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Development of Bile Salt-Resistant *Leuconostoc citreum* by Expression of Bile Salt Hydrolase Gene

Seung Kee Cho[1], Soo Jin Lee[1], So-Yeon Shin[1], Jin Seok Moon[1], Ling Li[1], Wooha Joo[1], Dae-Kyung Kang[2], and Nam Soo Han[1]*

[1]*Brain Korea 21 Center for Bio-Resource Development, Division of Animal, Horticultural, and Food Sciences, Chungbuk National University, Cheongju 28644, Republic of Korea*
[2]*Department of Animal Resources Science, Dankook University, Cheonan 31116, Republic of Korea*

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**Corresponding author*
Phone: +82-43-261-2567;
Fax: +82-43-271-4412;
E-mail: namsoo@chungbuk.ac.kr

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Probiotic bacteria must have not only tolerance against bile salt but also no genes for antibiotic resistance. *Leuconostoc citreum* is a dominant lactic acid bacterium in various fermented foods, but it is not regarded as a probiotic because it lacks bile salt resistance. Therefore, we aimed to construct a bile salt-resistant *L. citreum* strain by transforming it with a bile salt hydrolase gene (*bsh*). We obtained the 1,001 bp *bsh* gene from the chromosomal DNA of *Lactobacillus plantarum* and subcloned it into the pCB4170 vector under a constitutive P710 promoter. The resulting vector, pCB4170BSH was transformed into *L. citreum* CB2567 by electroporation, and bile salt-resistant transformants were selected. Upon incubation with glycodeoxycholic acid sodium salt (GDCA), the *L. citreum* transformants grew and formed colonies, successfully transcribed the *bsh* gene, and expressed the BSH enzyme. The recombinant strain grew in up to 0.3% (w/v) GDCA, conditions unsuitable for the host strain. In *in vitro* digestion conditions of 10 mM bile salt, the transformant was over 67.6% viable, whereas only 0.8% of the host strain survived.

Keywords: *Leuconostoc citreum*, probiotics, bile salt hydrolase

Leuconostoc is an important genus of lactic acid bacteria (LAB) that plays a major role in maintaining the quality of fermented milk, dairy products, vegetables, and meats [3, 8, 12]. *Leuconostoc* species are used as starter cultures for commercial food products, such as *L. mesenteroides* subsp. *cremoris* for *viili* in Finland [14], various *Leuconostoc* species for *kefir* in many countries, and *L. mesenteroides* DRC for *kimchi* in Korea [9]. However, despite its important role, *Leuconostoc* sp. is not regarded as a probiotic because of its low colonization of the large intestine, mainly due to the absence of resistance to acid and bile salt.

Probiotics are "live microorganisms which when administered in adequate amount confer health benefits to the host" [11]. A variety of microorganisms, typically food-grade LAB, have been evaluated for their probiotic potential and are applied in a variety of food products or therapeutic preparations [24]. Microorganisms used in probiotic products generally contain lactobacilli and bifidobacteria [27]. Before a probiotic can benefit human health, it must fulfill certain criteria: it must survive passage through the upper gastrointestinal tract and arrive alive at its site of action, and it must be able to function in the gut environment [25].

Food-grade expression systems must avoid antibiotic resistance markers because such markers risk the transfer of antibiotic resistance to the human intestinal microbiota [6, 21]. Several potential selection markers have been developed that fulfill the food-grade requirements and avoid the use of any harmful or toxic substances [16, 23]. Resistance markers used in food-grade approaches can be classified, based on the method of selection, into dominant or complementation selection markers [7, 20].

In this study, we attempted to construct a bile salt-resistant *L. citreum* strain that can survive in the small intestine. We cloned the bile salt hydrolase gene (*bsh*) from *Lactobacillus plantarum* and expressed it in *L. citreum* using the pCB4170 vector. To improve the BSH activity, we also employed the strong P710 promoter from *L. mesenteroides*

Table 1. Bacterial strains and plasmids.

