



# Expression, purification, and characterization of glutamate decarboxylase from human gut-originated *Lactococcus garvieae* MJF010

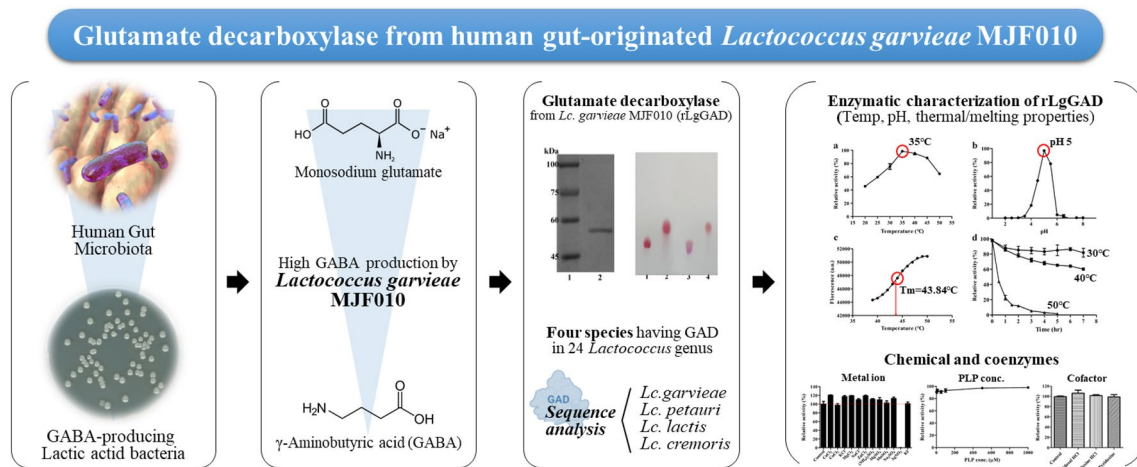
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## Abstract

Human gut-originated lactic acid bacteria were cultivated, and high  $\gamma$ -aminobutyric acid (GABA)-producing *Lactococcus garvieae* MJF010 was identified. To date, despite the importance of GABA, no studies have investigated GABA-producing *Lactococcus* species, except for *Lc. lactis*. A recombinant glutamate decarboxylase of the strain MJF010 (rLgGad) was successfully expressed in *Escherichia coli* BL21(DE3) with a size of 53.9 kDa. rLgGad could produce GABA, which was verified using the silylation-derivative fragment ions of GABA. The purified rLgGad showed the highest GABA-producing activity at 35 °C and pH 5. rLgGad showed a melting temperature of 43.84 °C. At 30 °C, more than 80% of the activity was maintained even after 7 h; however, it rapidly decreased at 50 °C. The kinetic parameters,  $K_m$ ,  $V_{max}$ , and  $k_{cat}$ , of rLgGad were 2.94 mM, 0.023 mM/min, and 12.3 min<sup>-1</sup>, respectively. The metal reagents of CaCl<sub>2</sub>, MgCl<sub>2</sub>, and ZnCl<sub>2</sub> significantly had positive effects on rLgGad activity. However, most coenzymes including pyridoxal 5'-phosphate showed no significant effects on enzyme activity. In conclusion, this is the first report of Gad from *Lc. garvieae* species and provides important enzymatic information related to GABA biosynthesis in the *Lactococcus* genus.

## Graphical abstract



**Keywords**  $\gamma$ -Aminobutyric acid · Expression · Glutamate decarboxylase · *Lactococcus garvieae*

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## Introduction

$\gamma$ -Aminobutyric acid (GABA) is a four-carbon non-protein amino acid widely distributed in nature and a primary suppressive neurotransmitter in the central nervous system of mammals (Sarasa et al. 2020). GABA is distributed throughout the human body and exerts therapeutic effects on disorders of the nervous system, including Alzheimer's, Parkinson's, and Huntington's diseases (Diez-Gutiérrez et al. 2020; Lee et al. 2005; Solas et al. 2015). It also plays a role in the regulation of cardiovascular conditions, such as blood pressure and heart rate, and reduction of anxiety and pain (Sarasa et al. 2020). In particular, the anti-hypertensive and anti-depressant functions of GABA were verified by oral administration of GABA-enriched foods in humans (Diana et al. 2014; Wu and Shah 2017). GABA is responsible for the regulation of motor activity and sleep-awake cycle. In addition, GABA is involved in the maintenance of memory formation, cognition, and high seizure threshold. GABA also has the metabolic functions including the provision of energy to the brain and the resistance to oxygen deficiency (Kalueff and Nutt 2007; Auteri et al. 2015; Yunes et al. 2016).

Because of the physiological effects of GABA, scientific interest and commercial demands for GABA have been increasing. In the pharmaceutical and food industries, it is applied as a bioactive component that positively affects physiological activity; therefore, the demand for GABA is immense. However, the current demand of GABA cannot be sufficiently satisfied by the amounts naturally present in plants and animals (Choi et al. 2006; Youn et al. 2011). In addition, the chemical synthesis of GABA is not suitable to be used in foods due to the use of harmful reagents and the corrosive reactants. However, the bioconversion method for GABA production is, not only, efficient and economic, but also, safer and eco-friendly (Diez-Gutiérrez et al. 2020). Bioconversion of GABA has a high transformation rate and convenient control over an enzyme reaction; hence, researchers are now using microbial and enzymatic methods to produce GABA.

Glutamate decarboxylase (Gad, EC. 4.1.1.15), a pyridoxal 5'-phosphate-dependent enzyme, is a primary enzyme for GABA production. GABA is produced through the irreversible  $\alpha$ -decarboxylation of L-glutamic acid in a reaction catalyzed by Gad (Lyu et al. 2021). Gad is an intracellular enzyme that induces an acid stress response (Villegas et al. 2016).

Numerous studies have reported GABA-producing microorganisms and their Gad activity. To date, studies have focused on lactic acid bacteria (LAB) as GABA-producing microorganisms, and many strains of GABA-producing LAB derived from food sources have been

reported. In particular, *Levilactobacillus brevis* is the most studied species. In addition, various *Lactobacillus* genera and a few other genera (*Streptococcus* sp., *Pseudomonas* sp., and *Enterococcus* sp.) have been studied (Sarasa et al. 2020).

Despite their minor contribution to the population of human gut microbiota, LAB have attracted considerable attention due to their positive effects on human health. Various species of LAB provide health-promoting benefits, such as anti-obesity, anti-inflammatory, anti-cancer, and anti-allergic activities, urogenital health care (Kerry et al. 2018), and GABA-producing ability. However, the ability of human-originated bacteria to produce GABA remains poorly studied. Moreover, only a few gut-originated strains have been shown to produce GABA. It has been recently reported that the strains of *Bacteroides* species isolated from human gut can produce GABA, of which biosynthesis is regulated by pH (Otaru et al. 2021). In addition, Yunes et al. (2016) reported the GABA-producing ability and the presence of the *gad* gene of human-originated *Lactiplantibacillus plantarum*, *Levi. brevis*, *Bifidobacterium adolescentis*, *B. angulatum*, *B. dentium*, and other gut-originated bacterial species, which can be considered as delivery vehicles for GABA to specific regions of the gut.

In this study, human gut-originated LAB were cultivated, and GABA-producing *Lactococcus garvieae* MJF010 was isolated. The Gad of strain MJF010 was successfully produced in *Escherichia coli*, and its enzymatic properties were investigated. This is the first study on the Gad of *Lc. garvieae* species, providing an important enzymatic information on GABA biosynthesis in the *Lactococcus* genus.

