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Synthesis of γ-aminobutyric acid (GABA) by glutamate decarboxylase and lactic acid bacteria isolated from Indonesian fermented foods

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ORIGINALITY STATEMENT

I hereby declare that this submission is my own original work and to the best of my knowledge it contains no materials previously published or written by another person, or substantial proportions of material which have been accepted for the award of any other degree or diploma at the University of Natural Resources and Life Sciences BOKU Vienna or any other educational institution, except where due acknowledgment is made in the thesis. Any contribution made to the research by others, with whom I made worked at BOKU or elsewhere, is explicitly acknowledged in the thesis. In addition, I declare that the intellectual content of this thesis is the product of my own work.

Signature 

Place and date Bali, Indonesia 1 July 2021

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Abstract

γ-aminobutyric acid (GABA) is a non-protein amino acids that acts as a potent neural signal transmitter and has been well studied in medical and pharmaceutical fields. GABA synthesis is catalyzed by glutamate decarboxylase (GAD; EC. 4.1.1.15) and the enzyme is widely distributed in microorganisms, various plants and the human brain. In the last decade, natural ways to synthesize GABA have been proposed as chemical synthesis has been rejected because of the corrosive reactants that are used. Currently, there is an increasing interest in GABA production using natural methods such as microbial fermentation and enzymatic synthesis. Most of these processes are based on the GABA-producing ability of lactic acid bacteria (LAB) and relevant enzymes derived from LAB. Fermented foods are considered as an isolation sources of GABA-producing LAB. Hence, it is of great interest to isolate GABA-producing LAB as well as their GAD to cope with the limitation of GABA synthesis using chemical means.

In this first part of this thesis, an overview of GAD from lactic acid bacteria is given. In this review paper, we outline the importance of *gad* genes in LAB along with phylogenetic analysis. Structural function and biochemical properties of GAD from LAB are also briefly discussed and summarized. in addition, various advanced techniques in GABA production and the potential of GAD in bio-based manufacture is also described.

In the second part of the thesis, the isolation, screening and identification of GABA-producing LAB from Indonesian fermented foods is covered. Several fermented foods were used as an isolation source in this study. *L. plantarum,* identified by partial 16S rDNA sequencing and MALDI-TOF MS, is shown to be an efficient GABA-producing LAB. In addition, rep-PCR fingerprinting using the $(GTG)_5$ primer was performed to classified GABA-producing LAB

isolated from fermented foods. These strains could be potential candidates for GABA-producing cultures in GABA-enriched foods.

The third step of this thesis was to produce GABA using microbial fermentation and enzymatic synthesis. The ability of *L. plantarum* FNCC 260 and its enzyme to produce GABA were studied and compared. The *gad*B genes from *L. plantarum* FNCC 260 were cloned and expressed in *E. coli* T7 harboring pGRO7. The results showed that the use of purified GAD was more efficient (5-fold higher) than that of microbial fermentation, suggesting that the use of purified GAD was more economical and should be considered in GABA production as functional foods ingredient. Furthermore, the purified GAD was characterized more detail. GAD from *L. plantarum* FNCC 260 showed optimum pH and temperature at 4.5 and 60^{o}C respectively. GAD activity was enhanced by the addition of 0.6 mM PLP and the presence of 2 mM $CaCl_2$. However, the enzyme showed poor thermostability at 20^{o}C and 30^{o}C respectively.

Zusammenfassung

γ-Aminobuttersäure (GABA) ist eine nicht-protein Aminosäure, die als potenter Neurotransmitter fungiert und in medizinischen und pharmazeutischen Bereichen gut untersucht wurde. Die Bildung von GABA wird durch Glutamat-Decarboxylase katalysiert (GAD; EC. 4.1.1.15). Das Enzym ist in Mikroorganismen, verschiedenen Pflanzen und dem menschlichen Gehirn weit verbreitet. In den letzten zehn Jahren wurden unterschiedliche natürliche Weg, GABA zu synthetisieren, vorgeschlagen, da die chemische Synthese wegen korrosiven Reaktanten, die verwendet werden, starke Nachteile aufweist. Derzeit gibt es ein wachsendes Interesse an der GABA-Produktion mit natürlichen Methoden wie mikrobielle Fermentation und enzymatische Synthese. Die meisten dieser Prozesse basieren auf der GABA-produzierenden Fähigkeit von Milchsäurebakterien (LAB) und denrelevanten Enzymen, die aus LAB gewonnen werden. Fermentierte Lebensmittel gelten als wichtige Quellen zu Isolierung von GABA-produzierenden LAB. Daher ist es von großem Interesse, neue GABA-produzierende LAB sowie deren GAD zu isolieren, um neue natürliche Verfahren zur GABA-Synthese zu etablieren.

Im ersten Teil dieser Dissertation wird ein Überblick über GAD aus Milchsäurebakterien gegeben. In diesem Review skizzieren wir die Bedeutung von *Gad*-Genen in LAB zusammen mit phylogenetischer Analyse, Funktion und biochemische Eigenschaften von GAD von LAB. Darüber hinaus werden verschiedene neue Techniken in der GABA-Produktion und das Potenzial von GAD in der biobasierten Fertigung beschrieben.

Im zweiten Teil der Dissertation wird das Screening, die Isolierung und Identifizierung von GABA-produzierenden LAB aus indonesischen fermentierten Lebensmitteln beschrieben. Unzterscheidliche fermentierte Lebensmittel wurden in dieser Studie als Quelle der Isolierung

verwendet. Ein *L. plantarum*-Stamm, der mittels partieller 16SrDNA-Sequenzierung und MALDI-TOF MS identifiziert wurde, wurde als effizientes GABA-produzierendes LAB nachgewiesen. Darüber hinaus wurde Rep-PCR-Fingerprinting mit $(GTG)_5$ Primern durchgeführt, um GABA-produzierendes LAB aus fermentierten Lebensmitteln zu klassifizieren. Diese Stämme stellen potenzielle Kandidaten zur GABA-Produktion oder neue Starterkulturen für GABA-angereicherten Lebensmitteln dar.

Der dritte Teil dieser Arbeit befasst sich mit der Herstellung von GABA durch mikrobielle Fermentation und enzymatischer Synthese. Die Fähigkeit von *L. plantarum* FNCC 260 sowie der aus diesem Stamm isolierten GAD, GABA zu produzieren, wurden untersucht und verglichen. Die *gad*BGene von *L. plantarum* FNCC 260 wurden geklont und in *E. coli* T7 mit pGRO7 exprimiert. Die Ergebnisse zeigten, dass die Verwendung von gereinigter GAD effizienter war (5-fach höher) als die mikrobiellen Fermentation, was darauf hindeutet, dass die Verwendung von gereinigtem GAD wirtschaftlicher sein könnte und für die GABA-Produktion in Betracht gezogen werden sollte. Darüber hinaus wurde die gereinigte GAD genauer charakterisiert. GAD von *L. plantarum* FNCC 260 zeigte seine optimale Aktivität bei pH 4,5 bzw. 60^{o}C. Die GAD-Aktivität wurde durch die Zugabe von 0,6 mM PLP und in Gegenwart von 2 mM $CaCl_2$ erhöht. Das Enzym zeigte jedoch eine geringe Thermostabilität bei 20°bzw. 30°C.

Publications arising from this dissertation

This dissertation is presented for examination as a cumulative dissertation containing published articles.

Chapter 3.

Ida Bagus Agung Yogeswara, Suppasil Maneerat, Dietmar Haltrich.. *Microorganisms* **2020**, *8*, 1923, DOI: 10.3390/microorganisms8121923.

Chapter 4

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Chapter 5

Dalin Ly, Sigrid Mayrhofer, **I.B. Agung Yogeswara**, Thu Ha Nguyen, Konrad, J. Domig. Identification, classification and screening for γ-aminobutyric acid production in lactic acid bacteria from Cambodian fermented foods. *Biomolecules* **2019**, *9*, 768 DOI: 10.3390/biom9120768.

Chapter 6

Ida Bagus Agung Yogeswara, Suwapat Kittibunchakul, Endang Sutriswati Rahayu, Konrad J. Domig, Dietmar Haltrich, Thu Ha Nguyen. Microbial production and enzymatic biosynthesis of γ-aminobutyric acid (GABA) using *Lactobacillus plantarum* FNCC 260 isolated from Indonesian fermented foods. *Processes* **2021**, *9*, 22 DOI: dx.doi/org/10.3390/pr9010022.

Table of contents

Chapter 1

Introduction

Introduction

Nowadays, consumers pay a lot of attention to the relation between food and health. Consequently, the development of foods with health-promoting properties, so called functional foods, has shown a remarkable growth over the last few years. One of which these functional food ingredients is γ-aminobutyric acid (GABA) as it exhibits several physiological functions and hence has a promising prospect for the Development of Foods for Specified Health Use (FOSHU) [1]. GABA is found naturally in microorganisms and plants, but its concentration in these matrices is low and it requires expensive processes for its isolation or enrichment [1]. Chemical synthesis has been suggested as a route to GABA; however, a drawback of this process is the use of hazardous reagents, which is not applicable for the use in the food industry [2,3]. Furthermore, the direct addition of chemically-synthesize GABA to food is considered unnatural and unsafe. Therefore, more natural methods are needed to introduce GABA in food products [4].

As also found in other parts of east Asia, Indonesia has many kinds of fermented foods. Indonesian people utilize a variety of raw ingredients such as fruit, vegetables, fish, milk and meat for food fermentation. These fermented foods have become a part of their regular diet and food supply. It has been shown that lactic acid bacteria (LAB) are predominant in Indonesian fermented foods [5,6,7]. Moreover, a number of strains of dietary LAB from Indonesian fermented foods showed beneficial physiological properties including bile and acid tolerant, assimilation of cholesterol, antibacterial activity and β-glucosidase activity [8,9]. In general, LAB have been used as probiotics and postbiotic due to their properties and the ability to control food spoilage pathogen by secreting acids, conjugated linoleic acid, vitamin, aromas, bacteriocins, exopolysaccharides, enzymes and GABA [10–16]]. Therefore, utilization of GABA-producing LAB in fermentation processes can offer a natural and safe method to achieve biosynthesis of GABA and at the same

time offering the consumer with new, attractive food products [17,18]. Moreover, the use of LAB as GABA-producing bacteria is a more sustainable approach and gained significant attention to be developed.

Microbial GABA formation involve decarboxylation step of glutamate to GABA, catalyzed by glutamate decarboxylase (GAD, EC. 4.1.1.15) [19]. The responsible enzyme GAD requires pyridoxal-5'-phosphate as cofactor [20]. Several studies suggested that fermented foods can serve as excellent isolation sources of GABA-producing LAB, since free glutamate is liberated during fermentation [16,17]. A number of GABA-producing LAB have thus been isolated from *kimchi* [21], cheese [22–24], fermented soybeans [25], fermented shrimp [26], fermented milk [27], and fermented seaweed [28]. Since LAB are also present in most of Indonesian fermented foods, it is of great interest to study their ability and potential in GABA production as well as their relevant enzyme, GAD. Moreover, studies of GABA-producing LAB and their enzyme (GAD) from Indonesian fermented foods have not been available yet. In this study, several fermented foods were considered as an isolation sources of GABA-producing LAB. GABA-producing LAB were isolated, screened, and identified. Furthermore, the existence of *gad* genes of GABA-producing LAB was revealed for cloning and expression of the GAD protein in *E. coli* T7 harboring pGRO7 vector for enzymatic synthesis and characterization. These preliminary results may provide useful information for potential expression of *gad* genes from LAB in other microbes. In addition, GABA biosynthesis ability from the strain and their GAD were compared.

1.1Lactic acid bacteria from Indonesian fermented foods

Fermentation is recognized as the oldest forms of food preservation in the world. Fermentation can extend the shelf life of highly perishable food products such as meat, vegetables, fish, and fruits, especially in tropical countries like Indonesia. As in parts of Southeast Asia, Indonesian fermented foods feature the use of a variety of raw materials including fish, tubers, soybeans, milk, vegetables, fruits and cereals [5,29,30]. Indonesian fermented foods mainly contain lactic acid (fruits, vegetables, cassava, meat, milk), acetic acid (cereals, vegetables), alcohol (rice, cassava) as natural preservative, and additionally involve mold fermentations (soybeans, peanut press cake) and high salt fermentations (fish, soy sauce, fermented bean slurry) [31,32]. Fermentation processes also enhance the organoleptic characteristics of foods including the changes of aromas, flavors and textures. Furthermore, fermentation can improve the digestibility and nutritional values of the food products with vitamins, proteins, essential amino acids, essential fatty acids and other bioactive components such as bioactive peptides and γ-aminobutyric acid (GABA) formed [33–35].

Lactic acid bacteria (LAB) are a group of Gram-positive, non-spore forming, coccus or rod shaped bacteria. They able to ferment carbohydrates to lactic acid (homofermentation) or to a combination of acetic acid, lactic acid, carbon dioxide and/or ethanol (heterofermentation) [36,37]. Other compounds such as diacetyl, acetaldehyde and hydrogen peroxide are also produced. These compounds contribute to the flavor and texture of fermented foods and may also contribute to the inhibition of undesirable microbes [38,39].

LAB identified in Indonesian fermented foods are dominated by *Lactobacillus* spp, followed by other genera such as *Pediococcus, Lactococcus, Streptococcus, Weisella* and *Leuconostoc*

(Table 1). Furthermore, lactobacilli have been found in plant-based and meat-based fermentation [7], while pediococci and streptococci have been found in fermented plant material and fermented food of fish origin [40,41]. LAB are involved to varying degrees in Asian fermented foods and can have positive and negative effects on food products. In cereal alcoholic fermentations, LAB contribute to the development of the unique flavor and taste. In fruit, vegetables, milk and meat fermentations, LAB play a major role in producing the acid necessary for the preservation and quality of the products [42]. It is interesting that LAB are generally present in *tempe* (fermented soybeans) in which, *Rhizopus oligosporus* is the primary microbe involved in the fermentation process. Acid fermentations involving LAB occur during the soaking of the soybeans overnight, and some growth of LAB commonly occurs during the stage of mold growth as well [32,43,44]. As mentioned above, lactic acid showed antibacterial activity which may lead to the safety of fermented foods.

Fermented food	**Raw materials**	**Fermentation process**	**LAB species**	**References**
Tempoyak	Durian flesh	lactic fermentation	*L. plantarum, L. coryneformis,L. casei, L. plantarum. Weisella paramesentroides, P. acidilactici, Enterococcus gallinarum, E. faecalis*	[45]
Tape starter culture	Rice flour		*P. pentosaceus, E. faecium, L. curvatus, W. confusa, W. paramesentroides*	[46]
Growol	Cassava	lactic fermentation	*L. plantarum, L. rhamnosus.*	[41,47]
Tempeh	Soybean	Mold fermentation	*L. fermentum, L plantarum, P. pentosaceus, W. confusa, L. delbrueckii subs delbrueckii*	[32]

rice wine	glutinuous rice (steam)	alcoholic fermentation	*P. pentosaceus, Weisella sp*	[48]
Soy sauce	Soybean	mold fermentation followed by brine fermentation	*Tetragenococcus halophillus*	[49]
Salted vegetable	Mustard cabbage leaf	lactic fermentation	*L. plantarum, L. fermentum, L. brevis, L. namurensis, L. farciminis, L. rhamnosus, L. curvatus, P. pentosaceus*	[50]
Bekasang	Fish	high salt fermentation	*P. acidilactici*	[51]
Urutan (traditional Balinese sausage)	Lean pork	lactic fermentation	*L. plantarum, P. acidilactici, L. farciminis*	[52]
Dadih	Buffalo milk	lactic fermentation	*L. mesentroides, L. brevis, L. casei, L. plantarum, E. faecium, L. fermentum. L. rhamnosus, L. lactis.*	[53,54]
Fermented Mare milk	Mare milk	lactic fermentation	*L. rhamnosus, L. fermentum, L. acidophilus, L. brevis.*	[55,56]

1.2GABA pathway in LAB

GABA biosynthesis by microorganisms is performed by the glutamic acid decarboxylase (GAD, EC 4. 1. 1. 15) system, consisting of the GAD enzyme (encoded by *gadA* or *gadB*) and glutamate/GABA antiporter GadC [57–61] . The biosynthetic pathway of GABA is shown in Figure 1.

L-glutamate is transported into the cell through GadC. L-glutamate is decarboxylated by GAD, a pyridoxal-5'-phosphate dependent enzyme, and which leads to the synthesis of GABA. This step is irreversible and the reaction will consume a proton and release CO_2 as byproduct. The resulting product GABA is subsequently exported to the extracellular matrix by GadC [62,63]. α-ketoglutarate is synthesized from glucose as a precursor of L-glutamate via the glycolysis pathway and part of the tricarboxylic acid (TCA) cycle, and then transformed by glutamate dehydrogenase

into L-glutamate (GDH, EC 1. 4. 1.4). The 2-oxoglutarate dehydrogenase complex (ODHC) is an essential enzyme of the TCA cycle and functions at the branching point of metabolic flux between the synthesis of L-glutamate and energy supply. In addition, it competes with GDH for the substrate of α-ketoglutarate.

In prokaryotes, such as *E. coli* and *L. monocytogenes,* the molecular mechanisms of GABA degradation have been revealed [64,65]. GABA is degraded to succinic semialdehyde (SSA) by GABA aminotransferase (GABA-AT, EC 2. 6. 1. 19). SSA is then converted to succinic acid by succinate semialdehyde dehydrogenase (SSADH, EC 1. 2. 1. 16) for entry into the TCA cycle.

The GABA-AT encoding gene *gadT has* been identified in some species of LAB such as *L. fermentum, L. frumenti, L. gastricus, L gorilla, L mucosae, L oris, L plantarum* [66], *L. pontis, L. reuteri* [67], *L. similis, L. vaginalis, Leuconostoc citreum* [68], *Leuconostoc kimchii* [69,70], *Leuconostoc mesentroides* subsp. *mesentroides* and *Oenococcus oeni* [71]. Recently, it was found that MSG enhanced GABA production of *L. plantarum*. It was clear that the activity of GABA-AT was inhibited and GABA-AT activity is strongly correlated with GABA production [72]. Synthesis of GABA can also proceed through polyamines (putrescine and spermidine) degradation [73] and under oxidative stress, it occurs by non-enzymatic reaction from proline [74].

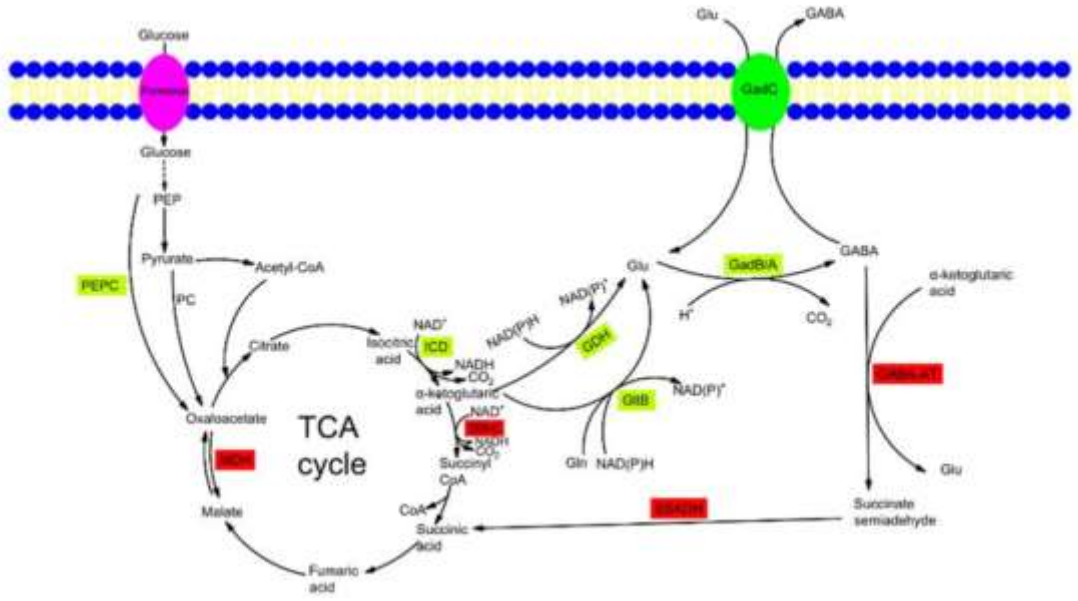

Figure. The biosynthetic pathway of GABA by microorganism. GABA-AT, GABA aminotransferase; GadB/GadA, glutamate decarboxylase; GadC, glutamate: γ-aminobutyrate antiporter; GDH, L-glutamate dehydrogenase; GltB, glutamate synthetase; Icd, isocitrate dehydrogenase; MDH. Malate dehydrogenase; ODHC. 2-oxoglutarate dehydrogenase complex; PC, pyruvate carboxylase; PEP, phosphoenolpyruvate; PEPC, phosphoenolpyruvate carboxylase; SSADH, succinate semialdehyde dehydrogenase; TCA cycle. The expression of enzymes in green font increase GABA production, and the expression of enzymes in red font decrease GABA production (adapted from [75]).

1.3 Isolation sources of GABA-producing LAB

A number of microorganisms including various bacteria, yeasts and fungi have been reported to produce GABA[76–80]. The most interesting and practical group of bacteria for GABA production are LAB, which produce high levels of GABA [81]. LAB possess' special physiological activities, are generally recognized as safe (GRAS), and have been extensively

utilized in food industries for a long time [82]. GABA production by LAB is thus natural and safe.

The GABA-producing LAB is mostly isolated from several fermented foods. To date, *L. brevis* was the predominant and most-studied species from fermented foods and, due to its ability to efficiently produced high amount of GABA [27,83]. GABA-producing *L. brevis* was isolated from *kimchi* [21], Chinese *paocai* [83], fresh milk [84], cheese [24] and fermented fish [85]. *L. farmicinis* was isolated from fermented fish [85], *L. delbrueckii* subsp. *bulgaricus* was isolated from cheese [24], *L. plantarum* was isolated from cheese and fermented fish [24,85], *L. paracasei* from fermented fish [86]. *L. acidophillus* was also isolated from fermented meat [16], *L. buchneri* from kimchi [87], *L. helveticus* from fermented mare milk [88]. In addition, *L. lactis* was isolated from cheese [89], kimchi and yoghurt [76,90]. These results indicate that Lactobacilli is the most dominant species were reported as GABA-producing LAB. Moreover, *Lactobacillus* spp is presence in the last stage of fermentation due to low acid production than cocci, but more tolerant to acid than cocci [91,92].

Although many GABA-producing LAB strains have been isolated and identified, a further isolation and characterization research is needed. In a further screening, the isolation sources should be expanded to as many as possible fermented foods to obtain GABA-producing LAB strains. This will lead to a wider application area and higher flexibility of starter cultures

1.4 Some LAB strains cannot produce GABA

It is well known that the property of GABA production is species/strain dependent [93–95]. Possible reason for this is the presence or absence of *gadCB* genes. Studies by Nomura et al. (2000) revealed that *gadCB* genes are present in *L. lactis subsp. cremoris* and that they are

not grossly rearranged by insertions or deletions of large fragments [96]. However, when a one-base deletion of adenine and a one-base insertion of thymine occurred within the coding region, this leads to frame shift mutations. As the mutations occurred within the coding region, the translated protein was not functional. The regions around these two mutations were sequenced in other *L. lactis subsp. cremoris* strains to confirm that the mutations are common [96]. These findings indicate that the development of polymerase chain reaction (PCR)-based methods for rapid detection of GABA-producing LAB is feasible.

1.5 Physiological functions of GABA and GABA-enriched foods

Several physiological functions of GABA have been studied and these results lead to the development of functional foods enriched with GABA. GABA is found in high concentrations in the mammalian brain where it acts as the most inhibitory neurotransmitter. GABA plays an important role in the regulation of local neuronal circuitry such as noradrenergic, dopaminergic and serotonergic neurons [97]. The alteration of GABAergic circuits or the GABA content in the brain is linked to Alzheimer disease, Parkinson disease, Huntington disease, stiff person syndrome and schizophrenia [97]. Glutamate decarboxylase converts L-glutamate in the human neuron to GABA and acts as inhibitor to prevent the alteration of the GABA concentration in the brain. Alteration of GABA plasma levels have been found in depression patients suffering from mood disorders. In addition, lower GABA plasma levels were also found in children and adolescents with mood disorders [97]. Seo et al. reported that the administration of 46.69 mg/ml of GABA significantly improved the neurological disorders of mice [98]. These studies also suggest that high doses of GABA intake may be necessary for the efficacy of GABA. Ko et al. demonstrated that fermented black soybeans enriched with

GABA exhibit antidepressant effect in rats subjected to a forced swimming test [99]. Moreover, fermented black soybeans had a similar antidepressant effect to that of fluoxetine and without any side effect [99]. In human trials, Okada et al. demonstrated that daily consumption of rice germ containing 26.4 mg of GABA 3 times a day was significantly effective in treating neurological disorders such as depression and sleeplessness in menopausal women [100]. Studies by Cho et al. reported that *Lactobacillus buchneri* MS isolated from *kimchi* showed complete protection of neuronal cells against neurotoxicant-induced cell death [87].

GABA can be used to improve other physiological functions such as long term memory [98]), sleeplessness and depression [100,101]. High GABA doses were also studied in tea and demonstrated sleep-promoting effect mainly mediated via GABAergic systems [102]. This bioactive compound could potentially increase insulin secretion and prevent diabetes [103]. Hagiwara et al demonstrated that feeding diabetic rats with pre-germinated brown rice containing GABA could ameliorated blood glucose elevation and decrease lipid peroxidation. Furthermore, the protective effect of pre-germinated rice containing GABA was effective to prevent diabetic complication [104]. Protection against oxidative stress was also studied by Xie et al. who showed that administration of 0.2% and 0.12% of GABA could prevent obesity by ameliorating oxidative stress and protect thyroid functions of mice with high fat diet [105]. Another study reported that GABA-rich yoghurt exhibits positive impact on diabetic rats. GABA-rich yogurt significantly decreased the concentration of total cholesterol and triacylglycerol, whereas remarkably increased the HDL concentration in serum of diabetic rats [106]. This study also suggested that novel GABA-rich yoghurt could delay the progression of diabetes complication and can be used to treat type 1 diabetes.

Daily consumption of GABA-enriched foods affected the development of cancer cells. Consumption of brown rice with high GABA content could delay the proliferation of leukemia cells and had stimulatory effect on cell apoptosis [107]. Other natural products such as rice germs possess antimutagenic activity in animal model. Dietary administration of GABA-enriched defatted rice germ could prevent the proliferation of colonic adenocarcinoma cells and also reduced the frequency of colonic adenocarcinoma by 71% [108]. The authors suggested that GABA-enriched rice germ is a possible dietary preventative for human colon cancers. Physiological functions of GABA are link to regulation of sleep latency, duration and total sleeping time. The administration of GABA increased the sleep quality and GABA content in plasma serum in vertebrate models [109]. Furthermore, long term administration increased GABA receptor and serotonin signaling. Mood disorders have a link to low GABA levels in plasma since this bioactive compounds may be considered as a biological marker of vulnerability to the development of various mood disorders [110]. Studied by Petty reported that plasma concentrations of GABA are significantly lower in patients with major depressive disorder, mania and bipolar disorder. In addition, these low concentrations of GABA appear to persist after recovery from depressions and are not increased after treatments [111]. However, GABA has been shown to reverse depression and is effective in unipolar and bipolar patients by increasing brain GABAergic activity [97]. Clinical evidence suggests that some human nervous disorders involving GABAergic systems are related to thyroid dysfunctions such as hyperthyroidism or hypothyroidism. Furthermore, thyroid hormones have effects on multiple components of the GABA system such as enzyme activities responsible for synthesis and degradation of GABA, levels of glutamate and GABA, GABA release and uptake, and GABA receptor expression and function. Hypothyroidism generally decreases enzyme

activities and GABA levels in the developing brain, whereas, in adult brain, hypothyroidism generally increased enzyme activities and GABA levels [111]. Xie et al. showed that a certain dose of GABA administration contributes to thyroid function through activating antioxidative enzyme pathways [105].

