

## *Lactobacillus futsaii* CS3, a New GABA-Producing Strain Isolated from Thai Fermented Shrimp (*Kung-Som*)

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**Abstract** *Kung-Som* is a popular traditional Thai fermented shrimp product. It is rich in glutamic acid, which is the major substrate for the biosynthesis of gamma-aminobutyric acid (GABA) by lactic acid bacteria (LAB). In the present study, LAB from *Kung-Som* were isolated, screened for GABA formation, and the two isolates that transform glutamic acid most efficiently into GABA were identified. Based on the API-CHL50 fermentation profile and a phylogenetic tree of 16S rDNA sequences, strain CS3 and CS5 were identified as *Lactobacillus futsaii*, which was for the first time shown to be a promising GABA producer. *L. futsaii* CS3 was the most efficient microorganism for the conversion of 25 mg/mL monosodium glutamate (MSG) to GABA, with a maximum yield of more than 99% conversion rate within 72 h. The open reading frame (ORF) of the glutamate decarboxylase (*gad*) gene was identified by PCR. It consists of 1410 bp encoding a polypeptide of 469 amino acids with a predicted molecular weight of

53.64 kDa and an isoelectric point (pI) of 5.56. Moreover, a good quality of the constructed model of *L. futsaii* CS3 was also estimated. Our results indicate that *L. futsaii* CS3 could be of interest for the production of GABA-enriched foods by fermentation and for other value-added products.

**Keywords** Gamma-aminobutyric acid (GABA) · Glutamate decarboxylase (*gad*) gene · *Kung-Som* · Lactic acid bacteria · *Lactobacillus futsaii*

### Introduction

Gamma-aminobutyric acid (GABA) is a four-carbon free amino acid that is widely present in bacteria, plants and vertebrates. GABA is well known as a bioactive substance with several physiological functions and hence a great application potential in functional foods and pharmaceuticals. It acts as major inhibitory neurotransmitter in the sympathetic nervous system, and shows anti-diabetic and antihypertensive effects in humans. In addition, consumption of GABA-enriched foods inhibits cancer cell proliferation and can control lipid levels in serum, as well as pain and anxiety [1, 2].

*Kung-Som* is a Thai fermented food made from shrimp, sugar, salt, and is typically fermented with a natural, spontaneous microbiota, mainly various lactic acid bacteria (LAB) [3]. In general, LAB can prolong the shelf life of food, enhance its safety and nutritional value, improve food texture, and contribute to a pleasant sensory profile of the final product. Therefore, the use of suitable GABA-producing LAB strains as starter cultures in food fermentation processes can help to achieve bio-synthetic in situ production of GABA in various products. This provides a way of replacing chemically synthesized GABA, and at the

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same time offering the consumer with new attractive, GABA-enriched food products. This approach also reduces the production cost because the addition of exogenous GABA is omitted [4].

Glutamate decarboxylase (GAD) catalyzes the decarboxylation of glutamic acid or glutamate to GABA and is thus the essential enzyme for GABA production. The enzyme needs pyridoxal 5'-phosphate (PLP) as a cofactor [5]. Glutamic acid is found in *Kung-Som* products in ample amounts since the main ingredient shrimp is a rich source of glutamic acid [6]. Yet, studies on GABA-producing LAB in *Kung-Som* have not been reported. The development of *Kung-Som* containing higher levels of GABA that will contribute additional functional properties to this product seems a promising application. In the present study, the isolation and the identification of GABA-producing LAB from *Kung-Som* were performed.

## Materials and Methods

### Isolation and Screening of GABA-Producing LAB

Twelve *Kung-Som* samples were purchased from different local markets in Songkhla Province, Thailand. Samples were homogenized in sterile 0.1% (w/v) peptone (HiMedia, Mumbai, India), spread onto MRS agar (LabM, Lancashire, UK) supplemented with 0.02% (w/v) bromocresol purple and incubated at 37 °C for 24 h. Bacterial colonies exhibiting a yellow zone on the plates were tested for catalase activity and Gram-staining. Catalase-negative and Gram-positive isolates were cultivated in MRS broth containing 25 mg/mL monosodium glutamate (MSG) (Ajinomoto, Bangkok, Thailand) at 37 °C for 24 h. The culture was then centrifuged at 8000×g at 4 °C for 5 min to obtain a clear supernatant. Presence of GABA in this supernatant was first analyzed by using thin-layer chromatography (TLC). Briefly, GABA was determined qualitatively using Silicagel 60 F<sub>254</sub> TLC plates (Merck, Darmstadt, Germany). One microliter of supernatant was spotted onto the TLC plates. TLC was conducted using an 1-butanol:acetic acid:distilled water (5:2:2) mixture, the plate was subsequently sprayed with 0.5% (w/v) ninhydrin solution and then heated at 105 °C for 2 min. The R<sub>f</sub> value was calculated as follows: R<sub>f</sub> = migration distance by component/migration distance by solvent. Cultures of bacterial strains showing the same R<sub>f</sub> value as the authentic GABA standard (Sigma-Aldrich, Steinheim, Germany) were selected for quantitation by HPLC.

