Original research article

Restoration of GABA production machinery in *Lactobacillus brevis* by accessible carbohydrates, anaerobiosis and early acidification

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Abstract

Lactobacillus brevis is an efficient cell factory for producing bioactive γ-aminobutyric acid (GABA) by its *gad* operon-encoded glutamic acid decarboxylase (GAD) system. However, little mechanistic insights have been reported on the effects of carbohydrate, oxygen and early acidification on GABA production machinery in *Lb. brevis*. In the present study, GABA production from *Lb. brevis* was enhanced by accessible carbohydrates. Fast growth of this organism was stimulated by maltose and xylose. However, its GABA production was highly suppressed by oxygen exposure, but was fully restored by anaerobiosis that up-regulated the expression of *gad* operon in *Lb. brevis* cells. Although the level of cytosolic acidity was suitable for the functioning of GadA and GadB, early acidification of the medium (ipH 5 and ipH 4) restored GABA synthesis strictly in aerated cells of *Lb. brevis* because the expression of *gad* operon was not up-regulated in them. We conclude that GABA production machinery in *Lb. brevis* could be restored by accessible carbohydrates, anaerobiosis and early acidification. This will be of interest for controlling fermentation for synthesis of GABA and manufacturing GABA-rich fermented vegetables.

Keywords: GABA, *Lactobacillus brevis*, carbohydrate, oxygen, acid resistance

1. Introduction

There are a variety of fermented vegetables in Asia such as Chinese sauerkraut, pickles, Japanese Natto and Korean kimchi. These fermented vegetables are prepared using several microorganisms including *Lactobacillus* for the formation of flavor, taste and bioactive compounds during the natural fermentation process (Leroy and De Vuyst, 2004). Among them, lactic fermentation of vegetables by their consortium of lactic acid bacteria (LAB) is an ancient method for the home-made healthy cuisines, but also an excellent preservation strategy to extend the shelf life of vegetables (Leroy and De Vuyst, 2004). Thus, LAB play a key role in the quality of fermented vegetables, and LAB flora is normally influenced by several environmental factors such as the carbohydrate source (from vegetables or added), oxygen and the acidity of juice during the fermentation process.

γ-Aminobutyric acid (GABA) has been documented as anti-hypertensive and anti-depressant compound in the past decade (Diana et al., 2014). However, its content in natural animal- and plant-based food products is very low, thus attentions have been paid to GABA-producing bacteria, especially food-grade bacteria such as LAB and bifidobacteria (Dhakal et al., 2012; Li and Cao, 2010; Wu and Shah, 2016). These microorganisms are preferable candidates as food starter cultures for manufacturing GABA-rich fermented foods (Wu et al., 2015; Wu and Shah, 2016). Among the identified isolates of GABA producers, *Lb. brevis* is a key species of high GABA-producing LAB (Wu and Shah, 2016). Our recent study highlighted the common presence of *gad* operon-encoded glutamic acid decarboxylase (GAD) system in *Lb. brevis* for GABA production; also its efficient GABA synthesis is associated with the GadA that retained its activity towards cytosolic near-neutral pH range of *Lb. brevis* (Wu et al., 2017). Thus, it was

found that *Lb. brevis* could be a promising cell factory for manufacturing GABA and GABA-rich fermented foods.

Usually, *Lb. brevis* could catabolize several carbohydrates based on the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways of completely sequenced strains of *Lb. brevis* ATCC 367 and KB290. This species could metabolize xylose simultaneously with glucose (Kim et al., 2009), maltose (Gobbetti et al., 1995), and fructose (Neveling et al., 2012). During the batch fermentations, glucose was normally added as the carbon source for the production of GABA from *Lb. brevis* (Li, Qiu, Gao, et al., 2010; Li, Qiu, Huang, et al., 2010; Zhang et al., 2012). A previous study applied maltose to the medium optimization for *Lb. brevis* (Binh et al., 2014). There are natural LAB flora in vegetables and the environment; these are vital to promote the growth of *Lb. brevis*, which is also one of dominant species in Korean kimchi products (Jung et al., 2014). However, catabolism of varying accessible carbohydrates by *Lb. brevis* and their effects of bacterial growth have not been well compared.

In addition, a rapid reduction in the transformation efficiency of glutamate to GABA was observed in the immobilized cells of *Lb. brevis* after exposure to oxygen by shaking incubation in sodium acetate buffer (Huang et al., 2007). Recently, overexpression of a heme-dependent catalase in *Lb. brevis* counteracted the inhibition of GABA production from this organism after the H_2O_2 treatment or long-term aerated growth in glucose-based de Man, Rogosa and Sharpe (MRS) broth (Lyu et al., 2016). However, genetically modified microorganism is normally prohibited for use in food fermentations such as Korean kimchi and yogurt, but may be allowed for the manufacture of functional compounds that can be further purified. Thus, it would be necessary to seek other solutions that are able to restore the GABA synthesis in aerated cells of

Lb. brevis. Our study has demonstrated that GABA synthesis was highly associated with acid resistance in *Lb. brevis* (Wu et al., 2017). However, the effects of early acidification on the GABA production from aerated and unaerated cells of *Lb. brevis* have not been well characterized. Thus, this study aimed to investigate the effects of environmental factors including carbon source, oxygen and acidification on the GABA production from *Lb. brevis* and provide mechanistic insights into this bio-machinery.

