Role of cysteine in the improvement of γ -aminobutyric acid production by nonproteolytic *Levilactobacillus brevis* in coculture with *Streptococcus thermophilus*

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ABSTRACT

Previous research has showed that nonproteolytic *Levilactobacillus brevis* 145 (L) in coculture with *Streptococcus thermophilus* 1275 (S), not *Lactobacillus delbrueckii* ssp. *bulgaricus* (Lbu), was able to produce γ -aminobutyric acid (GABA) during milk fermentation in the presence of monosodium glutamate (MSG). It was assumed that differences of casein hydrolysis patterns between *Strep. thermophilus* 1275 and *L. bulgaricus* caused the phenomenon. Moreover, the GABA content was low and residual MSG was high in SL-fermented milk. In our research, comparison of peptide profiles determined by liquid chromatography/tandem mass spectrometry showed that α_{S2} -casein, β -casein, and κ -casein degradation by *L. bulgaricus* and *Strep. thermophilus* varied. Importantly, the peptide number in the L and Lbu coculture group increased compared with the Lbu monoculture group, whereas the peptide number in the SL coculture group decreased in comparison with S monoculture group, suggesting that *L. bulgaricus* was not able to provide peptides for the growth of *Lb. brevis* 145. Furthermore, we found that after supplementation with cysteine (50 mg/L) during milk fermentation by SL, 10 g/L MSG was converted into 4.8 g/L GABA with a minimum level of residual MSG, viable cell counts of *Lb. brevis* and lactic acid production were increased, and the casein hydrolysis pattern was not influenced. Moreover, sulfhydryl group-containing chemicals including cystine, reduced glutathione, and oxidized glutathione showed effects similar to that of cysteine in improving GABA production. Finally, when *L. bulgaricus* YIB2 was combined with SL, supplementation of cysteine was also able to significantly improve GABA production.

Key words: γ -aminobutyric acid, cysteine, casein hydrolysis, bacteria coculture

INTRODUCTION

γ -Aminobutyric acid (GABA), a 4-carbon nonprotein amino acid, is a major inhibitory neurotransmitter in the central nervous system (Bowery and Smart, 2006), and GABA-rich food is of interest for the food industry as it provides various physiological functions, such as a neuroprotective effect (Li et al., 2016), attenuation of cardiac apoptosis in spontaneously hypertensive rats (Cherng et al., 2014), and reduction of blood pressure (Hayakawa et al., 2004; Shimada et al., 2009).

High GABA-producing lactic acid bacteria have commonly been applied to produce GABA-rich milk beverages, cheeses, and yogurts for promoting human health, as dairy products can serve as highly effective carriers of GABA (Sun et al., 2009; Song and Yu, 2018). Milk protein is a well-known precursor protein for the generation of peptides degraded by lactic acid bacteria. Development of liquid chromatography/tandem MS (LC/MS-MS) enables us to determine peptide sequences, peptide counts, and peptide intensities in casein hydrolysates. Miclo et al. (2012) found that numbers and types of peptides released from casein were strain-dependent. Hydrolysis appeared to be linked with the accessibility of different casein regions by protease. For *Lactobacillus helveticus*, rapid hydrolysis of pure β -CN was observed, and the hydrolysis kinetics of α_{S1} -CN varied based on the number of cell envelope proteinases. When only PrtH2 proteinase was present, 22 to 30% of the peptides originated from α_{S1} -CN. The percentage increased to 41 to 49% for strains in which both PrtH2 and PrtH proteinases were expressed (Sadat-Mekmene et al., 2011). For *Bifidobacterium longum* KACC9156, a total of 28 peptides were generated from the <3-kDa fraction, corresponding to 11 CN- β , 16 α_{S1} -CN, and 1 κ -CN peptides. In bovine kefir prepared with kefir grains, 124 peptides were identified, mainly released from CN- β , followed by α_{S1} -CN, κ -CN, and α_{S2} -CN (Ebner et al., 2015). A growing body of publications suggest the multifunctional roles of milk-derived peptides, which included antihypertensive (Rai et al., 2017), antioxidative (Pihlanto, 2006; Power

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et al., 2013), and immunoregulation effects (Lahov and Regelson, 1996), and more. These peptides are capable of acting as additives in food manufacturing for the development of functional dairy products with beneficial effects for human health.

Applying high GABA-producing lactic acid bacteria in fermenting GABA-enriched dairy products has been an increasing trend (Cui et al., 2020). *Levilactobacillus* species, especially *Levilactobacillus brevis*, have been confirmed as the highest GABA producers isolated from Korean kimchi and other fermented vegetables (Wu and Shah, 2017). It has been documented that *Lb. brevis* of plant origin may not be able to survive in milk because of the absence of genes coding extracellular or other wall-anchored proteinases. Wu et al. (2015) reported a novel and promising method for manufacturing GABA-rich fermented milk by coculturing *Lb. brevis* 145 and *Streptococcus thermophilus* 1275 in the presence of monosodium glutamate (MSG). However, in their research, the final GABA concentration was only 0.265 g/L, and the residual MSG (1.2 g/L) supplemented as substrate for GABA production was relatively high, which was reported to promote an undesirable taste (Jinap and Hajeb, 2010). It would be of great economic importance to improve the GABA yield and simultaneously reduce the level of residual MSG in fermented milk. Most milk proteins (α - and β -caseins) do not contain cysteine in their amino acid composition (Gordon et al., 1949); cysteine could be an essential amino acid for *Lb. brevis*, and this supplementation would enhance growth and consequent GABA production by *Lb. brevis*. Dave and Shah (1997) also reported that cysteine could improve viability of probiotics in fermented milk by providing essential growth factors and lowering the redox potential. Until now, very little information has been published on the effect of cysteine on improving GABA production by *Lb. brevis* during milk fermentation.