## Materials and methods

### Reagents and bacterial strains

Monosodium glutamate (MSG) and  $\gamma$ -aminobutyric acid (GABA) were purchased from Sigma-Aldrich (St. Louis, MO, USA). The media used in this study, De Man, Rogosa and Sharpe (MRS) broth and MRS agar, and Luria-Bertani (LB), were obtained from MCell (Seoul, Korea). All other chemicals used in this study were of analytical grade. *Lactococcus garvieae* MJF010 isolated in this study was deposited in the Korea Culture Center of Microorganisms (KCCM; Seoul, Korea) under the collection number KCCM12722P. *E. coli* DH5 $\alpha$  (Takara, Shiga, Japan) and *E. coli* BL21(DE3) (Stratagene, La Jolla, CA, USA) were used as hosts for construction and expression, respectively. The plasmid pET28a (Merck, Darmstadt, Germany) vector was used to express the *gad* gene from *Lc. garvieae* MJF010.

## Isolation and identification of GABA-producing LAB from human fecal microbiota

The human fecal sample was collected from a 45-year-old healthy Korean male adult (height and weight were 174 cm and 80 kg, respectively) with no history of gastrointestinal diseases and who had not taken any antibiotics in the last six months. The care and use of human derivatives were approved by the Institutional Review Board of Incheon National University (IRB 7007971-202006-001 A) and compliant with all relevant ethical guidelines. The fecal samples were serially diluted ten folds with 10 mL of 0.85% NaCl solution, followed by homogenization. To isolate LAB from human feces, 100  $\mu$ L of each prepared diluted feces sample was spread and incubated on MRS agar at 37 °C for 48 h. Fifty colonies were randomly picked and grown individually in MRS broth for 24 h at 37 °C under anaerobic conditions. All processes including the dilution of fecal samples and incubation step were performed in anaerobic chamber (Coy Laboratory Products, Grass Lake, MI, USA) filled with 85% N<sub>2</sub>, 10% CO<sub>2</sub>, and 5% H<sub>2</sub> gas.

To identify the isolated LAB strains, genomic DNA was extracted using the HiYield Genomic DNA Mini Kit (RBC, Taiwan). The full-length 16 S rRNA gene was amplified using recombinant EF-Taq DNA polymerase (Solgent, Daejeon, Korea) with universal primers (27 F and 1492R). The polymerase chain reaction (PCR) conditions were as follows: 95 °C for 5 min; 30 cycles of 30 s at 95 °C, 30 s at 55 °C, and 1.5 min at 72 °C; and 5 min at 72 °C. The amplified fragments were sequenced and identified using the EzBioCloud database ver. 2021.04.13 (<https://www.ezbiocloud.net/>). Phylogenetic tree of *Lc. garvieae* MJF010 was constructed using MEGA7 software (Kumar et al. 2016). Multiple alignment of the 16 S rDNA gene (mean length 1443 nt) was performed using EBI-Clustal Omega (Madeira et al. 2019). The Kimura 2-parameter model was applied with bootstrap replications of 1,000 datasets. The 16 S rRNA gene sequence of *Lc. garvieae* MJF010 was deposited in the NCBI GenBank database (<https://www.ncbi.nlm.nih.gov/genbank/>) under accession number MH057260.

To select the GABA-producing LAB strain, each strain isolated from human feces was individually grown in 10 mL of MRS broth containing 1% (w/v) MSG at 37 °C for 48 h in anaerobic chamber as mentioned above. The supernatant of each culture was harvested by centrifugation at 4000 $\times$ g for 20 min and analyzed using thin layer chromatography (TLC).

## Cloning and expression of glutamate decarboxylase from *Lc. garvieae* MJF010

The glutamate decarboxylase gene (*gad*) of *Lc. garvieae* MJF010 (hereafter referred to as *lggad*) was amplified using

a primer set, which was designed based on *Gad* sequence of *Lactococcus* species accessed from NCBI (protein ID: WP\_019292826.1), including restriction enzyme sites (forward: 5'-CGC CAT ATG TTA TAT GGA AAA GAA AAT C-3' and reverse: 5'-CGC GGA TCC TTA ATG TGT AAA TCC-3', *Nde*I and *Bam*HI underlined). The amplification of *lggad* was conducted using recombinant EF-Taq DNA polymerase (Solgent) under the following conditions: 95 °C for 5 min; 30 cycles of 30 s at 95 °C, 45 s at 58 °C, and 1.5 min at 72 °C; and 5 min at 72 °C. The 1.4 kb amplified *lggad* was ligated into the pET28a vector using T4 ligase (Enzymomics, Daejeon, Korea). The ligation mixture was transformed into *E. coli* DH5 $\alpha$  for the propagation of the construct, followed by transformation into *E. coli* BL21(DE3) for expression. The gene sequence of *lggad* was deposited in the NCBI GenBank database under accession number MH071407.

The growth of *E. coli* BL21(DE3) harboring pET28a-*lggad* and the purification of the recombinant *Gad* from *Lc. garvieae* MJF010 (rLgGad) was performed according to the previous report (Lim et al. 2018). Briefly, the recombinant *E. coli* BL21(DE3) harboring pET28a-*lggad* was cultivated in 250 mL of LB medium including kanamycin (50 mg/mL) at 37 °C. The expression of rLgGad with C-terminal His<sub>6</sub> tag was induced at an optical density (600 nm) of 0.6 by adding 0.5 mmol/L isopropyl  $\beta$ -D-1-thiogalactopyranoside (IPTG) and incubated at 18 °C for 18 h. After centrifuging the harvested cell broth at 7000 $\times$ g for 10 min at 4 °C, the cell pellets were resuspended in lysis buffer (20 mmol/L Tris-HCl, 300 mmol/L NaCl, 5 mmol/L  $\beta$ -mercaptoethanol, 0.1% triton X-100, 5% glycerol, and 10 mmol/L imidazole, pH 8.5). The lysed cells were disrupted by sonifier (Sonics & Materials, Inc., Newtown, CT, USA), followed by being harvested using centrifugation at 10,000 $\times$ g for 30 min at 4 °C to remove cell debris. The supernatant was loaded onto a Ni-NTA polypropylene column (Bio-Rad Laboratories, Inc., Hercules, CA, USA) filled with Ni-NTA agarose (QIAGEN, Hilden, Germany) and washed with 20 mmol/L imidazole in lysis buffer. The targeted rLgGad protein was finally eluted with 250 mmol/L imidazole in lysis buffer. The final purified protein was analyzed by 10% (w/v) sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Protein concentration was determined using a bicinchoninic acid protein assay kit (Thermo Fisher Scientific; Waltham, MA, USA), with bovine serum albumin as the standard.

## Analysis of rLgGad activity and its GABA production

The activity of rLgGad was measured by the production of GABA. To produce GABA, 25  $\mu$ L of rLgGad was mixed with 0.2 mL of buffer consisting of 70 mM Na<sub>2</sub>HPO<sub>4</sub>-citric acid (pH 5.0), 10 mM MSG, and 0.01 mM pyridoxal 5'-phosphate (PLP). The reaction mixture was incubated at 35 °C for 1 h and then boiled for 5 min

to stop the reaction. The supernatant containing GABA was harvested by centrifugation at  $10,000\times g$  for 20 min at 10 °C.