2. Materials and methods

2.1.Bacterial strain and cultivation conditions

High GABA-producing *Lb. brevis* NPS-QW-145 (hereafter *Lb. brevis* 145), a sequenced strain isolated from Korean kimchi (Wu and Shah, 2015; Wu et al., 2017), was cultivated in Lactobacilli MRS medium (BD Company, Franklin Lakes, NJ, USA). Unless otherwise stated, *Lb. brevis* 145 used in this study was anaerobically cultivated at 37 ℃ in the above medium.

2.2.Bacterial growth assay on different carbohydrates

The modified MRS (mMRS) broth (pH 6.5) was prepared by dissolving 10 g peptone, 10 g beef extract, 5 g yeast extract, 1 g Tween-80, 2 g sodium citrate, 5 g sodium acetate, 0.1 g magnesium sulfate, 0.05 g manganese sulfate, 2 g dipotassium phosphate and 20 g selected carbohydrate (glucose, lactose, galactose, sucrose, fructose, maltose, mannose, xylose or cellobiose) in 1 L distilled water, and the pH of the medium was adjusted to pH 6.5 with hydrochloric acid before autoclaving at 121℃ for 15 min. The mMRS without sugar was also prepared as the negative control for the bacterial cultivation. The growth of *Lb. brevis* 145 in mMRS broth supplemented with different carbohydrate was measured as previously described

with minor modifications (Alcantara et al., 2014). Briefly, a volume of 200 µL of broth after inoculation with *Lb. brevis* 145 (18-h fresh culture; 1% v/v) was loaded into 96-well microplates. Additional 50 µL of sterile mineral oil was added to cover the surface of the broth to create anaerobic condition and to avoid the contamination with air-borne bacteria during the incubation and measurement. The bacterial growth was monitored at the absorbance of 600 nm in Multiskan^{TM} GO Microplate Spectrophotometer (Thermo Scientific) at 37℃ within 48 h.

In addition, an aliquot of 10 mL of mMRS broth containing different carbohydrate inoculated with *Lb. brevis* 145 (18-h fresh culture; 1% v/v) was anaerobically incubated at 37℃ for 48 h. Subsequently, acids and amino acids in the culture supernatants were profiled.

2.3.Measurements of acids and amino acids

Concentrations of lactic acid, acetic acid, GABA and glutamate in the culture supernatants were analyzed by HPLC as previously described (Wu et al., 2017).

2.4.Assessment of anaerobic and aerobic conditions on GABA production

Culture of *Lb. brevis* 145 was propagated in Lactobacilli MRS broth for 18 h prior to the inoculation into 300 mL of the following two media. A glutamate-rich medium – Lactobacilli MRS broth containing 10 g/L monosodium glutamate (MSG) was used for monitoring the GABA production under aerobic and anaerobic conditions. In order to mimic the salty condition caused by supplementation of MSG to Lactobacilli MRS broth, 3.46 g/L of sodium chloride (NaCl) was supplemented to the MRS as a control medium (Lactobacilli MRS broth containing 3.46 g/L NaCl). The acidity of Lactobacilli MRS broth containing either MSG or extra NaCl decreased from pH 6.75 to pH 6.25 after the inoculation with 18-h fresh cultures at the size of 1%

(v/v). For the aerobic condition, the 1-L conical flask was covered with sterilized cotton and kept in a shaking incubator (37℃ and 200 rpm). A 2 cm-layer of sterilized mineral oil was used to cover the surface of the medium broth in a screw-topped bottle in order to create an anaerobic condition during the shaking incubation (37℃ and 100 rpm). Samples were collected every 2 hours for the measurement of cell viability, acids and amino acids during the 24-h course of the cultivation.

2.5.Assessment of early acidification on the GABA production from Lb. brevis under aerobic and anaerobic conditions

To investigate the effects of initial acidification on the GABA production from *Lb. brevis*, the initial pH (ipH) of Lactobacilli MRS broth containing 10 g/L of MSG was adjusted to pH 6, pH 5 and pH 4 with hydrochloric acid before autoclave at 121℃ for 15 min. After inoculation of the medium broth with the fresh 18-h cultures of *Lb. brevis* 145, anaerobic and aerobic incubation were carried out as described in the above section. Samples were collected after 24 h for the measurements of cell viability, acids and amino acids. However, due to the introduction of lactic acid from the 18-h fresh cultures into the fresh medium broth, the acidity of Lactobacilli MRS broth (ipH 6) decreased to pH 5.8, but there were no changes in the pH values of Lactobacilli MRS broth (ipH 5 and ipH 4) after inoculation.