Several reports have associated GABA with blood regulation. Numerous studies have shown that GABA can reduce blood pressure in animals and humans. In animal studies, administration of GABA was able to decrease blood pressure in spontaneously hypertensive rats (SHR). A study by Suwanmanon and Hsieh has shown that daily intake of fermented soybeans *natto* containing GABA was able to control the systolic blood pressure in SHR. In addition, systolic and diastolic blood pressure was significantly reduced after 8 weeks of administration [112]. Another GABA-enriched fermented soybean *tempe* was developed using *Rhizopus oligosporus* IFO 8631 and shown to lower blood pressure in SHR [80]. The development of novel functional foods contained GABA in dairy product was conducted by Hayakawa et al. A novel GABA-enriched fermented milk showed a decrease in systolic blood pressure in SHR and the hypotensive effect of fermented milk product depended on GABA [113]. Furthermore, reduction of blood pressured in SHR can be observed from 4 to 8 hours after administration of low-dose GABA-enriched fermented milk [113]. GABA also naturally occurs in mulberry leaves and tea leaves. Water extracts from mulberry leaves and tea leaves have been reported to exert antihypertensive effect. Mulberry leaf extract containing GABA decreased systolic blood pressure in SHR in a dose-dependent manner [114]. In addition, daily oral intake of green tea rich in GABA decreased blood pressure in young and old-sensitive rats fed with a high salt diet [115]. Mechanisms underlying the antihypertensive effect of GABA in SHR were shown by Hayakawa et al. A single oral administration of GABA significantly

lowered the systemic blood pressure in the mesenteric arterial bed from SHR. Moreover, GABA-induced antihypertensive effect may be due to the inhibition of noradrenaline release from sympathetic nerve fibers through an action of GABA receptors via GABAergic systems [116].

In human trials, dietary interventions containing GABA have been reported. Daily intake of 50 g of cheese containing 16 mg of GABA decreased significantly systolic blood pressure of men with slightly elevated blood pressure [117]. The author suggests that production of GABA in cheese could be enhanced through a longer ripening time. An effect of GABA in patients with mild hypertension was also observed [118]. Daily intake for 12 weeks of a fermented milk product containing GABA significantly reduced blood pressure within 2 or 4 weeks and it remained decreased throughout the 12 - week intake period. Antihypertensive effects of algae enriched with GABA were also reported in human. Daily interventions for 16 weeks of chlorella containing 20 mg of GABA reduced blood pressure in mildly hypertension subjects [119]. Furthermore, other studies suggest that consumption of one portion (30 mg) of GABA-enriched breakfast cereals can meet up to 55% of the daily requirement to lower blood pressure (ca. 10 mg) [120].

1.6 Potential application of GABA-producing lactic acid bacteria

Production of GABA using lactic acid bacteria has been extensively studied, since these microorganisms are generally considered as safe or GRAS (generally recognized as safe). The consumers awareness towards the selection of health-beneficial foods contributes to the significant growth of GABA-enriched foods as part of functional foods. Natural addition of GABA is demanded over the addition of chemical synthesized GABA since consumers prefer naturally-

occurring substances. The fermentation helps to reduce the cost of the foods due to the omission of chemical addition of GABA and also provides attractive foods with better taste. Therefore, GABA production by naturally-occurring microorganisms during fermentation is getting higher request.

Production of GABA by lactic acid bacteria has been studied in food products such as raspberry juice, cheese, kimchi, fermented adzuki milk, fermented sea tangle, *Nham* sausages and fermented soybean [18,24,80,120–123]. GABA-producing *Lactobacillus brevis* GABA100 was employed to manufacture fermented black raspberry juice. The fermentation at 30^{o}C generally showed higher production of GABA in the juices. GABA was produced continuously even when the viable bacterial counts markedly decreased. This indicated that black raspberry juice can be enriched with GABA using strain *L. brevis* GABA100 [122]. Some commercial cheeses have been found to contain GABA [89,117]. Di Cagno et al. manufactured a functional grape must beverage enriched GABA by a fermentation of *L. plantarum* DSM19463 (124). This functional beverage was reported to have potential antihypertensive effect and dermatological protective properties. Hence, the full use of the by-products based on LAB capacity for synthesizing GABA may open new perspectives of GABA-enriched food products.

GABA-producing lactic acid bacteria can also act as probiotics and this property adds functional traits for lactic acid bacteria as functional foods ingredients. Probiotics can only be effective if they remain viable as they survive through the stomach and colonize the intestine [125]. Conversion of glutamate to GABA by irreversible decarboxylation will consumes an intracellular proton in the LAB cells, hence, maintaining a neutral cytoplasmic pH when the extracellular pH drops. Due to its role in pH resistance, it was suggesting that LAB with high GAD activities have potential use as probiotics. Three strains *Lactobacillus pracasei* PF6, *Lactobacillus delbruekii*

subsp *bulgaricus* PR1 and *Lactobacillus plantarum* C48 isolated from cheese were subjected to pepsin and pancreatin digestion and they survived and synthesized GABA. These results suggest that the strains are able to survive and synthesize GABA under simulated gastrointestinal conditions [24].

Chapter 2

Aims and Dissertation Overview

2.1 Objectives and Dissertation Overview

γ-aminobutyric acid (GABA), as outlined above is an interesting inhibitor neurotransmitter and can be synthesized using microbial fermentation and enzymatic synthesis. Glutamate decarboxylase (GAD, EC. 4.1.1.15) is a key enzyme in catalyzing the decarboxylation reaction of glutamate to GABA, releasing CO_2 as by-product. It is the objective to study alternative LAB and GAD derived thereof as producing systems for GABA, since the main focus in the scientific literature had been on *L. brevis* and GAD derived from *L. brevis.* The overview regarding GAD from LAB is described in **chapter 3**. In this review paper, the presence of *gad* genes in LAB and a phylogenetic analysis of GAD are briefly described. Moreover, structural and biochemical properties of GAD from different LAB is summarized.

In **Chapter 4** presents GABA-producing LAB from Indonesian fermented foods. Fermented foods serve as an interesting and appropriate isolation source of GABA-producing LAB. Therefore, several Indonesian fermented foods were considered in this study. The fermented foods consisted of two kinds of fermented cassava, fermented soybeans (*tempe*), salted cabbage, fermented chicken and beef sausage, salted fruits and *bekasam* (fermented fish with rice). GABA-producing LAB were isolated, screened and quantified for their GABA production. In **chapter 5,** molecular (16S rDNA and rep-PCR) and proteomic methods (MALDI-TOF MS) were performed to classify GABA-producing LAB at strain level.

In **chapter 6,** the ability of LAB and their enzyme GAD to produce GABA were determined. The effect of various concentrations of MSG, pyridoxal-5'-phosphate (PLP) and pyridoxine on GABA production by *L. plantarum* FNCC 260 were determined. Furthermore, *gad*B genes from *L. plantarum* FNCC 260 were amplified, cloned and expressed in *E. coli* T7 harboring

pGRO7. Microbial production and enzymatic synthesis of GABA of this strain were compared. The biochemical properties of GAD were further characterized as supplementary materials in **Chapter 7**.

Chapter 3

Glutamate decarboxylase from lactic acid bacteria- A key enzyme in GABA synthesis

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Review

Glutamate Decarboxylase from Lactic Acid Bacteria—A Key Enzyme in GABA Synthesis

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Abstract: Glutamate decarboxylase (L-glutamate-1-carboxylase, GAD; EC 4.1.1.15) is a pyridoxal-5'-phosphate-dependent enzyme that 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, the biochemical properties of their GAD enzymes, and possible applications are reviewed here.

Keywords: γ-aminobutyric acid production; lactic acid bacteria; glutamate decarboxylase; fermented foods; *gad* genes

1. Introduction

Lactic acid bacteria (LAB) are Gram-positive, acid-tolerant, non-spore forming bacteria, with a morphology of either cocci or rods that share 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*, in addition to *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 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 the 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 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), and the reaction requires pyridoxal-5′-phosphate (PLP) as a cofactor (Figure 1). A wide range of LAB possess the ability to produce GAD, and the biochemical properties have been studied from a number LAB sources, 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 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 fed mice [14], and that it can effectively prevent diabetic conditions by acting as an insulin secretagogue [15,16]. Due to these properties, GABA or GABA-rich products are of interest as a food supplement or functional food.

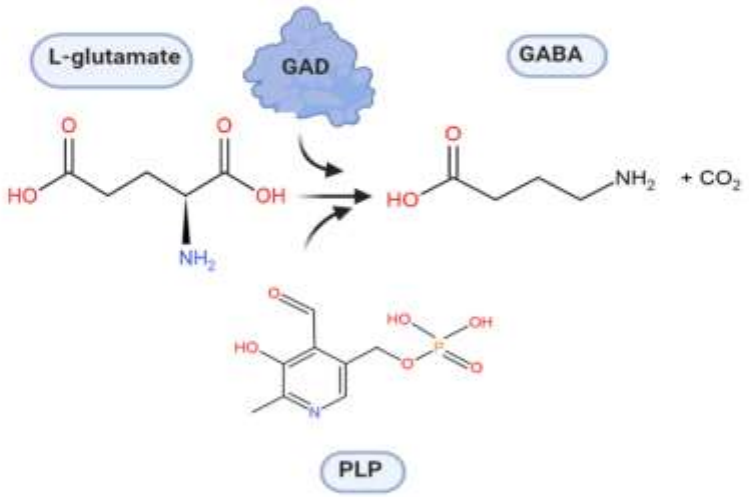

Figure 1. Decarboxylation of L-glutamate to GABA catalyzed by glutamate decarboxylase. PLP: pyridoxal- 5′-phosphate.

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, however, 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 in 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 sources had first been shown to have the ability to produce GABA, for example, *Lactobacillus namurensis* NH2 and *Pediococcus pentosaceus* NH8 from *nham* [19], *Lactobacillus paracasei*

NFRI 7415 from Japanese fermented fish [20], *L. paracasei* PF6, *Lactococcus lactis* PU1 and *Lactobacillus brevis* PM17 from cheese [21], *L. brevis* CGMCC 1306 from unpasteurized milk [22], *L. brevis* GABA100 from kimchi [23,24], *L. brevis* BJ20 from fermented sea tangle [25], *Lactobacillus futsaii* CS3 from Thai fermented shrimp [26] and *L. brevis* 119-2 and *L. brevis* 119-6 from *tsuda kabu* [12]. Recently, many studies have focused on the identification of novel GABA-producing LAB and investigated the biochemical properties of GAD from different strains in more detail [12,14,15,27–29].

Here, we outline the presence of *gad* genes in LAB as important and efficient GABA-producing organisms together with a phylogenetic analysis, we summarize the 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 Glutamate to γ-Aminobutyric Acid (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 [2,13,25,26,30] (Table 1).

Table 1. Diversity of glutamate to γ-aminobutyric acid (GABA)-converting lactic acid bacteria (LAB), isolation sources, GABA production, and fermentation conditions. GABA concentrations as found in food products fermented with this strain are given.

LAB Species and Strain	Sources	Fermentation Conditions	GABA Production	References
L. brevis HY1	*Kimchi*	30 °C, 48 h	18.76 mM	[27]
L. brevis NCL912	*Paocai*	pH 5.0, 32 °C, 36 h Fed-batch fermentation	149.05 mM	[28]
L. helveticus NDO1	*Koumiss*	pH 3.5, 30 °C, 30 h	0.16 g/L	[29]
L. brevis BJ20	Fermented *jotgal*	30 °C, 24 h	2.465 mg/L	[25]
L. paracasei 15C	Raw milk cheese	pH 5.5, 30 °C, 48 h, anaerobe	14.8 mg/kg	[30]
L. rhamnosus 21D-B	Raw milk cheese	pH 5.5, 30 °C, 48 h, anaerobe	11.3 mg/kg	[30]
S. thermophilus 84C	Raw milk cheese	pH 5.5, 30 °C, 48 h, anaerobe	80 mg/kg	[30]
L. plantarum DM5	*Marcha Sikkim*	pH 6.4, 30 °C, 30 h	NR	[31]
L. brevis L-32	*Kimchi*	30 °C, 24 h	38 g/L	[32]
L. buchneri WPZ001	Chinese fermented sausage	30 °C, 72 h	129 g/L	[33]
L. lactis	*Kimchi*	pH 5.5, 30 °C, 20 h	6.41 g/L	[34]
L. otakiensis	Pico cheese	30 °C, 48 h	659 mg/L	[35]
S. thermophilus Y2	Yoghurt	pH 4.5, 40 °C, 100 h	7.98 g/L	[36]
L. buchneri MS	*Kimchi*	pH 5.0, 30 °C, 36 h	251 mM	[37]
E. faecium JK29	*Kimchi*	30 °C, 72 h	14.86 mM	[38]
L. brevis 877G	*Kimchi*	30 °C, 24 h	18.94 mM	[39]
L. plantarum IFK 10	fermented soybean	pH 6.5, 37 °C, 48 h	2.68 g/L	[40]
Weissella hellenica	*ika-kurozukuri*	30 °C, 96 h	7.69 g/L	[41]
L. brevis K203	*Kimchi*	pH 5.25, 37 °C, 48 h	44.4 g/L	[42]
L. futsaii CS3	*Kung-som*	37 °C, 108 h	25 g/L	[26]
L. paracasei NFR7415	Fermented fish	30 °C, 144 h	302 mM	[20]
L. plantarum C48	Cheese	30 °C, 48 h	16 mg/kg	[21]

Table 1. *Cont.*

LAB Species and Strain	Sources	Fermentation Conditions	GABA Production	References
L. paracasei PF6	Cheese	30 °C, 48 h	99.9 mg/kg	[21]
L. brevis PM17	Cheese	30 °C, 48 h	15 mg/kg	[21]
L. lactis PU1	Cheese	30 °C, 72 h	36 mg/kg	[21]
L. delbrueckii subsp. *bulgaricus* PR1	Cheese	42 °C, 48 h	63 mg/kg	[21]
L. lactis subsp. *lactis*	Cheese starter	30 °C, 48 h	27.1 mg/L	[3]
L. brevis CECT 8183	Goat cheese	pH 4.7, 30 °C, 48 h	0.96 mM	[16]
L. brevis CECT 8182	Sheep cheese	pH 4.7, 30 °C, 48 h	0.94 mM	[16]
L. brevis CECT 8182	Goat cheese	pH 4.7, 30 °C, 48 h	0.99 mM	[16]
L. lactis CECT 8184	Goat cheese	pH 4.7, 30 °C, 48 h	0.93 mM	[16]
L. namurensis NH2	*Nham*	30 °C, 24 h	9.06 g/L	[17]
P. pentosaceus HN8	*Nham*	30 °C, 24 h	7.34 g/L	[17]
L. plantarum	*paork kampeus*	pH 6.5, 37 °C, 72 h	20 mM	[1]

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. plantarum*, *L. helveticus*, or *L. futsaii* [21,30–33,42,43]. Among these, *L. brevis*, a heterofermentative LAB, is one of the best-studied organisms [43] and is 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. Furthermore, the adjustment of the pH medium to an acidic condition (pH 4.5–5.5) could improve GABA production since GABA biosynthesis is closely related to the pH. 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]; Thai fermented fish *plaa-som* [45], or other fermented vegetable (kimchi) [46]; fermented shrimp paste [47]; cheese [16] or milk products as well as various fermented meat or fish products including sausages or traditional fermented Cambodian food, mainly based on fish, where six 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 a carbon source, was isolated from *kimchi* [46]. Recently, Sanchart et al. isolated the novel GABA-forming strain *L. futsaii* CS3 with probiotic properties from fermented shrimp (*Kung-som*) [26,47]. 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 the 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 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 the 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 to transform considerable amounts of glutamate into GABA, and identified Weissella hellenica as a novel GABA producer [41]. Thus, these new genera expand the list of GABA-producing bacteria, which can 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]. Production of GABA by different LAB together with fermentation conditions, yields, and productivities has recently been reviewed in detail [15,43,48].

3. Occurrence and Organization of Glutamic Acid Decarboxylase (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 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 the cytoplasm in the presence of L-glutamate (Figure 2) [2,6,53,54]. This system, the so-called glutamate-dependent acid-resistance system (GDAR), provides protection under the 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., *gadA*, *gadB*, and *gadC*) 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 [21]. Recently, cell numbers of the GABA-producing strain *L. futsaii* CS3 were 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 the potential as functional probiotic starter cultures [47].

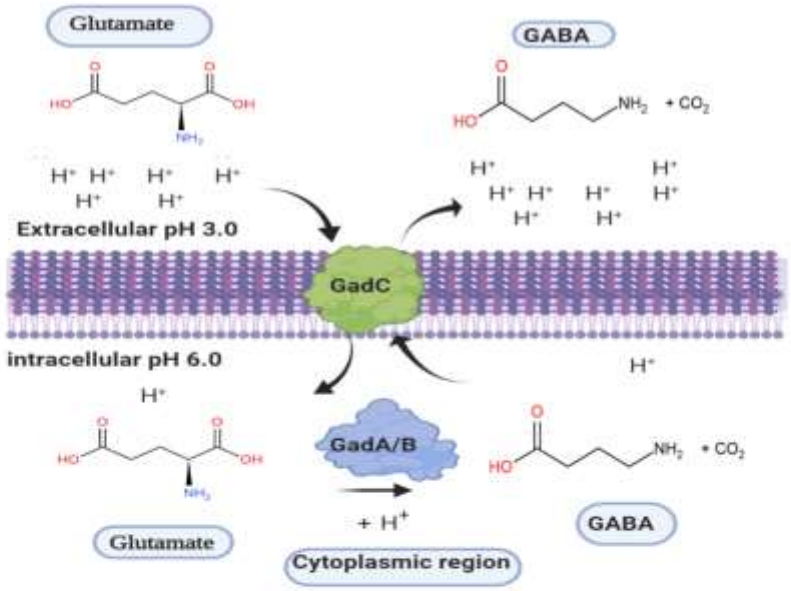

Figure 2. Schematic representation of the glutamate-dependent acid-resistance system. Glutamate (net charge 0) is taken up by the L-glutamate/GABA antiporter GadC, while concurrently GABA is exported by GadC as indicated by the arrows. Subsequently, GadA/B catalyze the decarboxylation of glutamate by consuming an intracellular proton (H^+) at each cycle and generate the proton motive force by GABA export (net charge +1).

GAD systems and the organization of the *gad* operons among LAB species are highly variable [56,57]. Numerous studies reported that some LAB species such as *Streptococcus thermophilus* [5], *L. brevis* [6,7],

or *L. lactis* [3] have one or two *gad* genes (i.e., *gadA*, *gadB*), together with the antiporter (*gad*C). Interestingly, *E. avium* 352 carries three *gad* genes [58]. Typically, *L. brevis* contains two GAD-encoding genes, *gadA* and *gadB*, which when expressed yield GAD enzymes that share approximately 50% amino acid sequence similarity [6]. In contrast, the *gad*B gene is absent in strain *L. brevis* CD0817 [59] and the amino acid sequence identities of GadA and GadC from *L. brevis* CD0817 against other *L. brevis* strains are 91% and 90%, respectively. The transcriptional regulator gene *gadR* plays a crucial role in GABA production and acid resistance in *L. brevis*. Gong et al. reported that deletion of *gadR* in *L. brevis* ATCC 367 resulted in lower expression of both the *gadB* and *gadC* gene, a concurrent reduction in GABA synthesis, and an increased sensitivity to acidic conditions [6]. Expression levels of *gadR* are varied among different LAB strains. The *gadR* gene was expressed 13–155-fold higher than *gadCB* in *L. brevis* NCL912 during the cultivation period [60]. In contrast, expression of *gadR* in *L. brevis* CGMCC1306 was observed to be much lower compared to *gad*CB. The role of GadA and GadB in *L. brevis* CGMCC1306 was investigated by disruption of the genes *gadA*, *gadB*, and *gadC*, 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* (six strains), *L. buchneri* (two strains), *L. oris* (three strains), *L. lactis* (29 strains), and *L. garvieae* (five strains) were shown to have an intact *gad* operon. The majority of these strains were shown to contain either *gadA* or *gadB*, whereas *gadC* 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 the 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 implies that the order of *gad*B and *gad*C in *S. thermophilus* ST110 is similar to *S. thermophilus* Y2 [63], but in a different order from that reported for *L. lactis* [64], *L. brevis* [60], and *L. plantarum* [62].

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 (*gadC1* and *gadC2*) as well as the glutaminase-encoding gene *gls3*, indicating that glutamine serves as a substrate for the synthesis of GABA. The organization of the *gad* operon is in a different order for other species of LAB (*L. lactis* and *L. plantarum*) as glutaminase (*gls3*) is in between the antiporters *gadC1* and *gadC2*, 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 *gadA* from *L. brevis* NCL912, and the whole gene fragment (4615 bp) including *gadR*, *gadC*, *gadA*, 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 *gadA* locus, forming a *gadCA* 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 *Escherichia 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 showed that amino acid sequences of GAD are highly conserved within the same species (Figure 3), 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 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. aviaries*, *L. coleohominis*, *L. farraginis*, *L. japonicas*, *L. koreensis*, *L. nuruki*, *L. oris*, *L. rossiae*, *L. rennini*, or *L. suebicus* (Figure 3). 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.

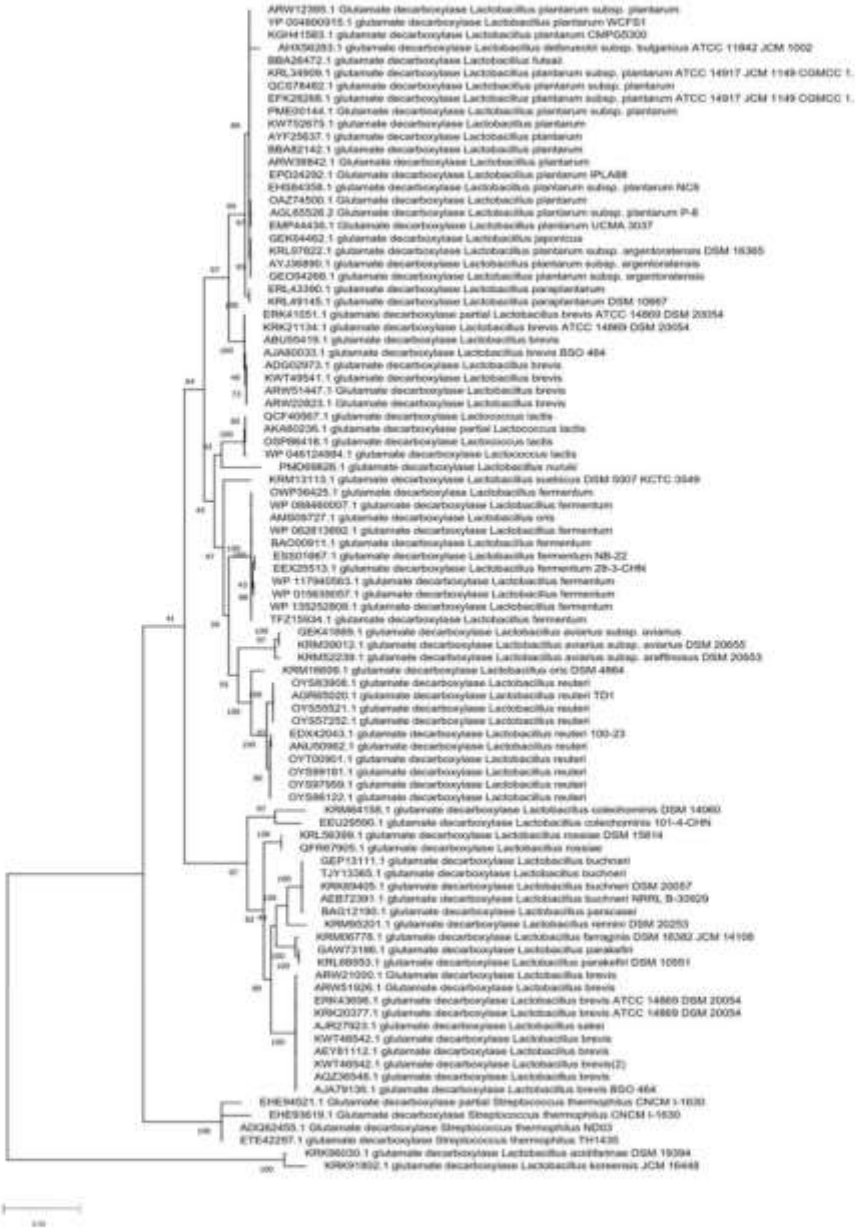

Figure 3. Phylogenetic analysis of glutamate decarboxylase from different species of LAB. The phylogenetic tree was calculated based on the amino acid sequences of glutamic acid decarboxylase (GAD) (maximum-likelihood method). The phylogenetic analysis was performed after the alignment of GAD sequences using MUSCLE in the MEGA X software.

4. Glutamate Decarboxylase

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 PLP-dependent enzyme and as such belongs to the PLP-dependent enzyme superfamily. This superfamily comprises 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* CGMCC 1306, the active site entrance is located at the *re*-face of the cofactor PLP. PLP is covalently attached to a lysine (K279) via an imine linkage (Figure 4), referred to as an internal aldimine [68,71]. 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 [72]. 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].

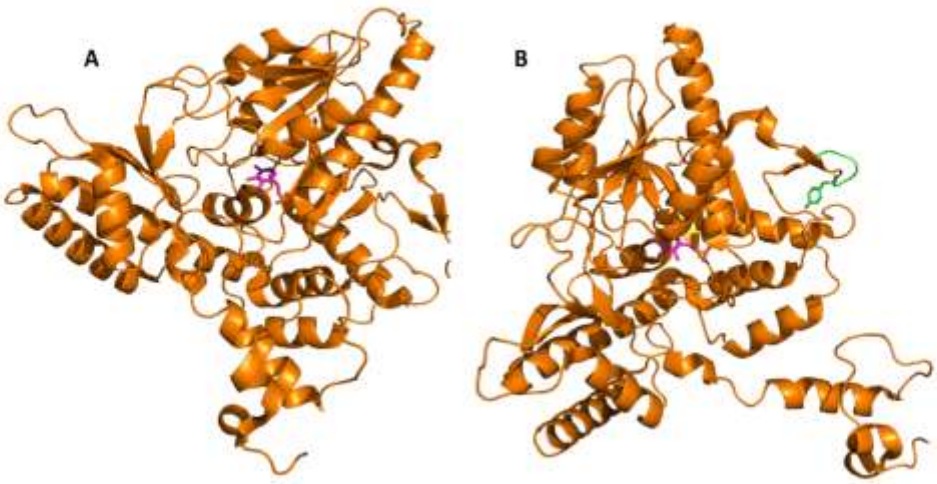

Figure 4. Overall secondary structure of the glutamate decarboxylase monomer from *L. brevis* (PDB code 5GP4). (**A**) Chain A is represented as an orange cartoon, and its prosthetic group PLP is represented as sticks colored by atom type, with carbons shown in magenta. (**B**) Position of the Y308-E312 flexible loop shown in green. The conserved Y308 is represented as sticks, colored by atom types, with carbons being green. All images were made using the PyMOL Molecular Graphics System, v. 2.3.0. for Linux.

