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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 antidepressant activities as the major functions in humans and animals. Hence, high GABAproducing 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.

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.

In this review, the biofunctionalities of GABA and GABA-rich foods, high GABAproducing 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 prodrugs 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, antihypertensive 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.

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

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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, prescreening 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.

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

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(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°C (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 GABAproducing 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 gadAencoding 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°C, which is not an optimal condition for bacterial growth. Thus, these GABA producers incubated at normal temperature (37°C) 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 $4.0 \sim 5.0$), 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 $4.5 \sim 3.5$) would be of importance since milk fermentation generates an acidic condition in this range.

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- κ B 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 longlasting 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

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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 metabolismgenerated 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 ACEinhibitory 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

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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.

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

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

N.A.: not applicable.

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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)

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

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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	Fermented beef	9.06 g/L	(Ratanaburee et al., 2013)
HN8			
Pediococcus pentosaceus	Fermented pork	8.386 g/L	(Ratanaburee et al., 2013)
NH102			
Pediococcus pentosaceus	Fermented pork	8.411 g/L	(Ratanaburee et al., 2013)
NH116			
Bifidobacterium	Infant feces	1.57 g/L	(Barrett, 2014)
adolescentis DPC6044			
Bifidobacterium dentium	Infant feces	4.31 g/L	(Barrett, 2014)
DPC6333			
Bifidobacterium dentium	Infant feces	6.24 g/L	(Barrett, 2014)
NFBC2243			
Bifidobacterium infantis	Infant feces	2.84 g/L	(Barrett, 2014)
UCC35624			

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

Starin	GAD	Optimal	Enzyme	Reference(s)	
Strain	length (aa)	conditions	kinetics		
Lb. brevis IFO 12005	480	рН 4.2, 30°С	$K_{\rm m} = 9.3 \ {\rm mM}$	(Uenoa et al., 1997)	
Lc. lactis 01-7	466	рН 4.7, 30°С	$K_{\rm m} = 0.5 \ {\rm mM}$	(Nomura et al., 1999b)	
Lb. paracasei NFRI7415	481	pH 5.0, 50°C	$K_{\rm m} = 5 \ {\rm mM}$	(Komatsuzaki et al.,	
		1 /		2008)	
Str. thermophilus Y2	459	рН 4.0, 55°С	$K_{\rm m} = 2.3 \ {\rm mM}$	(Yang et al., 2008a)	
Lb. brevis CGMCC 1306	479	pH 4.8, 48°C	$K_{\rm m} = 10.26$	(Fan et al., 2012)	
			mM		
Lb. brevis 877G	468	рН 5.2, 45°С	$K_{\rm m}$ = 3.6 mM	(Seo et al., 2013a)	
Lb. zymae GU240	479	pH 4.5, 41°C	$K_{\rm m} = 1.7 \ {\rm mM}$	(Park et al., 2014a)	
Lb. plantarum ATCC 14917	469	рН 4.5, 40°С	$K_{\rm m} = 22.8 \ {\rm mM}$	(Shin et al., 2014)	