2.6.Total RNA extraction, cDNA synthesis and real-time quantitative PCR assay

Hot SDS/phenol-based total RNA extraction method for Gram-positive bacteria, DNase I treatment of total RNA samples, cDNA synthesis of total RNA, and RT-qPCR assay were carried out as per previously described (Wu et al., 2017). The expression of target genes (*gadR*,

136 *gadA*, *gadB* and *gadC* in *Lb. brevis* 145) listed in Table 1 was quantified by RT-qPCR assay.
137 Reference gene, *tuf* in *Lb. brevis* (Table 1), was used to normalize the expression of target genes.
138 The efficacy of qPCR amplification using each pair of primers was in the range of 90-110% and
139 non-specific amplification products including primer dimers were not detected by melt curve
140 analysis and agarose gel electrophoresis (data not shown). The comparative critical threshold
141 method ($2^{-\Delta\Delta Ct}$) was used to calculate the relative gene expression. The RT-qPCR assay was
142 performed in duplicates for each cDNA sample and independent experiments were carried out in
143 triplicates.

144 ***2.7.Measurements of extracellular pH (pH_{ex}) and intracellular pH (pH_{in}) of bacterial cells***

145 Extracellular pH (pH_{ex}) of the cultures was directly measured by a pH meter. The fluorescent
146 probe, 5(6)-carboxyfluorescein diacetate N-succinimidyl ester (cFDA-SE; Thermo Fisher
147 Scientific), was used to stain bacterial cells for the pH_{in} measurement as previously described
148 (Wu et al., 2017). Briefly, the pH_{ex} of cultures was measured first, and bacterial cells were
149 centrifuged, washed and re-suspended in phosphate-buffered saline (PBS) where its pH level was
150 adjusted to the pH_{ex} of the cultures. The cell density was adjusted to an optical density (λ=600)
151 of 0.6-0.7 before cFDA-SE staining procedure. The stained cells were centrifuged to remove
152 residual cFDA-SE probe in the PBS, and were re-suspended in the PBS buffer again with the
153 same pH_{ex} prior to the fluorescence measurement. Fluorescence intensities of the stained cells
154 were measured in the Fluorescence Spectrophotometer F-7000 (Hitachi High-technologies,
155 Shenzhen, China) at the excitation wavelengths of 488 nm (pH-sensitive) and 435 nm (pH-
156 insensitive). The emission wavelength was in the range of 400–650 nm. The width of both
157 excitation and emission slit was 5 nm. For the calibration curve of *Lb. brevis* 145, the stained

cells were suspended in PBS buffer with different pH values. Valinomycin (0.2 mM in methanol; Sigma) and nigericin (0.2 mM in methanol; Sigma) were added to the cell suspension at the final concentrations of 5 µM and incubation of cell suspension at 37℃ for 10 min equilibrated both potassium and proton ions across cell membrane. The fluorescence intensity of the stained cells after equilibration was later recorded as described above.

2.8.Statistical analysis

All presented data in the bar charts and tables correspond to means ± standard deviation (SD). Significant difference analysis was carried out by using IBM SPSS Statistics 20.0 version.

3. Results

3.1.Carbohydrate catabolism apparatus affects the growth of Lb. brevis

The catabolism map of different carbohydrates was constructed based on the KEGG pathway database on *Lb. brevis* strains ATCC 367, KB290 and the availability of target genes in the genome (accession no. CP015398.1) of *Lb. brevis* NPS-QW-145 (Fig. 1A). Although these genes are present in the genomes of *Lb. brevis*, it is necessary to validate whether this organism could catabolize them efficiently. Thus, we grew *Lb. brevis* in the mMRS containing individual carbohydrate and its growth curves monitored as shown in Fig. 1B and Fig. 1C. Firstly, *Lb. brevis* 145 was still able to grow in mMRS broth without sugar supplementation though its OD_{600} value was low (up to ~0.12). However, although genes encoding β-galactosidase and mannose-specific PTS were available in the genome of *Lb. brevis* 145, it was observed that lactose and sucrose were not able to support its growth, while mannose and cellobiose could stimulate its growth weakly. Interestingly, carbohydrates, maltose and xylose, supported faster

179 growth of *Lb. brevis* 145 than other including common sugars, glucose and fructose (Fig. 1B and
180 1C). In addition, we surveyed the KEGG metabolism pathway map on xylose among sequenced
181 strains of *Lactobacillus* and found the common presence of enzymes 12 and 13 in *Lb. brevis* (Fig.
182 1A), *Lb. fermentum* and *Leuconostoc* spp., but not in *Lb. plantarum*, *Lb. acidophilus*, *Lb. casei*
183 group, *Lb. reuteri* and *Pediococcus* (data not shown).

184 ***3.2.GABA production from Lb. brevis correlates with its lactic acid production***

185 Different carbon sources were used to generate various levels of lactic acid from *Lb. brevis*
186 due to its varying capacity of carbohydrate catabolism (Fig. 1A). Lactic acid yields increased
187 significantly when cells catabolized glucose, galactose, fructose, maltose, and xylose, while a
188 limited amount of acetic acid was produced from *Lb. brevis* (Fig. 1D and 1E). Theoretically
189 acetic acid has a pKa value of 4.75 and that of lactic acid is 3.86, the latter contributes mostly to
190 acidify the environment since acetic acid production was very low (Fig. 1E). There was a strong
191 correlation coefficient ($r > 0.85$) between lactic acid production/bacterial biomass and GABA
192 yield from *Lb. brevis* (Fig. 1H and 1I).