Strains or plasmids	Genotype/relevant features	Source
Strains		
E. coli MC1061	F*frr*(wild-type (Wt)) *araD139* (*ara-leu*)*7679* (*lacIPOZYA*)*X74 galU galK hsdR2 mcrB1 rpsL* (Sm^r)	New England Biolabs
L. citreum CB2567	Wild-type strain isolated from *kimchi*	KACC91348P
Lb. plantarum	Wild-type strain; template of *bsh* gene	KCTC 3104
Plasmids		
pCB4170	*E. coli-Leuconostoc* shuttle vectors, Amp^R, Cm^R	This study

KACC, Korean Agricultural Culture Collection; KCTC, Korean Collection for Type Cultures.

ATCC 8293 for the expression of BSH [4]. The characteristics of the transformant strains were investigated by *in vitro* tests. The bacterial strains and plasmids used in this study are listed in Table 1. *L. citreum* was routinely grown at 30°C in MRS medium (Difco, Detroit, MI, USA), whereas other LAB were grown at 37°C in MRS medium. *Escherichia coli* was cultured in Luria-Bertani (LB) medium with vigorous shaking at 37°C. Ampicillin (50 μg/ml) and chloramphenicol (10 μg/ml) were used for *E. coli* and LAB, respectively.

Restriction enzymes, T4 DNA ligase, CIAP (calf intestinal alkaline phosphatase), and Ex Taq polymerase were purchased from TaKaRa (Kyoto, Japan). A molecular weight standard of DNA was obtained from Bioneer Co. (Daejeon, Korea). Ampicillin, chloramphenicol, and glycodeoxycholic acid sodium salt (GDCA; G9910) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Other chemicals were of reagent grade. Lactobacilli MRS broth and LB broth were purchased from Difco. Oligonucleotide primers were synthesized on an automated DNA synthesizer by Bioneer Co.

To obtain the *bsh* gene, the genomic DNA of *Lb. plantarum* was isolated. The *bsh* gene was amplified from the genomic DNA by PCR using the prepared primer sets (GenBank Accession No. AL935262). The purified PCR products were digested with *Sal*I and *Pst*I, extracted from a sliced gel, and ligated into *Sal*I- and *Pst*I-digested pCB4170 vectors, respectively. All of the above ligation mixture was transformed into $CaCl_2$-competent *E. coli* MC1061 cells using a standard heat-shock protocol. Transformants were grown on LB medium containing ampicillin (50 μg/ml) at 37°C. Recombinant plasmids were extracted, and the resulting construct was designated pCB4170BSH (Fig. 1).

RNA was isolated from exponentially growing cells (OD_{660nm} 0.3–0.4). Two volumes of RNA Protect (Qiagen, CA, USA) were added, and total RNA was isolated using an RNeasy Mini Kit. DNA was removed by digestion using

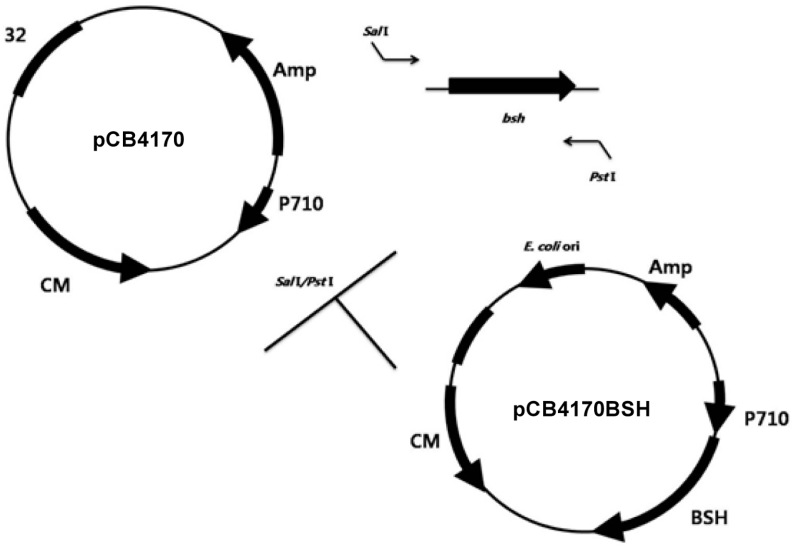

Fig. 1. Construction of pCB4170BSH vector harboring the bile salt hydrolase gene (*bsh*).

the RNase-Free DNase Set (Qiagen) at 37°C for 20 min. Total RNA (1 μg) was reverse-transcribed with 100 U of CycleScript Reverse transcriptase using 100 pmol random hexamer primers according to the manufacturer's instructions (Bioneer).

