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Sodium L-Glutamate-Induced Physiological Changes in *Lactobacillus Brevis* NCL912 During GABA Production Under Acidic Conditions

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Abstract: Previous studies showed that γ -aminobutyric acid (GABA) can protect some GABA-producing bacteria against acid stress through glutamate decarboxylation. However, the acid stress mechanism is a comprehensive network related to numerous genes and proteins. Other than the Glutamate Decarboxylase (GAD)/GABA antiporter system, limited information is available about the physiological modifications that occur in *Lactobacillus brevis* during GABA production under acidic conditions. Therefore, this study aims to investigate the physiological modifications that occur in a GABA-producing *L. brevis* NCL912 during GABA production under acidic conditions. The differential protein expression of *L. brevis* NCL912 under different culture conditions was determined by using proteome analysis. Eleven of the protein spots were identified by mass spectrometry. Among the 11 proteins, 10 were upregulated, whereas one was downregulated. The function of downregulated protein was unknown. The upregulated proteins were involved in stress response, protein synthesis, quorum sensing, glycolysis and membrane lipid synthesis. Stress response, protein synthesis, glycolysis-related proteins are general acid stress proteins, while LuxS-dependent quorum sensing system and membrane lipid synthesis-related proteins might be induced by sodium L-glutamate during GABA production under acid stress. In conclusion, sodium L-glutamate might trigger other acid-tolerance responses, except the GAD/GABA system in *L. brevis* NCL912 during GABA production under acidic conditions.

Keywords: *Lactobacillus brevis* NCL912, Sodium L-Glutamate, γ -Aminobutyric Acid, Differentially Expressed Proteins, Acid Stress

Introduction

Lactobacillus is generally regarded as safe to humans. These bacteria serve an important function in the food industry. Researchers have exerted considerable effort to select more new *Lactobacillus* strains with functional properties for fermented products because of the positive effects of lactobacillus on human health. However, only a limited number of *Lactobacillus* can be used in commercial food products or probiotics because *Lactobacillus* performance under optimal laboratory conditions might be difficult to achieve under typical processing conditions. For instance, starter strains have to resist adverse conditions encountered in industrial processes (i.e., low and high temperatures, osmotic stress, etc.) (Zamfir and Grosu-Tudor, 2014). As probiotics, *Lactobacillus* must pass the natural low pH barrier in the stomach to ensure that an

adequate number will reach the target site to induce a beneficial effect. Therefore, acid tolerance is one of the main characteristics that *Lactobacillus* should possess under stress conditions. Mechanisms contributing to the capacity of *Lactobacillus* to tolerate acidic pH are essential to the production and functionality of a probiotic culture (Azcarate-Peril *et al.*, 2004).

The γ -aminobutyric acid (GABA) is a non-protein amino acid that is widely distributed in nature and can be found in microorganisms, plants and animals. GABA is also the chief inhibitory neurotransmitter in the central nervous system of mammals (Li *et al.*, 2008). Studies showed that GABA is important to some bacteria, (such as *E.coli*, *Listeria monocytogenes*) that produce GABA through glutamate decarboxylation under acid stress (Tramonti *et al.*, 2006; Feehily *et al.*, 2014). Glutamate decarboxylase (GAD) (EC 4.1.1.15) is a pyridoxal 5'-

phosphate-dependent enzyme that catalyzes the proton-consuming conversion of L-glutamate or glutamine to GABA (Tramonti *et al.*, 2006). When bacteria are exposed to environments that cause acid stress, cytosolic acidification stimulates GAD activity and protons (H⁺) are consumed in the cytoplasm, thus increasing internal pH. The product of alkaline GABA is exported from the cell via the antiporter system. This product increases external pH and helps maintain a neutral cytoplasmic pH when external pH decreases. The GAD/GABA antiporter system is regarded as a defense mechanism of bacteria against acidic environments (Richard and Foster, 2004; Siragusa *et al.*, 2007; Small and Waterman, 1998).

Some studies reported that *Lactobacillus paracasei*, *Lactobacillus brevis*, *Lactobacillus reuti* and *Lactobacillus buchneri* are also assayed in culture media and/or in food matrices for GABA synthesis (Gobbetti *et al.*, 2010; Su *et al.*, 2011). In our previous study, *Lactobacillus brevis* NCL912 significantly converted sodium L-glutamate (L-MSG) to GABA and GAD activity was significantly enhanced under acid stress ($p < 0.05$). The putative acid stress response mechanism is referred to as the GAD/GABA antiporter system (Huang *et al.*, 2012). However, the acid stress mechanism is a comprehensive network related to numerous genes and proteins. Except for the GAD/GABA antiporter system, little is known about the physiological modifications that occur in *Lactobacillus brevis* during GABA production under acid stress. Therefore, this study investigates the physiological modifications that occur in GABA-producing *L. brevis* NCL912 during GABA production under acidic conditions. The results may provide new insights into the inducible acid stress response mechanisms of this bacterium.

