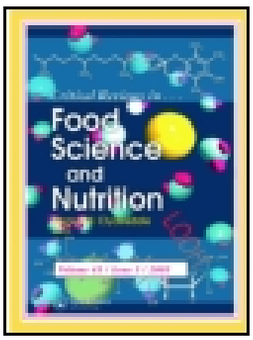

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Review

High γ-aminobutyric acid production from lactic acid bacteria: emphasis on *Lactobacillus brevis* as a functional dairy starter

Running title: GABA producer and GABA-rich fermented milk

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ABSTRACT

γ-Aminobutyric acid (GABA) and GABA-rich foods have shown anti-hypertensive and anti-depressant activities as the major functions in humans and animals. Hence, high GABA-producing lactic acid bacteria (LAB) could be used as functional starters for manufacturing novel fermented dairy foods. Glutamic acid decarboxylases (GADs) from LAB are highly conserved at the species level based on the phylogenetic tree of GADs from LAB. Moreover, two functionally distinct GADs and one intact *gad* operon were observed in all the completely sequenced

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Lactobacillus brevis strains suggesting its common capability to synthesize GABA. Difficulties and strategies for the manufacture of GABA-rich fermented dairy foods have been discussed and proposed, respectively. In addition, a genetic survey on the sequenced LAB strains demonstrated the absence of cell envelope proteinases in the majority of LAB including *Lb. brevis*, which diminishes their cell viabilities in milk environments due to their non-proteolytic nature. Thus, several strategies have been proposed to overcome the non-proteolytic nature of *Lb. brevis* in order to produce GABA-rich dairy foods.

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Keywords: γ-aminobutyric acid; *Lactobacillus brevis*; glutamic acid decarboxylase; cell envelope proteinase; dairy fermentation; dairy starter culture

INTRODUCTION

Lactic acid bacteria (LAB) are organisms that produce lactic acid as the dominant end product of carbohydrate fermentation and have been extensively used for food fermentation (Leroy et al., 2004). These include the core genus of *Lactobacillus*, *Leuconostoc*, *Pediococcus*, *Lactococcus* and *Streptococcus*, as well as the peripheral *Enterococcus*, *Oenococcus* and *Weisella* (Stiles et al., 1997). In LAB, catabolism of carbohydrates is via various pathways such as Embden-Meyerhof-Parnas (EMP) pathway, Leloir pathway and pentose phosphate pathway for producing lactic acid as the major end metabolite (Kandler, 1983). Lactic acid generated from above pathways affects the intracellular metabolic activities via altering the activities of enzymes

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under such acidic condition in bacterial cytoplasm. Also, exogenous proton enters into the bacterial cytoplasm if the condition in the environments is extremely acidic. Hence, in order to maintain the viability and cellular activities against the acidic condition, LAB have developed several acid resistance including the most common F_0F_1-ATPase system and cation/proton antiporters/symporters, and also including specific urease system, amino acid decarboxylase system and amino acid deiminase/deaminase system (Hutkins et al., 1993). Among them, glutamic acid decarboxylase (GAD) system in LAB such as *Lb. brevis*, has been documented recently, and serves as a fitness determinant in LAB for acid resistance (De Biase et al., 2012; Feehily et al., 2013; Li et al., 2010a; Teixeira et al., 2014). γ-Aminobutyric acid (GABA) is the end product of the decarboxylation of glutamic acid in LAB. It has shown several important physiological activities in humans and animals (Foster et al., 2006). Moreover, GABA or GABA-rich products have been successfully commercialized as food additives or functional food supplements, and some high GABA-producing LAB strains have been used to produce functional foods containing GABA.

In this review, the biofunctionalities of GABA and GABA-rich foods, high GABA-producing LAB including *Lb. brevis*, and the activities and phylogeny of their glutamic acid decarboxylases (GADs) have been discussed. However, the absence of genes encoding cell envelope proteinases (hence non-proteolytic in nature) has been found in the genomes of most LAB strains including *Lb. brevis*, these LAB strains may not be able to survive and even ferment milk. Hence, several strategies against this shortage have been proposed. Difficulties and strategies for manufacturing GABA-rich fermented dairy foods have also been discussed and proposed, respectively.

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BIOFUNCTIONALITIES OF GABA AND GABA-RICH FOODS

GABA is widely distributed in the animal and plant products. In animals, GABA is the major inhibitory neurotransmitter in central nervous system (CNS) (McCormick, 1989), while it also has multiple functions such as regulating cytosolic pH, protecting against oxidative stress, acting as an osmoregulator and a signalling molecule in plants (Bouche et al., 2004). Scientific and medicinal evidences suggest that GABA cannot cross blood-brain barrier (BBB) because of the absence of specific receptors in the human BBB (Kuriyama et al., 1971). However, certain pro-drugs of GABA after structural modifications, such as nicotinoyl-GABA, are able to permeate the BBB and thus exhibit its pharmacological effects in human (Matsuyama et al., 1984).

Despite the absence of certain receptors in BBB for binding GABA molecule, GABA or GABA-rich foods are still able to deliver benefits to the host after oral administration as indicated in Table 1. A pioneering study conducted in 2003 reported that daily intake of fermented milk containing 10-12 mg/100 mL of GABA could significantly lower blood pressure in patients with mild hypertension within 2 weeks (Inoue et al., 2003). In general, anti-hypertensive and anti-depressant activities are the major functions of GABA or GABA-rich foods. However, mechanisms of these activities are still unknown due to fewer studies on the pathway for GABA absorption. The proposed mechanism is that GABA may reach certain areas of brain such as periventricular nucleus with ineffective or reduced BBB function, thus increasing the concentration of GABA in GABAergic synapse of the neurons (Muller et al., 1999). Moreover, there is very limited information on the effective dosage of GABA for

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exhibiting anti-hypertensive and anti-depressant activities. Further studies are necessary to understand these mechanisms.

DIVERSITY OF GABA-PRODUCING LACTIC ACID BACTERIA – GENETIC SURVEY ON GLUTAMIC ACID DECARBOXYLASE AND EXPERIMENTAL FERMENTATION

Although GABA is widely found in the natural animal and plant based products, its content is very low, hence requiring enrichment process. As a result, products with low GABA level are not sufficient to deliver its benefits to human host. Currently, chemically synthesized GABA is prohibited as food additive, thus GABA produced by food-grade microorganisms, especially LAB, will be of great importance for manufacturing food-grade GABA or GABA-rich foods via various fermentations where food-grade substances are used as fermentative substrates.

Although there are many microorganisms that are capable of producing GABA, the main focus of this review is GABA production by LAB due to their food-grade nature and generally regarded as safe (GRAS) status. Thus, glutamic acid decarboxylase (GAD) in LAB has been searched in NCBI protein database, the phylogenetic tree of LAB GADs has been constructed, and are shown in Fig. 1. In general, the amino acid sequences of GADs are highly conserved at the species level (Fig. 1A). As indicated in Fig. 1A, it was found that the genes encoding GADs were mainly distributed in *Lactobacillus brevis*, *Lb. plantarum*, *Lb. fermentum*, *Lb. reuteri*, *Strrptococcus thermophilus*, *Lactococcus lactis* subsp. *cremoris*, *Lc. lactis* subsp. *lactis* and some *Bifidobacterium* species. This genetic evidence indicates that strains in above species with the GAD gene in them may be able to synthesize GABA. Some GABA-producing *Str. thermophilus* and *Lc. lactis* strains may be of great interest to dairy and starter culture industries, which may

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generate GABA-rich milk products if these strains were involved in the fermentation process. In particular, it was observed that the presence of GAD-encoding genes in both subspecies of *Lc. lactis* (Fig. 1A) did not support the previous finding that the ability of producing GABA could be able to distinguish *Lc. lactis* subsp. *lactis* from *Lc. lactis* subsp. *cremoris* (Nomura et al., 1999a).

