Use of *Streptococcus thermophilus* for the in situ production of γ -aminobutyric acid-enriched fermented milk

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ABSTRACT

γ -Aminobutyric acid (GABA) is a potentially bioactive ingredient with health-promoting properties that is added to functional foods. *Streptococcus thermophilus* was selected to produce naturally GABA-enriched fermented milk. This strain can yield a GABA concentration of 2.8 g/L after a 48-h fermentation. In the presence of 1 g/L food-grade casein hydrolysate as a nitrogen source, *S. thermophilus* yielded GABA concentrations as high as 5.4 g/L or even 8.3 g/L when cocultured with *Lactobacillus rhamnosus*. In other words, both of these added conditions promoted GABA enrichment. The GABA dose achieved with fermented milk was comparable to the doses of commercially available GABA supplements. Additionally, the in situ use of *S. thermophilus* to produce GABA-enriched fermented milk was cost effective. The complete genomic sequence of *S. thermophilus* GABA has been published and will be highly useful to other researchers studying the regulation of genes related to GABA accumulation. In conclusion, the *S. thermophilus* GABA-producing strain reported herein represents a natural method for the production of fermented milk containing high GABA concentrations.

Key words: *Streptococcus thermophilus* γ -aminobutyric acid, γ -aminobutyric acid, nitrogen source, co-culture

INTRODUCTION

γ -Aminobutyric acid (GABA), a 4-carbon non-protein-forming amino acid, acts as a major inhibitory neurotransmitter in the central nervous system and plays important roles in physiological functions (Abdou et al., 2006; Dyke et al., 2017; Sanchart et al., 2017). γ -Aminobutyric acid exerts hypotensive (Hayakawa et al., 2004; Watanabe and Washio, 2011; Suwanmanon and Hsieh, 2014), diuretic (Jakobs et al., 1993), and

tranquilizing effects (Okada et al., 2000; Wong et al., 2003) and has been classified as a health-promoting bioactive component in foods and pharmaceuticals (Li and Cao, 2010). Therefore, GABA-rich traditional and functional foods were on sale all over the world.

Screening of microorganisms used in traditional foods can identify those that possess the capacity to produce GABA through fermentation. This pathway provides a convenient and efficient means of producing GABA-enriched foods. Several microorganisms (Yokoyama et al., 2002; Lu et al., 2008; Yang et al., 2008; Sun et al., 2009; Di Cagno et al., 2010; Nejati et al., 2013; Wu et al., 2015; Brasca et al., 2016) generally regarded as safe, such as lactic acid bacteria (LAB), have been researched widely and applied to GABA production. However, these GABA-producing LAB are largely cultivated in synthetic or semisynthetic media (Komatsuzaki et al., 2005; Lu et al., 2008; Yang et al., 2008), and therefore procedures are needed to separate and purify GABA after fermentation. Additionally, the suitability of these strains for GABA production in fermented milk and yogurt has not yet been explored.

Streptococcus thermophilus is a commercially relevant starter used widely in the industrial production of yogurt. However, conventional strains of *S. thermophilus* largely lack the capacity for the high-yield production of GABA as a potentially bioactive component in foods and pharmaceuticals. In this study, we screened a high-GABA-producing *S. thermophilus* strain and developed an in situ method for the production of GABA-enriched fermented milk. This *S. thermophilus* GABA strain exhibits great potential as a starter for the production of GABA-enriched fermented milk, cheese, and other functional fermented foods.

MATERIALS AND METHODS

Isolation of GABA-Producing Strains and Gene Analysis

Twenty different traditional koumiss dairy products collected in Xinjiang province (China) were used to isolate GABA-producing strains. After proper dilution,

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100 μ L of sample was spread on M17 and de Man, Rogosa, and Sharpe (MRS), pH 4.5 plate to select *S. thermophilus* and *Lactobacillus*. Fifty strains of *S. thermophilus* and 50 strains of *Lactobacillus* were cultured in 1 mL of M17 or MRS broth as the seed. Then 0.2 mL of seed culture was switched to 10 mL of reconstituted skim milk (containing 10% skim milk powder and 10 g/L monosodium glutamate (MSG) sterilized at 105°C for 15 min) for GABA accumulation. The production of GABA was determined by HPLC. The strain with the highest yield was named *S. thermophilus* GABA (preservation number: CGMCC 14810) isolated by Mei Han. It was subjected to genomic sequencing by Majorbio (Shanghai, China). The complete sequence has been published in the National Center for Biotechnology Information database (accession number: CP025399). The sequence corresponding to *gadB*, which encodes glutamate decarboxylase β (GAD), was determined from the whole-genomic sequence. Phylogenetic analysis of *S. thermophilus* GABA based on the complete genomes was done using software MEGA 7 (<https://www.megasoftware.net/>).

