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Review

A brief review on glutamate decarboxylase from lactic acid bacteria

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Abstract: Glutamate decarboxylase (L-glutamate-1-carboxylase, GAD; EC 4.1.1.15) is a pyridoxal 5-phosphate-dependent enzyme, which catalyzes the irreversible α-decarboxylation of L-glutamic acid to γ-aminobutyric acid (GABA) and CO_2. The enzyme is widely distributed in eukaryotes as well as prokaryotes, where it – together with its reaction product GABA - fulfils very different physiological functions. The occurrence of *gad* genes encoding GAD has been shown for many microorganisms, and GABA-producing lactic acid bacteria (LAB) have been a focus of research during recent years. A wide range of traditional foods produced by fermentation based on LAB offer the potential of providing new functional food products enriched with GABA that may offer certain health-benefits. Different GAD enzymes and genes from several strains of LAB have been isolated and characterized recently. GABA-producing LAB, biochemical properties of their GAD enzymes, and possible applications are reviewed here.

Keywords: γ-aminobutyric acid, lactic acid bacteria, glutamate decarboxylase, fermented foods, GAD genes.

1. Introduction

Lactic acid bacteria (LAB) are Gram-positive, acid-tolerant, non-sporulating bacteria forming cocci as well as rods, and sharing common physiological and metabolic characteristics. Even though many genera of bacteria produce lactic acid as their primary or secondary metabolic end-product, the term 'lactic acid bacteria' is conventionally reserved for genera in the order *Lactobacillales*, which includes *Lactobacillus*, *Lactococcus*, *Leuconostoc*, *Pediococcus* and *Streptococcus*, and in addition *Carnobacterium*, *Enterococcus*, *Oenococcus*, *Tetragenococcus*, *Vagococcus*, and *Weisella*. LAB are important for a wide range of fermented foods, and are widely used as starter cultures in traditional and industrial food fermentations [1].

Lactic acid formed during the fermentation of carbohydrates as one of the main metabolic products can affect the physiological activities of LAB. Under acidic conditions, several LAB have developed different acid-resistance systems to maintain cell viability; these systems include for example the F_0F_1-ATPase system or cation/proton antiporter/symporter systems such as the K^+-ATPase, which contribute to pH homeostasis in the cytosol by the translocation of protons [2]. In addition, glutamate or arginine-dependent systems, which require the presence of glutamate and arginine, respectively, as substrates, contribute to acid resistance of LAB. The first enzyme in the arginine-dependent system is arginine deiminase, which degrades arginine to citrulline and NH_3. Citrulline is then further converted to ornithine and exported from the cell by an ornithine/arginine antiporter. While the arginine-dependent system is based on the production of an intracellular alkaline compound, the glutamate-dependent system consumes an intracellular proton by combining

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it with internalized glutamate to γ-aminobutyric acid (GABA), and then exchanging this product for another glutamate substrate. Thereby, an extracellular amino acid is converted to an extracellular compound at the expense of an intracellular proton, which results in an increase in the intracellular pH value. This conversion of glutamate to GABA is catalyzed by glutamate decarboxylase (GAD). A wide range of LAB possess the ability to produce GAD, and its biochemical properties have been studied from a number LAB species, namely *Lactobacillus* spp., *Lactococcus* spp., and *Streptococcus* spp. [2,3]. Typically, the *gad* operon is located on the chromosomes of LAB species, with its organization varying among different species and strains [4–6]. Thus, GAD is important for acid resistance of LAB but also for the formation of GABA in LAB-fermented food. GABA is the most abundant inhibitory neurotransmitter in the brain [7,8]. It has various physiological functions, and it is of interest as an antidepressant [9], for the induction of hypotension [10,11], and because of its cholesterol-lowering effect [12]. For example, studies by Inoue et al. and Mathieu-Pouliot et al. showed that GABA-enriched dairy products could significantly decrease the systolic blood pressure in mildly hypertensive men [10,13]. Furthermore, it was shown that GABA could prevent obesity by ameliorating oxidative stress in high-fat diet in mice [14], and that it can effectively prevent diabetic conditions by acting as an insulin secretagogue [15,16]. Because of these properties, GABA or GABA-rich products are of interest as a food supplement or functional food.

GABA is primarily produced via different biotechnological approaches using either isolated GAD in a biocatalytic approach or various microbial strains [17], rather than through chemical synthesis due to the corrosive nature of the reactant compound [18]. GABA is currently commercialized as a nutritional supplement, yet interest in GABA-enriched food, in which GABA is formed *in situ* via fermentation using appropriate microorganisms, has increased lately in parallel to a general interest in functional foods. As GABA is formed as a by-product of food fermentations, LAB, which play an eminent role the fermentation of a wide range of different products, are of particular importance when talking about GABA-enriched food. Hence it is not surprising that strains isolated from various fermented food source had first been shown to have the ability to produce GABA, for example *L. namurensis* NH2 and *P. pentosaceus* NH8 from *nham* [19], *L. paracasei* NFRI 7415 from Japanese fermented fish [20], *L. paracasei* PF6, *Lactococcus lactis* PU1 and *L. brevis* PM17 from cheese [28], *L. brevis* 119-2 and *L. brevis* 119-6 from *tsuda kabu* [12], and recently many studies focused on the identification of novel GABA-producing LAB and investigated the biochemical properties of GAD from different strains in more detail [12,14,15,26,27,28,29].

Here, we outline the presence of *gad* genes in LAB as important and efficient GABA-producing organisms together with a phylogenetic analysis, we summarize biochemical data available for GAD from different LAB, and finally we give an outlook on potential applications of GAD in the manufacture of bio-based chemicals.

2. Biodiversity of GABA-producing lactic acid bacteria

LAB are among the most important organisms when it comes to the fermentation of various food raw materials. They efficiently and rapidly convert sugars into lactic acid as their main metabolic product (or one of their main products), and thus contribute to the preservation of these fermented foods. Many of these raw materials or foods contain glutamate in significant amounts, which can be utilized by LAB to increase their tolerance against acidic conditions. Hence, a number of GABA-producing LAB have been isolated from a wide range of fermented foods including cheese, *kimchi*, *paocai*, fermented Thai sausage *nham* or various fermented Asian fish products [2,13,25,30] (Table 1).

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Table 1. Diversity of GABA-producing LAB, isolation sources and GABA production. GABA concentrations as found in food products fermented with this strain or from biocatalytic, while-cell transformations of glutamate are given

LAB species and strain	Sources	GABA production	References
L. brevis HY1	*Kimchi*	18.76 mM	[27]
L. brevis NCL912	*Paocai*	149.05 mM	[28]
L. helveticus NDO1	*Koumiss*	0.16 g/L	[29]
L. brevis BJ20	Fermented *jotgal*	2.465 mg/L	[25]
L. paracasei 15C	Raw milk cheese	14.8 mg/kg	[30]
L. rhamnosus 21D-B	Raw milk cheese	11.3 mg/kg	[30]
S. thermophillus 84C	Raw milk cheese	80 mg/kg	[30]
L. plantarum DM5	*Marcha Sikkim*	NR	[31]
L. brevis L-32	*Kimchi*	38 g/L	[32]
L. buchneri WPZ001	Chinese fermented sausage	129 g/L	[33]
L. lactis	*Kimchi*	6.41 g/L	[34]
L. futsaii CS3	*Kung-som*	25 g/L	[47]
L. brevis K203	*Kimchi*	44.4 g/L	[95]
L. paracasei NFR7415	Fermented fish	302 mM	[35]
S. thermophilus Y2	Yoghurt	7.98 g/L	[36]
L. buchneri MS	*Kimchi*	251 mM	[37]
L. namurensis NH2	*Nham*	9.06 g/L	[17]
P. pentosaceus HN8	*Nham*	7.34 g/L	[17]
L. plantarum C48	Cheese	16 mg/kg	[26]
L. paracasei PF6	Cheese	99.9 mg/kg	[26]
L. brevis PM17	Cheese	15 mg/kg	[26]
L. lactis PU1	Cheese	36 mg/kg	[26]
L. delbrueckii subsp. *bulgaricus* PR1	Cheese	63 mg/kg	[26]
L. lactis subsp. *lactis*	Cheese starter	27.1 mg/L	[3]
E. faecium JK29	*Kimchi*	14.86 mM	[38]
L. brevis 877G	*Kimchi*	18.94 mM	[39]
L. brevis CECT 8183	Goat cheese	0.96 mM	[16]
L. brevis CECT 8182	Sheep cheese	0.94 mM	[16]
L. brevis CECT 8182	Goat cheese	0.99 mM	[16]
L. lactis CECT 8184	Goat cheese	0.93 mM	[16]
L. plantarum IFK 10	fermented soybean	2.68 g/L	[40]
L. plantarum	*paork kampeus*	20 mM	[1]
Weissella hellenica	*ika-kurozukuri*	7.69 g/L	[41]

NR; Not Reported.

Lactobacillus spp. are the most predominant species that have been described as GABA-producing organisms, including for example *L. brevis*, *L. paracasei*, *L. bulgaricus*, *L. buchneri*, *L.*

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plantarum, L. helveticus, or L. futsaii [2,13, 31,32,33,47]. Among these, *L. brevis,* a heterofermentative LAB, is one of the best-studied organisms [43], known for forming high levels of GABA under appropriate conditions (Table 1). Traditionally, fermented food samples containing GABA are used to screen for and isolate GABA-producing LAB, and it is not surprising that food samples with high GABA content may result in the isolation of promising strains showing good GABA-forming properties. Typical fermented foods used for isolating GABA-producing LAB are *kimchi*, where in one study 68 out of 230 LAB isolates showed the ability to convert glutamate to GABA [44] or other fermented vegetable, cheese [16] or fermented milk products, as well as various fermented meat or fish products including sausages, Thai fermented fish *plaa-som* [45], or traditional fermented Cambodian food, mainly based on fish, where 6 out of 68 LAB isolates showed a significant GABA-producing ability [1]. These screening / isolation strategies often resulted in the identification of strains capable of efficiently converting glutamate or in the discovery of novel, not-yet-identified producers of GABA, which show promise as starter cultures for various fermented foods enriched in GABA. For example, the novel GABA producer *Lactobacillus zymae*, which can grow on up to 10% NaCl and is able to utilize D-arabitol as carbon source, was isolated from *kimchi* [46]. Recently, Sanchart et al. have isolated the novel GABA-forming strain *Lactobacillus futsaii* CS3 from fermented shrimp (*Kung-som*) [47]. This isolate was able to convert 25 mg/mL of monosodium glutamate to GABA with a yield of more than 99% within 72 h. These studies (Table 1) showed that genera *Lactobacillus* and *Lactococcus* are the predominant GABA-producing LAB, but also other genera such as *Enterococcus* were studied in this respect. A novel GABA-producing *Enterococcus avium* strain was isolated from the Korean traditional fermented anchovy and shrimp (*jeotgal*), and was shown to produce 18.47 mg/mL GABA within 48 h in a medium containing glutamate as substrate. A recent study looking at LAB isolated from traditional Japanese fermented fish products (*kaburazushi*, *narezushi*, *konkazuke*, and *ishiru*) showed that out of 53 randomly picked LAB isolates 10 showed the ability of transforming considerable amounts glutamate into GABA, and identified *Weissella hellenica* as a novel GABA producer [41]. Thus, these new genera expand the list of microorganisms as GABA-producing bacteria and can be open up new and different applications in the food industry. This may lead to a wider application and flexibility of starter cultures in the food industry [9].

