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FULL PAPER

Screening of *Lactobacillus rhamnosus*-producing gamma aminobutyric acid (GABA) isolated from Sumbawa mare milk and its potential application to increase GABA content in fermented milk

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Abstract

The broad role of gamma aminobutyric acid (GABA) in human health has triggered various efforts to increase its concentration in foodstuffs. The use of microorganisms to increase the GABA content in food is a rational choice. The objective of this study was to identify the GABA-producing *Lactobacillus rhamnosus* strain isolated from Sumbawa mare milk and its potential application as a starter to increase GABA content in fermented milk. A total of 34 *L. rhamnosus* strains were screened for their ability to produce GABA in MRS medium containing 2% monosodium glutamate (MSG). GABA presence in the culture broth was analysed qualitatively by using TLC, whereas GABA concentration was determined using UPLC. The results showed that 17 out of 34 *L. rhamnosus* strains produced GABA at varying concentrations from 0.047 to 0.384 mg/mL. Six selected strains were determined for their capacity in GABA production in skim milk without MSG addition. The results showed that GABA was produced by *L. rhamnosus* strains on skim milk ranging from 0.07 to 2.01 mg/mL *L. rhamnosus* SMM37 possessed the highest GABA production capability, which produced 2.01 mg/mL in skim milk after fermentation for 72 hrs at 37°C. These results indicated that *L. rhamnosus* strains isolated from Sumbawa mare milk have the potential to be developed as a starter culture to increase GABA content in fermented milk. Nevertheless, further studies are needed in optimizing fermentation conditions to improve GABA production from the *L. rhamnosus* strains isolated from Sumbawa mare milk.

1. Introduction

Gamma-aminobutyric acid (GABA) is a non-protein amino acid produced through the irreversible α-decarboxylation reaction of the amino acid L-glutamic acid catalysed by the glutamic acid decarboxylase enzyme (Li *et al*., 2010; Zhuang *et al*., 2018). GABA has the general structure of H_2N (CH_2) -CO_2H (Anju *et al*., 2014). GABA is a neurotransmitter, a major inhibitor of the central nervous system, and plays important role in regulating physiological processes such as blood pressure and heart rate, increasing plasma concentrations, growth hormone, and protein synthesis in the brain (Villegas *et al*., 2016). Consumption of foods enriched with GABA can inhibit the proliferation of cancer cells (Park and Oh, 2007), and improve memory and learning abilities (Miura *et al*., 2006). On the other hand, GABA deficiency can cause several diseases such as Huntington's, Parkinson's, Alzheimer's, schizophrenia, and depression (Diana *et al*., 2014).

The functional effects of GABA on various aspects of health have led to increasingly interesting studies on how to produce GABA and increase GABA content in food. Due to the difficulty in isolating GABA from natural resources, hampering its content in foodstuffs,

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recent studies are more focused on how to produce GABA using the activity of microorganisms (Dhakal *et al*., 2012) than isolating it from natural resources. Various types of microorganisms can produce GABA including bacteria, moulds, and yeasts. However, lactic acid bacteria (LAB) are of great interest in GABA production, because LAB plays an important role in the fermentation process of foods, which can improve aroma, taste, and its preservation. In addition, LAB has a long history of being used in traditional fermented food production so that it is considered generally recognized as safe (GRAS) for human consumption.

Several studies regarding the source and ability of LAB strains to produce GABA have been reported from *Lactobacillus paracasei* isolated from fish fermentation (Komatsuzaki *et al*., 2008), *Lactobacillus buchneri* isolated from kimchi (Cho *et al*., 2007), *Lactobacillus brevis* and *Bifidobacterium dentium* isolated from the human gastrointestinal tract (Barrett *et al*., 2012), *Lactobacillus namurensis* and *Pediococcus pentosaceus* isolated from fermented nham sausage (Ratanaburee *et al*., 2011), *Lactococcus lactis* isolated from cheese starter (Pouliot-Mathieu *et al*., 2013), *Lactobacillus plantarum* Taj-Apis362 from honey (Tajabadi *et al*., 2015) and *Lactococcus* spp. isolated from Mexican artisanal cheese (Santos-Espinosa *et al*., 2020).

The ability of LAB for GABA synthesis and the fermentation parameters were reported to vary in several specific studies. Recent studies have reported that LAB can synthesize GABA in high concentrations in Mexican artisanal cheese (Gonzalez-Gonzalez *et al*., 2019). *Lactobacillus rhamnosus* YS9 has monosodium glutamate (MSG) concentration limit of 200 mM, an incubation temperature of 43°C, 200 µM pyridoxal 5'-phosphat (PLP), and a medium pH of 4.4 which can produce 1.87 mM of GABA (Lin, 2013). This is different from *Lactobacillus rhamnosus* GG which is used in the fermentation of adzuki beans and uses 2.27% MSG, 1.44% galactose, 0.20% PLP at 37°C incubation temperature at a medium pH of 4.4 which can produce 1.12mg/mL GABA (Song and Yu, 2018). *Lactobacillus rhamnosus* has been isolated and identified from Sumbawa mare milk (Shi *et al*., 2012; Sujaya *et al*., 2008) and was shown to have the potential as a starter for fermented milk, with the main functional effect of improved lipid profiles (Nocianitri *et al*., 2017), the potential for this strain to produce GABA has never been elucidated. Considering the importance of GABA in various applications, it is important to screen GABA producing *L. rhamnosus* strain isolated from Sumbawa mare milk to improve GABA content in fermented milk. Therefore, this study aimed to identify *L. rhamnosus* from Sumbawa mare milk which is best capable to produce GABA when applied as a starter for fermented milk.