### Identification of GABA-Producing LAB

Morphological characterization of the best GABA-producing isolates was done by examining colony growth, cell

morphology, together with the Gram reaction and catalase test. Biochemical characterization was performed using the API 50 CHL fermentation strips (bioMérieux, Inc., Marcy l'Etoile, France).

Genomic DNA from the best GABA producing isolates was extracted and used as template for PCR to amplify the 16S rDNA by using the universal bacterial primers 8f (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492r (5'-GGTTACCTTGTTCAGACTT-3'). Purified PCR products were cloned into the pGEM T-Easy vector (Promega, Madison, USA) and transformed into *E. coli* JM109 by the heat shock method [7]. The sequencing results were compared with known sequences using the BLAST program ([www.ncbi.nlm.nih.gov/BLAST/](http://www.ncbi.nlm.nih.gov/BLAST/)).

### Growth Profile and GABA Production of *Lactobacillus futsaii* CS3

*L. futsaii* CS3 was cultivated in MRS broth with 25 mg/mL of total MSG containing 1% (v/v) inoculum (OD<sub>600</sub> ~ 0.4–0.5). Incubation was performed at 37 °C for 72 h. Growth of the strain was determined by measuring culture turbidity at 600 nm every 3 h. In addition, culture pH, lactic acid content [8] and GABA concentration were measured at the same time intervals. The GABA conversion efficiency (%) was calculated from the equation according to Ratanaburee et al. [9].

### GABA Analysis

#### Sample Preparation

Quantitative analysis of GABA in the culture broth was performed by derivatization with *o*-phthalaldehyde (OPA) (Sigma-Aldrich, Steinheim, Germany) before detection using RP-HPLC according to Populin et al. [10] with some modifications. The OPA solution (pH 9.3) was prepared by mixing 5.0 mL of methanolic OPA (0.25 g of OPA in 50 mL of methanol), 20 mL of borate buffer (pH 9.9) and 50 µL of 2-mercaptoethanol. The borate buffer solution was prepared by mixing 0.2 M boric acid (dissolved in 0.2 M potassium chloride) and 0.2 M sodium hydroxide in a ratio of 50:50 (v/v). Fifteen microliters of sample and 75 µL of the OPA solution were mixed and allowed to stand for 2 min before injection into the column.

#### Reversed-Phase High-Performance Liquid Chromatography (RP-HPLC) Analysis

RP-HPLC determination of GABA was performed with an Agilent Technologies 1200 series binary pump, autosampler and a fluorescence detector equipped with an automatic

liquid sampler, injector program. The column used was a Hypersil ODS C<sub>18</sub> reverse phase column (4.6 × 250 mm, 5 μm) thermostatted at 30 °C. The separation of OPA-derivatives was performed with a mobile phase, consisting of 370 mL of milli-Q water plus 90 mL of 0.1 M phosphate buffer at pH 7.0 as solvent A, while solvent B was acetonitrile. The gradient elution program was held at 13% of B for 15 min, ramped at 50% of B (40 min), then at 85% of B (60 min) and held until the end of the run (62 min) with a flow rate of 1.0 mL/min. Detection was performed with a spectrofluorometer, model G1321A set at 330 nm ( $\lambda$  excitation) and 440 nm ( $\lambda$  emission).

### Cloning of the *gad* Gene

Based on a sequence comparison of open reading frames (ORF) of the glutamate decarboxylase genes (*gad*) from *Lactobacillus* available in GenBank ([www.ncbi.nlm.nih.gov](http://www.ncbi.nlm.nih.gov)) and conserved sequences identified, a pair of primers, *gad*\_F (5'-ATGGCAATGTTATACGGTAAACAC-3') and *gad*\_R (5'-TCAGTGTGTGAATCCGTATTTCTT-3'), was designed to amplify the ORF of the *gad* gene from *L. futsaii*. The amplified fragments were cloned into the pGEM T-Easy vector and transformed into competent cells of *E. coli* JM109 by the heat shock method [7]. Similarity of the isolated *gad* gene with others was analyzed using the online BLAST program. The molecular mass and the predicted isoelectric point (pI) of the corresponding glutamate decarboxylase protein (GAD) were obtained through ExPASy.