Materials and Methods

Strain, Media and Culture Conditions

High GABA-producing *L. brevis* NCL912 (CCTCCM 208054) was isolated from *paocai*, a Chinese traditional fermented vegetable, in our laboratory. Modified MRS broth (MMRS) was used as the culture medium, which contained in g L⁻¹, glucose, 50; yeast extract, 12.5; soya peptone, 12.5; MgSO₄·7H₂O, 0.2; MnSO₄·4H₂O, 0.05 and Tween 80, 1 mL L⁻¹. The initial L-MSG concentration in the medium was 0.1 mol L⁻¹ (Huang *et al.*, 2012; Li *et al.*, 2008). The pH of the medium was adjusted with hydrochloric acid to pH 4.0.

Measurement of Growth Curves, GABA and pH

Strains were propagated overnight twice in MMRS at 32°C. The strains (4.3×10^8) were then added into the MMRS with or without L-MSG at pH 4.0 (± 0.1). Samples were obtained every 4 h. After that, cell growth, the variations in pH and GABA yield were measured. GABA yield was determined by pre-staining paper chromatography (Li *et al.*, 2009).

Protein Extraction and Two-Dimensional Gel Electrophoresis (2-DE)

The strain was incubated in the medium with or without L-MSG at pH 4.0 for 4 h. The cells were then collected by centrifugation at 8000×g for 10 min, washed twice with pH 7.0 phosphate-buffered saline and then dissolved in lysis buffer containing 7 M urea, 2 M thiourea, 4% 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate, 1% Isoelectric Focusing (IEF) buffer and 65 mM dithiothreitol (DTT). The lysates were centrifuged at 12,000×g for 60 min at 4°C and the supernatants were stored at -80°C until further use. Protein concentration was estimated using the Bradford assay.

A 200 µg protein sample was subjected to first-dimension IEF and loaded on pH 3.0~10.0 at 18 cm nonlinear Immobilized pH Gradient (IPG) strip (Sigma-Aldrich, St Louis, USA). IEF was carried out using the EttanIPGphor II IEF system (GE Healthcare, Piscataway, USA) at 20°C as follows: 6 h at 40 V (active rehydration), 6 h at 100 V, 0.5 h at 500 V, 1 h at 1,000 V, 1 h at 2,000 V, 1.5 h at 10,000 V and 60,000 Vh at 10,000 V. The minimal Vh applied was at least 60,000 units. After IEF, the strips were equilibrated for 15 min in an equilibration buffer (0.05 M Tris-HCl pH 8.8, 6 M urea, 20% glycerol, 2% SDS) supplemented with 2% DTT for disulfide bridge reduction. The strips were supplemented with 2.5% iodoacetamide for an additional 15 min in the same equilibration buffer for cysteine alkylation. A 2D SDS-PAGE was run in 12% polyacrylamide gels using the Bio-Rad Protean II xi cell (Bio-Rad, California, USA). Triplicate electrophoreses were performed to ensure reproducibility for each protein sample.

Gels were stained with silver, scanned using Image Scanner II (GE healthcare, Piscataway, USA) and then analyzed with Imagemaster 2D Elite 5.0 software (GE healthcare, Piscataway, USA). The chemicals for 2D electrophoresis were purchased from Sigma-Aldrich (St Louis, USA). The significant and differentially expressed protein spots ($p < 0.05$) with two fold or more increased or decreased intensity in the medium with or without L-MSG were selected and subjected to further identification using an Applied BiosystemSciex 4800 Matrix-Assisted Laser Desorption/Ionization (MALDI) Time-of-Flight (TOF)/TOF Mass Spectrometer (MS) (Applied Biosystems, Carlsbad, USA).

Protein Digestion

Silver-stained protein spots were excised from the gel, washed with MilliQ water for 10 min and then destained in a 1:1 solution of 100 mM ammonium bicarbonate and acetonitrile. After hydrating with acetonitrile and drying in a SpeedVac, all of the gel pieces were incubated with 20 µg mL⁻¹ sequencing-grade porcine trypsin (Promega, Madison USA) in 25 mM NH₄HCO₃ overnight at 37°C. The supernatants were transferred into a 200 µL microcentrifuge tube and the gels were extracted once with extraction buffer

(67% acetonitrile containing 1% trifluoroacetic acid). The peptide extract and the supernatant were pooled and completely dried by Speed Vac.