2. Materials and methods

2.1 Bacterial strain and culture conditions

The *L. rhamnosus* strain used in this study was from the Unud Culture Collection stored in 30% glycerol at -80°C (Table 1). The strain was grown by transferring a loopful of stock strain and inoculated into 5 mL of MRS broth (OXOID™, Thermo Fisher Scientific, USA) then incubated aerobically (in Gas Pack, Thermo, USA) at 37°C for 24 hrs. After that, a confirmation test was

Table 1. Production of GABA by *L. rhamnosus* strains

No	Strain	*Rf	GABA (mg/mL)
1	*L. rhamnosus* SKG34	0.50	ND
2	*L. rhamnosus* SMM5	0.47	ND
3	*L. rhamnosus* SMM5F	0.47	ND
4	*L. rhamnosus* SMM33	0.61	0.055±0.003^{b}
5	*L. rhamnosus* SMM34	0.61	0.335±0.007^{k}
6	*L. rhamnosus* SMM35	0.61	0.254±0.014^{i}
7	*L.* rhamnosus SMM36	0.61	0.251±0.012^{i}
8	*L. rhamnosus* SMM37	0.61	0.239±0.002^{h}
9	*L. rhamnosus* SMM38	0.61	0.071±0.006^{c}
10	*L. rhamnosus* SMM39	0.46	ND
11	*L. rhamnosus* SMM40	0.50	ND
12	*L. rhamnosus* SMM41	0.51	ND
13	*L. rhamnosus* SMM42	0.50	ND
14	*L. rhamnosus* SMM43	0.61	0.151±0.009^{e}
15	*L. rhamnosus* SMM44	0.47	ND
16	*L. rhamnosus* SMM45	0.61	0.153±0.009^{e}
17	*L. rhamnosus* SMM46	0.46	ND
18	*L. rhamnosus* SMM47	0.61	0.268±0.013^{j}
19	*L. rhamnosus* SMM49	0.61	0.384±0.001^{l}
20	*L. rhamnosus* SMM50	0.61	0.336±0.011^{k}
21	*L. rhamnosus* SMM51	0.48	ND
22	*L. rhamnosus* SMM53	0.61	0.334±0.005^{k}
23	*L. rhamnosus* SMM55	0.46	ND
24	*L. rhamnosus* SMM56	0.46	ND
25	*L. rhamnosus* SMM57	0.46	ND
26	*L. rhamnosus* SMM58	0.61	0.224±0.009^{g}
27	*L. rhamnosus* SMM59	0.61	0.276±0.004^{j}
28	*L. rhamnosus* SMM61	0.61	0.183±0.009^{f}
29	*L. rhamnosus* SMM68	0.61	0.092±0.008^{d}
30	*L. rhamnosus* SMM72	0.47	ND
31	*L. rhamnosus* SMM79	0.47	ND
32	*L. rhamnosus* SMM84	0.47	ND
33	*L. rhamnosus* SMM86	0.47	ND
34	*L. rhamnosus* SMM89	0.61	0.047±0.004^{b}
35	GABA	0.61	0.097±0.005^{d}
36	Na-glutamate	0.47	ND
37	MRS broth	0.54	ND
38	MRS broth contains 2% MSG	0.54	ND

*Rf = 0.61 were calculated as GABA determined using TLC; ND: not determined. Values are means±SD from 3 time replication (n = 3). Values with different superscript within the same column are significantly different (p<0.05)

carried out to ensure the characteristics of the strain including gas production tests from glucose metabolism, catalase test, Gram staining, and cell morphology.

2.2 Screening of GABA-producing Lactobacillus rhamnosus

A total of 50 µL of culture broth of *L. rhamnosus* that have been cultured in MRS broth for 24 hrs was inoculated into MRS broth containing 2% monosodium glutamate (MSG), a commercial product from Ajinomoto, purchased in the local market and was incubated at 37°C for 48 hrs. Cultures were centrifuged at 10,000 rpm at 5°C, for 10 mins. The supernatant was then taken and the transformation of NA glutamate to GABA was analysed using TLC. The supernatant (1 µL) was spotted on a TLC plate (Silica gel 60 F254, Merck No. 1.05554.0001) and the plate was eluted using a mobile phase containing butanol (Merck): glacial acetic acid (Merck): water (5:3:2 v/v). For colour development, 0.5% (w/v) ninhydrin solution in ethanol was used, followed by heating at 105°C for 5 mins. The presence of GABA spots was observed under UV rays at 254 nm and 366 nm (Qiu *et al*., 2010). The spots which have the same R_f as the spot of standard GABA were then calculated quantitatively.