### Computer Modeling

The molecular model of GAD from *L. futsaii* CS3 was obtained by homology modeling via the SWISS-MODEL server [11]. The structure of GadB from *E. coli* at low pH (PDB ID: 1PMM) was selected as the template for model building. Sequence alignment was performed using ClustalX [12]. The resulting model was evaluated with the SWISS-MODEL server.

## Results and Discussion

### Screening and Identification of GABA-Producing LAB

Among 600 single LAB isolates tested, only two isolates (CS3 and CS5) exhibited a strong spot for GABA by the TLC method when MSG was present in the growth medium (Fig. 1). In addition, GABA formation was quantitatively confirmed using RP-HPLC. The GABA concentration when using strain CS3 was 3.25 and 6.30 mg/mL at 24 and 48 h,

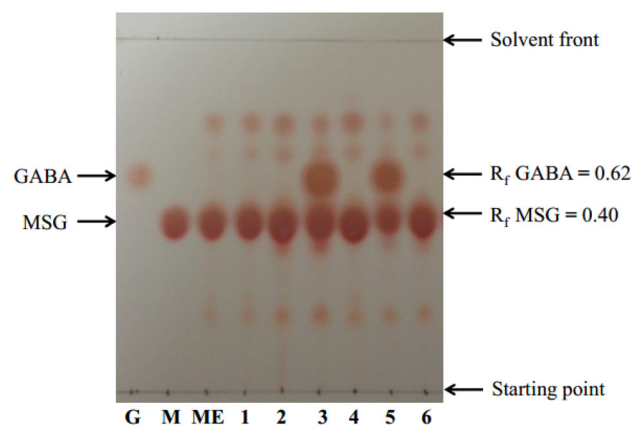
respectively. Strain CS5 produced GABA at 2.55 and 6.0 mg/mL after 24 and 48 h, respectively.

The two selected, GABA-producing isolates CS3 and CS5 were Gram-positive, rod-shaped, catalase negative and facultative anaerobic bacteria. The phenotypic results obtained with the API system (Table S1) indicated that both strain CS3 and CS5 correspond to the species *Lactobacillus futsaii* when compared with the type strains *L. futsaii* YM0097<sup>T</sup>, JCM 17355<sup>T</sup> or BCRC 80278<sup>T</sup> according to Chao et al. [13].

In addition, 16S rDNA sequence analysis (Fig. S1) showed that strains CS3 and CS5 belong to the species *L. futsaii* with 99.80 and 99.66% identity of the sequence from *L. futsaii* YM0097<sup>T</sup> (accession no. HQ322270), respectively. Therefore, the strains CS3 and CS5 were designated as *L. futsaii* CS3 and *L. futsaii* CS5, respectively. The nucleotide sequences of the 16S rDNA from *L. futsaii* CS3 and CS5 were deposited in the DNA Data Bank of Japan (DDBJ) under the accession numbers AB839950 and LC019014, respectively.

### Growth Profile and GABA Production of *L. futsaii* CS3

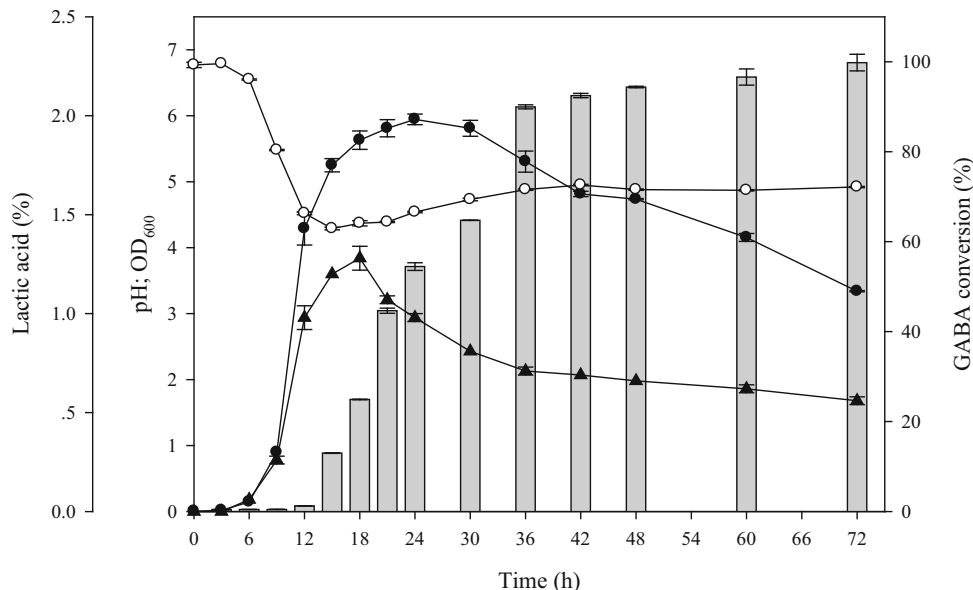
Growth and GABA production of *L. futsaii* CS3 are shown in Fig. 2. The bacterium grew rapidly within the first 24 h (~9 log CFU/mL), which was correlated with a sharp decrease in pH due to lactic acid formation. The lactic acid concentration reached a maximum of ~1.3% after the pH had dropped to 4.5. Only when the pH value of the medium dropped to pH 4.5, GABA was produced. Subsequently, the concentration of GABA reached a plateau with a maximum concentration around 36 h. We noted that *L. futsaii* CS3 could almost completely convert MSG to GABA with a maximum yield of more than 99% MSG



