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Growth stimulation of *Clostridium butyricum* in the presence of *Lactobacillus brevis* JL16 and *Lactobacillus parabuchneri* MH44

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Abstract

Certain strains of *Clostridium butyricum* exhibit characteristics of probiotics that are now sold in eastern Asian countries such as Korea and Japan. Integral improvement in *C. butyricum* functions can be achieved in the presence of *Lactobacillus* spp. through the process of attentive selection. This study aimed to select specific strains of lactic acid bacteria that could synergistically enhance the probiotic functions of *C. butyricum*. The supernatants of 249 lactic acid bacteria were examined, and we observed that 24 strains did not inhibit the growth of *C. butyricum*. Additionally, 4 of these 24 strains induced over 2-fold promotion in *C. butyricum* growth rates during co-culture with this bacterial strain. This growth promotion was verified using qPCR. In particular, *Lactobacillus brevis* JL16 and *Lactobacillus parabuchneri* MH44 stimulated *C. butyricum* more effectively than did other strains. We determined the level of *buk* (butyrate kinase) expression, the survival rate in the presence of bile salt, the ability of auto-aggregation, and the survival rate after freeze-drying.

Keywords: *Clostridium butyricum*; butyric acid; probiotics; *Lactobacillus* spp.; co-culture.

Practical Application: Co-culture of *C. butyricum* with *Lactobacillus* strains and its application to growth stimulation.

1 Introduction

Optimal candidates for market probiotics must exhibit the ability to undergo affordable fermentation and cost-effective separation. Probiotics should also possess characteristics that make them suitable for healthcare purposes. Although a large number of research papers have claimed various efficacies, a clear mechanism underlying the functions of these probiotics has not been confirmed. Among the probiotics, *Clostridium butyricum* is a particularly notable probiotic species. *C. butyricum* is an anaerobic bacterium and strengthens the immune system and decreases the number of pathogenic bacteria, thus indicating that *C. butyricum* possesses the potential for future use as a probiotic (Takahashi et al., 2004; Yasueda et al., 2016; Kong et al., 2011). In previous studies, *Clostridium* spp. (*Clostridium botulinum, Clostridium diffile, Clostridium tetani,* etc.) were demonstrated to act as pathogenic bacteria that generate toxic chemicals that disturb the nervous system in mammals However, some strains of *C. butyricum* were adequately regarded as possible probiotics, as the capability and safety of *C. butyricum* have both been validated. In Asia, promising clinical results indicated that *C. butyricum* (MIYAIRI 588) could be useful for curing inflammation or diarrhea caused by antibiotic treatment, and this bacterial strain did not cause any significant side-effects during treatment (Yasueda et al., 2016). *C. butyricum* produces butyric acid (butyrate), which is a short-chain fatty acid that exerts various functions in the body. Butyrate is a major energy source for epithelial cells and helps epithelial cells to multiply to reduce intestinal damage and permeability and also accelerates anti-inflammatory effects (Kong et al., 2011). Additionally, butyrate has been reported to alleviate intestinal disease and to prevent metabolic syndrome. Patients suffering from intestinal diseases are known to generate less butyrate due to a reduction in the number of *C. butyricum* within the intestine (Rivera-Chávez et al., 2016). It must be noted that the interactions between intestinal microorganisms and the brain nervous system are affected by several types of interference that originate from the endocrine system, nerve system, and cell immune signal system. For example, the metabolic chemicals generated from microorganisms can act as signal molecules in the brain (Stilling et al. 2016). As a type of signal molecule, butyrate has been evaluated as an adjuvant therapy to treat vascular dementia (Liu et al., 2015), ischemic stroke (Sun et al., 2016), and depression (Hsiao et al., 2013; Tian et al., 2019). In animal tests, *C. butyricum* in combination with *Lactobacillus* spp. or other probiotics improved some of these factors in the animals. When *C. butyricum* was examined in chickens, it aided in the maturation and growth of beneficial probiotics when it was fed with *Bacillus subtilis* and *L. acidophilus* (Phuoc and Jamikorn, 2017).