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 (Figure 5), respectively, were proposed [68]. The flexible loop residue Tyr308-Glu312 in *L. brevis* GAD is located near the substrate-binding site (Figure 4). This loop is important for the catalytic reaction, and the conserved residue Tyr308 plays a crucial role in decarboxylation of L-Glu. Thr 215 and Asp246 are the two catalytic residues in *L. brevis* GAD (Figure 5), which are also highly conserved and promote decarboxylation of L-Glu [68,71,73].

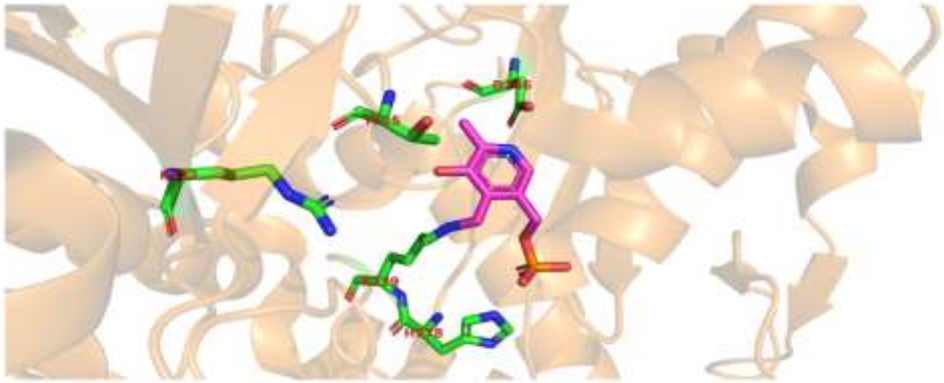

Figure 5. Active site of glutamate decarboxylase from *L. brevis* (PDB code 5GP4). The conserved catalytic residues T215 and D246 are shown in sticks, colored by atom type, with carbons shown in green, nitrogen in blue, and oxygen in red. The prosthetic group PLP is represented as sticks colored by atom type with carbons in magenta. The residues R422, H278, and K279, proposed to be involved in electrostatic interactions with the substrate glutamate [68], are represented as sticks colored by atom type. The rest of the chain is shown as a transparent orange cartoon. K279 is also involved in forming the imine linkage to PLP. The image was made using the PyMOL Molecular Graphics System, v. 2.3.0. for Linux.

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. This Schiff base 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 in inactive apoGAD (Figure 6), 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 be inactivated as a function of time and substrate concentration [62,67–69,74–77].

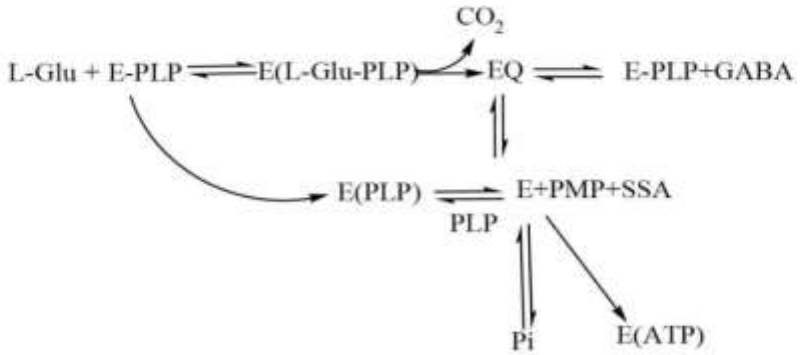

Figure 6. The interconversion of holo- and apoGAD. The primary reaction results in the formation of GABA and holoGAD remains intact and active. holoGAD reacting with PLP will activate a secondary reaction resulting in the formation of apoGAD. E, apoGAD; E-PLP, holoGAD; Pi, inorganic phosphate; EQ, quinonoid intermediate; PMP, pyridoxamine phosphate; PLP, pyridoxal-5′-phosphate; SSA; succinic semialdehyde (modified from [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 kDa and is formed in its mature holo-form, 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 resulted in an active tetrameric form with the enzyme from *L. brevis* IFO12005 GAD [78]. The presence 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 [71]. Subsequent structural studies on this enzyme revealed, however, that GAD from *L. brevis* CGMCC 1306 is active as a dimer (Figure 7), even though elucidation of the crystal structure resulted in a distorted asymmetric trimer. The authors concluded that this observed trimer only resulted from the crystallographic packing and not the biological form [68].

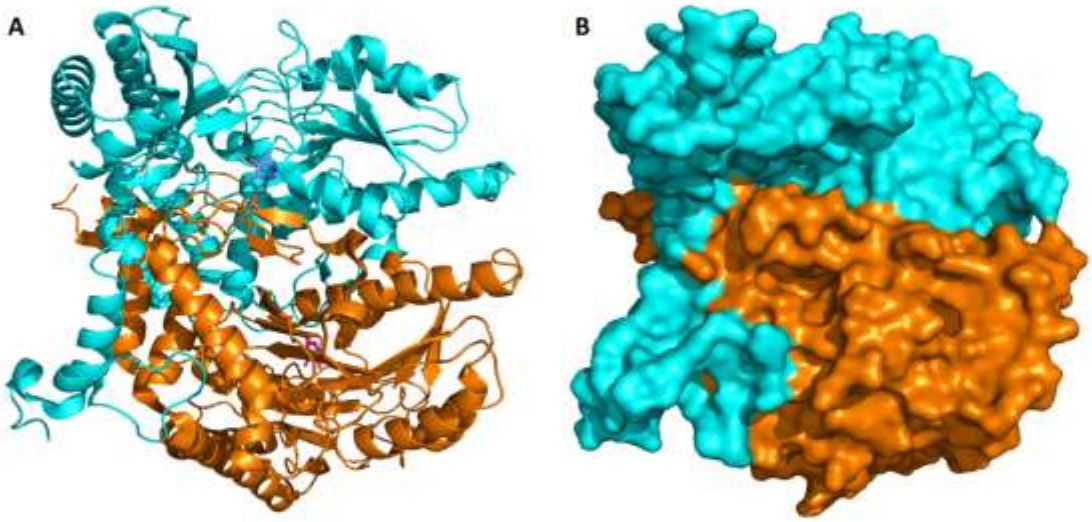

Figure 7. Structure of homodimeric glutamate decarboxylase from *L. brevis* CGMCC 1306 (PDB code: 5GP4). (**A**): overall secondary structure with chains A and B represented as orange and cyan cartoons, respectively. The prosthetic group PLP is represented as sticks, colored by atom type, with carbons shown in magenta or blue. (**B**). Surface of GAD with chains A and B of the crystal structure represented as orange and cyan surfaces, respectively. Images were made using the PyMOL Molecular Graphics System, v. 2.3.0. for Linux.

As above-mentioned, a number of LAB carry two GAD-encoding genes, *gadA* and *gadB*. Frequently, studies have focused on the purification and characterization of GadB (e.g., from *L. plantarum* [79], *L. sakei* [80], *L. brevis* [78], *Enterococcus raffinosus* [75], and *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 the *gadA* transcript was highly upregulated (55-fold) in strain *L. brevis* NPS-QW-145 at the stationary phase of growth [7]. 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 efficient maintenance of pH homeostasis. These findings suggest that extending the activity of GadA to the near-neutral pH region offers 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 8), the sequence HVD(A/S)A(S/F)GG was highly conserved among LAB GAD, and a lysine residue (Lys279 in *L. brevis* GAD) played a crucial role in the PLP binding site. Table 2 summarizes the 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 have been 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 modeling, since these residues exhibit different interactions 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, when 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 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].

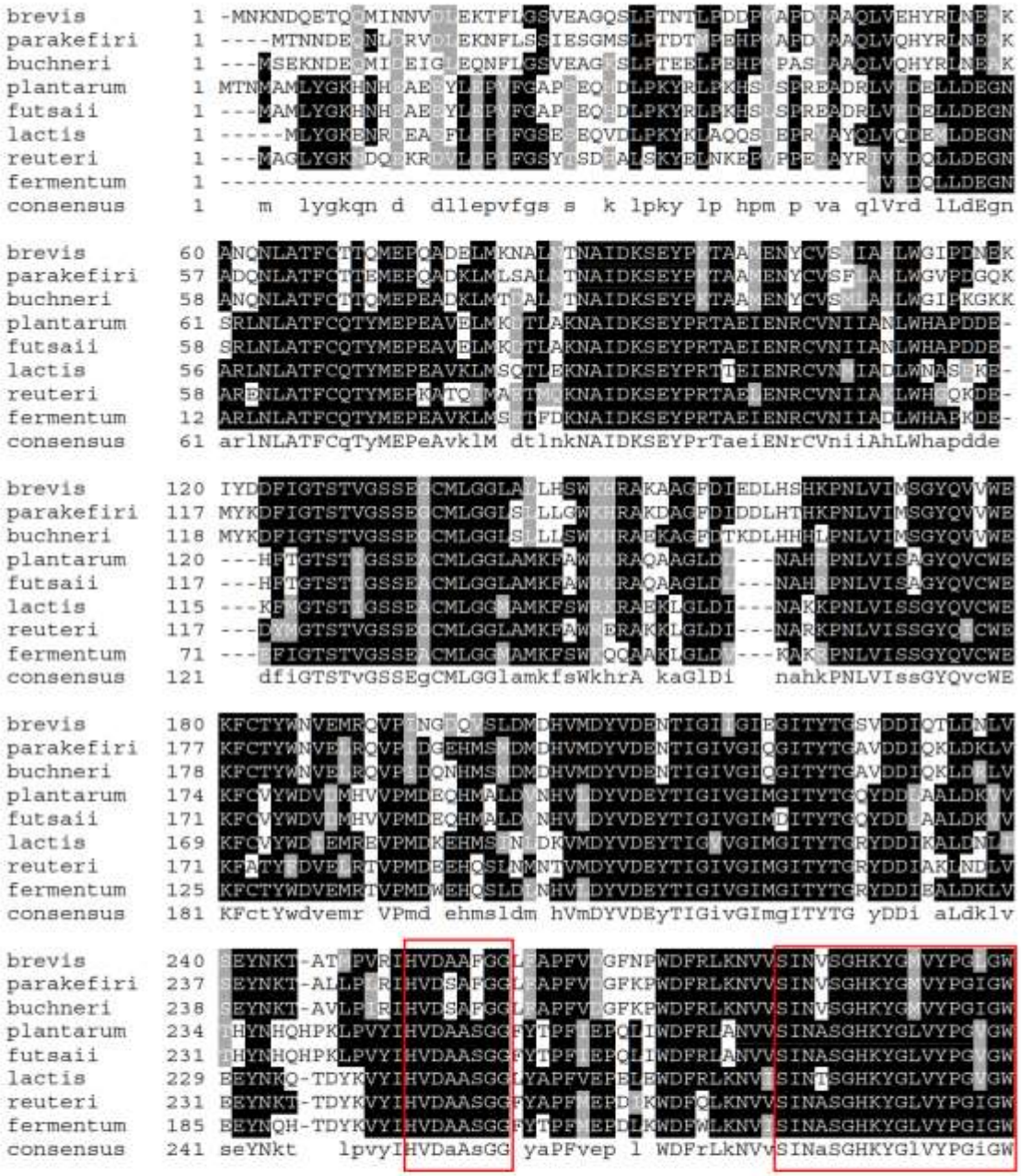

Figure 8. *Cont.*

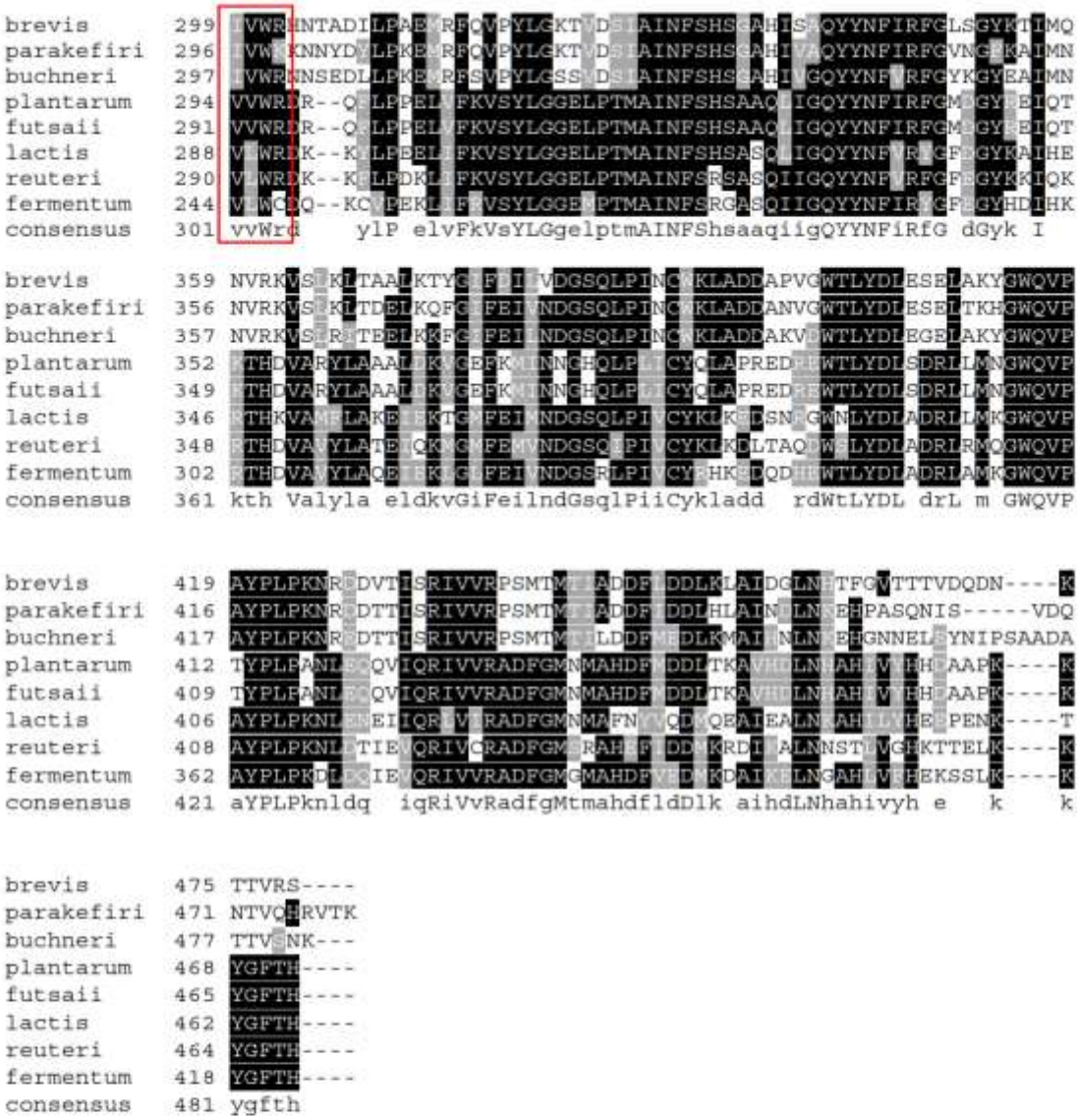

Figure 8. Comparison of amino acid sequences of GAD from *L. brevis*, *L. parakefiri*, *L. buchneri*, *L. plantarum*, *L. futsaii*, *L. lactis*, *L. reuteri*, and *L. fermentum*. The accession numbers of these sequences are GAW73186.1, ERK43696.1, KRL34909.1, AEB72391.1, OYT00901.1, ESS01667.1, BBA26472.1, and 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]. Furthermore, the residues SINA/V/TSGHKYGM/LVYPGI/V/LGWI/VV/LW/R/K/V are part of the PLP-binding domain [26].

Table 2. Biochemical properties of glutamate decarboxylase from various LAB.

Source	Molecular Mass of Subunit (kDa)	Optimal pH	Optimal Temperature	Effect of Metal Ions (Increased Activity)	Effect of Metal Ions (Decreased Activity)	K_m (Mm)	V_{max}	References
L. zymae	53	4.5	41	NH_4^+, Ca^{2+}, Mg^{2+}, Mn^{2+}, Na^+	Co^{2+}, Cu^{2+}, Ag^+	1.7	0.01 mM/min	[46]
L. paracasei NFRI 7415	57	5	50	NH_4^+, Ca^{2+}	EDTA, Na^+	5	NR	[18]
L. sakei A156	54.4	5	55	Mn^{2+}, Co^{2+}, Ca^{2+}, Zn^{2+}	NH_4^+, Mg^{2+}, Ag^+	0.045	0.011 mM/min	[80]
L. brevis CGMCC 1306	53	4.8	48	NR	NR	10.26	8.86 U/mg	[22]
S. salivarius subsp. *thermophilus* Y2	46.9	4	55	Ba^{2+}	Fe^{2+}, Zn^{2+}, Cu^{2+}, Mn^{2+}, Na^+, Ag^+, Co^{2+}, Li^+, K^+	2.3	NR	[83]
Enterococcus avium M5	53	4.5	55	Ca^{2+}, Mg^{2+}, Mn^{2+}, Zn^{2+}	Cu^{2+}, Ag^+	3.26	0.012 mM/min	[82]
E. raffinosus TCCC11660	55	4.6	45	Mo^{6+}, Mg^{2+}	Fe^{2+}, Zn^{2+}, Cu^{2+}, Co^{2+}	5.26	3.45 μM/min	[75]
Lactococcus lactis	NR	4.7	NR	NR	NR	0.51	NR	[3]
L. brevis 877G	50	5.2	45	Ca^{2+}, Mg^{2+}, Mn^{2+}, Na^+	Ag^+, Zn^{2+}, Cu^{2+}, K^+	3.6	0.06 mM/min	[81]

NR; Not reported.

6. Improvement of GAD Activities and GABA Production

GABA biosynthesis can be achieved by using whole cell reactions, recombinant bacteria, and purified GAD (Table 3). *gad* genes from various sources of LAB have been overexpressed in different hosts including *E. coli* [86], *L. sakei* [87], *L. plantarum* [88], *Corynebacterium glutamicum* [89], and *Bacillus subtilis* [90]. 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 [91], fed-batch fermentation [92] as well as immobilized cell technology [93–95] have been employed. All of these approaches effectively increased GABA productivity by improving cell viability resulting in extended 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 temperature, nitrogen sources, cofactor, and feeding time [34,94]. A study by Lim et al. showed that under optimized conditions, *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 the supplementation of PLP to the medium 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 [42]. 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 the growth medium of *S. thermophilus*, GABA production could not be enhanced. It was suggested that this strain is not able to tolerate high glutamate concentrations [36]. 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 key factors 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 occurs in a time-dependent manner [96].

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 *gad*B gene from *L. lactis* was used as the starting point of this engineering approach. An engineered strain was constructed by (i) introducing mutations into this GadB to shift its decarboxylation activity toward a neutral pH; (ii) by modifying the glutamate/GABA antiporter GadC to facilitate transport at neutral pH; (iii) by enhancing the expression of soluble GadB through overexpression of the GroESL molecular chaperones; and (iv) by inhibiting the degradation of GABA through inactivation of *gadA* and *gadB* from the *E. coli* genome. This engineered strain achieved a productivity of 44.04 g/L of GABA per h with an almost quantitative conversion of 3 M glutamate [97].

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 [84,97–101] and were 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 *C. glutamicum* grows best around neutral pH [84]. *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 [84].

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 indicating flexible regions can be used, and subsequently, these regions are strengthened [102]. Identification of the consensus sequences can also improve the thermostability of proteins [103]. 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 that of 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 [102].

The use of purified GAD seems to be economically more feasible than whole-cell biocatalysis when aiming at producing pure GABA due to simplified downstream purification of this compound from less complex reaction mixtures. A number of immobilization techniques have been applied for re-use of the biocatalyst such as immobilization of GadB in calcium alginate beads that are then employed in a bioreactor [104], a GAD/cellulose-binding domain fusion protein immobilized onto cellulose [105], and GAD immobilized to metal affinity gels [106]. 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 in enzyme activities was observed during the reaction when the inactivation reaction of PLP to succinic semialdehyde and pyridoxamine-5′-phosphate during catalysis was avoided by the addition of a small amount of α-ketoglutaric acid to the reactor, which regenerated PLP [101]. 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]. These results suggest that immobilization gives advantageous results for industrial application when using (partially) purified GAD for GABA production from glutamate.

Table 3. Various approaches to improve GABA production.

Strain	GABA Enhancement Techniques	Reaction Conditions	GABA Production	References
L. plantarum Taj-apis 362	GAD was expressed in pMG36e vector	Resting cells, reaction mixtures contain 1.32 mM glutamic acid and 200 mM sodium acetate, incubated at 37 °C for 60 min	1.14 g/L	[88]
L. plantarum ATCC 14917	*L. sakei* expression host	MRS supplemented with 1% MSG, incubated at 30 °C for 48 h, initial pH 6.0	27.36 g/L	[87]
S. salivarius ssp, *thermophilus* Y2	*B. subtilis* expression host	Resting cells, reaction mixtures contain 0.4 M sodium glutamate and 0.4 M acetate buffer, incubated at 37 °C for 6 h	5.26 g/L	[90]
L. brevis NCL 912	Continuous cultivation method	Fermentation medium with glucose, yeast extract, soy peptone, $MnSO_4$, Tween 80 and MSG, initial pH 5.0. incubated at 32 °C with 150 rpm agitation.	5.11 g/L	[91]
L. brevis NCL 912	Fed-batch fermentation	Seed medium containing glucose, soya peptone, $MnSO_4$, $4H_2O$, L-glutamate. Incubated at 32 °C for 84 h with initial pH 5.0	103.72 g/L	[92]
L. brevis RK03	Cell immobilization with hydrogels 2-hydroxyethyl methacrylate/polyethylene glycol diacrylate (HEMA/PEGDA)	MRS medium containing 450 mM MSG, incubated for 84 h at 30 °C.	39.7 g/L	[93]
L. brevis GABA 057	Cell immobilization with alginate beads + isomaltooligosaccharide	GYP medium (pH 4.5) containing MSG incubated for 48 h at 37 °C.	23 g/L	[94]
L. lactis	optimizing fermentative condition (temperature 31.9 °C, pH 7.1, 15 g/L of MSG)	Growth on optimized MRS medium containing brown rice, germinated soy bean and skim milk.	7.2 g/L	[34]
L. brevis CRL 1942	optimizing culture conditions (30 °C, 48 h, 270 mM MSG)	Growth on optimized MRS medium	26.30 g/L	[96]
E. faecium JK29	optimizing MRS medium (0.5% sucrose, 2% yeast extract, 0.5% MSG, pH 7.5, 30 °C)	Growth on optimized MRS medium	1.53 g/L	[38]
L. brevis HYE1	optimizing MRS medium (2.14% maltose, 4.01% tryptone, 2.38% MSG, pH 4.74)	Growth on optimized MRS medium	2.21 g/L	[27]
L. brevis	modifiying MRS medium containing 6% L-glutamic acid, 4% maltose, 2% yeast extract, 1% NaCl, 1% $CaCl_2$, 2 g Tween 80, 0.02 mM PLP, pH 5.25, 37 °C, 72 h	Growth on optimized MRS medium	44.4 g/L	[42]
L. brevis Lb85	directed evolution and mutagenesis	Growth on LBG medium supplemented with glucose, kanamycin and L-glutamate, incubated at 30 °C with 200 rpm agitation.	7.13 g/L	[84]
L. lactis FJNUGA01	whole-cell bioconversion with pET28a	Resting cells in deionized water with 2 mol/L glutamate, incubated at 45 °C for 6 h	34 g/L	[99]
L. plantarum WCFS1	immobilized enzymes to porous silica beads	Enzymatic conversion of 0.5 M MSG, 0.2 mM PLP and 0.02 μg GAD/μL in sodium acetate buffer (pH 5.0), incubated at 37 °C for 20 min.	41.7 g/L	[106]

7. The Role of Glutamate Decarboxylase in the Manufacturing of Bio-Based Industrial Chemicals

Agricultural waste and waste streams 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 [107]. 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 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 acids 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 the 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 rugosa*, and in a second step, *L. futsaii* GAD converted glutamate to GABA. Both steps were catalyzed by immobilized whole cells [108]. Recently, it was shown that supplementation of arginine to media containing glutamate could 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) [109].

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 *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 [110]. Another interesting material synthesized by ring-opening polymerization of 2-pyrrolidone is Nylon 4 [111], a four-carbon polyamide suitable for application as an engineering plastic due to its superior thermal and mechanical properties [112]. Contrary to other nylon polymers, Nylon 4 is heat-resistant, biodegradable, biocompatible, and compostable [112].

8. Future Trends and Conclusions

The demand for 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* and the encoded enzymes GadA and GadB has led 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 toward 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 are 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 yields at genetic and metabolic levels.

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Chapter 4

Isolation and identification of lactic acid bacteria from Indonesian fermented foods as γ-aminobutyric acid-producing bacteria

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Isolation and identification of lactic acid bacteria from Indonesian fermented foods as γ-aminobutyric acid-producing bacteria

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Abstract

γ-aminobutyric acid is the most abundant inhibitory neurotransmitter in brain and has various physiological functions. The aim of this study was to isolated and screened GABA-producing lactic acid bacteria originally from Indonesia. Twelve fermented foods were considered in this study and the ability of LAB as GABA-producing bacteria were analyzed using TLC cellulose plates and pre-staining chromatography method. Six isolates (IFK-10, IFK-11, IFK-12, FN-12, FN-14, FN-15) were able to convert MSG to GABA during 24 h of cultivation. Two strains IFK-10 and IFK-11 showed the highest amount of GABA concentration i.e 2.68 and 2.06 mg/ml, respectively. These strains were identified as *Lactobacillus plantarum* IFK-10 and *Pediococcus pentosaceus* IFK-11. Two strains of LAB from Indonesian as GABA-producing bacteria have a promising prospect and could support the development of functional foods.

Introduction

γ-Aminobutyric acid (GABA) is the most abundant inhibitory neurotransmitter in brain and widely distributed in plants, animals and microorganisms (Brambilla *et al.*, 2013). GABA has various physiological functions such as antidepressant (Chih *et al.*, 2013), induction of hypotension (Inoue *et al.*, 2003), and cholesterol lowering effect (Watanabe *et al.*, 2012). GABA is also could prevent obesity by ameliorating oxidative stress in high-fat diet in mice (Xie *et al.*, 2014) and effectively prevent diabetic condition by strongly secretagogue insulin from pancreas (Hagiwara *et al.*, 2004; Adeghate and Ponery, 2012). A study by Inoue *et al*. (2003) and Mathieu-Pouliot *et al*. (2013) reported that GABA-enriched dairy product was significantly decrease systolic blood pressure in men and mildly hypertensive men.

GABA is primarily formed by glutamic acid decarboxylase enzyme (GAD, EC 4.1.1.15) which catalyzes the irreversible α-decarboxylation of glutamic acid to produce GABA (Li and Chao, 2010). Many microorganisms can produced GAD such as bacteria (Komatsuzaki *et al.*, 2005), fungi (Kono and Himeno, 2000) and yeast (Masuda *et al.*, 2008). Several strains of lactic acid bacteria (LAB) have an ability to produce GAD and its biochemical properties have been characterized (Komatsuzaki *et al.*, 2005; Siragusa *et al.*, 2007; Komatsuzaki *et al.*, 2008).