Notably, GAD-encoding gene in various microorganisms can be detected by using polymerase chain reaction (PCR) method, which may be able to indicate their capacity to produce GABA at genetic level. However, in this review, we only focused on the high GABA producers in which their GABA yield has been confirmed. As shown in Table 2, several high GABA-producing LAB strains have been isolated from various environments in the last decade. It was found that acid-based fermented foods such as Korean kimchi and pickled vegetables could be the habitat of high GABA producers. Those high GABA producers could eliminate intracellular protons during the decarboxylation of glutamate by GAD to maintain the intracellular pH homeostasis under acidic conditions (Hutkins et al., 1993). Since the acid fermentation is a natural process, acid-based fermented foods could be an important source for the screening of high GABA producer.

The GABA yield in Table 2 is not comparable because these isolates have been cultivated in respective media under certain incubation conditions. However, based on those author's interpretations, these isolates were claimed as being high GABA producers because they produced more GABA as compared to other LAB strains. In addition, it has been found that most of the high GABA producers belong to *Lb. brevis* and *Lb. plantarum* (Table 2). Importantly, several important species such as *Lc. lactis*, *Str. thermophilus* and *Lb. bulgaricus* isolated from

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milk environments also exhibit abilities to produce GABA. This could be used to generate GABA-rich fermented milk products. However, to our knowledge, these dairy isolates produced less GABA than those of *Lb. brevis* and *Lb. plantarum*. Thus, use of *Lb. brevis* as functional starter for dairy fermentation to manufacture GABA-rich cultured dairy foods such as cheese and yogurt could be promising.

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METHODS FOR THE MEASUREMENT OF GABA AND FOR THE SCREENING OF GABA PRODUCER

A rapid and accurate analytical method is critical for differentiating the level of GABA content in foods and for screening GABA producers. Currently, chromatography-based techniques such as amino acid analyzer (AAA), liquid chromatography (LC), gas chromatography (GC) and thin layer chromatography (TLC) are still the first choice for determining GABA content in various food samples (Li et al., 2010a). AAA and TLC techniques do not require the derivatization process for GABA, but TLC method does not provide enough accuracy for quantifying GABA; LC method normally requires pre-column derivatization with dansyl chloride by an addition of chromophore to GABA that could be detected by fluorescence spectroscopy (Wu et al., 2015b); For GC method, pre-column derivatization of GABA is usually achieved with norvaline to form a volatile compound, which could be separated and detected by GC equipped with a flame ionization detector (FID) or mass spectrometry (MS) (Kagan et al., 2008). Above methods are time-consuming due to tedious sample preparation processes according to analytical protocols; this requires high work-load for screening GABA-producing microorganisms, and is not economical nor rapid. Hence, a pre-screening method is necessary for

identifying GABA producers prior to determining their capability to produce GABA by chromatography-based methods. Many researchers used TLC as a pre-screening method, but this requires a number of plates and a large volume of solvents (Li et al., 2010a). Currently, pre-screening methods are normally developed based on the decarboxylation of glutamate into GABA by glutamate decarboxylase; this reaction eliminates a proton and also produces carbon dioxide. Thus, glutamate decarboxylase-based microtiter plate assay (Tsukatani et al., 2005), pH indicator method (Yang et al., 2006) and gas release-based assays (Wu et al., 2015b) are suitable for high-throughput pre-screening of GABA producers with remarkable improvements in testing time and economic practice.

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HIGH GABA-PRODUCING LACTOBACILLUS BREVIS

As shown in Table 2, the high GABA production from *Lb. brevis* has been confirmed by several individual studies on various *Lb. brevis* strains. In addition to the experimental data, we conducted a survey in regards to the presence or absence of *gad* operons in the sequenced *Lb. brevis* strains at the genomic level. Till 1st November 2015, a total of 14 complete or incomplete (at contigs or scaffolds level) genome sequences of *Lactobacillus brevis* isolated from various environments have been released in NCBI database; among them, 13 strains have the intact *gad* operon in their genomes except for *Lb. brevis* subsp. *gravesensis* ATCC 27305. It appears that *Lb. brevis* appears to be a common cell factory to produce GABA. Furthermore, we have demonstrated that the GAD system is more efficient to eliminate protons than both agmatine deiminase (AgDI) system and arginine deiminase (ADI) system in *Lb. brevis* during late stationary growth phase; this is evidenced by the content of end product of above systems

(GABA from GAD system, putrescine from AgDI system and ornithine from ADI system) in *Lb. brevis* when incubated in de Man, Rogosa and Sharpe (MRS) broth at 37℃ (unpublished data). This implies that GAD system in *Lb. brevis* is an important and efficient system under acidic conditions in *Lb. brevis* thus producing high content of GABA in the medium.

Moreover, two GAD-encoding genes – *gadA* (~ 479 aa) and *gadB* (~ 468 aa) encoding two distinct GADs were found in *Lb. brevis* (Fig. 1A). Attentions should be paid to the names of two GAD-encoding genes in *Lb. brevis*. Some studies used the same name of *gadB* for both two GAD-encoding genes in *Lb. brevis* if both genes existed in their genomes. This is confusing to differentiate these two GAD-encoding genes in the same organism. In this review, the classification of GADs from *Lb. brevis* is based on one previous comparative study where single but a different GAD gene (*gadA*) in the *intact* operon has been amplified from high GABA-producing *Lb. brevis* NCL912 (Li et al., 2013). In general, the *gadA*-encoding GAD from *Lb. brevis* shows a distinct relationship with other GADs found in *Lb. plantarum*, *Lc. lactis*, *Lb. reuteri* and *Lb. fermentum* (Fig. 1A), which suggests the occurrence of independent evolution in *Lb. brevis*. Some high GABA producers isolated have been identified as *Lb. plantarum* (Table 2) and the genetic distance of their GADs is close to the *gadB*-encoding GAD, but not *gadA*-encoding GAD from *Lb. brevis* (Fig. 1A). Moreover, it was observed that only one GAD gene (*gadA* or *gadB*) was present in the *gad* operon in *Lb. brevis*, whereas another GAD gene in *Lb. brevis* (*gadB*) is far away from the *gad* operon in their complete genomes (Fig. 1B). Based on the information from the phylogenetic tree, it suggests that *Lb. brevis* may integrate *gadB* from other bacteria into its genome via gain-of-function theory, e.g. horizontal gene transfer (HGT), but not

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due to the gene duplication theory because the translated amino acids sequences of *gadA* and *gadB* do not have high score in identity (Fig. 1C).