In this study, we screened potential probiotic lactic acid bacteria (LAB) that could enhance the probiotic functions of *C. butyricum*. First, the growth of *C. butyricum* was evaluated in the presence of LAB (249 strains). To confirm the effect of co-culture with LAB on butyrate production by *C. butyricum*, we quantified the expression of *buk* gene. The *buk* gene is known to play an important role in the pathway of butyrate in *C. butyricum*. To evaluate the probiotic characteristics of the

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selected LAB, we examined bile resistance, auto-aggregation, and resistance to the freeze-drying process. The discussion was followed in regards to the stimulating growth of *C. butyricum* in the presence of candidate LAB strains.

2 Materials and methods

2.1 Bacterial strains and growth conditions

We obtained collections of LAB isolated from Korean women in 2001 (83 strains) and 166 strains isolated from fish and shellfish in the West Sea between 2008 and 2019 (Lee et al., 2010; Kang et al., 2016). The collected LAB were grown in MRS broth at 35 °C under non-shaken conditions, and they were then maintained in 10% skim milk and stored at -80 °C. *C. butyricum* KCTC1786 was obtained from the Korean Culture Center of Microorganisms and incubated on Reinforced Clostridium medium (RCM; Merck, Darmstadt, Germany) agar and Brain-Heart Infusion (BHI; Bacto, Spark, USA) broth at 35 °C under anaerobic conditions.

2.2 Stimulation of C. butyricum with supernatants from Lactic acid bacteria

Pre-cultured media were further cultured for one day after inoculation. Inoculation was controlled to 2% by adjusting the value of OD_{600} = 1 (optical density at 600 nm). Supernatants were filtered to remove the cell of LAB using 0.2 μm filter paper. *C. butyricum* was inoculated after mixing a LAB supernatant at a ratio of 7:3 and cultured in an anaerobic chamber. The value for OD and the number of strains were both determined to evaluate the growth rate. For comparison, MRS and BHI broths were added separately and compared using the same procedure.

2.3 Stimulation of C. butyricum with cell of Lactic acid bacteria

Equally measured numbers (10^6 CFU/mL) of *Lactobacillus* and *C. butyricum* were mixed and cultured for 24 h in an anaerobic chamber. Dilution of LAB was also used when different ratios of these mixtures (1:1 and 1:0.2) were needed. Quantification of *C. butyricum* was performed by analyzing the *spo0A* gene and by qPCR. Absolute quantification was recorded according to a standard curve by determining the qPCR Nano drop DS-11 (Denovix, Wilmington, DE, USA). The plate count method was employed to count the number of LAB present in MRS broth.

2.4 Quantification of buk gene expression

Gene expression and real productivity were determined to evaluate the number of *C. butyricum* and the concentration of butyrate. Specimens were prepared using the Tissue Total RNA Purification Mini Kit (Favogen, Ping-Tung, Taiwan). A DNase I solution was used to remove genomic DNA. Extracted RNA was assayed using a Nanodrop DS-11. cDNA was synthesized using the AMPIGENETM cDNA Synthesis kit (Enzo, NY, USA). Relative quantification of qPCR results was performed using BioRAD CFX96 (BioRAD, Hercules, CA, USA).

2.5 Acid and bile resistances

Acid resistance was determined in phosphate buffered saline (PBS) solutions at different pH values (pH 2.5, 3, and 7) that were adjusted using 5 M HCl. LAB were cultured overnight and then centrifuged at 4,045 x g for 5 min. This was followed by washing and then inoculation using 10% sample after dilution to 10^8 CFU/mL. Bacterial enumeration was performed after 1 h of culture at 37 °C. MRS broths containing 0, 0.3, and 1% (w/v) of Oxgall (Difco, Sparks, MD, USA) were prepared for the bile resistance test. Inoculation was controlled to 2% of the strains (OD_{600} = 1.0). Viable cell count was obtained by plate counting after a 24 h culture at 37 °C.

2.6 Auto-aggregation

As previously proposed in the literature (Kos et al., 2003), we used a modified auto-aggregation test to evaluate the indirect adhesive property between cells. Cultured LAB were centrifuged at 4,045 x g (5 min at 4 °C) and then washed before being suspended in PBS (pH 7.2, OD_{600}=1). Auto-aggregation activity was calculated using Equation 1.

$$Auto-aggregation(\%) = \left(1 - \frac{A_t}{A_0}\right) \times 100 \quad (1)$$

Here, A_0 is the initial value of OD, and A_t is the determined value after 1, 3, and 5 h.