193 ***3.3.Aerobiosis suppresses GABA production from Lb. brevis***

194 Effects of aerobic and anaerobic conditions on GABA production from *Lb. brevis* were
195 investigated in a 24-h course of cultivation. In this study, anaerobiosis improved cell viability of
196 *Lb. brevis* (Fig. 2A) and subsequently its GABA production significantly ($p < 0.001$; Fig. 2C and
197 2D). Moreover, glutamate supplementation significantly ($p < 0.001$) decreased extracellular
198 acidity of *Lb. brevis* cells in both conditions than those without glutamate supplementation (Fig.
199 2B). However, the presence of oxygen reduced lactic acid production largely but not acetic acid

by *Lb. brevis* (Fig. 2E). Since lactic acid production correlated with GABA production (Fig. 1I), less lactic acid production may explain its low GABA production by *Lb. brevis* under aerobic condition.

3.4.Anaerobiosis up-regulates gene expression of gad operon in Lb. brevis

The intact *gad* operon in *Lb. brevis* includes *gadR*, *gadC* and *gadA* which maybe co-regulated under the same promoter (Fig. 3A). Both *gadA*- and *gadB*-encoded glutamate decarboxylases could decarboxylase glutamate into GABA (Fig. 3B). However, *gadB* is located far away from the *gad* operon (Fig. 3A) and is possibly regulated together with other operons. Under anaerobic condition, *gadR*, *gadC* and *gadA* were up-regulated by at least 10-fold change indicating the active status of GAD system in *Lb. brevis*, while the *gadB* expression was slightly down-regulated (Fig. 3C). This suggests that the expression of *gad* operon in the aerated cells of *Lb. brevis* was highly inhibited as evidenced by the low GABA yield (Fig. 2C and 2D). Moreover, it indicated that GadA played the central role in GABA synthesis in *Lb. brevis* since *gadB* expression was weakly altered under anaerobic condition.

3.5.Early acidification does not fully restore GABA production from Lb. brevis under oxygen challenge

In order to assess the effect of early acidification on the GABA production, the pH of Lactobacilli MRS broth was initially adjusted to pH 6, 5 and 4. As shown in Fig. 4A, viable counts of *Lb. brevis* were not significantly ($p > 0.05$) changed after 24-h incubation under aerobic condition. It appears that acidic condition (ipH 4) reduced cell viability of *Lb. brevis* and thus affected its GABA production from glutamate decarboxylation under anaerobic condition

(Fig. 4C and 4D). GABA yields increased significantly ($p < 0.05$) in more acidic conditions (ipH 5 and ipH 4), but the yields were largely lower in aerobic condition than that in anaerobic cultivation (ipH 6 and ipH 5; Fig. 4C). Since lactic acid production correlated with the GABA production, it was also observed that lactic acid production from *Lb. brevis* was still significantly ($p < 0.05$) lower in aerobic cultivation (ipH 6.0 and ipH 5.0) than that in anaerobic cultivation (Fig. 4E and 4F).

3.6.Effects of early acidification on the gene expression of gad operon in Lb. brevis

In ipH 6 and ipH 5 conditions, cell viability of *Lb. brevis* was ~ 9.2 Log10 CFU/mL (Fig. 4A) and lactic acid production by *Lb. brevis* was ~ 6 g/L generating sufficient acidic environment for GABA biosynthesis (Fig. 4B & 4E) under aerobic condition, but less GABA production was observed in aerated cells. Thus it is necessary to understand the changes of GABA production machinery in aerated and unaerated cells of *Lb. brevis* in different acidic media. Thus, real-time qPCR assay was used to evaluate the effects of early acidification on the expression of *gad* operon in *Lb. brevis* under anaerobic and aerobic conditions. It was found that the *gad* operon in *Lb. brevis* in ipH 6 and ipH 5 conditions under anaerobic condition were highly up-regulated by at least 8 folds, and 2-fold up-regulation of *gad* operon was also observed in ipH 4 under anaerobic condition when compared to that under aerobic condition (Fig. 5). In addition, *gadA* and *gadC* were up-regulated more in ipH 5 condition than that in ipH 6 and ipH 4 conditions (Fig. 5). This may indicate a better GABA production in the unaerated cells of *Lb. brevis* under ipH 5 condition (Fig. 4C).