In our research, we aim to find (1) the contribution of specific casein hydrolysis pattern by *Strep. thermophilus* 1275 and *Lactobacillus bulgaricus* coculture (SL) to the growth of *Lb. brevis* during milk fermentation; (2) ways to increase GABA content and reduce the amount of MSG in SL-fermented milk; (3) the effects of coculture and cysteine on casein hydrolysis; and (4) whether the

mixture of *L. bulgaricus* and SL are capable of producing GABA-enriched milk in the presence of cysteine. The successful resolution of these issues would lay a foundation for the development of functional dairy products.

MATERIALS AND METHODS

Bacterial Strains and Cultivation Conditions

Levilactobacillus brevis NPS-QW-145 and 3 different *Lactobacillus delbrueckii* ssp. *bulgaricus* strains (Table 1) were activated in Difco lactobacilli de Man, Rogosa, and Sharpe (MRS) broth (Becton, Dickinson and Co.), and *Strep. thermophilus* strain 1275 was cultivated in M17 broth (Becton, Dickinson and Co.). Working cultures were propagated 3 times consecutively using 1% inoculation in the previously listed media (MRS or M17) at 37°C for 16 h. Cell counts of the working cultures were enumerated on MRS or M17 agar plates using the plate counting method before inoculation in reconstituted skim milk (RSM). The initial cell counts for both *L. brevis* 145 and *Strep. thermophilus* were $\sim 1 \times 10^9$ cfu/mL.

Parameter Selection and Optimization for GABA-Rich Milk

For coculture fermentation, *Strep. thermophilus* 1275 (1%, vol/vol) and *Lb. brevis* 145 (3%, vol/vol) were inoculated together into 10% (wt/vol) RSM for 48-h fermentation at 37°C. The one-factor-at-a-time strategy was employed to identify concentration ranges of MSG (1–5 g/L), cysteine (25–200 mg/L), pyridoxal phosphate (PLP; 0.01–0.05 mM), and whey protein hydrolysate (WPH; 2–10 g/L) for improving GABA production during milk fermentation. Thereafter, the individual effects of cysteine, PLP, and WPH in the listed concentrations on GABA production in the presence of 1 g/L of MSG was examined. Finally, the best combination of MSG (concentration ranging from 0 to 10 g/L) and cysteine (concentrations ranging from 0 to 300 mg/L) for improving the GABA production level by *Lb. brevis* 145 coculture with *Strep. thermophilus* 1275 was determined. The conversion ratio of MSG to

Table 1. Starter bacteria strains used for milk fermentation in this study

Species	Strain ID	Origin
<i>Streptococcus thermophilus</i>	ASCC1275	Australian Starter Culture Research Center
<i>Lactobacillus delbrueckii</i> ssp. <i>bulgaricus</i>	ASCC756	Australian Starter Culture Research Center
	ASCC859	Australian Starter Culture Research Center
	YIB2	Commercial yogurt isolate
<i>Levilactobacillus brevis</i>	NPS-QW-145	High γ -aminobutyric acid producer isolated from Korean kimchi

GABA was calculated by the following formula: conversion ratio (%) = $100 \times [\text{Final GABA concentration (mM)} / \text{Initial MSG concentration (mM)}]$.

Effect of Sulfur-Containing Chemicals on GABA Improvement

Sulfur-containing chemicals, including cystine, methionine, reduced glutathione, and oxidized glutathione, were individually supplemented at 50 mg/L for milk fermentation at 37°C with coculture of *Strep. thermophilus* 1275 and *Lb. brevis* 145 in the presence of 2 g/L MSG, after 48-h fermentation, and GABA production was determined by reversed-phase HPLC, as will be discussed.

Sample Preparation from 48-h Milk Fermentation With or Without Cysteine

Milk fermentation was carried out in 10% (wt/vol) RSM supplemented with MSG at the level of 2 g/L. First, an aliquot of 40 mL of the RSM and MSG mixture was divided into 50-mL sterile centrifuge tubes, and 3% (vol/vol) *Lb. brevis* 145 and 1% (vol/vol) *Strep. thermophilus* 1275 were inoculated with and without 50 mg/L cysteine. After mixing, 3.5 mL of the inoculated RSM containing MSG, with or without cysteine, was distributed into 5-mL sterile polystyrene round-bottom tubes and incubated statically at 37°C. Each tube was withdrawn at 0, 3, 6, 9, 12, 16, 20, 24, 36, and 48 h to check bacterial growth, pH of the fermented milk, GABA and MSG concentrations, lactic acid production, and lactose and galactose utilization.

Determination of GABA and Residual MSG by Reversed-Phase HPLC

The concentrations of GABA and MSG were measured as previously described with a minor modification (Wu and Shah, 2015). Briefly, Carrez solutions were used to remove milk proteins before reversed-phase HPLC analysis for residual MSG and generated GABA. One milliliter of fermented milk was added into 4.0 mL of distilled water, followed by addition of 0.25 mL of Carrez I solution (0.25 M potassium ferrocyanide) and 0.25 mL of Carrez solution II (0.50 M zinc acetate). Then, the mixture was thoroughly mixed using a vortex, and kept for 1 h at 25°C until the precipitation of milk proteins, followed by centrifugation at $5,000 \times g$ and 25°C for 40 min. Supernatants were collected and filtered through a 0.20- μm Millipore filter. Filtrates were then freeze-dried, followed by re-dissolving in 1 mL of double-distilled water and removing residues by centrifugation at 4°C and $5,000 \times g$ for 40 min. Dansyl

derivatization of free amino acids including MSG and GABA in the supernatants was carried out, followed by HPLC analysis of dansyl amino acids.