Due to physiological effect of GABA, many studies have been pursued and recent research undertaken to increase GABA content in food since direct addition of GABA to food is considered unnatural (Kim *et al.*, 2009; Villegas *et al.*, 2016). Therefore, it is necessary to produce and increase GABA naturally in food. Various LAB have been reported as GABA- producing bacteria. LAB are the most important microorganisms and play important role in food fermentation. LAB generally regarded as safe (GRAS) and have been extensively studied their role in food industries, bioactive compound producing-bacteria, dairy industries and as probiotic. Most research on GABA production has focused on the production of microbial rather than chemical synthesis due to corrosive nature of the reactant compound (Diana *et al.*, 2014). Several LAB strains isolated from food source have been shown to have an ability to produce GABA including *L. namurensis* NH_2 and *P. Pentosaceus* NH8 from Nham (Ratanaburee *et al.*, 2013), *L. paracasei* NFRI 7415 from Japanese fermented fish (Komatsuzaki *et al.*, 2005), *L.paracasei* PF6, *Lactococcus lactis*

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PU1 and *L. brevis* PM17 from cheese (Siragusa *et al.*, 2007), *L. brevis* 119-2 and *L. brevis* 119-6 Tsuda Kabu (Watanabe *et al.*, 2012).

Indonesia has many kinds of fermented foods which are fermented spontaneously by indigenous LAB and other microorganisms such as yeast and molds. Some species of LAB have been isolated from dadih, tempoyak, salted cabbage, gatot, growol etc. A previous study (Rahayu *et al.*, 2015) reported that LAB isolated from Indonesian fermented foods could potentially be probiotics as they are able to survive in acidic conditions, resistance to high bile salts and has an antibacterial activity. However, there is a little information about LAB from fermented foods Indonesian origin as GABA-producing bacteria. In this study LAB were isolated from Indonesian fermented foods and the ability to produce GABA were screened. A new strain of LAB from Indonesian could support the development of functional foods fortified with GABA.

Materials and Methods

Materials

Twelve fermented foods were considered in this study, consisting of two kinds of fermented cassava (namely growol and tape ubi), fermented soy beans, tempeh, salted cabbage, salted fruits, chicken sausage, beef sausage, and bekasam (fermented fish with rice). These samples will be used for selecting GABA-producing LAB. Medium of de Man Rogosa agar was from Oxoid. GABA standard were purchased from Sigma Aldrich. All chemicals reagent used in this study were analytical grade.

Isolation and screening GABA-producing LAB by thin layer chromatography

One mg of samples were serially diluted and inoculated on MRS agar containing 1% calcium carbonate ($CaCO_3$) (Oxoid) then incubated at 37°C for 24 h under aerobic conditions. The isolates which formed clear zone were considered as LAB. Single colonies were streaks onto MRS agar to obtain pure cultures. In order to select LAB with high GABA-producing ability, bacteria were grown on MRS supplemented with MSG 5% and incubated at 37°C for 24 h, and then the cultured broth was centrifuged at 1000 rpm for 10 min. The resulting supernatants were filtered using membrane filter.

A 2 µl of supernatants were spotted on TLC silica plates activated using butanol: acetic acid: aquades (5:3:2) containing 0.4% ninhidryn (Qiu *et al.*,2010). After development, the plate was directly dried at 90°C for 5 min. The GABA-producing strain were identified based on gram staining, observation under microscope and by 16S rDNA sequence determination.

Quantification of GABA-producing bacteria

GABA concentrations in cultured broths were determined by pre-staining paper chromatography (Li *et al.*, 2009). A 2 µl of supernatants were spotted onto cellulose plates and developed at 30°C with n-butanol-acetic acid-water (5:3:2) containing 1.2% ninhydrin. After development, GABA spots were scratched out from the paper and were extracted with 5 ml of 75% alcohol (v/v):0.6% cupric sulfate (w/v) (38:2) at 40°C. The absorbance was read using spectrophotometer at 512 nm. The purity of GABA was also determined by HPLC as reference procedures.

Identification of GABA-producing bacteria

Identification was based on 16S RNA according to Ivanova *et al.* (2008). Genomic DNA was isolated using Kit Isoplant II (isoplant code No. 310-04151, Nippon Gene, Toyama, Japan). A 1 µl of supernatant was used as a template in PCR. Amplification was conducted with infinigen thermocycler machine. The 16S rRNA genes were amplified using a pair of universal primers corresponds to positions 27F (5' AGAGTTTGATCMTGGCTCAG 3') and 1492R (5' TACGGYTACCTTGTTACGACTT 3').The amplification procedures were as follows: initial denaturation at 94°C for 5 min, followed by 40 cycles at 94°C for 20 sec, the annealing temperature at 40°C for 2 min and the elongation temperature at 72°C for 30 sec, and additional final elongation temperature at 72°C for 5 min. A 5 µl PCR product were analysed using gel electrophoresis. The partial 16S rDNA sequence was compared with the GenBank database in the NCBI using BLAST and phylogenetic tree was performed using MEGA 4.

Results and Discussion

Screening and identification of GABA-producing LAB

GABA-producing LAB were isolated from fermented foods and isolated strains which formed a clear zone on MRS plates containing $CaCO_3$ at 37°C for 24 h, were considered as LAB. Six isolates (IFK-10, IFK-11, IFK-12, FN-12, FN-14, FN-15) originally from fermented soy beans and fermented fish showed high GABA production by TLC (Figure 1). The isolates were able to convert MSG during 24 h of incubation and showed the same Rf as that of GABA standard (Rf = 0.61). Two isolates IFK-10

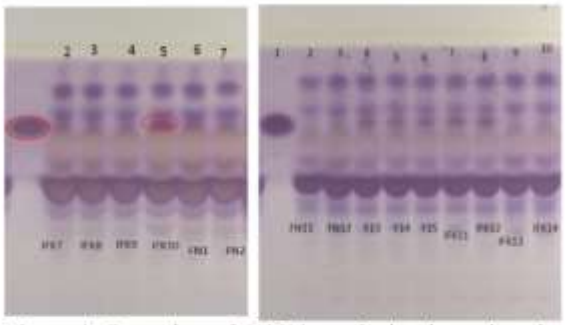

Figure 1. Screening of GABA producing bacteria using TLC cellulose plate. Plate A, lane 1. GABA standard, lane 2.supernatant of IFK-7, lane 3. Supernatant of IFK-8, 4. Supernatant of IFK-9, 5. Supernatant of IFK-10, lane 6. Supernatant of FN-1 and lane 7. Supernatant of FN-2. Plate B, lane 1, GABA standard, lane 2. Supernatant of FN-11, lane 3. Supernatant of FN-12, lane 4. Supernatant of F-13, lane 5. Supernatant of F-14, lane 6, Supernatant of F-15, lane 7. Supernatant of IFK-11, lane 8. Supernatant of IFK-12, lane 9. Supernatant of IFK-13 and lane 10. Supernatant of IFK-14. All isolates were grown in MRSB supplemented with 5% of MSG and incubated for 24 h at 37^{0}C.

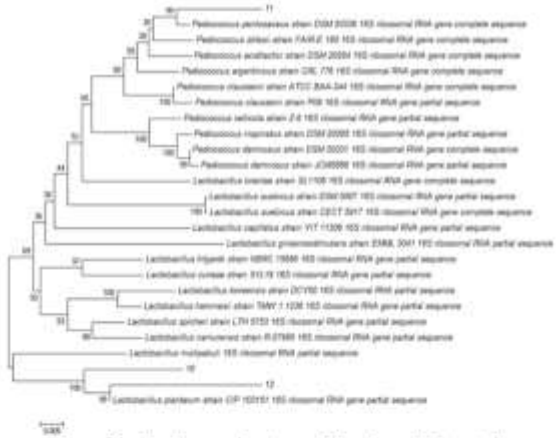

Figure 2. phylogenetic tree of lactic acid bacteria

and IFK-11 showed high surface area determined by TLC scanner. The surface area of strains IFK-10 and IFK-11 have reached to 66,712.2 and 30,435.7. These results showed that strains IFK-10 and IFK-11 could convert monosodium glutame after 24 hours of incubation. The amounts of GABA in supernatant were further analyzed by pre-staining chromatography and the purity of this product was determined with HPLC.

Two isolates produced the highest amount of GABA were Gram positive, short rod and cocci cell type. The isolates were identified as *Lactobacillus plantarum* and *Pediococcus pentosaceus* (IFK-10 and IFK-11, respectively) according to phylogenetic tree (Figure 2). The partial complete sequence (1492

Table 1. GABA content of some strains as measured by pre-staining chromatography. The absorbance was read at 512 nm.

Isolates	Rf	surface area (AU)	GABA (mg/ml)
IFK 10	0.61	66712.2	2.68
IFK 11	0.61	30435.7	2.06
IFK 12	0.6	28790.7	1.11
FN 13	0.61	25618.8	1.37
FN 14	0.61	14352.2	1.72
FN 15	0.61	28995.9	1.12

bp) of the 16S rRNA gene of strain IFK-10 and IFK-11 were amplified by PCR. The results were indicated that isolates IFK-10 and IFK-11 exhibited high similarity value (99%) to *Lactobacillus plantarum* CIP 103151 and *Pediococcus pentosaceus* DSM 20336. The result of phenotypic characters and phylogenetic analysis clearly indicated that strain IFK-10 and IFK-11 belonging to *L. plantarum* and *P. pentosaceus,* respectively. These results were in agreement with Siragusa *et al.,* (2007) and Ratanaburee *et al*., (2013) reported that strains *L. plantarum* C48 and *P. pentosaceus* NH8 isolated from Italian cheese and Nham were considered as a high GABA – producing lactic acid bacteria

Several strains or species of LAB have been reported as GABA - producing bacteria and the ability to convert MSG to GABA is a strain dependent. Almost all strains or species of GABA – producing bacteria were isolated from traditional fermented foods such Nham (Ratanaburee *et al.*, 2013), Italian cheese (Siragusa *et al*., 2007), and paocai (Li *et al.*, 2008). Moreover, all isolation sources are rich in glutame which is an essential source to screen GABA – producing bacteria. Almost all isolates reported are belongs to lactobacilli species. This is our first report strains *L. plantarum* IFK-10 and *P. pentosaceus* IFK-11 from fermented soy beans as an isolation source.

Quantification of GABA

The amount of GABA in supernatant was determined using pre-staining chromatography method. Pre-staining chromatography are suitable for detection of GABA and the method is more clean, simple, convenient, inexpensive and reproducible (Li and Chao, 2010). This method has almost the same Rf values to those of traditional method. Two strains IFK-10 and IFK-11 produced high concentration of GABA in supernatant during 24 h of incubation and reached to 2.68 and 2.06 mg/ml in MRSB containing 5% of MSG compare to other four strains (Table 1). Thanh-Binh *et al*. (2014) reported that *L. brevis* isolated from kimchi was able to produce GABA up to 44.4 g/l after 72 h of incubation in MRSB containing

6% of MSG. Several studies have reported that LAB isolated from fermented foods is able to convert MSG as substrate to GABA.

High content of MSG are required to improve the production of GABA by LAB. Recently, *L. brevis* CRL 1942 isolated from Real Hornillos quinoa sourdough was able to convert 270 mM of MSG to GABA reached to 255 mM after 48 h of cultivation at 300C (Villegas *et al.*, 2016). Siragusa *et al.* (2007) reported that strain *Lactobacillus brevis* PM17, *Lactobacillus plantarum* C48, *Lactobacillus paracasei* PF6, *Lactobacillus delbrueckii* subsp. *bulgaricus* PR1 and *Lactococcus lactis* PU1 isolated from 22 Italian cheese varieties produce GABA up to 15-63 mg/kg in different culture media. In addition, *L. brevis* CECT8183 isolated from artisan Spanish cheese produced 100 mg/l of GABA (Diana *et al.*, 2014). LAB with high GABA production is related with GAD enzyme activity. The concentration of glutamic acid in food matrix should be high enough to increase GABA production. In this study suggest that almost all isolates may exhibit different GABA – producing ability depending on glutamate/GABA content in the samples. GABA-producing bacteria could exhibit high production of GABA in samples with high glutamate/GABA content than those from the samples with low glutamate/GABA content. In addition, Siragusa *et al.* (2007) reported that only four isolates of LAB such as *L. paracasei* PF6, *L. bulgaricus* PR1, *L. lactis* PU1 and *L. brevis* PM17 isolated from different variety of cheese with the highest GABA production and exhibit the highest level of GABA.

Conclusion

In conclusion, six isolate of LAB from fermented soy beans have the ability to convert MSG to GABA during 24 h of cultivation. Two strains IFK-10 and IFK-11 showed the highest amount of GABA concentration during 24 h of cultivation i.e 2.68 and 2.06 mg/ml, respectively. These strains were identified as *Lactobacillus plantarum* (IFK-10) and *Pediococcus pentosaceus* (IFK-11), short rod shapes, cocci cell type and gram positive bacteria. In this study we obtained two novel LAB strains from fermented soy bean as GABA-producing bacteria hence could be used as starter culture that has a functional trait to develop functional foods.

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Chapter 5

Identification, classification and screening for γ-aminobutyric acid production in lactic acid bacteria from Cambodian fermented foods

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Identification, Classification and Screening for γ-Amino-butyric Acid Production in Lactic Acid Bacteria from Cambodian Fermented Foods

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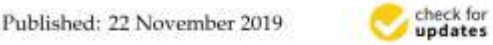

Abstract: Screening for various types of lactic acid bacteria (LAB) that form the biological agent γ-amino-butyric acid (GABA) is important to produce different kinds of GABA-containing fermented foods. So far, no GABA-producing LAB have been reported from Cambodian fermented foods. Most small-scale fermentations and even some industrial processes in this country still rely on indigenous LAB. The application of GABA-producing autochthonous starters would allow the production of Cambodian fermented foods with an additional nutritional value that meet the population's dietary habits and that are also more attractive for the international food market. Matrix-assisted laser desorption/ionizing time-of-flight mass spectrometry (MALDI-TOF MS) and partial 16S rDNA sequencing were used to identify 68 LAB isolates from Cambodian fermented foods. These isolates were classified and grouped with $(GTG)_5$ rep-PCR, resulting in 50 strains. Subsequently, all strains were investigated for their ability to produce GABA by thin layer chromatography. GABA-positive strains were further analyzed by the GABase assay. Of the six GABA-positive LAB strains—one *Lactobacillus futsaii*, two *Lactobacillus namurensis*, and three *Lactobacillus plantarum* strains—two *Lactobacillus plantarum* strains produced high amounts of GABA (20.34 mM, 16.47 mM). These strains should be further investigated for their potential application as GABA-producing starter cultures in the food applications.

Keywords: lactic acid bacteria; fermented foods; 16S rDNA sequencing; MALDI-TOF MS; $(GTG)_5$ rep-PCR fingerprinting; GABA; Cambodia

1. Introduction

Fermented foods are widely consumed in Cambodia. In particular, fermented fish are eaten in almost every meal in most parts of the country and fermented fruits and vegetables are popular among females who eat them as a snack. Most of these food products are produced at household and small-scale levels. The majority of small-scale fermentations and even some industrial processes are still done as natural processes involving lactic acid bacteria (LAB) that are indigenously present in raw materials and the production environment. This represents a low-cost and reliable preservation technique [1]. Although LAB are generally recognized as safe (GRAS) by the US Food and Drug Administration

(FDA) and partly have the qualified presumption of safety (QPS) status provided by the European Food Safety Authority (EFSA) [2,3], the growth of this group is completely uncontrolled and unpredictable, resulting in less uniform sensory characteristics and compositions [4]. Tailored commercial starter cultures would guarantee obtaining products with constant hygienic and organoleptic qualities in a shorter time and might also improve the stability and shelf life [5]. Since there is no commercial production of starter cultures for Cambodian products, universal cultures from other sources have to be used. Such cultures, however, are tailored to the needs of other markets and are not typical for Cambodian foods [4]. Moreover, many small-scale manufacturers are unwilling to accept changes and to modify fermentation processes [1]. Using autochthonous starter cultures, which can effectively preserve typical characteristics of fermented products, would be better accepted by producers [5]. A recent trend in food preservation is the use of safe starter cultures that show additional positive functions apart from technological and possible antimicrobial properties as well as stress resistance [4,5]. Such starter cultures would allow the production of Cambodian fermented foods with an additional nutritional value that meet the population's dietary habits and that are also more attractive for the international food market.

There is currently considerable research and industrial interest in the potential biological activity of LAB, either as probiotics themselves or as producers of bioactive agents [6]. One of these safe and eco-friendly bioactive agents is γ-aminobutyric acid (GABA), which is a non-protein amino acid [7]. It is formed by the decarboxylation of L-glutamic acid in a reaction catalysed by the enzyme glutamate decarboxylase (GAD) [8]. Although GABA is found in many plants and animals, its content is generally low [9]. The application of concentrated GABA is wide and versatile, as this component has many physiological functions such as the induction of hypotension, neurotransmission, diuretic and sedative effects as well as the stimulation of immune cells [6]. Consequently, GABA is used in pharmaceuticals and functional/fermented foods as active component [10].

Because of the increasing commercial demand, there have been many attempts for synthesizing GABA chemically or biologically [10]. Since biological methods using microorganisms are more promising [11,12], many GABA products are obtained by fermentation [8]. Ongoing efforts in the molecular evolutions of GADs offer new prospects for effective GABA biosynthesis [13]. Next to LAB, other microorganisms have been reported for GABA production, including bacteria, fungi and yeasts [12,14]. However, LAB are the most interesting and practical group for this fermentation as they can produce high levels of GABA due to a high cellular GAD activity [10]. Although many GABA-producing LAB strains have already been isolated and identified, further research on the isolation and characterization of LAB is needed. Screening for new strains of LAB that can produce GABA still attracts attention because LAB with different physiological characteristics show potential for use as starters in the food industry to produce GABA-containing fermented foods with different acid and flavor profiles [14].

So far, no GABA-producing LAB starter cultures have been reported from Cambodian fermented foods. Thus, the objective of the present study was the identification of LAB from various Cambodian fermented fishery and vegetable products followed by an investigation of their ability to produce GABA. Hence, the first stage is to establish the precise identity of an isolate at genus and species level [4]. Currently, molecular techniques provide an important contribution to the identification and classification of microorganisms [15,16]. The most commonly used target for bacterial identification is the 16S rDNA (16S rRNA gene). Sequencing this gene is considered to be the 'gold standard' for solving bacterial phylogeny and taxonomy issues in different contexts [17]. Additionally, proteomic analysis based on protein profiling using matrix-assisted laser desorption/ionizing time-of-flight mass spectrometry (MALDI-TOF MS) has been recognized as a tool for microbial identification with high sensitivity and throughput [18,19]. To exclude possible duplicates from further analyses, a discrimination at strain level was performed using repetitive element palindromic (rep)-PCR. Subsequently, well-identified and characterized strains were screened for GABA production by thin layer chromatography (TLC), and the level of GABA produced was determined by GABase assay.

2. Materials and Methods

2.1. Fermented Food Samples and Sampling

Eight types of naturally prepared Cambodian fermented foods (mainly fermented fish and vegetables) were randomly purchased from wet markets (Chamkadaung, Oreusey, Thmey, Chas, Phumreusey, and Limcheanghak) in Phnom Penh, the capital city of Cambodia. These products originated from various provinces in the country. The samples included fish paste (*prahok*; $n = 1$), fermented fish (*paork chav*; $n = 3$ and *mam trey*; $n = 1$), salted fish (*trey proheum*; $n = 2$), shrimp paste (*kapi*; $n = 1$), fermented papaya (*mam lahong*; $n = 2$), fermented mustard (*spey chrouk*; $n = 2$) and fermented tiny freshwater shrimp (*paork kampeus*; $n = 3$). The information about each fermented product is provided in Table 1. After purchasing fermented foods from the wet markets in Phnom Penh, samples were immediately packed into hygienic plastic boxes. The samples were taken to the Food Microbiology and Hygiene Laboratory of the Department of Food Science and Technology at BOKU in Vienna, Austria, and kept in their original containers at 4 °C until analysis. Cambodian fermented foods have mostly no shelf-life indicated and these foods are usually stored until completely consumed [1]. Generally, fermented fish can be stored for a few months up to a year and fermented vegetables are still fine up to two or three weeks if they are stored at 4 °C. To cover the purpose of the project, all analyses were carried out as soon as possible within the usual shelf life of the products (e.g., three months for fermented fishery products and two weeks for fermented vegetables after purchasing).

Table 1. Selected Cambodian fermented food samples.

No.	Local Name (Number of Samples)	English Name	Ingredients	Usage	Market Origin
1	*Prahok* ($n = 1$)	Fish paste	Freshwater fish, salt	Main dish, side-dish, condiment, seasoning	Chamkadaung
2	*Paork chav* ($n = 3$)	Fermented fish	Freshwater fish, brown glutinous rice, salt	Main dish, side-dish	Oreusey
3	*Mam trey* ($n = 1$)	Fermented fish	Freshwater fish, palm sugar, salt	Main dish, side-dish	Thmey
4	*Trey proheum* ($n = 2$)	Salted fish	Freshwater fish, salt	Main dish, seasoning	Thmey
5	*Kapi* ($n = 1$)	Shrimp paste	Tiny marine shrimp, salt	Side-dish, condiment, seasoning	Chas
6	*Paork kampeus* ($n = 3$)	Fermented tiny freshwater shrimp	Tiny freshwater shrimp, salt, roasted rice, slightly green papaya, galangal	Side-dish	Phumreusey, Limcheanghak
7	*Mam lahong* ($n = 2$)	Fermented green papaya	Green papaya, slightly tiny fermented fish, salt, roasted rice, galangal	Side-dish	Limcheanghak
8	*Spey chrourk* ($n = 2$)	Fermented mustard	Chinese mustard, salt	Side-dish	Phumreusey, Limcheanghak

2.2. Growth Conditions and LAB Isolation

Ten grams (10 g) of each sample were aseptically taken, transferred into a stomacher bag, and homogenized (Stomacher 400 Circulator, Seward Ltd, Worthing, UK) with 90 mL buffered peptone water for 45 s at 230 rpm. Appropriate decimal dilutions of the samples were prepared using the same medium. From each dilution, 0.1 mL were inoculated on DeMan Rogosa Sharpe (MRS) agar (Merck, Darmstadt, Germany) by the spread plate method. Inoculated plates were incubated at 30 °C for 72 h in an anaerobic chamber (80% N_2, 10% CO_2, 10% H_2, Scholzen Technik, Kriens, Switzerland). Subsequently, colonies with different morphologies were selected and streaked onto MRS agar for purification. After three days of anaerobic incubation at 30 °C, pure isolates were Gram-stained. Only colonies with gram-positive cocci or rods were transferred into 3 mL MRS broth (Merck) and anaerobically incubated for 24 h at 30 °C. The incubated MRS broth of each isolate was mixed with

glycerol (99.5%, Roth, Karlsruhe, Germany) to obtain a final concentration of 20% glycerol (*v*/*v*) and stored at −80 °C.

2.3. LAB Identification by Partial 16S rDNA Sequencing and MALDI-TOF MS

2.3.1. DNA Extraction and Identification of LAB by Partial 16S rDNA Sequencing

Before DNA extraction, LAB isolates were resuscitated in MRS broth at 30 °C for 48 h. Genomic DNA of the isolates was then extracted using the peqGOLD Bacterial DNA Mini Kit (PeqLab, Erlangen, Germany) according to the manufacturer's instructions. Afterwards, the DNA preparations were stored at −20 °C until use. The extracted DNA was used as template for 16S rDNA sequencing. PCR amplifications were performed in a total volume of 25 µL containing 1 µL of DNA, respectively. Moreover, 1 µL each of the forward primer bak4 (5'-AGG AGG TCA TCC ARC CGCA-3'; 10 pmol/µL) and the reverse primer bak11w (5'-AGT TTG ATC MTG GCT CAG-3'; 10 pmol/µL), 2.5 µL of 10× PCR buffer (Dynazyme buffer 10×; Thermo Scientific, Waltham, MA, USA), 0.5 µL of deoxynucleoside triphosphate (dNTP) mix (10 nmol/µL of each dNTP; GE Healthcare, Buckinghamshire, UK), 0.5 µL of DNA polymerase (2 U/µL; Dynazyme II; Thermo Scientific), and 18.5 µL of sterile distilled water were added. The following PCR program was applied: an initial denaturation at 95 °C for 3 min, 30 cycles of denaturation at 95 °C for 30 s, annealing at 56 °C for 30 s, extension at 72 °C for 2 min and a final extension at 72 °C for 7 min. PCR was conducted in an Eppendorf Mastercycler (Eppendorf, Hamburg, Germany). The obtained PCR products were analysed with a DNA ladder (GeneRuler 100 bp DNA ladder extended) by electrophoresis on a 2% (*w*/*v*) agarose gel in 0.75 × TAE buffer at 80 V for 110 min, stained with GelRed Nucleic Acid Gel Stain (Biotium, Fremont, CA, USA), and visualized with an ultraviolet transilluminator (Bio-RAD, Hercules, CA, USA).

PCR products thereof were purified with the QIAquick PCR Purification Kit (Qiagen, Venlo, The Netherlands) and sent to commercial sequencing (Eurofins MWG Operon, Ebersberg, Germany). Upon receipt of the data, sequences were aligned to the National Center for Biotechnology Information (NCBI) database using the Basic Local Alignment Search Tool (BLAST) with the BLASTn program. An unknown isolate was generally assigned to a species in the database whose sequence had a nearest neighbour exhibiting the highest similarity score of ≥97%.

2.3.2. Identification of LAB by MALDI-TOF MS

The isolates were identified using MALDI-TOF MS with the Bruker Biotyper (Bruker Daltonics, Bremen, Germany). The identification was conducted by the "extended direct transfer" and the "formic acid extraction" procedure according to the manufacturer's instruction. For the "extended direct transfer" technique, a single colony of each isolate was deposited directly on a steel MSP96 target plate and subsequently overlaid with 1 µL of 70% formic acid (Roth) and air-dried. For the "formic acid extraction" method, a single colony or several colonies were placed into an Eppendorf tube containing 300 µL deionized water and mixed thoroughly. Then, 900 µL of ethanol (99.7%, VWR Chemicals, Fontenay-sous-Bois, France) was added and mixed. After that, samples were centrifuged at 14,000 rpm for 2 min and the supernatant was decanted. To remove the residual ethanol, the centrifugation step was repeated under the same conditions. Subsequently, 10 µL (depending on the size of the pellet) of 70% formic acid was added together with 10 µL pure acetonitrile (99.9%, VWR Chemicals, Fontenay-sous-Bois, France) and mixed thoroughly. After a further centrifugation step for 2 min at 14,000 rpm, 1 µL of the supernatant was applied onto a steel MSP96 target plate and air-dried at ambient temperature. Next, samples on the MSP96 target plate from both procedures were overlaid with 1 µL of matrix solution (10 mg/mL of α-cyano-4-hydroxycinnamic acid (HCCA) in acetonitrile:water:trifluoroacetic acid, 50:47.5:2.5 [*v*/*v*/*v*]). After the matrix solution was air-dried at ambient temperature, the plate was immediately applied to the MALDI-TOF Biotyper chamber (Bruker Daltonics) for analysis. Measurements were taken using a Microflex LT bench-top mass spectrometer (Bruker Daltonics) controlled by the FlexControl software (version 3.4; Bruker Daltonics). Three

independent experiments were conducted for each isolate. Mass spectra were processed using the Biotyper software (version 4.1; Bruker Daltonics) and the BioTyper database containing 8223 reference MALDI-TOF MS profiles. The reliability of identification by the MALDI Bruker Biotyper system was expressed in points. A log(score) of ≥2.00 [green color (+++)] indicated identification to the species level and a log(score) of ≥1.70 and <2.00 [yellow color (+)] indicated identification to the genus level, while a score value under 1.70 [red color (-)] means no significant similarity between the unknown profile and any reference profile. If the log(score) was <2.00 (+), the "formic acid extraction" procedure was applied.