Lactobacillus_plantarum_subspplantarum_ATCC_14917_(469_aa_EFK28268.1)	ATCC 367 020	Aben 1 Aben 2ben 9
A Lactobacillus_plantarum_subspplantarum_ST-III_(469_aa_ADO00011.1) B	Alec 307 gau	
Lactobacillus_plantarum_2165_(469_aa_ERJ51396.2)	LVIS_RS1	1920 LVIS_RS11925 LVIS_RS11930 LVIS_RS20565
Lactobacillus_plantarum_GB_01-21_(469_aa_AEL29212.1)		
Lactobacillus_plantarum_WCFS1_(469_aa_CCC80401.1)	KB290 - gao	IR > gadC > gadA > for gadB >
— Lactobacillus_plantarum_ZJ3443_(469_aa_AID16351.1)		
 Lactobacillus_plantarum_Taj-Apis362_(469aa_AHG59384.1) 	LVISKB_R	S00330 LVISKB_RS00335 LVISKB_RS00340 LVISKB_RS09240
- Lactobacillus reuteri ATCC 23272 (469 aa AHX56280.1)		
Streptococcus thermophilus ATCC 19258 (470 aa AHX56282.1)	BSO 464 — gao	IR ≻ gadC ≻ gadA ≻ / → gadB >→
Lactobacillus delbrueckii subsp. bulgaricus ATCC 11842 (470 aa AHX56283.1)	1747 016	1747 01685 1747 01690 1747 08320
Lactococcus lactis subsp. lactis ATCC 11454 (466 aa AHX56281.1)	2141_010	
Lactobacillus plantarum IPLA88 (467 aa EPD24292.1)	(Note: based on	NCBI complete genome on 15 June 2015.)
Lactobacillus plantarum Z1316 (469 aa AGE37597 1)		1 0
Lactobacillus plantarium L n90 (469 aa. KEZ12254.1)		10 20 30 40
Lactobacillus plantarium OL-14 (469 aa AHV847271)		
Lactobacillus plantarium 2025 $(467, a_2, FR160202)$	gadB (468 aa)	MAMLYGKHTH ETDETLKPIFGASA ERHDLPKYKL
Lactobacillus plantarum LICMA 3037 (467 ap EMD44436 1)	gadA (480 aa)	MMNKNDQ ETQQMINNVD LEKTFLGSVE AGQSLPTYTL
Lactobacillus plantarum $16 (469 ag A CO003261)$	Consensus	M K ET K G LPYL
Lactobacillus plantarum WHE 0/40-2a_AG009350.1)		
Lactobacillus plantarum write $22 (409)$ aa $= 1 \times (2418.1)$		
Lactoracting planarum subs. planarum NC8 (409 aa EHS4353.1)		50 60 70 80
- Lactobachus piantarum subs, piantarum P-8 (469 aa AGL65526.2)		
Lactobacillus plantarum AY01 (469 aa ERL43390.1)	gadB (468 aa)	AKHALEPREA DRLVRDQLSD EGNSRLNLAT FCQTYMEPEA
Lactobacillus_paraplantarum_L-ZS9_(467_aa_KGE/6605.1)	gadA (480 aa)	PDDPMAPDVA AQLVEHYRLN EAKANQNLAT FCTTQMEPQA
Lactobacillus brevis BH2 (galB 468 aa ABU55419.1)	Consensus	P A LV E NLAT FC T MEP A
Lactobacillus_brevis_BSO_464_(gadB_468_aa_AJA80033.1)		N-torminal domain
Lactobacillus_brevis_EW_(gadB_466_aa_NZ_AUTD01000013.1)		W CEIMINAI GOMAIN
Lactobacillus brevis DmCS 003 (gadB 468 aa KID43237.1)		
Lactobacillus_brevis_877G_(gadB_468_aa_AFU61547.1)	10 1460	
Lactobacillus_brevis_OPK-3_(gadB_466_aa_AAZ95185.1)	gads (468 aa)	VELMEDILEE NAIDESEYPE TAEIENROVN IIANLWHAPE
Lactobacillus_brevis_CGMCC_1306_(gadB_468_aa_ADG02973.1)	gadA (480 aa)	DELMKNALNT NAIDKSEYPK TAAMENYCVS MIAHLWGIPD
Lactobacillus_brevis_KB290_(gadB_468_aa_BAN07465.1)	Consensus	ELMK L NAIDKSEYP TA EN CV IA LW P
Lactobacillus_brevis_ATCC_367_(gadB_468_aa_ABJ64910.1)		
Lactobacillus_brevis_AG48_(gadB_466_aa_NZ_JAGR01000001.1)		130 140 150 160
Lactococcus_garvieae_MT14_(463_aa_CEF54855.1)		
Lactococcus garvieae M14 (463 aa CEF51573.1)	and P (469 an)	NE SETC TETTCESENC MINCINNER NEWDAWANCT
Lactococcus lactis subsp. cremoris NZ9000 (466 aa ADJ60179.1)	gadb (468 aa)	ALSFIG ISIIGSSEAC MLAGLAMKFA WKKRAKANGL
Lactococcus lactis subsp. cremoris GE214 (466 aa KEY62213.1)	gadA (480 aa)	NEKIYDDFIG TSTVGSSEGC MLGGLALLHS WKHRAKAAGF
Lactococcus lactis subsp. cremoris MG1363 (466 aa AAC46188,1)	Consensus	E F G TST GSSE C ML GLA W RAKA G
Lactococcus lactis subsp. cremoris A17 (466 aa KGH34330 l)		
Lactococcus lactis subsp. cremoris KW2 (466 aa AGV73208.1)		170 180 190 200
Lactococcus lactis subsp. lactis KF147 (466 aa ADA650341)		
Lactococcus lactis subsp. lactis NCDO 2118 (466 aa AII12798.1)	gadB (468 aa)	DITAHOP NIVISAGYOV CWEKECVYWD IDMHVUPMDD
Lactococcus lactis subsp. lactis CNCM I-1631 (466 aa EHE94113.1)	gad3 (490 as)	DIEDIUCUED NILITMCCVOU UNEVECTIVAN UEMDOUDINC