The transcription levels of *bsh* were compared by real-time PCR using the iQ5 Multicolor Real-Time PCR Detection System (Bio-Rad Laboratories, Hercules, CA, USA). The phosphoketolase gene (*pho*), a single-copy gene from the *L. citreum* CB2567 chromosome, was used as the reference gene (GenBank Accession No. NC010471). A 120 bp fragment of the *pho* gene was amplified with primers pho-F (5′-ACACAACTAA CCGTCAATGGATG-3′) and pho-R (5′-CCTTCAAGCCAACCTTCAGC-3′), and a 166 bp fragment of the *bsh* gene was amplified with primers bsh-F (5′-TCAAATAGCACACCCCAAAA-3′) and bsh-R (5′-TGCCACTCTCTGTCTGCATC-3′). The primers (0.05 pmol each) were added to a master mix containing 10 μl of iQ SYBR Green Supermix (Bio-Rad), 2 μl of cDNA, and RNase-free water in a final volume of 20 μl. Total RNA preparations were used as negative controls to verify the absence of chromosomal DNA in the cDNA libraries. The *pho* gene was used as a reference, and chromosomal DNA was used as a positive control. Melting curve analysis and amplicon size determination were performed to verify amplification of the appropriate transcripts. Transcription levels were quantified according to the method described by Pfaffl [22]. The threshold cycle number (C_T) of target reference genes was determined using the iQ5 Optical System Software (Bio-Rad) and used for further analysis.

To investigate heterologous expression of *bsh* in *L. citreum*, the plasmid pCB4170BSH was introduced by electroporation with chloramphenicol selection. BSH activity was examined by a bile salt plate assay as described by Dashkevicz and Feighner [5]. Briefly, overnight cultures grown in MRS broth were streaked on MRS agar plates supplemented with 0.3% (w/v) GDCA. BSH activity was indicated by precipitate halos of deconjugated bile acid around active colonies. A bile salt survival assay was also performed to investigate the resistance of *L. citreum* to GDCA. Briefly, overnight cultures were inoculated (1%) into fresh MRS broth containing 0–0.5% (w/v) GDCA. Cell growth was measured by viable cell count.

In addition, the tolerance of *L. citreum* harboring pCB4170BSH against bile salts was determined using a simulated intestinal fluid (SIF) electrolyte stock solution (6.8 mM KCl, 0.8 mM KH_2PO_4, 85 mM $NaHCO_3$, 38.4 mM NaCl, 0.33 mM $MgCl(H_2O)_6$, 8.4 mM HCl, and 0.6 mM $CaCl_2(H_2O)_2$) with 10 mM GDCA [18]. Cells from all strains