2.7 Freeze-drying stress resistance

Skim milk was used to determine the resistance to freeze-drying stress. It is established that skim milk can play an important role in protecting live cells during freeze-stress testing. Cultured strains were washed and suspended completely in PBS, and they were then mixed with 20% skim milk at a ratio of 1:1. Bacterial enumeration was performed after the drying process was complete. The viable cell counts were compared between the cases before and after the freeze-drying process.

3 Results

3.1 Stimulation of C. butyricum growth by lactic acid bacteria

C. butyricum was cultured in the presence of 249 different LAB supernatants, and the results of OD values were presented in Table 1. Among 249 LAB supernatants, 24 LAB supernatants showed the positive result of *C. butyricum* growth. In Figure 1, the increase of viable cells *C. butyricum* as well as OD values were presented. For contrast, MRS and BHI were included instead of LAB supernatant.

qPCR was employed to verify the effectiveness of co-cultures of *C. butyricum* and the LAB strains. Figure 2 presents the growth level and indirect results of gene expression when *C. butyricum* were co-cultured with selected 24 LAB. It is known that the *spo0A* gene induces spore formation only in *C. butyricum* and not in LAB. It was speculated that qPCR-based quantification could provide clues regarding *C. butyricum* growth, as the copy level of the *spo0A* gene is proportional to the concentration of

Table 1. Optical Density of *C. butyricum* with supernatant of lactic acid bacteria.

Strain	OD	Strain	OD	Strain	OD	Strain	OD	Strain	OD	Strain	OD	Strain	OD	Strain	OD	Strain	OD
KLB13	-	KLB227	-	KLB265	-	HL 2	-	HL 34	-	JL29	-	MH71	-	QL9	-	ML28	-
KLB14	-	KLB228	-	KLB266	-	**HL 3**	+	HL 36	-	**JL30**	+	MH74	-	QL10	-	YJ1	-
KLB30	-	KLB229	-	KLB267	-	HL 4	-	JL1	-	JL31	-	MH92	-	QL11	-	YJ2	-
KLB58	-	KLB230	-	KLB268	-	**HL 7**	+	JL2	-	JL32	-	D1	-	QL12	-	YJ3	-
KLB62	-	KLB231	-	KLB270	-	HL 8	-	JL3	-	JL33	-	D3	-	ML1	-	YJ4	-
KLB63	-	KLB233	-	KLB271	-	HL 9	-	JL4	-	JL34	-	D4	-	ML3	-	**YJ5**	+
KLB68	-	KLB234	-	KLB272	-	HL 10	-	JL5	-	**YG1**	+	D5	-	ML4	-	YJ6	-
KLB79	-	KLB235	-	KLB277	-	HL 11	-	JL7	-	**YG2**	+	D6	-	ML5	-	YJ7	-
KLB100	-	KLB236	-	KLB279	-	HL 12	-	JL8	-	YG3	-	M1	-	ML6	-	YJ8	-
KLB101	-	KLB237	-	KLB281	-	HL 13	-	JL9	-	YG4	-	M3	-	ML7	-	YJ9	-
KLB103	-	KLB239	-	KLB282	-	HL 14	-	JL10	-	YG5	-	M4	-	ML8	-	YJ10	-
KLB201	+	**KLB240**	+	KLB283	-	HL 15	-	JL11	-	**YG6**	+	M5	-	ML10	-	YJ11	-
eKLB202	-	KLB241	-	**KLB285**	+	**HL 16**	+	JL12	-	MH5	-	M6	-	ML11	-	YJ12	-
KLB203	-	KLB242	-	KLB286	-	**HL 17**	+	JL13	-	MH6	-	M7	-	ML12	-	YJ13	-
KLB207	-	KLB243	-	KLB287	-	HL 18	-	JL14	-	MH8	-	O1	-	ML13	-	YJ14	-
KLB209	-	KLB244	-	KLB288	-	HL 19	-	**JL15**	+	MH15	-	S6	-	ML14	-	YJ15	-
KLB210	-	KLB245	-	KLB289	-	HL 20	-	**JL16**	+	MH19	-	S7	-	ML15	-	YJ16	-
KLB212	-	KLB246	-	KLB290	-	HL 21	-	JL17	-	**MH21**	+	T1	-	ML16	-	YJ17	-
KLB213	-	**KLB247**	+	KLB292	-	HL 22	-	JL18	-	MH22	-	T2	-	ML17	-	YJ18	-
KLB214	-	KLB248	-	KLB293	-	HL 23	-	JL19	-	**MH33**	+	T3	-	ML18	-	YJ19	-
KLB215	-	KLB249	-	KLB294	-	**HL 25**	+	JL21	-	**MH44**	+	T4	-	ML19	-	YJ20	-
KLB217	-	KLB251	-	KLB295	-	HL 26	-	JL22	-	MH49	-	QL1	-	ML20	-	YJ21	-
KLB218	-	KLB254	-	KLB296	-	HL 27	-	JL23	-	MH51	-	QL2	-	ML21	-	JY1	-
KLB219	-	KLB258	-	KLB298	-	HL 28	-	JL24	-	MH53	-	**QL3**	+	ML22	-	JY2	-
KLB220	-	KLB260	-	KLB300	-	HL 29	-	JL25	-	MH55	-	QL5	-	ML23	-	JY3	-
KLB221	-	KLB261	-	KLB302	-	HL 31	-	JL26	-	MH58	-	**QL6**	+	ML25	-		
KLB224	-	KLB262	-	KLB306	-	HL 32	-	JL27	-	MH62	-	**QL7**	+	**ML26**	+		
KLB225	-	KLB263	-	**HL 1**	+	HL 33	-	JL28	-	MH67	-	QL8	-	ML27	-		