Lactococcus lactis subsp. lactis CV56 (466 aa ADZ63898.1)	gaux (100 aa)	DI U D W UT OUOU MENTOTINA VERACUTING
Lactococcus lactis subsp. lactis S0 (466 aa AJA57089.1)	Consensus	DD H P N VI GIQV WEREC IW M VP
Lactococcus lactis subsp. lactis 1AA59 (466 aa KHE775791)		
Lactococcus lactis subsp. lactis KLDS 4 0325 (466 aa AGY443101)		210 220 230 240
Lactococcus lactis subsp. lactis 11/403 (466 aa AAK05388.1)		······································
Lactococcus lactis subsp. lactis Dephy 1 (466 aa CDI465191)	gadB (468 aa)	DHMSLNVDHV LDYVDDYTIG IVGIMGITYT GQYDDLARLD
Lactococcus lactis subsplating by diacetulactis str. TIEN2 (466 as EOC924311)	gadA (480 aa)	DOVSLOMDHV MDYVDENTIG IIGIEGITYT GSVDDIQTLD
I actococcus lactis subsplactus by diacetulactis at TIENA (466 as EOC901951)	Consensus	D SL DHY DYVD TIG I GI GITYT G DD LD
La octobacilla formatium 28.2-CHN (422 as EEX255131)		
Lactobacillus formatium NB_{22} (22 as ESS016671)		
Lastohadhus formantum F.6 (467 as AGL 80002.1)		250 260 270 280
Lactobactures remember 1020PB (207 as (D1687021))		
Lactobaching Crimentum L253067 as PA0000111)	gadB (468 aa)	AVVERYNRTT KFPVYIHVDA ASGGFYTPFI EPELKWDFRL
Lactobacillus_termicinum_152_(407 as ERC050911.1)	gadA (480 aa)	NLVTEYNKTA TMPVRIHVDA AFGGLFAPFV DGFNPWDFRL
Lactobacillus rais DR012 T2 2 (468 as EE0523081)	Consensus	V YN T PV IHVDA A GG PF WDFRL
Lactobacillus oris FD015-12-5_(406_aa_ErQ52506.1)		*
Lactobacillus onts F0425 (466 at E0553904.1)		
- Lactobachitis and LOSM_10041 (406 as AEEW34005.1)		250 300 310 320
Lactobachius return $1D_{-}(406)$ aa $-AG(65)020.1$		NUTRINA CO UNVCI UNDOLL CHUTHODO OVI DEPI UPP
Lactobachus reduct TUIS449 (469 aa KEK15/30.1)	gadb (400 aa)	NNVISINASG HRIGDVIPGV GWVIWRDQ QILPREDVFR
Lactoracinus return L113446 (406 at KCr/2303.1)	gadA (480 aa)	KNVVSINVSG HKYGMVYPGL GWIVWRHDTA DILPAEMRFQ
Lactobacillus reducti 100 22 (468 as EQ176251)	Consensus	NV SIN SG HKYG VYPG GW WR LP E F
Lactobachius retien 100-25 (468 au EDA2045.1)		** DID-binding domain
Lactobachus coleonominis 101-4-CHN (458 aa EEU29590.1)		330 340 350 360
Lactobacillus Duciner INRRL 7-30929 (462 az AED/2391.1)		····· [·····] ····· [·····] ····· [·····] ···· [·····]
Lactobactinus paracaset NFRI 7413 (461 au BAU12190.1)	gadB (468 aa)	VSYLGGELPT MAINFSHSAS OLIGOYYNFI RFGFDGYREI
Lactobactilus tarragins DSM 18382 (434 aa GAF38011.1)	gadà (480 aa)	UDVLCKTUDS TATNESHSCA HISAOVYNET DECLSCYKTT
Lactoracinus Devis NCL912 (gadA 481 aa AFP61/22.1)	Conconcilla	U VIC ATNECUS OVVNET DEC CV T
Lactobacinus of even in ATO 12005 (gadA 480 ga BAF99157.2)	Sousensus	A A A A A A A A A A A A A A A A A A A
Lactobacillus Drevis A ICC 14809 (gadA 4/9 aa EKK45096.1)		
Lactobacinus Drewis CUMUC 1500 (gadA 4/9 a AET81112.1)		370 380 390 400
Lactopacitius previs DmCS 003 (gadA 4/9 aa KiD43894,1)		
Lactobacinus Drevis BSO 404 (gadA 4/9 aa AJA/9130.1) gadA	gadB (468 aa)	QEKTHDVARY LAKSLTKLGG FSLINDGHEL PLICYELTAD
Lactobachius brevis KB290 (gatA 480 aa BAR05709.1)	gadA (480 aa)	MQNVRKVSLK LTAALKTYGI FDILVDGSQL PINCWKLADD
Lactobachius brevis ATCC_507 (gadA_479_aa_ABJ65255.1)	Consensus	V L L G F DG L P C L D
Lactobacilius brevis AG48 (gadA 4/9 aa NZ JAGR010000011)		
Lactobacillus brevis EW (gadA 469 aa NZ AUTD01000011.1)		
Lactobacillus_zymae_GU240_(4/9_aa_AHF/2525.1)		410 420 430 440
1 11 ND00 (150 D00 (150		the second secon
Streptococcus_thermophilus_ND03_(459_aa_ADQ62455.1)		
Streptococcus_thermophilus_ND03_(459 aa_ADQ62455.1) Streptococcus_thermophilus_TH1435_(459 aa_ETE42297.1)	gadB (468 aa)	SDREWTLYDL SDRLLMKGWQ VPTYPLPKNM TDRVIQRIVV
Streptococcus thermophilus IND03 (459 aa ADQ62455.1) Streptococcus thermophilus IN1433 (459 aa ETE4297.1) Streptococcus thermophilus IF8CT (459 aa EWM55323.1)	gadB (468 aa) gadA (480 aa)	SDREWTLYDL SDRLLMKGWQ VPTYPLPKNM TDRVIQRIVV APVGWTLYDL ESELAKYGWQ VPAYPLPKNR DDVTISRIVV