Preparation of GABA-Enriched Fermented Milk In Situ Via *S. thermophilus* GABA Culture

Streptococcus thermophilus GABA (CGMCC no. 14810) was cultured in a 100-mL flask containing 50 mL of M17 broth (Oxoid, Basingstoke, UK) at 37°C for 24 h, which yielded approximately 9 log viable cfu/mL. The culture was then centrifuged at $2,500 \times g$ for 30 min at 4°C to obtain a bacterial pellet, which was washed in sterile water and used to seed the fermentation reaction. The fermentation medium consisted of reconstituted skim milk (Brightdairy, Shanghai, China) containing MSG (Sigma-Aldrich, St. Louis, MO) and was sterilized at 105°C for 15 min. The fermentation reaction was inoculated at a concentration of 2×10^6 cfu/mL and incubated without shaking. All fermentation experiments were performed in triplicate.

Detection and Quantification of GABA Production Using HPLC

Fermented milk was centrifuged and filtered through a Millex-HA 0.22- μ m pore size filter to collect the supernatant. The concentration of GABA in the supernatant was analyzed using HPLC according to the dabsyl chloride (Sigma-Aldrich) derivatization method (Syu et al., 2008). The GABA reagent standard was purchased from Sigma-Aldrich.

Briefly, the determination of dabsyl-GABA was carried out by HPLC (Agilent 1200, Santa Clara, CA).

A reversed-phase column (Agilent ZORBAX SB-C18, 250 mm $\times$ 4 mm, 5 μ m particle size) coupled with a C18 cartridge was used. The column temperature was maintained at 35°C. The detection wavelength was 425 nm. The composition of the optimized mobile phase was methanol/CH₃COONa solution (0.045 mol/L, pH 4) 65:35. The mobile phase was filtered through a 0.22- μ m filter membrane and degassed before use.

Optimization of Conditions Required for a High GABA Yield

The effects of different temperatures on GABA production were tested by culturing the bacteria in medium containing 10% skim milk powder and 10 g/L MSG at 37 and 42°C. The effects of different nitrogen sources were tested at 37°C in medium containing 10% (wt/wt) skim milk powder, 10 g/L MSG, and one of the following: casein hydrolysate (CH), yeast extract (YE), or soy protein hydrolysate (SH) (Oxoid).

The coculture characteristics were tested by inoculating medium containing 10% (wt/wt) skim milk powder and 15 g/L MSG simultaneously with *Lactobacillus* and *S. thermophilus* GABA at a 1:1 ratio (10^6 cfu/mL each). *Lactobacilli* are *L. delbrueckii* (Wecare-Bio, Jiangsu, China), *L. rhamnosus* (Wecare-Bio), *L. helveticus* (Danisco, New Century, KS), and *L. paracasei* (Chr. Hansen, Hørsholm, Denmark).

Viable colony-forming units of *S. thermophilus* GABA were tested using M17 agar (Oxoid) at 43°C for 48 h in aerobic conditions. Viable colony-forming units of *Lactobacillus* were tested using MRS agar pH 4.5 (Oxoid), at 37°C for 48 h in anaerobic conditions.

RESULTS AND DISCUSSION

Analysis of the *S. thermophilus* GABA *gadB* Sequence

The GABA-producing ability of 100 strains isolated from Xinjiang koumiss (containing 50 strains of *S. thermophilus* and 50 strains of *Lactobacillus*) was tested by HPLC. Only 2 *S. thermophilus* strains isolated from koumiss produced GABA when cultured in milk. The GABA-producing gene, *gadB*, was present in only 10 of the 50 *S. thermophilus* strains. The GAD system converts 1 molecule of glutamate into 1 molecule of GABA (Di Cagno et al., 2010). Therefore, a method based on the production of GABA from GAD via fermentation is considered a convenient and efficient screening method. In a previous study of 191 *S. thermophilus* strains, Brasca and colleagues found that only 20 (10%) harbored *gadB*. Of those 20, only 5 were capable of