Alignment of the amino acids sequences of *gadA*- and *gadB*-encoding GADs from two representative GABA-producing *Lb. brevis* strains (IFO 12005 and 877G) is shown Fig. 1C. It was found that *gadA* (*Lb. brevis* IFO 12005) and *gadB* (*Lb. brevis* 877G) show only ~ 50% identity in amino acids sequences, however, the PLP-binding domain where the active site residues (D, H & K; Fig. 1C) of both GADs are highly conserved. This suggests that both GADs from *Lb. brevis* may show the core function.

ENZYMATIC ACTIVITIES OF LAB GLUTAMIC ACID DECARBOXYLASES

Several important studies have shown the activities of hetero-expressed GADs from several species as exhibited in Table 3. It was observed that these GADs have different optimal conditions and activities. In general, the pH between 4.0 ~ 5.0 is preferred for exhibiting their optimal activities. The GADs from *Lb. paracasei* NFRI7415, *Str. thermophilus* Y2, *Lb. brevis* CGMCC 1306 and *Lb. brevis* 877G exhibited highest activities at a temperature over 45℃, which is not an optimal condition for bacterial growth. Thus, these GABA producers incubated at normal temperature (37℃) may have an effect on their GABA biosynthesis.

In addition, the *gadB*-encoding GAD from *Lb. brevis* 877G had a lower K_m than *gadA*-encoding GADs from *Lb. brevis* CGMCC 1306 and *Lb. brevis* IFO 12005 under their optimal conditions. This may be explained by the different conformational structures of *gadA*- and *gadB*-encoding GADs, though they have similar core structure including PLP-binding sites and active

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residues (Fig. 1C). This implies that other regional structures of GADs have an impact on their activities. Also, LAB GADs exhibit an acidic optimum (pH 4.0 ~ 5.0), which in turn will affect the bacterial viability. Hence, mutations to the GADs have been performed via site-directed mutagenesis approach in order to improve the enzyme activities under neutral acidic conditions (Shi et al., 2014; Yu et al., 2012). Further studies on the improvement of GAD activity via mutation under extreme acidic conditions (pH 4.5 ~ 3.5) would be of importance since milk fermentation generates an acidic condition in this range.

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PROBIOTIC SPECTRA OF WHOLE CELLS OF LACTOBACILLUS BREVIS IN ADDITION TO ITS HIGH GABA BIOSYNTHESIS

As shown in Table 2, *Lb. brevis* is an important source of high GABA producers. Hence, its probiotic properties would need to be documented before adopting it as functional dairy starter. Recently, it was observed that *Lb. brevis* ATCC 8287 exhibited intestinal immunomodulatory effects in piglets (Lahteinen et al., 2014); *Lb. brevis* OK56 showed anti-obesity property through inhibiting the lipopolysaccharides production from gut microbiota, colonic NF-κB signaling pathway, and macrophage infiltration into the adipose tissue in mice (Kim et al., 2015); probiotic *Lb. brevis* CD2 inhibited periodontitis via modulatory effects on the host response and the periodontal microbiota in mice (Maekawa et al., 2014). Further human subject trail showed a reduction in plaque acidogenicity, salivary and bleeding on probing by *Lb. brevis* CD2 (Campus et al., 2014); *Lb. brevis* KB290 protected mice against influenza virus infection via the long-lasting enhancement of interferon-α production and the augmentation of IFV-specific IgA production (Waki et al., 2014b). A preliminary intervention study conducted on 1089 elementary

school children demonstrated that *Lb. brevis* KB290 was capable of reducing the incidence of influenza in school children (Waki et al., 2014a). Moreover, dietary heat-killed *Lb. brevis* SBC8803 could modulate circadian locomotion and sleep rhythms, which might benefit individuals with circadian rhythms disrupted by stress or ageing (Miyazaki et al., 2014). In addition, polyphosphate, an active molecule derived from *Lb. brevis*, suppressed intestinal inflammation and fibrosis by down regulating the expression of inflammation- and fibrosis-associated molecules in the intestinal epithelium in murine with colitis (Kashima et al., 2015). Although *Lb. brevis* is involved in spoilage of beer during fermentation (Suzuki et al., 2006), based on above recent investigations in terms of its health-promoting effects on the host suggest, it is generally accepted as the candidate for probiotics.

This highlights the possibility of *Lb. brevis* used as functional starter cultures for dairy fermentations or as probiotics for human consumption. However, there are selective criteria for the candidate applied as dairy starter including acid production, acidification rate, bacterial viability, and proteolytic activity, which shall be clearly demonstrated in dairy fermentations before commercialization (Buckenhuskes, 1993).

TECHNO-FUNCTIONAL PROPERTIES OF LACTOBACILLUS BREVIS

It is generally recognized that *Lb. brevis* is a heterofermentative LAB as evidenced by the ability to produce lactic acid, acetic acid, ethanol and carbon dioxide as the by-products from carbohydrate metabolism. According to the pyruvate metabolism of two complete sequenced *Lb. brevis* strains - ATCC 367 and KB 290 as per Kyoto Encyclopedia of Genes and Genomes (KEGG) database, pyruvate is the precursor for the synthesis of lactic and acetic acids, which are

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the main metabolites that give sour taste to foods. Additionally, carbohydrate metabolism-generated lactic acid from *Lb. brevis* could accelerate the gel formation of set-type yogurt during fermentation. Moreover, pathways for other metabolites such as acetaldehyde, diacetyl and acetoin in above two sequenced strains were also found in KEGG pathway map. These compounds synthesized by LAB contribute to the flavor of fermented foods and have been detected in various sour milk products (Smit et al., 2005). In addition to the antimicrobial property of lactic acid, a novel bacteriocin called "brevicin" from *Lb. brevis* has been isolated and characterized. Brevicin 925A was able to inhibit *Listeria monocytogenes* and *Streptococcus mutans* (an oral pathogen) (Wada et al., 2009). Brevicin 286 has a narrow inhibition spectrum against *Listeria* sp. (Coventry et al., 1996) and is inhibitory mainly to closely related *Lb. brevis* and *Lb. buchneri* (Benoit et al., 1997). Brevicin SG1 shows a broad spectrum activity against pathogens targeting fungal cell wall and cell membrane (Adebayo et al., 2011). These studies highlight that *Lb. brevis* may be able to produce different types of bacteriocins based on their inhibitory spectra against pathogens. This will contribute to the shelf life and safety of fermented foods. Above important functional properties and the capability to produce GABA suggests that *Lb. brevis* could be a potential candidate as adjunct starter culture for milk fermentation.

CHALLENGES FOR MANUFACTURING GABA OR GABA-RICH FERMENTED DAIRY FOODS – CONCERNS ON GABA YIELD AND RESIDUAL GLUTAMATE

Fermented dairy products such as yogurt and cheese have been consumed for thousands of years and are still one of the most important sources of diets in modern communities. Based on numerous studies, fermented dairy foods can provide protection against cancer, at the mean time

some studies also reported that these foods may increase the risk of cancer, although the latter claims are inconclusive (Davoodi et al., 2013). Thus the proven health-promoting benefits of fermented dairy foods greatly outweigh the unproven harmful effect (Davoodi et al., 2013). Studies also suggest that high intake of fermented dairy foods such as yogurt is associated with a reduced risk of type 2 diabetes (Chen et al., 2014; O'Connor et al., 2014). Importantly, fermented dairy products with probiotics affected the activity of brain regions that regulate the central processing of emotional and sensational activities (Tillisch et al., 2013). Moreover, GABA-rich fermented milk generated by high GABA-producing LAB could regulate our blood pressure in addition to other properties as indicated in Table 1.