Estimation of Lactose, Glucose, Galactose, and Lactic Acid

The concentrations of lactose, glucose, and galactose and lactic acid were measured as previously described with a minor modification (Padmanabhan et al., 2018). Utilization of lactose, glucose and galactose, and production of lactic acid were simultaneously quantified from 10 μL of sample injected into a Shimadzu model LC-2010A (Shimadzu Corp.) system equipped with HPX-87H anion exchange column (300×7.8 mm, 9 mm, Bio-Rad Laboratories Inc.). An isocratic elution was performed using 5 mM H_2SO_4 at a flow rate of 0.6 mL/min for 30 min and column temperature of 65°C. A refractive index detector and UV-Vis detector (220 nm) were connected to simultaneously determine the concentrations of lactose, glucose, galactose, and lactic acid.

Measurement of the pH of Fermented Milks

The pH of the fermented milks was measured using an Orion Model 250A portable pH meter (Thermo Scientific).

Enumeration of Viable *Lb. brevis* 145 and *Strep. thermophilus* 1275 in Fermented Milk

Samples were taken during milk fermentation as previously described, to enumerate the counts of *Strep. thermophilus* 1275 and *Lb. brevis* 145 using selective media as previously described (Wu et al., 2015). The M17 agar plates for *Strep. thermophilus* were aerobically incubated at 37°C for 48 h, and MRS agar plates for enumerating *Lb. brevis* 145 were aerobically incubated at 37°C for 48 h, followed by counting of colonies.

Peptide Extract

Milk fermentation applying monoculture of *L. bulgaricus* 859 (8), *L. bulgaricus* YIB2 (Y), and *Strep. thermophilus* 1275 (S), and coculture of *Lb. brevis* (L) 145 with one of *L. bulgaricus* 859 (8L), *L. bulgaricus* YIB2 (YL), and *Strep. thermophilus* 1275 (SL), as well as cocultures with cysteine (8LC, YLC, SLC), were carried out at 37°C for 48 h. An aliquot of 4 mL of fermented milk in each batch (8, 8L, 8LC, Y, YL, YLC, S, SL, and SLC) was diluted with an equal amount of deionized water, followed by centrifugation at $5,000 \times g$ and 4°C for 30 min to remove coagulated caseins and

bacteria. Ultrafiltration centrifugal tube with 10-kDa cutoff (Millipore) was used to ultrafilter the supernatants at $5,000 \times g$ and 20°C for 30 min, and ultrafiltrate was loaded onto a C18 solid-phase extraction column (Waters Corp.) to perform desalting 3 times for each sample. The eluate containing target peptides was freeze-dried to powder.

LC-MS/MS and Data Analysis

The lyophilized peptide fractions were resuspended in double-distilled water containing 0.1% formic acid, and an aliquot of 5 μL of peptide-containing solvent was loaded into a nanoViper C18 (3- μm , 100- $\text{\AA}$, Thermo Scientific) trap column for analysis. The generated RAW files of MS/MS data were converted to MGF by Proteome Discoverer (version 2.1.0.81, Thermo) and were searched against a *Bos taurus*-reviewed database (6,008 sequences) using Mascot Daemon (version 2.3.2). No enzyme or static modification was set for database searching. Precursor and fragment mass tolerance were set to 10 ppm and 0.05 Da, respectively. The obtained peptide sequence data set was visualized and compared with a novel online tool, Peptigram (Manguy et al., 2017), which is implemented as a web application and is freely available for academic use at <http://bioware.ucd.ie/peptigram>.

Effects of Cysteine on GABA Yield in Mixtures of SL and *L. bulgaricus* Strains

Milk fermentation using culture *Lb. brevis* 145 and different *L. bulgaricus* strains with or without *Strep. thermophilus* and with and without cysteine was also carried out. Fermentation was carried out in 10% (wt/vol) RSM with 2 g/L of MSG at 3% (vol/vol) *Lb. brevis* 145 inoculation level. For coculturing of *Lb. brevis* 145 with *L. bulgaricus* in RSM, the inoculation level of *Lb. brevis* 145 was 3% (vol/vol) and that of *L. bulgaricus* was 1% (vol/vol). For the 3-bacteria coculture batch, the inoculation levels of *L. bulgaricus* and *Strep. thermophilus* 1275 were each 0.5% (vol/vol), and that of *Lb. brevis* 145 was 3% (vol/vol). All the fermentation experiments were carried out at 2 occasions under static conditions at 37°C for 48 h.

Statistical Analysis

All data in the bar charts, line charts, and tables are means $\pm$ standard deviation. Significant differences ($P < 0.05$, $P < 0.01$, $P < 0.001$) among the groups were calculated by one-way ANOVA using IBM SPSS Statistics version 20.0.

RESULTS

Strain-Specific Casein Hydrolysis Patterns of *Strep. thermophilus* and *L. bulgaricus*

Liquid chromatography/tandem MS was performed to investigate the differences of casein hydrolysis patterns between *L. bulgaricus* and *Strep. thermophilus*. By comparing the peptide components in *L. bulgaricus* and *Strep. thermophilus* single-culture groups (Table 2), it was observed that the total number of the peptide degraded by *L. bulgaricus* was higher than that degraded by *Strep. thermophilus*. *Lactobacillus bulgaricus* and *Strep. thermophilus* showed different preferences to digest α_{S1} -CN, α_{S2} -CN, CN- β , and κ -CN. In the *Strep. thermophilus* 1275 single-culture group, peptides from CN- β and α_{S1} -CN accounted for 33%, respectively, whereas peptides from α_{S2} -CN and κ -CN accounted for 12 to 17%. In *L. bulgaricus* 859 and *L. bulgaricus* YIB2 single-culture groups, peptides from CN- β accounted for 54.3% and 60.8%, respectively, suggesting that the *L. bulgaricus* strain preferred to digest CN- β . The percentage of peptides from α_{S1} -CN in *L. bulgaricus* 859 and *L. bulgaricus* YIB2 groups was in the second position, falling into the range of 20 to 25%. Peptides from α_{S2} -CN and κ -CN showed similar percentages, 8 to 10%.