3. Occurrence and organization of GAD genes

The conversion of glutamate to γ-aminobutyric acid is catalyzed by glutamate decarboxylase [glutamic acid decarboxylase, GAD, systematic name L-glutamate 1-carboxy-lyase (4-aminobutanoate-forming), EC 4.1.1.15], which catalyzes the irreversible α-decarboxylation of glutamate [5,48]. GAD employs pyridoxal 5′-phosphate (PLP) as its cofactor, and is found in numerous microorganisms such as bacteria [3], fungi [49] and yeasts [50]; furthermore, GAD is found in plants [51], insects and vertebrates (52). GAD is an intracellular enzyme that is utilized by LAB to encounter acidic stress by decreasing the proton concentration in cytoplasm in the presence of L-glutamate [2,6,54]. This system, the so-called glutamate-dependent acid-resistant system (GDAR), provides protection under acidic condition, and therefore the ability of LAB to perceive and cope with acid stress is crucial for successful colonization of the gastrointestinal tract (GIT) and survival under acidic environments such as in fermented food. The GDAR system consists of two homologous inducible glutamate decarboxylases, GadA and GadB, and the glutamate/γ-aminobutyrate antiporter GadC [20,48]. The corresponding genes, i.e., *gad*A, *gad*B and *gad*C, are expressed upon entry into the stationary phase when cells are growing in rich media independently of pH, and are further induced upon hypoosmotic and hyperosmotic stress, or in the log-phase of growth in minimal medium containing glucose at a pH of 5.5 [53,55]. Siragusa et al. demonstrated that three strains with a GDAR system, *L. bulgaricus* PR1, *L. lactis* PU1 and *L. plantarum* C48, were able to survive and synthesize GABA under simulated gastrointestinal conditions [26]. Recently, the GABA-producing strain *L. futsaii* CS3 was shown to be only decreased by 1.5 log cycles under simulated gastrointestinal conditions, indicating that the GDAR system contributes to resistance to the conditions in the GIT and that GABA-producing LAB thus have a potential as functional probiotic starter cultures [47].

GAD systems and the organization of the *gad* operons among LAB species are highly variable [57]. Numerous studies reported that some LAB species such as *S. thermophilus* [5], *L. brevis* [6,7], *L. lactis* [42] have one or two *gad* gene (i.e., *gad*A, *gad*B) together with the antiporter (*gad*C). Interestingly, *E. avium* 352 carries 3 *gad* genes [58]. Typically, *L. brevis* contains two GAD-encoding genes, *gad*A and *gad*B, sharing approximately 50% amino acids sequence identity [6]. In contrast, the *gad*B genes is absent in strain *L. brevis* CD0817 [59], and the amino acid sequences identities of *gad*A and *gad*C from *L. brevis* CD0817 against other *L. brevis* strain are 91% and 90%, respectively. The transcriptional regulator gene *gad*R plays a crucial role in GABA production and acid resistance in *L. brevis*. Gong et al. reported that deletion of *gad*R in *L. brevis* ATCC 367 resulted in lower expression of both the *gad*B and *gad*C gene, a concurrent reduction in GABA synthesis and an increased sensitivity to acidic conditions [6]. Expression levels of *gad*R are varied among different LAB strains. The *gad*R gene was expressed 13–155-fold higher than *gad*CB in *L. brevis* NCL912 during the cultivation period [60]. In contrast, expression of *gad*R in *L. brevis* CGMCC1306 was observed to be much lower compared to *gad*CB. The role of *gad*A and *gad*B in *L. brevis* CGMCC1306 was investigated by disruption of the genes *gad*A, *gad*B and *gad*C resulting in complete elimination of GABA formation and increased sensitivity to acidic conditions, suggesting that both GAD proteins and the antiporter are essential for GABA production and acid resistance [61].

A genomic survey was conducted by Wu et al. to gain insight on the distribution of the *gad* operon and genes encoding glutamate decarboxylase in LAB [7]. Most strains of *L. brevis* (14 strains) as well as some strains of *L. reuteri* (6 strains), *L. buchneri* (2 strains), *L. oris* (3 strains), *L. lactis* (29 strains), and *L. garvieae* (5 strains) were shown to have an intact *gad* operon, the majority of these strains were shown to contain either *gadA* or *gadB*, whereas *gad*C is only present in the genomes of certain strains and noticeably lacking in *L. plantarum*, suggesting that the characteristic of GABA production is strain-dependent. Similar results were obtained by Yunes et al. who showed that *L. fermentum* (9 strains), *L. plantarum* (30 strains) and *L. brevis* (3 strains) typically contain *gad*B genes. In addition, no antiporter gene was observed next to *gad*B in *L. plantarum* 90sk and the expression of *gad*B was increased in early stationary phase and at low pH (3.5-5) [62]. The *gad*B gene from *S. thermophilus* encoding 459 amino acids has been investigated. The transposase genes Tn1216 (5′ and 3′) and Tn1546 are located downstream and upstream of hydrolase genes flanking the *gad*B/*gad*C operon as a result from horizontal gene transfer. This sequence implied that the order of *gad*B and *gad*C in *S. thermophilus* ST110 is similar to *S. thermophilus* Y2 [63], yet in different order from that reported for *L. brevis* [60], *L. plantarum* [62] and *L. lactis* [64].

The *L. reuteri* 100-23 genome was investigated by Su et al. for its *gad* operon [65]. This genome contains *gad*B and two genes for the antiporter (*gad*C1 and *gad*C2), as well as the glutaminase-encoding gene *gls3*, indicating that glutamine serves as a substrate for the synthesis of GABA. The organisation of the *gad* operon is in different order for other species of LAB (*L. lactis* and *L. plantarum*) as glutaminase (*gls3*) is in between the antiporters *gad*C1 and *gad*C2, while *gad*B is accompanied by *gad*C1 [65]. The full length of *gad* genes has been cloned and sequenced for several species and strains of LAB. Li et al. cloned *gad*A from *L. brevis* NCL912, and the whole gene fragment (4615 bp) including *gad*R, *gad*C, *gad*A and *gts* (glutamyl t-RNA synthetase) was successfully amplified. Their work suggested that the high GABA production capacity of *L. brevis* NCL912 may be linked to the *gad*A locus forming a *gad*CA operon complex that ensures the coordinated expression of GAD and the antiporter [60]. A core fragment of the *gad* gene from *L. brevis* OPK3 was cloned and successfully expressed in *E. coli*. The nucleotide sequence revealed that the open reading frame of the *gad* gene consisted of 1401 bases encoding 467 amino acid residues. The sequence showed 83%, 71% and 60% homology to GAD from *L. plantarum*, *L. lactis* and *Listeria monocytogenes,* respectively [66].

A phylogenetic tree constructed from available GAD sequences in the NCBI protein database shows that amino acids sequences of GAD are highly conserved within the same species (Figure 1), and that GAD is widely distributed in a number of LAB including *L. brevis, L. buchneri, L. delbrueckii* subsp. *bulgaricus, L. fermentum, L. futsaii, L. paracasei, L. parakefiri, L. paraplantarum, L. plantarum, L. plantarum* subsp. *argentoratensis, L. reuteri, L. sakei, L. lactis,* and *S. thermophilus.* All of these LAB are commonly found in fermented foods and with some of these are commonly used as starter cultures in food industries. In addition, GAD is also found in other lactobacilli including *L. acidifarinae, L.*

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aviaries, *L. coleohominis*, *L. farraginis*, *L. japonicas*, *L. koreensis*, *L. nuruki*, *L. oris*, *L. rossiae*, *L. rennini*, or *L. suebicus* (Figure 1). These organisms have not been studied for their capacity to synthesize GABA nor have their GAD system been studied, and hence they could be of interest with respect to GABA production and GABA-enriched food.