Supplementation of yeast extract, black soybean extract and germinated soybean extract to the milk base could improve GABA biosynthesis in GABA producers including *Lb. brevis* (Ko et al., 2013; Nejati et al., 2013; Park et al., 2007b). Those peptides from various extracts provide sufficient amino acids to support or trigger the replication and growth of *Lb. brevis* and other GABA producers in the milk environment. Thus, these practices should be taken into account for the manufacture of GABA-rich fermented milk. In addition to the nutrient supply, providing substrate, glutamate and a co-factor, pyridoxal-5-phosphate (PLP), also accelerate the GABA biosynthesis in GABA producers (Shan et al., 2015). However, all of these supplements to milk base should be of food-grade level to ensure the safety of end products. There have been only few studies pertaining to improving GABA content by using functional GABA producer in fermented milk products without supplying extra nutrients. Use of *Lb. casei* Shirota and *Lc. lactis* YIT 2027 (GABA producer) for milk fermentation without glutamate supplementation produced 10-12 mg/100 mL GABA, which exhibited anti-hypertensive effect on mildly

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hypertensive patients (Inoue et al., 2003). A similar study indicates that milk fermented by *Lb. helveticus* ND01 contained 165.11 mg/kg of GABA in fermented milk in addition to ACE-inhibitory activity (Sun et al., 2009). Both above studies did not involve the supplementation of glutamate as the substrate for producing GABA by GABA-producing LAB. In order to improve the GABA yield in fermented milk, three studies reported enhanced GABA yield in milk at 144.5 mg/kg of milk fermented by *Lc. lactis* DIBCA2 and *Lb. plantarum* PU11 (GABA producer) with addition of 3.38 g/L monosodium glutamate (MSG) into milk (Nejati et al., 2013), *Lb. plantarum* NDC75017 yielded 314.56 mg/100 g of GABA when 80 mM of MSG and 18 μM of PLP were added to the milk (Shan et al., 2015), and *Str. thermophilus* YI-B1 and *Lb. brevis* NPS-QW-145 (GABA producer) produced 314.97 mg/kg of GABA in milk containing 2 g/L of MSG after 24 h fermentation (Wu et al., 2015a). However, MSG was not fully metabolized by these starters added to the milk in the above three studies.

Residual glutamate affect the flavor of the final fermented milk products as glutamate is a strong flavoring agent, which has been approved as food additive. This merits further investigation on the reduction of its content in these functional fermented milks. At least one study suggests that dairy *Str. thermophilus* could metabolize more MSG than dairy *Lb. bulgaricus* resulting in a reduced MSG content, and co-culture of *Str. thermophilus* and *Lb. brevis* in milk also showed an improved GABA yield (Wu et al., 2015a). Thus, using high GABA producer could be a strategy to generate an enhanced GABA content in fermented dairy foods, while *Str. thermophilus* has the potential to metabolize more glutamate; this still needs to be elucidated in the future.

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ABSENCE OF EXTRACELLULAR PROTEINASES IN THE MAJORITY OF LACTIC ACID BACTERIA

Although *Lb. brevis* and other novel LAB could be a probiotic candidate as well as a dairy starter bacterium, their proteolytic activity needs to be demonstrated at least at genetic level. Cell envelope proteinase has been proven to be a key component for maintaining protein metabolism and bacterial survival under protein-rich environments (Kunji et al., 1996; Savijoki et al., 2006). Firstly, cleavage of large proteins such as milk casein into peptides is achieved by cell envelope proteinase, and these peptides are then transported into the bacterial cells via the membrane oligopeptide permease, iron-like transporters or ABC transporters for further utilization (Savijoki et al., 2006).

In order to understand the diversity and distribution of cell envelope proteinases in LAB, we constructed the phylogenetic tree based on the amino acids sequence of cell envelope proteinases and performed a genetic survey regarding to the distribution of cell envelope proteinases as shown in Fig. 2. As shown in Fig. 2C, five types of LAB cell envelope proteinases have been classified based on the structure of enzyme domains (Kunji et al., 1996; Savijoki et al., 2006). However, it was observed that several LAB species such as *Lb. casei* (two types; functionally distinct) and *Lb. helveticus* (two types; functionally distinct) possess more than one type of cell envelope proteinases, while dairy isolates including *Str. thermophilus*, *Lc. lactis* and *Lb. bulgaricus* have only one type of highly conserved cell envelope proteinase (Fig. 2A). As shown in the figure, cell envelope proteinase (PrtP) from *Lc. lactis* shows high identity in amino acids sequences with that from *Lb. casei*. This may suggest that *Lb. casei* may integrate the gene

encoding PrtP from *Lc. lactis* via HGT events based on the phylogenetic tree (Fig. 2A). This may explain the two types of cell envelope proteinases in *Lb. casei*.

As shown in Fig. 2B, most completely sequenced LAB strains lack genes encoding cell envelope proteinases in their genomes. Attentions should be paid to LAB strains because they were selected to be sequenced but were not randomly chosen for sequencing. Due to the cost for complete sequencing, there are not many LAB strains that have been completely sequenced. However, there are numerous strains sequenced but with different sequencing levels: 1) chromosome with gaps; 2) scaffolds; 3) contigs. These incomplete sequencing projects cannot provide full genomic information because there were gaps left (unknown fragment sequences). However, these strains with complete genomes could be able to provide preliminary information because some properties in the same species may be specific and highly conserved. It was found that most strains of *Lb. casei* group including *Lb. casei*, *Lb. paracasei* and *Lb. rhamnosus* may have proteolytic activity as evidenced by the presence of genes encoding cell envelope proteinases (Fig. 2B). All the four completely sequenced *Lb. delbrueckii* strains which are normally used for milk fermentation possess cell envelope proteinases, while most completely sequenced strains of *Lc. lactis* and *Str. thermophilus* lack cell envelope proteinases; however, genes encoding exported serine proteinase (htrA), which is able to hydrolyze milk proteins, are present in the two latter species. Also, genes encoding another typical proteinase – extracellular zinc proteinases were observed in most sequenced strains of *Lb. plantarum*. However, we did not identify any extracellular proteinases including cell envelope proteinase, exported serine proteinase and extracellular zinc proteinases in the completely sequenced strains of *Lb. brevis*, *Lb. reuteri*, and *Lb. fermentum*. It appears that *Lb. brevis*, the most important high GABA

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producer, is incapable of hydrolyzing milk proteins, which may lead to failure of milk fermentation.

As mentioned above, *Lb. brevis* could be used as a functional starter bacterium for its high GABA production. However, we observed that one representative isolate, *Lb. brevis* NPS-QW-145 (Table 2), a high GABA producer, was not able to ferment milk in the presence of various supplemented sugars (Fig. 3). As shown in the figure, additional sugars including glucose, galactose and fructose were supplemented to the milk base in addition to its original lactose. To our knowledge, glucose is the most common sugar that could be metabolized by bacteria including LAB. However, it was observed that this isolate could not significantly acidify milk in the presence of various sugars including glucose, galactose, lactose and fructose, whereas the viable count of *Lb. brevis* NPS-QW-145 did not significantly increase after 24 h of fermentation. As for the bacterial growth, the carbon source and nitrogen source are the two important factors for bacterial replication and metabolic activities. This indicates that *Lb. brevis* could not break down milk proteins (casein) due to its absence of genes-encoding cell envelope proteinases (Wu et al., 2015a). In short, *Lb. brevis* is not able to utilize large peptides as evidenced by the absence of cell envelope proteinases in its genome and experimental fermentation results.