2.4. Fingerprinting and Typing of LAB by $(GTG)_5$-PCR

Repetitive element palindromic (rep-PCR) using the $(GTG)_5$ primer (5′-GTG GTG GTG GTG GTG-3′) was performed [20] with a few modifications. Briefly, 1 µL of DNA was pipetted into 24 µL of a PCR mixture containing 1 µL of the $(GTG)_5$ primer (50 pmol/µL), 0.5 µL of dNTP mix (10 nmol/µL of each dNTP; GE Healthcare), 0.5 µL of DNA polymerase (2 U/µL; Dynazyme II; Thermo Scientific), 2.5 µL of 10× PCR buffer (100 nmol/µL Tris-HCl, 15 nmol/µL $MgCl_2$, 150 nmol/µL KCl, 0.1% Triton X-100; pH 8.8, Thermo Scientific), and 19.5 µL sterile distilled water. The cycling program of the Eppendorf Mastercycler (Eppendorf, AG) consisted of an initial denaturation step at 94 °C for 7 min, 30 cycles of denaturation at 90 °C for 30 sec, annealing at 40 °C for 1 min, extension at 65 °C for 8 min and a final extension at 65 °C for 16 min. Obtained PCR products were separated on a 2% agarose gel and stained with GelRed Nucleic Acid Gel Stain.

The cluster analysis of the rep-PCR profiles was performed on similarity matrices, which were produced using the Dice's coefficient [21] and subjected to the unweighted pair group method with arithmetic mean (UPGMA) clustering algorithm using the BioNumerics software version 7.6.1 (Applied-Maths, Saint-Martens-Latem, Belgium). A tolerance level of 1% and an optimization of 0.5% were chosen for creating the dendrogram.

2.5. Screening LAB Strains for GABA Production Using Thin Layer Chromatography (TLC)

All identified LAB strains were screened by TLC using the method described [22] with some modifications. Briefly, all strains were cultured in 4 mL MRS broth supplemented with 2% monosodium glutamate (MSG; Sigma-Aldrich, St. Louis, MO, USA) and anaerobically incubated at 30 °C for 2–3 days. After incubation, the culture broth was centrifuged at 8700 rpm at 4 °C for 5 min. One microliter of supernatant from each strain was spotted on a silica TLC plate (Aluminum Sheets Silica gel 60 F254, Merck). GABA separation by TLC was conducted using a solvent mixture (1-butanol:acetic acid:distilled water, 5:2:2 *v/v/v*). GABA spots were detected after spraying 0.5% (*w/v*) ninhydrin on the plates and heating at 105 °C for 5 min. GABA standard (Sigma-Aldrich) and MSG were used as control standards. This analysis was performed in triplicate. The retention factor (Rf), defined as the ratio of the distance traveled by the center of a spot to the distance traveled by the solvent front, was calculated. Cultures of strains showing the same Rf value as the GABA standard were selected for quantification by the GABase assay.

2.6. Quantification of GABA Production

The GABA concentration of GABA-producing strains was determined by a spectrophotometric GABase assay as described [23] with modifications. Briefly, the reaction mixture (190 µL) contained 140 µL of 100 mM $K_4P_2O_7$ buffer (pH 8.6), 30 µL of 4 mM NADP+, 10 µL of GABase (1 unit/mL, Sigma-Aldrich), and 10 µL of the standard solution (GABA) or culture supernatant (see 2.5.). This mixture was dispensed into each well of a 96-well plate. Before adding 10 µL of 20 mM α-ketoglutarate, the initial absorbance was read at 340 nm in a Multiskan FC plate reader (Thermo Fisher Scientific). The final absorbance was read again after 60 min incubation at room temperature at the same wavelength. The difference of both A340 values due to the conversion of NADP+ to NADPH was used to calculate the GABA content in the sample. The equation of the GABA standard curve was

A340 = 0.0341x − 0.0154 (R^2 = 0.9995), where x is the GABA concentration of the sample in mM. This analysis was done in triplicate.

2.7. Statistical Analysis

All statistical analyses were conducted with Microsoft Excel 2016 (Microsoft, Redmond, WA, USA). The data were checked for plausibility and validity. Descriptive statistics were applied by calculating the mean and standard deviations (SD) of the test results.

3. Results and Discussion

3.1. LAB Identification by Partial 16S rDNA Sequencing and MALDI-TOF MS

3.1.1. Identification of LAB by Partial 16S rDNA Sequencing

Isolating LAB from different types of Cambodian fermented foods, 96 isolates were gram-positive cocci (19 isolates) or rods (77 isolates). Of these, 68 isolates were confirmed as LAB, including 56 *Lactobacillus* isolates [*Lb. fermentum* (18), *Lb. acidipiscis* (17), *Lb. plantarum/paraplantarum/pentosus* (13), *Lb. namurensis* (four), *Lb. futsaii* (two), *Lb. zymae* (one), *Lb. sucicola* (one)], eight *Pediococcus* isolates [*P. pentosaceus* (eight)], and four *Enterococcus* isolates [*E. faecium* (two), *E. pseudoavium/E. avium* (one), *E. viikkiensis/E. durans/E. malodoratus/E. pseudoavium* (one)] (Figure 1). The remaining 28 isolates were bacilli, clostridia and staphylococci (data not shown). Our results strongly support previous findings, which stated that in some cases sequencing of the 16S rDNA has a limited discriminating and low phylogenetic power for several closely related lactobacilli and enterococcal species [24–28] due to substantial similarities of their 16S rDNA sequences [29–32]. Correspondingly, it was impossible to distinguish the three species *Lb. plantarum*, *Lb. paraplantarum* and *Lb. pentosus* in our study because their partial 16S rDNA sequences were highly similar (≥99%) (Table S1). The identification of enterococcal species is also challenging. *E. pseudoavium* and *E. avium* as well as *E. viikkiensis*, *E. durans*, *E. malodoratus*, and *E. pseudoavium* showed similarities of 96% and 99%, respectively (Table S1). Therefore, a few *Lactobacillus* spp. and *Enterococcus* spp. can in some cases only be correctly identified by combining several methods [33,34].

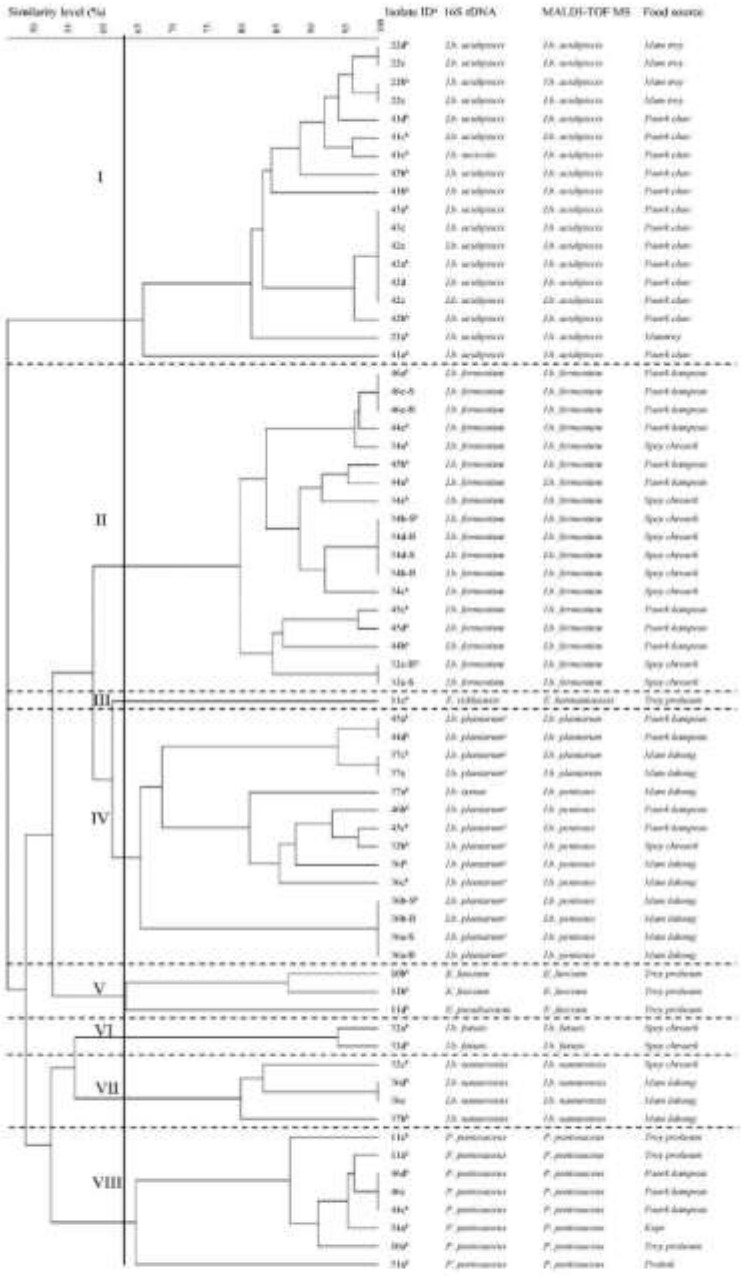

Figure 1. Dendrogram based on cluster analysis of rep-PCR $(GTG)_5$ fingerprints obtained for LAB isolated from Cambodian fermented foods and identified at species level by partial 16S rDNA sequencing and MALDI-TOF MS (Bruker Biotyper). The dendrogram was constructed by the unweighted pair group method using arithmetic (UPGMA) mean with similarity levels expressed as percentage values of the Dice correlation coefficient. All isolates with a similarity of 100% and the same source were regarded as multiple isolates representing a single strain. [a] Identity. [b] Single strain used for GABA screening by TLC. [c] *Lb. plantarum/Lb. paraplantarum/Lb. pentosus* cannot be distinguished by partial 16S rDNA sequencing.

3.1.2. Identification of LAB by MALDI-TOF MS

MALDI-TOF MS has been widely used for the rapid identification and taxonomic characterization of *Lactobacillus* spp. [15,30,34–42], *Enterococcus* spp. [33], and *Pediococcus* spp. [43] of different origin. The 68 LAB isolates of this study were firstly investigated using the "extended direct transfer" procedure. Hence, 38 isolates (55.9%) were identified at species level (score ≥2.00). For the remaining 30 isolates (44.1%), which had a primary score of <2.00, the "formic acid extraction" procedure was applied. Hence, it is known that the simple and rapid "extended direct transfer" protocol is inferior in accuracy because of insufficient cell wall disruption [44]. In contrast, acetonitrile is used together with formic acid in the "formic acid extraction" procedure to improve cell wall disruption [45]. Of the 30 isolates, 23 (76.7%) obtained a score of ≥2.00, indicating an identification at species level (Table S1). Overall, MALDI-TOF MS with the Bruker Biotyper identified 61 (89.7%) LAB isolates at species level. For the remaining seven isolates, MALDI-TOF scores in the range of 1.70–1.99 were obtained (Table S1) based on a comparison with the producer's reference database. Andersen et al. (2014) even considered MALDI-TOF (Bruker Biotyper) scores in this range as acceptable for identification of *Lactobacillus* species [30]. Accordingly, six remaining isolates could be assigned to the species *Lb. acidipiscis*. As previously reported [35], the extension of the reference database could probably improve the performance of MALDI-TOF MS with the Bruker Biotyper for the classification of *Lb. acidipiscis* and *E. viikkiensis* isolates, which had the lowest average scores in this study. For example, two *Lb. futsaii* isolates were originally identified as *Lb. farciminis* with an average score of 1.88. After establishing an in-house database using the two *Lb. futsaii* reference strains CS3 and CS5 [46], they were identified as *Lb. futsaii* with an average log(score) of 2.28 (Table S1).

3.2. Fingerprinting and Typing of LAB by $(GTG)_5$-PCR

The 68 LAB isolates were subjected to $(GTG)_5$-PCR fingerprinting technique for genotypic grouping. The dendrogram of all obtained $(GTG)_5$-PCR patterns is shown in Figure 1. Setting a cut-off value at 63% similarity, the 68 isolates were grouped into seven separate clusters (I, II, IV–VIII) and one singleton (III; Figure 1). According to the results of 16S rDNA sequencing and MALDI-TOF MS, each cluster is well-differentiated and represents an individual species [*Lb. acidipiscis* (I), *Lb. fermentum* (II), *Lb. plantarum* (IV), *E. faecium* (V), *Lb. futsaii* (VI), *Lb. namurensis* (VII), and *P. pentosaceus* (VIII)]. The only singleton is an *Enterococcus* strain (III) either belonging to the species *E. viikkiensis* (16S rDNA sequencing) or *E. hermaniensis* (MALDI-TOF MS). Both species are closely related [26]. As previously reported, these results demonstrate that $(GTG)_5$-PCR fingerprinting is a useful tool for grouping lactobacilli [20,47,48].

A high concordance of 95.6% (65/68) was assessed between 16S rDNA sequencing and MALDI-TOF MS with the Bruker Biotyper. However, one isolate (41e) was identified as *Lb. sucicola* by 16S rDNA sequencing and as *Lb. acidipiscis* by MALDI-TOF MS. Interestingly, this isolate was displayed in the *Lb. acidipiscis* cluster by $(GTG)_5$-PCR fingerprinting (Figure 1). According to the literature both species are members of the phylogenetic *Lb. salivarius* group [49]. Furthermore, this data supports the finding of Dušková et al. (2012), who proved that MALDI-TOF MS with Bruker Biotyper is superior in the identification of lactobacilli species [35]. Similarly, the *Lb. plantarum*/*Lb. paraplantarum*/*Lb. pentosus* cluster determined by 16S rDNA sequencing could be divided into one *Lb. plantarum* and two *Lb. pentosus* sub-clusters based on the dendrogram and MALDI-TOF MS (Figure 1). Next to these three phylogenetically similar sub-clusters, a *Lb. zymae* strain was also assigned to this cluster. This species was recently transferred from the *Lb. buchneri* clade to the *Lb. brevis* clade [50], which is close to the *Lb. plantarum* clade [51,52].

Only strains with different fingerprints (e.g., 41b, 41d) or the same fingerprint and different origins (e.g., 44d, 45a) were considered for further evaluations. Isolates with a 100% similarity and the same origin were regarded as multiple isolates representing a single strain (e.g., 34d-B, 34d-S, 34b-B, 34b-S). Choosing only one of these multiple isolates (e.g., 34b-S), the initial 68 LAB isolates were reduced to 50 strains by $(GTG)_5$-PCR fingerprinting technique. These 50 strains were used for GABA

screening and quantification. For simplicity, only species names determined by MALDI-TOF MS were furthermore applied.

3.3. Prevalence of LAB in Cambodian Fermented Foods

Strains of *Lb. acidipiscis* were only found in Cambodian fermented fish (*paork chav* and *mam trey*). The species *Lb. fermentum*, *Lb. plantarum*, *Lb. pentosus*, *Lb. namurensis*, *Lb. futsaii*, and *Lb. zymae* were detected in *paork kampeus*, *mam lahong*, and *spey chrourk*, fermented foods mainly made of tiny freshwater shrimp, green papaya, and mustard (Table 1, Table 2).

Table 2. Identification of LAB from different fermented foods by partial 16S rDNA sequencing and MALDI-TOF MS.

Food Samples	Total Number of Isolates	Identification Technique			
		Partial 16S rDNA	Number of Identified Isolates	MALDI-TOF MS (Bruker Biotyper)	Number of Identified Isolates
Prahok (n = 1)	1	*P. pentosaceus*	1	*P. pentosaceus*	1
Paork chav (n = 3)	13	*Lb. acidipiscis* *Lb. sucicola*	12 1	*Lb. acidipiscis* –	13 0
Mam trey (n = 1)	5	*Lb. acidipiscis*	5	*Lb. acidipiscis*	5
Trey proheum (n = 2)	7	*P. pentosaceus* *E. faecium* – *E. viikkiensis/E. durans/E. malodoratus/E. pseudoavium* *E. pseudoavium/E. avium*	3 2 0 1 1	*P. pentosaceus* *E. faecium* *E. hermanniensis* – –	3 3 1 0 0
Kapi (n = 1)	1	*P. pentosaceus*	1	*P. pentosaceus*	1
Paork kampeus (n = 3)	16	*Lb. fermentum* *Lb. plantarum/Lb. paraplantarum/Lb. pentosus* *P. pentosaceus*	9 4 3	*Lb. fermentum* *Lb. pentosus* *Lb. plantarum* *P. pentosaceus*	9 2 2 3
Mam lahong (n = 2)	12	*Lb. plantarum/Lb. paraplantarum/Lb. pentosus* *Lb. namurensis* *Lb. zymae*	8 3 1	*Lb. pentosus* *Lb. plantarum* *Lb. namurensis* *Lb. zymae*	6 2 3 1
Spey chrourk (n = 2)	13	*Lb. fermentum* *Lb. plantarum/Lb. paraplantarum/Lb. pentosus* *Lb. namurensis* *Lb. futsaii*	9 1 1 2	*Lb. fermentum* *Lb. pentosus* *Lb. namurensis* *Lb. futsaii*	9 1 1 2

(–) species that was not identified by the respective technique.

These findings are in agreement with previous studies [18,39,53–55]. Thus, Lb. acidipiscis was originally determined in fermented fish [53]. In general, LAB are identified as important components of the gut microbiota of fish. Members of the Lactobacillus, Lactococcus, Leuconostoc, Enterococcus, Carnobacterium, Pediococcus, Streptococcus and Weissella genera have already been isolated including Lb. fermentum, Lb. plantarum, E. durans, E. faecalis, E. pseudoavium, E. faecium, and P. pentosaceus [56,57]. Besides fermented fish, Lb. fermentum and Lb. plantarum were also found in fermented mustard and onion [14,18,39,54,55]. Followed by Lb. plantarum, Lb. pentosus was the predominant species in fermented olives [58]. Also Lb. namurensis and Lb. zymae seem to be plant-associated as these species were detected in sourdough [59]. In this context, it is assumed that LAB come from flour and may originate from wheat [60]. Furthermore, Lb. namurensis was present in fermented rice bran [61] and Lb. zymae in kimchi [62] and fermented onion [54]. In this study, Lb. zymae and Lb. namurensis were detected in fermented green papaya (mam lahong), whereas Lb. namurensis was additionally found in fermented mustard (spey chrourk) (Table 2). Likewise,

Lb. futsaii was identified in fermented mustard, which corresponds to the literature [63]. It is well known that LAB represent a subdominant part of the microbiota of raw vegetables and fruits [60]. Table 2 shows that P. pentosaceus strains were present in fermented fish (prahok and trey proheum) and shrimp products (kapi and paork kampeus). This species was already isolated from fermented vegetables [18], fermented fish [55,57], and seafoods [64,65]. Next to P. pentosaceus, enterococci were detected in salted fish (Table 2). While P. pentosaceus and E. faecium can grow in the presence of ≥6.5% NaCl [66,67], salt resistance is strain-dependent in the species E. hermanniensis [68]. As enterococci are found in a variety of different ecological environments including surface and waste waters, their association with fermented seafood [64,69,70] can be explained.

3.4. Screening LAB Strains for GABA Production and GABA Quantification

All LAB strains were screened for their potential to produce GABA on TLC silica plates (Figure S1). Only strains with a Rf value corresponding to that of the GABA standard (0.27 cm) were selected for GABA quantification. Compared to this value, just six strains (12%) are GABA producers (Figure S1). These strains belong to the species (number of strains) *Lb. plantarum* (three), *Lb. namurensis* (two), and *Lb. futsaii* (one). Previous studies also reported mainly strains of the genus *Lactobacillus* as GABA-producing LAB [10]. Thus, *Lb. plantarum* strains from kimchi [71], cheese [72] and other traditional fermented food products [14] have already been recognized to produce GABA. In addition, *Lb. namurensis* and *Lb. futsaii* strains, isolated from Thai fermented pork sausages and shrimp products, have been identified as GABA-producing LAB [22,73,74]. According to the literature [62,75], *Lb. fermentum* and *Lb. zymae* strains have also been indicated as GABA producers, but within this work no GABA production could be determined for these *Lactobacillus* species as well as for *Lb. acidipiscis* and *Lb. pentosus*. Some studies also described GABA-producing *P. pentosaceus* strains, which were found in various fermented foods such as fermented beef or pork and alpine cheeses [6,73,74,76]. There have been only few studies on GABA-producing enterococci from fermented foods. This might be due to lower GABA production levels of *Enterococcus* strains compared to those of the genus *Lactobacillus*. Therefore, a GABA production of 1.56 mM by an *E. faecium* strain from Korean traditional fermented food was considered as high-level GABA production [77]. Nevertheless, single *E. durans* and *E. avium* strains with higher GABA yields were reported from Italian cheese and Korean fermented seafood [72,78]. In this study, however, no GABA production could be verified for *P. pentosaceus* or *Enterococcus* strains.

The two *Lb. plantarum* strains 45a and 44d showed stronger spots for GABA production on TLC silica plates than the other four strains (Figure S1). These strains were isolated from *paork kampeus*. Thus, they may originate from tiny freshwater shrimp, but roasted rice, green papaya and galangal could also be their sources, as they are all main ingredients of *poark kampeus*. The origin of the *Lb. plantarum* strain 37e with a weaker spot is probably green papaya. Similar to *paork kampeus*, however, it can also be tiny fermented fish, roasted rice or galangal because these are components of *mam lahong* as well (Table 1). When incubated at 30 °C for 48 h in MRS broth supplemented with MSG (2%, *w/v*), the two *Lb. plantarum* strains 45a and 44d also produced higher concentrations of GABA (20.34 ± 1.41 mM and 16.47 ± 1.91 mM, respectively), whereas the *Lb. plantarum* strain 37e was only able to produce 5.63 ± 0.68 mM GABA (Table 3). Even lower GABA concentrations were obtained from the *Lb. futsaii* (4.68 ± 0.87 mM) and the two *Lb. namurensis* (1.62 ± 0.43 mM and 1.19 ± 0.66 mM, respectively) strains, which were isolated from fermented green papaya and mustard (Table 1).

To our best knowledge, the highest GABA-producing *Lb. plantarum* strain isolated from fermented food produced 30.54 mM GABA (3.15 g/kg) [79]. Furthermore, one *Lb. namurensis* and two *Lb. futsaii* strains from Thai fermented shrimp and Thai fermented sausages synthesizing higher GABA concentrations [71.18 mM (7.34 g/L) and >77.58 mM (>8.00 g/L), respectively] were previously described [46,74]. However, such comparisons should be treated with caution as results from different studies are received under various conditions. Thus, the food source of the strain might have an influence on the GABA production level. In this respect, it was found that acidic food could be the

habitat of high GABA producers as these can maintain the intracellular pH under acidic conditions by eliminating intracellular protons during the decarboxylation of glutamate [80]. Of course, GABA-rich foods themselves can also be a good origin of high GABA producing LAB. In addition to a wide range of traditional fermented foods such as yogurt, cheese, kimchi, sourdough and paocai [10], substantial amounts of GABA were also found in germinated edible seeds and sprouts as well as in tomato during the mature green stage [81]. However, it is believed that the accumulation of GABA in these foods is due to the presence and activity of enzymes and not to microorganisms [82]. Moreover, LAB are often selectively isolated from food based on their capacity to form high levels of GABA by supplementing culture media with MSG [46]. Also, the applied detection method (e.g. enzyme assay, chromatography or automatic amino acid analyzer) has an effect on the determination of GABA level [83,84]. It was noted that the GABA levels determined by high-performance liquid chromatography (HPLC) were lower than those measured by the GABase assay [85]. According to the authors, media with different amounts of MSG as well as the extraction and derivatisation processes required for HPLC might have been the reason for this difference [85]. Finally, the production of GABA itself can be affected and optimized by different factors, of which the most common and essential ones are pH, temperature, the ingredients and additives in the media, as well as the fermentation time [10]. For example, the GABA production of the aforementioned *E. faecium* strain was increased from 1.56 mM to 14.86 mM when this strain was cultivated in a specially designed medium under optimal conditions [77]. These requirements vary among microorganisms due to the different properties of their GADs [10]. Hence, the recommended step for optimizing GABA production is the characterization of these properties in the relevant *Lactobacillus* strains and the development of efficient production processes. When fermenting glutamate-rich foods with well-characterized starter cultures and the addition of exogenous MSG, GABA-concentrations of approximately 101.82 mM (10.5 g/kg) could be finally achieved [86].

Table 3. LAB with GABA-producing abilities after 48 h cultivation.

Strain	LAB Species Identified by MALDI-TOF MS (Bruker Biotyper)	Corresponding Lanes of TLC Analysis	Rf (cm) [a]	GABA (mM) [b] Production
45a	*Lb. plantarum*		0.27	20.34 ± 1.41
44d	*Lb. plantarum*		0.27	16.47 ± 1.91
37e	*Lb. plantarum*		0.27	5.63 ± 0.68
32d	*Lb. futsaii*		0.27	4.68 ± 0.87
37b	*Lb. namurensis*		0.27	1.62 ± 0.43
32c	*Lb. namurensis*		0.27	1.19 ± 0.66
-	GABA-negative strain [c]		0.16	n.d.
-	GABA standard		0.27	n.d.
-	MSG [d]		0.16	n.d.

[a] Rf = retention factor, defined as the ratio of the distance traveled by the center of a spot to the distance traveled by the solvent front; only strains showing the same Rf value as the GABA standard (=0.27 cm) were selected for GABA (mM) quantification. [b] mean ± SD. [c] GABA-negative strain = any strain that does not produce a GABA spot with a Rf value equal to that of the GABA standard (e.g. all tested strains except of 45a, 44d, 37e, 32d, 37 b, 32c). [d] MSG = monosodium glutamate. n.d.= not determined.

4. Conclusions

A total of 68 LAB isolates from different Cambodian fermented foods were identified by genotypic and proteomic techniques. Applying rep-PCR $(GTG)_5$, the initial number of LAB was reduced to 50 strains, which were screened for GABA production. Six strains belonging to the species *Lb. plantarum*, *Lb. futsaii*, and *Lb. namurensis* were able to produce GABA, in particular one *Lb. plantarum* strain showed the highest GABA concentration (20.34 mM), followed by another *Lb. plantarum* strain (16.47 mM). Since GABA is used as an active component in foods and pharmaceuticals, these GABA-producing strains could be of interest for the production of GABA-enriched fermented foods and beverages.

However, in order to further increase and optimize the GABA production, detailed characterization of these strains is needed, including research on their safety, technological performance and other probiotic attributes. Such starter cultures would allow the production of Cambodian fermented foods with an additional nutritional value that meet the population's dietary habits and are also more attractive for the international food market.

Supplementary Materials: The following are available online at http://www.mdpi.com/2218-273X/9/12/768/s1, Figure S1: Thin-layer chromatography (TLC) analysis of GABA producing-LAB, Table S1: List of LAB isolates identified by partial 16S rDNA sequencing (% similarity, accession number) and MALDI-TOF MS (Bruker Biotyper) log(score) and their source.