The amount of GABA in the supernatant was analyzed by a spectrophotometric GABA assay using GABase enzyme (5 units/mL; Sigma-Aldrich, St. Louis, MO, USA) as described previously (Zhang and Bown 1997). The assay mixture contained 234  $\mu\text{L}$  of 100 mM  $\text{K}_4\text{P}_2\text{O}_7$  buffer (pH 8.6), 42  $\mu\text{L}$  of 10 mM  $\text{NADP}^+$ , 12  $\mu\text{L}$  of the supernatant of the reaction mixture, and 5  $\mu\text{L}$  of GABase. The initial absorbance was measured at 340 nm using a Multiskan FC plate reader (Thermo Fisher), and 6  $\mu\text{L}$  of 100 mM  $\alpha$ -ketoglutarate was added and incubated at room temperature for 1 h. The final absorbance was measured at the same wavelength, and the GABA concentration was calculated based on the difference in these absorbances and the GABA standard curve. One unit of rLgGad activity was defined as the amount of enzyme that produced 1  $\mu\text{mol}$  of GABA per min under the assay conditions.

### Qualitative analysis of GABA produced by rLgGad

GABA production was verified by TLC and gas chromatography-mass spectrometry (GC-MS). For TLC analysis, one  $\mu\text{L}$  of culture supernatant was loaded onto a TLC silica gel plate (Merck). The samples were developed with a mixed solvent consisting of *n*-butanol, acetic acid, and water (4:1:1, v/v/v). After development, the plate was dried completely at 115 °C for 5 min and visualized using 0.2% (w/v) ninhydrin solution and dried again.

For GC-MS analysis, the supernatant was filtered using a 0.2  $\mu\text{m}$  syringe filter and then freeze-dried. Samples (10 mg) were extracted with 1 mL of a solvent mixture consisting of methanol, chloroform, and water (2.5:1:1, v/v/v), and subjected to two-stage derivatization (oximation with trimethylsilyl etherification). The separation of GABA and MSG from the derivative (1  $\mu\text{L}$ ) was performed with a DB-5 capillary column (0.25 mm I.D. $\times$ 30 m, 1.0  $\mu\text{m}$  film thickness; J&W Scientific, Folsom, CA, USA) by using the GCMS-QP2010 Ultra system equipped with an AOC20i auto-sampler (Shimadzu, Kyoto, Japan). The carrier gas was helium with a flow rate of 1 mL/min and split ratio of 1:10. The temperatures of the injector, ion source, and interface were 280 °C, 200 °C, and 280 °C, respectively. The temperature program was operated as follows: 100 °C for 4 min, followed by an increase of 10 °C/min to 320 °C, and holding for 11 min at 320 °C. The standard spectra were recorded in scan mode within a scanned mass range of 45–600 m/z, and selected ion monitoring (SIM) mode was used for identification (m/z 304 for GABA).

### Biochemical characterization of rLgGad

The biochemical characterization of rLgGad, including the effects of temperature, pH, metal reagents ( $\text{CaCl}_2$ ,  $\text{CuCl}_2$ ,  $\text{KCl}$ ,  $\text{MgCl}_2$ ,  $\text{NaCl}$ ,  $\text{ZnCl}_2$ ,  $(\text{NH}_4)_2\text{SO}_4$ ,  $\text{MgSO}_4$ ,  $\text{MnSO}_4$ ,  $\text{Na}_2\text{SO}_4$ ,  $\text{AgNO}_3$ , and  $\text{KI}$ ), PLP, and other coenzymes was performed under the enzyme reaction conditions as mentioned above, except for changes in the parameter to be measured. The temperature was changed from 20 to 50 °C, and the pH was adjusted from 2 to 8 by controlling the ratio of the buffer components ( $\text{Na}_2\text{HPO}_4$  and citric acid).

Two millimolar of each metal reagent was added to the reaction mixture to study their effects on rLgGad. The effect of PLP on rLgGad was measured at different PLP concentrations (0–1 mM). The effect of coenzymes was also measured by adding 0.5 mM each of pyridoxal HCl, pyridoxine HCl, and pyridoxine, respectively, at 35 °C and pH 5.

The thermostability was determined by measuring the residual activities after standing at 30–50 °C for 0–7 h. In addition, the fluorescence data to calculate melting temperature were measured using differential scanning fluorimetry (Niesen et al. 2007). The sample mixture containing 10  $\mu\text{L}$  of the enzyme solution (0.01 mg/mL) and 10  $\mu\text{L}$  of SYPRO orange (10 $\times$ ; Invitrogen, Waltham, MA, USA) was incubated at a temperature gradient ranging from 25 to 95 °C in 1 °C increment by using a StepOnePlus Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). Melting temperature was calculated using GraphPad Prism 5.0 software (GraphPad, San Diego, CA, USA) with a Boltzmann Sigmoidal curve. Non-linear fitting of truncated fluorescence data were applied to the following equation (Huynh and Partch 2015).

$$Y = \text{Bottom} + (\text{Top} - \text{Bottom}) / (1 + \exp(T_m - X / \text{Slope}))$$

Y = fluorescence emission in arbitrary units; X = temperature; Bottom = baseline fluorescence at low temperature; Top = maximal fluorescence at top of the truncated dataset; Slope = describes the steepness of the curve, with larger values denoting shallower curves; and  $T_m$  = melting temperature of the protein.

The kinetic parameters ( $K_m$  and  $V_{\text{max}}$ ) were determined under optimum conditions (35 °C and pH 5) with changes in the MSG concentrations (5–20 mM). The data were analyzed using a Lineweaver-Burk plot to calculate the kinetic parameters.

### Statistical analysis

In the biochemical characterization of rLgGad, data are expressed as the mean  $\pm$  standard error of mean of three independent measurements. Tukey's multiple-comparison

test analyzed significant differences from control. All statistical analyses were performed using GraphPad Prism 5.0 (GraphPad, San Diego, CA, USA).

## Results

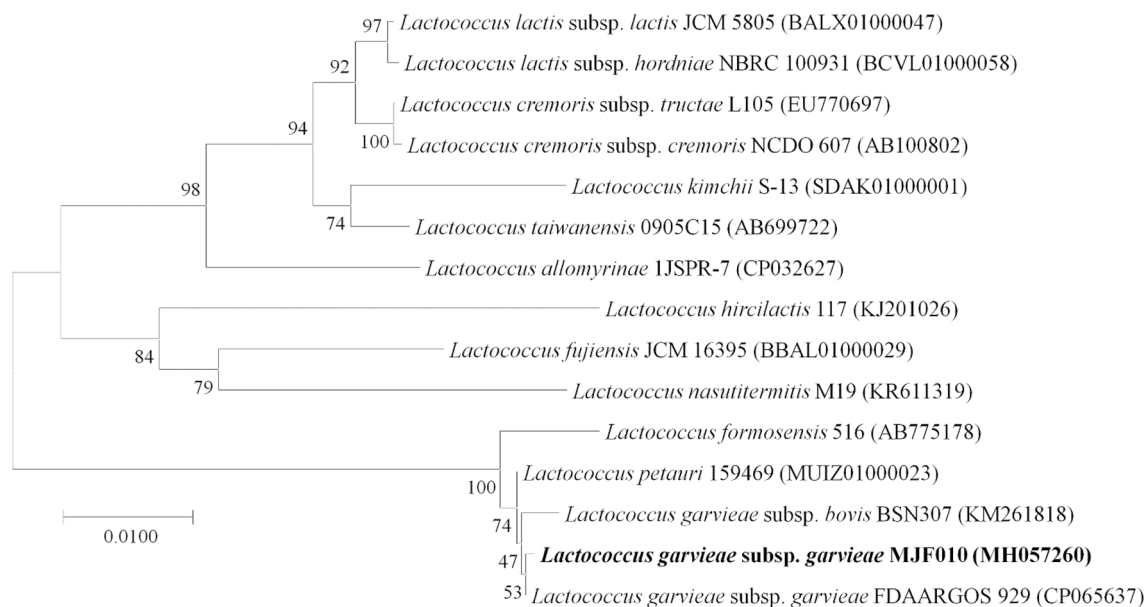
### Isolation of GABA-producing *Lactococcus garvieae* MJF010 from human fecal microbiota