**Fig. 1** Thin-layer chromatography analysis of GABA production by isolated LAB. Lane G, GABA standard; M, MSG standard; ME, MRS medium containing 25 mg/mL MSG (control); 1–6, isolate CS1–CS6, respectively

conversion within 72 h. Initially, the pH value decreased significantly due to lactic acid formation by *L. futsaii* during growth, especially during the exponential phase, whereas the pH gradually increased once GABA formation started. Our results show a trend similar to that reported by Cho et al. [14] who indicated that increased growth and a decreased pH had a significant effect on GABA production. In addition, Lin [15] reported that GABA synthesis is related to bacterial GAD, which plays a major role in the

**Fig. 2** Time course of a cultivation of *Lactobacillus futsaii* CS3 in MRS broth containing 25 mg/mL MSG. Symbols: optical density at 600 nm (filled circle), GABA production (% GABA conversion) (grey bars), broth pH value (open circle) and lactic acid concentration (filled triangle)



**Fig. 4** Alignment of the amino acid sequence of *Lactobacillus futsaii* CS3 GadB (LfuCS3) with GadB proteins from other LAB (LpWCFS1, *Lactobacillus plantarum* WCFS1; LpJDM1, *Lactobacillus plantarum* JDM1; LbCGMCC1306, *Lactobacillus brevis* CGMCC1306; LrATCC23272, *Lactobacillus reuteri* ATCC23272; LdATCC11842, *Lactobacillus delbrueckii* subsp. *bulgaricus* ATCC11842; LcATCC11454, *Lactococcus lactis* subsp. *lactis* ATCC11454; LfYS2, *Lactobacillus fermentum* YS2). The amino acid residues (HVDAASGG) marked by a box are highly conserved in pyridoxal 5'-phosphate-dependent decarboxylases; the amino acid residues in the second box represent the pyridoxal 5'-phosphate binding domain