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Chapter 6

Microbial production and enzymatic biosynthesis of γ-aminobutyric acid (GABA) using *Lactobacillus plantarum* FNCC 260 isolated from Indonesian fermented foods

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Microbial Production and Enzymatic Biosynthesis of γ-Aminobutyric Acid (GABA) Using *Lactobacillus plantarum* FNCC 260 Isolated from Indonesian Fermented Foods

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Abstract: In the present study, we isolated and screened thirty strains of GABA (γ-aminobutyric acid)-producing lactic acid bacteria (LAB) from traditional Indonesian fermented foods. Two strains were able to convert monosodium glutamate (MSG) to GABA after 24 h of cultivation at 37 °C based on thin layer chromatography (TLC) screening. Proteomic identification and 16S rDNA sequencing using MALDI-TOF MS identified the strain as *Lactobacillus plantarum* designated as *L. plantarum* FNCC 260 and FNCC 343. The highest yield of GABA production obtained from the fermentation of *L. plantarum* FNCC 260 was 809.2 mg/L of culture medium after 60 h of cultivation. The supplementation of 0.6 mM pyridoxal 5′-phosphate (PLP) and 0.1 mM pyridoxine led to the increase in GABA production to 945.3 mg/L and 969.5 mg/L, respectively. The highest GABA production of 1226.5 mg/L of the culture medium was obtained with 100 mM initial concentration of MSG added in the cultivation medium. The open reading frame (ORF) of 1410 bp of the *gad*B gene from *L. plantarum* FNCC 260 encodes 469 amino acids with a calculated molecular mass of 53.57 kDa. The production of GABA via enzymatic conversion of monosodium glutamate (MSG) using purified recombinant glutamate decarboxylase (GAD) from *L. plantarum* FNCC 260 expressed in *Escherichia coli* was found to be more efficient (5-fold higher within 6 h) than the production obtained from fermentation. *L. plantarum* FNCC 260 could be of interest for the synthesis of GABA.

Keywords: GABA; Indonesian fermented foods; glutamate decarboxylase; lactic acid bacteria; *L. plantarum*

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1. Introduction

γ-aminobutyric acid (GABA), which is a non-protein amino acid and plays a major role as a suppressive neurotransmitter, is widely present in plants, microorganisms, and the mammalian brain [1–3]. GABA has been extensively studied due to its physiological and pharmacological effects including anti-depressant, hypotensive activity, anti-diabetic in humans [4–6]. Recently, GABA administration in fluoride-exposed mice showed protective effects against hypothyroidism and maintained lipid and glucose levels in vivo [4]. Furthermore, GABA-enriched foods have been developed [5–11]. GABA-rich chlorella

has been shown to significantly lower high blood pressure in hypertensive subjects [12]. A number of GABA-enriched foods such as soymilk [13], fermented milk [14], natto [15], green tea [16], and cheese [5] have been reported to suppress the elevation of blood pressure in spontaneously hypertensive rats (SHR) and hypertensive subjects.

GABA can be synthesized using chemical or biochemical means, of which the latter involves enzymatic conversion, whole-cell biocatalysts, or microbial fermentation. The chemical synthesis is considered hazardous due to corrosive nature of used reagents [17–19], hence the application in the food industry is limited. Moreover, the supplementation of synthetic GABA to food system is considered unnatural and unsafe [14]. Therefore, it is important to develop a natural and safe method to increase GABA in foods, since there are no side effects of natural GABA supplementation [16]. Recent studies showed that several strains of lactic acid bacteria (LAB) are promising candidates as GABA-producing bacteria [1–3,5] due to their GRAS (generally recognized as safe) status. A number of GABA-producing bacteria has been isolated from fermented foods such as *L. brevis* (from kimchi) [20], *L. rhamnosus* (from fermented pickles) [21], *L. plantarum* (from fermented dairy products) [22], *L. helveticus* (from koumis fermented milk) [23], *L. buchneri* (from kimchi) [24], *L. otakiensis* (from Pico cheese) [25], *L. namurensis* (from fermented green papaya) [11], and *L. paracasei* (from Italian cheese) [3]. These reports have shown that fermented foods are promising sources of GABA-producing bacteria. In addition, screening GABA-producing LAB from various fermented foods might open the possibilities to obtain newly isolated strains for the use as functional starter cultures in the food industry.

Several fermented foods from Indonesia namely *gatot*, *growol*, *tape ubi*, *bekasam*, and *tempoyak* are spontaneously fermented by LAB, which mainly involve the strains of the genera *Lactobacillus*, *Pediococcus*, and *Streptococcus* [26,27]. However, the potential of these LAB strains from Indonesian fermented foods to be used as GABA-producing bacteria as well as their relevant enzymes have not yet been studied. Therefore, the development of GABA-enriched foods using suitable LAB is a promising strategy, to bring new functional foods to the market. In addition, biosynthesis of GABA using LAB also provides advantageous effects including probiotic activity and extension of the shelf-life of food products [9].

The biosynthesis of GABA involves irreversible decarboxylation reaction of glutamate to GABA and carbon dioxide catalyzed by glutamate decarboxylase (GAD). GAD (EC 4.1.1.15) is a major enzyme for GABA synthesis and it requires pyridoxal 5′-phosphate (PLP) as a cofactor [28–30]. The GAD genes from various sources have been cloned, expressed and their biochemical properties have been characterized [20,31–34]. The use of purified GAD for the biosynthesis of GABA is also of interest because only simple downstream purification of GABA is required and yet, the process could overcome the limitation of microbial fermentation (i.e., GABA catabolism). In the present study, we describe the screening of GABA-producing LAB from Indonesian fermented foods (fermented soybeans, *growol*, *gatot*, *tempeh*, and *bekasam*) and GABA productions using microbial fermentation of the isolated strain and the purified GAD of this strain for the conversion of glutamate to GABA.

2. Materials and Methods

2.1. Screening of GABA-Producing LAB

Thirty isolates of *Lactobacillus* spp. were previously isolated from Indonesian fermented foods such as fermented soybeans, *growol*, *gatot*, *tempeh*, and *bekasam* (fermented fish) [35]. *Lactobacillus* spp. were the predominant genus according to cell morphology, Gram reactions and catalase tests. All strains were obtained and stored in the Food and Nutrition Culture Collection, Universitas Gadjah Mada (Yogyakarta, Indonesia). Prior to screening, all strains were grown in MRS broth containing 118 mM monosodium glutamate (MSG) (Ajinomoto, Tokyo, Japan) for 24–48 h at 37 °C under microaerophilic conditions. The cultures broth was then centrifuged at 8000× *g* for 5 min at 4 °C. GABA formation in the supernatant was analyzed using thin layer chromatography (TLC). Briefly, 0.5–1.0 µL of supernatants were spotted onto TLC plates Silica gel 60 F_{254} (Merck, Darmstadt, Germany).

The mobile phase consists of a mixture of 1-butanol: acetic acid: distilled water (5:2:2). Subsequently, the plates were sprayed with 0.5% ninhydrin and heated at 105 °C for 5 min to visualize the spots. GABA (Sigma Aldrich, St. Louis, MO, USA) was used as a standard, and the Rf values were calculated. LAB cultures showing the same Rf values as GABA standard were considered as positive GABA-producers. Positive GABA-producing strains were identified using proteomic and genotype techniques. Furthermore, the amount of GABA produced was determined by the GABase assay [36]. All chemicals were of the highest grade.

2.2. Identification of GABA-Producing LAB

Proteomic and genotype techniques were performed to identified GABA-producing LAB. Genomic DNA of GABA-producing LAB was extracted using peqGOLD Bacterial DNA Mini Kit (PeqLab, Erlangen, Germany) according to manufacturer's instructions. The extracted DNA was used as a template for partial 16S rDNA amplification. The amplifications of 16S rDNA were performed using forward primer bak4 (5′-AGGAGGTCATCCARC CGCA-3′) and reverse primer bak11w (5′-AGTTTGATCMTGGCTCAG-3′) [37], The PCR reaction mixtures consisted of 10× PCR buffer (Dynazyme buffer 10× Thermo scientific, Waltham, MA, USA), 10 nmol/µL dNTP mix (GE Healthcare Buckinghamshire, UK), 2 U/µL DNA polymerase (Dynazime II, Thermo scientific) and high-quality sterile water to a total volume of 25 µL. The conditions for PCR amplification were as follows: initial denaturation at 95 °C for 3 min, followed by 30 cycles of denaturation at 95 °C for 30 s, annealing at 56 °C for 30 s, extension 72 °C for 2 min, and a final extension at 72 °C for 7 min. After PCR amplification, the amplified products were visualized by gel electrophoresis. The gel was stained with GelRed Nucleic Acid (Biotium, Hayward, CA, USA) and subsequently visualized with an ultraviolet transilluminator (BioRad, Hercules, CA, USA). The PCR products were purified using QIAquick PCR purification Kit (Qiagen, Venlo, The Netherlands) and sent for sequencing (Eurofins MWG Operon, Ebersberg, Germany). Subsequently, the partial 16S rDNA sequence was compared with the National Center for Biotechnology Information (NCBI) sequence database using Basic Local Alignment Search Tool (BLAST) program.

Proteomic identification was performed using matrix-assisted laser desorption/ionizing time-of-flight mass spectrometry (MALDI-TOF MS). GABA-producing bacteria were identified by the extended direct transfer method. A single colony was directly spread onto a MALDI target plate. The spot was overlayed with 1 µL of 70% formic acid and allowed to dry at room temperature. Furthermore, 1 µL of 10 mg/µL HCCA (α-cyano-4-hydroxycinnamic acid) solution was then added to the spot and allowed to dry at room temperature. The target plate was immediately applied to MALDI-TOF MS and analyzed using Microflex LT bench-top mass spectrometer (Bruker Daltonics, Bremen, Germany) equipped with the FlexControl 3.4 software. A mass spectrum was processed using Bio-Typer software (version 3.0, Bruker Daltonics, Bremen, Germany). MALDI-TOF MS profiles were obtained from bacteria isolates and matched with a database containing 8223 reference MALDI-TOF MS profiles.

2.3. Determination of GABA Production and GAD Assay

The GABAse method was performed to determine GABA concentration in culture supernatants. Briefly, the culture broth was centrifuged at 8000× *g* for 5 min at 4 °C. 10 µL of supernatants were mixed with 140 µL of 100 mM $K_4P_2O_7$ buffer (pH 8.6), 30 µL of 4 mM $NADP^+$, 10 µL of 1 U/mL GABase (Sigma-Aldrich, St. Louis, MO, USA). The mixtures were dispensed into each well of 96-well plate. The initial absorbance was read at 340 nm in PerkinElmer plate reader (PerkinElmer, Buckinghamshire, UK). After the initial reading, 10 µL of 20 mM α-ketoglutarate were added and the mixtures were incubated for 1 h. The final absorbance was read after 1 h at the same wavelength. GABA concentrations were determined based on the difference of A_{340} values and the standard curve of GABA.

The GAD assay was carried out using colorimetric method [38]. The reaction mixtures consist of 200 mM Na_2HPO_4-citric acid buffer (pH 5.0), 20 mM L-MSG, 0.2 mM PLP, and 20 µL of purified GAD. The mixtures were thoroughly mixed and incubated at 37 °C for 1 h and then deactivated by boiling for 5 min. The reaction mixtures were used to determined GAD activity using the Berthelot reaction method, which was composed of 100 µL of reaction sample, 250 µL of H_2O, 50 µL of 200 mM sodium borate (pH 9.0), 250 µL of 6% phenol and 200 µL of 5% (*w*/*v*) sodium hypochlorite. Subsequently, the reaction mixtures were thoroughly mixed and boiled for 10 min until the blue color developed, then immediately placed on ice for 15 min. The mixtures were analyzed colorimetric at 630 nm to determine the absorption value. One unit of GAD activity was defined as the amount of enzyme that liberates 1 µmol of GABA per minute under activity assay conditions.

The concentrations of GABA formed in supernatants and after enzymatic conversions were confirmed using Ultra Performance Liquid Chromatography (UPLC Acquity H- Class, Waters Corporation, Milford, MA, USA) equipped with a PDA detector and an AccQ. Tag Ultra C18 column (1.7 µm particle, 2.1 × 100 mm). The samples were hydrolyzed using 6 N HCL and followed by derivatization of the samples and the GABA standard using AccQ-Tag ultra-derivatization kit (Waters, Milford, MA, USA) according to the manufacturer's instructions. For UPLC analysis, the derivatized samples were injected to Acquity UPLC H class [39]. The system was operated at a flow rate of 0.5 mL/min at 49 °C with a wavelength of 260 nm. The mobile phase used were AccQ. Tag Ultra Eluent A 100%; Accq. Tag Ultra Eluent B (Aquabides 90:10); Aquabides Eluent C; AccQ. Tag Ultra Eluent B 100%.

2.4. GABA Production

The MRS medium was inoculated with 5% inoculum of GABA-producing LAB and incubated at 37 °C for 108 h. The optical densities (OD_{600}) of the cultures were measured every 12 h. A concentration of MSG (25–100 mM) (Sigma Aldrich, St. Louis, MO, USA), pyridoxal 5-phosphate (PLP, 0.2 and 0.6 mM) and pyridoxine (vitamin B6, 0.1–0.3 mM) were added to the MRS medium and GABA production under these conditions was investigated subsequently.

2.5. Cloning of gadB Gene

The glutamate decarboxylase gene (*gad*) from *L. plantarum* FNCC260 was amplified using degenerated primers *gad*_FwdNdeI (5′-CATATGATGGCAATGTTRTAYGGTAAAC-3′) and *gad*_RevEcoRI (5′-GAATTCCAGTGTGTGAATMSGTATTTC-3′), which were designed based on the sequences of the *gad* genes of *Lactobacillus* spp. available in GenBank (Accession numbers JN248358.1, KU214639.1, AB986192.1, CP029349.1, AL935263.1, CP018209.1, CP028977.1, GU987102.1, JX545343.1).

The primers were supplied by VBC-Biotech Service (Vienna, Austria) and the appropriate endonuclease restriction sites were introduced in the forward and reverse primers (underlined sequences). The conditions for PCR reactions were as follows: initial denaturation at 98 °C for 20 s; 30 cycles of denaturation at 98 °C for 20 s, annealing at 58 °C for 20 s, extension at 72 °C for 1 min 45 s, and final extension at 72 °C for 2 min. The amplified PCR products were purified using the Monarch DNA Gel Extraction Kit (New England Biolabs, Ipswich, MA, USA), digested with *Nde*I and *Eco*RI and cloned into the pET 21(+a) vector (Novagen, Merck KGaA, Darmstadt, Germany) resulting in the plasmid pET21GAD. *E. coli* NEB5α was used as a host for obtaining the plasmids in sufficient amounts. The sequence of the insert was confirmed by DNA sequencing performed by a commercial provider (Microsynth, Vienna, Austria). The alignment tool (BLAST) from the National Center for Biotechnology Information BLAST website was used for the alignment of the nucleotide sequence of the *gad* gene from *L. plantarum* FNCC 260 with the available *gad* sequences from LAB. The comparison of glutamate decaroboxylases (GAD) from different LAB species was carried out using the program Clustal Omega (https://www.ebi.ac.uk/Tools/msa/clustalo/) [31,33].

2.6. Overexpression of GADLbFNCC260 in E. coli and Protein Purification

The expression vector of pET21GAD harboring the *gad* gene from *L. plantarum* FNCC 260 was transformed into *E. coli* T7 Express GRO carrying the plasmid pGRO7, which encodes the chaperones GroEL and GroES (Takara, Shiga, Japan). Subsequently, *E. coli* T7 Express GRO carrying the plasmid pET21GAD was cultivated in LB broth medium supplemented with 100 µg/mL ampicillin, 20 µg/mL chloramphenicol, and 1 mg/mL arabinose until OD_{600nm} of 0.6 was reached. Thereafter, the isopropyl β-D-1-thiogalactopyranoside (IPTG) was added to a final concentration of 0.5 mM for induction. The culture was further incubated at 18 °C for 20 h with shaking at 180 rpm. The cells were harvested, washed twice with 50 mM sodium phosphate buffer (pH 6.5), and resuspended in buffer A (50 mM NaH_2PO_4, 300 mM NaCl, and 10 mM imidazole, pH 7.0). The resuspended cells were disrupted using a French press (Aminco, Silver Spring, MD, USA) and centrifuged at 15,000× *g* for 20 min at 4 °C. The cell-free extracts were collected and loaded to a prepacked 1 mL HisTrap HP Ni-immobilized metal ion affinity chromatography (IMAC) column (GE Healthcare, Uppsala, Sweden) that was pre-equilibrated with buffer A (50 mM NaH_2PO_4, 300 mM NaCl, and 10 mM imidazole, pH 7.0). The His-tagged protein was eluted at a rate of 1 mL/min with a 15 mL linear gradient from 0 to 100% buffer B (20 mM NaH_2PO, 500 mM imidazole, 500 mM NaCl, pH 6.5). Active fractions were pooled, desalted, and concentrated by ultrafiltration using an Amicon Ultra centrifugal filter unit with a 30 kDa cut-off membrane (Millipore, Burlington, MA, USA). The purified enzyme was stored in 50 mM citrate-phosphate buffer (pH 5.0) for further characterization and enzymatic conversion. The molecular masses of purified GAD were determined by SDS-PAGE and Native PAGE. Protein bands were visualized by staining with Bio-safe Coomassie (Bio-Rad). The determination of protein mass was carried out using Unstained Precision plus Protein Standard (Bio-Rad, Hercules, CA, USA).

The size of the protein was also confirmed by LC-ESI-MS analysis. The proteins were S-alkylated with iodoacetamide and digested with Trypsin (Promega, Madison, WI, USA). The digested proteins were directly injected to LC-ESI-MS (LC: Dionex Ultimate 3000 LC). A gradient from 10 to 80% acetonitrile in 0.05% trifluoroacetic acid (using a Thermo ProSwiftTM RP-4H column (0.2 × 250 mm) at a flow rate of 8 µL/min was applied (30 min gradient time). Detection was performed with a Q-TOF instrument (Bruker maxis 4G, Billerica, MA, USA) equipped with standard ESI source in positive ion, MS mode (range: 400–3000 Da). Instrument calibration was performed using ESI calibration mixture (Agilent, Santa Clara, CA, USA). Data were processed using Data analysis 4.0 (Bruker) and the spectrum was deconvoluted by MaxEnt.

2.7. Enzymatic Synthesis of GABA

Batch conversion reactions were carried out in 2 mL scale with 0.64 U/mL purified GAD using 100 mM MSG in 50 mM citrate-phosphate buffer (pH 4.5) containing 0.2 mM of PLP as cofactor. Decarboxylation reactions were performed at 30 °C with 300 rpm agitation using a Thermomixer (Eppendorf, Hamburg, Germany). The samples were withdrawn at time intervals and the enzyme GAD was inactivated at 100 °C for 5 min. The samples were stored at −20 °C for subsequent analysis. GABA content in the reaction mixtures was determined using the GABase assay and confirmed with UPLC analysis as described in Section 2.3.

3. Results and Discussion

3.1. Screening and Identification of GABA-Producing LAB

Thirty isolates of *Lactobacillus* spp. from Indonesian fermented foods were screened for the formation of GABA in the culture medium using the TLC method and only two isolates showed clear spots on TLC plate (Figure 1), which have similar Rf value (0.78) as the GABA standard. These two isolates were FNCC 260 and FNCC 343 isolated from fermented cassava and fermented fish, respectively. The two strains FNCC 260 and FNCC 343 were cultivated in MRS broth containing 118 mM MSG for 48 h to determine the GABA

production in the culture medium, which was analyzed to be 352 mg and 328 mg of GABA per liter of culture medium, respectively. Based on morphological observation, these two strains were Gram positive, rod-shape, microaerophilic, and catalase negative.

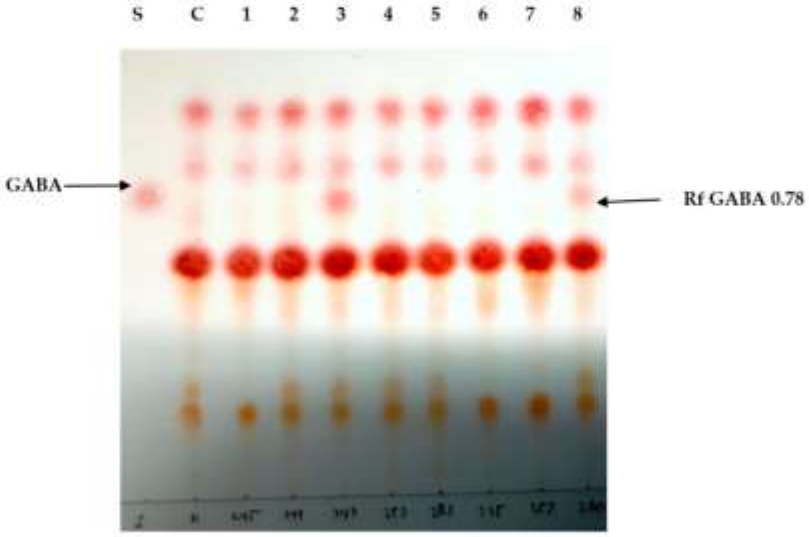


Figure 1. Thin layer chromatography (TLC) screening of GABA–producing LAB. Lane S: GABA standard, Lane C: MRS with 118 mM monosodium glutamate (MSG), Lane 1–8: the strains FNCC 245, FNCC 344, FNCC 343, FNCC 253, FNCC 283, FNCC 235, FNCC 257, and FNCC 260, respectively. All strains were cultivated in MRS broth supplemented with 118 mM MSG and incubated at 37 °C for 48 h.

Subsequently, these two GABA-producing LAB were identified using 16S rDNA and MALDI-TOF MS (Bruker Biotyper). Proteomic identification by MALDI-TOF MS was performed since this technique is very effective in identifying species and subspecies of LAB. A number of species and subspecies of LAB have been successfully identified using proteomic-based identification technique [40–43]. Based on MALDI-TOF MS identification, both strains FNCC 260 (2.15 log score) and FNCC 343 (2.12 log score) were identified to be *L. plantarum*, which matches with the strain in a reference database. The log scores also indicate the accuracy and reliability of MALDI-TOF MS identification. A log score between 2.00 and 2.30 indicates accurate identification to the genus and species level [44,45]. Subsequently, GABA-producing LAB were subjected to 16S rDNA sequencing.

Based on partial 16S rDNA sequencing (~1400 bp), both strains belong to the species *L. plantarum*, with 99.81% sequence identity with *L. plantarum* strain CIP 103,151 (accession number, NR_104573.1) for the strain FNCC 260, and 99.81% sequence identity with *L. plantarum* strain NBRC 15,891 (accession number, NR_113338.1) for the strain FNCC 343, respectively. The results confirmed that both strains FNCC 260 and FNCC 343 are indeed *L. plantarum*.

3.2. Time-Course of GABA Production by L. plantarum FNCC 260

L. plantarum FNCC 260 was cultivated in MRS medium supplemented with 118 mM MSG at 37 °C. The time courses of GABA production, the pH value and the growth profile of *L. plantarum* FNCC 260 are shown in Figure 2. GABA production started when cell growth reached the stationary phase after 12 h of cultivation. A slightly higher GABA production was obtained after 48 h (450 mg/L) compared to the GABA production mentioned above in the screening experiment (352 mg/L). This was due to different MSG used in these two experiments (see Materials and Methods). The highest GABA production was 809.2 mg/L

of cultivation medium after 60 h of cultivation, at which cell growth is still in the stationary phase. This observation agrees with previous reports in the literature. The maximum GABA production of *L. brevis* L-32 was observed between 36 to 72 h of cultivation and GABA was mainly produced during the stationary growth phase [46–48]. However, GABA production decreased when the cultivation time was prolonged further. This might be due to the activity of the enzyme GABA transaminase (GABA-T), which degrades GABA. This enzyme catalyzes GABA degradation to succinic semialdehyde by using either pyruvate or α-ketoglutarate as the amino acceptors and succinic semialdehyde is irreversibly oxidized to succinate by succinic semialdehyde dehydrogenase [46,49,50]. Interestingly, we observed that the cell growth did not show a decreasing trend when the cultivation time was extended up to 108 h since GABA is utilized as a nutrient during prolonged cultivation [49]. Ko et al. (2013) reported a similar observation of cell growth of *L. brevis* FPA 3709 during GABA synthesis when GABA production decreased [49].

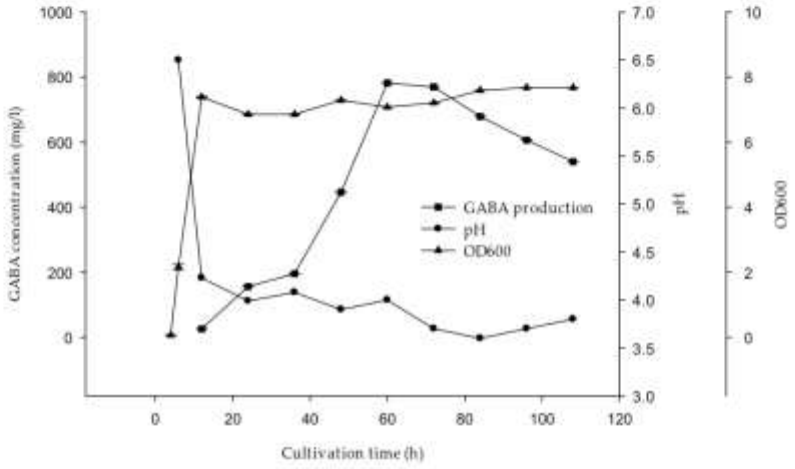


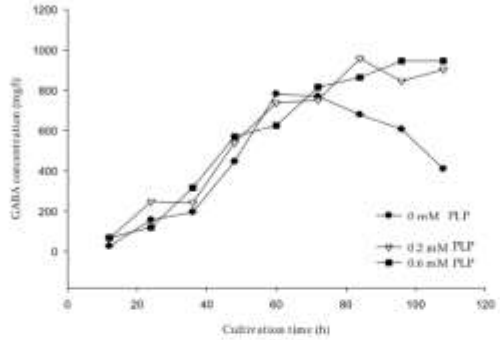
Figure 2. Time course of γ-aminobutyric acid (GABA) production, bacterial growth, and change in pH during cultivation of *L. plantarum* FNCC 260 strain in MRS broth supplemented with 118 mM MSG at 37 °C. Data shown as mean ±SD with SD less than 5%. The experiments were conducted at least in duplicates.

During GABA production, a decrease in pH of MRS medium was observed. The pH of the cultivation medium rapidly decreased from the initial pH 6.5 to pH 4.1 after 12 h of cultivation. The decrease in pH was due to lactic acid and acetic acid formation during the cultivation of the organism [50]. GABA production involves cytoplasmic decarboxylation, which results in extracellular proton consumption after the uptake of glutamate by its specific transporter [28]. This may lead to the removal of hydrogen ions and an increase of pH in the cytoplasm [51]. Apparently, we observed that the decrease in pH of the cultivation medium (to below pH 4.0) correlated with the increase in GABA production during cultivation of *L. plantarum* FNCC 260. Similarly, the maximum GABA production of *L. buchneri* was achieved when the pH of the cultivation medium decreased to pH 5.0 [24]. In this study, although GABA production started to decrease after 60 h of cultivation, which might be due to the activity of the enzyme GABA transaminase, the pH of the cultivation medium kept decreasing until 84 h of cultivation indicating that decarboxylation of glutamate still occurred.