Fifty LAB were isolated from the feces of a healthy Korean male adult and identified using 16 S rRNA genes. Each species was closest to the following bacteria: *E. coli*, *Streptococcus agalactiae*, *Lactococcus garvieae*, *Lactobacillus salivarius*, *Weissella cibaria*, and *Leuconostoc lactis*, and was organized into 23 strains. To select the GABA-producing strain, each strain was cultivated in MRS broth containing MSG and screened by TLC analysis. Consequently, one bacterium, strain MJF010, was observed to produce the darkest and largest spot on the TLC plate, indicating that a significant amount of a compound expected to be GABA was produced from MSG (data not shown). The 16 S rRNA sequence of strain MJF010 showed 99.93%, 99.86%, 99.64%, 98.91%, and 93.64% similarity to *Lc. garvieae* subsp. *garvieae* FDAARGOS 929<sup>T</sup>, *Lc. petauri* 159,469<sup>T</sup>, *Lc. garvieae* subsp. *bovis* BSN307<sup>T</sup>, *Lc. formosensis* 516<sup>T</sup>, and *Lc. lactis* subsp. *lactis* JCM 5805<sup>T</sup>, respectively. The strain MJF010 had the closest sequence identity with that of *Lc. garvieae* subsp. *garvieae* FDAARGOS\_929<sup>T</sup> in the

phylogenetic tree (Fig. 1). However, it should be noted that the strain MJF010 might correspond to one of the already described strains, because its 16 S rRNA sequence similarity to *Lc. garvieae* subsp. *garvieae* FDAARGOS\_929<sup>T</sup> showed more than 98.5%, and its phylogenetic analysis was evaluated at the species level, not the strain level. Nevertheless, the GABA-producing strain MJF010 in this study could be designated as *Lc. garvieae* MJF010. In the future, the ortho average nucleotide identity and *in silico* DNA-DNA hybridization analyses based on the whole genome of the strain MJF010 will be conducted to identify the novelty of the strain MJF010 at the strain level.

### Sequence analysis of glutamate decarboxylase (Gad) from *Lc. garvieae* MJF010

The full-length coding sequence of Gad from *Lc. garvieae* MJF010 was composed of 1,401 bp with 35% G + C content, which could be translated into 466 amino acids. The mature protein of LgGad showed a predicted molecular weight (MW) of 53.9 kDa and a theoretical isoelectric point of 5.37. In the BlastP analysis, LgGad contained a highly conserved catalytic domain of PLP-dependent Gad, and was identical to Gad from *Lactococcus* species. Among the 24 species, including eight subspecies of *Lactococcus*, only *Lc. petauri* (98.28% similarity), *Lc. lactis* (90.77–92.70%), *Lc. cremoris* (91.85%) were observed to have Gad. Therefore, it was considered that this type of Gad is observed in four out of 24 *Lactococcus* species. In



**Fig. 1** Phylogenetic tree of *Lc. garvieae* MJF010. The tree was constructed using MEGA X software using the 16 S rRNA genes (mean length of 1443 nt) of a closely related *Lactococcus* species from EzBioCloud database ver. 2021.04.13 (<https://www.ezbiocloud.net/>).

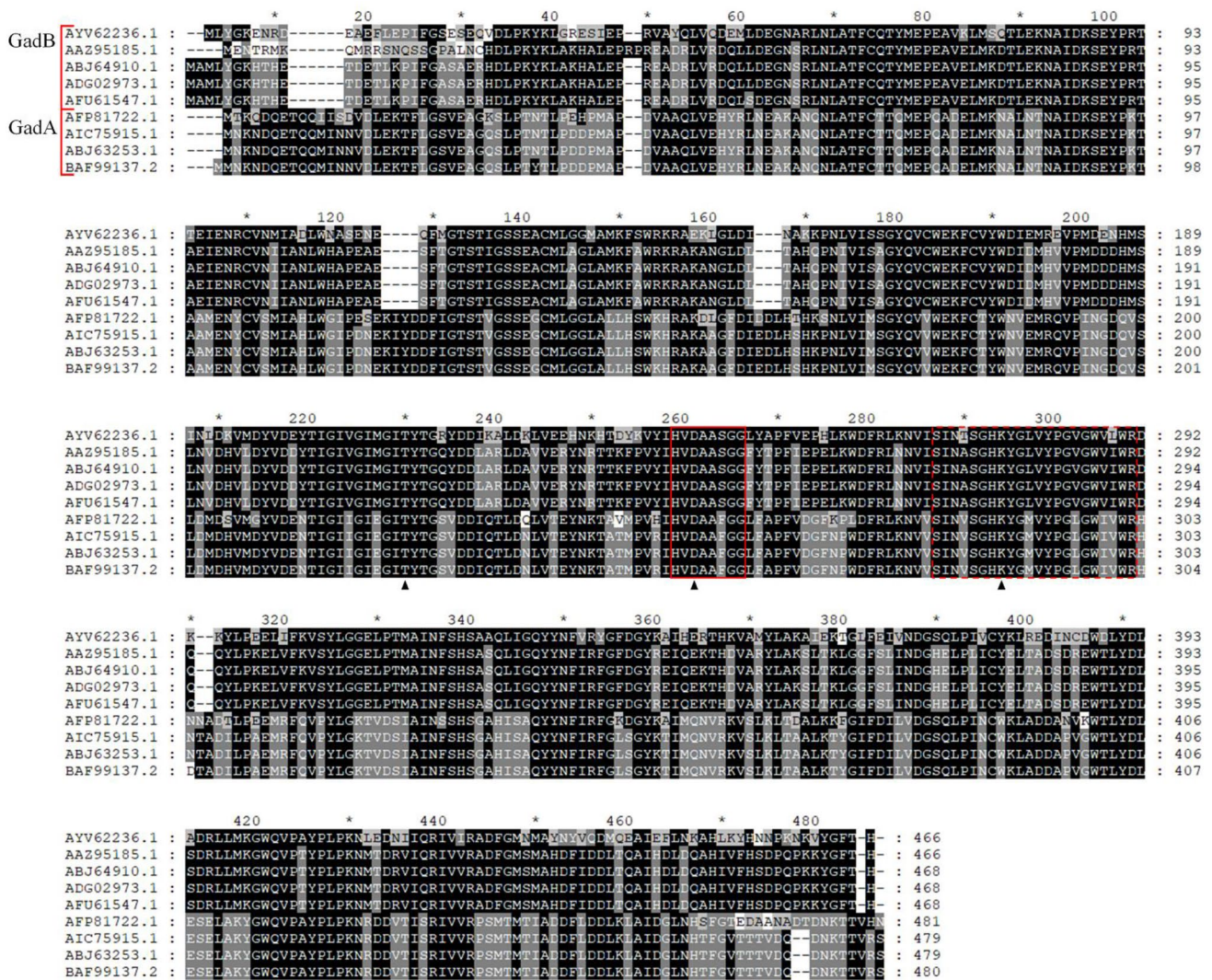
The neighbor-joining statistical method was applied with bootstrap replications of 1,000 datasets using *kimura 2-parameter* model. The bar represents 0.01 substitutions per site. The accession number for 16 S rRNA genes of each strain is in parentheses