Streptococcus, thermophilus, ND03 (459 aa, ADQ62455.1) Streptococcus, thermophilus, TH1435 (459 aa, ETE42297.1) Streptococcus, thermophilus, IF8CT (459 aa, EWM55323.1) Streptococcus, thermophilus, TH985 (459 aa, EWM55821.1)	gadB (468 aa) gadA (480 aa) Consensus	SDREWTLYDL SDRLLMKGWQ VPTYPLPKNM TDRVIQRIVV APVGWTLYDL ESELAKYGWQ VPAYPLPKNR DDVTISRIVV WTLYDL L GWQ VP YPLPKN D I RIVV
Streptococcus thermophilus [ND03] (459 aa ADQ62455.1) Streptococcus thermophilus [TH1435 (459 aa ETE42297.1) Streptococcus thermophilus [F8CT (459 aa EWM55323.1) Streptococcus thermophilus [TH955 (459 aa EWM55821.1) Streptococcus thermophilus [TH952 (459 aa EWM55821.1) Streptococcus thermophilus [TH954 (459 aa EWM55821.1)	gadB (468 aa) gadA (480 aa) Consensus	SDREWILYDL SDRLIMKGWQ VPTYPLPKNM TDRVIQRIVV APVGWILYDL ESELAKYGWQ VPAYPLPKNR DDVIISRIVV WILYDL L GWQ VPYPLPKN D I RIVV
Streptococcus thermophilus TH435 (459 aa ADQ62455.1) Streptococcus thermophilus TH435 (459 aa ETE4297.1) Streptococcus thermophilus TF8CT (459 aa EWM55323.1) Streptococcus thermophilus 72 (459 aa EWM55821.1) Streptococcus thermophilus 72 (459 aa AB31651.2) Streptococcus thermophilus 72 (459 aa EWM60131.1)	gadB (468 aa) gadA (480 aa) Consensus	SDREWTLYDL SDRLIMKGWQ VPTYPLPKNM TDRVIQRIVV APVGWTLYDL ESELAKYGWQ VPAYPLPKNR DDVTISRIVV WTLYDL L GWQ VP YPLPKN D I RIVV 450 460 470 480
Streptococcus, thermophilus_ID03_(459 aa_ADQ62455.1) Streptococcus, thermophilus_IF4CT_(459 aa_ETE42297.1) Streptococcus, thermophilus_IF8CT_(459 aa_EWM55323.1) Streptococcus, thermophilus_IY2_(459 aa_EWM55321.1) Streptococcus, thermophilus_IY2_(459 aa_ABI31651.2) Streptococcus, thermophilus_IT11477_(459 aa_EWM60131.1) Bifidobacterium_angulatum_DSM_20098_(478 aa_EEP21189.1)	gadB (468 aa) gadÀ (480 aa) Consensus	SDREWTLYDL SDRLIMKCMQ UPTYPLPKNM TDRUTQRIVV APVGWTLYDL ESELAKYGWQ VPAYPLPKNN DUVISRIVV WTLYDL L GWQ VPAYPLPKNN D I RIVV 450 460 470 480 490 490 490 400
Streptococcus thermophilus TM143 (459 aa LADQ62455.1) Streptococcus thermophilus TH143 (459 aa ETEV2297.1) Streptococcus thermophilus TH88 (459 aa EWM55323.1) Streptococcus thermophilus TH88 (459 aa EWM55221.1) Streptococcus thermophilus TV2 (459 aa AB31651.2).1) Streptococcus thermophilus TV2 (459 aa EWM60131.1) Bifidobacterium angulatum DX0008 (478 aa EEP21189.1) Bifidobacterium angulatum LMG 11039 (478 aa KF138755.1)	gadB (468 aa) gadA (480 aa) Consensus gadB (468 ac)	SDREWTLYDL SDRLIMKGWQ VPTYPLPKNM TDRVIQRIVV APVGWTLYDL ESLAKYGWQ VPAYPLPKNR DDVTISRIVV WTLYDL L GWQ VP YPLPKN D I RIVV 450 460 470 480 RADFGKSMAH DFIDDITOAI HDLFOAHTUF HSDPAPUYG
Streptococcus, thermophilus_IN03_(459 aa_IDQ62455.1) Streptococcus, thermophilus_ITH1435_(459 aa_EEWA552297.1) Streptococcus, thermophilus_IF8CT_(459 aa_EWM55323.1) Streptococcus, thermophilus_Y2_(459 aa_BAB31651.2) Streptococcus, thermophilus_Y2_(459 aa_BAB31651.2) Streptococcus, thermophilus_Y2_(459 aa_BAB31651.2) Infidobacterium_angulaturu_IA06_11039_(478 aa_EFP138755.1) Bifdobacterium_merycicum_LMG_11341_(483_aa_EFP138755.1) Bifdobacterium_merycicum_LMG_11341_(483_aa_EFP13875.1)	gadB (468 aa) gadA (480 aa) Consensus gadB (468 aa) gadB (468 aa)	SDREWTLYDL SDRLIMKGMQ VPTYPLPKNM TDRVTQRIVV APVGWTLYDL ESELAKYGMQ VPAYPLPKNR DDVTISRIVV WTLYDL L GWQ VP YPLPKN D I RIVV 450 460 470 480 RAPFGKSMAH DFIDDLTQAI HOLDQAHIVF HSDPQPKKYG DBSNTWTLAD DFLDDLKAL DEINHEGUT TTUDANATT