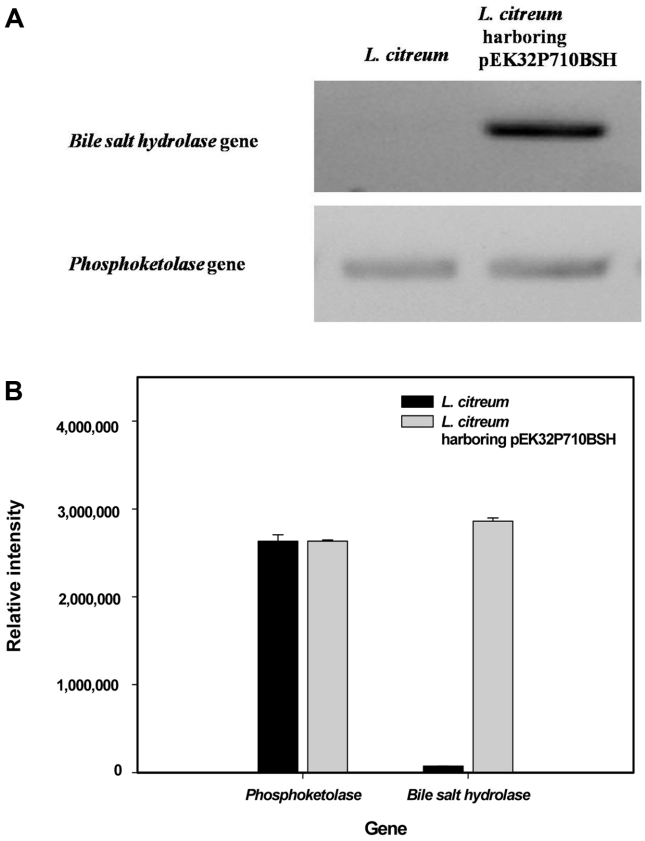

Fig. 2. Comparison of *bsh* transcription levels.
(**A**) Comparison of transcription levels of the *bsh* gene in *L. citreum*. (**B**) Relative intensity of the *bsh* gene in *L. citreum*.

grown overnight in MRS broth were harvested by centrifugation, washed, and resuspended in 2 ml of the SIF electrolyte stock solution with 10 mM GDCA. After incubation for 0, 60, or 120 min at 30°C, viable cells were counted by standard plate counting. Measurements were performed in triplicate, and the mean values are reported.

To construct an expression vector for *Leuconostoc* sp., *bsh* was amplified from the template *Lb. plantarum* genome, and the generated PCR fragment was subcloned into the pCB4170 vector containing a constitutive P710 promoter (Fig. 1). The constructed plasmid was transformed into *L. citreum* CB2567. Colonies carrying pCB4170BSH were selected on an MRS agar plate containing chloramphenicol.

The transcription level of *bsh* in *L. citreum* CB2567 harboring pCB4170BSH was determined by RT-qPCR analysis. Fig. 2 shows that *bsh* was expressed in the transformant at a higher level than that of *pho* (housekeeping gene). When the host and transformant strains were cultured in medium containing bile salt, growth of the wild-type strain was obviously inhibited at GDCA concentrations as

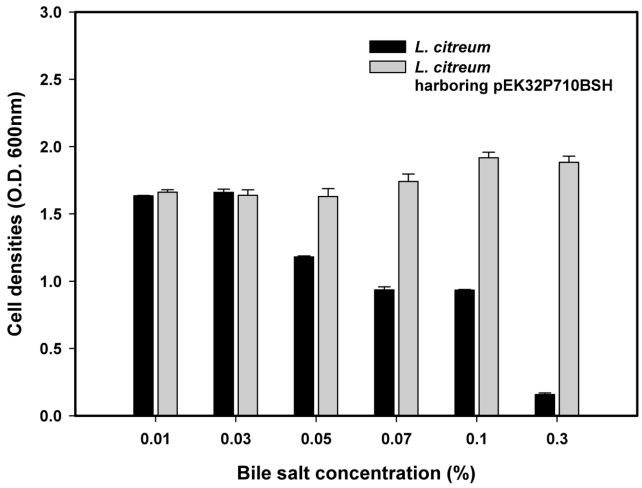

Fig. 3. Growth of wild-type *L. citreum* CB2567 and the transformant harboring pCB4170BSH in various bile salt concentrations.

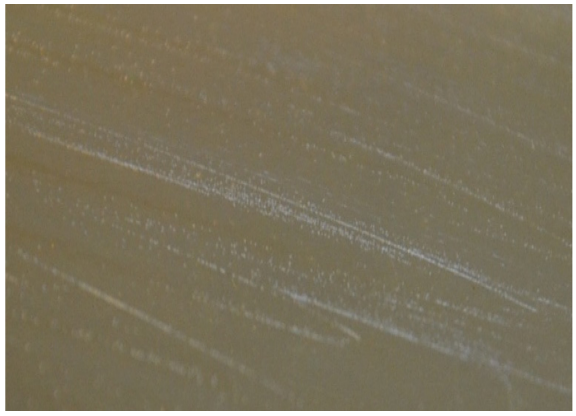

Fig. 4. BSH activity assay in *L. citreum* CB2567 harboring pCB4170BSH.
Overnight culture cells were streaked onto MRS agar plates supplemented with 0.3% (w/v) GDCA and incubated for 48 h.