general, Gad can be divided into two isoforms, GadA and GadB (Yunes et al. 2016). LgGad was aligned with other GadA and GadB (Fig. 2) and was identified as belonging to GadB. The LgGad contains a motif [H(V/I)DAASGG], which is highly conserved in PLP-dependent decarboxylases, and a PLP-binding domain (Murzin 1996). In LgGad, the catalytic amino acid residues (Thr<sup>215</sup>, Asp<sup>246</sup>, and Lys<sup>279</sup>) were confirmed. The active site residues (Thr<sup>215</sup> and Asp<sup>246</sup>) are known to promote decarboxylation, and the highly conserved lysine residue (Lys<sup>279</sup>) is indispensable for PLP binding (Kim et al. 2007; Li et al. 2013).

### Production and verification of GABA by rLgGad

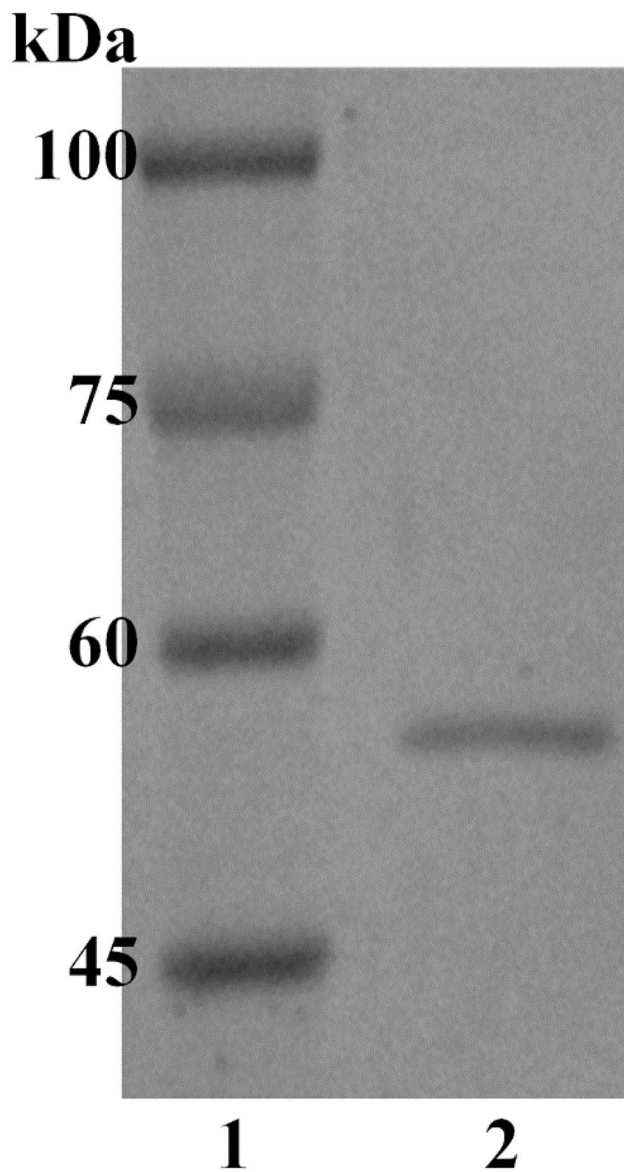
Recombinant LgGad (rLgGad) was overexpressed in *E. coli* and purified successfully. The purified rLgGad carrying C-terminal His<sub>6</sub> tag showed a clear single band of approximately 53 kDa, which was identical to the theoretical MW of 53.9 kDa (Fig. 3). Then, biosynthesis of GABA was carried out by rLgGad using MSG as the sole substrate.

GABA was identified using thin-layer chromatography (Fig. 4a). It was confirmed that rLgGad produced a compound from MSG, which was presumed to be GABA. The MW was determined using gas chromatography-mass spectrometry, as shown in Fig. 4b and c. The



**Fig. 2** Amino acid sequence alignment of Gad from *Lc. garvieae* MJF010. The multiple alignment with other GadA and GadB was performed by Clustal Omega (<http://www.ebi.ac.uk/Tools/msa/clustalo/>). The motif [H(V/I)DAASGG] and the PLP binding domain are in the solid and dotted line boxes, respectively. Arrows represent the catalytic amino acid residues (Thr<sup>215</sup>, Asp<sup>246</sup>, and Lys<sup>279</sup>). The Gad

sequences used for comparison were from *Lc. garvieae* MJF010 (AYV62236.1) and *Levi. brevis* [strain OPK-3 (AAZ95185.1); ATCC 367 (ABJ64910.1); CGMCC 1306 (ADG02973.1); 877G (AFU61547.1); NCL912 (AFP81722.1); BH2 (AIC75915.1); ATCC 367 (ABJ63253.1); IFO 12,005 (BAF99137.2)]



**Fig. 3** SDS-PAGE analysis of rLgGad. Lane 1: protein marker and lane 2: rLgGad purified by Ni-NTA resin. The estimated size of purified rLgGad was 53.9 kDa

sialylation-derivative of GABA appeared 319 MW and had an important fragment ion at  $m/z$  304  $[M-CH_3]^-$  (Iwase et al. 1979). The prominent peak at  $m/z$  174 of GABA was assigned to the fragment  $(CH_2=N^+(TMS)_2)$  of the *N,N,O*-trimethylsilyl derivative of GABA (Cattabeni et al. 1976). Thus, the MW of the compound produced from MSG by rLgGad (spot in lane 4) was identical to that of GABA.

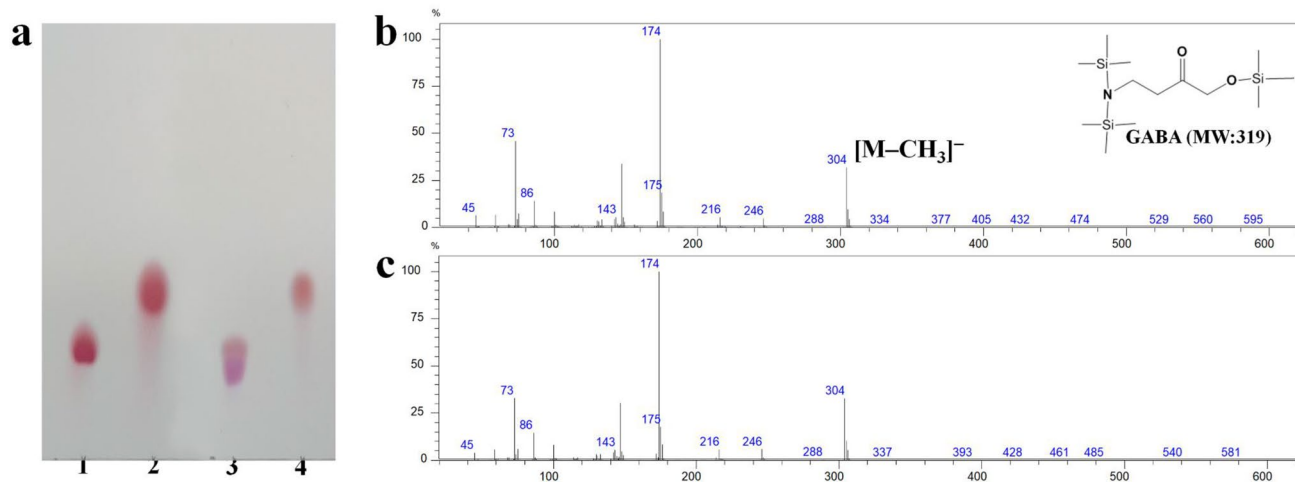
### Enzymatic characterization of rLgGad