Visualization of Casein Hydrolysis Patterns in Mono- and Coculture Groups

With the visualization tool (Manguy et al., 2017), peptide coverage on precursor casein, α_{S1} -CN, α_{S2} -CN, CN- β , and κ -CN in mono- and coculture groups is clearly displayed in Figure 1.

As shown in Figure 1A, distribution of peptides from hydrolyzed α_{S1} -CN in the *Strep. thermophilus* group was generally similar to that of the *L. bulgaricus* group, except for a higher count of overlapping peptides with a higher sum of intensities at C terminal in the *Strep. thermophilus* groups. When *L. bulgaricus* 859 and *Strep. thermophilus* were cocultured with *Lb. brevis*, the α_{S1} -CN hydrolyzation pattern was not influenced. For *L. bulgaricus* YIB2, a higher count of peptides with a higher sum of intensities (amino acid position 38–45, approximately) was observed in the coculture group compared with the monoculture group.

As shown in Figure 1B, *Strep. thermophilus* and *L. bulgaricus* displayed distinct profiles on precursor casein CN- β , and overall differences in peptide intensity were also clearly observed. The peptide count and intensity from the *Strep. thermophilus* group were much lower than those of the 2 *L. bulgaricus* groups, which was consistent with the analysis results in Table 2.

Table 2. Peptide number and percentages of peptides (in parentheses) in different milk fermentation groups¹

Item	S	SL	SLC	8	8L	8LC	Y	YL	YLC
α_{S1} -Casein	150 (33%)	140 (36%)	141 (33%)	127 (24%)	139 (25%)	150 (26%)	105 (21%)	132 (23%)	140 (24%)
α_{S2} -Casein	66 (15%)	54 (14%)	60 (14%)	48 (9%)	58 (10%)	47 (8%)	38 (8%)	43 (7%)	37 (6%)
β -Casein	150 (33%)	123 (32%)	146 (34%)	291 (54%)	282 (50%)	282 (49%)	307 (61%)	313 (54%)	315 (54%)
κ -Casein	71 (16%)	52 (14%)	65 (15%)	45 (8%)	51 (9%)	53 (9%)	39 (8%)	60 (10%)	63 (11%)
β -Lactoglobulin	16 (4%)	12 (3%)	14 (3%)	13 (2%)	16 (3%)	22 (4%)	13 (3%)	15 (3%)	10 (2%)
α -Lactalbumin	0 (0%)	3 (1%)	0 (0%)	12 (2%)	18 (3%)	17 (3%)	3 (1%)	12 (2%)	19 (3%)
All	453	384	426	536	564	571	505	575	584

¹Fermentations were carried out at 37°C for 48 h. S = *Streptococcus thermophilus* 1275; L = *Levilactobacillus brevis* 145; C = cysteine; 8 = *Lactobacillus bulgaricus* ASCC859; Y = *L. bulgaricus* YIB2.

When *L. bulgaricus* or *Strep. thermophilus* were cocultured with *Lb. brevis*, the overall CN- β hydrolyzation pattern was not influenced. However, a lower count of peptides (amino acid position 180–220, approximately) was observed in the SL group in comparison with the S group, whereas the peptide reduction did not appear in *L. bulgaricus* and *Lb. brevis* coculture groups.

As shown in Figure 1C, *Strep. thermophilus* and *L. bulgaricus* displayed distinct profiles on precursor casein α_{S2} -CN. For the *Strep. thermophilus* group, a high count of overlapping peptides was found at the C terminal, whereas for the *L. bulgaricus* 859 group, a high count of overlapping peptides with a high sum of intensities was found at amino acid positions 115 to 125. The pep-tigram for the *L. bulgaricus* YIB2 group was similar to that of the *L. bulgaricus* 859 group, except for a low sum of intensities at amino acid positions 115 to 125. When *L. bulgaricus* or *Strep. thermophilus* were cocultured with *Lb. brevis*, overall α_{S2} -CN hydrolyzation pattern was not significantly influenced. However, the count of peptides in SL declined compared with the S group; specifically, a lower count of peptides (amino acid positions 115–140 and 180–220, approximately) was observed in the SL group. However, the reduction of peptide count did not appear in the *L. bulgaricus* and *Lb. brevis* coculture groups; conversely, the α_{S2} -CN hydrolysis level was even higher at amino acid positions 185 to 200 in *L. bulgaricus* and *Lb. brevis* coculture groups.

As shown in Figure 1D, *Strep. thermophilus* and *L. bulgaricus* displayed distinct profiles on precursor casein κ -CN. For the *Strep. thermophilus* groups, peptides were mainly distributed at the C terminal, whereas peptides were evenly distributed in the green shaded area for 2 *L. bulgaricus* groups, and the peptide coverage for the *L. bulgaricus* YIB2 group was similar to that of *L. bulgaricus* 859. When *L. bulgaricus* or *Strep. thermophilus* were cocultured with *Lb. brevis*, overall κ -CN hydrolyzation pattern was not significantly influenced. However, the count of peptides in SL declined compared with the S group, whereas the peptide count was higher in the *L. bulgaricus* and *Lb. brevis* coculture groups, especially for the *L. bulgaricus* YIB2 coculture group.