+: higher OD values that determined to the effective growth of *C. butyricum*; -: Compared to '+', not effective growth of *C. butyricum*.

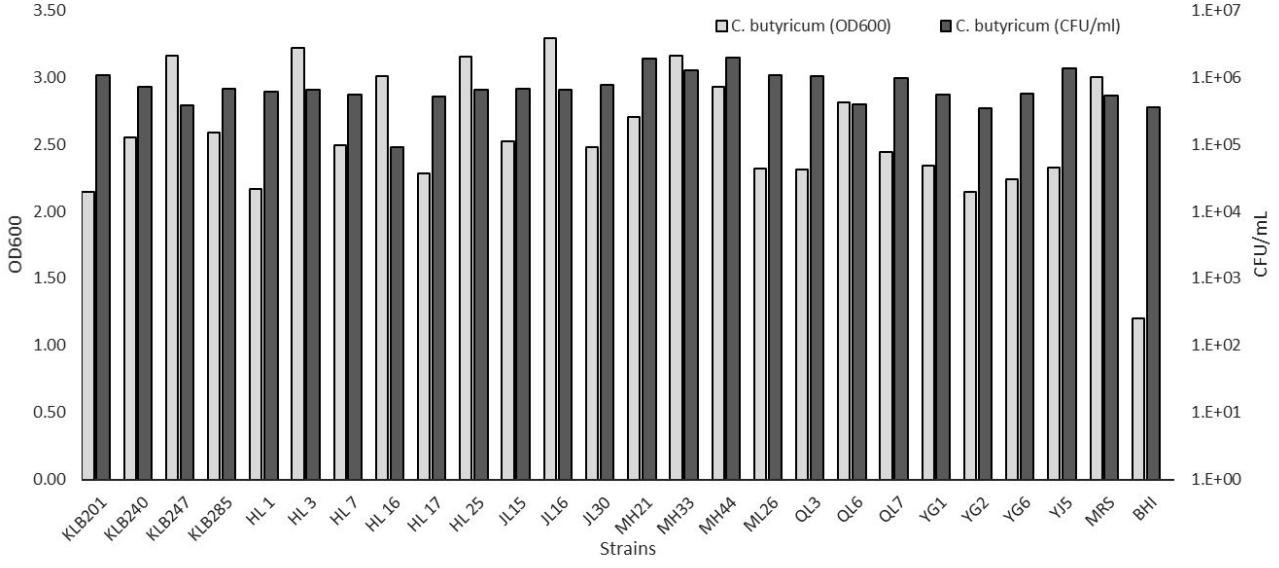

Figure 1. Growth of *C. butyricum* co-cultured with supernatants of lactic acid bacteria.