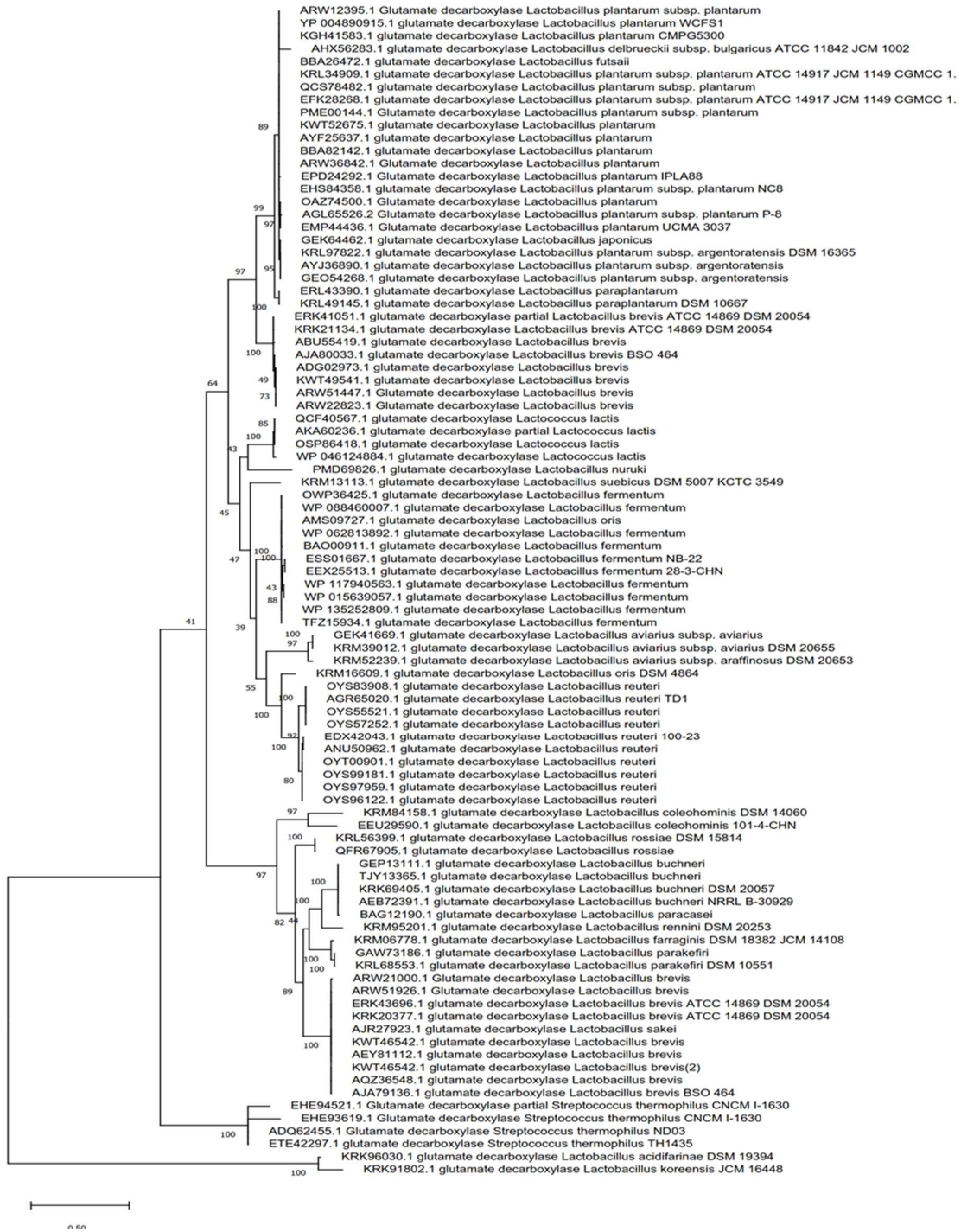

Figure 1. Phylogenetic analysis of glutamate decarboxylase from different species of LAB (maximum-likelihood method). The phylogenetic analysis was performed after the alignment of GAD sequences using MUSCLE in MEGA X software.

4. Glutamate decarboxylase

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Glutamate decarboxylase is an intracellular enzyme that is found ubiquitously in eukaryotes and prokaryotes. GAD exhibits different physiological roles especially in vertebrates and plants, and its presence is highly variable among organisms [52]. GAD is a pyridoxal 5'-phosphate (PLP) dependent enzyme and as such belongs to the PLP-dependent enzyme superfamily, which contains at seven different folds [67], with GAD from LAB showing the type-I fold of PLP-dependent enzymes [68]. A number of important catalytic reactions including α- and β-eliminations, decarboxylation, transamination, racemization and aldol cleavage are catalyzed by various members of this superfamily of enzymes [69]. GAD activity relies on the binding of its co-factor PLP, and belongs to group II of PLP-dependent decarboxylases [70]. In GAD from *L. brevis* GCMCC 1306, the active site entrance is located at the reface of the cofactor PLP, and PLP is covalently attached to a lysine (K279) via an imine linkage, referred to as an internal aldimine [85]. This lysine is strictly conserved in group II PLP-dependent decarboxylases. The corresponding lysine in *E. coli* GAD is at position 276, and when mutating this residue, the variant has less flexibility and affinity to both its substrate and the cofactor [71]. In addition to this covalent attachment, PLP is positioned in the active site via a number of H bonds between the phosphate group of PLP and surrounding amino acids, while the pyridine ring of PLP forms hydrophobic interactions with side chains of various amino acids in the active site [68]. Molecular docking of the substrate glutamate into the active-site of the holo form of *L. brevis* GAD showed several noncovalent interactions including hydrogen bonds between the O2, the O3 and the O4 atoms of the substrate L-Glu to various parts of the GAD polypeptide chain. Furthermore, electrostatic interactions between the negatively charged oxygen atom of the α-carboxyl and the γ-carboxyl group of L-Glu and the positively charged nitrogen atom of residue R422 as well as H278 and K279, respectively, were proposed [72]. The flexible loop residues Tyr308-Glu312 in *L. brevis* GAD is located near the substrate-binding site, and is important for its catalytic reaction. Furthermore, the conserved residue Tyr308 play crucial role in decarboxylation of L-Glu. Thr 215 and Asp246 are the two catalytic residues in *L. brevis* GAD, which are also highly conserved and promote decarboxylation of L-Glu [71, 73].

During catalysis a transamination reaction occurs, and PLP, which is covalently attached to a Lys in the active site of GAD in its resting state, now becomes covalently bonded to the substrate glutamate, forming a Schiff base or what is referred to as an external aldimine, which can then be transformed to a quinonoid intermediate [67,74]. In a small fraction of catalytic cycles when glutamate is decarboxylated, a subsequent alternative transamination of the quinonoid intermediate of the reaction can occur, and succinic semialdehyde (SSA) and pyridoxamine-5'-phosphate (PMP) are formed. The latter will immediately be released from the enzyme, resulting inactive apoGAD (Figure 2), which can be regenerated to the active GAD-PLP complex when free pyridoxal 5'-phosphate is present, thus completing a cycle of inactivation and activation. However, when free PLP is not present, GAD will not be reactive as a function of time and substrate concentration [62,67–69,74, 77].

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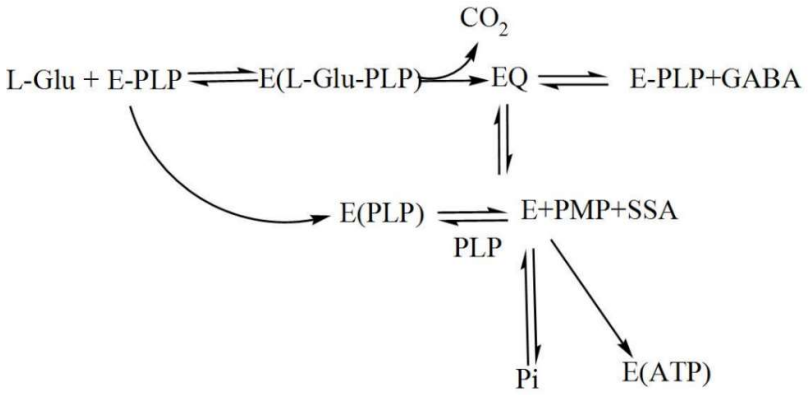

Figure 2. The interconversion of holo- and apoGAD. The primary reaction results in the formation of GABA and holoGAD remains intact. holoGAD reacting with PLP will activate a secondary reaction resulting in the formation of apoGAD. E, apoGAD; E-PLP, holoGAD; Pi, inorganic phosphate; EQ, quinonoid intermediate; PMP, pyridoxamine phosphate; PLP, pyridoxal 5'-phosphate; SSA; succinic semialdehyde (adapted from Ref. [76]).

5. Biochemical insights into glutamate decarboxylase from lactic acid bacteria

GAD from LAB typically consists of identical subunits with molecular masses ranging from 54 to 62 kD, and is formed in its mature holoform even when produced heterologously. The oligomerization, typically resulting in the formation of a homodimer, is crucial for activity of the *Lactobacillus* spp. enzymes. Some ambiguity about the active form of GAD isolated from different isolates of *L. brevis* and its quaternary structure exists in the scientific literature. Hiraga et al. reported that treatment with high concentrations of ammonium sulfate results in an active tetrameric form with the enzyme from *L. brevis* IFO12005 GAD [72]. The presences of ammonium sulfate apparently stabilized GAD from this source as the purified enzyme was found to be rather unstable, and the dimeric form showed no activity. Moreover, the presence of ammonium sulfate apparently did not affect the overall conformation but had effects on the active site of the protein. Studies by Yu et al. showed that GAD from *L. brevis* CGMCC 1306 is active as a monomer, while GAD from other LAB are generally active as dimers [85]. Subsequent structural studies on this enzyme revealed, however, that GAD from *L. brevis* CGMCC 1306 is active as a dimer, even though elucidation of the crystal structure resulted in a distorted asymmetric trimer. The authors concluded that this observed trimer is only the result from crystallographic packing and not the biological form [68].

As mentioned above, a number of LAB carry two GAD-encoding genes, *gad*A and *gad*B. Frequently, studies have focused on the purification and characterization of GadB, e.g., from *E. raffinosus* [75], *L. plantarum* [79], *L. brevis* [78], *L. sakei* [80], *L. paracasei* [18] since the expression levels of recombinant GadB are typically higher than those for GadA [55]. A recent study by Wu et al. showed that *gadA* transcript was highly upregulated (55-fold) in strain *L. brevis* NPS-QW-145 at the stationary phase of growth. Subsequently, both GadA and GadB were recombinantly produced and characterized. GadA showed a pH profile of activity near the neutral region, with the optimal activity found in the range of pH 5.5-6.6, in contrast to GadB, which is more active under acidic conditions (3.0-5.5). Presence of both of these two enzymes, GadA and GadB, in the *L. brevis* genome will give the organism a significant advantage to produce GABA over a broad range of pH (3.0-6.0) and thus to more efficiently maintain pH homeostasis. These findings suggest that extending the activity of GadA to the near-neutral pH region offer a novel genetic diversity of *gad* genes from LABs [7].

A number of GAD have been expressed and characterized from a variety of LABs. In general, the N- and C-terminal regions of GAD from different sources show significant differences, and this might affect recombinant GABA production. As shown in a sequence alignment (Figure 3), the sequence HVD(A/S)A(S/F)GG is highly conserved among LAB GAD, and a lysine residue (Lys279

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in) plays crucial role in the PLP binding site. Table 2 summarizes biochemical properties of GAD from different strains [18,42,81, 82]. Typically, the pH optima of GAD are found between 4.0 and 5.0. GAD from *L. zymae, E. avium* M5, *S. salivarius* subsp. *thermophilus* Y2 and *L. paracasei* NFRI 7415 have an optimum activity of above 40°C, which does not coincide with the optimal temperature for growth of these strains [46,72,82,83]. Different ions can affect the stability and activity of GAD from different sources (Table 2). GAD from *E. avium* M5 is activated in the presence of $CaCl_2$ and $MnCl_2$ but the activity is decreased by $CuSO_4$ and $AgNO_3$ [82]; comparable results were also obtained for GAD from other LAB sources, *L. zymae* [46] and *L. sakei* A156 [80].