low as 0.1% (w/v). In contrast, the recombinant strain harboring pCB4170BSH sustained normal growth in MRS containing 0.1% (w/v) GDCA and could grow in the presence of 0.3% (w/v) GDCA. Therefore, 0.3% (w/v) GDCA can be used to screen transformants harboring the *bsh* gene. Furthermore, the agar plate containing 0.3% bile salt clearly showed sustained growth of only *L. citreum* CB2567 harboring pCB4170BSH, indicating that resistance to GDCA can be used as a food-grade selection marker (Fig. 3).

The bile salt resistance of *L. citreum* was examined to investigate whether it can survive in the small intestine environment. The cell viability of strains exposed to 10 mM GDCA for 120 min is shown in Table 2. After exposure for 60 min, the viable cell numbers of transformant decreased to 8.84 log CFU/ml, and the transformant was still viable (8.77 log CFU/ml) after 120 min exposure. The number of viable transformant cells was higher than that of the host strain, which decreased to 6.90 log CFU/ml at 60 min (Fig. 4). This result reveals that the recombinant strain was over 67.6% viable after incubation with 10 mM bile salt in *in vitro* digestion conditions, whereas only 0.8% of the host strain survived.

Several genes or gene clusters have been used as selection markers for construction of food-grade expression systems: D-xylose catabolism of *Lb. pentosus* [23], sucrose transporter system of *Pediococcus pentosaceus* [16], nisin immunity of *Lactococcus lactis* [26], lactacin F immunity of *Lb. acidophilus* [1], purine biosynthesis of *Lc. lactis* [7], alanine racemase of *Lb. plantarum* [20], and glutamate racemase of *Lc. lactis* [2]. Yin *et al.* [28] also demonstrated that the *bsh* gene from *Lb. plantarum* was functional in lactobacilli, permitting simple selection of transformant cells in the presence of bile salt. In our study, the *bsh* gene was newly used to provide bile salt resistance to bile-sensitive *L. citreum* to make it a potential probiotic. Indeed, our results show that expression of *bsh* was effective to confer bile tolerance to *L. citreum* and, at the same time, it was useful for selection of transformants.

In order to improve the BSH expression level in *L. citreum*, we also employed the strong P710 promoter from *L. mesenteroides* ATCC 8293. Transcriptomic analysis using microarrays revealed that genes of Leum_209, 710, and

Table 2. Survival of wild-type *L. citreum* CB2567 and the transformant harboring pCB4170BSH after incubation in 10 mM bile salt for 60 and 120 min.

Strain	Initial mean counts (Log CFU/ml)	Mean counts after 60 min (Log CFU/ml)	Mean counts after 120 min (Log CFU/ml)
L. citreum CB2567	9.08 ± 0.01	6.90 ± 0.01	6.94 ± 0.01
L. citreum CB2567 harboring pCB4170BSH	8.94 ± 0.01	8.84 ± 0.01	8.77 ± 0.03

All values indicate the mean ± standard deviation.

1694 were expressed most strongly and constitutively in *L. mesenteroides* ATCC 8293 in both glucose- and sucrose-MRS media [4]. When the three promoter sequences (p209, p710, and p1694) were fused to the promoterless *β-gal* gene in pCB4170 and expressed in *L. citreum* CB2567, p710 resulted in the highest level of β-galactosidase activity, showing a strong and constitutive transcription of the heterologous gene. Annotation using NCBI BLAST analysis revealed that Leum_710 is an integral membrane protein and its functional information was not yet known.