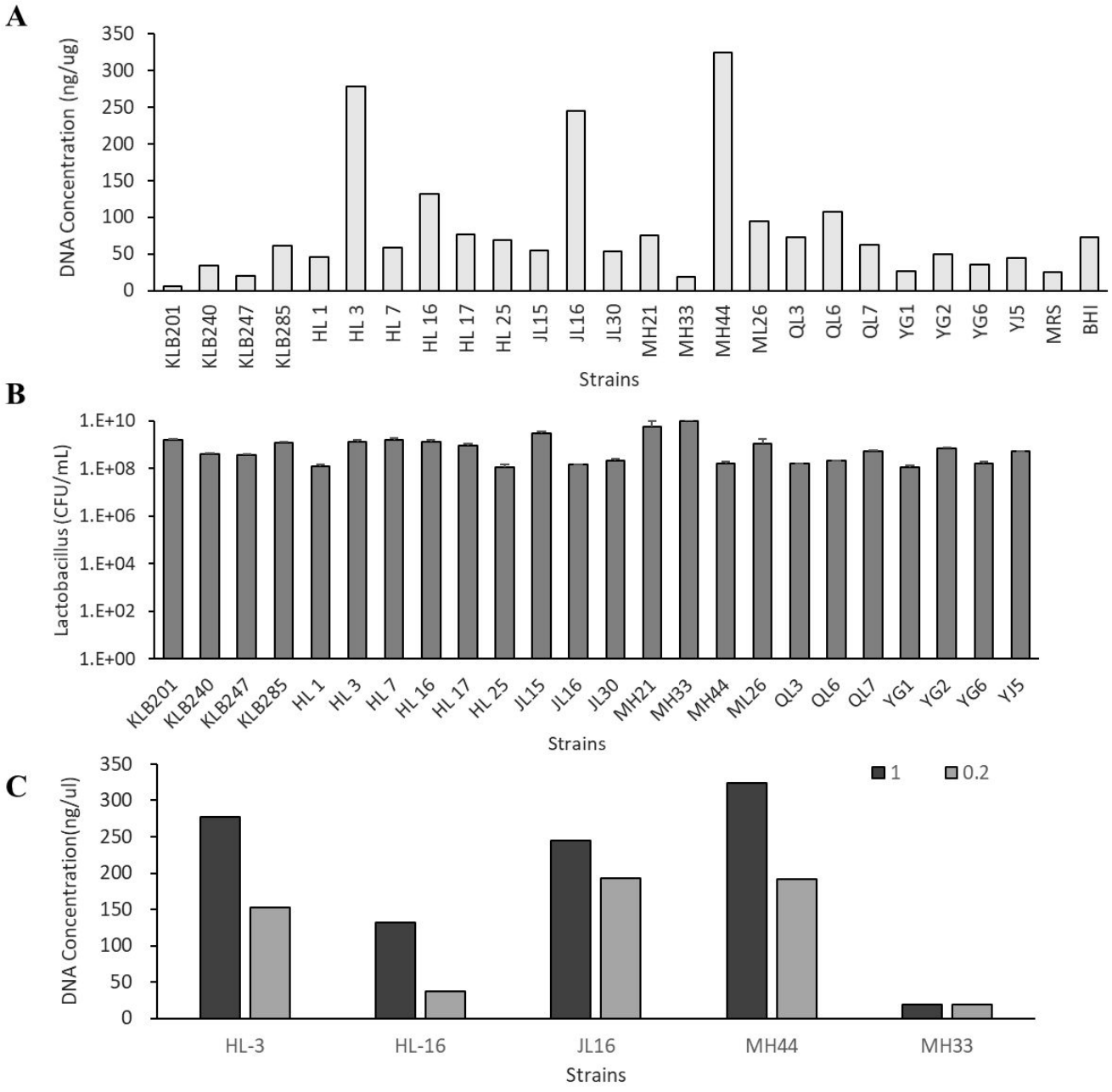

Figure 2. Growth of *C. butyricum* and lactic acid bacteria after co-culture. Quantification of the *spo0A* gene copy level in *C. butyricum* (A), cell growth of lactic acid bacteria (B), and quantification of *spo0A* gene copy level in *C. butyricum* according to the initial dose ratio of *C. butyricum* to lactic acid bacteria (C).