The enzymatic reaction conditions of rLgGad carrying C-terminal His<sub>6</sub> tag such as reaction temperature and pH and thermal stability, were evaluated. rLgGad showed GABA-producing

activity over a wide range of temperatures (Fig. 5a). It showed the highest activity at 35 °C, but showed at least 40% activity at 20–50 °C. As the strain MJF010 is a human gut-originated bacterium, it can be considered to exhibit optimal activity under mesophilic conditions. In contrast, GABA-producing activity was observed in a very narrow pH range (Fig. 5b). The optimum activity of rLgGad was shown at pH 5, and no activity was shown in the acidic pH range (pH 2–4) and the pH range from 6 to 8. The alkaline pH range (pH 9–10) also resulted in no activity of rLgGad (data not shown). The optimal reaction pH for other Gads, found in various LAB reported previously, was approximately near 4 to 5, which is mostly acidic condition (Table 1). Therefore, it could be anticipated that most LAB-derived Gads optimally convert glutamate to GABA under acidic conditions. According to previous reports, in bacteria, the Gad, together with Glu/GABA antiporter, forms the glutamate-dependent acid resistance systems; the Gad catalyzes the decarboxylation of glutamate by consuming protons to maintain the intracellular pH in the acidic environment (Wu et al. 2017). The rLgGad showed a melting temperature of 43.84 °C, which was slightly above the optimum temperature (35 °C) (Fig. 5c). The rLgGad retained 60% of its initial activity after 7 h at 40 °C, whereas the enzyme activity remarkably decreased to 1.5% at 50 °C after 5 h (Fig. 5d). The activity gradually decreased as time elapsed and decreased to 60% after 7 h of exposure at 40 °C. At 50 °C, approximately 80% of the activity was diminished after 1 h, and almost all the activity was lost after 3 h. But more than 80% of the activity was maintained even after 7 h of exposure at 30 °C. The kinetic parameters,  $K_m$ ,  $V_{max}$ , and  $k_{cat}$ , were estimated using a Lineweaver-Burk plot (data not shown) and calculated as 2.94 mM, 0.023 mM/min, and 12.3 min<sup>-1</sup>, respectively.

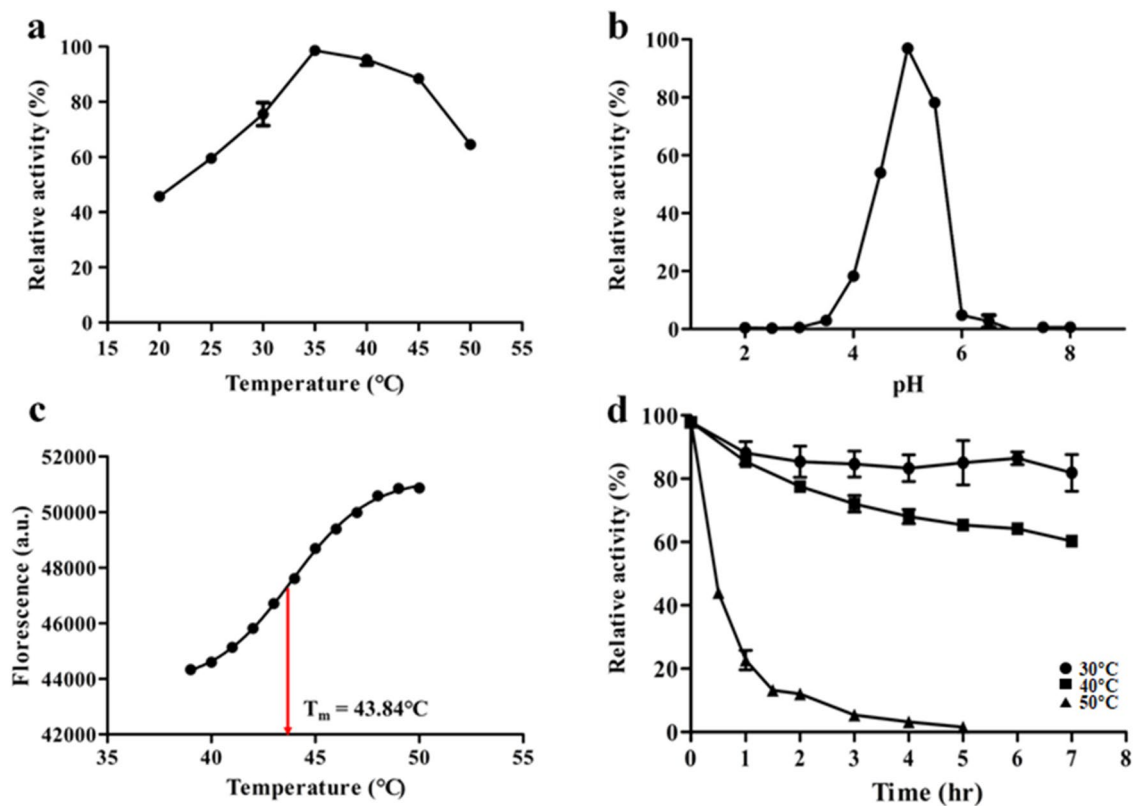
### Effects of chemical reagents and coenzymes on rLgGad activity

The chemical reagents and coenzymes affecting the activity of rLgGad were evaluated under optimum conditions (35 °C and pH 5) (Fig. 6). Statistically significant positive effects on the GABA-producing activity of rLgGad were observed in three chemical reagents [ $CaCl_2$  (120%),  $MgCl_2$  (119%), and  $ZnCl_2$  (119%)] ( $p < 0.05$ ) (Fig. 6a). In contrast,  $AgNO_3$  completely inhibited the activity of rLgGad ( $p < 0.001$ ). The effect of exogenous PLP concentrations on rLgGad was investigated under optimum conditions (Fig. 6b). It was seen that the GABA-producing activity of rLgGad was not dependent on exogenous PLP, suggesting that rLgGad is mostly expressed as a Gad-PLP complex in *E. coli*. It was also investigated if the Gad-PLP complex could be affected by other coenzymes including pyridoxal HCl, pyridoxine HCl, and pyridoxine (Fig. 6c). Most of the tested coenzymes showed no significant increase in catalytic activity; pyridoxal HCl exhibited a very slight positive effect on activity (107%).