The genome sequences of several *Leuconostoc* spp. have recently been determined, with growing industrial attention: *L. mesenteroides* ATCC 8293 [17], *L. citreum* KM20 [15], *L. lactis* [19], and *L. gasicomitatum* [13]. However, few molecular-level manipulations have been performed on this genus and this is due to the absence of efficient tools for genetic engineering works. In this study, the pCB4170 vector was employed to express gene *bsh* in *L. citreum*, after size minimization of the pCB42 plasmid as described by Eom *et al*. [10]. The pCB42 plasmid was first obtained from *L. citreum* CB2567 and it was capable of replicating in various LAB, including *Lb. plantarum, Lb. reuteri, Lc. lactis, Streptococcus thermophilus, Weissella confusa,* and *Oenococcus oeni*. Our results reveal that vector pCB4170 is applicable to construct food-grade transformants of the above LAB cells. In particular, *W. confusa* and *O. oeni* are important for food fermentation, and no appropriate tools are developed for genetic engineering. It is supposed that the expression of *bsh* by using pCB4170 can be further performed in various bile-sensitive LAB to give bile tolerance.

In this study, the *bsh* gene from *Lb. plantarum* was cloned and expressed in BSH-deficient *L. citreum* CB2567. The recombinant strain acquired the ability to deconjugate GDCA, revealing a strong correlation between the presence of the *bsh* gene and resistance to GDCA. Heterologous expression of the *bsh* gene in *L. citreum* CB2567 was under the transcriptional control of the P710 promoter. The recombinant strain acquired resistance to GDCA and grew in the presence of up to 0.3% (w/v) GDCA. In conclusion, gene *bsh* not only acted as a potential selection marker in *L. citreum* but also conferred probiotic properties.

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References

1. Allison G, Klaenhammer T. 1996. Functional analysis of the gene encoding immunity to lactacin F, *lafI*, and its use as a *Lactobacillus*-specific, food-grade genetic marker. *Appl. Environ. Microbiol.* **62:** 4450-4460.
2. Bohmer N, Dautel A, Eisele T, Fischer L. 2013. Recombinant expression, purification and characterisation of the native glutamate racemase from *Lactobacillus plantarum* NC8. *Protein Expr. Purif.* **88:** 54-60.
3. Budde BB, Hornbæk T, Jacobsen T, Barkholt V, Koch AG. 2003. *Leuconostoc carnosum* 4010 has the potential for use as a protective culture for vacuum-packed meats: culture isolation, bacteriocin identification, and meat application experiments. *Int. J. Food Microbiol*. **83:** 171-184.
4. Cho SK. 2015. Development of food-grade vector for constitutive and inducible gene expression in *Leuconostoc citreum*. PhD Thesis, Chungbuk National University, Chungbuk, South Korea.
5. Dashkevicz MP, Feighner SD. 1989. Development of a differential medium for bile salt hydrolase-active *Lactobacillus* spp. *Appl. Environ. Microbiol*. **55:** 11-16.
6. De Vos WM. 1999. Gene expression systems for lactic acid bacteria. *Curr. Opin. Microbiol.* **2:** 289-295.
7. Dickely F, Nilsson D, Hansen EB, Johansen E. 1995. Isolation of *Lactococcus lactis* nonsense suppressors and construction of a food-grade cloning vector. *Mol. Microbiol.* **15:** 839-847.
8. Eom HJ, Seo DM, Han NS. 2007. Selection of psychrotrophic *Leuconostoc* spp. producing highly active dextransucrase from lactate fermented vegetables. *Int. J. Food Microbiol.* **117:** 61-67.
9. Eom HJ, Park JM, Seo MJ, Kim MD, Han NS. 2008. Monitoring of *Leuconostoc mesenteroides* DRC starter in fermented vegetable by random integration of chloramphenicol acetyltransferase gene. *J. Ind. Microbiol. Biotechnol.* **35:** 953-959.
10. Eom HJ, Moon JS, Cho SK, Kim JH, Han NS. 2012 Construction of theta-type shuttle vector for *Leuconostoc* and other lactic acid bacteria using pCB42 isolated from *kimchi*. *Plasmid* **67:** 35-43.
11. FAO/WHO. 2002. Guidelines for the evaluation of probiotics in food. Report of a joint FAO/WHO Working Group. Available at http://www.fao.org/es/ESN/food/foodandfood_probio_en.stm.
12. Hemme D, Foucaud-Scheunemann C. 2004. *Leuconostoc*, characteristics, use in dairy technology and prospects in functional foods. *Int. Dairy J.* **14:** 467-494.
13. Johansson P, Paulin L, Säde E, Salovuori N, Alatalo ER, Björkroth KJ, Auvinen P. 2011. Genome sequence of a food spoilage lactic acid bacterium, *Leuconostoc gasicomitatum* LMG18811T, in association with specific spoilage reactions. *Appl. Environ. Microbiol.* **77:** 4344-4351.
14. Kahala M, Mäki M, Lehtovaara A, Tapanainen JM, Katiska R, Juuruskorpi M, *et al*. 2008. Characterization of starter lactic acid bacteria from the Finnish fermented milk product *viili*.