Since GAD is mainly active under acidic conditions, several engineering approaches were employed to broaden its activity, especially at the near-neutral pH region. To this end, Shi et al. applied both directed evolution and site-directed mutagenesis at the β-hairpin region and C- terminal end of *L. brevis* GAD [84]. By using a plate-based screening assay employing a pH indicator as assay principle, they could identify several variants and positions that improved activity at pH 6.0. Furthermore, they selected three residues (Tyr308, Glu312, Thr315) in the β-hairpin region for site directed mutagenesis based on homology modelling, since these residues exhibit different interaction with surrounding amino acids in the model at different pH values. By combining various positive mutations, they could increase the catalytic efficiency of GAD from *L. brevis* 13.1- and 43.2-fold at pH 4.6 and 6.0, respectively, as compared to the wild-type enzyme [84]. The role of the C-terminus for the pH dependence of catalysis of *L. plantarum* GAD was investigated by Shin et al. employing mutagenesis [79]. Deletions of three and eleven residues in the C-terminal region Ile454-Thr468 of this enzyme increased activity in the pH range of 5 to 7, with the Δ11 variant showing significantly better results, increasing the catalytic efficiency of the variant at pH 5.0 and 7.0 by a factor of 1.26 and 28.5, respectively. The authors concluded that the C-terminal region is involved in decreasing the activity of *L. plantarum* GAD at higher pH values by closing up the catalytic site as a result of pH-induced conformational changes [79]. In a similar way, a C-terminally truncated variant of *L. brevis* GAD, in which the terminal 14 amino acids had been removed by site-directed mutagenesis, showed improved activity at higher, around neutral pH values [85]. These studies point to the importance of the C-terminus of GAD for improved accessibility of the active site and this increased activity especially at higher pH values, and thus the C-terminal loop is an essential target for enzyme engineering for GABA production at fluctuated pH conditions [79,85].

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Table 2. Biochemical properties of glutamate decarboxylase from various LAB

Source	Molecular mass of subunit (kDa)	Optimal pH	Optimal temperature	Effect of various metal ions	K_m (mM)	v_{max}	references
L. zymae	53	4.5	41	NH_4^+, Ca^{2+}, Mg^{2+}, Mn^{2+}, Na^+	1.7	0.01 mM/min	[46]
L. paracasei NFRI 7415	57	5	50	NH_4^+, Ca^{2+}	5	NR	[18]
L. sakei A156	54.4	5	55	Mn^{2+}, Co^{2+}, Ca^{2+}, Zn^{2+}	0.045	0.011 mM/min	[80]
L. brevis CGMCC 1306	53	4.8	48	NR	10.26	8.86 U/mg	[22]
S. salivarius subsp. *thermophilus Y2*	46.9	4	55	Ba^{2+}	2.3	NR	[36]
Enterococcus avium M5	53	4.5	55	Ca^{2+}, Mg^{2+}, Mn^{2+}, Zn^{2+}	3.26	0.012 mM/min	[82]
E. raffinosus TCCC11660	55	4.6	45	Mo^{6+}, Mg^{2+}	5.26	3.45 μM/min	[75]
Lactococcus lactis	NR	4.7	NR	NR	0.51	NR	[42]
L. brevis IFO12005	60	4.2	30	NR	9.3	NR	[85]
L. brevis 877G	50	5.2	45	Ca^{2+}, Mg^{2+}, Mn^{2+}, Na^+	3.6	0.06 mM/min	[81]

NR; Not reported

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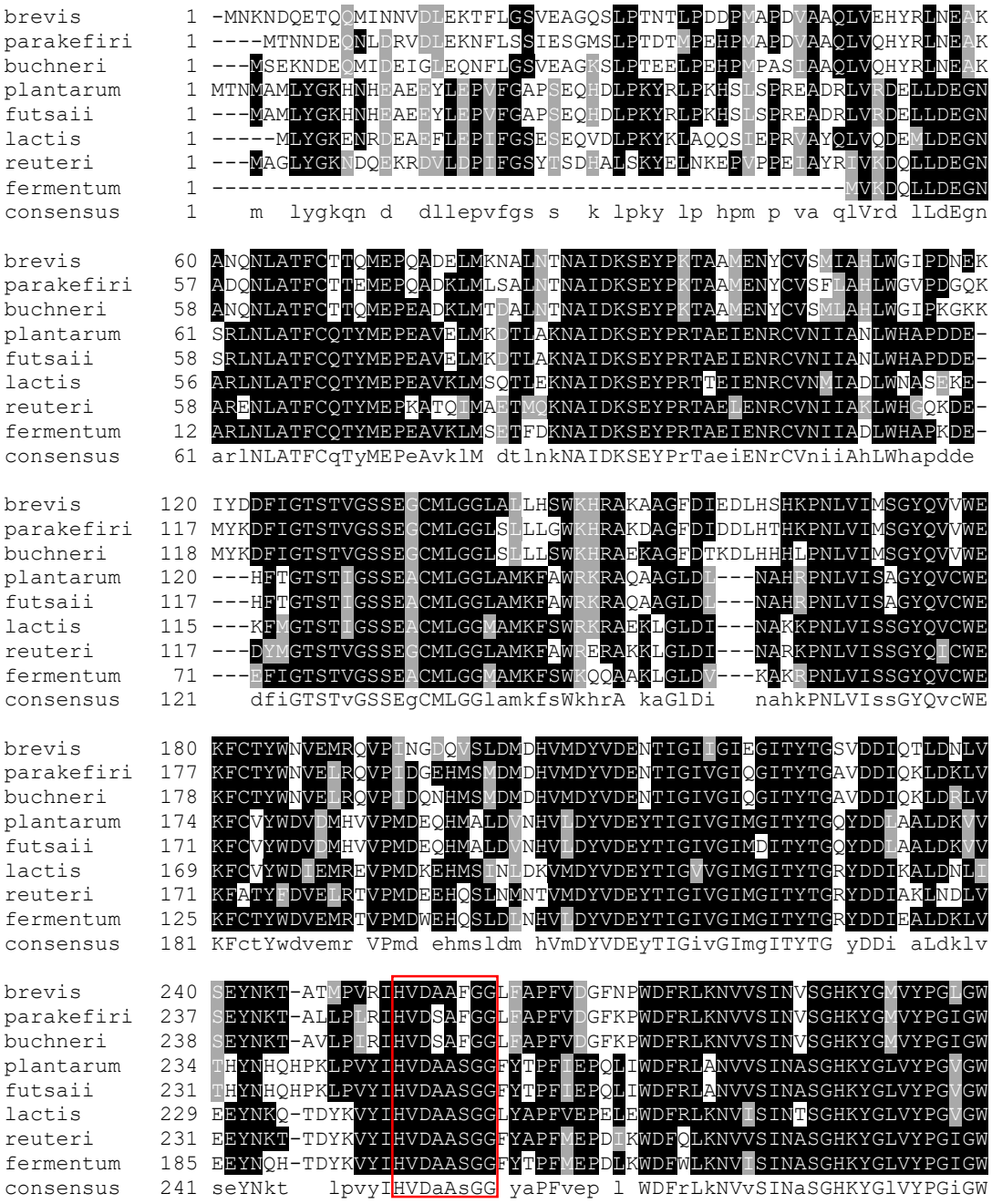

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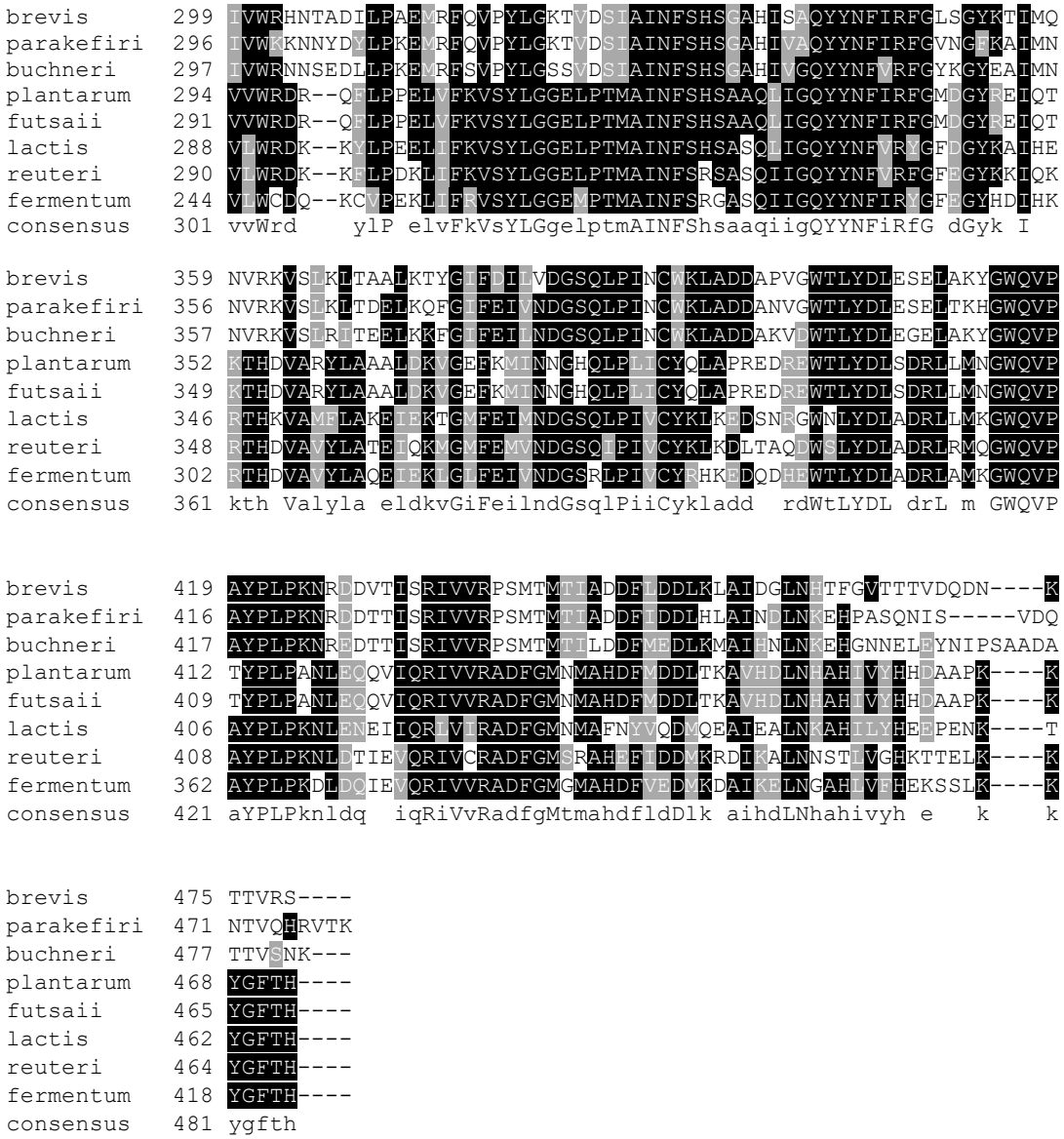

Figure 3. Comparison of amino acid sequences of GAD from *L. brevis, L. parakefiri, L. buchneri, L. plantarum, L. futsaii, L. lactis, L. reuteri* and *L. fermentum*. The accession numbers of these sequences are GAW73186.1, ERK43696.1, KRL34909.1, AEB72391.1, OYT00901.1, ESS01667.1, BBA26472.1, OSP86418.1, respectively. The alignment of amino acids was generated using the Clustal Omega software. The boxed sequence indicates residues HVD(A/S)A(S/F)GG; this sequence is highly conserved in PLP-dependent decarboxylases [48, 55].