Effects of PLP, WPH, and Cysteine on Improving GABA Production in SL-Fermented Milk in Presence of MSG

Monosodium glutamate was found to be necessary for GABA production by *Lb. brevis* 145 (Supplemental Table S1, <https://doi.org/10.6084/m9.figshare.17701424.v1>); thus, the effects of PLP, WPH, and cysteine on GABA production in the presence of 1 g/L MSG were exam-

Figure 1. Peptide coverage on precursor caseins α_{S1} -casein (A), β -casein (B), α_{S2} -casein (C), and κ -casein (D) in different milk fermentation groups. The height of the bars in these images is proportional to the count of peptides overlapping each position. Intensity of the color (green) is proportional to the sum of the peptide intensities overlapping each position. S = *Streptococcus thermophilus* 1275; L = *Levilactobacillus brevis* 145; C = cysteine; 8 = *Lactobacillus bulgaricus* ASCC859; Y = *Lactobacillus bulgaricus* YIB2.

Table 3. Effects of pyridoxal phosphate (PLP), whey protein hydrolysate (WPH), and cysteine (Cys) on γ -aminobutyric acid (GABA) production in presence of 1 g/L monosodium glutamate (MSG)¹

Additive	Residual MSG (g/L)	GABA production (g/L)
Blank milk	0.60 $\pm$ 0.010	0.13 $\pm$ 0.004 ^a
25 mg/L Cys	0.14 $\pm$ 0.017	0.55 $\pm$ 0.003 ^b
50 mg/L Cys	0.11 $\pm$ 0.004	0.52 $\pm$ 0.019 ^b
75 mg/L Cys	0.10 $\pm$ 0.000	0.56 $\pm$ 0.029 ^b
100 mg/L Cys	0.09 $\pm$ 0.001	0.58 $\pm$ 0.016 ^b
200 mg/L Cys	0.12 $\pm$ 0.005	0.47 $\pm$ 0.025 ^c
2 g/L WPH	ND	ND
4 g/L WPH	ND	ND
6 g/L WPH	ND	ND
8 g/L WPH	ND	ND
10 g/L WPH	ND	ND
0.01 mM PLP	ND	ND
0.02 mM PLP	ND	ND
0.03 mM PLP	ND	ND
0.04 mM PLP	ND	ND
0.05 mM PLP	ND	ND

^{a-c}Values with different letters are significantly different ($P < 0.05$).

¹Fermentations were carried out at 37°C for 48 h. Blank milk = only MSG added. ND = not detectable.

ined. As shown in Table 3, cysteine supplementation increased GABA content and simultaneously decreased MSG level. In the presence of 50 mg/L cysteine, GABA production increased from 0.13 g/L to 0.58 g/L in fermented milk; meanwhile, the remnant MSG decreased to 0.12 g/L. The residual MSG was higher when cysteine concentration was at either 25 mg/L or more than 200 mg/L. Combining GABA production and residual MSG, 50 to 100 mg/L cysteine was regarded as appropriate range for GABA production. In our research, PLP and WPH had no influence on GABA production in the presence of MSG (Table 3).

Influence of Different Concentrations of Cysteine and MSG on Improving GABA Production in SL-Fermented Milk

To confirm whether cysteine has greater influence on GABA production, the effects of different concentrations of cysteine (0, 50, 100, 150, 200, or 300 mg/L) and MSG (0, 2, 4, 6, 8, or 10 g/L) on improving GABA production after 48-h milk fermentation at 37°C were examined. The results in Table 4 showed that in the cocultured RSM with *Strep. thermophilus* 1275 and *Lb. brevis* 145 with cysteine at 50 mg/L, MSG ranging from 2, 4, 6, 8, and 10 g/L could be converted into 1.03, 2.11, 3.14, 3.93, and 4.80 g/L GABA, respectively. Meanwhile, the corresponding amounts of residual MSG after fermentation were 0.03, 0.04, 0.08, 0.15, and 0.19 g/L, respectively. In the presence of cysteine, the highest level of GABA after 48-h milk fermentation was 4.80 g/L (46.6 mM), and the maximum GABA conversion

ratio from MSG during milk fermentation was 86.61% at the level of 4 g/L MSG supplementation (Table 4). Without cysteine, the highest level of GABA after 48-h milk fermentation was 0.18 g/L (1.75 mM), and the maximum GABA conversion ratio was only 7.38% at the level of 6 g/L MSG supplementation. Moreover, supplementation of different concentrations of cysteine showed no significant influence on GABA yield.

Determination of Relationship Between MSG Utilization and GABA Production in Presence of Cysteine

To understand the association between GABA production and MSG utilization, a 48 h-course of RSM fermentation with cocultured *Lb. brevis* and *Strep. thermophilus* with or without cysteine was carried out at 37°C in presence of 2 g/L MSG. The results in Figure 2 showed that without cysteine, GABA production increased slowly during the 48-h fermentation, reaching 0.2 g/L, and MSG concentration simultaneously decreased at a slow rate similar to that of GABA production. For the fermentation batch with cysteine (50 mg/L), GABA production dramatically increased from 0.061 g/L to 0.908 g/L after 48 h of fermentation. Accordingly, the residual MSG concentration declined, and MSG utilization was proportionate to GABA production. The results systematically confirmed that MSG was the major reactive substrate for GABA production and cysteine could significantly promote GABA production.