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M A M L Y G K H N H E A E E Y L E P V F G A P S E Q H D L P
1 ATGGCAATGTTATACGGTAAACACAAATCATGAAGCTGAAGAATACTTGGAAACAGTCTTTGGTGCGCCTTCTGAGCAACATGATCTTCCT
  K Y R L P K H S L S P R E A D R L V R D E L L D E G N S R L
91 AAGTATCGGTTACCAAAGCATTTCATTATCCCCTCGAGAAGCCGATCGCTTAGTTCGTGATGAATTATTAGATGAAGGCAATTCACGACTG
  N L A T F C Q T Y M E P E A V E L M K D T L A K N A I D K S
181 AACCTGGCAACTTTTTGTCAGACCTATATGGAACCCGAAGCCGTGGAATTGATGAAGGATACGCTGGCTAAGAATGCCATCGACAAATCT
  E Y P R T A E I E N R C V N I A N L W H A P D D E H F T G
271 GAGTACCCCGCAGCGCCGAGATTGAAAATCGGTGTGTGAACATTATGCCAACTCTGTGGCAGCACCTGATGACGAACACTTTACGGGT
  T S T I G S S E A C M L G G L A M K F A W R K R A Q A A G L
361 ACCTCTACGATTGGCTCCTCTGAAGCTTGTATGTTAGGCGGTTTAGCAATGAAATTCGCTGGCGTAAACGCGCTCAAGCGGCAGGTTTA
  D L N A H R P N L V I S A G Y Q V C W E K F C V Y W D V D M
451 GATCTGAATGCCATCGACCTAACCTCGTTATTTGCGCTGGCTATCAAGTTTGTCTGGGAAAAGTTTGTGTCTACTGGGACGTTGACATG
  H V V P M D E Q H M A L D V N H V L D Y V D E Y T I G I V G
541 CACGTGGTCCCAATGGATGAGCAACACATGGCCCTTGACGTTAACCCAGTCTTAGACTACGTGGACGAATACACAATGGTATCGTCGGT
  I M D I T Y T G Q Y D D L A A L D K V V T H Y N H Q H P K L
631 ATCATGGACATCACTTATACCGGTCAATATGACGACCTAGCCGCACTGATAAGGTCGTTACTCACTACAATCATCAGCATCCCAAATTA
  P V Y I H V D A A S G G F Y T P F I E P Q L I W D F R L A N
721 CCAGTCTACATTCAGTTCAGCAGCGTTCAGTGGCTTCTATACCCATTATTAGCCGCAACTCATCTGGGACTTCCGGTGGCTAAC
  V V S I N A S G H K Y G L V Y P G V G W V V W R D R Q F L P
811 GTCGTTTCGATCAACGCTCCGGGCACAAGTACGTTTTAGTTTTATCTGGGGTCCGGTGGTTCGTTTGGCGTGATCGTCAGTTTTTACCG
  P E L V F K V S Y L G G E L P T M A I N F S H S A A Q L I G
901 CCAGAATTAGTCTTCAAAGTTAGTTATTTAGTGGGGAGTTGCCGACAAATGGCGATCAATTTCTCACATAGTGCAGCCCAGCTCATTGGA
  Q Y Y N F I R F G M D G Y R E I Q T K T H D V A R Y L A A A
991 CAATACTATAATTTTCATTCGCTTTGGTATGGACGGTTACCGCGAGATTCAAACAAAGACTCACGATGTTGCCCGCTACCTGGCAGCCGCT
  L D K V G E F K M I N N G H Q L P L I C Y Q L A P R E D R E
1081 CTGGATAAAGTTGGTGAAGTTAAGATGATCAATAACGGACCAACTCCCTGATTTGTTACCAACTAGCCCCGCGGAAGATCGTGAA
  W T L Y D L S D R L L M N G W Q V P T Y P L P A N L E Q Q V
1171 TGGACCTTTATGATTTATCGGATCGCCTATTAATGAACGGTTGGCAAGTACCAAGTATCCTTTACCTGCTAATCTGGAACAACAAGTC
  I Q R I V V R A D F G M N M A H D F M D D L T K A V H D L N
1261 ATCCAACGAATCGTTCGGCTGACTTTGGCATGAATATGGCCACGATTTCTGGATGACCTGACCAAGGCTGTCCATGACTTAAAC
  H A H I V Y H H D A A P K K Y G F T H *
1351 CACGCCACATTGTCTATCATCATGACGGGCACCTAAGAAATACGGATTACACACTGA
    
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**Fig. 3** Nucleotide and deduced amino acid sequence of the *Lactobacillus futsaii* CS3 *gadB* gene. The predicted amino acids sequence (single-letter abbreviation) is shown above the nucleotide sequence.

The presumed catalytic amino acid residues (Thr215 and Asp247, and Lys280) are shown in bold

1 75  
 LfuCS3 (1) MAMLYGKHNHEAEEYLEPVFGAPSEQHDLPKYRLPKHSLSPREADRLVRDELLDEGNSRLNLATFCQTYMEPEAV  
 LpWCF51 (1) MAMLYGKHNHEAEEYLEPVFGAPSEQHDLPKYRLPKHSLSPREADRLVRDELLDEGNSRLNLATFCQTYMEPEAV  
 LpJDM1 (1) MAMLYGKHNHEAEEYLEPVFGAPSEQHDLPKYRLPKHSLSPREADRLVRDELLDEGNSRLNLATFCQTYMEPEAV  
 LbCGMCC1306 (1) MAMLYGKHNHEAEEYLEPVFGAPSEQHDLPKYRLPKHSLSPREADRLVRDELLDEGNSRLNLATFCQTYMEPEAV  
 LrATCC23272 (1) MAMLYGKHNHEAEEYLEPVFGAPSEQHDLPKYRLPKHSLSPREADRLVRDELLDEGNSRLNLATFCQTYMEPEAV  
 LdATCC11842 (1) MAMLYGKHNHEAEEYLEPVFGAPSEQHDLPKYRLPKHSLSPREADRLVRDELLDEGNSRLNLATFCQTYMEPEAV  
 LcATCC11454 (1) MAMLYGKHNHEAEEYLEPVFGAPSEQHDLPKYRLPKHSLSPREADRLVRDELLDEGNSRLNLATFCQTYMEPEAV  
 LfYS2 (1) -MSLYGKYDQFERNTLDEHFGSFEEDHPLPKYKLNERPVEPRITAYQVVKDQLLDEGNARLNLATFCQTYMEPEAV  
 Consensus (1) MAMLYGKHNHEAEEYLEPVFGAPSEQHDLPKYRLPKHSLSPREADRLVRDELLDEGNSRLNLATFCQTYMEPEAV