6. Improvement of GAD activities and GABA production

GABA biosynthesis can be employed using whole cell reaction, recombinant bacteria and purified GAD. GAD from various sources of LAB have been overexpressed in different hosts including *E. coli* [86], *L. plantarum* [87], *L. sakei* [88], *Corynebacterium glutamicum* [21] and *B. subtilis* [89]. Utilization of whole cells for the biocatalytic conversion of glutamate to GABA has some drawbacks including the conversion of GABA to succinic semialdehyde by the enzyme GABA transaminase (GABA-T), which is often found in bacteria and might decrease GABA yields during cultivation. To prolong and thereby increase GABA production, continuous cultivation [90], fed-batch fermentation [91], as well immobilized cell technology [92,93] have been employed. All of these

approaches effectively increased GABA productivity by improving cell viability resulting in longer periods of cultivation.

GABA biosynthesis and production could be enhanced by optimizing fermentation conditions, with attention given to different factors including the carbon source, concentration of added glutamate, pH regulation, incubation temperatures, nitrogen sources, cofactor and feeding time [34,94]. A study by Lim et al. showed that under optimized conditions, strain *L. brevis* HYE1 produced 18.8 mM of GABA. Monosodium glutamate (MSG) or L-glutamate are the main substrate for the production of GABA using either appropriate GAD-containing cells or pure GAD [27]. LAB with GAD activity may furthermore require pyridoxal 5′-phosphate (PLP) as a coenzyme to enhance GABA production. The addition of 0.5% MSG increased GABA production by *E. faecium* JK29, which reached 14.9 mM after 48 h of cultivation [38]. A concentration of 6% MSG and the addition of 0.02 mM PLP were found to be optimal conditions for *L. brevis* K203 for GABA production [95]. This strategy of increasing glutamate supplementation could not be used for all strains though; when L-glutamate was added at concentrations of 10 to 20 g/L to a medium of *S. thermophilus* GABA production could not be enhanced. It was suggested that this strain is not able to tolerate high glutamate concentrations [35]. High glutamate concentrations increase the osmotic pressure in the cells, and this stress can disturb the bacterial metabolism [39]. Fermentation time and temperature are also a key factor for GABA production. Villegas et al. investigated GABA formation by *L. brevis* CRL 1942, and found that 48 h of fermentation at 30°C employing 270 mM of MSG resulted in a maximum GABA production of 255 mM in MRS medium, indicating that the GABA production is time-dependent manner [94].

Metabolic pathway engineering has been performed to achieve enhanced GABA production. The key points here are the direct modulation of GABA metabolic pathways. A whole-cell biocatalyst based on *E. coli* cells expressing the *gadB* gene from *L. lactis* was used as the starting point of this engineering approach. By introducing mutations into this GadB to shift its decarboxylation activity toward a neutral pH, by modifying the glutamate/GABA antiporter GadC to facilitate transport at a neutral pH, by enhanced the expression of soluble GadB by introducing the GroESL molecular chaperones, and by inhibited the degradation of GABA by inactivation of *gadA* and *gadB* from the *E. coli* genome an engineered strain was constructed that achieved a productivity of 44.04 g GABA per L and h with an almost quantitative conversion of 3 M glutamate [96].

Several mutational approaches such as directed evolution and site-specific mutagenesis are considered as powerful tools for optimizing or improving enzyme properties. Several researchers have applied these approaches to improve GAD activity [83,97–100] that was applied in whole-cell biocatalysts. In order to improve GAD activity over an expanded pH range, recombinant *C. glutamicum* cells were obtained by expressing *L. brevis* Lb85 GadB variants. These variants were constructed by combining directed evolution and site-specific mutagenesis of GadB to improve activity at higher pH values (see above) since *L. glutamicum* grows best around neutral pH [83]. *C. glutamicum* is an industrial producer of glutamate, and by introducing these GadB variants into this organism, GABA could be produced without the need of exogenous glutamate on a simple glucose-based medium, with yields of up to 7.13 g/L [83].

Insufficient thermostability is often a common problem associated with industrial enzymes, and most GAD show low stability even at moderate temperatures. A rational strategy for improving thermostability is to identify critical regions or amino acid residues by sequence alignments. Alternatively, structural information can be used which indicates flexible regions, and to subsequently strengthen these regions [101]. Identification of the consensus sequences can also improve thermostability of proteins [102]. Recently, Zhang et al. developed a parallel strategy to engineer *L. brevis* CGMCC 1306 GAD. They compared the sequence and structure of this mesophilic GAD with homologous thermophilic enzymes to identify amino acid residues that might affect stability. Two mutant enzymes were obtained and showed higher thermostability with their half-inactivation temperature 2.3°C and 1.4°C higher than the wild-type enzyme. Furthermore, the activity of the variants was 1.67-fold increased during incubation at 60°C for 20 min. They suggested that this approach can be an efficient tool to improve the thermostability of GAD [101].

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The use of purified GAD seems to be economically more feasible then whole-cell biocatalysis when aiming at producing pure GABA due to simplified downstream purification of this compound from less complex reaction mixtures. A numbers of immobilization technique have been performed for re-use purposes, such as GadB immobilized on calcium alginate beads that are then employed in a bioreactor [103], a GAD/cellulose-binding domain fusion protein immobilized onto cellulose [104], and GAD immobilized to metal affinity gels [105]. The performance of immobilized GAD in a fed-batch reactor was evaluated, which showed high productivity of GABA as the substrate concentration in the medium was kept constant by feeding solid glutamate. Moreover, no significant decrease of enzyme activities were observed during the reaction when the inactivation reaction of PLP to succinic semialdehyde and pyridoxamine-5′-phosphate during catalysis was avoided by adding addition of a small amount of α-ketoglutaric acid to the reactor, which regenerates PLP [100]. Sang-Jae Lee et al. performed immobilization of *L. plantarum* GAD using silica beads and showed high stability under acidic and alkaline conditions with improved thermostability [105]. In addition, the immobilized GAD converted 100% of glutamate to GABA [106]. The results suggest that immobilization gives an advantageous result for industrial application when using (partially) purified GAD for the GABA from glutamate.

7. The role of glutamate decarboxylase in the manufacturing of bio-based industrial chemical

Agricultural waste and waste stream from biofuel production are now being considered as a low-cost source of glutamate for biotechnological conversion into GABA, and production of bio-based chemicals [106]. These protein-rich materials are mainly bioethanol by-product streams including dried distiller's grains with solubles (DDGS) from maize and wheat, or vinasse from sugarcane or sugar beet, but also plant leaves, oil or biodiesel by-products, and slaughterhouse waste. In the future, algae could also provide an additional source for biodiesel and thus become a natural source low-cost source of glutamic acid.

The protein-rich fraction of plants can be further split into more- and less-nutritious fractions, for example by hydrolyzing the proteins and separating the essential (nutritious) amino acids from the non-essential (less nutritious) ones. Non-essential amino acid such as glutamic acid and aspartic acid, which have no significant value in animal feed, can be utilized for preparing functionalized chemicals. Recently, a by-product from tuna canning industry, tuna condensate, was shown to be a useful material for the production of GABA. Tuna condensate contains significant amounts of glutamine but relatively little glutamate. Glutamine was first converted to glutamate by a glutaminase from *Candida rugasa*, and in a second step *L. futsaii* GAD converted glutamate to GABA. Both steps were catalyzed by immobilized whole cells [107]. Recently, it was shown that supplementation of arginine to media containing glutamate can enhance GABA production, and that the simultaneous addition of arginine, malate, and glutamate enabled GABA production already during exponential growth at relatively high pH (6.5) [108].

The structure of glutamic acid resembles many industrial intermediates, so it can be transformed into a variety of chemicals using a relatively limited number of steps. Decarboxylation of glutamic acid to GABA, enzymatically performed by GAD, is an important reaction of the pathway from glutamic acid to a range of molecules. GABA is for example an intermediate for the synthesis of pyrrolidones. Such an approach can be used to produce for example *N*-methyl-2-pyrrolidone (NMP), which is used as an industrial solvent. Combining the enzymatic decarboxylation of glutamate performed by GAD with the one-pot cyclization of GABA to 2-pyrrolidone and subsequent methylation will thus yield NMP [109]. Another interesting material synthesized by ring-opening polymerization of 2-pyrrolidone is Nylon 4 (110), a four-carbon polyamide suitable for application as an engineering plastic due to its superior thermal and mechanical properties [111]. Contrary to other nylon polymers, Nylon 4 is heat-resistant, biodegradable, biocompatible and compostable [111].

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8. Future trends and conclusions

The demand of functional foods is increasing and marked by the awareness of consumers in maintaining health and prevention of degenerative diseases. Therefore, exploration of bioactive compounds such as GABA are important. The GAD system plays a crucial role in GABA biosynthesis. A number of studies on cloning, expression and characterization of both *gadA* and *gadB* lead to deciphering the role of the *gad* genes in the GABA metabolic pathway and its importance for LAB. Since the production of GABA is dependent on the biochemical properties of GAD, more study on the biochemical properties of GAD are important especially for those enzymes derived from LAB isolated from food fermentation processes as this will facilitate the optimization of the fermentation process and support the selection of suitable starter cultures for these processes that will bring more GABA-enriched food to the consumer. Recent structural information of GAD from LAB will facilitate enzyme-engineering approaches to improve GAD towards enhanced thermostability or improved activity over a broad range of pH. However, structural information is currently only limited to GAD from *L. brevis*, and thus structural studies on GAD from other GABA-producing LAB is needed in order to understand their catalytic and structural properties in more depth. The elucidation of molecular mechanisms and roles of GABA production, knowledge of the regulatory aspects of GABA production, and profound comprehension of GABA producing cell physiology will offer the basis and tools to increase GABA yield at genetic and metabolic levels.