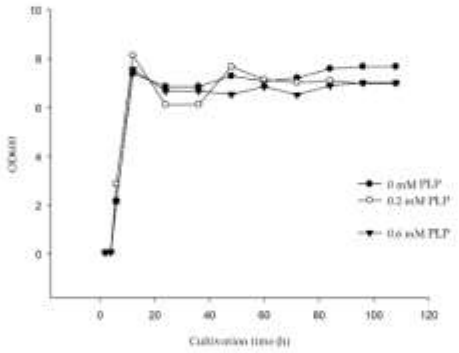
3.3. The Effect of Cofactors on GABA Production and Cell Growth

Glutamate decarboxylase is a pyridoxal 5′-phosphate (PLP) dependent enzyme. Theoretically, the addition of PLP to the medium could increase GAD activity and GABA production [2,21,52,53]. PLP and pyridoxine were added into the medium at various

concentrations. As expected, the addition of 0.2 mM and 0.6 mM PLP led to the increase in GABA production, reaching 903.0 mg/L and 945.3 mg/L after 108 h of cultivation, respectively (Figure 3a). Furthermore, GABA was still produced when cultivation time was prolonged to 108 h. In contrast, GABA production in a medium without PLP rapidly decreased after 72 h. it was clear that the strain was able to utilize PLP to produced GABA. The addition of PLP to the medium did not inhibit cell growth during the cultivations (Figure 3b). Previous studies by Komatsuzaki et al. [2] and Yang et al. [53] reported that the addition of 0.1 mM and 0.02 mM PLP significantly enhanced GABA production and GAD activity in the culture media of *L. paracasei* NFRI 7451 and *S. thermophillus* Y2, respectively.



(**a**)

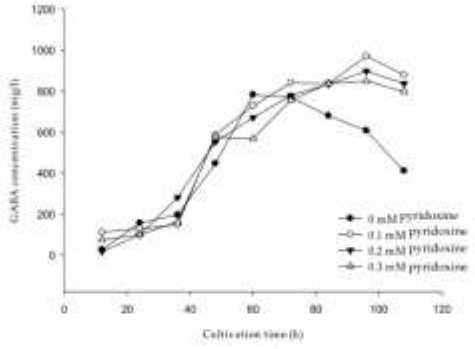


(**b**)

Figure 3. Effect of pyridoxal 5′-phosphate (PLP) on (**a**) GABA production and (**b**) cell growth during the cultivation of *L. plantarum* FNCC 260. The MRS medium were supplemented with 118 mM MSG and PLP at concentrations 0, 0.2 mM and 0.6 mM. The strain was incubated at 37 °C for 108 h. Data expressed as means ±SD with SD less than 5%. The experiments were conducted at least in duplicates.

Pyridoxine (vitamin B6) is a water-soluble vitamin that is ubiquitously found in nature. Pyridoxine can be taken by the cells at the plasma membrane and is subsequently phosphorylated to form PLP within the cytoplasm [52]. The utilization of pyridoxine could be an alternative to replace PLP since PLP is significantly more expensive with low availability. Therefore, we hypothesized that the addition of pyridoxine would improve GABA production. It is shown that the addition of 0.1 mM pyridoxine had a better enhancement on GABA production compared to higher concentrations of pyridoxine tested. The addition of 0.1 mM pyridoxine enhanced GABA production reaching 839.5 mg/L and 969.5 mg/L after 60 h and 96 h of cultivation, respectively (Figure 4a), which are higher compared to the cultivation without pyridoxine (754 mg/L and 606 mg/L, respectively). This observation

suggests that pyridoxine can be taken up by the cells and is phosphorylated to form PLP, which is essential for GAD activity. However, GABA production decreased to 878.8 mg/L after 108 h of cultivation (Figure 4a). This could be due to the degradation of pyridoxine during cultivation and hence it lost its activity as a cofactor [53]. The addition of pyridoxine did not have notable effects on cell growth during the cultivation of *L. plantarum* FNCC 260 (Figure 4b). In both cases of PLP and pyridoxine additions, we observed that the growth reached OD_{600} ~ 8 within 12 h and then maintained at OD ~ 6–7 during the entire cultivation time up to 108 h. Li et al. reported that the addition of cofactor did not improve or inhibit the cell growth of *L. brevis* NCL912 [28].



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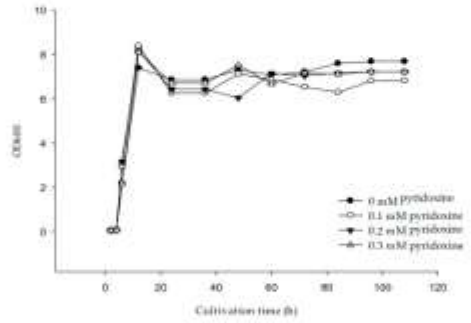


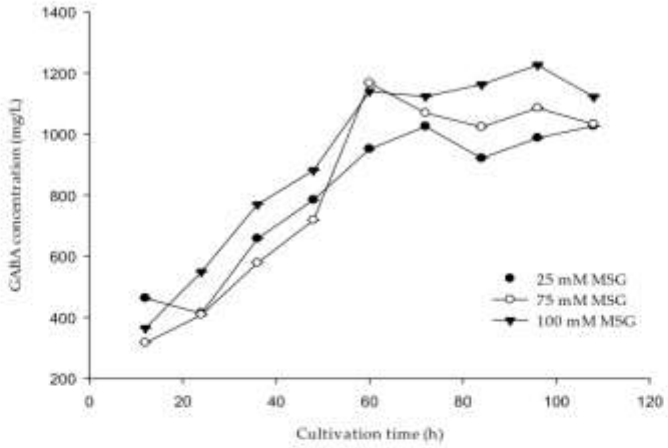
(**b**)

Figure 4. Effect of pyridoxine on (**a**) GABA production and (**b**) cell growth during the cultivation of *L. plantarum* FNCC 260. The MRS medium was supplemented with 118 mM MSG and various concentrations of pyridoxine 0, 0.1 mM, 0.2 mM, 0.3 mM. The strain was incubated at 37 °C for 108 h. Data expressed as mean ± SD with SD less than 5%. The experiments were conducted at least in duplicates.

3.4. The Effect of Monosodium Glutamate on GABA Production

The presence of MSG is a key factor in producing GABA. The optimal culture conditions for GABA production were determined by measuring the GABA content in the cultivation medium of *L. plantarum* FNCC 260 with different initial MSG concentrations in the MRS medium. As shown in Figure 5 increasing MSG concentrations increased GABA production (Figure 5a) and maintained cell viability (Figure 5b). The maximum GABA production was achieved at 1226 mg/L at 96 h in an MRS medium containing 100 mM MSG. It appears that a prolonged incubation time did not increase GABA productivity of the strain. A possible reason led to such circumstances was due to GABA catabolism,

resulting from GABA transaminase activity. The activity of GABA transaminase could decrease GABA production by converting GABA to succinic semi-aldehyde (SSA).



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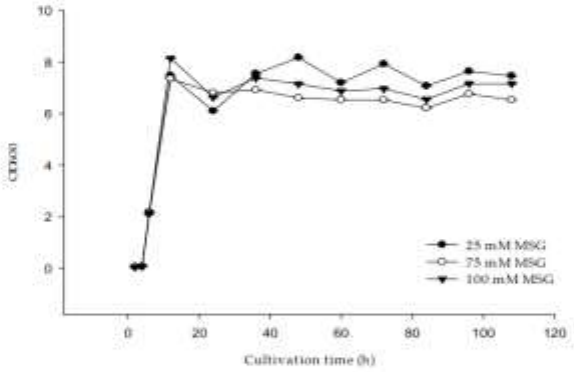


(**b**)

Figure 5. Effect of various MSG concentrations on (**a**) GABA production and (**b**) the growth of *L. plantarum* FNCC 260 cultivated in MRS medium at 37 °C. Data expressed as mean ± SD with SD less than 5%. The experiments were conducted at least in duplicates.

Similarly, high concentrations of MSG resulted in decreased GABA production of the strains *L brevis* CRL 1942, *S. thermophillus* Y2 and *L. paracasei* NFRI 7415 [2,54,55]. It was suggested that high glutamate concentrations become more toxic to some strains of LAB and suppressed the expression of *gad*B genes [56]. In this study, we observed that MSG concentration up to 100 mM did not have effects on bacterial growth (Figure 5b) indicating that GABA is consumed by the cells to maintain its viability during the cultivation period. However, as it was shown in Figure 2, when the initial concentration of MSG in cultivation medium was 118 mM, the production of GABA was significantly lower compared to the production obtained with 100 mM MSG. This suggests that the observations from previous studies [2,28,53] about the negative effects of high glutamate concentrations on bacterial growth of some LAB strains and the expression of *gad*B genes could be an explanation for a similar observation in our study.

3.5. Cloning, Expression of Glutamate Decarboxylase from L. plantarum FNCC 260 in E. coli and Purification of the Enzyme

The *gad*B gene from *L. plantarum* FNCC 260 was cloned and its complete open reading frame consists of 1410 bp, encoding 469 amino acids. The predicted molecular mass of GAD is 53. 57 kDa and the theoretical isoelectric point (pI) is 5.62 as calculated using ExPASy program (www.expasy.org). The GadB sequence from *L. plantarum* FNCC 260 shared 98% homology with the GadB from *L. futsaii* CS3 (accession number AB839950), *L. plantarum* Taj-Apis362 (accession number AHG59384) and *L. plantarum* WCFS1(accession number CCC80401.1).

The *gad*B gene was cloned into the expression vector pET-21a(+) (Novagen, Merck KGaA, Darmstadt, Germany). The resulting vector pET21Gad was subsequently transformed into *E. coli* T7 Express carrying the plasmid pGRO 7 for the enhanced expression of the chaperones GroEL/GroES (*E. coli* T7 Express GRO). *E. coli* cells were cultivated in LB medium and induced with 0.5 mM IPTG as described in Section 2. The obtained expression yield was 1.38 kU/L fermentation medium with a specific activity of 0.24 U/mg. The recombinant GAD was purified with a single-step purification using the His-trap HP column and the specific activity of the purified enzyme was 1.12 U/mg with a purification factor of 4.5. The apparent molecular mass as judged by SDS-PAGE and native PAGE was estimated to be ~51 kDa and ~140 kDa, respectively (Figure 6a,b). The size of the protein was also confirmed by LC-ESI-MS and it was determined to be 51.79 kDa (data not shown). Several bands were found in native PAGE with the largest band was ~140 kDa. The LC-ESI-MS analysis revealed that these bands on native PAGE contain components of the subunit. The first band represent the intact dimeric enzyme and the other bands with lower molecular masses result from degradation of the intact protein. It also suggested that GadB from *L. plantarum* FNCC 260 is a homodimeric enzyme. GadB from *L. plantarum* ATCC 14917 was also reported as a homodimer [56].

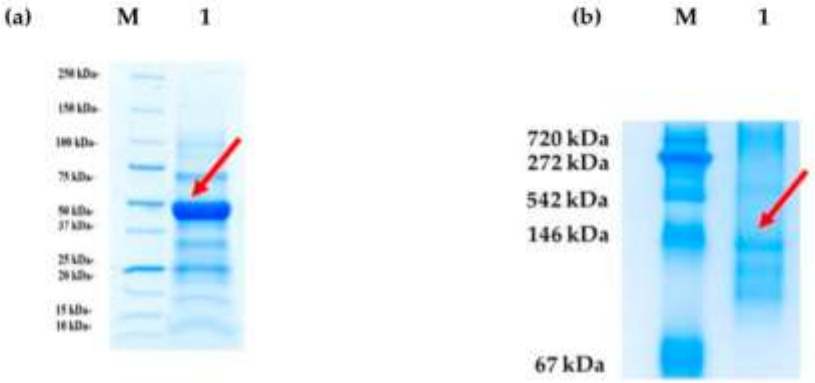


Figure 6. (**a**) SDS-PAGE analysis of purified recombinant GAD from *L. plantarum* FNCC 260 expressed in *E. coli*. Lane M; protein marker, 1; purified GAD. The arrow indicated GAD with molecular masses of approximately 53 kDa. (**b**) Native PAGE analysis of purified recombinant GAD. The molecular masses of GAD were estimated to be 140 kDa, Lane M; protein marker, 1; purified GAD.

The deduced amino acid sequence of *L. plantarum* FNCC 260 GAD contains a highly conserved catalytic domain that belongs to the PLP-dependent decarboxylase superfamily (Figure 7). A lysine residue (K280) is considered as the PLP-binding site for most bacterial GADs [51,56]. Lysine residue is also found in plant GADs since high homology between bacterial and plant GADs has been revealed [57,58]. In addition, the two residues T215 and D247 are crucial to promote decarboxylation [50]. Furthermore, the consensus sequence HVDAASGG is highly conserved in many bacterial GADs (Figure 7) and is also found in several GADs from Lactobacillus spp including *L. futsaii* CS3, *L. brevis* HYE1, *L. zymae*, and *L. paracasei* NFRI 7415 [19,34,36,51].

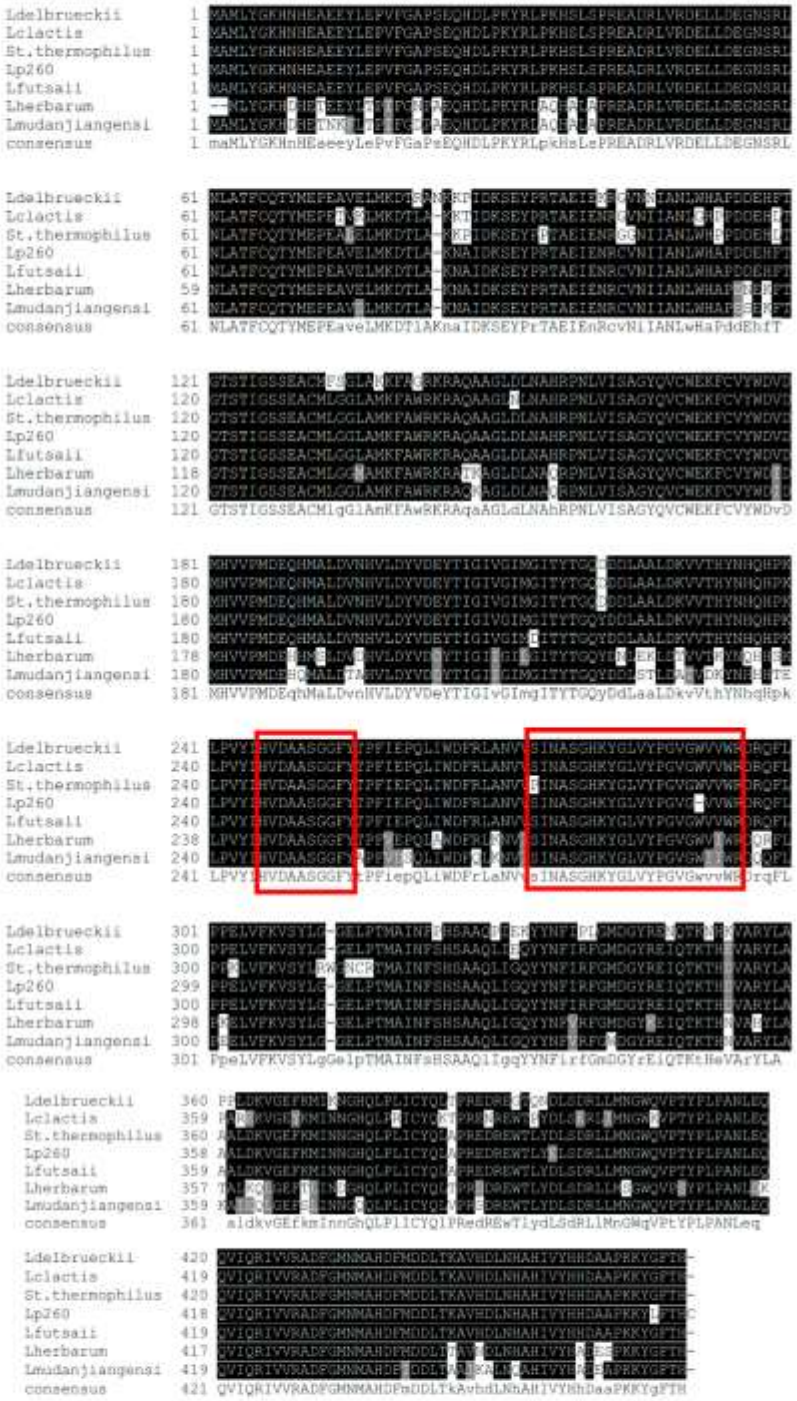


Figure 7. Alignment of amino acid sequences of GAD from *L. plantarum* FNCC 260 and six other GADs from LAB. The consensus sequence HVDAASGGF indicated by a smaller red box is highly conserved in GAD sequences. The sequence SINASGHKYGLVYPGVGWVVWR in the bigger red box is the PLP-binding domain [49]. GadB sequences shown are from *L. delbrueckii* (Ldelbrueckii), *Lactococcus lactis* (Lclactis), *S. thermophilus* (St. thermophilus), *L. plantarum* FNCC 260 (Lp260), *L. futsaii* (Lfutsaii), *L. herbarum* (Lherbarum), and *L. mudajiangensi* (Lmudanjiangensi).

3.6. GABA Synthesis by Recombinant Glutamate Decarboxylase from L. plantarum FNCC 260

For enzymatic GABA synthesis, we performed the conversion of MSG using 0.64 U/mL purified recombinant GAD in a 2-mL scale of reaction mixtures. Most GADs from *Lactobacillus* spp. have optimum activities at acidic pH values [33,36,57,58], and the recombinant GAD from *L. plantarum* FNCC 260 showed an optimum pH at pH 4.5 as expected (data not shown). MSG (100 mM) in 50 mM citrate-phosphate buffer (pH 4.5) containing 0.2 mM PLP was used as substrate and the reaction was performed at 30 °C. GABA synthesis reached its highest yield at 6450 mg/L (63 mM) within 6 h of reaction (Figure 8), and the enzyme retained 73% of its initial activity after 6 h of reaction. It was clear that the use of purified GAD was more efficient in terms of both GABA production and conversion time. Enzymatic synthesis of GABA using purified recombinant GAD from *L. plantarum* FNCC 260 showed 5 to 7-fold higher product concentrations than microbial fermentations in a significantly shorter time. This suggests that the use of purified GAD is crucial to overcome the limitations in GABA production due to GABA-degrading enzymes in the cells, slow reaction rate, and low production yield [28,53,59,60]. Furthermore, UPLC analysis was performed to confirm GABA production in both microbial fermentation and enzymatic conversion with a retention time of GABA at 8.5 min (Figure 9).

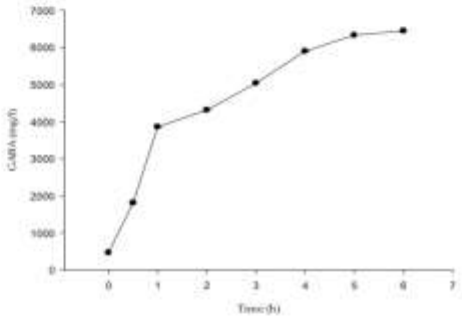
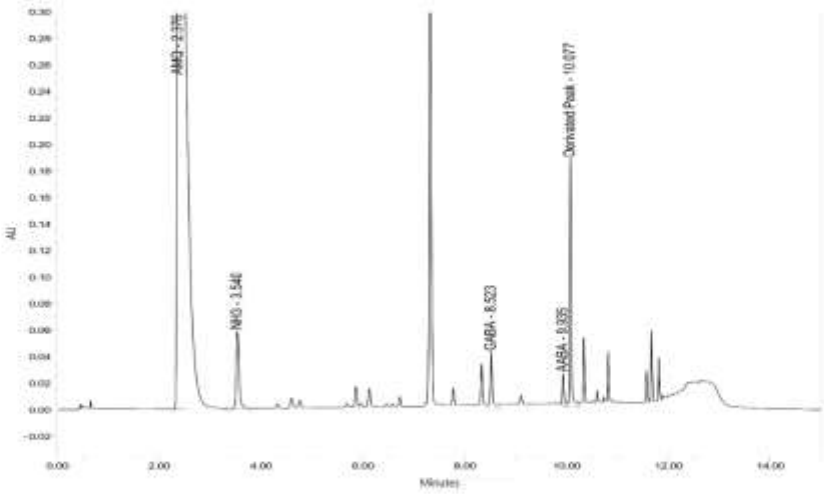
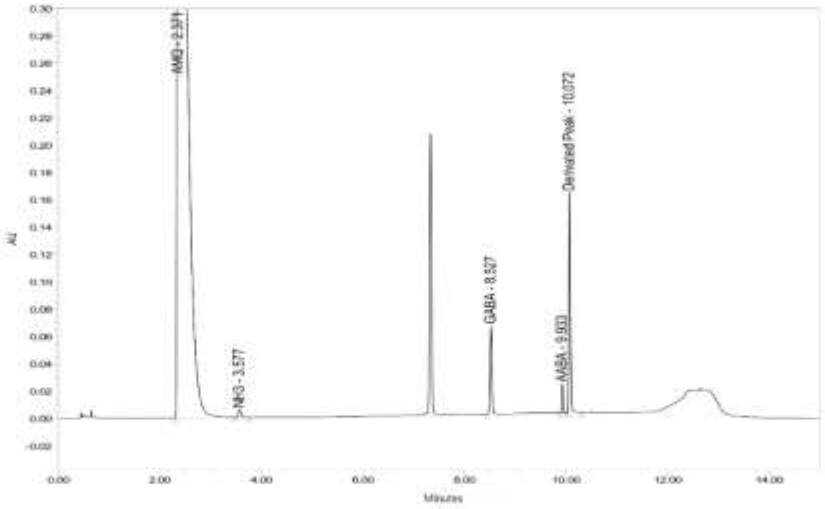


Figure 8. Enzymatic conversion of MSG to GABA using purified recombinant GAD from *L. plantarum* FNCC 260. The conversion was performed using 0.64 U/mL of GAD in 2 mL of 50 mM citrate-phosphate buffer (pH 4.5) containing 100 mM MSG and 0.2 mM PLP at 30 °C. Data expressed as mean ± SD with SD less than 5%. The experiments were conducted at least in duplicate.



(a)

Figure 9. *Cont.*



(**b**)

Figure 9. UPLC analysis of GABA in the culture medium of microbial fermentation (**a**) and in the reaction mixture of enzymatic conversion of MSG (**b**). The strain was cultivated in MRS broth containing 118 mM MSG and incubated at 37 °C for 48 h. The culture broth was centrifuged and the supernatans were collected (**a**). Enzymatic conversion was performed with 0.64 U/mL purified GAD using 100 mM MSG in 50 mM citrate-phosphate buffer (pH 4.5) containing 0.2 mM of PLP. The reactions were carried out at 30 °C with 300 rpm agitation (**b**). All samples (microbial fermentations and enzymatic conversion) were hydrolyzed and derivatized prior to UPLC analysis.

4. Conclusions

In the present study, we compared GABA synthesis between microbial fermentation of *L. plantarum* FNCC 260, which was isolated from Indonesian fermented cassava, and enzymatic conversion of glutamate using recombinant glutamate decarboxylase (GAD) from *L. plantarum* FNCC 260 expressed in *E. coli*. MSG, PLP, and pyridoxine were shown to positively affect GABA production during the cultivations of *L. plantarum* FNCC 260. Enzymatic synthesis of GABA using purified recombinant GAD from *L. plantarum* FNCC 260 showed at least 5-fold higher GABA titres than microbial fermentations in a significantly shorter time. The newly isolated GABA-producing LAB is of great interest to extend the area of applications. *L. plantarum* FNCC 260 should be considered as a potential candidate for GABA production via both fermentation and enzymatic synthesis and can be also developed as functional starter culture.

Author Contributions: Conceptualization, I.B.A.Y., E.S.R., D.H. and T.H.N.; methodology, I.B.A.Y., S.K., T.H.N. and D.H.; software, I.B.A.Y.; validation, I.B.A.Y., S.K. and T.H.N.; formal analysis, I.B.A.Y. and S.K.; investigation, I.B.A.Y., S.K. and T.H.N.; resources, I.B.A.Y.; data curation, I.B.A.Y. and S.K.; writing—original draft preparation, I.B.A.Y.; writing—review and editing, I.B.A.Y. and T.H.N., D.H., K.J.D.; visualization, I.B.A.Y.; supervision, T.H.N., D.H., K.J.D. and E.S.R.; project administration, I.B.A.Y.; funding acquisition, D.H. and T.H.N. All authors have read and agreed to the published version of the manuscript.

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Informed Consent Statement: This study did not involve any human/animal interventions.

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Conflicts of Interest: The authors declare no conflict of interest.

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Chapter 7

Biochemical characteristic of glutamate decarboxylase from *Lactobacillus plantarum* FNCC 260*

Supplementary material

7.1 Motivation of the study

Glutamate decarboxylase (GAD, EC 4.1.1.15) is the key enzyme that catalyzes the irreversible decarboxylation reaction of glutamate to GABA and CO_2. GAD is also member of the pyridoxal 5'-phosphate (PLP)-dependent decarboxylases family [126]. GAD is widely distributed in eukaryotes and prokaryotes [127]. Several GAD from *E. coli* [128], *Lactococcus lactis* [96], *Streptococcus thermophilllus*Y2 [129], *L. fermentum* YS2 [130], *Lactobacillus brevis* [21,84,131], *L. zymae* [132] and *E. avium* M5 [133] have been purified and characterized to date. Furthermore, biochemical properties of GAD are varied among different sources in terms of chemical and physical properties.

In the previous study, the *gadB* gene from newly isolated *L. plantarum* FNCC 260 was cloned, expressed in *E. coli* T7 harboring pGRO7 and subsequently, purified using Ni-immobilized metal ion affinity chromatography (IMAC) column. However, the biochemical properties of purified GAD from *L. plantarum* FNCC 260 have not yet been determined. The aim of this study was to determine the biochemical properties of GAD from *L. plantarum* FNCC 260.

The effect of pH on GAD activity was determined by incubating GAD in Britton-Robinson buffer (mixture of 20 mM each of acetic acid, phosphoric acid, and boric acid, adjusted with 1 M NaOH to desired pH values) for 1 h at 37^{o}C and then measuring the relative activity of GAD by colorimetric method as previously described in Chapter 6. By the same method, the effect of temperature (20-90^{o}C) was determined after 1 h incubation at pH 4.5. The effect of PLP concentration ranging from 0 – 1.8 mM and various chemicals (2 mM) were determined. The pH and temperature stability of GAD was also evaluated. The pH stability measurements were performed by incubating GAD in Britton-Robinson buffer at various pH values (pH 3-7) at 30^{o}C

for 72 h. At certain time intervals, samples were withdrawn and the residual activity of GAD was measured. The thermostability of GAD was determined by incubating GAD in citrate-phosphate buffer (pH 5.0) at various temperatures (4-60^{o}C), and the residual activity of GAD was measured.

7.2 Results and discussion

7.2.1 Characterization of recombinant GAD

The optimum pH of GAD from *L. plantarum* FNCC 260 was 4.5 (Figure 2), which is in agreement with other GAD enzymes from other microorganisms. However, the enzyme is more stable at pH 4.0 and exhibit 70% of the maximal activity after 72 h of incubation. Most of bacterial GAD are active at pH 4 – 5.5; for example, *L. brevis* IFO 12005 [131], pH 4-4.5, *L. lactis*, pH 4.7 [96] , *S. thermophilus* Y2, pH 4,2 [129] , *L. paracasei* NFRI 7415, pH 5.02 [134], *L. brevis* CGMCC 1306, pH 4.8 and pH 5.2 for *L. brevis* 877G [84,135]. GAD plays a crucial role in maintaining the intracellular pH under acidic condition and its role is essential for LAB to maintained its viability.