C. butyricum. A standard curve for copy level variance was attempted to determine the concentration of *C. butyricum.* In Figure 2A, 24 *Lactobacillus* strains were co-cultured to examine their effectiveness in regard to the growth of *C. butyricum*. A growth acceleration of greater than 3-fold was observed in co-cultures containing the HL3, JL16, and MH44 strains. The growth of 2-fold increase was observed in case of HL16. After the culture process, bacterial enumeration of LAB was also performed; however, no significant correlations were observed (Figure 2B). This suggests that the growth of *C. butyricum* could be affected by complicated factors, including the growth rate and the concentration of LAB.

To examine the initial mixing ratio between *C. butyricum* and LAB, the ratio of LAB and *C. butyricum* was adjusted to 0.2:1. We observed that the copy level of the *spo0A* gene was reduced when the ratio was 0.2:1 (Figure 2C). MH33 was used as a control group. Relative disproportions were observed where HL3 (45%, 152.7 ng/μL) > HL16 (72.1%, 36.8 ng/μL) > JL16 (21.3%, 192.3 ng/μL) > MH44 (40.8%, 191.8 ng/μL). The clear effectiveness was observed in co-cultures containing the JL16

and MH44 strains. With the help of 16S sequencing analysis, JL16 and MH44 were identified to *Lactobacillus brevis and Lactobacillus parabuchneri*, respectively.

3.2 Expression of the butyric acid production gene of C. butyricum in co-culture with Lactobacillus spp.

The primer sequences of *buk* and *recA* genes are listed in Table 2. It was expected that analyzing the *buk* gene provides the clues regarding butyrate production by *C. butyricum*, while *recA* gene expresses as a housekeeping gene. Here, two cases of MRS culture (without bacteria) and MH33 culture were selected and compared to the two other cases of JL16 and MH44 cultures (Figure 3). When compared to MRS culture, JL16 and MH44 exhibited 1.6-fold and 1.2-fold higher concentrations of the *buk* gene, respectively. While, MH33 culture exhibited a 20% reduction in the number of *buk* genes compared to that in the MRS culture.

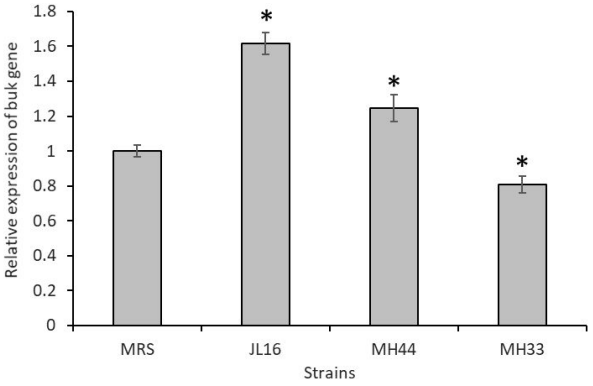

Figure 3. Relative expression of the *buk* gene in *C. butyricum* in the absence (MRS only) and presence of *Lactobacillus* strains (MH44, JL16 and MH33). Error bars indicate SEM; *P < 0.05 vs. MRS group.

3.3 Characterization of LAB for probiotics

Bacterial enumeration was performed after JL16 and MH44 were cultured in different acidic conditions (Figure 4A). For JL16, a live number of approximately 10^7 (pH 2) was reduced to 10^4 (pH 3) and 10^2 (pH 2.5) CFU/mL under these experimental conditions. When MH44 was cultured in acidic conditions (pH 2), the numbers were reduced by approximately 10^3. However, MH44 barely survived at the same level when the pH ranged from 2.5 to 3.