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References

1. Ly, D.; Mayrhofer, S.; Agung Yogeswara, I.B.; Nguyen, T.H.; Domig, K.J. Identification, classification and screening for γ-amino-butyric acid production in lactic acid bacteria from cambodian fermented foods. *Biomolecules* **2019**, 9(12).
2. Cotter, P.D.; Hill, C. Surviving the acid test: responses of Gram-positive bacteria to low pH. *Microbiol Mol Biol Rev* **2003**, 67(3), 429–53.
3. Nomura, M.; Kimoto, H.; Someya, Y.; Furukawa, S.; Suzuki, I. Production of γ -aminobutyric acid by cheese starters during cheese ripening. *J Dairy Sci* **1998**, 81(6), 1486–1491.
4. Komatsuzaki, N.; Shima, J.; Kawamoto, S.; Momose, H.; Kimura, T. Production of γ-aminobutyric acid (GABA) by *Lactobacillus paracasei* isolated from traditional fermented foods. *Food Microbiol* **2005**, 22(6), 497–504.
5. Somkuti, G.A.; Renye, J.A.; Steinberg, D.H. Molecular analysis of the glutamate decarboxylase locus in *Streptococcus thermophilus* ST110. *J Ind Microbiol Biotechnol* **2012**, 39(7), 957–963.
6. Gong, L.; Ren, C.; Xu, Y. Deciphering the crucial roles of transcriptional regulator GadR on γ -aminobutyric acid production and acid resistance in *Lactobacillus brevis. Microb Cell Fact* **2019**, 18(1), 1–12.
7. Wu, Q.; Tun, H.M.; Law, Y.S.; Khafipour, E.; Shah, N.P. Common distribution of *gad* operon in *Lactobacillus brevis* and its GadA contributes to efficient GABA synthesis toward cytosolic near-neutral pH. *Front Microbiol* **2017**, 8, 1–16.
8. Brambilla, P.; Perez, J.; Barale, F.; Schettini.; G, Soares, J.C. GABAergic dysfunction in mood disorders. *Mol Psychiatry* **2003**, 8(8), 721–737.
9. Ko, C.Y.; Lin, H.T.V.; Tsai, G.J. γ-aminobutyric acid production in black soybean milk by

Lactobacillus brevis FPA 3709 and the antidepressant effect of the fermented product on a forced swimming rat model. *Process Biochem* **2013**, 48(4), 559–568.

10. Inoue, K.; Shirai, T.; Ochiai, H.; Kasao, M.; Hayakawa, K.; Kimura, M.; et al. Blood-pressure-lowering effect of a novel fermented milk containing γ-aminobutyric acid (GABA) in mild hypertensives. *Eur J Clin Nutr* **2003**, 57(3), 490–495.
11. Tsai, J.S.; Lin, Y.S.; Pan, B.S.; Chen, T.J. Antihypertensive peptides and γ-aminobutyric acid from prozyme 6 facilitated lactic acid bacteria fermentation of soymilk. *Process Biochem* **2006**, 41(6), 1282–1288.
12. Watanabe, S.; Katsube, T.; Sonomoto, K. Cholesterol-lowering effects of *Lactobacillus brevis* isolated from turnip *"tsuda kabu." Food Sci Technol Res* **2012**, 18(6), 825–834.
13. Pouliot-Mathieu, K.; Gardner-Fortier; C., Lemieux, S.; St-Gelais, D.; Champagne, C.P.; Vuillemard, J.C. Effect of cheese containing γ-aminobutyric acid-producing lactic acid bacteria on blood pressure in men. *PharmaNutrition* **2013**, 1(4),141–148.
14. Xie, Z.; Xia, S.; Le, G.W. γ-aminobutyric acid improves oxidative stress and function of the thyroid in high-fat diet fed mice. *J Funct Foods* **2014**, 8(1), 76–86.
15. Xu, N.; Wei, L.; Liu, J. Biotechnological advances and perspectives of γ-aminobutyric acid production. *World J Microbiol Biotechnol*. **2017**, 33, 64.
16. Diana, M.; Tres, A.; Quílez, J.; Llombart, M.; Rafecas, M. Spanish cheese screening and selection of lactic acid bacteria with high gamma-aminobutyric acid production. *LWT - Food Sci Technol* **2014**, 56(2), 351–355.
17. Ratanaburee, A.; Kantachote, D.; Charernjiratrakul, W.; Sukhoom, A. Selection of γ-aminobutyric acid-producing lactic acid bacteria and their potential as probiotics for use as starter cultures in Thai fermented sausages (*Nham*). *Int J Food Sci Technol* **2013**, 48(7), 1371–1382.
18. Komatsuzaki, N.; Nakamura, T.; Kimura, T.; Shima, J. Characterization of glutamate decarboxylase from a high γ-aminobutyric acid (GABA)-producer, *Lactobacillus paracasei. Biosci Biotechnol Biochem* **2008**, 72(2), 278–285.
19. Le Vo, T.D.; Kim, T.W.; Hong, S.H. Effects of glutamate decarboxylase and γ-aminobutyric acid (GABA) transporter on the bioconversion of GABA in engineered *Escherichia coli*. *Bioprocess Biosyst Eng* **2012**, 35(4), 645–650.
20. Ma, D.; Lu, P.; Shi, Y. Substrate selectivity of the acid-activated glutamate/γ-aminobutyric acid (GABA) antiporter GadC from *Escherichia coli. J Biol Chem* **2013**, 288(21),15148–15153.
21. Choi, J.W.; Yim, S.S.; Lee, S.H.; Kang, T.J.; Park, S.J.; Jeong, K.J. Enhanced production of γ-aminobutyrate (GABA) in recombinant *Corynebacterium glutamicum* by expressing glutamate decarboxylase active in expanded pH range. *Microb Cell Fact* **2015**, 14:21.
22. Fan, E.; Huang, J.; Hu, S.; Mei, L.; Yu, K. Cloning, sequencing and expression of a glutamate decarboxylase gene from the GABA-producing strain *Lactobacillus brevis* CGMCC 1306. *Ann Microbiol* **2012**,62(2), 689–698.
23. Kim, J.Y.; Lee, M.Y.; Ji, G.E.; Lee, Y.S.; Hwang, K.T. Production of y-aminobutyric acid in black raspberry juice during fermentation by *Lactobacillus brevis* GABA100. *Int J Food Microbiol* **2009**,130(1), 12–16.
24. Kim, J.A.; Park, M.S.; Kang, S.A.; Ji, G.E. Production of γ-aminobutyric acid during fermentation of *Gastrodia elata* Bl. by co-culture of *Lactobacillus brevis* GABA 100 with

Bifidobacterium bifidum BGN4. *Food Sci Biotechnol* **2014**, 23(2),459–466.

25. Lee, B.J.; Kim, J.S.; Kang, Y.M.; Lim, J.H.; Kim, Y.M.; Lee, M.S.; et al. Antioxidant activity and γ-aminobutyric acid (GABA) content in sea tangle fermented by *Lactobacillus brevis* BJ20 isolated from traditional fermented foods. *Food Chem* **2010**, 122(1), 271–276.
26. Siragusa, S.; De Angelis, M.; Di Cagno, R.; Rizzello, C.G.; Coda, R.; Gobbetti, M. Synthesis of γ-aminobutyric acid by lactic acid bacteria isolated from a variety of Italian cheeses. *Appl Environ Microbiol* **2007**, 73(22), 7283–7290.
27. Lim, H.S.; Cha, I.T.; Roh, S.W.; Shin, H.H.; Seo, M.J. Enhanced production of γ-aminobutyric acid by optimizing culture conditions of *Lactobacillus brevis* HYE1 isolated from kimchi, a Korean fermented food. *J Microbiol Biotechnol* **2017**, 27(3), 450–459.
28. Li, H.; Gao, D.; Cao, Y. A high γ-aminobutyric acid-producing *Lactobacillus brevis* isolated from Chinese traditional paocai. *Ann Microbiol* **2008**, 58(4), 649–653.
29. Sun, T.; Zhao, S.; Wang, H.; Cai, C.; Chen, Y.; Zhang, H. ACE-inhibitory activity and γ-aminobutyric acid content of fermented skim milk by *Lactobacillus helveticus* isolated from Xinjiang *koumiss* in China. *Eur Food Res Technol* **2009**, 228(4), 607–612.
30. Franciosi, E.; Carafa, I.; Nardin, T.; Schiavon, S.; Poznanski, E.; Cavazza, A.; et al. Biodiversity and γ-aminobutyric acid production by lactic acid bacteria isolated from traditional alpine raw cow's milk cheeses. *Biomed Res Int* **2015**, 625740.
31. Das, D.; Goyal, A. Antioxidant activity and γ-aminobutyric acid (GABA) producing ability of probiotic *Lactobacillus plantarum* DM5 isolated from Marcha of Sikkim. *LWT - Food Sci Technol* **2015**, 61(1), 263–268.
32. Han, S.H.; Hong, K.B.; Suh, H.J. Biotransformation of monosodium glutamate to γ-aminobutyric acid by isolated strain *Lactobacillus brevis* L-32 for potentiation of pentobarbital-induced sleep in mice. *Food Biotechnol* **2017**, 31(2), 80–93.
33. Zhao, A.; Hu, X.; Pan, L.; Wang, X. Isolation and characterization of a γ-aminobutyric acid producing strain *Lactobacillus buchneri* WPZ001 that could efficiently utilize xylose and corncob hydrolysate. *Appl Microbiol Biotechnol* **2015**, 99(7), 3191–3200.
34. Xiaoxue, L.U.; Chunyan, X.I.E.; ZhenXin.; G.U. Optimisation of fermentative parameters for GABA enrichment by *Lactococcus lactis. Czech J Food Sci* **2009**, 27(6), 433–442.
35. Komatsuzaki, N.; Shima, J.; Kawamoto, S.; Momose, H.; Kimura, T. Production of γ-aminobutyric acid (GABA) by *Lactobacillus paracasei* isolated from traditional fermented foods. *Food Microbiol* **2005**, 22(6), 497–504.
36. Yang, S.Y.; Lü, F.X.; Lu, Z.X.; Bie, X.M.; Jiao, Y.; Sun, L.J.; et al. Production of γ-aminobutyric acid by *Streptococcus salivarius* subsp. *thermophilus* Y2 under submerged fermentation. *Amino Acids* **2008**, 34(3), 473–478.
37. Cho, Y.R.; Chang, J.Y.; Chang, H.C. Production of γ-aminobutyric acid (GABA) by *Lactobacillus buchneri* isolated from Kimchi and its neuroprotective effect on neuronal cells. *J Microbiol Biotechnol* **2007**, 17(1), 104–109.
38. Lim, H.S.; Cha, I.T.; Lee, H.; Seo, M.J. Optimization of γ-aminobutyric acid production by *Enterococcus faecium* JK29 isolated from a traditional fermented foods. *Korean J Microbiol Biotechnol* **2016**, 44(1), 26–33.
39. Seo, M.J.; Nam, Y.D.; Park, S.L.; Lee, S.Y.; Yi, S.H.; Lim, S.I. γ-aminobutyric acid production in skim milk co-fermented with *Lactobacillus brevis* 877G and *Lactobacillus sakei* 795. *Food Sci Biotechnol* **2013**, 22(3), 751–755.