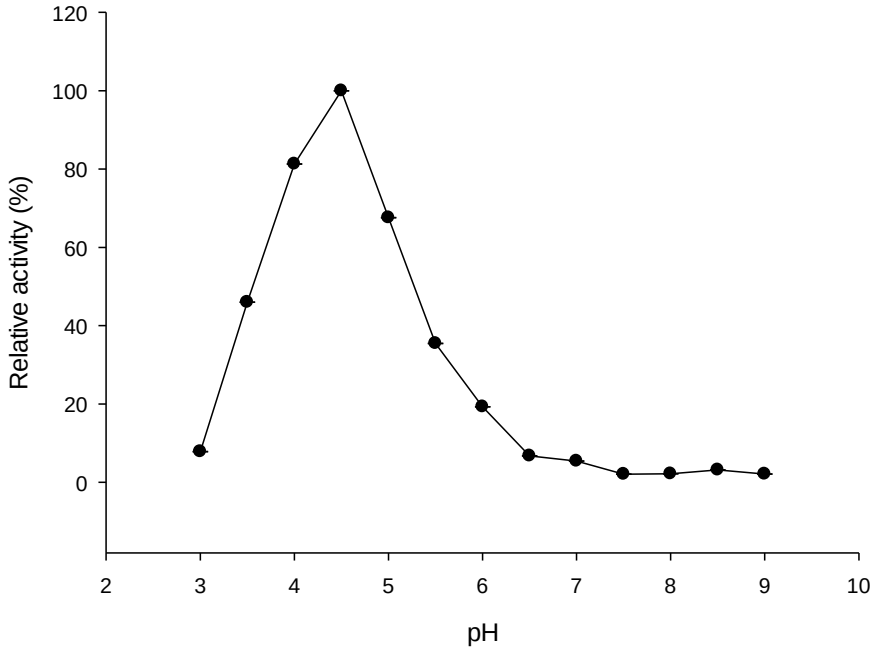

Figure 2. Effect of pH on GAD activity. GAD was incubated in Britton-Robinson buffer at various pH values for 1 h at 37ºC. The experiments were performed at least in duplicates. Data are expressed as the mean value with SD less than 5%.

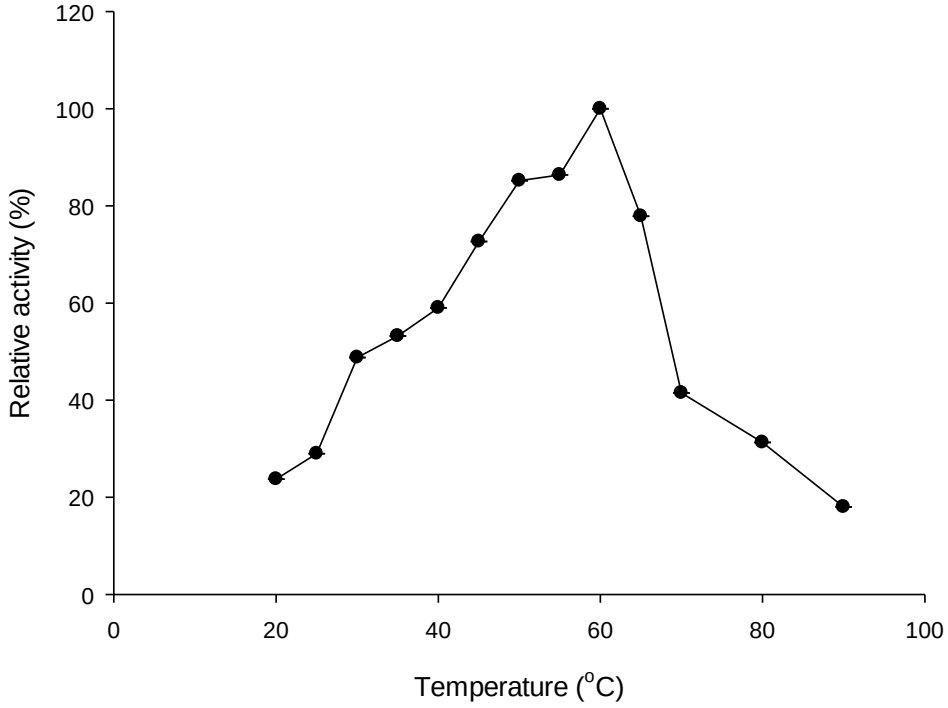

Figure 3. Effect of temperature on GAD activity. GAD was incubated at various temperatures in citrate-phosphate buffer (pH 4.5) for 1 h. The experiments were performed at least in duplicates. Data are expressed as the mean value and the SD was always less than 5%.

Table 2. Stability of GAD at various temperatures and pH. GAD was incubated in Britton-Robinson buffer (pH stability) and citrate-phosphate buffer pH 4.5 (temperature stability) for 72 h at 30^{o}C. The experiments were performed at least in duplicates. Data expressed as SD ± Mean with SD less than 5%.

Temperature (^{o}C)	**Residual activity (%)**	**pH**	**Residual activity (%)**
4	94.73	3	52.65
20	42.2	4	70.85
30	41.95	5	48.45
40	8.61	5.5	48.2
50	1.2	6	47.31
60	1.2	6.5	43.82
		7	42.51

The optimum temperature of GAD activity was 60^{o}C, higher than that of *l. brevis* 877G and *L. plantarum* ATCC 14917 (45 and 40^{o}C). The enzyme exhibit 94% of residual activity when store at 4^{o}C for 3 days. In contrast, the activity of the enzyme was decreasing at 20-60^{o}C (Table 2) after 72 h of incubation. Recombinant GAD from *L. plantarum* FNCC 260 depends on PLP for its activity (Figure 4). As it was shown in Figure 4, the activity of the recombinant GAD was increased rapidly with the addition of PLP at increasing concentrations ranging from 0.1-0.6 mM. The highest activity was observed at concentration of 0.6 mM PLP. However, PLP concentrations above 0.6 mM did not increase GAD activity [86,94,136]. Similarly, the addition of PLP above 0.6 mM did not affect GAD activity of *L. zymae* [132], *E. avium* M5 [133] and *L. sakei* A156 [137].

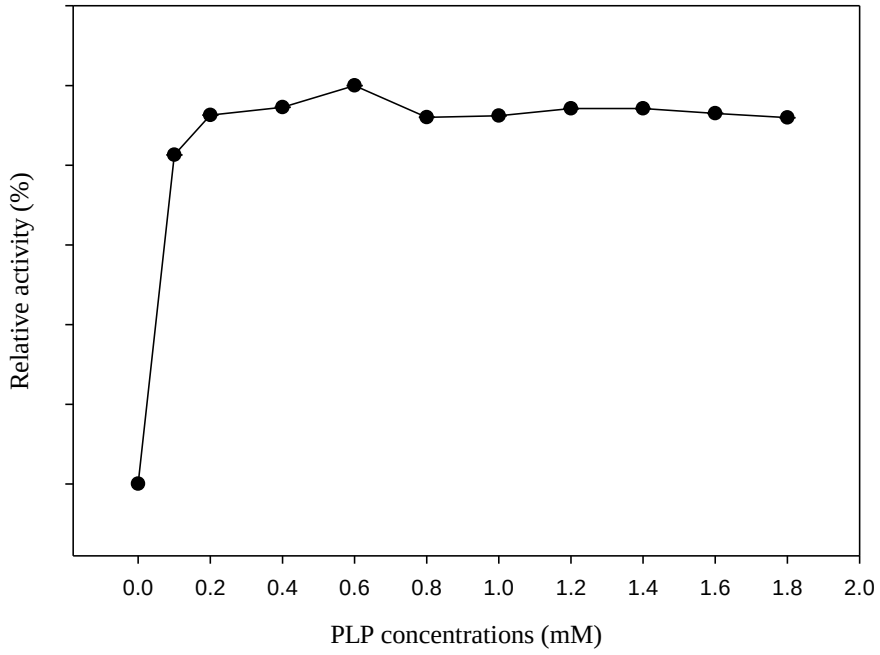

Figure 4. effect of PLP on GAD activity. GAD was incubated in citrate-phosphate buffer (pH 4.5) supplemented with various concentrations of PLP for 1 h at 37^{o}C. The experiments were performed at least in duplicates. Data are expressed as the mean value and the SD was always less than 5%.

The effect of various chemicals (2 mM) on GAD activity was determined. The highest activity was observed by adding $CaCl_2$ (150%) and $MgCl_2$ addition did not affect the activity (100%), whereas, it was decreased by $FeCl_3$ (42%), $CuSO_4$ (51%), $AgNO_3$ (51%) and $MnCl_2$ (54%) respectively. The results were similar accordance to other reports. In the presence of 2 mM $CaCl_2$, the activity of GAD increased slightly in *L. paracasei* NFRI 7415 (114%) [134], *L. sakei* A156 (113%) [137] and *L. zymae* GU240 (121%) [132].

In summary, the *gad*B gene from newly isolated *L. plantarum* FNCC 260 was cloned and the corresponding enzyme investigated. The pH and temperature optimum were 4.5 and 60^{o}C

respectively. The recombinant GAD was more stable at pH 4.0 and 4ºC. The activity of GAD was increased by the addition of 0.6 mM PLP. The presence of 2 mM $CaCl_2$ rapidly increased GAD activity and its activity was decreased by the presence of $FeCl_3$ (28%), $CuSO_4$ (34.41%), $AgNO_3$ (34.01%) and $MnCl_2$ (36%) respectively. The results suggesting that *L. plantarum* FNCC 260 and its GAD have the potential for use in the food industry when GABA-enriched foods are attempted to be produced. although the recombinant enzyme can be produced sufficiently in *E. coli*, food-grade microorganisms such as LAB is preferred if GAD is introduced in the food industry. Furthermore, the improvement of GAD activity by protein engineering techniques or metabolic engineering is needed to obtain high performance of the recombinant enzyme.

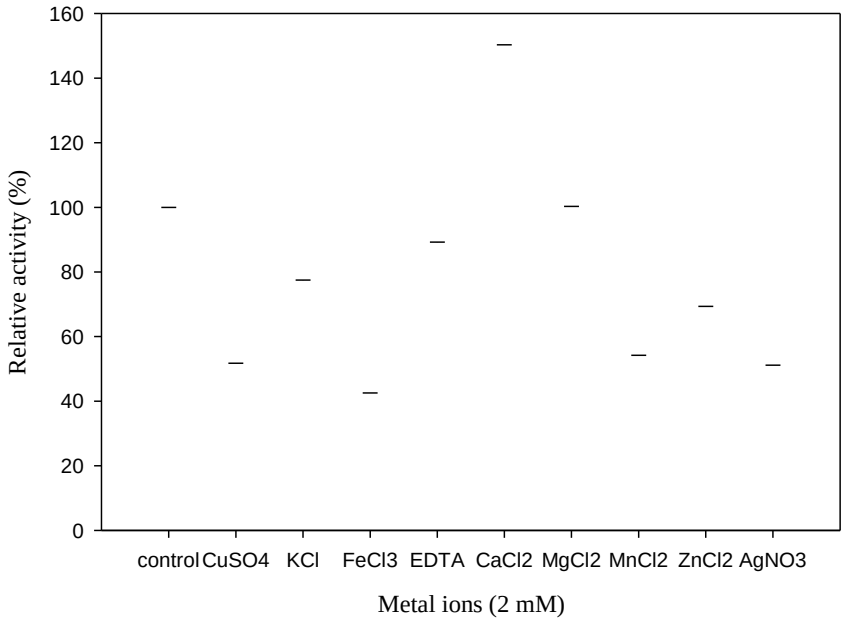

Figure 5. The effect of various metal ions (2 mM) on GAD activity. GAD was incubated in citrate-phosphate buffer (pH 4.5) supplemented with various metal ions (2 mM) for 1 h at 37ºC. The experiments were performed at least in duplicates. Data are expressed as the mean value and the SD was always less than 5%.

Conclusion and outlook

The first part of this work was to provide an overview of GAD from LAB. 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. The occurrence of *gadA* and *gadB* genes were also revealed to understand their role in GABA synthesis. The review also provided structural information of GAD from *L. brevis*, since the structural information is only limited to GAD from *L. brevis* and hence, structural studies of GAD from other GABA-producing LAB is needed to understand their catalytic and structural properties in more detail.

In the second part of this work, GABA-producing LAB have been isolated from various Indonesian fermented foods namely fermented cassava, fermented fish with rice, fermented soybeans, salted cabbage, salted fruits, chicken sausage and beef sausage. All isolates were screened and identified using molecular and proteomic methods. It was found that *L. plantarum* species are shown to be GABA-producing LAB in this respect. Moreover, *L. plantarum* are predominant species in Indonesian fermented foods. Hence, it is of great interest to develop *L. plantarum* as starter cultures in GABA-enriched traditional fermented foods. Since, most of Indonesian fermented foods are still rely on indigenous cultures. In addition, the use of LAB may overcome the limitation of GABA synthesis using chemical mean. As the use of LAB as a more sustainable route for postbiotic production is gaining interest among the scientific community and industrial sector.

The third part of this work, *gad*B genes from *L. plantarum* FNCC 260 were cloned and expressed in *E. coli* T7 harboring pGRO7. Subsequently, GadB were purified for further GABA synthesis and characterization. The obtained expression yield was 1.38 kU/L fermentation medium with specific activity of purified enzyme was 1.12 U/mg. In this part, microbial production and enzymatic synthesis of GABA were performed and compared. It was clear that GABA synthesis using purified GAD was 5 – 7 - fold higher than that of microbial fermentation using *L. plantarum* FNCC 260. The results suggesting that purified GAD may help to increase the yield and reduce the production time. In this part, purified GAD was also characterized. pH and temperature optimum of GAD were 4.5 and 60^{o}C respectively. Furthermore, GAD activity was enhanced by the addition of 0.6 mM PLP and 2 mM $CaCl_2$, and unfortunately the recombinant GAD showed poor thermostability at 20^{o}C and 30^{o}C respectively.

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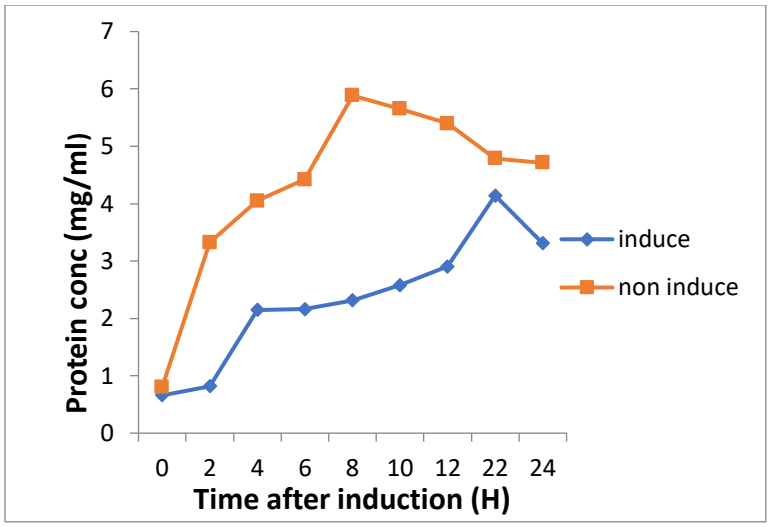
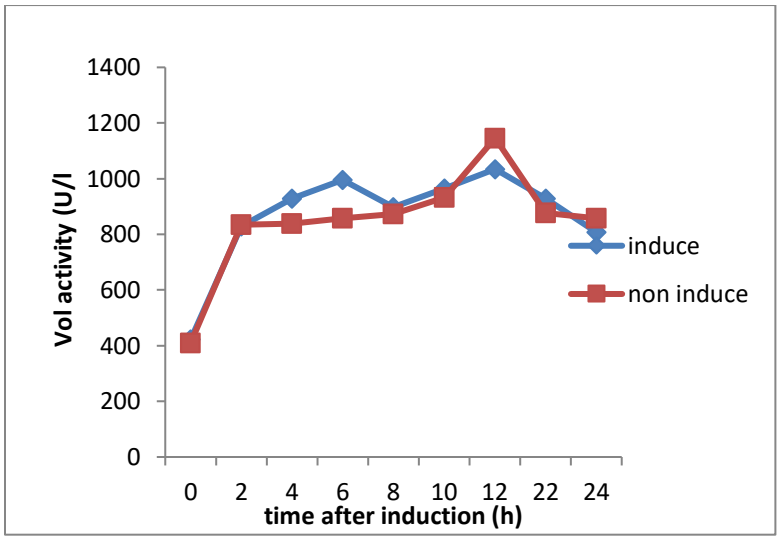
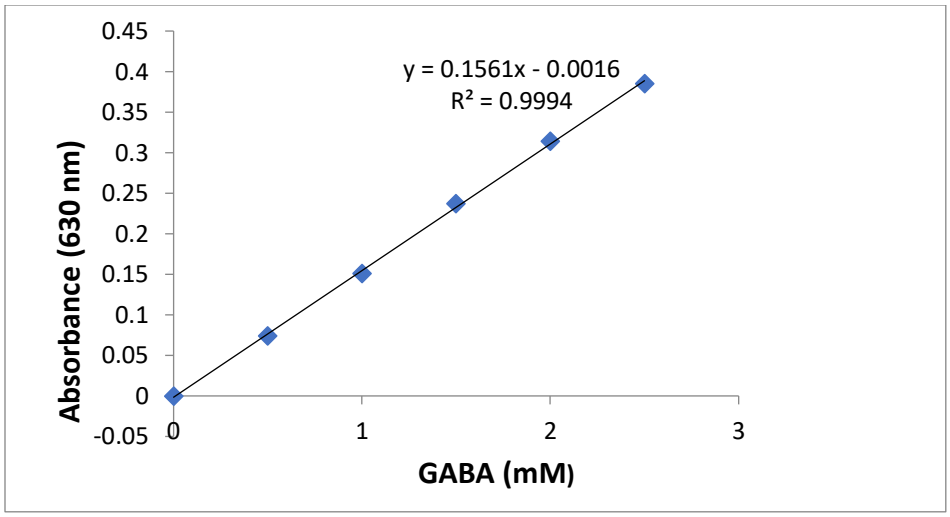
Appendix



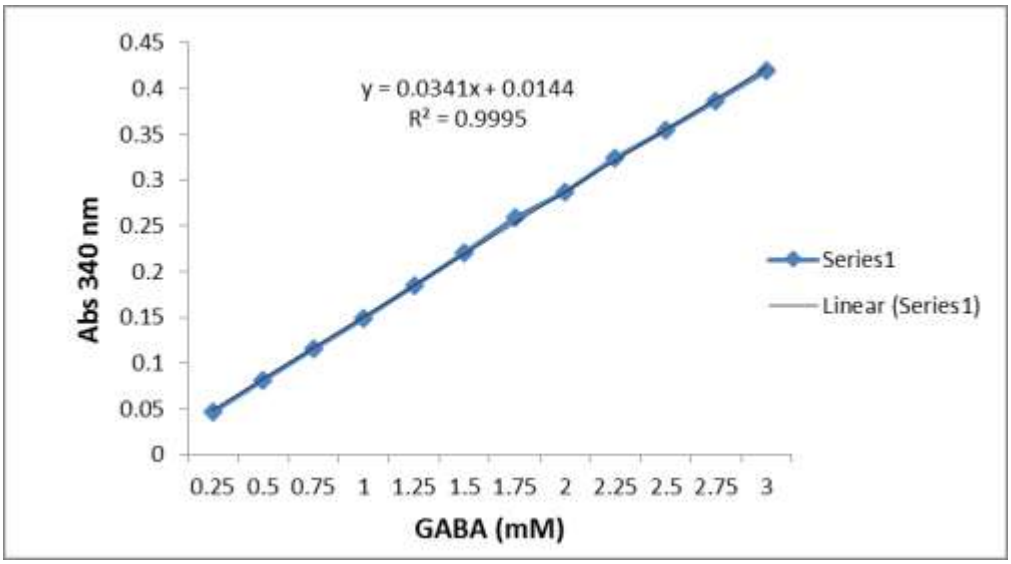
Appendix 1. Protein concentration of induce and non-induce



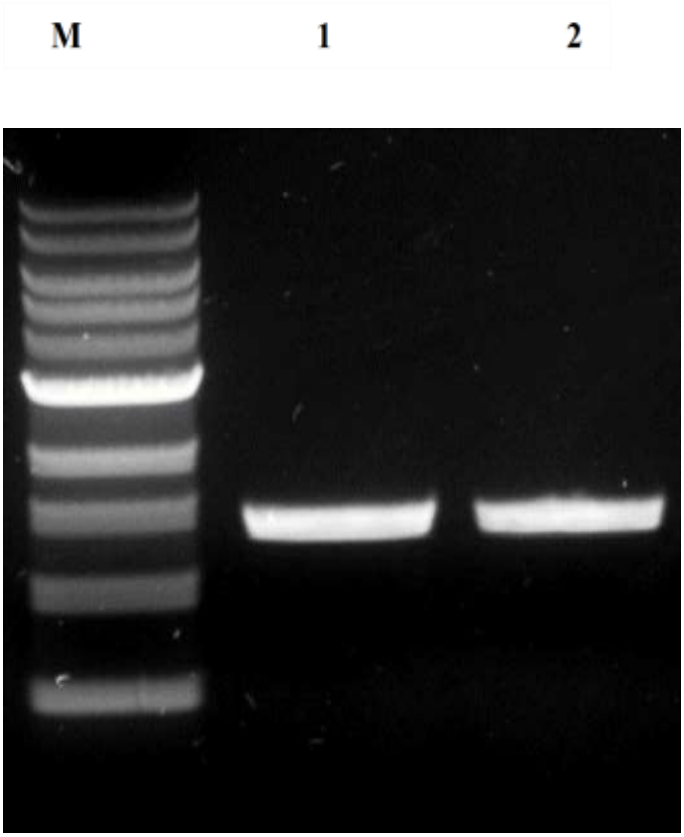
Appendix 2. GAD crude activity after induction



Appendix 3. Standard curve of GAD assay



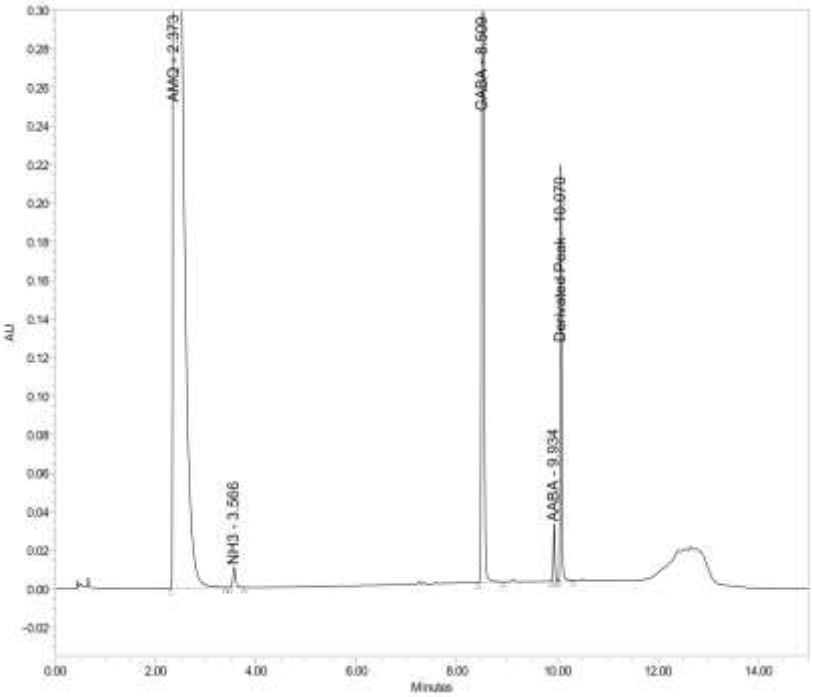
Appendix 4. GABase standard curve.



Appendix 5. Amplified *gad*B gene from *Lb. plantarum* FNCC 260 and FNCC 343. The amplified gene consist of 1410 bp. M; marker ladder is 2kb, 1; FNCC 260, 2; FNCC 343.

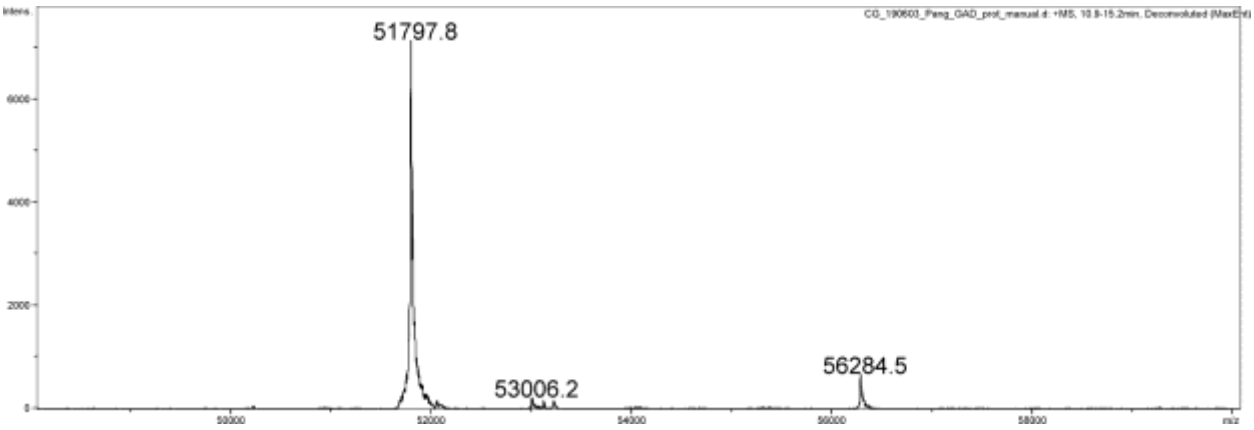
Strains	**API**	**Maldi-Tof**	**16S RNA**
245	*Lb. murinus*	*Lb. fermentum*	*Lb. fermentum*
282	*Lb. confusa*	*Weissella confusa*	*Weissela confusa*
257	*Lb. delbrueckii*	*Pediococcus pentosaceus*	*Pediococcus pentosaceus*
253	*Lb. delbrueckii*	*Lb. plantarum*	*Lb. plantarum*
235	*Lb. plantarum*	*Lb. fermentum*	*Lb. fermentum*
343	*Lb. casei*	*Lb. plantarum*	*Lb. plantarum*
322	*Lb. fermentum*	*Weissella confusa*	*Weissella confusa*
242	*Lb. plantarum*	*Lb. plantarum*	*Lb. plantarum*
246	*Lb. sake*	*Lb. plantarum*	*Lb. plantarum*
260	*Lb. sake*	*Lb. plantarum*	*Lb. plantarum*
283	*Lb. sake*	*Lb. pentosus*	*Lb. herbarum*
344	*Lb. plantarum*	*Lb. pentosus*	*Lb. herbarum*

Appendix 6. Identification of LAB from fermented foods



	Peak Name	RT	Area	% Area	Height
1	AMQ	2.373	7312074.75	75.57	692077
2	NH3	3.566	40016.96	0.41	10474
3	L-Histidine	4.375			
4	L-Serine	5.736			
5	L-Arginine	5.913			
6	Glycine	6.171			
7	L-Aspartic Acid	6.764			
8	L-Glutamic Acid	7.369			
9	L-Threonine	7.805			
10	L-Alanine	8.363			
11	GABA	8.509	1811371.50	18.72	669911
12	L-Proline	9.141			
13	AABA	9.934	59146.48	0.61	29732
14	Derivated Peak	10.070	452825.23	4.68	215752
15	L-Cystine	10.268			
16	L-Lysine	10.347			
17	L-Tyrosine	10.610			
18	L-Methionine	10.745			
19	L-Valine	10.828			
20	L-Isoleucine	11.589			
21	L-Leucine	11.687			
22	L-Phenylalanine	11.825			
Sum			9675434.93		

Appendix 7. UPLC analysis of GABA standard.



Appendix 8. Protein analysis of GAD using LC-ESI-MS system. One major variant with 51797.8 Da could be identified. Additionally a protein with 56284.5 Da was detected