76 150  
 LfuCS3 (76) ELMKDTLA-KNAIDKSEYPRTAETENRCVNIANLWHAPDDEHFTGTSTIGSSEACMLGGIAKFAWRKRAQAAG  
 LpWCF51 (76) ELMKDTLA-KNAIDKSEYPRTAETENRCVNIANLWHAPDDEHFTGTSTIGSSEACMLGGIAKFAWRKRAQAAG  
 LpJDM1 (76) ELMKDTLA-KNAIDKSEYPRTAETENRCVNIANLWHAPDDEHFTGTSTIGSSEACMLGGIAKFAWRKRAQAAG  
 LbCGMCC1306 (76) ELMKDTLA-KNAIDKSEYPRTAETENRCVNIANLWHAPDDEHFTGTSTIGSSEACMLGGIAKFAWRKRAQAAG  
 LrATCC23272 (76) ELMKDTLA-KNAIDKSEYPRTAETENRCVNIANLWHAPDDEHFTGTSTIGSSEACMLGGIAKFAWRKRAQAAG  
 LdATCC11842 (76) ELMKDTLA-KNAIDKSEYPRTAETENRCVNIANLWHAPDDEHFTGTSTIGSSEACMLGGIAKFAWRKRAQAAG  
 LcATCC11454 (76) ELMKDTLA-KNAIDKSEYPRTAETENRCVNIANLWHAPDDEHFTGTSTIGSSEACMLGGIAKFAWRKRAQAAG  
 LfYS2 (75) KLMSETFED-KNAIDKSEYPRTAETENRCVNIANLWHAPDDEHFTGTSTIGSSEACMLGGIAKFAWRKRAQAAG  
 Consensus (76) ELMKDTLA-KNAIDKSEYPRTAETENRCVNIANLWHAPDDEHFTGTSTIGSSEACMLGGIAKFAWRKRAQAAG

151 225  
 LfuCS3 (150) LDLNAHRPNLVISAGYQVCWEKFCVYWDVDMHVVPMDQHMALDVNHVLDYVDYTTIGIVGIMGITTYTGQYDDLA  
 LpWCF51 (150) LDLNAHRPNLVISAGYQVCWEKFCVYWDVDMHVVPMDQHMALDVNHVLDYVDYTTIGIVGIMGITTYTGQYDDLA  
 LpJDM1 (150) LDLNAHRPNLVISAGYQVCWEKFCVYWDVDMHVVPMDQHMALDVNHVLDYVDYTTIGIVGIMGITTYTGQYDDLA  
 LbCGMCC1306 (150) LDLNAHRPNLVISAGYQVCWEKFCVYWDVDMHVVPMDQHMALDVNHVLDYVDYTTIGIVGIMGITTYTGQYDDLA  
 LrATCC23272 (150) LDLNAHRPNLVISAGYQVCWEKFCVYWDVDMHVVPMDQHMALDVNHVLDYVDYTTIGIVGIMGITTYTGQYDDLA  
 LdATCC11842 (151) LDLNAHRPNLVISAGYQVCWEKFCVYWDVDMHVVPMDQHMALDVNHVLDYVDYTTIGIVGIMGITTYTGQYDDLA  
 LcATCC11454 (150) LDLNAHRPNLVISAGYQVCWEKFCVYWDVDMHVVPMDQHMALDVNHVLDYVDYTTIGIVGIMGITTYTGQYDDLA  
 LfYS2 (149) LDVKAHRPNLVISAGYQVCWEKFCVYWDVDMHVVPMDQHMALDVNHVLDYVDYTTIGIVGIMGITTYTGQYDDLA  
 Consensus (151) LDLNAHRPNLVISAGYQVCWEKFCVYWDVDMHVVPMDQHMALDVNHVLDYVDYTTIGIVGIMGITTYTGQYDDLA