Streptococcus thermophilus [ND03] (459 aa_ADQ62455.1) Streptococcus thermophilus [TH1435 (459 aa_ETE4297.1) Streptococcus thermophilus [F8CT (459 aa_EWM55823.1) Streptococcus thermophilus [TH985 (459 aa EWM55821.1) Streptococcus thermophilus [TH1477 (459 aa_BEM55812.1) Streptococcus thermophilus [TH1477 (459 aa_EWM5131.1) Bifidobacterium angulatum [DMG 11034 (478 aa_EFF12189.1) Bifidobacterium merycicum [LMG 11341 (483 aa_EFF1215.1) Bifidobacterium [LMG 11341 (487 aa_EF12155.1)] Bifidobacterium [LMG 11341 (487 aa_EF121555.1)] Bifidobacterium [LMG 11341 (487 aa_EF121555.1)] Bifidobacterium [LMG 11341 (487 aa_EF121555.1)] Bifidobacterium [LMG 11341 (487 aa_EF121555.1)] Bifidobacterium [LMG 11341 (487 aa_EF1215555.1)] Bifidobacterium [LMG 11341 (487 aa_EF1215555.1)] Bifidobacterium [LMG 11341 (487 aa_EF1215555.1)] Bifidobacterium [LMG 11341 (487 aa_EF12155555.1)] Bifidobacterium [LMG 11341 (487 aa_EF12155555555555555555555555555555555555	gadB (468 aa) gadA (480 aa) Consensus gadB (468 aa) gadA (480 aa)	SDREWTLYDL SDRLLMKCMQ VPTYPLPKNM TDRVLQRIVV APVGWTLYDL ESELAKYGMQ VPAYPLPKNM DDVTISRIVV WTLYDL L GWQ VP YPLPKN D I RIVV 455 460 476 480 RADFGMSMAH DFIDDLTQAI HDLDQAHIVF HSDPQPKKYG RPSMTMTIAD DFLDDLKLAI DGLNHTFGVT TIVDDNKTT
Streptococcus thermophilus TH435 (459 aa EADQ62455.1) Streptococcus thermophilus TH435 (459 aa ETE4297.1) Streptococcus thermophilus TH485 (459 aa EWM55323.1) Streptococcus thermophilus Y2 (459 aa EWM55821.1) Streptococcus thermophilus Y2 (459 aa AB31651.2) Streptococcus thermophilus Y2 (459 aa EWM60131.1) Bifidobacterium angulatum LMG 11039 (478 aa EFP21189.1) Bifidobacterium aneruita MCG 11039 (478 aa EFP2115.1) Bifidobacterium dentium ATCC 27678 (476 aa EDF45572.1) Bifidobacterium dentium ATCC 27679 (476 aa EDF45572.1)	gadB (468 aa) gadA (480 aa) Consensus gadB (468 aa) gadA (480 aa) Consensus	SDREWTLYDL SDRLIMKGMQ VPTYPLFKNM TDRVTQRTVV APVGWTLYDL ESELAKYGMQ VPAYPLFKNR DDVTISRIVV WTLYDL L GRQ VP YPLFKN D I RIVV 455 465 475 485 RADFGMSMAH DFIDDLTQAI HDLDQAHIVF HSDPQPKKYG RPSMTWTLAD DFLDDLKLAI DGLMHTFGVT TTVDQDNKTT R M A DF DDL AI L V Q K
Streptococcus thermophilus TH1435 (459 aa EADQ62455.1) Streptococcus thermophilus TH1435 (459 aa ETF4227.1) Streptococcus thermophilus F1458 (459 aa EWM55323.1) Streptococcus thermophilus TH985 (459 aa EWM5821.1) Streptococcus thermophilus TH1477 (459 aa EWM60131.1) Bifidobacterium angulatum DSM 20098 (478 aa EFF21189.1) Bifidobacterium angulatum LMG 11039 (478 aa EFF21181.1) Bifidobacterium dentium ATCC 27678 (476 aa EFF4572.1) Bifidobacterium dentium ATCC 27678 (476 aa EFF4572.1) Bifidobacterium modkalaberse DSM 27321 (476 aa EFF4522.1)	gadB (468 aa) gadA (480 aa) Consensus gadB (468 aa) gadA (480 aa) Consensus	SDREWTLYDL SDRLIMKCMQ VPTYPLPKNM TDRVTQRIVV APVGWTLYDL ESELAKYGWQ VPAYPLPKNR DDVTISRIVV WTLYDL L GWQ VP YPLPKN D I RIVV 455 465 476 480 RADFGMSMAH DFIDDLTQAI HDLDQAHIVF HSDPQPKKYG RPSMTMTIAD DFLDDLKLAI DGLNHTFGVT TIVDQDNKTT R M A DF DDL AI L V Q K C-terminal end
Streptococcus thermophilus TH035 (459 aa EADQ62455.1) Streptococcus thermophilus TH1435 (459 aa EEWA55323.1) Streptococcus thermophilus TH885 (459 aa EEWA55323.1) Streptococcus thermophilus T2 (459 aa EWM55323.1) Streptococcus thermophilus T2 (459 aa AB31651.2) Streptococcus thermophilus T1477 (459 aa EEMA5521.1) Bifidobacterium angulatum DXD 20098 (478 aa EEP21199.1) Bifidobacterium angulatum LMG_11039 (478 aa EFP21195.1) Bifidobacterium dentium ATCC 27678 (476 aa EEP44525.1) Bifidobacterium moukalabense DSM 2732.1 (476 aa EFV4525.1) Bifidobacterium moukalabense DSM 2732.1 (476 aa EFV71896.1) Bifidobacterium moukalabense DSM 2732.1 (476 aa EFV71896.1) Bifidobacterium moukalabense DSM 2732.1 (476 aa EFV71896.1) Bifidobacterium moukalabense DSM 2732.1 (476 aa EFV71896.1)	gadB (468 aa) gadA (480 aa) Consensus gadB (468 aa) gadA (480 aa) Consensus	SDREWTLYDL SDRLIMKCHQ VPTYPLFRMM TDRUTQRIVV APVGWTLYDL ESELAKYGWQ VPAYPLFMNR DDVTISRIVV WILYDL L GWQ VP YPLFMN D I RIVV 455 445 475 485 RADFGMSMAH DFIDDLTQAI HDLDQAHIVF HSDPQFKKYG RPSMIMTIAD DFLDDLKLAI DGLMHFGVT TTVDQDNKTT R M A DF DDL AI L V Q K C-terminal end