2.3 Quantitative analysis of GABA concentration produced by Lactobacillu rhamnosus

Quantitative GABA analysis was performed using UPLC (Waters) according to the procedure described by Wu and Shah (2015) with slight modifications. The details are described as follows. A total of 0.1-1.0 g of fermented milk sample was put into 20 mL vials headspace (Waters No.186000384C) and added with 5 mL of 5N HCL. It was then heated at 110°C for 22 hrs. The entire sample was transferred into a 50 mL measuring flask, homogenized, and successively filtered using an ash-free filter paper No. 42 and using a GHP / RC 0.2 µm filter. The filtrate (500 µL) was transferred into 2 mL tubes, added with 400 µL of 2.5 mM ABAA solution as the internal standard, and 460 µL of distilled water. Derivatization of the sample was carried out by taking 10 µL of standard solution or sample solution that has been added to the internal standard into the insert vial, and then added with 70 µL of AccQ-Tag Flour Borate Buffer, and was mixed vigorously. After that, the solution was added with 20 µL of AccQ- Tag Reagent 2A followed by heating at 60°C for 10 mins. After cooling, 1 µL of the sample was injected into the UPLC. The analytical conditions were as follows. The column was AccQ-Tag Ultra C18 1.7 µm (2.1×100 mm), which was run at 49°C throughout the detection time. The mobile phase consisted of four solutions namely: solution A, AccQ-Tag Ultra Eluent A 100%; solution B, a mixture of AccQ-Tag Ultra Eluent B with double distilled water in a ratio of 90:1; solution C, double distilled water; and solution D, AccQ-Tag Ultra Eluent B 100%. The mobile phase flow rate was adjusted at 0.5 mL/min. with gradient pump system. The injection volume of the sample was 1 µL, and GABA was detected using a PDA detector at 260 nm.

2.4 Assessment of the ability of Lactobacillus rhamnosus to produce GABA in skim milk

2.4.1 Preparation of starter culture

The selected *L. rhamnosus* strains were grown in 5 mL of MRS broth at 37°C for 24 hrs. Broth culture was vortexed and 1 mL was put into 2 mL Eppendorf tubes and centrifuged at 7,000 rpm for 5 mins at 5°C to separate the cell mass and the supernatant. The supernatant was discarded and the cell mass obtained was washed twice using saline solution (0.85% NaCl) and then suspended using 5 mL of skim milk, henceforth referred to as inoculum. The population of *L. rhamnosus* strains (CFU/mL) in Culture broth and CFU/gr in starter culture on fermented milk, were enumerated by culturing them on MRS agar, incubated at 37°C for 24 hrs anaerobically using a gas pack.

2.4.2 Fermentation of skim milk by Lactobacillus rhamnosus

The process of making fermented milk with GABA-producing *L. rhamnosus* using skim milk (Diamond brand, commercial skim milk purchased at a local supermarket) was preceded by making a starter culture and milk fermentation using an active starter culture. Liquid skim milk (Diamond brand commercial milk) was pasteurized at 80°C for 30 mins, then as much as 100 mL was put into a glass jar and cooled down to 45°C. Skim milk in a jar was inoculated with 2% v/v inoculum and incubated for 48 hrs at 43°C. A total of 10 g of fermented milk in a glass beaker was added with 10 mL of distilled water, stirred, and then the pH was measured using a pH meter (Martini instruments Mi-1050), previously calibrated using pH 4.1 and 7.0 buffers. Then, the starter culture is ready to be used for making fermented milk.

Liquid skim milk was pasteurized at 80°C for 30 mins, then as much as 100 mL was put into a glass jar and cooled down to 45°C. Skim milk in a jar was inoculated with a 5% w/v starter and then incubated at 43°C for 72 hrs (to reach the pH of fermented milk according to the pH of yoghurt pH <4.5). The pH of the fermented milk was measured as previously described.

2.4.3 Determination of GABA content in fermented milk

A total of 1 mL of fermented milk was centrifuged at

13,000 rpm at 5°C for 10 mins, then the supernatant was taken and tested quantitatively by UPLC (Seo *et al*., 2013) according to the method described above.

2.5 Data analysis

The data were analysed using SPSS 16 package and results were expressed as mean ± standard deviation from three replications. Significant differences were assessed by one-way analysis of variance (ANOVA) at a *p*-value of ≤0.05.

3. Results and discussion

3.1 Bacterial strain and culture conditions

A total of 34 strains of *L. rhamnosus* isolates from Sumbawa mare milk, retrieved from the Unud Culture Collection (UNCC) were screened in this study (Table 1). The strains were previously identified, and their resistance to the gastrointestinal tract *in vitro* for probiotic application was characterized (Shi *et al*., 2012). Moreover, its potential as a starter of fermented milk has also been carried out (Dewi *et al*., 2012). Before using any strains for further study, the strains were cultured in MRS broth and the purity of the strains was checked by observing the shape and cell morphology by Gram staining, gas formation, and catalase. The results confirmed that all strains were long rods appearing in long-chains cell arrangement, Gram-positive, catalase-negative, and there was no gas production from glucose (homofermentative). All strains showed good growth performance and good purity and are ready to be used for further research.

