lac.VFE-04, Lac.VFE-08, and Lac.VFE-14, respectively. (M: DNA marker).

Each of the *bsh*1-deficient derivatives displayed GDC-mediated growth inhibition at concentrations as low as 0.1% (wt/vol) GDC. In contrast, strains containing an intact *bsh*1 gene

were capable of sustaining normal growth characteristics at 0.5% to 0.7% (wt/vol) GDC. Analogous with the limited level of hydrolytic activity relating to bile salts, *bsh*2, *bsh*3, and *bsh*4 did not appear to contribute significantly to tolerance of GDC (Zhang *et al.*, 2009; Kumar *et al.*, 2013).

L. plantarum appeared to be remarkably more sensitive to GDC, with obvious differences visible between the strains. The results clearly established that the presence of all *bsh*1, *bsh*2, *bsh*3, and *bsh*4 in *L. plantarum* VFE-04 and *L. plantarum* VFE-14 enhances GDC bile salt deconjugate. Moreover, in our study, besides these *L. plantarum*, *L. rhamnosus* VFE-08 was also able to deconjugate GDC.

In next studies, overexpression of BSH1, BSH2, BSH3, and BSH4 of *L. plantarum* VFE-04, *L. rhamnosus* VFE-08 and *L. plantarum* VFE-14 will be carried out to verify which *bsh* homologs are responsible for GDC deconjugation of these bacteria.

CONCLUSION

Intestinal microbes in the small intestine are capable of deconjugation of glycine or taurine from bile salts using BSH. The obtained characteristics of *L. plantarum* VFE-04, *L. rhamnosus* VFE-08, and *L. plantarum* VFE-14 indicated that these bacteria might be excellent candidates to improve the health of patients suffering from hypercholesterolemia.

Conflicts of Interest: *The authors declare that they have no conflicts of interest.*

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