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STRATEGIES FOR OVERCOMING THE NON-PROTEOLYTIC NATURE OF LACTOBACILLUS BREVIS IN MILK

In order to apply high GABA-producing *Lb. brevis* as the functional starter culture for milk fermentation, supplementation of small peptides to the milk base could provide sufficient growth factors for growing *Lb. brevis*. Whey, a by-product of cheese making, contains high amount of

small peptides, which could be concentrated and used as a source of small peptides (4-20 amino acid residues) and supplemented to the milk base as a nitrogen source for *Lb. brevis* since there are various types of peptidases that are encoded in its genomes.

Another solution is to apply conventional dairy starter that is proteolytic in nature to co-culture with *Lb. brevis* during milk fermentation. Since most dairy starters such as *Lc. lactis*, *Str. thermophilus* and *Lb. bulgaricus* are capable of breaking down milk casein into peptides; such cocktail may produce sufficient peptides and can be further metabolized by the intracellular peptidases in *Lb. brevis*. At least, there is one study that suggests that dairy *Str. thermophilus*, but not dairy *Lb. bulgaricus*, could be used as a co-culture with *Lb. brevis* resulting in an enhanced viability of *Lb. brevis* and its GABA biosynthesis in milk (Wu et al., 2015a). It is important to examine whether dairy starters compete with *Lb. brevis* during co-culture. Such selection is important before adopting the latter for milk fermentation.

The third approach is through metabolic engineering for improving proteolytic nature of *Lb. brevis*. This could incorporate the genes-encoding cell envelope proteinase, exported serine proteinase or extracellular zinc proteinases into the genome of *Lb. brevis*, or construct a shuttle plasmid which could express above proteinases and transfer it into *Lb. brevis* for expression. This modified *Lb. brevis* is generally accepted to produce food-grade GABA during fermentation using food raw materials. However, this method may not be suggested for manipulating *Lb. brevis* because of its genetically modified status for manufacturing GABA-rich food. One of the important concerns is that the selection markers in the plasmids or host genomes during such engineering process should not involve the genes encoding antibiotics. If antibiotic genes are

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used as selection marker in these engineered bacteria, this may increase the risk of antibiotic transmission in the gut among people after consumption because the fermented milk is the carrier of these bacteria. Thus, it is highly necessary to remove those selection markers in the engineered strains.

CONCLUDING REMARKS & FUTURE TRENDS

Based on numerous human and animal studies, GABA or GABA-rich foods regulate blood pressure and emotion of the host. Due to the low GABA content in natural animal- and plant-associated food products, high GABA-producing LAB isolates are of great importance to produce food-grade GABA and GABA-rich fermented dairy foods via fermentations. Among all of GABA producers isolated from various environments, *Lb. brevis* is an important source of GABA-producing microorganism and has been found to exhibit the capability of producing high level of GABA. In addition, two functionally distinct GAD-encoding genes (*gadA* & *gadB*) and one intact *gad* operon were found in all the sequenced strains of *Lb. brevis* suggesting its common capability of high GABA biosynthesis at both genetic and experimental levels. Thus, *Lb. brevis* could be a functional starter for manufacturing GABA-rich fermented dairy foods. However, strategies for the reduction of residual glutamate and enhanced GABA biosynthesis in milk by co-culturing *Lb. brevis* with conventional dairy starters and their interactions need to be further demonstrated. In addition, the absence of cell envelope proteinases in *Lb. brevis* and experimental data demonstrate its non-proteolytic nature. Little efforts have been made to overcome this shortage. Future studies regarding the improvement of enzymatic activities of

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GADs in LAB under extreme acidic conditions and improvement of proteolytic activity of *Lb. brevis* merit further investigations.

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Table 1 Biofunctionalities of GABA-rich foods

Subject	Type of food	GABA yield	Function(s)	Reference(s)
Mildly hypertensive patients	GABA-rich fermented milk	10 ~ 12 mg/100 mL	Blood-pressure-lowering effect	(Inoue et al., 2003)
Spontaneously hypertensive rats	GABA-enriched tempeh-like fermented soybean	1.3 % (w/w) in dry powder form	Retarded blood pressure	(Aoki et al., 2003)
Spontaneously hypertensive rats	GABA-enriched soybean	53.76 mg/g in dry powder form	Blood-pressure-lowering effect	(Shizuka et al., 2004)
Rats with glycerol-induced acute renal failure	Pure GABA	N.A.	Against the renal damage involved in acute renal failure	(Kim et al., 2004)
Streptozotocin-induced diabetic rats	Pure GABA	N.A.	Impaired glucose metabolism; enhanced oxidative stress	(Nakagawa et al., 2005)
Spontaneously hypertensive rats	GABA-rich soy sauce	1.0 g/100 mL	Reduced overall cardiovascular risk	(Yamakoshi et al., 2007)
Male	Pure GABA	N.A.	Increased human growth hormone	(Powers et al., 2008)

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Human with high-normal blood pressure and borderline hypertension	GABA-rich *Chlorella*	500 mg/100 g in the fresh form	Decreased high-normal blood pressure and borderline hypertension	(Shimada et al., 2009)
Healthy male	GABA-rich chocolate	0.28% (w/w)	Psychological stress-reducing effect	(Nakamura et al., 2009)
Spontaneously hypertensive rats	GABA-rich tomato	179 mg/100 g in the fresh form	Anti-hypertensive effect	(Yoshimura et al., 2010)
Forced swimming rat	GABA-rich Monascus-fermented product	16.4 mg/g in dry powder form	Anti-depressant effect	(Chuang et al., 2011)
Spontaneously hypertensive rats	GABA-rich mulberry leaf water extract	3.8 mg/g in water extract	Anti-hypertensive effect	(Yang et al., 2012)
Forced swimming rat	GABA-rich fermented black soybean milk	5.42 mg/mL	Anti-depressant effect	(Ko et al., 2013)
Streptozotocin-induced diabetic rats	GABA-rich tea extract	1.72 mg/g in the dry powder form	Anti-diabetic property	(Cherng et al., 2014)

N.A.: not applicable.