Mass Spectrometry Analysis and Database Searching

Protein digestion extracts (tryptic peptides) were resuspended with 5 μ L 0.1% trifluoroacetic acid. The peptide samples were mixed (1:1 ratio) with a saturated solution of alpha-cyano-4-hydroxycinnamic acid in 50% acetonitrile/water containing 0.1% trifluoroacetic acid; 0.8 μ L liquid was spotted onto the stainless steel sample target plates. Mass spectrometry analysis was conducted using MALDI TOF/TOF MS. The instrument was calibrated using a CalMix5 standard in reflection positive-ion mode (ABI4700 Calibration Mixture). The MS and MS/MS data were interpreted and processed using the Global Proteome Server ExplorerTM software (Version 3.6, Applied Biosystems, Carlsbad, USA). The MS spectra were obtained from each sample spot by accumulating 600 to 800 laser shots in an 800 to 4000 mass range. On the other hand, the MS/MS spectra, which were the five most abundant precursor ions per sample, were selected for subsequent fragmentation. About 900 to 1200 laser shots were accumulated per precursor ion. The 20 most intense precursors per spot were selected with a minimum signal-to-noise ratio of 50.

A combined MS and MS/MS search was first performed against the NCBI database without taxonomic restriction using the MASCOT search engine (Version 2.1, Matrix Science, London, UK). Database search parameters were set as follows: trypsin of the digestion enzyme, one missed cleavage, $p < 0.05$ significance threshold, partial modification of cysteine carboamidomethylated and methionine oxidized, non-fixed modifications, 50 ppm peptide mass tolerance and MS/MS tolerance of 0.25 Da. Known contaminant ions (keratin) were excluded. MASCOT protein scores (based on combined MS and MS/MS spectra) greater than 51 were considered to be statistically significant ($p < 0.05$). The individual MS/MS spectrum with a statistically significant (confidence interval > 95%) best ion score (based on MS/MS spectra) was also accepted.

Verification at the Transcription Level by Real-Time Fluorescence Quantitative PCR

Differentially expressed proteins required further validation at the mRNA level. The mRNA expression levels of selected proteins were determined using Real-Time Fluorescence Quantitative Polymerase Chain Reaction (RTFQ-PCR). The total RNA was extracted using a TRIzol reagent (Tiangen Biotech, Beijing, China). The Super Script III first-strand synthesis kit was used for reverse transcription-PCR (Invitrogen, Carlsbad, USA). The gene-specific primer pairs used for RTFQ-PCR were designed as shown in Table 1. The RTFQ-PCR was performed using the FTC-2000 Real-time PCR System (Funglyn, Shanghai, China) and was analyzed with a Fast Start Universal SYBR Green

Master (Roche, Basel, Switzerland) according to the manufacturer's instructions. Relative gene expression levels were determined with the comparative threshold cycle ($\Delta\Delta C_t$) method and 16S rRNA gene was used as the endogenous reference gene.

Statistical Analysis

All of the experiments were done in triplicate. Data are expressed as mean \pm S.D. ($n \geq 3$). Statistical comparisons were performed with SPSS 17.0. One-way ANOVA and student *t*-test were carried out to determine statistical significance, wherein $p < 0.05$ was considered statistically significant.

Results

Growth Curves of L. brevis NCL912, GABA Yield and pH Change in the Medium with or Without L-MSG at pH 4.0

L. brevis NCL912 was incubated in the medium with or without L-MSG at pH 4.0 for 36 h. The growth curves, production of GABA and pH change were measured and results are shown in Fig. 1. *L. brevis* NCL912 had similar growth patterns in the medium with or without L-MSG. The exponential phase of *L. brevis* NCL912 was from 4 h to 24 h and the OD_{600nm} of the strain in the medium with L-MSG was higher than that in the medium without L-MSG at the same growth period. This result suggested that L-MSG might improve the growth of *L. brevis* NCL912. In the medium with L-MSG, GABA yields increased from 0.72 to 10.46 g L⁻¹ and pH was 3.99, 4.21, 4.51, 5.18 and 6.42 at different culture times (0, 4, 8, 12 and 24 h), respectively (Fig. 1.). In the medium without L-MSG, GABA content slightly increased (from 0.68 to 1.14 g L⁻¹) before 8 h, which might be related to the minute amount of glutamate in the medium. Thus, pH slightly increased at 4 h, with 3.96, 4.10, 3.91, 3.73 and 3.41 at different culture times (0, 4, 8, 12 and 24 h), respectively. These results (Fig. 1.) suggested that *L. brevis* NCL912 tolerated acid condition by GABA accumulation and increasing the pH of the medium. However, at 4 h, similar pH and GABA content were observed and the OD_{600nm} in the medium with L-MSG was higher than that in the medium without L-MSG. This result suggested that GABA accumulation might not be the only response of *L. brevis* NCL912 in resisting acid stress.