The resistance behavior of JL16 and MH44 to the bile salts was measured and is presented in Figure 4B. The results are presented as the survival rates based on the reference state (0%). Both JL16 and MH44 possessed improved rates in response to 0.3% exposure compared to those in response to 1.0% exposure. The survival rates for JL16 and MH44 and were 43% and 85%, respectively, when they were evaluated in the presence of 0.3% bile salt. For 1.0% bile salt, the survival rates decreased to 36% for JL16 and 21% for MH44.

As an *in vitro* test, auto-aggregation was substantially examined to evaluate the capability of biofilm formation. Both JL16 and MH44 exhibited rapid aggregation values of 87.8% for JL16 and 96.4% for MH44 in 5 hr. A freeze-drying technique is commonly used in the process of probiotic production, and this technique often harms these probiotics and limits their survival. The survival rates may rapidly decrease after the freeze-drying process. The survival rates of JL16 and MH44 were determined to 46% and 41%, respectively.

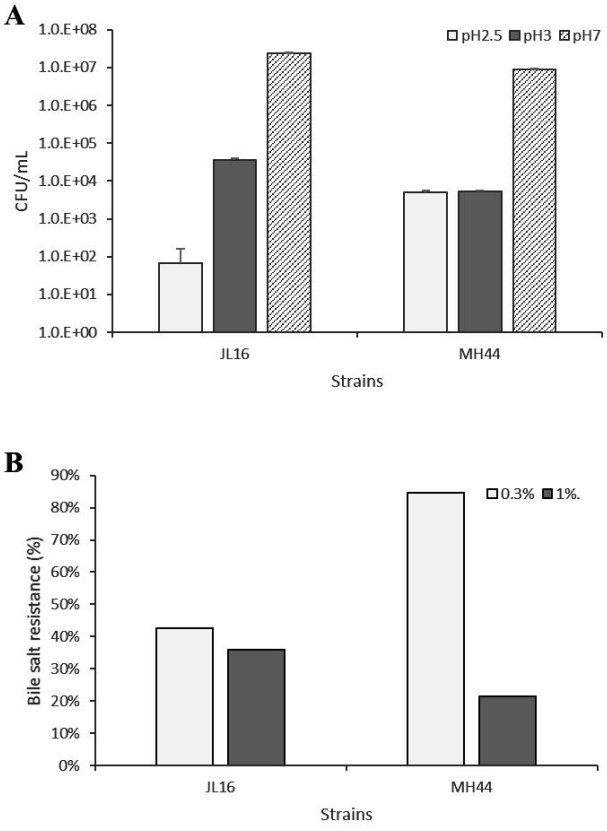

Figure 4. Two resistant behaviors of the MH44 and JL16 strains. Acid resistance (A) and bile resistance (B).

Table 2. *C. butyricum* genes examined by qPCR in this study.

Primer	Sequence (5′ to 3′)	Reference
RTSpo0A_Fw	AGTGCTCCAACAATACAAGA	This study
RTSpo0A_Rv	AATATGAGCAGGTACACCG	
G_buk_F	TGCTGTWGTTGGWAGAGGYGGA	Vital et al. (2013)
G_buk_R	GCAACIGCYTTTTGATTTAATGCATGG	
recA-fw	GCAGAGCATGCATTAGATCCT	This study
recA-rv	GAATCTCCCATTTCCCCTTC	