226 Motif PLP-binding domain 300  
 LfuCS3 (225) ALDKVVTTHYNHQPPLVYIHVDAASGGIYTPFTEPOLWDFRLANV[SINASGHKYGLVYPGVVWRDRQFL  
 LpWCF51 (225) ALDKVVTTHYNHQPPLVYIHVDAASGGIYTPFTEPOLWDFRLANV[SINASGHKYGLVYPGVVWRDRQFL  
 LpJDM1 (225) ALDKVVTTHYNHQPPLVYIHVDAASGGIYTPFTEPOLWDFRLANV[SINASGHKYGLVYPGVVWRDRQFL  
 LbCGMCC1306 (225) RLDAVVERYN-RTTKFVYIHVDAASGGIYTPFTEPOLWDFRLANV[SINASGHKYGLVYPGVVWRDRQFL  
 LrATCC23272 (225) ALDKVVTTHYNHQPPLVYIHVDAASGGIYTPFTEPOLWDFRLANV[SINASGHKYGLVYPGVVWRDRQFL  
 LdATCC11842 (226) ALDKVVTTHYNHQPPLVYIHVDAASGGIYTPFTEPOLWDFRLANV[SINASGHKYGLVYPGVVWRDRQFL  
 LcATCC11454 (225) ALDKVVTTHYNHQPPLVYIHVDAASGGIYTPFTEPOLWDFRLANV[SINASGHKYGLVYPGVVWRDRQFL  
 LfYS2 (224) ALDKVVTTHYN-QHTDYKYVYIHVDAASGGIYTPFTEPOLWDFRLANV[SINASGHKYGLVYPGVVWRDRQFL  
 Consensus (226) ALDKVVTTHYNHQPPLVYIHVDAASGGIYTPFTEPOLWDFRLANV[SINASGHKYGLVYPGVVWRDRQFL

301 375  
 LfuCS3 (300) PPELVFKVSYLGGELPTMAINFSHSAQQLIGQYNNFIRFGMDGYREIQTKTHDVARYLAAALDKVGEFKMINNGH  
 LpWCF51 (300) PPELVFKVSYLGGELPTMAINFSHSAQQLIGQYNNFIRFGMDGYREIQTKTHDVARYLAAALDKVGEFKMINNGH  
 LpJDM1 (300) PPELVFKVSYLGGELPTMAINFSHSAQQLIGQYNNFIRFGMDGYREIQTKTHDVARYLAAALDKVGEFKMINNGH  
 LbCGMCC1306 (299) PPELVFKVSYLGGELPTMAINFSHSAQQLIGQYNNFIRFGMDGYREIQTKTHDVARYLAAALDKVGEFKMINNGH  
 LrATCC23272 (300) PPELVFKVSYLGGELPTMAINFSHSAQQLIGQYNNFIRFGMDGYREIQTKTHDVARYLAAALDKVGEFKMINNGH  
 LdATCC11842 (301) PPELVFKVSYLGGELPTMAINFSHSAQQLIGQYNNFIRFGMDGYREIQTKTHDVARYLAAALDKVGEFKMINNGH  
 LcATCC11454 (300) PPELVFKVSYLGGELPTMAINFSHSAQQLIGQYNNFIRFGMDGYREIQTKTHDVARYLAAALDKVGEFKMINNGH  
 LfYS2 (298) PPELVFKVSYLGGELPTMAINFSHSAQQLIGQYNNFIRFGMDGYREIQTKTHDVARYLAAALDKVGEFKMINNGH  
 Consensus (301) PPELVFKVSYLGGELPTMAINFSHSAQQLIGQYNNFIRFGMDGYREIQTKTHDVARYLAAALDKVGEFKMINNGH

376 450  
 LfuCS3 (375) QLPLICYQLAPREDREWTLYDLSDRLLMNGWVPTYPPLPANLEQQVQRIIVVRADFGMMAHDFMDDLTKAVHDL  
 LpWCF51 (375) QLPLICYQLAPREDREWTLYDLSDRLLMNGWVPTYPPLPANLEQQVQRIIVVRADFGMMAHDFMDDLTKAVHDL  
 LpJDM1 (375) QLPLICYQLAPREDREWTLYDLSDRLLMNGWVPTYPPLPANLEQQVQRIIVVRADFGMMAHDFMDDLTKAVHDL  
 LbCGMCC1306 (374) QLPLICYQLAPREDREWTLYDLSDRLLMNGWVPTYPPLPANLEQQVQRIIVVRADFGMMAHDFMDDLTKAVHDL  
 LrATCC23272 (375) QLPLICYQLAPREDREWTLYDLSDRLLMNGWVPTYPPLPANLEQQVQRIIVVRADFGMMAHDFMDDLTKAVHDL  
 LdATCC11842 (376) QLPLICYQLAPREDREWTLYDLSDRLLMNGWVPTYPPLPANLEQQVQRIIVVRADFGMMAHDFMDDLTKAVHDL  
 LcATCC11454 (375) QLPLICYQLAPREDREWTLYDLSDRLLMNGWVPTYPPLPANLEQQVQRIIVVRADFGMMAHDFMDDLTKAVHDL  
 LfYS2 (373) RLPLICYQLAPREDREWTLYDLSDRLLMNGWVPTYPPLPANLEQQVQRIIVVRADFGMMAHDFMDDLTKAVHDL  
 Consensus (376) QLPLICYQLAPREDREWTLYDLSDRLLMNGWVPTYPPLPANLEQQVQRIIVVRADFGMMAHDFMDDLTKAVHDL