Figure 1. Phylogenetic analysis of *Streptococcus thermophilus* γ -aminobutyric acid (GABA) based on the complete genomes using software MEGA 7 (<https://www.megasoftware.net/>).

producing GABA in microfiltered pasteurized milk (Brasca et al., 2016). Therefore, only a small fraction of even *gadB*-positive *S. thermophilus* strains can produce GABA. The complete genomic sequences of *S. thermophilus* GABA (accession number: CP025399) and 16 other *S. thermophilus* strains have been published in the National Center for Biotechnology Information. A phylogenetic analysis based on the complete genomes of these 17 strains is shown in Figure 1. Notably, *S. thermophilus* GABA is most closely related to *S. thermophilus* CP016877. A Basic Local Alignment Search Tool (BLAST) analysis of the translational product of the *gadB* locus in *S. thermophilus* GABA and the other 16 strains revealed that only the strains GABA, ACA-DC 2 (accession number: LT604076.1), KLDS 3.1003 (accession number: CP016877.1), APC151 (accession

number: CP019935.1), and ND03 (accession number: CP002340.1) harbor this locus. An AA sequence analysis of these 5 strains yielded almost identical *gadB* and glutamate/GABA antiporter (*gadC*) sequences. However, it is unknown whether these other *gadB*-positive strains actually make GABA. In addition, future genomic analysis of *S. thermophilus* GABA may reveal why this strain makes more GABA than other tested strains.

Growth Dynamics and GABA Production During Milk Fermentation

To produce GABA-fortified fermented milk, we cultivated *S. thermophilus* GABA in skim milk supplemented with MSG. Production of GABA was monitored using

Table 1. Effect of temperature on viable count and γ -aminobutyric acid (GABA) production

Fermentation condition	Count cfu/mL	GABA production (g/L $\pm$ SD)
37°C		
24 h	5.2×10^8	1.1 ± 0.1
48 h	2.1×10^9	2.8 ± 0.2
72 h	1.2×10^9	3.4 ± 0.2
42°C		
24 h	1.5×10^9	1.3 ± 0.1
48 h	3.2×10^8	2.3 ± 0.2
72 h	2.2×10^5	3.1 ± 0.2

HPLC. Notably, temperature was among the most influential factors affecting bacterial growth, consistent with previous observations that this factor affects the fermentation time of exopolysaccharide production in *S. thermophilus* (Khanal and Lucey, 2018). The rate of milk acidification is a highly important technological factor in the manufacturing of fermented milk. Accordingly, the pH curves of GABA-fermented milk products were recorded during the fermentation process. Figure 2 demonstrates that the lag phases of *S. thermophilus* GABA decreased as the temperature increased, suggesting a strong inverse relationship. The acidification rate of milk also depended greatly on temperature, as this factor increased with increasing temperature. Our finding was consistent with a previous report by Haque and colleagues in which increasing fermentation temperature was shown to increase the rate at which

the pH decreased (Haque et al., 2001). Moreover, the production of GABA was higher at 42°C than at 37°C during a 24-h fermentation period because of the rapid growth rate at high temperature, but decreased over longer periods (48 or 72 h) due to more rapid bacterial death at the higher temperature (Table 1). Accordingly, we determined that 37°C is the optimal culture temperature for maximum GABA production. A change in the fermentation temperature would affect the production of GABA and lactic acid by *S. thermophilus* strains because the fermentation temperature affects both the growth rate and growth stress.

Supplementation with Food-Grade Organic Nitrogen Promotes GABA Production

Organic nitrogen sources are widely used to promote microbial growth during fermentation. Dave and Shah (1998) reported that although a 12-h period was needed to reach a pH of 4.5 in control fermented milk incubated at 37°C, this incubation time decreased to 9 h in a culture supplemented with CH or tryptone. Moreover, the inclusion of a low concentration of YE (0.1%) in liquid medium favored rapid growth and high viability in cultures of *Rhizobium* sp. (Skinner et al., 2010). Although nitrogen sources can be used to enhance the fermentation rate, these chemicals are unpalatable and are rarely added to fermented foods. Therefore, the nitrogen level must be controlled within an acceptable

Figure 2. Effect of culture temperatures on pH changes at 37°C (●) and 42°C (▲). Error bars indicate SD.