4 Discussion

In literature, *C. butyricum* in combination with *Lactobacillus* spp. improved some factors in the animals such as promotion in intestinal functions, the stimulation of antioxidant pathway in combination with an effective reduction of lipid levels in its serum (Long et al., 2018), and effectiveness in curing acute colitis in a rat (Wang et al., 2018a). In this study, 2 LAB exhibited the potential that could enhance the probiotic functions of *C. butyricum* and identified *L. brevis* JL16 and *L. parabuchneri* MH44. *L. brevis* and *L. parabuchneri* are classified as obligate heterofermentative, where they convert sugars with 6 carbons to lactic acid, acetic acid, CO_2, and two species that often occur in Kimchi and cheese (Coton et al., 2008; Park et al., 2016). A previous study indicated that *L. brevis* was effective in production of γ–aminobutyric acid, and it has been reported that *L. brevis* may be applicable to oral healthcare (Fang et al., 2018). In bacterial butyrate metabolism, butyrate kinase is recognized as one of the key enzymes in the pathway that produces butyrate (Chai et al., 2019). Butyrate-producing bacteria such as *C. butyricum* convert carbohydrates to butyryl-CoA through several processes, and butyryl-CoA is finally converted to butyrate through two channels (Esquivel-Elizondo et al., 2017). The key enzymes in these two channels are butyrate kinase and butyryl-CoA:acetate CoA transferase, respectively. Butyryl-CoA:acetate CoA transfer occurs in the presence of acetate. Meanwhile, Equivel-Elizondo *et al.* speculated that one of the two primary channels of butyryl-CoA:acetate CoA transfer may not represent the main butyrate production route. When they compared the gene expression levels of the two major enzymes that mediated each response, they predicted that the expression of butyryl-CoA:acetate CoA transferase would remain relatively low in both batch culture and continuous culture. Thereafter, our comparison of the gene expression of butyrate kinase appears to be more reliable than that from butyryl-CoA:acetate CoA transferase, as butyrate production was more accurately predicted. It has also been reported the interchanges of chemicals were possible between different species of bacteria (Louis et al., 2004; Detman et al., 2019). For example, one of these nutrient-based interactions between bacteria can produce lactate, and another neighboring species of bacteria can consume lactate to produce butyrate. In literature, it was reported that *C. butyricum* can consume lactate and produce butyrate (Louis et al., 2004). The pathway responsible for generating butyrate requires the presence of two enzymes, butyrate kinase and butyryl-CoA:acetate CoA transferase. It is known that numerous steps are involved in the conversion from lactate to butyrate (Detman et al., 2019).

It is important to maintain a higher survival rate of probiotics when they enter the intestines. The first obstacle to survival occurs in a stomach where the pH is estimated to be 2, although the pH can increase to approximately 2.5 to 3 when food is present. It has been reported that *Lactobacillus* spp. possess innate resistance to mild acidic environments (Šeme et al., 2015; Wang et al., 2018b); however, some species are rapidly weakened when exposed to a strongly acidic environment (pH < 2) (Jin et al., 1998). In other cases, the use of either encapsulation or adaptation pretreatment can increase the resistance of probiotics to an acidic environment. The second obstacle to probiotic survival is the exposure of these organisms to bile salt. Bile salts can easily destroy the walls of probiotic bacteria and induce antibiotic functions such as oxidation stress following damage to DNA (Ruiz et al., 2013; Oleinikova et al., 2020). It was reported that the initial concentration of bile salt was 2–1.5% within 1 h and then decreased to 0.3% (Noriega et al., 2004). In this study, the concentration effect of bile salt was examined in the range of 0.3-1.0%. It has been reported that the inhibitive concentration of bile salt is the most influential factor for the survival of strains, despite the knowledge that these concentration ranges vary according to strains. As mentioned earlier, the major objective of probiotics would be their safe delivery to intestines where they can activate their functions. It was speculated that biofilm formation of probiotics would provide a favorable strategy for them to remain and to activate within intestines (Juárez Tomás et al., 2005). In this study, skim milk was used to protect and stabilize the cell membranes of both strains, as suggested in an earlier research paper (Carvalho et al., 2004). In other cases, the survival rates of the six strains were 62%–92% after freeze-drying. The addition of sugars to skim milk may enhance the survival rates based on previously published results (Juárez Tomás et al., 2009). Additionally, sugar may remove activated oxygen molecules during the thaw process by inducing the protective roles of cell collapse (Leslie et al., 1995).

5 Conclusion

Clostridium butyricum was co-cultured in the presence of supernatants of 249 lactic acid bacteria. The gene expression of butyrate kinase was evaluated to verify the growth stimulation of *C. butyricum* in the presence of *Lactobacillus* spp. It was determined that *Lactobacillus brevis* JL16 and *Lactobacillus parabuchneri* MH44 stimulated *C. butyricum* more effectively than did other strains. Meanwhile, the acidic tolerances of both JL16 and MH44 were not adequate in a strongly acidic environment and needed to be overcome for use as probiotics such as capsulation. Probiotics might eventually reach in the intestine and interact with the intestinal microbe. This study is the basic research about potential probiotics for use with *C. butyricum* and interaction with the intestinal microorganisms.

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