Streptococcus thermophilus TH1435 (459 aa EFL297.1) Streptococcus thermophilus TH1435 (459 aa EFL4297.1) Streptococcus thermophilus TH854 (549 aa EFWA5523.1) Streptococcus thermophilus TH955 (459 aa EWM6131.1) Streptococcus thermophilus TH1477 (459 aa EWM60131.1) Bifidobacterium angulatum DSM 20098 (478 aa EEF21189.1) Bifidobacterium mervicum LMG 11030 (478 aa EFF21185.1) Bifidobacterium dentium ATCC 27678 (476 aa EFF4572.1) Bifidobacterium dentium ATCC 27678 (476 aa EFF4572.1) Bifidobacterium mervicum LMG 21811 (481 aa KF10852.1) Bifidobacterium munianatium LMG 21811 (481 aa KF10852.1) Bifidobacterium munianatium LMG 21811 (481 aa KF10825.1) Bifidobacterium munianatium LMG 21811 (481 aa KF10825.1) Bifidobacterium adolescentis L2-32 (482 aa EDN82390.1)	gadB (468 aa) gadA (480 aa) Consensus gadB (468 aa) gadA (480 aa) Consensus	SDREWTLYDL SDRLIMKCHQ VPTYPLPKNM TDRVTQRIVV APVGWTLYDL ESELAKYGWQ VPAYPLPKNR DDVTISRIVV WTLYDL L GWQ VP YPLPKN D I RIVV 450 440 470 480 RADFCMSMAH DFIDDLTQAI HOLDQAHIVF HSDPQPKKYG RPSMIMIIAD DFLDDLKAI DGLNHTFVT TIVDQDNKTT R M A DF DDL AI L V Q K C-terminal end
Streptococcus thermophilus TH1435 (459 aa EADQ62455.1) Streptococcus thermophilus TH1435 (459 aa ETEV297.1) Streptococcus thermophilus [F8CT (459 aa EWM55823.1) Streptococcus thermophilus [TH385 (459 aa EWM55821.1) Streptococcus thermophilus [TH1477 (459 aa EWM55821.1) Streptococcus thermophilus [TH1477 (459 aa EWM5131.1) Bifidobacterium angulatum DSM 20098 (478 aa EFP21189.1) Bifidobacterium angulatum LMG [1039 (478 aa EFF1875.1) Bifidobacterium dentium ATCC 27678 (476 aa EFW18259.1) Bifidobacterium dentium ATCC 27679 (476 aa EFW18259.1) Bifidobacterium moukialabenes DSM 27321 (476 aa EFV71896.1) Bifidobacterium adolescentis [2-32 (428 aa AE176825.1) Bifidobacterium adolescentis [2-32 (428 aa AE176842.1)] Bifidobacterium adolescentis [2-32 (452 aa AE176842.1)]	gadB (468 aa) gadA (480 aa) Consensus gadB (468 aa) gadA (480 aa) Consensus gadB (468 aa)	SDREWTLYDL SDRLIMKCMQ VPTYPLPRNM TDRVIQRIVV APVGMTLYDL ESELAKYGMQ VPAYPLPRNR DDVTISRIVV WTLYDL L GWQ VP YPLPRN D I RIVV 450 460 470 480 RADFGMSMAH DFIDDLTQAI HDLDQAHIVF HSDPQPKKYG RPSMIMTIAD DFIDDLTQAI HDLDQAHIVF HSDPQPKKYG RPSMIMTIAD DFIDDLKLAI DGLNHTFGVT TTVDQDNKTT R M A DF DDL AI L V Q K C-terminal end FTH
Streptococcus thermophilus TH435 (459 aa ELADQ62455.1) Streptococcus thermophilus TH435 (459 aa ETE4297.1) Streptococcus thermophilus TP485 (459 aa EWM55323.1) Streptococcus thermophilus T2 (459 aa EWM55821.1) Streptococcus thermophilus T2 (459 aa EWM512.1) Streptococcus thermophilus T2 (459 aa EWM60131.1) Bifidobacterium angulatum LMG_11039 (478 aa EFP21199.1) Bifidobacterium aneulatum LMG_11314 (483 aa KF1315.1) Bifidobacterium dentium ATCC 27678 (476 aa EDF24572.1) Bifidobacterium moukalabense DSM_27321 (476 aa ETF21896.1) Bifidobacterium moukalabense DSM_27321 (476 aa ETF21896.1) Bifidobacterium andelscentis L2-32 (482 aa AJF06825.1) Bifidobacterium adolescentis ZL (482 aa AJF06825.1) Bifidobacterium adolescentis ZL (482 aa AJF06825.1) Bifidobacterium adolescentis ZL (482 aa AJF06833.1)	gadB (468 aa) gadA (480 aa) Consensus gadB (468 aa) gadA (480 aa) Consensus gadB (468 aa) gadA (480 aa)	SDREWTLYDL SDRLIMKCMQ VPTYPLPKNM TDRVTQRTVV APVGWTLYDL ESELAKYGMQ VPAYPLPKNR DDVTISRIVV WTLYDL L GWQ VP YPLPKN D I RIVV 455 465 470 480 RAPFGMSMAH DFIDDLTGAI HOLDGAHIVF HSDPQPKKYG RPSMTMTIAD DFLDDLKLAI DGLNHTFGVT TTVDQDNKTT R M A DF DDL AI L V Q K C-terminal end FTH YZS [Note: Lb. brevis IFO 12005 (gadA; 480 aa:
Streptococcus thermophilus TH1435 (459 aa ET4297.1) Streptococcus thermophilus TH1435 (459 aa ET4297.1) Streptococcus thermophilus TH1435 (459 aa EWM55812.1) Streptococcus thermophilus TH1477 (459 aa EWM55812.1) Streptococcus thermophilus TH1477 (459 aa AB131651.2) Streptococcus thermophilus TH1477 (459 aa EWM5131.1) Bifidobacterium angulatum DSM (20098 (478 aa EF121189.1) Bifidobacterium angulatum CMG (1134) (443 aa EF121151.1) Bifidobacterium dentium, ATCC 27678 (476 aa ED741572.1) Bifidobacterium moukialberse DSM 27321 (476 aa EF741572.1) Bifidobacterium moukialberse DSM 27321 (476 aa EF741896.1) Bifidobacterium dolescentis L2-32 (428 aa EF19219.1) Bifidobacterium adolescentis L2-32 (478 aa EF136432.1) Bifidobacterium adolescentis Z21 (482 aa AE16633.1) Bifidobacterium stercis BBMN23 (482 aa AE16633.1) Bifidobacterium stercoris JCM _15918_(482 aa KF197074.1)	gadB (468 aa) gadA (480 aa) Consensus gadB (468 aa) gadA (480 aa) Consensus gadB (468 aa) gadB (468 aa) gadB (468 aa)	SDREWTLYDL SDRLIMKCHQ UPTYPLPKNM TDRUTQRIUV APUGWTLYDL ESELAKYGWQ UPAYPLPKNR DDVTISRIUV WTLYDL L GWQ UP YPLPKN D I RIVV 455 465 476 480 RADFCMSMAH DFIDDLTQAI HDLDQAHIVF HSDPQPKKYG RPSMIMTIAD DFLDDLKLAI DELNHTFGVI TIVDQDNKTI R M A DF DDL AI L V Q K C-terminal end FTH FTH FTH FTH FTH FTH [Note: Lb. brevis IFO 12005 (gadA; 480 aa; BAF99137 2) & L b brevis 877G (andB-A68
Streptococcus thermophilus TH143; (459 aa ET4297.1) Streptococcus thermophilus TH143; (459 aa ET4297.1) Streptococcus thermophilus TH143; (459 aa EWM55323.1) Streptococcus thermophilus T2 (459 aa EWM55323.1) Streptococcus thermophilus T1447; (459 aa EWM60131.1) Bifidobacterium angulatum DX0098 (478 aa EF21875.1) Bifidobacterium angulatum LMG_11039 (478 aa EF718755.1) Bifidobacterium dentium ATCC 27678 (476 aa ED74572.1) Bifidobacterium dentium ATCC 2770; (476 aa ED74572.1) Bifidobacterium moukialabenes DSM 27321 (476 aa ET71896.1) Bifidobacterium adolescentis L2-32; (482 aa KF108259.1) Bifidobacterium adolescentis L2-32; (482 aa KF108259.1) Bifidobacterium adolescentis L2-32; (482 aa KF10784.1) Bifidobacterium adolescentis BMN23 (482 aa KF107074.1) Bifidobacterium adolescentis BMN23 (482 aa KF107074.1) Bifidobacterium adolescentis BMN23 (482 aa KF107074.1)	gadB (468 aa) gadA (480 aa) Consensus gadB (468 aa) gadA (480 aa) Consensus gadB (468 aa) gadA (480 aa) Consensus	SDREWTLYDL SDRLIMKCMQ VPTYPLFNM TDRUTQRTUV APVGWTLYDL ESELAKYGWQ VPAYPLFNN DDVTISRIVV WTLYDL L GWQ VP YPLFNN D I RIVV 455 465 475 485 RADFCMSMAH DFIDDLTQAI HOLDQAHIVF HSDPQPKKYG RPSMTHTIAD DFLDDLKAI DGLMHFGVT TIVDQDNKTT R M A DF DDL AI L V Q K C-terminal end FTH VPS [Note: Lb. brevis IFO 12005 (gadA; 480 aa; BAF99137.2) & Lb. brevis 877G (gadB; 468
Streptococcus thermophilus TH1435 (459 aa ET4297.1) Streptococcus thermophilus TH1435 (459 aa ET4297.1) Streptococcus thermophilus TH258 (459 aa ET4297.1) Streptococcus thermophilus TH258 (459 aa EW45821.1) Streptococcus thermophilus TH25 (459 aa EW45821.1) Streptococcus thermophilus TH1477 (459 aa EW45013.1.1) Bifdobacterium angulatum DSM 20098 (478 aa EF12185.1) Bifdobacterium derium ATCC 27678 (476 aa EF741515.1) Bifdobacterium dentium ATCC 27678 (476 aa EF741515.1) Bifdobacterium dentium ATCC 27679 (476 aa EF741529.1) Bifdobacterium dentium ATCC 27679 (476 aa EF741592.1) Bifdobacterium dentium ATCC 27679 (476 aa EF741592.1) Bifdobacterium dentium ATCC 27679 (476 aa EF741592.1) Bifdobacterium adolescentis L2-32 (428 aa AL79842.1) Bifdobacterium adolescentis Z212 (482 aa AL79842.1) Bifdobacterium adolescentis Z212 (482 aa AL79842.1) Bifdobacterium ateriorius BBM232 (482 aa AL79843.1) Bifdobacterium ateriorius BBM232 (482 aa AL79633.1) Bifdobacterium stercoris JCM_15918 (482 aa KF197074.1) O.1	gadB (468 aa) gadA (480 aa) Consensus gadB (468 aa) gadA (480 aa) Consensus gadB (468 aa) gadA (480 aa) Consensus	SDREWTLYDL SDRLIMKCHQ VPTYPLPKNM TDRUTQRIVV APVGWTLYDL ESELAKYGWQ VPAYPLPKNR DDVTISRIVV WTLYDL L GWQ VP YPLPKN D I RIVV 450 460 470 480 RAPFCMSMAH DFIDDLTQAI HOLDQAHIVF HSDPQPKKYG RPSMTMTIAD DFIDDLKAI DGLNHTGVT TIVQDNKTT R M A DF DDL AI L V Q K C-terminal end FTH INote: Lb. brevis IFO 12005 (gadA; 480 aa; BAF99137.2) & Lb. brevis 877G (gadB; 468 aa; AFU61547.1) were compared.]