40. Agung Yogeswara, I.B.; Kusumawati, I.G.A.W.; Sumadewi, N.L.U.; Rahayu, E.S.; Indrati, R. Isolation and identification of lactic acid bacteria from Indonesian fermented foods as γ-aminobutyric acid-producing bacteria. *Int Food Res J* **2018**, 25(4), 1753–1757.
41. Barla, F.; Koyanagi, T.; Tokuda, N.; Matsui, H.; Katayama, T.; Kumagai, H.; Michihata, T.; Sasaki, T.; Tsuji, A.; Enomoto, T. The γ-aminobutyric acid-producing ability under low pH conditions of lactic acid bacteria isolated from traditional fermented foods of Ishikawa Prefecture, Japan, with a strong ability to produce ACE-inhibitory peptides. *Biotechnol Reports* **2016**, 10, 105–110.
42. Nomura, M.; Nakajima, I.; Fujita, Y.; Kobayashi, M.; Kimoto, H.; Suzuki, I.; Aso, H. *Lactococcus lactis* contains only one glutamate decarboxylase gene. *Microbiology* **1999**, 145, 1375–1380.
43. Wu, Q.; Shah, N.P. High γ-aminobutyric acid production from lactic acid bacteria: Emphasis on *Lactobacillus brevis* as a functional dairy starter. *Crit Rev Food Sci Nutr* **2017**, 57(17), 3661–3672.
44. Kim, M.J.; Kim K.S. Isolation and identification of γ-aminobutyric acid (GABA)-producing lactic acid bacteria from Kimchi. *J Korean Soc Appl Biol Chem* **2012**, 55, 777-785.
45. Tanamool, V.; Hongsachart, P.; Soemphol, W. Screening and characterisation of γ-aminobutyric acid (GABA) producing lactic acid bacteria isolated from Thai fermented fish (*Plaa-som*) in Nong Khai and its application in Thai fermented vegetables (Som-pak). *Food Sci Technol* **2019**; 2061, 1–8.
46. Park, J.Y.; Jeong, S.J.; Kim, J.H. Characterization of a glutamate decarboxylase (GAD) gene from *Lactobacillus zymae*. *Biotechnol Lett* **2014**, 36(9), 1791–1799.
47. Sanchart, C.; Rattanaporn, O.; Haltrich, D.; Phukpattaranont, P.; Maneerat, S. Technological and safety properties of newly isolated GABA-producing *Lactobacillus futsaii* strains. *J Appl Microbiol* **2016**, 121(3), 734–745.
48. Li, H.; Cao, Y. Lactic acid bacterial cell factories for γ-aminobutyric acid. *Amino Acids* **2010**, 39(5), 1107–1116.
49. Kono, I.; Himeno, K. Changes in γ-aminobutyric acid content during *beni-koji* making. *Biosci Biotechnol Biochem* **2000**, 64, 617–619.
50. Masuda, K.; Guo, X.; Uryu, N.; Hagiwara, T.; Watabe, S. Isolation of marine yeasts collected from the Pacific Ocean showing a high production of γ-aminobutyric acid. *Biosci Biotechnol Biochem* **2008**, 72(12), 3265–3272.
51. Kinnersley, A.M.; Turano, F.J. γ-aminobutyric acid (GABA) and plant responses to stress. *Crit Rev Plant Sci* **2000**, 19(6), 479-509.
52. Ueno H. Enzymatic and structural aspects on glutamate decarboxylase. *J Mol Catal - B Enzym* **2000**, 10(1–3), 67–79.
53. Cotter, P.D.; O'Reilly, K.; Hill, C. Role of the glutamate decarboxylase acid resistance system in the survival of *Listeria monocytogenes* LO28 in low pH foods. *J Food Prot* **2001**, 64(9), 1362–1368.
54. De Biase, D.; Pennacchietti, E. Glutamate decarboxylase-dependent acid resistance in orally acquired bacteria: Function, distribution and biomedical implications of the *gadBC* operon. *Mol Microbiol* **2012**, 86(4), 770–786.
55. Lim, H.S.; Seo, D.H.; Cha, I.T.; Lee, H.; Nam, Y.D.; Seo M.J. Expression and characterization

of glutamate decarboxylase from *Lactobacillus brevis* HYE1 isolated from kimchi. *World J Microbiol Biotechnol* **2018**, 34(3), 1–10.

56. Capitani, G.; De Biase, D.; Aurizi, C.; Gut, H.; Bossa, F.; Grutter, M.G. Crystal structure and functional analysis of *Escherichia coli* glutamate decarboxylase. *EMBO J* **2003**, 22(16), 4027-4037.
57. Cui, Y.; Miao, K.; Niyaphorn, S.; Qu, X. Production of γ-aminobutyric acid from lactic acid bacteria : A systematic review. *Int J Mol Sci* **2020**, 21:995.
58. Yu, T.; Li, L.; Zhao, Q.; Wang, P.; Zuo, X. Complete genome sequence of bile-isolated *Enterococcus avium strain* 352. *Gut Pathog* **2019**, 11(16), 1–5.
59. Gao, D.; Chang, K.; Ding, G.; Wu, H.; Chen, Y.; Jia, M.; Liu, X.; Wang, S.; Jin, Y.; Pan, H.; Li, H. Genomic insights into a robust γ-aminobutyric acid-producer *Lactobacillus brevis* CD0817. AMB Express. **2019**, 9(72), 1-11.
60. Li, H.; Li, W.; Liu, X.; Cao, Y. *gadA* gene locus in *Lactobacillus brevis* NCL912 and its expression during fed-batch fermentation. *FEMS Microbiol Lett* **2013**, 349(2), 108–116.
61. Lyu, C.; Zhao, W.; Peng, C.; Hu, S.; Fang, H.; Hua, Y.; Yao, S.; Huang, J.; Mei, L. Exploring the contributions of two glutamate decarboxylase isozymes in *Lactobacillus brevis* to acid resistance and γ-aminobutyric acid production. *Microb Cell Fact*. **2018**, 17:180.
62. Yunes, R.A.; Poluektova, E.U.; Dyachkova, M.S.; Klimina, K.M.; Kovtun, A.S.; Averina, O.V.; Orlova, V.S.; Danilenko, V.N. GABA production and structure of *gadB*/*gadC* genes in *Lactobacillus* and *Bifidobacterium* strains from human microbiota. *Anaerobe* **2016**, 42, 197–204.
63. Lin, Q.; Yang, S.; Fengxia, L.; Lu, Z.; Bie, X.; Jiao, Y.; Zou, X. Cloning and expression of glutamate decarboxylase gene from *Streptococcus thermophilus* Y2. *J Gen Appl Microbiol* **2009**, 55(4), 305–310.
64. Sanders, J.W.; Leenhouts, K.; Burghoorn, J.; Brands, J.R.; Venema, G.; Kok, J. A chloride-inducible acid resistance mechanism in *Lactococcus lactis* and its regulation. *Mol Microbiol* **1998**, 27(2), 299–310.
65. Su, M.S.; Schlicht, S.; Gänzle, M.G. Contribution of glutamate decarboxylase in *Lactobacillus reuteri* to acid resistance and persistence in sourdough fermentation. *Microb Cell Fact* **2011**, 10:S8.
66. Park, K.B.; Oh, S.H. Cloning, sequencing and expression of a novel glutamate decarboxylase gene from a newly isolated lactic acid bacterium, *Lactobacillus brevis* OPK-3. *Bioresour Technol* **2007**, 98(2), 312–319.
67. Steffen-Munsberg, F.; Vickers, C.; Kohls, H.; Land, H.; Mallin, H.; Nobili, A.; Skalden, L.; van den Bergh, T.; Joosten, H.J.; Berglund, P.; Hohne, M.; Bornscheur, U.T. Bioinformatic analysis of a PLP-dependent enzyme superfamily suitable for biocatalytic applications. *Biotechnol Adv* **2015**, 33(5), 566–604.
68. Huang, J.; Fang, H.; Gai, Z.C.; Mei, J.Q.; Li, J.N.; Hu, S.; Lv, C.J.; Zhao, W.R.; Mei, L.H. *Lactobacillus brevis* CGMCC 1306 glutamate decarboxylase: Crystal structure and functional analysis. *Biochem Biophys Res Commun* **2018**, 503(3), 1703–1709.
69. Rocha, J.F.; Pina, A.F.; Sousa, S.F.; Cerqueira, N.M.S.A. PLP-dependent enzymes as important biocatalysts for the pharmaceutical, chemical and food industries: A structural and mechanistic perspective. *Catal Sci Technol* **2019**, 9(18), 4864–4876.
70. Sandmeier, E.; Hale, T.; Christen, P. Multiple evolutionary origin of pyridoxal-5′-phosphate-dependent amino acid decarboxylases. *Eur J Biochem* **1994**, 221(3), 997–1002.