Differentially Expressed Proteins of L. Brevis NCL912 during GABA Production under Acidic Conditions

Differentially expressed proteins of *L. brevis* NCL912 during GABA production under acidic conditions were determined in the study. The total proteins of high GABA-producing *L. brevis* NCL912 in the medium with or without L-MSG at pH 4.0 were separated by 2-DE. Image analysis revealed 26 protein spots, which clearly showed differential expressions under the two culture conditions (Fig. 2).

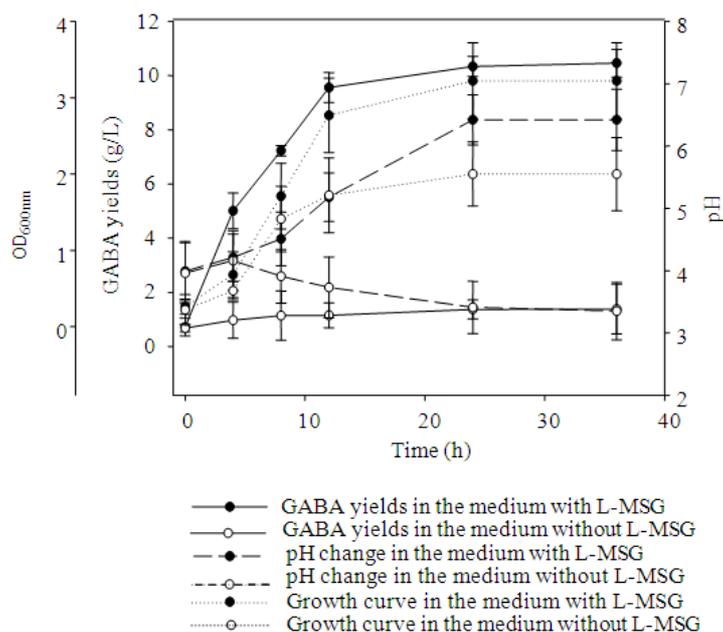


Fig. 1. The growth curves of *L. brevis* NCL912, GABA yield and pH change in the medium with or without L-MSG at pH 4.0 (Huang *et al.*, 2012)

Table 1. Primer sequences used for RTFQ-PCR

Gene	Accession no.	Primers direction	Reverse primer sequence (5' to 3')	Amplicon size (bp)
16s rRNA	4413327	Sense	CC TAC GGG AG G CAG CAG	103
		Antisense	ATT ACC GCG GCT GCT GG	
UspA	4414418	Sense	GTGGTGCCGTTAGTGGTGAC	148
		Antisense	CGGGCAATCACGGGTTTA	
GrpE	4414470	Sense	TCAGCAACTGGCTAAGGACG	146
		Antisense	GGTTACGCTTCAGGGCATCT	
S-rib	4413194	Sense	AGAAACTGGGACGAAGGGTG	112
		Antisense	CGCTAATAAATGCTCGATGGTA	
ndse	4414358	Sense	AGCAACTCGCAGCAACTGG	122
		Antisense	ATCTTCAGCGGTCCCTAACAG	

Eleven of the 26 spots were identified by matrix-assisted laser desorption/ionization time-of-flight/time-of-flight mass spectrometry. The expression levels of spots 1, 4, 5, 6, 7, 9, 10, 15, 20, 22 were upregulated in the medium with L-MSG, whereas that of spot 21 was downregulated in the medium without L-MSG (Table 2).

Cellular Localization Analysis of Identified Proteins

PSORTb version 3.0.2 (<http://www.psort.org/psortb/index.html>) was used to predict the cellular localization of the 11 identified proteins (Table 2). Six proteins localized in the cytoplasm accounting for 54.5% of the total proteins. RNA-Binding Protein (RBP) was in the outer membrane, hypothetical protein was in the periplasm and other three proteins had an unknown cellular location (Table 3).