Figure 3. Effect of edible nitrogen content on the production of γ -aminobutyric acid (GABA). Yeast extract, casein hydrolysate, and soy protein hydrolysate were tested at the content of 0.5, 1, and 2 g/kg reconstituted milk after 24 h (black) and 48 h (gray) fermentation. Error bars indicate SD.

level, and the nitrogen source must be edible. Some food-grade organic nitrogen sources, such as CH, YE, and SH, were tested. All 3 sources led to remarkable increases in bacterial growth and GABA production even at low concentrations. As shown in Figure 3, when CH, YE, and SH were tested at a concentration of 1 g/L, CH led to the highest GABA yield of 2.9 g/L after a 24-h fermentation. This represented a 160% increase in GABA production relative to the control. After a 48-h fermentation, the CH-supplemented culture produced a GABA yield of 5.4 g/L, representing a 93% increase over the control. These results support the use of CH as an effective edible nitrogen source in traditional foods.

Coculture with Different LAB Strains to Produce GABA Yogurt

In most countries, the term yogurt can only be applied legally to milk products fermented simultaneously using *S. thermophilus* and *Lactobacillus delbrueckii* (Han et al., 2014). The best example of mutualism during food processing involves the fermentation of yogurt by *S. thermophilus* and *L. delbrueckii*. The proto-cooperative process of yogurt fermentation enhances the growth rate and size of each bacterial population (Sieuwerts et al., 2010). These previously described interactions are trophic, wherein the species feed each other by producing formic acid, pyruvate, folate, long-chain fatty acids, ornithine, carbon dioxide, peptides, AA, and putrescine (Sieuwerts et al., 2010). Accordingly, we evaluated

GABA production in cocultures of *S. thermophilus* with different *Lactobacillus* strains.

Four different LAB strains (*L. delbrueckii*, *L. rhamnosus*, *L. helveticus*, and *L. paracasei*) were cocultured with *S. thermophilus* GABA, and the production of GABA-enriched fermented milk was evaluated. Although *L. helveticus* had a rapid acidification rate (Figure 4), this culture contained the lowest number of living cells after a 48-h fermentation (Table 2). Production of GABA was enhanced in all cocultures with *Lactobacillus* spp., with the weakest and strongest enhancement observed in cultures containing *L. delbrueckii* and *L. rhamnosus*, respectively. Notably, GABA production increased by 260% when *S. thermophilus* GABA was cocultured with *L. rhamnosus* for 48 h (Table 2). Using the 4 *Lactobacillus* individually, the production of GABA was less than 0.1 g/L. Consequently, the high production of GABA was due to the promoted activity of *S. thermophilus* GABA. According to the production of GABA enhanced by organic nitrogen source (Figure 3). We speculated that the enhancement may be due to the milk protein peptide hydrolyzed by *Lactobacillus* or other small promoter molecules such as formic acid, pyruvate, folate, and ornithine secreted by *Lactobacillus*.

Table 3 lists the other GABA-enriched fermented products generated using GABA-producing LAB strains as starter cultures. Notably, *S. thermophilus* GABA exhibited superior production and productivity when compared with other LAB. Additionally, CH supplementation and coculture technology increased

Table 2. *Lactobacillus* cocultured with *Streptococcus thermophilus* γ -aminobutyric acid (GABA) for 48 h

Strain	pH	Titration acidity (°T $\pm$ SD)	Production (g/L $\pm$ SD)	cfu/mL	
				<i>Lactobacillus</i>	<i>S. thermophilus</i>
<i>Streptococcus thermophilus</i>	4.62	117 $\pm$ 10	2.3 $\pm$ 0.2		2.1 $\times 10^9$
<i>Lactobacillus delbrueckii</i>	4.41	141 $\pm$ 10	2.5 $\pm$ 0.2	1.6 $\times 10^8$	2.1 $\times 10^9$
<i>Lactobacillus rhamnosus</i>	4.46	141 $\pm$ 10	8.3 $\pm$ 0.6	2.8 $\times 10^9$	2.1 $\times 10^9$
<i>Lactobacillus helveticus</i>	3.78	326 $\pm$ 20	5.2 $\pm$ 0.4	6.1 $\times 10^2$	6.2 $\times 10^7$
<i>Lactobacillus paracasei</i>	4.01	163 $\pm$ 10	6.2 $\pm$ 0.4	2.5 $\times 10^9$	2.1 $\times 10^9$