Changes in Cell Counts, Lactic Acid Production, pH, and Lactose Utilization in Presence of Cysteine

Viable cell counts, lactose and galactose utilization, lactic acid production, and pH values were determined during 48-h fermentation. The results in Figure 3B showed that the exponential growth period of bacteria in both batches was from 3 to 6 h, and cell counts of *Lb. brevis* 145 increased from 6.7×10^7 to 5.78×10^8 cfu/mL in the SL group, and from 6.94×10^8 to 7.02×10^8 cfu/mL in the SLC group. After 12-h fermentation, viable cell counts of *Lb. brevis* 145 decreased from 5.78×10^8 to 1.94×10^8 cfu/mL in the SL group but declined from 7.02×10^8 to 3.28×10^8 cfu/mL in the SLC group. Moreover, it was observed that *Lb. brevis* 145 cell counts in the SLC group were always higher than those in the SL group during 48-h fermentation.

As shown in Figure 3C, lactic acid accumulated during the first 6 h of fermentation, and it was observed that the amount of lactic acid produced in the SLC group was always higher than that in the SL group. However, pH values for the SL and SLC batches were

Table 4. Effects of different concentration of cysteine and monosodium glutamate (MSG) on improving γ -aminobutyric acid (GABA) production¹

MSG level (g/L)	Cysteine level (mg/L)	Residual MSG (g/L)	GABA production (g/L)	GABA conversion ratio from MSG (%)
2	0	1.75 $\pm$ 0.228	0.06 $\pm$ 0.061 ^a	4.92
	50	0.03 $\pm$ 0.002	1.03 $\pm$ 0.011 ^b	84.55
	100	0.05 $\pm$ 0.016	1.00 $\pm$ 0.036 ^b	82.09
	150	0.08 $\pm$ 0.002	0.98 $\pm$ 0.046 ^b	80.45
	200	0.09 $\pm$ 0.004	0.99 $\pm$ 0.014 ^b	81.27
	300	0.13 $\pm$ 0.004	0.94 $\pm$ 0.051 ^b	77.17
4	0	3.70 $\pm$ 0.199	0.13 $\pm$ 0.103 ^a	5.33
	50	0.03 $\pm$ 0.001	2.10 $\pm$ 0.107 ^b	86.19
	100	0.04 $\pm$ 0.003	2.11 $\pm$ 0.118 ^b	86.61
	150	0.05 $\pm$ 0.002	2.02 $\pm$ 0.020 ^b	82.91
	200	0.09 $\pm$ 0.016	1.98 $\pm$ 0.059 ^b	81.27
	300	0.14 $\pm$ 0.010	2.07 $\pm$ 0.003 ^b	84.96
6	0	4.74 $\pm$ 0.518	0.18 $\pm$ 0.126 ^a	7.38
	50	0.08 $\pm$ 0.042	3.14 $\pm$ 0.039 ^b	85.92
	100	0.04 $\pm$ 0.003	2.83 $\pm$ 0.184 ^b	77.44
	150	0.05 $\pm$ 0.001	2.99 $\pm$ 0.102 ^b	81.82
	200	0.07 $\pm$ 0.001	3.10 $\pm$ 0.015 ^b	84.83
	300	0.15 $\pm$ 0.001	3.00 $\pm$ 0.069 ^b	82.09
8	0	7.26 $\pm$ 0.487	0.12 $\pm$ 0.051 ^a	2.46
	50	0.11 $\pm$ 0.010	3.84 $\pm$ 0.041 ^b	78.81
	100	0.18 $\pm$ 0.043	3.89 $\pm$ 0.021 ^b	79.83
	150	0.09 $\pm$ 0.014	3.78 $\pm$ 0.119 ^b	77.58
	200	0.14 $\pm$ 0.028	3.86 $\pm$ 0.399 ^b	79.22
	300	0.15 $\pm$ 0.000	3.93 $\pm$ 0.088 ^b	80.65
10	0	8.93 $\pm$ 0.172	0.10 $\pm$ 0.065 ^a	1.65
	50	0.24 $\pm$ 0.059	4.53 $\pm$ 0.381 ^b	74.37
	100	0.20 $\pm$ 0.019	4.65 $\pm$ 0.014 ^b	76.35
	150	0.13 $\pm$ 0.048	4.76 $\pm$ 0.321 ^b	78.15
	200	0.19 $\pm$ 0.071	4.80 $\pm$ 0.126 ^b	78.81
	300	0.29 $\pm$ 0.060	3.93 $\pm$ 0.358 ^c	64.52

^{a-c}Values with different letters are significantly different ($P < 0.05$).¹Fermentations were carried out at 37°C for 48 h.

similar; pH dropped from 6.6 to 4.8 during the first 6 h of fermentation, followed by a slight decline, finally remaining steady at 4.3.

As shown in Figure 3D, no significant differences in lactose and galactose levels occurred between the SL and SLC groups during 48-h fermentation, indicating that cysteine showed no significant effect on sugar utilization. During early 6-h fermentation, the lactose level decreased from 49.89 to 8.82 g/L, and galactose concentration went up from 0.72 to 15.28 g/L in the SL group; lactose level decreased from 50.01 to 6.43 g/L, and galactose concentration went up from 0.81 to 14.62 g/L in the SLC group. After 6-h fermentation, lactose levels in both groups remained unchanged, whereas galactose utilization increased.

Effect of Cysteine on Casein Hydrolysis Patterns

As shown in Figure 1A–D, there were no significant differences in α_{S1} -CN, α_{S2} -CN, CN- β , and κ -CN hydrolyzation patterns for *Strep. thermophilus* and *L. bulgaricus* in coculture with *Lb. brevis* with or without cysteine.