Classification and Function Analysis of Identified Proteins

To determine the acid stress response mechanisms of that occurs in *L. brevis* NCL912 during GABA

production under acidic conditions, biological process and molecular functional data of the identified proteins were obtained from the UniProtKB protein knowledgebase (<http://www.uniprot.org>). The identified upregulated proteins were related to stress response, protein synthesis, glycolysis, cell membrane formation and cell communication. The function of the hypothetical protein BRAFLDRAFT_64486 (21) was unclear (Table 3).

RTFQ-PCR Analysis of Selected Proteins

The expression levels of the UspA family nucleotide-binding protein (UspA), molecular chaperone GrpE, S-ribosylhomocysteinase (LuxS) and Nucleoside-Diphosphate-Sugar Epimerase (NDSE) were estimated at the mRNA level by RTFQ-PCR. The RTFQ-PCR results indicated that the RTFQ-PCR analysis data matched the protein expression levels from 2-DE (Fig. 3). The mRNA expression levels of UspA protein, GrpE and NDSE significantly increased in the medium with L-MSG ($p < 0.05$).

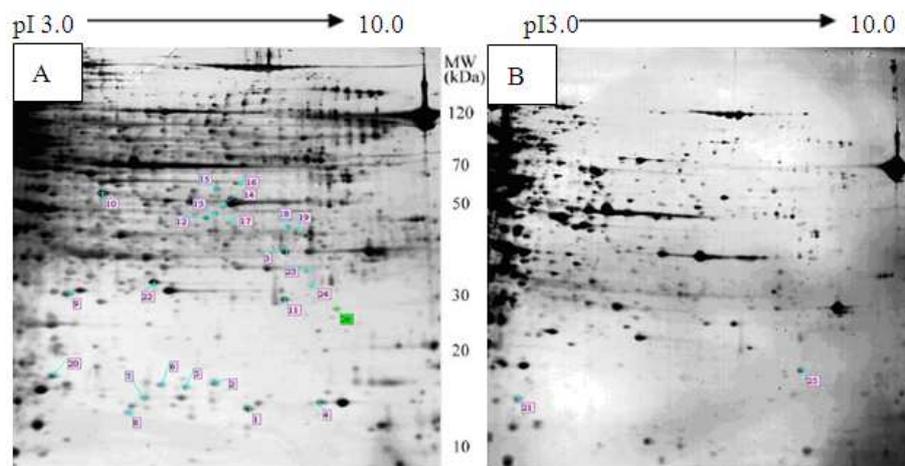


Fig. 2. 2-DE maps of proteins extracted from *L. brevis* NCL912 in the medium (A) with or (B) without L-MSG at pH 4.0. The spots were visualized by silverstaining. Differentially expressed protein spots for each treatment were marked with numbers (Huang *et al.*, 2012)

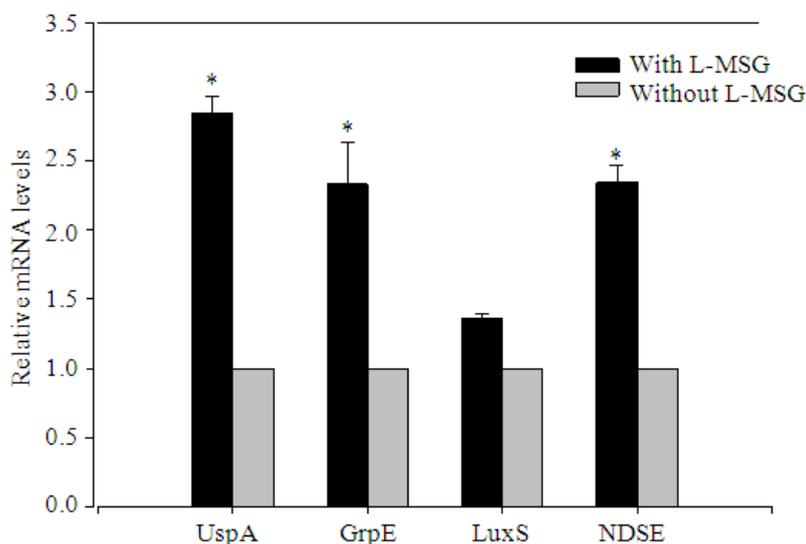


Fig. 3. Relative mRNA levels of selected differentially expressed proteins in *L. brevis* NCL912 as revealed by RTFQ-PCR. Significant differences in gene expression are indicated with asterisks (*) ($p < 0.05$). UspA, UspA family nucleotide-binding protein; GrpE, molecular chaperone GrpE; LuxS, S-ribosylhomocysteinase; NDSE, nucleoside-diphosphate-sugar epimerase