3.7.Effects of early acidification on the cytosolic acidity of Lb. brevis

242 We measured the pH_{in} of *Lb. brevis* cells under both aerobic and anaerobic conditions. As the
243 GABA yield was significantly ($p < 0.05$) increased after 12 h during the 24-h course of
244 cultivation of *Lb. brevis* in Lactobacilli MRS broth containing 10 g/L of MSG under anaerobic
245 condition (Fig. 2D), we measured the intracellular and extracellular pH of *Lb. brevis* from 12 h
246 to 24 h. In general, it was found that the values of pH_{in} and pH_{ex} were higher under anaerobic
247 condition than that under aerobic condition (Fig. 6B, 6C and 6D). This evidence together with
248 the improved GABA yield and the enhanced expression of *gad* operon in *Lb. brevis* under
249 anaerobic condition (Fig. 2C and 2D, Fig. 4C and Fig. 5) suggests that GABA synthesis
250 improved the pH level either intracellularly or extracellularly. Also, the value of pH_{in} in *Lb.*
251 *brevis* was always higher than that of pH_{ex} in ipH 6, ipH 5 and ipH 4 conditions under both
252 anaerobic and aerobic conditions (Fig. 6B, 6C and 6D). In addition, the pH_{in} values of *Lb. brevis*
253 under aerobic condition was pH 5.0-5.5 (ipH 6 and ipH 5) and pH 4.4-5.0 (ipH 4); these were
254 preferable acidity for the functioning of both GadA and GadB (Fig. 6). GABA synthesis
255 improved the value of pH_{in} of *Lb. brevis*, specifically pH 6.0-6.2 (ipH 6), pH 5.8-6.1 (ipH 5) and
256 pH 4.6-5.5 (ipH 4) under anaerobic condition (Fig. 6); such pH level decreased the activity of
257 GadA, especially GadB since GadA retains its activity towards near-neutral pH range while
258 GadB shows an acidic activity spectrum (Fig. 6A). The enzymatic activities of both GadA and
259 GadB would increase in such acidic range (4.4-5.0), which may explain the improved GABA
260 yield under this condition (Fig. 4C).

261 **4. Discussion**

262 Our previous genomic survey and biochemical analysis have demonstrated the common
263 presence of GAD system in *Lb. brevis* suggesting its species-specific characteristic of GABA

production (Wu et al., 2017). However, limited mechanistic studies have been carried out to investigate environmental determinants including carbohydrates, anaerobiosis and acidification on the GABA production by *Lb. brevis*. These determinants are key parameters for GABA production by *Lb. brevis* either during batch fermentation or natural food fermentations due to several reasons: 1) different capacities of the catabolism pathways and membrane transporters for varying carbohydrates (Fig. 1A); 2) catalase-negative status of *Lb. brevis* for detoxifying or scavenging reactive oxygen species generated from environmental oxygen during shaking incubation (Lyu et al., 2016); and 3) GABA synthesis is associated with acid resistance in *Lb. brevis* (Fig. 3B), and medium acidification may promote its GABA yield.

The first factor is the carbohydrate catabolism apparatus in *Lb. brevis*. It is the first time that we compared different effects of accessible sugars on the growth of *Lb. brevis* (Fig. 1B-C). Fast growth of *Lb. brevis* was stimulated by maltose and xylose; these could be applied as selective sugars that may help this organism dominate the microbiota during natural vegetable fermentations (i.e., Korean Kimchi) and even shorten the duration of fermentation process. The high biomass also synthesized more GABA (Fig. 1H). Maltose and xylose could be firstly catalyzed by maltose phosphorylase and xylose isomerase, and later via phospho-ketolase pathway and pentose phosphate pathway in *Lb. brevis* (Fig. 1A); these sugars generated from starch or xylan after enzymatic hydrolysis could be also applied for large-scale industrial batch fermentations for the production of GABA (Egloff et al., 2001; Zhao et al., 2015). The common sugars, glucose and fructose, found in our diets frequently could be catabolized by *Lb. brevis* during its transit or even colonization in human gastrointestinal (GI) tract.

285 Lactic acid bacteria are known for their excellent ability to produce lactic acid (Fig. 1A),
286 which acidify cells intracellularly and extracellularly. Importantly, the function of GAD system
287 including the GadC is activated under acidic conditions (below pH 6.5) in bacteria (Ma et al.,
288 2013; Ma et al., 2012). However, the correlation between lactic acid production and GABA
289 production has not been well understood. Based on this carbohydrate catabolism apparatus of *Lb.*
290 *brevis*, we adopted this apparatus generating varying levels of lactic acid production and
291 observed a strong positive correlation between lactic acid production and GABA production (Fig.
292 1I). In addition, GABA production and cell growth of *Lb. brevis* was affected by the carbon
293 source added to the growth medium; in general, the increase in GABA production was
294 concomitant with the decrease in the concentration of residual glutamate indicating that the event
295 of decarboxylation occurred in the organism (Fig. 1F and 1G). This indicates that the
296 intracellular lactic acid in *Lb. brevis* could stimulate GABA production via activation of GadC
297 function and GABA synthesis thus consuming protons to maintain intracellular pH homeostasis
298 (De Biase and Pennacchietti, 2012; Wu et al., 2017).

299 One study reported the low efficacy of glutamate decarboxylation in immobilized cells of *Lb.*
300 *brevis* in the acetate buffer, but not in medium broth or food matrix, during short-term shaking
301 incubation (Huang et al., 2007). In the present study, the second factor, oxygen, showed extreme
302 inhibitory effect on GABA production from *Lb. brevis* cultivated in MRS-based broth, and a
303 reduced biomass accompanying low GABA yield during shaking process was also observed for
304 *Lb. brevis* (Fig. 2). Thus, we conclude that aerobiosis reduced the growth of *Lb. brevis* and its
305 lactic acid production thus affecting its GABA production. To understand the mechanism behind
306 this, we found that the mRNA transcripts of *gadR*, *gadA*, *gadB* and *gadC* in aerated cells were

307 detected by qPCR assay, but enhanced levels of these genes were observed in *Lb. brevis* under
308 anaerobiosis (Fig. 3). This may explain the low GABA yields in aerated cells of *Lb. brevis*.