451 470  
 LfuCS3 (450) NHAHIVYHHDAAPKKYGFTH  
 LpWCF51 (450) NHAHIVYHHDAAPKKYGFTH  
 LpJDM1 (450) NHAHIVYHHDAAPKKYGFTH  
 LbCGMCC1306 (449) DCAHIVYHSHDAPKKYGFTH  
 LrATCC23272 (450) NHAHIVYHHDAAPKKYGFTH  
 LdATCC11842 (451) NHAHIVYHHDAAPKKYGFTH  
 LcATCC11454 (450) NHAHIVYHHDAAPKKYGFTH  
 LfYS2 (448) NCAHIVYHHEKSSLKKYGFTH  
 Consensus (451) NHAHIVYHHDAAPKKYGFTH

acid-resistance mechanism. Cytoplasmic decarboxylation results in the consumption of an intracellular proton after the uptake of glutamate by its specific transporter. The reaction product GABA is exported from cells by an antiporter, and the net result is an increase in the pH of the cytoplasm, due to the removal of hydrogen ions, and a slight increase in the extracellular pH, due to the exchange of extracellular glutamate for the more alkaline GABA [14]. Of the three acid resistance systems known in *E. coli*, the *gad* system is by far the most potent in conferring acid resistance to the bacteria in the stationary phase, giving them survival capacity for at least 2 h in a strongly acidic environment (pH < 2.5), such as that of the stomach [16]. It is hence conceivable that the *gad* system plays a similar role in lactobacilli.

### Identification of the *gad* Gene in *L. futsaii* CS3

The presence of *gad* genes in LAB has been shown in several previous reports [17, 18]. Its presence suggests an increased ability to produce GABA, since GAD must be active to convert glutamate into GABA. Using primers for conserved regions of published *gad* genes we cloned a DNA fragment from *L. futsaii*. Sequence analysis showed that this cloned fragment of the *gad* gene from *L. futsaii* CS3 contained a complete ORF of 1410 nucleotides encoding a protein of 469 amino acids (Fig. 3) with a predicted molecular weight of 53.64 kDa and an isoelectric point (pI) of 5.56 as estimated by the ExPASy program.

In addition, the deduced amino acid sequence corresponding to the CS3 *gad* gene belongs to the *gadB* family of pyridoxal-dependent decarboxylases. Sequence alignment of the deduced amino acid sequence with other GAD sequences showed a highly conserved catalytic domain that belong to the PLP-dependent decarboxylase superfamily. The deduced amino acid sequence of the *gadB* gene from *L. futsaii* CS3 showed a conserved lysine residue (Lys280) that is known to be crucial for the binding of PLP as well as the proposed active site residues (Thr215 and Asp247) that promote decarboxylation. Moreover, the motif HVDAASGG, which is highly conserved in PLP-dependent decarboxylases, was discovered in the *L. futsaii* CS3 *gad* sequence (Fig. 4) [17, 19]. Furthermore, a modeled structure of *L. futsaii* CS3 GAD was constructed using the SWISS-MODEL server (Fig. S2), which was proven statistically to be a good model according to the Global Model Quality Estimation (GMQE) value of 0.74. Since GAD is localized exclusively in the cytoplasm at neutral pH, but is recruited to the membrane when the pH falls [16], the subunit A of GadB from *E. coli* at low pH was used as the template for modeling. Correspondingly, GAD of *L. futsaii* CS3 is composed of three domains, the N-terminal domain (residues 1–59), the large domain (residues 60–350) and

the small domain (residues 351–469). The nucleotide sequence of the *gadB* gene from *L. futsaii* CS3 was submitted to the DDBJ nucleotide sequence database under the accession no. AB986192.

### Conclusion

*Lactobacillus futsaii* CS3 isolated from *Kung-Som* is a strain with potential application in novel, GABA-enriched functional foods since a number of fermented foods are based on fermentation with different *Lactobacillus* spp. In addition, we report here for the first time the sequence of the *gadB* gene from *L. futsaii*. These results may provide useful information for the potential expression of LAB *gad* genes in other microbes. The modeled structure of *L. futsaii* CS3 GAD was based on the sequence of this gene, and should be useful for future work with the enzyme. It will further expand the application area of GABA-producing LAB to GABA-enriched foods and other value-added products.

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### Compliance with ethical standards

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

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