Table 2. Identified differentially expressed proteins of *L. brevis* NCL912 during GABA production under acid conditions

Spots	Protein	Accession no.	Locus	Theor. Mr (Da)/Pi	Number of peptides matched	Protein score	Sequence coverage (%)	Total ion score	Cellular localization
1	UspA	gi116332842	YP_794369.1	16474.8/6.82	22	234	14	109	Unknown
4	UspA	gi116334159	YP_795686	16883/9.16	16	86	10	60	Cytoplasm
5	RBP	gi221056514	XP_002259395.1	183415.4/5.79	12	92	6	55	Outer Membrane
6	PBP	gi116332861	YP_794388.1	18565.4/5.71	16	72	9	58	Unknown
7	LuxS	gi116333010	YP_794537.1	17653.8/5.48	18	80	9	61	Cytoplasm
9	GrpE	gi116333936	YP_795463.1	21231.7/4.6	11	101	5	52	Cytoplasm
10	GAPDH	gi42784288	NP_981535.1	35757.3/5.37	45	320	13	209	Cytoplasm
15	GPAT	gi116333592	YP_795119.1	36704/5.48	22	88	6	88	Cytoplasm
20	EF-Tu	gi116333991	YP_795518	43612.1/4.73	16	219	4	154	Cytoplasm
21	HPB	gi219408088	XP_002201859.1	729515.9/5.62	14	94	6	63	Periplasm
22	NDSE	gi116332989	YP_794516.1	22722.7/5.47	14	119	6	100	Unknown

UspA, UspA family nucleotide-binding protein; GrpE, molecular chaperone GrpE; LuxS, S-ribosylhomocysteinase; NDSE, nucleoside-diphosphate-sugar epimerase; RBPs, RNA-binding proteins; EF-Tu, elongation factor Tu; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GPAT, putative glycerol-3-phosphate acyltransferase PIsX, PBP, phospholipid-binding protein; HPB, Hypothetical protein BRAFLDRAFT_64486

Table 3. Cellular localization and function of identified differentially expressed proteins of *L. brevis* NCL912 during GABA production under acid conditions

Protein	Spots	Cellular localization	Biological process	Molecular Function annotation
pH 4.0 stress-induced proteins				
UspA	1	Unknown	Response to stress	Required for resistance to DNA-damaging agents; Involved in stress response
UspA	4	Cytoplasm	Response to stress	Required for resistance to DNA-damaging agents; Involved in stress response
GrpE	9	Cytoplasm	Response to stress; protein folding	Chaperone
Protein synthesis-related proteins				
RBP	5	OuterMembrane	Translation	Nucleic acid binding
EF-Tu	20	Cytoplasm	Protein biosynthesis	Translation elongation factor activity
Glycolysis-related proteins				
GAPDH	10	Cytoplasm	Glucose metabolic process	Oxidoreductase activity
Quorum sensing-related proteins				
LuxS	7	Cytoplasm	Quorum sensing	S-ribosylhomocysteine lyase activity
Membrane lipid synthesis-related proteins				
GPAT	15	Cytoplasm	Fatty acid biosynthetic process	Transferase activity, transferring acyl groups other than amino-acyl groups
NDSE	22	Unknown	-	-
PBP	6	Unknown	-	-
pH 5.0 Unknown function				
HPB	21	Periplasm	-	-

UspA, UspA family nucleotide-binding protein; GrpE, molecular chaperone GrpE; LuxS, S-ribosylhomocysteinase, NDSE, nucleoside-diphosphate-sugar epimerase; RBPs, RNA-binding proteins; EF-Tu, elongation factor Tu; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GPAT, putative glycerol-3-phosphate acyltransferasePlsX, PBP, phospholipid-binding protein; HPB, Hypothetical protein BRAFLDRAFT_64486

The LuxS mRNA expression level was also upregulated, although not significantly, in the medium with L-MSG (Fig. 3). These findings are attributed to the differences in the regulation mechanisms, such as synthesis and degradation rates, that act on mRNA and protein synthesis and ultimately affect the molecular levels (Jianke *et al.*, 2010).