309 Optimization of the pH of the medium have been carried out for *Lb. brevis* under anaerobic
310 cultivation (Komatsuzaki et al., 2005; Li, Qiu, Huang, et al., 2010). However, limited evidence
311 has been provided on the effects of acidification on its GABA production from aerated cells of
312 *Lb. brevis*. During natural food fermentations, i.e., Korean kimchi fermentation, some oxygen
313 still remains trapped in the container, and additional acids, i.e., acetic acid, are added to acidify
314 the cabbage matrix for inhibiting the growth of contaminated pathogens prior to fermentation.
315 Although the cell viability of *Lb. brevis* was high (Fig. 4A) and its intracellular pH was also
316 suitable for GABA synthesis (Fig. 6), early acidification of the medium still did not counteract
317 the oxygenic inhibition of GABA biosynthesis in *Lb. brevis* (Fig. 4C). It was concluded that
318 acidification of medium did not fully restore GABA production capacity in *Lb. brevis* under
319 aerobic condition. This may be explained by the down-regulation of *gad* operon in *Lb. brevis* by
320 oxygen exposure (Fig. 5). However, GABA contents from aerated cells were significantly ($p <$
321 0.05) enhanced under ipH 5 and ipH 4 media compared to that under the ipH 6 condition (Fig.
322 4C), this may be due to the elevated GABA biosynthesis against acidification challenge. Our
323 qPCR assay detected the presence of mRNA transcripts of the *gad* operon in the aerated cells of
324 *Lb. brevis* (Fig. 3C and Fig. 5). This suggests that the GAD system may still be functioning in
325 aerated cells as evidenced by their low GABA yield (Fig. 4C). The enzyme activities of GadA
326 and GadB are highly dependent on the cytosolic acidity in *Lb. brevis* (Fig. 6A) (Wu et al., 2017).
327 Thus, acid challenge could be an activator of GABA production from *Lb. brevis*. Since GadC
328 exhibited stringent pH dependence for substrate transport and was activated under acidic
329 conditions (Ma et al., 2013; Ma et al., 2012), acid challenge not only promoted GABA synthesis

but also activated GadC function immediately in bacteria thus improving GABA production under both aerobic and anaerobic conditions.

5. Conclusions

In the present study, we assessed the effects of carbohydrates, oxygen and acidity on the GABA production from *Lb. brevis*. Accessible carbohydrates (maltose and xylose) could stimulate the fast growth of *Lb. brevis* for high GABA production. Aerobiosis suppressed GABA production by down-regulating the expression of *gad* operon in *Lb. brevis*, but this could be fully restored by anaerobiosis. Also, GABA production could be partially restored in the aerated cells of *Lb. brevis* by suitable early acidification of the medium (ipH 5) since the cytosolic acidity was suitable for the functioning of GAD system. These mechanistic insights will be of importance for the industrial manufacture of GABA-rich fermented vegetables or GABA by batch fermentations.

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Figure captions

Figure 1. Carbohydrate catabolism capacity alters GABA biosynthetic capacity in *Lb. brevis*. (**A**) KEGG metabolic pathways of selected carbohydrates in *Lb. brevis*. Denotation: PK pathway, phosphoketolase pathway; PP pathway, pentose phosphate pathway; GAP, Glyceraldehyde-3P; 1, maltose phosphorylase; 2, β-glucosidase; 3, glucokinase; 4, phosphoglucomutase; 5, glucose-6P isomerase; 6, transketolase; 7, α-glucosidase; 8, β-galactosidase; 9, fructokinase; 10, mannose-6P isomerase; 11, mannose-specific PTS; 12, xylose isomerase; 13, xylulose kinase; 14, phosphoketolase; 15, lactate dehydrogenase; 16, pyruvate oxidase; 17, acylphosphatase or acetate kinase; 18, phosphate acetyltransferase. (**B-C**) Growth curves of *Lb. brevis* 145 on different sugars. (**D-E**) Production of lactic acid and acetic acid from *Lb. brevis* 145 grown in modified MRS (mMRS) broth containing different carbohydrates after 48 h. Initial concentrations of lactic acid and acetic acid after bacterial inoculation were 0.25 g/L and 2.48 g/L, respectively. (**F-G**) GABA and glutamate concentration in the broth after 48 h (glutamate content was calculated in the form of MSG). (**H-I**) Correlation coefficient analysis of GABA production and lactic acid production/biomass from *Lb. brevis* 145. Values (a, b, c, d, e, f & g) with no letters in common at the top of each bar are significantly different ($p < 0.05$). The experiment was carried out in triplicates and data was presented as mean ± standard derivation (SD).