3.2 Screening of GABA-producing Lactobacillus rhamnosus

Isolated *L. rhamnosus* was cultured in MRS broth media containing 2% MSG and incubated at 37°C for 24 hrs. The results showed that only 17 out of 34 strains were able to produce GABA (R_f = 0.61) at detectable concentrations (Table 1). The other strains did not result in detectable GABA under our experimental protocols, even though glutamate, a precursor of GABA, was added to the medium. This result implied that activities of glutamate decarboxylation (GAD) enzyme in those strains were diminished, which might be altered by the growth conditions. GABA is reported to be synthesized by LAB through decarboxylation of glutamic acid molecules catalyzed by the glutamate decarboxylase enzyme (Li *et al*., 2010; Diana *et al*., 2014; Zhuang *et al*., 2018). The differences in GABA production by different strains have been reported to be influenced by various factors. The series of previous research results show that GABA is produced depending on the concentration of glutamate (as a precursor) and the presence of pyridoxal 5'-phosphate (PLP) as a cofactor to catalyze α-decarboxylation of glutamic acid or its salts in growth media (Shi and Li, 2011; Xu *et al*., 2017). Therefore, in the early screening, L-glutamate is deliberately added to induce the activity of the GAD enzyme, although GABA may also be formed through the other metabolic pathways of microorganisms (Cui *et al*., 2020). We examined the *L. rhamnosus* population (Table 2) and showed that the population of strains was not much different and the pH of fermented milk was at an optimum value of GAD activity (pH 3.8-4.6) (Capitani *et al*., 2003). This evidence suggested that the GABA production was merely due to the characteristic of individual strains investigated.

GABA is mainly formed by the α-decarboxylation reaction of L-glutamic acid or its salts, which is catalyzed by the glutamic acid decarboxylase enzyme (GAD; EC 4.1.1.15) (Li *et al*., 2010; Diana *et al*., 2014; Zhuang *et al*., 2018). The biochemical properties of GAD have been well characterized (Nomura *et al*., 1999). GAD enzymes are found in various types of microorganisms such as LAB (Bertoldi *et al*., 1999), *Escherichia coli* (Rice *et al*., 1993), *Streptococcus, Aspergillus* (Kato *et al*., 2002), and *Neurospora* (Kubicek and Hampel, 1979). GAD is also found in plants such as tea (Zhao *et al*., 2011), tomatoes, soybean (Serraj *et al*., 1998), mulberry leaves (Yang *et al*., 2012), germinated brown rice (Yong-qiang, 2008), and petunias (Johnson *et al*. 1997). Even GAD is also found in the brains of mammals (Nathan *et al*., 1994). However, for commercial purposes and its application in food and pharmaceuticals, bacterial groups such as the LAB group (Maras *et al*., 1992) and yeasts (Hao and Schmit, 1993), are very important producers of GABA because they can also be applied in the food fermentation process.

As given in Table 1, the activity of GAD to synthesize GABA without PLP addition was different from each of the screened strains. It was also seen that 17/34 (50%) of the *L. rhamnosus* strains screened did not show GABA formation at detectable concentrations. To optimize the formation of GABA, it is necessary to carry out further studies, especially in the optimization of growth conditions and the addition of PLP as an activator of the GAD enzyme in the *L. rhamnosus* strain used in this study.

3.3 Production of GABA in fermented milk by Lactobacillus rhamnosus

A total of 17 strains capable of producing GABA (Table 1) were screened first by observing their growth rate on the MRS broth medium. Based on the screening results, 6 strains of *L. rhamnosus* (Table 2) were found to be potential as fermented milk starters (Dewi *et al*.,

2012).

The pH value of fermented milk produced by these 6 strains ranged from 3.91-4.14 (Table 2). In the context of GABA production, the optimal activity of GAD to produce GABA is at pH 4.4 (Huang *et al*., 2016), which means that GABA may be produced during the milk fermentation process in this study. Furthermore, it was reported that in using *Lactococcus* spp. in the manufacture of fermented milk, GABA was produced at a pH range of 4-6 at 37^{o}C (Santos-Espinosa *et al*., 2020).

The production of GABA by *L. rhamnosus* in this study on MRS media ranged from 0.24 to 2.27 mg/mL. Meanwhile, the addition of 2% glutamate in MRS increased GABA production, ranging from 0.24 to 0.29 mg/mL. In addition, the GABA production in milk ranged from 0.07-2.01 mg/mL (Tables 1 and 2). The production of GABA, which is different from each strain, is influenced by the fermentation activity. The fermentation process is reported to cause various LAB stresses, especially due to exposure to low pH, changes in osmotic pressure, and the diminishing presence of nutrients. The conversion activity of primary acid to GABA by GAD is accompanied by intracellular consumption of protons (hydrogen ions) (Feehily and Karatzas, 2013), which causes a decrease in pH (Le Vo *et al*., 2012).

Lactobacillus is the most frequently reported as GABA-producing LAB, which is widely applied in several food products such as raw milk, cheese, fermented milk, pickles, tea, and vegetable drinks with their GAD well characterized (Lin, 2013; Nejati *et al*., 2013; Franciosi *et al*., 2015; Song and Yu, 2018; Santos-Espinosa *et al*., 2020). The fermented milk made in this study used skim milk and *L. rhamnosus* without the addition of MSG or PLP. However, the activity of GAD on *L. rhamnosus* used may have also been stimulated by the presence of PLP in skim milk which may exist in a low concentration of approximately 3 µM as reported by Li *et al*. (2016) and Schmidt *et al*. (2017). From the results, it was also found that the highest GABA was produced by *L. rhamnosus* SMM37 at about 2.01 mg/mL. Even though the produced GABA concentration was lower than that produced by other *L. rhamnosus* strains, it is still an opportunity to optimize GABA yield through optimizing the growth condition.