Discussion

In this present study, *L. brevis* NCL912 could grow at pH 4.0, GABA yields and pH increased with culture time. This finding suggested that the acid tolerance properties of *L. brevis* NCL912 might be related to pH increase and GABA accumulation. The putative acid stress response mechanism is referred to as the GAD/GABA antiporter system and the mechanism was reported in our previous study (Huang *et al.*, 2012). However, the strain grew better in the medium with L-MSG than in the medium without L-MSG at 4 h, during which the pH and GABA content were similar. This result suggested that the GAD/GABA antiporter system might not be the only response of *L. brevis* NCL912 in resisting acid stress. At present, except for the GAD/GABA antiporter system, little is known about the physiological modifications that occur in *L. brevis* NCL912 during GABA production under acid conditions. Therefore, the physiological modifications that occur in GABA-producing *L. brevis* NCL912 during GABA production under acidic conditions were investigated in this study. Here, the expressions of stress response, protein synthesis, glycolysis, LuxS-dependent QS

system and membrane lipid synthesis-related proteins were up-regulated. All these suggested that stress response, protein synthesis, glycolysis-related proteins are general acid stress proteins, while LuxS-dependent QS system and membrane lipid synthesis-related proteins might be induced by L-MSG during GABA production under acid stress. The up-regulation of these proteins protects *L. brevis* NCL912 against acid stress. However, proteomic analyses failed to detect GAD among the differentially expressed proteins, although this result does not absolutely exclude that GAD expression was modulated by glutamate because GAD might be among the unidentifiable spots in very low amounts or because the differential expression level of GAD is less than twofold under L-MSG-stimulated conditions than under control conditions. The result is consistent with the mRNA levels of *gadA* in *L. brevis* NCL912 reported by Li *et al.* (2013), who found that *gadA* is only GAD encoding gene in *L. brevis* NCL912. The mRNA levels of *gadA* in *L. brevis* NCL912 were almost identical during the 0 h to 12 h culture period in the medium with or without glutamate.

Stress Response Proteins

Proteomic results indicated that the expressions of UspA protein and GrpE upregulated in this study. Previous studies showed that UspA protein and GrpE are essential stress-response proteins and are both essential components of the acid stress response network (Chen *et al.*, 2006; Licandro-Seraut *et al.*, 2008; Zhang and Griffiths, 2003; Zininga and Shonhai, 2014).

UspA serves a regulatory function in biofilm formation and DNA repair, as well as affects the motility and adhesion of bacterium under stress conditions (Chen *et al.*, 2006). Meanwhile, GrpE contributes to the maturation of newly synthesized polypeptides, promotion of protein refolding and degradation of denatured proteins under stress (Fukuda *et al.*, 2002). The observed over expression of UspA and GrpE suggests that *L. brevis* NCL912 tolerates acid stress conditions during GABA production under acidic conditions by regulating the expression of *L. brevis* NCL912 stress-response proteins.

Protein Synthesis-Related Proteins

RNA-Binding Proteins (RBPs) and elongation factor Tu (EF-Tu) in *L. brevis* NCL912, which are related to protein synthesis, were upregulated in this study. RBPs serve key functions in the post-transcriptional regulation of gene expression that involves editing, degradation and translation (Lee and Schedl, 2006). These processes can occur at many different steps during RNA metabolism. EF-Tu, one of the prokaryotic elongation factors that participate in the protein translation of ribosome, is remarkably conserved and is responsible for achieving accurate ribosome translation. This factor transiently mediates the selection and binding of the cognate aminoacyl-tRNA to the acceptor site of the ribosome, thus serving a crucial function in protein synthesis (Dallo *et al.*, 2012). The upregulation of these proteins indicates protein synthesis is activated. Such activation may have protected *L. brevis* NCL912 by allowing it to tolerate acidic conditions.

Glycolysis-Related Proteins

The expression of glyceraldehyde-3-phosphate dehydrogenase (GAPDH), a key enzyme in glycolysis that reversibly catalyzes the oxidation and phosphorylation of glyceraldehyde-3-phosphate to 1,3-diphospho-glycerate, was altered under different conditions in the study. This finding indicates that glycolysis may be an essential metabolic pathway for anaerobic bacteria and facultative anaerobes in response to various stressful conditions. GAPDH is also a multifunctional protein that affects membrane fusion, gene transcription, DNA replication, DNA repair and nuclear RNA export (Wouters *et al.*, 2000).

The observed upregulation of GAPDH suggests that *L. brevis* NCL912 obtained additional energy for growth and survival by affecting the glycolysis pathway during GABA production under acidic conditions. The result is the same as that observed in *Lactococcus lactis* NCDO 2118 (Mazzoli *et al.*, 2010).