Figure 2. Aerobiosis suppresses GABA production from *Lb. brevis*. (**A**) Growth curves of *Lb. brevis* 145 in the Lactobacilli MRS broth containing 3.46 g/L of NaCl (control group) or 10 g/L of MSG (glutamate group). Extra sodium chloride (3.46 g/L) was added to the Lactobacilli MRS medium to mimic the condition that the extra sodium was introduced by MSG supplementation to the medium. (**B**) Acidity changes. (**C**) Effects of aerobic and anaerobic conditions on GABA production from *Lb. brevis* in Lactobacilli MRS broth containing 3.46 g/L of NaCl. (**D**) Effects of aerobic and anaerobic conditions on GABA production from *Lb. brevis* in Lactobacilli MRS broth containing 10 g/L of MSG. (**E**) Lactic acid and acetic acid production. Values (a, b, c, d & e) with no letters in common at the top of each bar are significantly different ($p < 0.05$). *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$. Aerobic condition was generated by shaking process to incorporate the filtered air into the medium broth. Glutamate content was calculated in the form of MSG. The experiment was carried out in triplicates and data was presented as mean ± standard derivation (SD).

Figure 3. GAD system in *Lb. brevis* is highly up-regulated under anaerobic condition. (**A**) The *gad* operon in *Lb. brevis* 145. The locus tag of each gene is indicated in the pentagon. Promoter and transcription terminator were predicated by PePPER server (de Jong et al., 2012) and ARNold server (Naville et al., 2011), respectively. (**B**) Schematic diagram of GAD system in *Lb. brevis*. (**C**) Relative gene expression of *gadR*, *gadA*, *gadB* and *gadC* in *Lb. brevis* 145 in anaerobic condition normalized to that in aerobic condition. Bacterial cells of *Lb. brevis* 145 were inoculated (1%, v/v) in Lactobacilli MRS broth containing 10 g/L of MSG, and cells were collected for gene expression study after 12 h of aerobic or anaerobic cultivation. Comparative critical threshold method ($2^{-\Delta\Delta Ct}$) was used to calculate the relative gene expression. The experiment was carried out in triplicates and data was presented as mean ± standard derivation (SD).

Figure 4. Acidification does not fully restore GABA production from *Lb. brevis*. (**A**) Viable counts of *L. brevis* 145. (**B**) The pH of culture supernatant. (**C**) GABA yield. (**D**) MSG content. (**E**) Lactic acid production. (**F**) Acetic acid production. The concentration of acetic acid in pH-adjusted Lactobacilli MRS medium was about 4.20 g/L. *Lb. brevis* was cultivated in Lactobacilli MRS medium (initial pH-adjusted) containing 10 g/L MSG at 37℃ for 24 h. Gray bar, cultivation in the medium broth of ipH 6; white bar, cultivation in the medium broth of ipH 5; black bar, cultivation in the medium broth of ipH 4. Values (a, b, c, d, e & f) with no letters in common at the top of each bar are significantly different ($p < 0.05$). The experiment was carried out in triplicates and data was presented as mean ± standard derivation (SD).

Figure 5. Early acidification alters the gene expression of GAD system in *Lb. brevis* under anaerobic condition. Bacterial cells of *Lb. brevis* 145 were inoculated (1%, v/v) in initial-pH adjusted Lactobacilli MRS broth containing 10 g/L of MSG, and cells were collected for gene expression study after 12 h of aerobic or anaerobic cultivation. Comparative critical threshold method ($2^{-\Delta\Delta Ct}$) was used to calculate the relative gene expression. Relative gene expression of *Lb. brevis* in anaerobic condition was normalized to that in aerobic condition. The experiment was carried out in triplicates and data was presented as mean ± standard derivation (SD).

Figure 6. Early acidification alters intracellular pH (pH_{in}) of *Lb. brevis* cells during 24-h course of cultivation. (**A**) Effects of pH on the enzyme activities of GadA and GadB from *Lb. brevis* 145. This figure was adopted from our previous study (Wu et al., 2017). (**B**) pH_{in} (intracellular pH)and pH_{ex} (extracellular pH) of *Lb. brevis* 145 under aerobic and anaerobic cultivation in pH-adjusted MRS broth (ipH 6). (**C**) pH_{in} and pH_{ex} of *Lb. brevis* 145 under aerobic and anaerobic cultivation in pH-adjusted MRS broth (ipH 5) (**D**) pH_{in} and pH_{ex} of *Lb. brevis* 145 under aerobic and anaerobic cultivation in pH-adjusted MRS broth (ipH 4). The measurements were carried out in triplicates and data was presented as mean ± standard derivation (SD).

478 **Table 1. List of primers used for qPCR assay in this study.**

Primer name	Sequence (5'→3')	Amplicon size (bp)	Target gene	Reference
Lb-tuf-F	CGTGAGCTCTTGTCTGAATAC	152	*tuf* (reference gene) in *Lb. brevis* 145	(Wu et al., 2017)
Lb-tuf-R	CGTTCTGGAGTTGGGATATAAT			
Lb-gadR-F	CAACTGGCTGGCTAGTTATC	151	*gadR* in *Lb. brevis* 145	This study
Lb-gadR-R	ACTCTGTTTCAATCGCTCTAC			
Lb-gadA-757F	CAGGTTACAAGACGATCATGC	188	*gadA* in *Lb. brevis* 145	(Wu et al., 2015)
Lb-gadA-945R	ATACTTAGCCAGCTCGGACTC			
Lb-gadB-364F	GGACAATACGACGACTTAGC	135	*gadB* in *Lb. brevis* 145	(Wu et al., 2015)
Lb-gadB-499R	CTTGAGCTCGGGTTCAATAA			
Lb-gadC-F2	TGGGATTGTTTACGCCTATG	122	*gadC* in *Lb. brevis* 145	This study
Lb-gadC-R2	CGACCCAACTGCTGATTT			