**Fig. 4** Verification of GABA production using rLgGad. **a** TLC analysis of produced GABA. Lane 1: MSG standard, lane 2: GABA standard, lane 3: rLgGad reactant without MSG, and lane 4: rLgGad

reactant with MSG. **b** GC-MS analysis of GABA standard. **c** GC-MS analysis of rLgGad reactant with MSG



**Fig. 5** Enzymatic characteristics of rLgGad at different **a** reaction temperatures, **b** pH, **c** melting temperature, and **d** thermal stability of rLgGad (wherein, triangle, square, and circle symbols represent

50 °C, 40 °C, and 30 °C, respectively). The residual enzyme activity was expressed as the relative percentage rLgGad activity



**Table 1** Characteristics on different Gads from various LABs

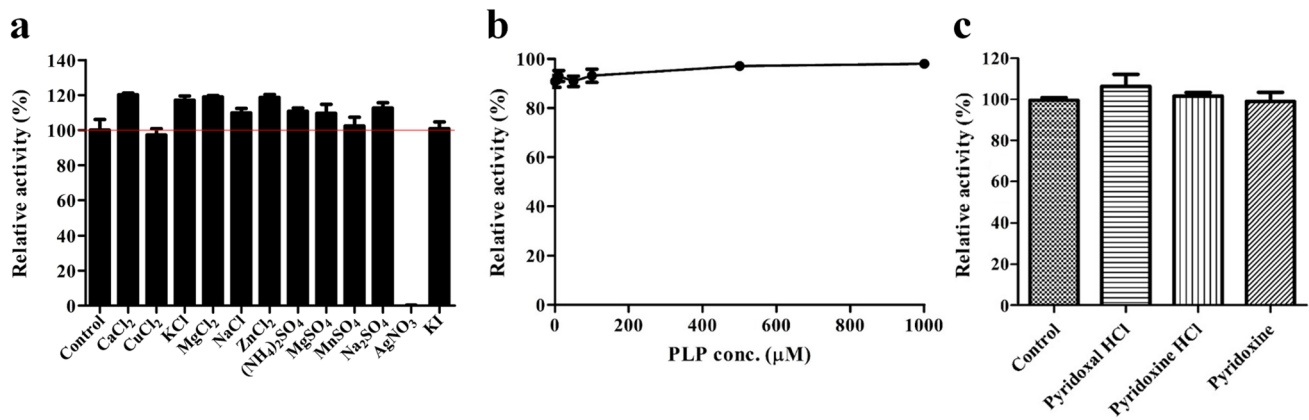
Reported strain	Optimal pH	Optimal temperature (°C)	Activator ion	Inhibitor ion	$K_m$ (mM)	$V_{max}$	Reference
<i>Lactococcus garvieae</i> MJF010*	5	35	Ca <sup>2+</sup> , K <sup>+</sup> , Mg <sup>2+</sup> , Zn <sup>2+</sup>	Ag <sup>+</sup>	2.94	0.023 mM/min	This study
<i>Enterococcus avium</i> M5*	5.5	45	Ca <sup>2+</sup> , Mn <sup>2+</sup>	Ag <sup>+</sup> , Cu <sup>2+</sup>	3.26	0.012 mM/min	(Lee et al. 2017)
<i>Levilactobacillus brevis</i> 877G*	5.2	45	Ca <sup>2+</sup>	Ag <sup>+</sup> , Cu <sup>2+</sup> , Zn <sup>2+</sup> , K <sup>+</sup>	3.6	0.06 mM/min	(Seo et al. 2013)
<i>Levilactobacillus brevis</i> CGMCC 1306*	4.8	48	NR	NR	10.26	8.86 U/mg	(Fan et al. 2012)
<i>Levilactobacillus brevis</i> HYE1*	4	55	NR	Ag <sup>+</sup> , Cu <sup>2+</sup>	4.99	0.224 mM/min	(Lim et al. 2018)
<i>Levilactobacillus brevis</i> IFO 12,005	4.5-5.0	NR	NH <sub>4</sub> <sup>+</sup>	NR	1.4	NR	(Hiraga et al. 2008)
<i>Levilactobacillus brevis</i> NPS-QW-145*	4.8	40	NR	NR	21.39	32.56 μmol/min/mg (0.64 mM/min)	(Wu et al. 2017)
<i>Limosilactobacillus fermentum</i> YS2*	4.5	40	Ca <sup>2+</sup> , Mg <sup>2+</sup>	Cu <sup>2+</sup> , Fe <sup>3+</sup> , Ag <sup>+</sup> , Fe <sup>2+</sup>	10.6	NR	(Lin et al. 2017)
<i>Lacticaseibacillus paracasei</i> NFRI 7415	5	50	NH <sub>4</sub> <sup>+</sup> , Ca <sup>2+</sup>	Na <sup>+</sup> , EDTA	5	7.5 U/mg	(Komatsuzaki et al. 2008)
<i>Lactiplantibacillus plantarum</i> ATCC 14,917*	5	40	NR	NR	22.8	NR	(Shin et al. 2014)
<i>Lactiplantibacillus plantarum</i> WCFS1*	4.8	60	NR	NR	20.02	73.33 μmol/min/mg (1.4 mM/min)	(Lee et al. 2015)
<i>Latilactobacillus sakei</i> A156*	5	55	Mn <sup>2+</sup> , Co <sup>2+</sup> , Ca <sup>2+</sup> , Zn <sup>2+</sup>	Ag <sup>+</sup> , Mg <sup>2+</sup> , NH <sub>4</sub> <sup>+</sup>	16	0.011 mM/min	(Sa et al. 2015)
<i>Latilactobacillus sakei</i> OPK2-59	5	30	Ca <sup>2+</sup> , Fe <sup>3+</sup> , Mg <sup>2+</sup>	Mn <sup>2+</sup>	NR	NR	(Yu and Oh 2011)
<i>Levilactobacillus zymae</i> GU240*	4.5	41	NH <sub>4</sub> <sup>+</sup> , Ca <sup>2+</sup> , Mg <sup>2+</sup> , Na <sup>+</sup>	Cu <sup>2+</sup> , Ag <sup>+</sup> , Co <sup>2+</sup>	1.7	0.01 mM/min	(Park et al. 2014)
<i>Lactococcus lactis</i> ssp. <i>lactis</i> CV56*	4.7	50	NR	NR	3.9	15.1 U/mg	(Lyu et al. 2020)

The mark \* represent the recombinant glutamate decarboxylase expressed in *E. coli*

## Discussion

GABA is a major inhibitory neurotransmitter in the mammalian central nervous system. GABA is widely distributed in bacteria, plants, and vertebrates. LAB are the main GABA-producers among bacteria (Sarasa et al. 2020). GABA-producing LAB derived from food sources have been reported in many strains, such as *Companilactobacillus farciminis*, *Com. futsaii*, *Lacticaseibacillus paracasei*, *Lacti. plantarum*, *L. helveticus*, *Lactococcus lactis*, *Latilactobacillus sakei*, *Lentilactobacillus bunchneri*, *Len. Curieae*, *Len. otakiensis*, *Levilactobacillus zymae*, and *Limosilactobacillus fermentum* have been studied. In addition, *Streptococcus thermophilus*, *Pseudomonas*,

*Saccharomyces cerevisiae*, *Enterococcus raffinosus*, *E. avium*, *E. faecium*, *Asepergillus oryzae*, *Candida utilis*, and *C. fermani* have been reported (Sarasa et al. 2020). However, GABA-producing LAB from the human body remain poorly studied. In fact, only a few gut-originated strains have been shown to produce GABA. Recently, Otaru et al. (2021) investigated the GABA production by human gut-derived *Bacteroides* in culture and metabolic assays combined with genomics and phylogenetics, resulting in a high prevalence of the Gad-system among *Bacteroides* genomes harboring all genes of the Gad-system. Yunes et al. (2016) screened the collection of 135 human-derived *Lactobacillus* and *Bifidobacterium* strains for their ability to produce GABA from its precursor, monosodium