J. Appl. Microbiol. **105:** 1929-1938.
15. Kim JF, Jeong H, Lee JS, Choi SH, Ha M, Hur CG, *et al*. 2008. Complete genome sequence of *Leuconostoc citreum* KM20. *J. Bacteriol.* **190:** 3093-3094.
16. Leenhouts K, Bolhuis A, Venema G, Kok J. 1998. Construction of a food-grade multiple-copy integration system for *Lactococcus lactis*. *Appl. Microbiol. Biotechnol.* **49:** 417-423.
17. Makarova K, Slesarev A, Wolf Y, Sorokin A, Mirkin B, Koonin E, *et al*. 2006. Comparative genomics of the lactic acid bacteria. *Proc. Natl. Acad. Sci. USA* **103:** 15611-15616.
18. Minekus M, Alminger M, Alvito P, Ballance S, Bohn T, Bourlieu C, *et al*. 2014. A standardised static *in vitro* digestion method suitable for food – an international consensus. *Food Funct*. **5:** 1113-1124.
19. Moon JS, Choi HS, Shin SY, Noh SJ, Jeon CO, Han NS. 2015. Genome sequence analysis of potential probiotic strain *Leuconostoc lactis* EFEL005 isolated from *kimchi*. *J. Microbiol*. **53:** 337-342.
20. Nguyen TT, Mathiesen G, Fredriksen L, Kittl R, Nguyen TH, Eijsink VG, *et al*. 2011. A food-grade system for inducible gene expression in *Lactobacillus plantarum* using an alanine racemase-encoding selection marker. *J. Agric. Food Chem.* **59:** 5617-5624
21. Peterbauer C, Maischberger T, Haltrich D. 2011. Food-grade gene expression in lactic acid bacteria. *Biotechnol. J.* **6:** 1147-1161.
22. Pfaffl MW. 2001. A new mathematical model for relative quantification in real-time RT–PCR. *Nucleic Acids Res.* **29:** e45.
23. Posno M, Heuvelmans P, Van Giezen M, Lokman B, Leer R, Pouwels P. 1991. Complementation of the inability of *Lactobacillus* strains to utilize D-xylose with D-xylose catabolism-encoding genes of *Lactobacillus pentosus*. *Appl. Environ. Microbiol.* **57:** 2764-2766.
24. Rodgers S. 2008. Novel applications of live bacteria in food services: probiotics and protective cultures. *Trends Food Sci. Technol.* **19:** 188-197.
25. Saarela M, Mogensen G, Fondén R, Mättö J, Mattila-Sandholm T. 2000. Probiotic bacteria: safety, functional and technological properties. *J. Biotechnol.* **84:** 197-215.
26. Takala T, Saris P. 2002. A food-grade cloning vector for lactic acid bacteria based on the nisin immunity gene *nisI*. *Appl. Microbiol. Biotechnol*. **59:** 467-471.
27. Tannock GW. 1997. Probiotic properties of lactic-acid bacteria: plenty of scope for fundamental R & D. *Trends Biotechnol.* **15:** 270-274.
28. Yin S, Zhai Z, Wang G, An H, Luo Y, Hao Y. 2011 A novel vector for lactic acid bacteria that uses a bile salt hydrolase gene as a potential food-grade selection marker. *J. Biotechnol* **152:** 49-53.