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Table 2 List of representative high GABA-producing lactic acid bacteria and bifidobacteria

Strain	Origin	GABA yield	Reference
Lb. brevis NPS-QW-145*	Korean kimchi	25.831 g/L	(Wu et al., 2015b)
Lb. brevis NPS-QW-171	Korean kimchi	19.631 g/L	(Wu et al., 2015b)
Lb. brevis NPS-QW-177	Korean kimchi	24.098 g/L	(Wu et al., 2015b)
Lb. brevis NPS-QW-193	Korean kimchi	23.33 g/L	(Wu et al., 2015b)
Lb. brevis NPS-QW-216	Korean kimchi	21.694 g/L	(Wu et al., 2015b)
Lb. brevis NPS-QW-242	Korean kimchi	22.986 g/L	(Wu et al., 2015b)
Lb. brevis NPS-QW-255	Korean kimchi	19.072 g/L	(Wu et al., 2015b)
Lb. brevis NPS-QW-267	Korean kimchi	24.992 g/L	(Wu et al., 2015b)
Lb. brevis NPS-QW-281	Korean kimchi	23.638 g/L	(Wu et al., 2015b)
Lb. brevis NCL912	Chinese paocai	345.83 mM	(Li et al., 2010b)
Lb. brevis IFO-12005	N.M.	10.18 mM	(Yokoyama et al., 2002)
Lb. brevis OPY-1*	Korean kimchi	424.67 μg/g	(Park et al., 2007b)
Lb. brevis GABA100	Korean kimchi	26.9 g/L	(Kim et al., 2009)
Lb. brevis BJ20	Fermented Jot-gal	2.465 g/L	(Lee et al., 2010)
Lb. brevis BH2	Korean kimchi	194 mM	(Kim et al., 2007)
Lb. brevis GABA 057	N.M.	223 mM	(Choi et al., 2006)
Lb. brevis TCCC13007	Pickled vegetables	61 g/L	(Zhang et al., 2012)
Lb. brevis CGMCC 1306	Fresh milk	N.M.	(Huang et al., 2007)
Lb. brevis OPK-3	Korean kimchi	84.292 mg/L/h	(Park et al., 2007a)
Lb. brevis DPC6108	Infant feces	16.16 g/L	(Barrett, 2014)

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Lb. brevis FPA 3709	Fish intestine	2.45 g/L	(Ko et al., 2013)
Lb. brevis PM17*	Cheese	15 mg/kg	(Siragusa et al., 2007)
Lb. brevis K203	Korean kimchi	44.4 g/L	(Binh et al., 2014)
Lb. brevis 877G*	Korean kimchi	22.51 mM	(Seo et al., 2013b)
Lb. brevis CECT 8183	Goat cheese	0.96 mM	(Diana et al., 2014)
Lb. brevis CECT 8181	Sheep cheese	0.94 mM	(Diana et al., 2014)
Lb. brevis CECT 8182	Goat cheese	0.99 mM	(Diana et al., 2014)
Lb. paracasei NFRI 7415	Fermented fish	302 mM	(Komatsuzaki et al., 2005)
Lb. paracasei PF6*	Cheese	99.9 mg/kg	(Siragusa et al., 2007)
Lb. plantarum NTU 102	Cabbage pickles	629 mg/L	(Tung et al., 2011)
Lb. plantarum C48*	Cheese	16 mg/kg	(Siragusa et al., 2007)
Lb. plantarum DW12	Fermented foods	4 g/L	(Ratanaburee et al., 2011)
Lb. plantarum K154	Korean kimchi	201.78 mg/L	(Park et al., 2014b)
Lb. plantarum NDC75017*	Fermented milk	3.15 g/kg	(Shan et al., 2015)
Lb. plantarum NMZ	Fermented foods	1.032 mM	(Zareian et al., 2012)
Lb. buchneri MS	Korean kimchi	251 mM	(Cho et al., 2007)
Lb. buchneri WPZ001	Fermented sausages	129 g/L	(Zhao et al., 2015)
Lb. sakei B2-16	N.M.	660 mM	(Kook et al., 2010)
Lb. rhamnosus YS9	Pickled vegetables	187 mM	(Lin, 2013)
Lb. bulgaricus PR1*	Cheese	63 mg/kg	(Siragusa et al., 2007)
Lb. helveticus ND01	Koumiss	165.11 mg/L	(Sun et al., 2009)
Lb. namurensis NH2	Fermented pork	7.339 g/L	(Ratanaburee et al., 2013)

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Lc. lactis subsp. *lactis* B	Korean kimchi	6.41 g/L	(Lu et al., 2008)
Lc. lactis CECT 8184	Goat cheese	0.93 mM	(Diana et al., 2014)
Lc. lactis PU1*	Cheese	36 mg/kg	(Siragusa et al., 2007)
Str. thermophilus Y2	N.M.	7.98 mg/L	(Yang et al., 2008b)
Str. thermophilus ST110*	Yogurt starter	655 mg/L	(Somkuti et al., 2012)
Pediococcus pentosaceus HN8	Fermented beef	9.06 g/L	(Ratanaburee et al., 2013)
Pediococcus pentosaceus NH102	Fermented pork	8.386 g/L	(Ratanaburee et al., 2013)
Pediococcus pentosaceus NH116	Fermented pork	8.411 g/L	(Ratanaburee et al., 2013)
Bifidobacterium adolescentis DPC6044	Infant feces	1.57 g/L	(Barrett, 2014)
Bifidobacterium dentium DPC6333	Infant feces	4.31 g/L	(Barrett, 2014)
Bifidobacterium dentium NFBC2243	Infant feces	6.24 g/L	(Barrett, 2014)
Bifidobacterium infantis UCC35624	Infant feces	2.84 g/L	(Barrett, 2014)

Note: GABA yield reported in the reference was associated with cultivation conditions and supplemented MSG level. Strains highlighted with asterisk (*) indicated that GABA production by this strain has been detected in milk.

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Table 3 Biochemical properties and kinetics of hetero-expressed and purified GADs from lactic acid bacteria

Strain	GAD length (aa)	Optimal conditions	Enzyme kinetics	Reference(s)
Lb. brevis IFO 12005	480	pH 4.2, 30℃	K_m = 9.3 mM	(Uenoa et al., 1997)
Lc. lactis 01-7	466	pH 4.7, 30℃	K_m = 0.5 mM	(Nomura et al., 1999b)
Lb. paracasei NFRI7415	481	pH 5.0, 50℃	K_m = 5 mM	(Komatsuzaki et al., 2008)
Str. thermophilus Y2	459	pH 4.0, 55℃	K_m = 2.3 mM	(Yang et al., 2008a)
Lb. brevis CGMCC 1306	479	pH 4.8, 48℃	K_m = 10.26 mM	(Fan et al., 2012)
Lb. brevis 877G	468	pH 5.2, 45℃	K_m = 3.6 mM	(Seo et al., 2013a)
Lb. zymae GU240	479	pH 4.5, 41℃	K_m = 1.7 mM	(Park et al., 2014a)
Lb. plantarum ATCC 14917	469	pH 4.5, 40℃	K_m = 22.8 mM	(Shin et al., 2014)