Positive Effect of Sulfhydryl Group-Containing Chemicals on GABA Production in SL-Fermented Milk

To investigate whether the sulfhydryl group in cysteine exerted a positive effect on GABA production, sulfur-containing chemicals, including cystine, methionine, reduced glutathione, and oxidized glutathione, were individually supplemented in milk fermentation to determine their effects on GABA production. The results in Figure 3A showed that addition of cystine, reduced glutathione, and oxidized glutathione showed positive effects similar to cysteine in improving GABA production, with GABA reaching 0.6 g/L after 48-h fermentation. Conversely, methionine was observed to inhibit GABA production.

Improvement of GABA Production by *Lb. brevis* Cocultured with *Strep. thermophilus* and *L. bulgaricus* YIB2 in Presence of Cysteine

As shown in Figure 4, when *L. bulgaricus* YIB2 was combined with *Lb. brevis* 145, 0.08 g/L of GABA was

Figure 2. Monosodium glutamate (MSG) consumption and γ -aminobutyric acid (GABA) production by SL coculture with or without cysteine during 48-h milk fermentation. MSG level exogenously supplemented in milk medium was 2 g/L. SL = *Streptococcus thermophilus* 1275 + *Levilactobacillus brevis* 145. ** $P < 0.01$; *** $P < 0.001$. The experiment was carried out in triplicate, and data are presented as mean $\pm$ SD.

produced; supplementation of cysteine in the 2-bacteria coculture system showed no effect on GABA production. When *Strep. thermophilus* 1275 was added in the 2-bacteria coculture batch, the GABA level reached 0.16 g/L. Supplementation with cysteine in the 3-bacteria coculture system significantly improved GABA production at 0.99 g/L.

Lactobacillus bulgaricus 756 was also combined with *Lb. brevis* 145 to improve GABA production; however, no GABA was produced. Supplementation with cysteine in the 2-bacteria coculture system improved GABA production to 0.12 g/L. When *Strep. thermophilus* 1275 was added in the 2-bacteria coculture batch, GABA production reached 0.14 g/L, and supplementation of cysteine in the 3-bacteria coculture system significantly improved GABA production at 0.55 g/L.

When *L. bulgaricus* 859 was combined with *Lb. brevis* 145 to improve GABA production, no GABA was produced. Supplementation of cysteine in the 2-bacteria coculture system increased GABA production only to 0.05 g/L. When *Strep. thermophilus* 1275 was added in the 2-bacteria coculture batch, GABA concentration reached 0.02 g/L, and supplementation of cysteine in the 3-bacteria coculture fermentation system showed no effect on GABA production.

DISCUSSION

Wu et al. (2015) found that nonproteolytic *Lb. brevis* 145 in coculture with *Strep. thermophilus*, but not *L. bulgaricus*, was able to produce GABA during milk fermentation in the presence of MSG. To understand why only *Strep. thermophilus*, not *L. bulgaricus*, was able to help *Lb. brevis* 145 survive in milk and produce GABA during milk fermentation, LC-MS/MS was performed to investigate whether different casein hydrolysis patterns by *L. bulgaricus* and *Strep. thermophilus* contributed to the feasibility and efficiency of coculture systems for GABA production. Specific casein hydrolyzation patterns could not be explored with SDS-PAGE alone (Abraham et al., 1993); thus, LC-MS/MS was applied to determine the peptide profiles from different bacteria, as well as to compare the differences in peptide profiles among different groups to explore the mechanisms behind the coculture. Our results showed that *L. bulgaricus* and *Strep. thermophilus* showed different preferences to digest α_{S1} -CN, α_{S2} -CN, CN- β , and κ -CN. In the *Strep. thermophilus* 1275 single-culture group, CN- β and α_{S1} -CN contributed to 33% of total peptides, respectively, followed by κ -CN and α_{S2} -CN. Miclo et al. (2012) reported that CN- β was preferentially hydro-

Figure 3. Changes of pH and metabolites during 48-h milk fermentation. (A) Effect of sulfur group-containing chemicals on GABA improvement; (B) cell counts of *Levilactobacillus brevis* 145; (C) pH and lactic acid production; (D) lactose and galactose utilization. S = *Streptococcus thermophilus* 1275; L = *Lb. brevis* 145; C = cysteine; GSH = reduced glutathione; GSSH = oxidized glutathione. *** $P < 0.001$. The experiment was carried out in triplicate, and data are presented as mean $\pm$ SD.

lyzed by *Strep. thermophilus* strains, however, followed by α_{S2} -CN and then α_{S1} -CN, which exhibited a little difference compared with our results. For *L. bulgaricus*, peptide profiles for *L. bulgaricus* 859 and *L. bulgaricus* YIB2 single-culture groups showed that CN- β was the most susceptible casein component to be degraded, followed by the α_{S1} -CN and κ -CN fractions, which was in agreement with the previous study (Chandan et al., 1982). Importantly, comparative analysis of peptide profiles among different groups showed that peptide numbers increased in the *L. bulgaricus* and *Lb. brevis* coculture batch but decreased in the SL coculture batch compared with the corresponding monoculture groups. Moreover, Xiao et al. (2020) reported that particular peptides hydrolyzed by *Strep. thermophilus* ASCC1275 were transported and biodegraded with peptidase in

Lb. brevis 145 to meet the growth needs of the latter, all of which could explain why *Strep. thermophilus*, but not *L. bulgaricus*, was capable of coculturing with *Lb. brevis* to produce GABA in milk.