Various studies have been conducted to evaluate the production of GABA on various growth factors such as substrates in culture media, as well as in food products such as vegetable drinks, meat products, seafood, and dairy products (Ratanaburee *et al*., 2013; Shan *et al*., 2015; Taoka *et al*., 2019). All of these study reports state that natural GABA production depends mainly on glutamate concentration in the food matrix, the LAB strain used, and the fermentation conditions. It was reported that the yoghurt added with *Lactobacillus plantarum* NDC75017 affects GABA production, which reaches 3.15 g/kg (Shan *et al*., 2015). In another study, *Lactobacillus brevis* 877G produced 1.95 g/L GABA in skim milk. Using a mixture of *L. brevis* 877G and *Lactobacillus sakei* 795 (in the proportion of 1:1) GABA production was increased to 2.32 g/L (Seo *et al*., 2013). On the other hand, *Lactobacillus rhamnosus* GG used in the fermentation of adzuki beans can produce 1.12 mg/mL GABA (Song and Yu, 2018). In this study, however, the production of GABA by *L. rhamnosus* is lower than the *L. rhamnosus* GG, which was probably caused by the growth conditions of the strain characteristics (Minervini *et al*., 2009) or the expression of the GAD enzyme (Li *et al*., 2013). In addition, some strains may have deficiencies in the glutamate-GABA transport system to the inside and outside of the cell (Feehily and Karatzas, 2013) or the assimilation of glutamate in other metabolic pathways, which will limit GABA production (Fernández and Zúñiga, 2006). Therefore, these results need to be further investigated.

4. Conclusion

It can be concluded from the results that 17 *L. rhamnosus* strains isolated from Sumbawa mare milk were able to produce GABA in MRS without and with the addition of monosodium glutamate. Further studies on 6 selected strains to ferment skim milk showed that the highest GABA was produced by *L. rhamnosus* SMM37 at about 2.01 mg/mL. Further studies are needed

Table 2. Production of GABA in skim milk

Strain	Population (log_{10} CFU/mL)		pH	GABA (mg/mL)
	Milk starter	Fermented milk		
L. rhamnosus SMM36	9.580±7.653^{d}	9.476±7.328^{d}	4.10±0.032^{c}	0.07±0.005^{a}
L. rhamnosus SMM37	9.185±7.422^{a}	9.369±7.061^{b}	4.16±0.041^{d}	2.01±0.005^{c}
L. rhamnosus SMM43	9.480±7.661^{c}	9.403±7.795^{c}	3.92±0.057^{a}	0.09±0.011^{a}
L. rhamnosus SMM53	9.638±7.025^{e}	9.332±7.539^{e}	3.90±0.010^{a}	1.97±0.011^{b}
L. rhamnosus SMM58	9.176±7.883^{a}	9.330±7.699^{a}	3.97±0.020^{b}	0.08±0.005^{a}
L. rhamnosus SMM59	9.362±7.484^{b}	9.386±7.606^{a}	4.11±0.032^{cd}	0.07±0.005^{a}

Values are means±SD from 3-time replications (n = 3). Values with different superscript within the same column are significantly different (p<0.05).

to optimize the growth conditions of *L. rhamnosus* SMM37 to increase the activity of GABA formation so that it can increase the GABA content in fermented milk.

Conflict of interest

The authors declare no conflict of interest.

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References

Barrett, E., Ross, R.P., O' Toole, P.W., Fitzgerald, G.F. and Stanton, C. (2012). γ-Aminobutyric acid production by culturable bacteria from the human intestine. *Journal of Applied Microbiology*, 113(2), 411–417. https://doi.org/10.1111/j.1365-2672.2012.05344.x

Bertoldi, M., Frigeri, P., Paci, M. and Voltattorni, C.B. (1999). Reaction specificity of native and nicked 3,4-dihydroxyphenylalanine decarboxylase. *Journal of Biological Chemistry*, 274(9), 5514–5521. https://doi.org/10.1074/jbc.274.9.5514

Capitani, G., De Biase, D., Aurizi,C., Gut,H., Bossa, F. and Grütter, M.G. (2003). Crystal structure and functional analysis of *Escherichia coli* glutamate decarboxylase. *EMBO Journal*, 22(16), 4027–4037. https://doi.org/10.1093/emboj/cdg403

Cho, Y.R., Chang, J.Y. and Chang, H.C. (2007). Production of γ-aminobutyric acid (GABA) by *Lactobacillus buchneri* isolated from kimchi and its neuroprotective effect on neuronal cells. *Journal of Microbiology and Biotechnology*, 17(1), 104–109. https://doi.org/10.1271/nogeikagaku1924.61.1449

Cui, Y., Miao, K., Niyaphorn, S. and Qu, X. (2020). Production of gamma-aminobutyric acid from lactic acid bacteria: A systematic review. *International Journal of Molecular Sciences*, 21(3), 1–21. https://doi.org/10.3390/ijms21030995

Dewi, I.G.A.K., Putra, I.G.N.A.D. and Sujaya, I.N. (2012). Pengembangan Stater dari *Lactobacillus* spp. isolat susu kuda Sumbawa untuk pembuatan susu terfermentasi. *Farmasi, Universitas Udayana*

Dhakal, R., Bajpai, V.K. and Baek, K.H. (2012). Production of GABA (γ-aminobutyric acid) by microorganisms: A review. *Brazilian Journal of Microbiology*, 43(4), 1230–1241. https://doi.org/10.1590/S1517-83822012000400001 [In Bahasa Inodnesia].