the production of GABA to a level comparable to that achieved by LAB cultured in synthetic nutrient broth medium. The use of *S. thermophilus* GABA as described in this work would yield fermented milk with a GABA concentration of 8.3 mg/mL after 48 h of fermentation. The ingestion of 1 unit (100 mL) of this fermented milk would provide the consumer with 830 mg of GABA, which exceeds the bioactivity threshold previously reported to yield health benefits.

In human patients with hypertension, the daily intake of fermented milk containing 10 mg of GABA for 12 wk was shown to decrease blood pressure by 17.4 mmHg (Inoue et al., 2003). Another study tested the effect of a naturally GABA-enriched Cheddar cheese on blood pressure levels in men with mild hypertension. In that study, a GABA-producing *Lactococcus lactis* ssp. *lactis* strain was used as the starter for experimental cheese production. The test group consumed 50 g of the experimental cheese, which contained 16 mg of GABA,

for 12 wk, and exhibited decreases in the mean and systolic blood pressure of 3.5 ± 2.8 and 5.5 ± 3.9 mmHg, respectively. For comparison, the placebo group exhibited corresponding decreases of 1.6 ± 3.0 and 3.6 ± 4.0 mmHg, respectively (Pouliot-Mathieu et al., 2013). Another previous study reported that the daily oral administration of 26.4 mg of GABA derived from rice germ could treat neurological disorders effectively (Okada et al., 2000). Moreover, streptozotocin-induced diabetic mice were orally administered GABA-rich yogurt for 6 wk, and the results showed that oral administration of GABA-rich yogurt improved both hyperglycaemia and impaired glucose tolerance of streptozotocin-induced diabetic mice in a dose-dependent manner. Notably, GABA-rich yogurt led to significant decreases in the concentrations of serum total cholesterol (TC) and triacylglycerol (TG) and remarkable increases in the concentrations of high-density lipoprotein cholesterol and insulin in these mice (Chen et al., 2016). In the

Figure 4. The acid response curves of *Lactobacillus helveticus* (■), *Lactobacillus delbrueckii* (●), *Lactobacillus rhamnosus* (◆), and *Lactobacillus paracasei* (▲) cocultured with *Streptococcus thermophilus* γ -aminobutyric acid (GABA).

Strain	Culture medium	Production and fermentation time	Reference
<i>Lactobacillus helveticus</i>	Milk with monosodium glutamate (MSG)	165.1 mg/L, 30 h	Sun et al., 2009
<i>Lactococcus lactis</i> DIBCA1 and <i>Lactococcus plantarum</i> PU11	Milk with MSG and 5 g/L yeast extract	144.5 mg/L, 48 h	Nejati et al., 2013
<i>Streptococcus thermophilus</i> YI-B1 and <i>Lactococcus brevis</i> 145	Milk with MSG	314 mg/L, 24 h	Wu et al., 2015
<i>S. thermophilus</i>	Milk with MSG	83–108 mg/L, 24 h	Brasca et al., 2016
<i>S. thermophilus</i> APC151	Milk with MSG	2 g/L, 72 h	Linares et al., 2016
<i>Streptococcus salivarius</i> ssp. <i>thermophilus</i> Y2	Nutrient broth medium with MSG and pyridoxal 5'-phosphate	7.98 g/L, 84 h	Yang et al., 2008
<i>L. lactis</i> B	Brown rice juice, germinated soybean juice, and enzymolyzed skim milk was 33:58:9 (vol: vol:vol)	6.41 g/L, 144 h	Lu et al., 2008
<i>S. thermophilus</i> GABA	Milk with 10 g/L MSG	2.8 g/L, 48 h 3.4 g/L, 72 h	This study
<i>S. thermophilus</i> GABA	Milk with 15 g/L MSG, 1 g/L casein hydrolysate	5.4 g/L, 48 h	This study
Cocultured with <i>Lactobacillus</i> <i>rhamnosus</i>	Milk with 15 g/L MSG	8.3 g/L, 48 h	This study

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