71. Tramonti, A.; John, R.A.; Bossa, F.; De Biase, D. Contribution of Lys276 to the conformational flexibility of the active site of glutamate decarboxylase from *Escherichia coli*. *Eur J Biochem* **2002**, 269(20), 4913–4920.
72. Hiraga, K.; Ueno, Y.; Oda, K. Glutamate decarboxylase from *Lactobacillus brevis*: activation by ammonium sulfate. *Biosci Biotechnol Biochem* **2008**, 72(5), 1299–1306.
73. Kim, S.H.; Shin, B.H.; Kim, Y.H.; Nam, S.W.; Jeon, S.J. Cloning and expression of a full-length glutamate decarboxylase gene from *Lactobacillus brevis* BH2. *Biotechnol Bioprocess Eng* **2007**, 12(6), 707–712.
74. Wang, C.; Zhu, R.; Sun, H.; Li, B. Quantum chemistry studies of the catalysis mechanism differences between the two isoforms of glutamic acid decarboxylase. *J Mol Model* **2013**, 19(2), 705–714.
75. Chang, C.; Zhang, J.; Ma, S.; Wang, L.; Wang, D.; Zhang, J.; Gao, Q. Purification and characterization of glutamate decarboxylase from *Enterococcus raffinosus* TCCC11660. *J Ind Microbiol Biot* **2017**, 44, 817-824.
76. Battaglioli, G.; Liu, H.; Martin, D.L. Kinetic differences between the isoforms of glutamate decarboxylase: Implications for the regulation of GABA synthesis. *J Neurochem* **2003**, 86(4), 879–887.
77. Jansonius, J.N. Structure, evolution and action of vitamin B6-dependent enzymes. *Curr Opin Struct Biol* **1998**, 8(6), 759–769.
78. Wang, Q.; Liu, X.; Fu, J.; Wang, S.; Chen, Y.; Chang, K.; Li, H. Substrate sustained release-based high efficacy biosynthesis of GABA by *Lactobacillus brevis* NCL912. *Microb Cell Fact* **2018**, 17:80.
79. Shin, S.; Kim, H.; Joo, Y.; Lee, S.; Lee, Y.; Lee, S.J.; Lee, D.W. Charactrization of glutamate decarboxylase from *Lactobacillus plantarum* and its C-terminal function for the pH dependence of activity. *J Agric Food Chem* **2014**, 62, 12186–12193.
80. Sa, H.D.; Park, J.Y.; Jeong, S.J.; Lee, K.W.; Kim, J.H. Characterization of glutamate decarboxylase (GAD) from *Lactobacillus sakei* A156 isolated from *jeot-gal*. *J Microbiol Biotechnol* **2015**, 25(5), 696–703.
81. Seo, M.J.; Nam, Y.D.; Lee, S.Y.; Park, S.L.; Yi, S.H.; Lim, S.I. Expression and characterization of a glutamate decarboxylase from *Lactobacillus brevis* 877G producing γ-aminobutyric acid. *Biosci Biotechnol Biochem* **2013**, 77(4), 853–856.
82. Lee, K.W.; Shim, J.M.; Yao, Z.; Kim, J.A.; Kim, H.J.; Kim, J.H. Characterization of a glutamate decarboxylase (GAD) from *Enterococcus avium* M5 isolated from *Jeotgal*, a Korean fermented seafood. *J Microbiol Biotechnol* **2017**, 27(7), 1216–1222.
83. Shi, F.; Xie, Y.; Jiang, J.; Wang, N.; Li, Y.; Wang, X. Directed evolution and mutagenesis of glutamate decarboxylase from *Lactobacillus brevis* Lb85 to broaden the range of its activity toward a near-neutral pH. *Enzyme Microb Technol* **2014**, 61–62, 35–43.
84. Yu, K.; Lin, L.; Hu, S.; Huang, J.; Mei, L. C-terminal truncation of glutamate decarboxylase from *Lactobacillus brevis* CGMCC 1306 extends its activity toward near-neutral pH. *Enzyme Microb Technol* **2012**, 50(4–5), 263–269.
85. Ueno, Y.; Hayakawa, K.; Takahashi, S.; Oda, K. Purification and characterization of glutamate decarboxylase from *Lactobacillus brevis* IFO 12005. *Biosci Biotechnol Biochem* **1997**, 61(7), 1168–1171.

86. Wang, Q.; Xin, Y.; Zhang, F.; Feng, Z.; Fu, J.; Luo, L.; Yin, Z. Enhanced γ-aminobutyric acid-forming activity of recombinant glutamate decarboxylase (*gadA*) from *Escherichia coli*. *World J Microbiol Biotechnol* **2011**, 27(3), 693–700.
87. Tajabadi, N.; Baradaran, A.; Ebrahimpour, A.; Rahim, R.A.; Bakar, F.A.; Manap, M.Y.A.; Mohammed, A.S.; Saari, N. Overexpression and optimization of glutamate decarboxylase in *Lactobacillus plantarum* Taj-Apis362 for high γ-aminobutyric acid production. *Microb Biotechnol* **2015**, 8(4), 623–632.
88. Kook, M.C.; Seo, M.J.; Cheigh, C.I.; Lee, S.J.; Pyun, Y.R.; Park, H. Enhancement of γ-aminobutyric acid production by *Lactobacillus sakei* B2-16 expressing glutamate decarboxylase from *Lactobacillus plantarum* ATCC 14917. *J Appl Biol Chem* **2010**, 53(6), 816–820.
89. Zhang, C.; Lu, J.; Chen, L.; Lu, F.; Lu, Z. Biosynthesis of γ-aminobutyric acid by a recombinant *Bacillus subtilis* strain expressing the glutamate decarboxylase gene derived from *Streptococcus salivarius* ssp. *thermophilus* Y2. *Process Biochem* **2014**, 49(11), 1851–1857.
90. Li, H.; Qiu, T.; Liu, X.; Cao, Y. Continuous cultivation of *Lactobacillus brevis* NCL912 for production of γ-aminobutyric acid. *Ann Microbiol* **2013**, 63(4), 1649–1652.
91. Li, H.; Qiu, T.; Huang, G.; Cao Y. Production of γ-aminobutyric acid by *Lactobacillus brevis* NCL912 using fed-batch fermentation. *Microb Cell Fact* **2010**, 9:85.
92. Hsueh, Y.H.; Liaw, W.C.; Kuo, J.M.; Deng, C.S.; Wu, C.H. Hydrogel film-immobilized *Lactobacillus brevis* RK03 for γ-aminobutyric acid production. *Int J Mol Sci* **2017**, 18(3):2324.
93. Choi, S.M.; Lee, J.W.; Park, S.M.; Lee, M.Y.; Ji, G.E.; Park, M.S.; Heo, T.R. Improvement of γ-aminobutyric acid (GABA) production using cell entrapment of *Lactobacillus brevis* GABA 057. *J Microbiol. Biotechnol* **2006**, 16(4), 562-568.
94. Villegas, J.M.; Brown, L.; Savoy de Giori, G.; Hebert, E.M. Optimization of batch culture conditions for GABA production by *Lactobacillus brevis* CRL 1942, isolated from quinoa sourdough. *LWT - Food Sci Technol*. **2016**, 67, 22–26.
95. Binh, T.T.T.; Ju, W.T.; Jung, W.J.; Park, R.D. Optimization of γ-amino butyric acid production in a newly isolated *Lactobacillus brevis. Biotechnol Lett* **2014**, 36(1), 93–98.
96. Yang, X.; Ke, C.; Zhu, J.; Wang, Y.; Zeng, W.; Huang, J. Enhanced productivity of γ-amino butyric acid by cascade modifications of a whole-cell biocatalyst. *Appl Microbiol Biotechnol* **2018**, 102(8), 3623–3633.
97. Fan, L.Q.; Li, M.W.; Qiu, Y.J.; Chen, Q.M.; Jiang, S.J.; Shang, Y.J.; Zhao, L.M. Increasing thermal stability of glutamate decarboxylase from *Escherichia coli* by site-directed saturation mutagenesis and its application in GABA production. *J Biotechnol* **2018**, 278, 1–9.
98. Ke, C.; Wei, J.; Ren, Y.; Yang, X.; Chen, J.; Huang, J. Efficient γ-aminobutyric acid bioconversion by engineered *Escherichia coli*. *Biotechnol Biotechnol Equip* **2018**, 32(3), 566–573.
99. Thu, H.N.; Hou, C.Y.; Kim, W.H.; Kang, T.J. Expanding the active pH range of *Escherichia coli* glutamate decarboxylase by breaking the cooperativeness. *J Biosci Bioeng* **2013**, 15(2), 154–158.
100. Lin, L.; Hu, S.; Yu, K.; Huang, J.; Yao, S.; Lei, Y.; Hu, G.; Mei, L. Enhancing the activity of glutamate decarboxylase from *Lactobacillus brevis* by directed evolution. *Chinese J Chem Eng* **2014**, 22(11), 1322–1327.
101. Zhang, Q.; Hu, S.; Zhao, W.; Huang, J.; Mei, J.; Mei, L. Parallel strategy increases the thermostability and activity of glutamate decarboxylase. *Molecules* **2020**, 25(3):690.
102. Lehmann, M.; Pasamontes, L.; Lassen, S.F.; Wyss, M. The consensus concept for thermostability engineering of proteins. *Biochim Biophys Acta* **2000**, 1543, 408–415.

103. Lammens, T.M.; De Biase, D.; Franssen, M.C.R.; Scott, E.L.; Sanders, J.P.M. The application of glutamic acid α-decarboxylase for the valorization of glutamic acid. *Green Chem* **2009**, 11(10), 1562–1567.
104. Park, H.; Ahn, J.; Lee, J.; Lee, H.; Kim, C.; Jung, J.K.; Lee, H.; Lee, E.G. Expression, immobilization and enzymatic properties of glutamate decarboxylase fused to a cellulose-binding domain. *Int J Mol Sci* **2012**, 13(1), 358–368.
105. Lee, S.J.; Lee, H.S.; Lee, D.W. Production of γ-aminobutyric acid using immobilized glutamate decarboxylase from *Lactobacillus plantarum*. *Microbiol Biotechnol Lett* **2015**, 43(3), 300-305.
106. Lammens, T.M.; Franssen, M.C.R.; Scott, E.L.; Sanders, J.P.M. Availability of protein-derived amino acids as feedstock for the production of bio-based chemicals. *Biomass Bioenergy* **2012**, 44, 168–181.
107. Sanchart, C.; Watthanasakphuban, N.; Boonseng, O.; Nguyen, T.H.; Haltrich, D.; Maneerat, S. Tuna condensate as a promising low-cost substrate for glutamic acid and GABA formation using *Candida rugosa* and *Lactobacillus futsaii*. *Process Biochem* **2018**, 70, 29–35.
108. Laroute, V.; Yasaro, C.; Narin, W.; Mazzoli, R.; Pessione, E.; Cocaign-Bousquet, M.; Loubiere, P. GABA production in *Lactococcus lactis* is enhanced by arginine and co-addition of malate. *Front Microbiol* **2016**, 7, 1–11.
109. Lammens, T.M; Franssen, M.C.R.; Scott, E.L.; Sanders, J.P.M. Synthesis of biobased N-methylpyrrolidone by one-pot cyclization and methylation of γ-aminobutyric acid. *Green Chem* **2010**, 12(8), 1430–1436.
110. Park, S.J.; Kim, E.Y.; Noh, W.; Oh, Y.H.; Kim, H.Y.; Song, B.K.; Cho, K.M.; Hong, H.S.; Lee, H.S.; Jegal, J. Synthesis of nylon 4 from γ-aminobutyrate (GABA) produced by recombinant *Escherichia coli*. *Bioprocess Biosyst Eng* **2013**, 36(7), 885–892.
111. Tokiwa, Y.; Calabia, B.P.; Ugwu, C.U.; Aiba S. Biodegradability of plastics. *Int J Mol Sci* **2009**, 10(9), 3722–3742.