Diana, M., Quílez, J. and Rafecas, M. (2014). Gamma-aminobutyric acid as a bioactive compound in foods: A review. *Journal of Functional Foods*, 10, 407–420. https://doi.org/10.1016/j.jff.2014.07.004

Feehily, C. and Karatzas, K.A.G. (2013). Role of glutamate metabolism in bacterial responses towards acid and other stresses. *Journal of Applied Microbiology*, 114(1), 11–24. https://doi.org/10.1111/j.1365-2672.2012.05434.x

Fernández, M. and Zúñiga, M. (2006). Amino acid catabolic pathways of lactic acid bacteria. *Critical Reviews in Microbiology*, 32(3), 155–183. https://doi.org/10.1080/10408410600880643

Franciosi, E., Carafa, I., Nardin, T., Schiavon, S., Poznanski, E., Cavazza, A., Larcher, R. and Tuohy, K.M. (2015). Biodiversity and γ -aminobutyric acid production by lactic acid bacteria isolated from traditional alpine raw cow's milk cheeses. *BioMed Research International*, 2015, 625740. https://doi.org/10.1155/2015/625740

Gonzalez-Gonzalez, C.R., Machado, J., Correia, S., McCartney, A.L., Stephen Elmore, J. and Jauregi, P. (2019). Highly proteolytic bacteria from semi-ripened Chiapas cheese elicit angiotensin-I converting enzyme inhibition and antioxidant activity. *LWT- Food Science and Technology*, 111, 449–456. https://doi.org/10.1016/j.lwt.2019.05.039

Hao, R. and Schmit, J.C. (1993). Cloning of the gene for glutamate decarboxylase and its expression during conidiation in *Neurospora crassa*. *Biochemical Journal*, 293(3), 735–738. https://doi.org/10.1042/bj2930735

Huang, Y., Su, L. and Wu, J. (2016). Pyridoxine supplementation improves the activity of recombinant glutamate decarboxylase and the enzymatic production of gamma-aminobutyric acid. *PLoS ONE*, 11(7), e0157466. https://doi.org/10.1371/journal.pone.0157466

Johnson, B.S. Narendra, K.S., Cherry, J.H. and Locy, R.D. (1997). Purification and characterization of glutamate decarboxylase from cowpeda. *Phytochemistry*, 46(1), 39–44

Kato, Y., Furukawa, K. and Hara, S. (2002). Cloning and nucleotide sequence of the glutamate decarboxylase-encoding gene gadA from *Aspergillus*. *Bioscience, Biotechnology and Biochemistry*, 66(12), 2600–

2605. https://doi.org/10.1271/bbb.66.2600

Komatsuzaki, N., Nakamura, T., Kimura, T. and Shima, J. (2008). Characterization of glutamate decarboxylase from a high γ-aminobutyric acid (GABA)-producer, *Lactobacillus paracasei*. *Bioscience, Biotechnology and Biochemistry*, 72(2), 278–285. https://doi.org/10.1271/bbb.70163

Kubicek, C.P., Hampel, W. and Röhr, M. (1979). *Manganese deficiency leads to elevated amino acid pools in citric acid accumulating Aspergillus niger*. *Archives of Microbiology*, 123, 73-79. https://doi.org/10.1007/BF00403504

Le Vo, T.D., Kim, T.W. and Hong, S.H. (2012). Effects of glutamate decarboxylase and gamma-aminobutyric acid (GABA) transporter on the bioconversion of GABA in engineered *Escherichia coli*. *Bioprocess and Biosystems Engineering*, 35(4), 645–650. https://doi.org/10.1007/s00449-011-0634-8

Li, H., Li, W., Liu, X. and Cao, Y. (2013). gadA gene locus in *Lactobacillus brevis* NCL912 and its expression during fed-batch fermentation. *FEMS Microbiology Letters*, 349(2), 108–116. https://doi.org/10.1111/1574-6968.12301

Li, H., Qiu, T., Huang, G. and Cao, Y. (2010). Production of gamma-aminobutyric acid by *Lactobacillus brevis* NCL912 using fed-batch fermentation. *Microbial Cell Factories*, 9, 85. https://doi.org/10.1186/1475-2859-9-85

Li, W., Wei, M., Wu, J., Rui, X. and Dong, M. (2016). Novel fermented chickpea milk with enhanced level of γ -aminobutyric acid and neuroprotective effect on PC12 cells. *PeerJ*, 4, e2292. https://doi.org/10.7717/peerj.2292

Lin, Q. (2013). Submerged fermentation of *Lactobacillus rhamnosus* YS9 for γ-aminobutyric acid (GABA) production. *Brazilian Journal of Microbiology*, 44 (1), 183–187. https://doi.org/10.1590/S1517-83822013000100028

Maras, B., Sweeney, G., Barra, D., Bossa, F. and John, R.A. (1992). The amino acid sequence of glutamate decarboxylase from *Escherichia coli*. *European Journal of Biochemistry*, 204(1), 93–98.