⁴² ACCEPTED MANUSCRIPT

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.

⁴³ ACCEPTED MANUSCRIPT

A

4	Lactobacillus_casei_LC2W_(1902_aa_AEA54764.1) Lactobacillus_casei_BD-II_(1902_aa_AEA57945.1) Lactobacillus_casei_BL23_(1902_aa_CAO67518.1)]	B Species	No. of strains with complete genome	No. of strains with cell envelope proteinase(s)	Strain(s)
	Lactobacillus_casei_UW4_(1902_aa_EKQ20054.1)		La lastia			
	Lactobacillus_paracasci_subspparacasci_NCDO_151_(1902_aa_AAA25248.1) Lactobacillus_casci_Lpc-37_(1902_aa_EKQ29015.1)		LC. IACUS	14	2	MG1363, SK11
	Lactobacillus_casei_M36_(1902_aa_EKQ08436.1) Lactobacillus_casei_32G_(1902_aa_EKP97825.1)		Str. thermophilus	9	3	LMD-9, JIM 8232, ASCC 1275
	Lactobacillus_casei_A2-362_(1902_aa_EKQ09094.1) Lactobacillus_casei_CRP28_(1902_aa_EKQ05671.1)		Lb. paracasei	3	2	JCM 8130, 8700:2
	Lactobacillus_casei_ 171499_(1902_aa_EKQ12697.1) Lactobacillus_casei_Lc-10_(1902_aa_EKQ25934.1) Lactobacillus_casei_UWI (1902_aa_EKQ19325.1)	PrtP	Lb. rhamnosus	5	3	GG, Lc 705, ATCC 8530
	Lactobacilluc_casei_UCD174_(1902_aa_EKQ17440.1) Lactococcus_lactis_subsplactis_bvdiacetylactis_HD9B_(1962_aa_ACF37494.1) Lactococcus_lactis_subsperemoris_HP_(1902_aa_EUN33251.1) Lactococcus_lactis_subsperemoris_W22_(1902_aa_AAA17677.1) Lactococcus_lactis_subsperemoris_NCD0763_(1902_aa_P15293.1) Lactococcus_lactis_S2_(1962_aa_AAA17672.1)		Lb. casei	9	9	ATCC 334, Zhang, BL23,BD-II, LC2W, 12A, W56, LOCK919, ATCC 393
	Lactococcus_lactis_subsperemoris_SK11_(1962_aa_P15292.2)		Lb. planrarum	8	2	P-8, CMPG5300
	Lactobacillus_hominis_DSM_23910 (2095_aa_CX50765.1) Lactobacillus_hominis_DSM_23910 (2095_aa_CX51856.1) Lactobacillus_helveticus_BST_11087_(2011_aa_BAO23451.1) Lactobacillus_helveticus_H10 (2011_aa_ADX70200.1) Lactobacillus_hefenenform_7W2 (2012_aa_ADX70202.1)		Lb. delbrueckii	4	4	ATCC 11842, ATCC BAA-365, ND02, 2038
	Lactobacillus_helveticus_CIRM-BIA_951_(1843_aa_CD58085.1)		Lb. reuteri	5	0	-
	Lactobacillus_helveticus_CIRM-384_(169_aa_AAD30063.1) Lactobacillus_helveticus_CIRM-381A_103_(1926_aa_CDI61811.1) — Lactobacillus_ruminis_DPC_6832_(1536_aa_KIC04660.1)		Lb. helveticus	7	4	CNRZ32, R0052, H10, H9
	Lactobacillus_delbrueckii_subsp_bulgaricus_ND02_(1965_aa_ADQ61106.1) Lactobacillus_delbrueckii_subsp_lactis_DSM_20072_(1942_aa_EGD28142.1)]	Lb. acidophilus	4	2	La-14, 30SC
	Lactobacillus_delbrueckii_subsplactis_CRL581_(1924_aa_EPB98635.1) Lactobacillus_delbrueckii_subspbulgaricus_(1813_aa_CDR72915.1)	PrtB	Lb. gasseri	2	0	-
	Lactobacillus_delbrueckii_subspbulgaricus_ATCC_11842_(1946_aa_CAI97991.1) Lactobacillus_delbrueckii_subspbulgaricus_2038_(1823_aa_ADY85200.1))	Lb. fermentum	3	0	-
	¹ Lactobacillus_delbrueckii_subspbulgaricus_ATCC_BAA-365_(1813_aa_ABJ5864 Lactobacillus_acidophilus_CIRM-BIA_442_(1627_aa_CDF69973.1)	8.1)	Lb. salivarius	4	0	
	Lactobacillus_acidophilus_DSM_9126_(1627_aa_CDF73591.1) Lactobacillus_acidophilus_DSM_20242_(1627_aa_CDF75588.1)		Lb. brevis	2	0	
	Lactobacillus_acidophilus_CIRM-BIA_443_(162/_aa_CDF/1/6/.1) Lactobacillus_acidophilus_CIP_76.13_(1627_aa_CDF68295.1)		Lb. johnsonii	4	0	-
	Lactobacillus_helveticus_CIRN-BIA_953_(1727_aa_CDI43077.1)		Lb. ruminis	1	0	-
	Lactobacillus_helveticus_CNR2.3_(178_aa_ABI13574.1) Lactobacillus_helveticus_CIRM-BIA_103_(1786_aa_CDI63718.1) Lactobacillus_helveticus_CIRM-BIA_104_(1794_aa_CD160919.1) Lactobacillus_helveticus_CIRM-BIA_104_(1794_aa_CD160919.1)		Lb. buchneri	2	2	CD034, NRRL B-30929
	- Lactobacillus salivarius_G1/24_(1542_aa_EGM49753.1)		Lb. sakei	1	0	-
	Lactobacillus_chamnesus_Df.Lt.2(197_aa_Df.L2(197.1)) Lactobacillus_chamnesus_Cf.(1494_aa_CAR91577.1) Lactobacillus_chamnesus_Cf.(1494_aa_CAR91577.1)		Lb. amylovorus	1	0	-
	Lactobacillus_chamnosus_GG_(1494_aa_CAA03029.1) Lactobacillus_chamnosus_GG_(1494_aa_CAA03138.1) Lactobacillus_champosus_R0011_(1480_aa_CAD43138.1)		Lb. sanfranciscen	sis 1	0	-
	Lactobacillus_plantarum_CMPG5300_(1482_aa_KGH41323.1)(2) Lactobacillus_plantarum_CMPG5300_(1482_aa_KGH41323.1)		Lb. kefiranofacien:	s 1	1	ZW3
	Lactobacillus classi 5 (231 as ERMA88(21)		Lb. hokkaidonensi	is 1	0	-
	Lactobacillus_paracasei_NRIC_1981_(2231_aa_GAN41075.1) Lactobacillus_paracasei_R123_(2232_aa_GA065658_1)		Lb. mucosae	1	0	-
	Lactobacillus_paracasci_subsp. paracasci_8700:2_(2178_aa_EEQ67197.1)		Lb. pentosus	1	0	-
	Lactobacillus_paracasei_NRIC_1917_(2033_aa_GAN38401.1) Lactobacillus_paracasei_NRIC_0644_(2033_aa_GAN37999.1)		Note: data was ger	nerated from NC	BI database on 2 Ju	ine 2015.
	Lactobacillus_paracasei_subspparacasei_8700:2_(1809_aa_AGT63635.1)	С	Representative strain(s)	Type Structure	of domains of different cell	envelope proteinases
I	Lactobacillus_casci_5b_(1806_aa_ERN48863.1) Lactobacillus_casci_LOCK919_(1808_aa_AGP67309.1) Lactobacillus_casci_BL23_(1809_aa_CAQ65659.1) Lactobacillus_casci_BL23_(1809_aa_CAQ65659.1)	•	Lc. lactis subsp. cremoris Wg2 Lb. paracasei NCDO 151	PrtP PP	PR A B	H
	Lactobacillus_casci_LC2W_(1809_aa_AEA52866.1) Lactobacillus_casci_BD-11_(1809_aa_AEA56029.1)		Lb. helveticus CNRZ32	PrtH PP		cell wall cell wall embrane ytoplasm
	Streptococcus_thermophilus_TH1435_(1618_aa_ETE42609.1) Streptococcus_thermophilus_JIM_8232_(1618_aa_ACL98068.1)		bulgaricus ATCC 11842	PrtB (F		B C Z C
	Streptococcus_thermophilus_CNRZ_385_(1585_aa_AAG09771.1) Streptococcus_thermophilus_ASCC_1275_(1618_aa_AIC24310.1)		Str. thermophilus CNRZ 385	PrtS	PP PR A	
0.2	(Note: protein sequences were downloaded from NCBI database on 1 June 2015.)		Lb. rhannosus BGT10	PrtR F	PP PR A	BWA

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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°C for 24 h. Triplicate analysis was performed. Data in the figure was presented as mean ± standard deviation.

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