Quorum Sensing-Related Proteins

Quorum Sensing (QS) is a system of stimulus and response correlated to population density. It is the best characterized as a means of communication within bacteria (Sun *et al.*, 2014). Many species of bacteria use

QS to coordinate gene expression and regulate quorum behavior according to the density of their local population which allows bacteria to rapidly adapt to surrounding changes (Gölz *et al.*, 2012).

Bacteria use QS constitutively produce and secrete certain signaling molecules: (1) production of small biochemical signal molecules by bacterial cell; (2) release of the signal molecules into the surrounding environment; and (3) recognition of the signal molecules by specific receptors once they exceed a threshold concentration, leading to (4) changes in gene regulation (Galloway *et al.*, 2011). In the Gram-negative bacterium, autoinducer-2 (AI-2)-mediated system is the main signaling system of QS. The enzyme LuxS, which is widely conserved throughout the bacterial kingdom, is responsible for AI-2 biosynthesis (Plummer, 2012). LuxS catalyzes the cleavage of the thioether bond in *S*-ribosylhomocysteine (SRH) to produce homocysteine and 4,5-dihydroxy-2,3-pentanedione (DPD), which undergoes spontaneous rearrangements to form a variety of DPD derivatives that interconvert and exist in equilibrium (known as the AI-2 pool). AI-2 responses in bacteria can be triggered by different members of the AI-2 compound pool (Rajan *et al.*, 2005).

In the present study, LuxS in *L. brevis* NCL912 were up-regulated during GABA production under acidic conditions. Therefore, activated LuxS enzyme is assumed to produce more AI-2, the signal molecule is transported into the external environment to trigger QS system, which protects *L. brevis* NCL912 to tolerate low acid conditions. Moslehi-Jenabian *et al.* (2009) also considered that AI-2 signaling system is induced by acidic shock in probiotic strains of *Lactobacillus spp.*

Membrane Lipid Synthesis-Related Proteins

Cell membrane plays critical roles in the maintenance of cell structure and in the modulation of cell function which serves as a barrier of separating the interior of all cells from the outside environment. It is one of the most sensitive parts in response to stressful conditions. Stressful conditions could lead to the damage of cell membrane (Miajlovic and Smith, 2014). In this study, the expressions of Phospholipid-Binding Protein (PBP), glycerol-3-phosphate acyltransferase PlsX (GPAT), GAPDH and NDSE were up-regulated.

GAPDH and GPAT are the key enzymes of phospholipid synthesis. Phospholipid is the major component of cell membrane (Roy, 2009). GAPDH catalyzes dihydroxyacetone phosphate (DHAP) into glycerol-3-phosphate and DHAP is from the glycolytic pathway. GPAT transfers fatty acid from fatty acyl coenzyme A to glycerol-3-phosphate to produce lysophosphatidic acid (LysoPA). LysoPA is further acylated by a separate acyltransferase to produce Phosphatidic Acid (PA), a key intermediate in membrane

phospholipid synthesis (Lu *et al.*, 2006). PBP transfers phospholipid to cell membrane.

A previous report showed that NDSE catalyzes the interconversion of Uridine Diphosphate Galactose (UDP-Gal) and Uridine Diphosphate Glucose (UDP-Glu). UDP-Gal serves a function in the biosynthesis of monogalactosyldiacyl glycerol (MGDG), a major galactolipid of cell membrane (Li *et al.*, 2011).

In the present study, L-MSG induces the up-regulations of PBP, GPAT, GAPDH and NDSE expressions in *L. brevis* NCL912 during GABA production under acidic conditions. The cluster of proteins is related to the synthesis of phospholipid and glycolipids which are the major components of cell membrane. That is, L-MSG may protect *L. brevis* NCL912 against acid stress by regulating lipid synthesis related to cell membrane formation.

Conclusion

In conclusion, the physiological changes observed in *L. brevis* NCL912 during GABA production under acidic conditions include the over expression of particular stress proteins, regulation of protein synthesis and cell communication and participation in glucose catabolism and cell membrane formation. These findings suggest that during GABA production under acidic conditions, L-MSG might trigger other acid stress response mechanisms aside from the GABA/GAD system.

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Author's Contributions

The contribution made by each author to the research as follow:

Gui-Dong Huang: was responsible for the whole study and wrote paper.

Zhongwei Ji: Participated in qRT-PCR analysis.

Askaer Alati: Participated in the bio information analysis.

Jian Mao: Modified paper and was responsible for all the things.

Conflict of Interest

The authors declare no competing financial interest.

Ethics

All authors have read and approved the manuscript.

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