Minervini, F., Bilancia, M.T., Siragusa, S., Gobbetti, M. and Caponio, F. (2009). Fermented goats' milk produced with selected multiple starters as a potentially functional food. *Food Microbiology*, 26 (6), 559–564. https://doi.org/10.1016/j.fm.2009.03.008

Miura, D., Ito, Y., Mizukuchi, A., Kise, M., Aoto, H. and Yagasaki, K. (2006). Hypocholesterolemic action of pre-germinated brown rice in hepatoma-bearing rats. *Life Sciences*, 79(3), 259–264. https://doi.org/10.1016/j.lfs.2006.01.001

Nathan, B., Bao, J., Hsu, C.C., Aguilar, P., Wu, R., Yarom, M., Kuo, C.Y. and Wu, J.Y. (1994). A membrane form of brain L-glutamate decarboxylase: identification, isolation, and its relation to insulin-dependent diabetes mellitus. *Proceedings of the National Academy of Sciences of the United States of America*, 91(1), 242–246. https://doi.org/10.1073/pnas.91.1.242

Nejati, F., Rizzello, C.G., Di Cagno, R., Sheikh-Zeinoddin, M., Diviccaro, A., Minervini, F. and Gobbetti, M. (2013). Manufacture of a functional fermented milk enriched of Angiotensin-I Converting Enzyme (ACE)-inhibitory peptides and γ -amino butyric acid (GABA). *LWT - Food Science and Technology*, 51(1), 183–189. https://doi.org/10.1016/j.lwt.2012.09.017

Nocianitri, K.A., Antara, N.S., Sugitha, I.M., Sukrama, I.D.M., Ramona, Y. and Sujaya, I.N. (2017). The effect of two *Lactobacillus rhamnosus* strains on the blood lipid profile of rats fed with high fat containing diet. *International Food Research Journal*, 24(2), 795–802

Nomura, M., Kimoto, H., Someya, Y. and Suzuki, I. (1999). Novel characteristic for distinguishing. *International Journal of Systematic Bacteriology*, 49 (1), 163–166. https://doi.org/10.1099/00207713-49-1-163

Park, K.B. and Oh, S.H. (2007). Production of yogurt with enhanced levels of gamma-aminobutyric acid and valuable nutrients using lactic acid bacteria and germinated soybean extract. *Bioresource Technology*, 98(8), 1675–1679. https://doi.org/10.1016/j.biortech.2006.06.006

Pouliot-Mathieu, K., Gardner-Fortier, C., Lemieux, S., St-Gelais, D., Champagne, C.P. and Vuillemard, J.C. (2013). Effect of cheese containing gamma-aminobutyric acid-producing lactic acid bacteria on blood pressure in men. *PharmaNutrition*, 1(4), 141–148. https://doi.org/10.1016/j.phanu.2013.06.003

Qiu, T., Li, H. and Cao, Y. (2010). Pre-staining thin layer chromatography method for amino acid detection. *African Journal of Biotechnology*, 9(50), 8679–8681. https://doi.org/10.4314/ajb.v9i50

Ratanaburee, A., Kantachote, D., Charernjiratrakul, W., Penjamras, P. and Chaiyasut, C. (2011). Enhancement of γ-aminobutyric acid in a fermented red seaweed beverage by starter culture *Lactobacillus plantarum* DW12. *Electronic Journal of Biotechnology*, 14(3), 2. https://doi.org/10.2225/vol14-issue3-fulltext-2

Ratanaburee, A., Kantachote, D., Charernjiratrakul, W.

and Sukhoom, A. (2013). Selection of γ-aminobutyric acid-producing lactic acid bacteria and their potential as probiotics for use as starter cultures in Thai fermented sausages (Nham). *International Journal of Food Science and Technology*, 48(7), 1371–1382. https://doi.org/10.1111/ijfs.12098

Anju, P., Moothedath, I. and Rema Shree, A.B. (2014). Gamma amino butyric acid accumulation in medicinal plants without stress. *Ancient Science of Life*, 34(2), 68-72. https://doi.org/10.4103/0257-7941.153459

Rice, E.W., Johnson, C.H., Dunnigan, M.E. and Reasoner, D.J. (1993). Rapid glutamate decarboxylase assay for detection of *Escherichia coli*. *Applied and Environmental Microbiology*, 59 (12), 4347–4349. https://doi.org/10.1128/aem.59.12.4347-4349.1993

Santos-Espinosa, A., Beltrán-Barrientos, L.M., Reyes-Díaz, R., Mazorra-Manzano, M.Á., Hernández-Mendoza, A., González-Aguilar, G.A., Sáyago-Ayerdi, S.G., Vallejo-Cordoba, B. and González-Córdova, A.F. (2020). Gamma-aminobutyric acid (GABA) production in milk fermented by specific wild lactic acid bacteria strains isolated from artisanal Mexican cheeses. *Annals of Microbiology*, 70, 12. https://doi.org/10.1186/s13213-020-01542-3

Schmidt, A., Schreiner, M.G. and Mayer, H.K. (2017). Rapid determination of the various native forms of vitamin B6 and B2 in cow's milk using ultra-high performance liquid chromatography. *Journal of Chromatography A*, 1500, 89–95. https://doi.org/10.1016/j.chroma.2017.04.009

Seo, M.J., Lee, J.Y., Nam, Y.D., Lee, S.Y., Park, S.L., Yi, S.H., Lee, M.H., Roh, S.W., Choi, H.J. and Lim, S.I. (2013). Production of γ-Aminobutyric Acid by *Lactobacillus brevis* 340G Isolated from Kimchi and Its Application to Skim Milk. *Food Engineering Progress*, 17(4), 418–423. https://doi.org/10.13050/foodengprog.2013.17.4.418