**Fig. 6** Effects of chemical reagents and coenzymes on rLgGad. Effects of **a** metal reagents, **b** PLP concentrations, and **c** various coenzymes. The residual enzyme activity was expressed as the relative percentage rLgGad activity

glutamate. In their study, only 58 human gut-originated *Lactobacillus* (*Lacti. plantarum* and *Levi. brevis*), and *Bifidobacterium* (*B. adolescentis*, *B. angulatum*, and *B. dentium*) are able to produce GABA (Yunes et al. 2016). *Lacti. plantarum* and *Levi. brevis* is the most widely found species capable of producing GABA (Sarasa et al. 2020). The gastrointestinal (GI) tract of mammalian hosts is inhabited by numerous and diverse gut microbiota. The human gut is a complex ecosystem and a good habitat for commensal microbiota, which is more than 1,000 species with a population of up to  $10^{14}$  (Sender et al. 2016). The human gut microbiota is mainly composed of *Firmicutes* and *Bacteroidetes*, accounting for more than 90% of the entire phylogenetic classification, and significant numbers of *Actinobacteria* and *Proteobacteria* (Eckburg et al. 2005; El Kaoutari et al. 2013). The numbers of human gut-commensal LABs were less than 1% of the total bacterial population of the human gut (Heeney et al. 2018). However, from a probiotic perspective, research on human gut-commensal LAB producing GABA is of great importance. An extensive literature confirmed GABA as a mediator within the enteric nervous system controlling GI function, and therefore the presence of GABA in the human gut is imperative (Auteri et al. 2015). Probiotic strains derived from the human gut are inherently hypothesized to have great advantages in adaptability in the host gut. Therefore, human gut-commensal probiotic strains can be considered as delivery vehicles for GABA to specific regions of the gut (Ghatge et al. 2012).

As a beneficial symbiotic gut microbiota, LAB have been extensively studied for their functional elucidation. In this study, we screened and isolated GABA-producing LAB from the human fecal microbiota. A high GABA-producing *Lactococcus* species was isolated and identified as belonging to *Lactococcus garvieae*. To date, few studies have described GABA-producing *Lactococcus* species (for example, *Lc.*

*lactis*), and Gads. Several GABA-producing *L. lactis* strains isolated from cheese, kimchi, frozen peas, yam pickle, and dairy have been reported (Lyu et al. 2021).

So far, various Gads from various LABs have been characterized as recombinant enzyme and natural forms (Table 1). In fact, the direct recovery of microbial Gads in natural form is time-consuming and needs the expensive tasks with further processes using protein precipitation and purification. However, the production of heterologous proteins using an expression host (e.g. *E. coli*) has the advantages of efficient and mass production (Hannig and Makrides 1998). In this study, the *lggad* gene encoding a Gad from human gut-originated *Lc. garvieae* MJF010 was cloned and overexpressed in *E. coli*. Thereafter, the resulting rLgGad protein carrying C-terminal His<sub>6</sub> tag was purified Ni-NTA chromatography before enzymatic characterization. The purified rLgGad finally showed that the optimal temperature and pH for its activity was 35 °C and pH 5, respectively. The reported Gads-derived LAB including rLgGad showed optimal activity at 30–45 °C and under acidic conditions (pH range 4–5). The main function of Gad is to encounter acidic stress by consuming the hydrogen ions in the cytoplasm in the presence of L-glutamate, of which is called the glutamate-dependent acid-resistance system (Cotter and Hill 2003; Gong et al. 2019). Therefore, the expression of Gad requires LAB to survive under acidic pH, following that the increased Gad activity is critical to survive in acidic condition (Sarasa et al. 2020; Yogeswara et al. 2020). Consequently, most LAB-derived Gads exhibit optimal activity at pH 4–5, whereas they significantly lose the activity at near-neutral pH. The activity of rLgGad was significantly increased by adding metal reagents, in particular CaCl<sub>2</sub>, whereas AgNO<sub>3</sub> completely inhibited its activity. These results were consistent with the previously-reported LAB-derived Gads with reporting that Ca<sup>2+</sup> enhanced the GABA conversion activity, but

$\text{Ag}^+$  inhibited it. The other ions such as  $\text{K}^+$ ,  $\text{Mg}^{2+}$ ,  $\text{Zn}^{2+}$ ,  $\text{NH}_4^+$ ,  $\text{Co}^{2+}$ ,  $\text{Fe}^{3+}$ ,  $\text{Na}^+$  were inconsistent, either as activators or inhibitors. The  $K_m$  value of rLgGad characterized in this study was determined to be 2.94 mM, which was lower than those of corresponding Gads from most LABs except for *Levi. brevis* IFO 12,005 (Higara et al. 2008) and *Lactobacillus zymae* GU240 (Park et al. 2014), indicating that rLgGad has higher affinity for MSG compared to other previously reported Gads.

Gad has been known to be a PLP-dependent enzyme and apoGad can be converted to an active Gad-PLP complex in the presence of free PLP (Yogeswara et al. 2020). Therefore, the addition of PLP could enhance GABA biosynthesis by acting as a cofactor for the Gad enzyme. Several studies have reported that the supplement with PLP in the bacterial culture could increase the Gad biosynthesis in *Lacti. Paracasei* and *E. coli* (Su et al. 2015), and the GABA production in *Bacillus subtilis*, *Lacti. Plantarum*, and *Streptococcus salivarius* (Cui et al. 2020). Since PLP is required by a number of key enzymes, cells probably synthesize PLP at a sufficient rate when the Gad is expressed at relatively acceptable levels (Li et al. 2010; Yang et al. 2008). However, under most of the overexpression conditions described in previous reports, the PLP concentration generated in the normal growth of the cells was not sufficient for Gad overexpression (Su et al. 2015). Whereas, it has been reported that *E. coli* can biosynthesize PLP by *de novo* and salvage pathways (Ghatge et al. 2012). Therefore, it could be suggested that the rLgGad produced in recombinant *E. coli* was expressed as a Gad-PLP complex. The further rationale for this suggestion is that the GABA-producing activity of rLgGad in this study was not affected by exogenous PLP, despite that Gad is a PLP-dependent enzyme. Similar results have also been previously reported for Gads from *Levi. brevis* (Fan et al. 2012; Li et al. 2013). Perhaps, the expression condition applied in these studies produced adequate amount of PLP to form Gad-PLP complex with host-generated PLP. In addition, it should be noted that the addition of another coenzymes (pyridoxal HCl, pyridoxine HCl, and pyridoxine) did not affect the activity of rLgGad expressed in Gad-PLP complex.

In this study, as the first strain of GABA-producing *Lc. garvieae* species, the *gad* gene of *Lc. garvieae* MJF010 was first expressed in *E. coli*. In addition, the enzymatic properties of recombinant Gad were determined. Various methods are used to produce GABA, among which biosynthetic approaches, mainly microbial and enzymatic methods, are considered more effective. In this regard, the ability to synthesize GABA is an important criterion in the selection of microbial strains. The results of this study provide information on the Gad of the *Lactococcus* genus. Thus, the results of this study will contribute to the ongoing discovery of Gads.

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## Declarations

**Conflict of interest** The authors declare that they have no conflicts of interest.

**Ethical approval** All applicable international, national, and/or institutional guidelines for the care and use of human derivatives were followed. The study of human derivatives was approved by the Institutional Review Board of Incheon National University University (IRB 7007971-202006-001 A).


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