One problem pertaining to the SL coculture system is that the content of GABA in fermented milk was below the physiological level for humans (Abdou et al., 2006), and the high level of residual MSG may bring an undesirable taste. The aim of our research was to explore methods to increase GABA and reduce MSG content in fermented milk. We selected MSG, PLP, WPH and cysteine for our research. According to the genome map, an intact *gad* operon was identified in *Lb. brevis* 145 of plant origin (Wu et al., 2017), and the deficiency of the glutamate dehydrogenase-encoding gene (*gdhA*) to form glutamate was also observed in *Lb. brevis* 145. Hence,

exogenous supplementation of MSG was required for the decarboxylation reaction to produce GABA. It was observed that the maximum GABA conversion ratio reached 7.38% without cysteine, and the GABA conversion ratio declined when MSG addition exceeded 6 g/L. Pyridoxal phosphate was reported as an essential co-factor for the GAD enzyme (Li et al., 2016). Shan et al. (2015) found that the yield of GABA reached the highest level at 165.6 $\mu\text{g}/100\text{ g}$ after about 20 μM of PLP supplementation. However, in our research, PLP supplementation had no effect on GABA improvement. Whey protein hydrolysate was reported to promote the viability and growth of starter and probiotic bacteria in yogurt (Dąbrowska et al., 2017) by directly providing peptides for growth in milk and producing a high level of GABA. However, our research showed that WPH did not influence GABA production by *Lb. brevis* 145 in coculture with *Strep. thermophilus* 1275. Wu et al. (2015) reported that *Strep. thermophilus* 1275 encodes one intact PrtS (T303_05205), which is involved in the cleavage of casein to oligopeptides, and an abundance of intracellular protease and peptidase was also found in *Strep. thermophilus* 1275, all of which helped *Strep.*

thermophilus 1275 cells break down oligopeptides into free amino acids for cellular metabolism or direct utilization by the cocultured bacteria. Hence, there was no need to add extra WPH for growth of *Lb. brevis* 145 in milk.

Cysteine is not available in most milk proteins (α - and β -caseins) in their amino acid composition (Gordon et al., 1949), and may act as an essential amino acid for *Lb. brevis* to enhance growth and consequent GABA production. Dave and Shah (1997) also reported that cysteine was able to improve the viability of yogurt and probiotic bacteria in yogurt by lowering redox potential. Therefore, cysteine was expected to increase the bacteria cell counts in milk and thus GABA yield. In our study, a small amount of cysteine supplementation was able to increase cell counts, efficiently produce a high level of GABA (4.8 g/L, 46.6 mM), and significantly increase the GABA conversion ratio from 5.33% to 86.61%. Moreover, the residual MSG in all fermented milk groups was minimal, below 0.2 g/L, which may potentially bring a better taste to GABA-rich fermented milk. For *Levilactobacillus brevis* HYE1, the maximum GABA production (18.60 mM) was ob-

Figure 4. Improvement of γ -aminobutyric acid (GABA) production by *Levilactobacillus brevis* cocultured with *Streptococcus thermophilus* and *Lactobacillus bulgaricus* YIB2 in presence of cysteine. We added 2 g/L monosodium glutamate (MSG) in every treatment, and fermentations were carried out at 37°C for 48 h. 756 = *L. bulgaricus* ASCC756; 859 = *L. bulgaricus* ASCC859; YIB2 = *L. bulgaricus* YIB2; 145 = *Lb. brevis* 145; 1275 = *Strep. thermophilus* ASCC1275; Cys = cysteine. The experiment was carried out in triplicate, and data are presented as mean $\pm$ SD.

tained with addition of 10 g/L MSG in the modified MRS medium (Lim et al., 2017). The maximum yield of GABA from available glutamate by *Lb. brevis* 6018 was 75%, with 10 g/L (59 mM) MSG formulation in modified MRS (Banerjee et al., 2021). For *Lb. brevis* CRL 2013, the GABA conversion ratio from MSG in modified MRS was above 90%, and the maximum GABA production reached 258 mM with addition of 267 mM MSG (Cataldo et al., 2020). Compared with methods applying optimization of culture conditions and genetic engineering (Cui et al., 2020), the addition of a small amount of cysteine during milk fermentation is much more efficient and convenient. Additionally, sulfhydryl group-containing chemicals exhibited effects similar to cysteine in improving GABA production. Our work is the first study finding that cysteine showed great effect in improving GABA production by *Lb. brevis* 145 in coculture with *Strep. thermophilus* 1275 during milk fermentation, which may have particular interest to dairy industry.

Furthermore, combining *Lb. brevis* 145 with 2 yogurt bacteria for GABA-rich milk fermentation attracts our attention because this may improve the performance and market competitiveness of functional yogurts in the food industry. In our research, a 3-bacteria coculture system was successfully created for producing GABA-rich milk after fermentation in the presence of cysteine. Each probiotic bacterium offers different and specific health benefits: *Lb. brevis* 145 may act as a functional dairy starter for GABA production (Wu and Shah, 2017), and *L. bulgaricus* YIB2 is known for lactose utilization and acidification and for releasing free amino acids such as proline, which could bring a sweet flavor to yogurt (Deutsch et al., 2000). High exopolysaccharide-producing *Strep. thermophilus* 1275 showed faster growth and acidification rates, resulting in yogurt with low syneresis and high flow behavior (Purwandari et al., 2007), and provided nutrients for the growth of cocultured *Lb. brevis* in milk fermentation (Xiao et al., 2020).

CONCLUSIONS

Streptococcus thermophilus and *L. bulgaricus* showed different casein hydrolysis patterns; *Strep. thermophilus*, but not *L. bulgaricus*, was capable of providing peptides for the growth of *Lb. brevis* 145 in a coculture batch. Supplementation of cysteine helped achieve GABA-rich milk with a minimum level of residual MSG in the SL coculture group, increasing viable cell counts of *Lb. brevis* and lactic acid production, but without influencing casein hydrolysis patterns. Moreover, cysteine significantly improved GABA production when *L. bulgaricus* YIB2 was combined with SL.

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