Serraj, R., Shelp, B.J. and Sinclair, T.R. (1998). Accumulation of γ-aminobutyric acid in nodulated soybean in response to drought stress. In *Physiologia Plantarum*, 102(1), 79–86. https://doi.org/10.1034/j.1399-3054.1998.1020111.x

Shan, Y., Man, C.X., Han, X., Li, L., Guo, Y., Deng, Y., Li, T., Zhang, L.W. and Jiang, Y.J. (2015). Evaluation of improved γ-aminobutyric acid production in yogurt using *Lactobacillus plantarum* NDC75017. *Journal of Dairy Science*, 98(4), 2138–2149. https://doi.org/10.3168/jds.2014-8698

Shi, F. and Li, Y. (2011). Synthesis of γ-aminobutyric acid by expressing *Lactobacillus brevis*-derived glutamate decarboxylase in the *Corynebacterium glutamicum* strain ATCC 13032. *Biotechnology Letters*, 33(12), 2469–2474. https://doi.org/10.1007/s10529-011-0723-4

Shi, T., Nishiyama, K., Nakamata, K., Aryantini, N.P.D., Mikumo, D., Oda, Y., Yamamoto, Y., Mukai, T., Sujaya, I.N., Urashima, T. and Fukuda, K. (2012). Isolation of potential probiotic *Lactobacillus rhamnosus* strains from traditional fermented mare milk produced in Sumbawa Island of Indonesia. *Bioscience, Biotechnology and Biochemistry*, 76(10), 1897–1903. https://doi.org/10.1271/bbb.120385

Song, H.Y. and Yu, R.C. (2018). Optimization of culture conditions for gamma-aminobutyric acid production in fermented adzuki bean milk. *Journal of Food and Drug Analysis*, 26(1), 74–81. https://doi.org/10.1016/j.jfda.2016.11.024

Sujaya, I.N., Ramona, Y., Widarini, N.P., Suariani, N.P., Dwipayanti, N.M.U., Nocianitri, K.A. and Nursini, N.W. (2008). Isolasi dan karakteristik bakteri asam laktat dari susu kuda Sumbawa. *Jurnal Veteriner*, 9 (2), 1–10 [In Bahasa Indonesia].

Tajabadi, N., Ebrahimpour, A., Baradaran, A., Rahim, R.A., Mahyudin, N.A., Manap, M.Y.A., Bakar, F.A. and Saari, N. (2015). Optimization of γ-aminobutyric acid production by *Lactobacillus plantarum* Taj-Apis362 from honeybees. *Molecules*, 20(4), 6654–6669. https://doi.org/10.3390/molecules20046654

Taoka, Y., Nakamura, M., Nagai, S., Nagasaka, N., Tanaka, R. and Uchida, K. (2019). Production of anserine-rich fish sauce from giant masu salmon, *Oncorhynchus masou masou* and γ-aminobutyric acid (GABA)-enrichment by *Lactobacillus plantarum* strain N10. *Fermentation*, 5(2),45-61. https://doi.org/10.3390/fermentation5020045

Villegas, J.M., Brown, L., Savoy de Giori, G. and Hebert, E.M. (2016). Optimization of batch culture conditions for GABA production by *Lactobacillus brevis* CRL 1942, isolated from quinoa sourdough. *LWT - Food Science and Technology*, 67, 22–26. https://doi.org/10.1016/j.lwt.2015.11.027

Wu, Q. and Shah, N.P. (2015). Gas release-based prescreening combined with reversed-phase HPLC quantitation for efficient selection of high-γ-aminobutyric acid (GABA)-producing lactic acid bacteria. *Journal of Dairy Science*, 98(2), 790–797. https://doi.org/10.3168/jds.2014-8808

Xu, N., Wei, L. and Liu, J. (2017). Biotechnological advances and perspectives of gamma-aminobutyric acid production. *World Journal of Microbiology and Biotechnology*, 33(3), 64-74. https://doi.org/10.1007/s11274-017-2234-5

Yang, N.C., Jhou, K.Y. and Tseng, C.Y. (2012).

Antihypertensive effect of mulberry leaf aqueous extract containing γ-aminobutyric acid in spontaneously hypertensive rats. *Food Chemistry*, 132(4), 1796–1801. https://doi.org/10.1016/j.foodchem.2011.11.143

Yong-qiang, C. (2008). Development of Laozao enriched with GABA. *Food Science and Technology*, 1, 22–25

Zhao, M., Ma, Y., Wei, Z.Z., Yuan, W.X., Li, Y.L., Zhang, C.H., Xue, X.T. and Zhou, H.J. (2011). Determination and comparison of γ-aminobutyric acid (GABA) content in Pu-erh and other types of Chinese tea. *Journal of Agricultural and Food Chemistry*, 59(8), 3641–3648. https://doi.org/10.1021/jf104601v

Zhuang, K., Jiang, Y., Feng, X., Li, L., Dang, F., Zhang, W. and Man, C. (2018). Transcriptomic response to GABA-producing *Lactobacillus plantarum* CGMCC 1.2437T induced by L-MSG. *PLoS ONE*, 13(6), 1–18. https://doi.org/10.1371/journal.pone.0199021

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