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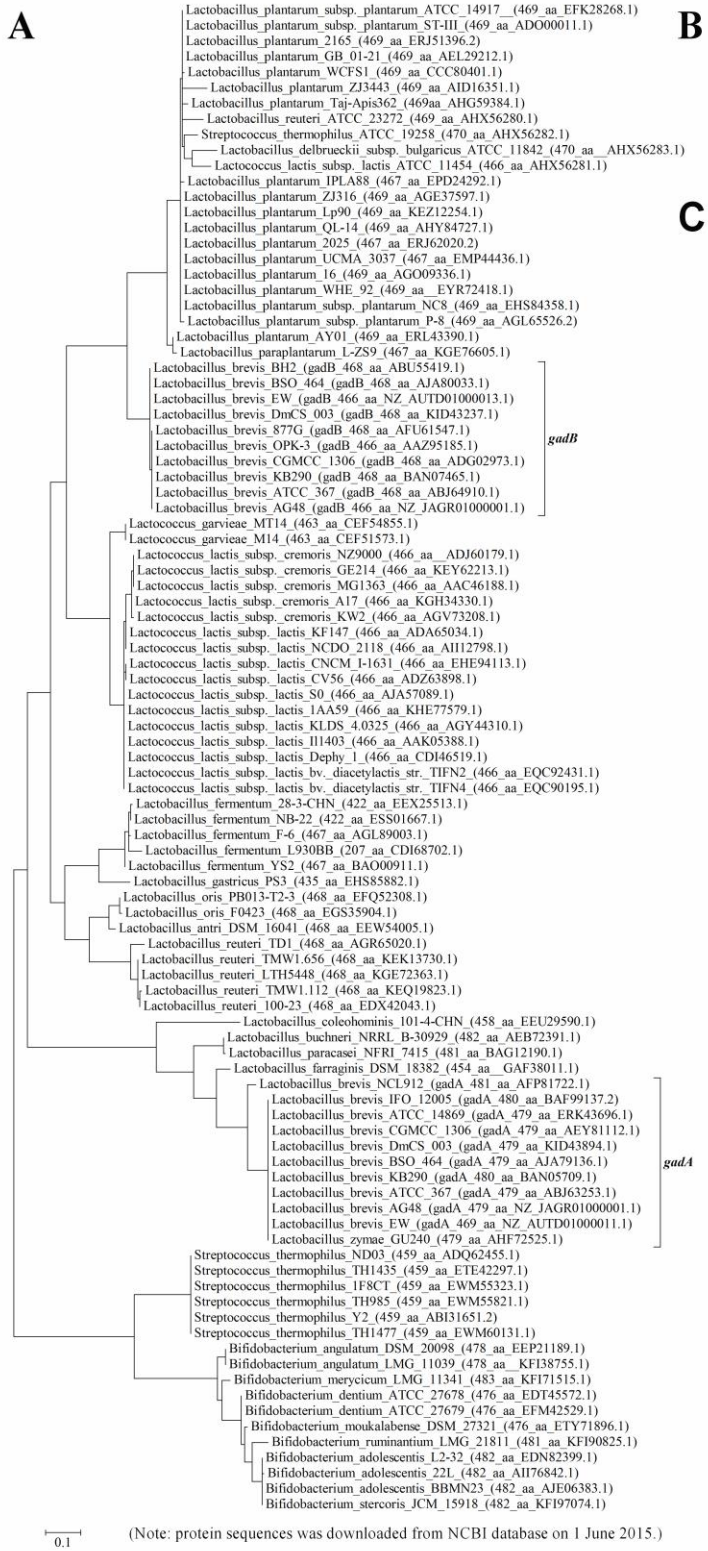
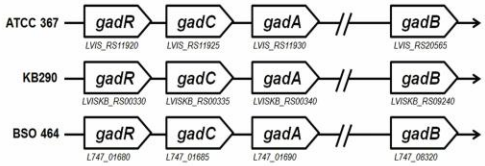
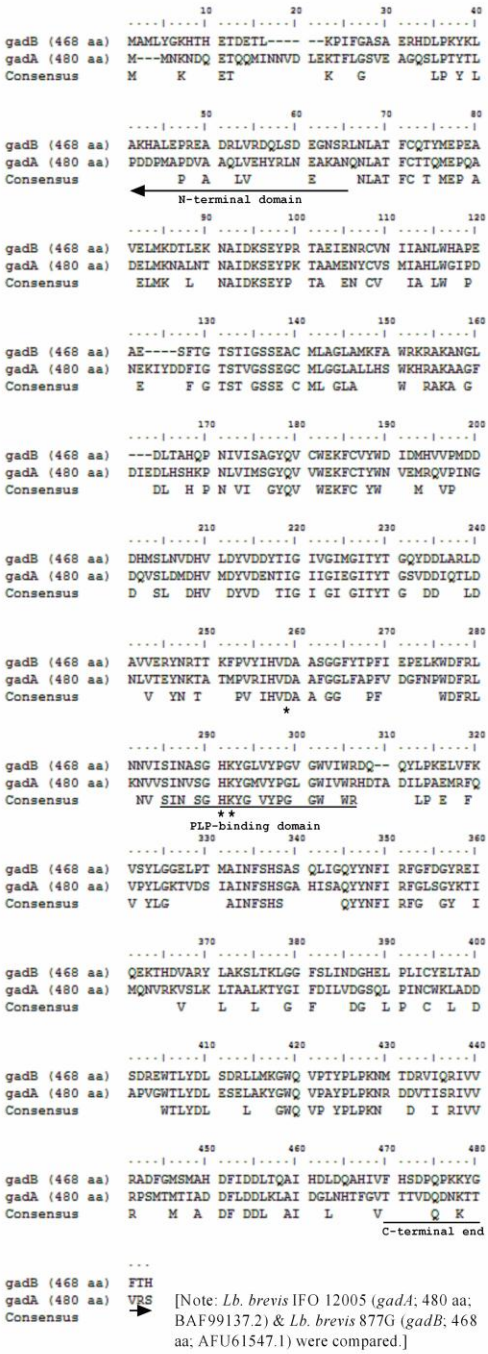




(Note: based on NCBI complete genome on 15 June 2015.)



[Note: *Lb. brevis* IFO 12005 (*gadA*; 480 aa; BAF99137.2) & *Lb. brevis* 877G (*gadB*; 468 aa; AFU61547.1) were compared.]

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Figure 1. *Lactobacillus brevis* possesses two distinct functionally glutamic acid decarboxylases and one intact *gad* operon. (A) Phylogenetic tree (maximum-likelihood method) based on amino acids sequences of glutamic acid decarboxylases from lactic acid bacteria and bifidobacteria. (B) Orientation of *gad* operon and GADs-encoding genes (*gadA* and *gadB*) in complete genomes of three *Lb. brevis* strains. (C) Alignment of two representative GADs from *L. brevis*. The catalytic residues were indicated by stars. Figures were generated from MEGA (version 6.0) after MUSCLE alignment of GADs. The length of GAD from each strain and its GenBank accession numbers are indicated in the braces.

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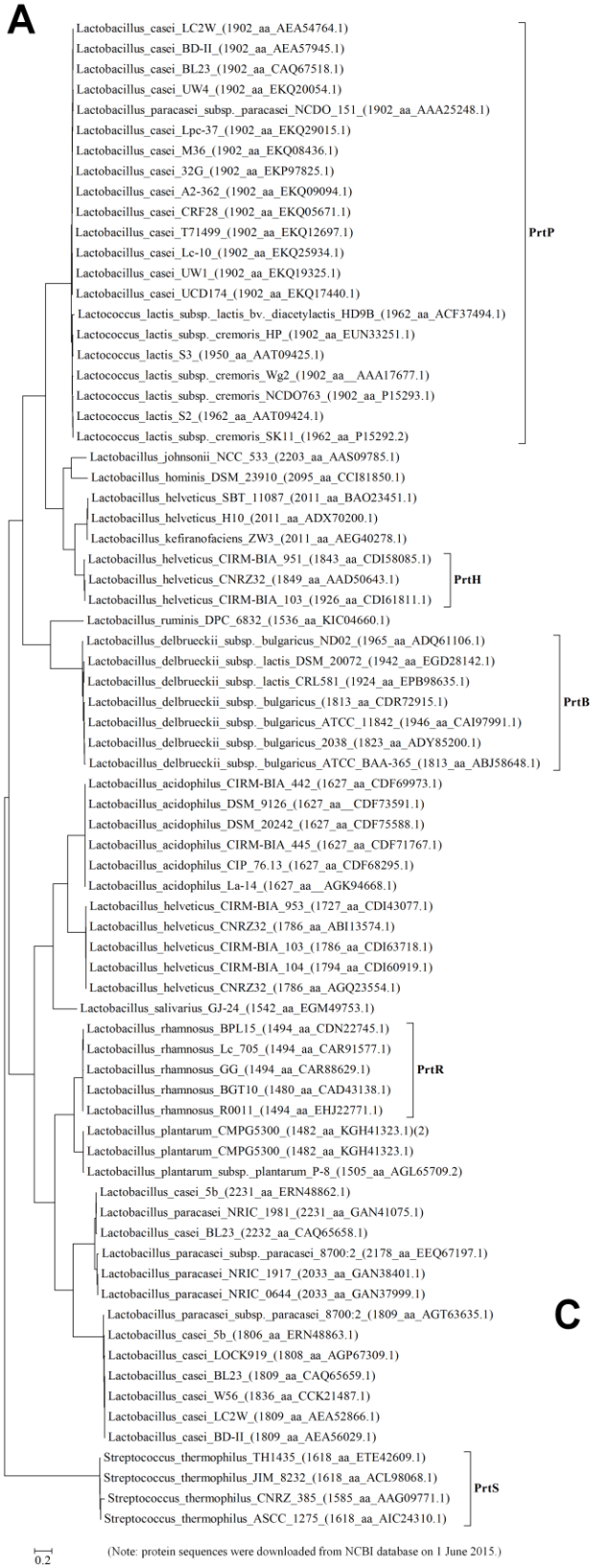

(Note: protein sequences were downloaded from NCBI database on 1 June 2015.)

B

Species	No. of strains with complete genome	No. of strains with cell envelope proteinase(s)	Strain(s)
Lc. lactis	14	2	MG1363, SK11
Str. thermophilus	9	3	LMD-9, JIM 8232, ASCC 1275
Lb. paracasei	3	2	JCM 8130, 8700:2
Lb. rhamnosus	5	3	GG, Lc 705, ATCC 8530
Lb. casei	9	9	ATCC 334, Zhang, BL23,BD-II, LC2W, 12A, W56, LOCK919, ATCC 393
Lb. planrarum	8	2	P-8, CMPG5300
Lb. delbrueckii	4	4	ATCC 11842, ATCC BAA-365, ND02, 2038
Lb. reuteri	5	0	-
Lb. helveticus	7	4	CNRZ32, R0052, H10, H9
Lb. acidophilus	4	2	La-14, 30SC
Lb. gasseri	2	0	-
Lb. fermentum	3	0	-
Lb. salivarius	4	0	-
Lb. brevis	2	0	-
Lb. johnsonii	4	0	-
Lb. ruminis	1	0	-
Lb. buchneri	2	2	CD034, NRRL B-30929
Lb. sakei	1	0	-
Lb. amylovorus	1	0	-
Lb. sanfranciscensis	1	0	-
Lb. kefiranofaciens	1	1	ZW3
Lb. hokkaidonensis	1	0	-
Lb. mucosae	1	0	-
Lb. pentosus	1	0	-

Note: data was generated from NCBI database on 2 June 2015.

C

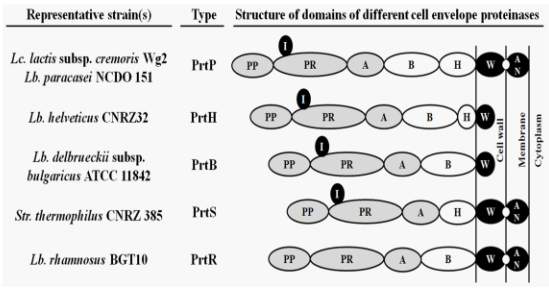

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Figure 2. Absence of cell envelope proteinases in majority of lactic acid bacteria. (A) Phylogenetic tree (maximum-likelihood method) based on amino acids sequences of LAB cell envelope proteinase. The figure was generated from MEGA (version 6.0) after MUSCLE alignment. The length of proteinase from each LAB strain and the GenBank accession number are indicated in the braces; (B) Distribution of cell envelope proteinase(s) in complete sequenced lactic acid bacteria; (C) classification of LAB cell envelope proteinases based on the domains of certain proteinases (Savijoki et al., 2006; Siezen, 1999). Denotation: PP, pre-pro domain; PR, catalytic domain; I, insert domain; A, A domain; B, B domain; H, helix domain; W, cell wall spacer domain; white dot, sorting signal; AN, anchor domain.

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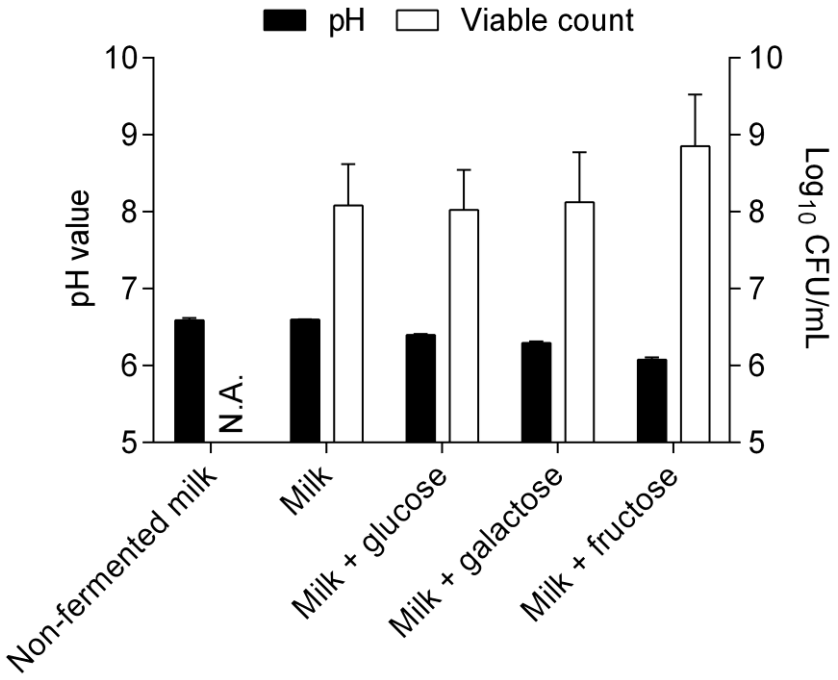

Figure 3. A representative high GABA-producing *Lactobacillus brevis* NPS-QW-145 could not significantly acidify and ferment milk in the presence of lactose, glucose, galactose and fructose. Initial bacterial density in 10% (w/v) skimmed milk after inoculation was about 7.81 Log_{10} CFU/mL. Conditions for fermentation were at 37℃ for 24 h. Triplicate analysis was performed. Data in the figure was presented as mean ± standard deviation.

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