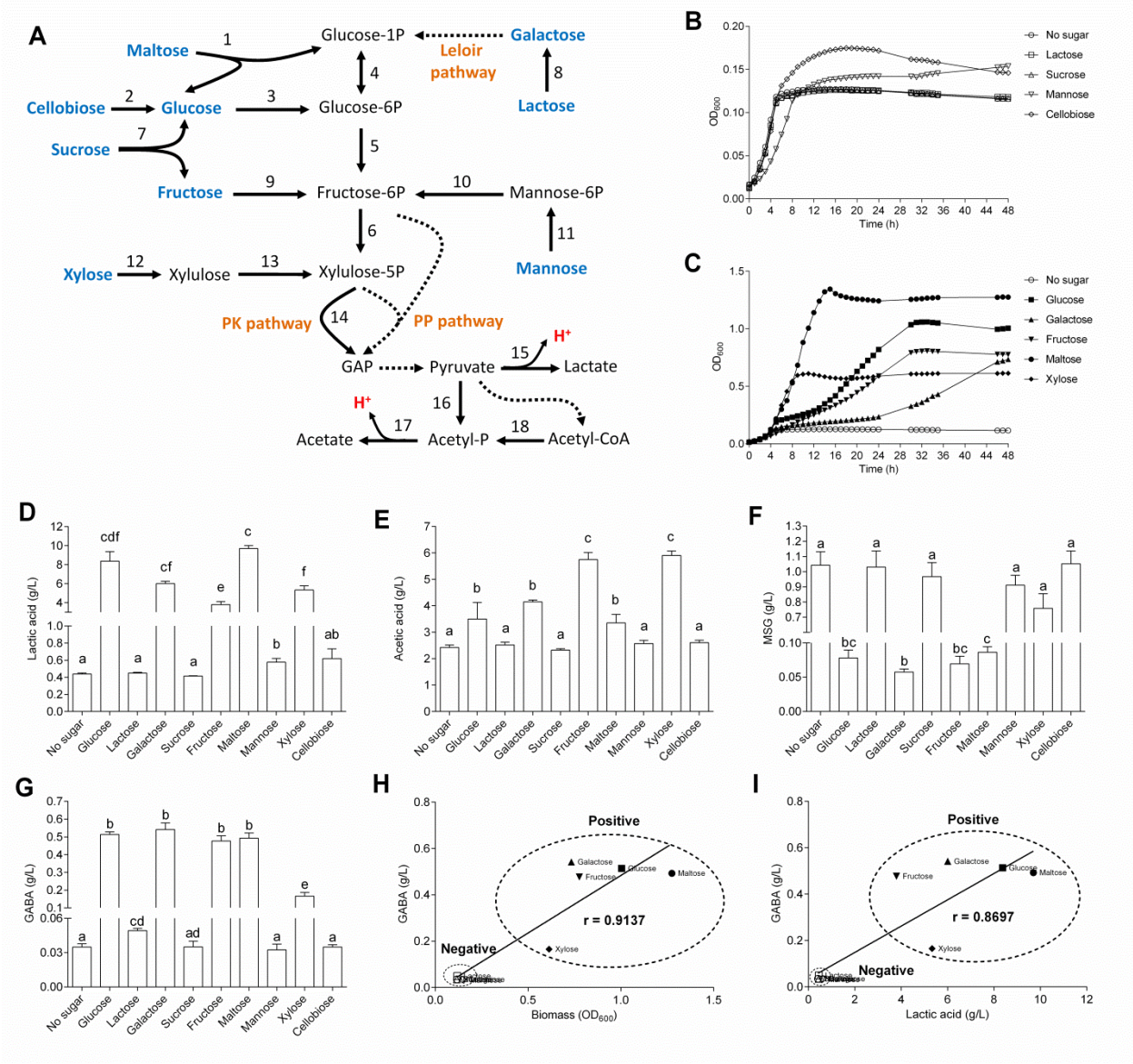

A
Maltose
Cellobiose
Glucose
Sucrose
Fructose
Xylose
Xylulose
Glucose-1P
Glucose-6P
Fructose-6P
Xylulose-5P
Galactose
Lactose
Mannose-6P
Mannose
Leloir pathway
PK pathway
PP pathway
GAP
Pyruvate
Lactate
Acetate
Acetyl-P
Acetyl-CoA
H+
B
No sugar
Lactose
Sucrose
Mannose
Cellobiose
Time (h)
C
No sugar
Glucose
Galactose
Fructose
Maltose
Xylose
Time (h)
D
Lactic acid (g/L)
E
Acetic acid (g/L)
F
MSG (g/L)
G
GABA (g/L)
H
Positive
Negative
r = 0.9137
Biomass (OD600)
I
Positive
Negative
r = 0.8697
Lactic acid (g/L)

Fig. 1. Wu et al.

f

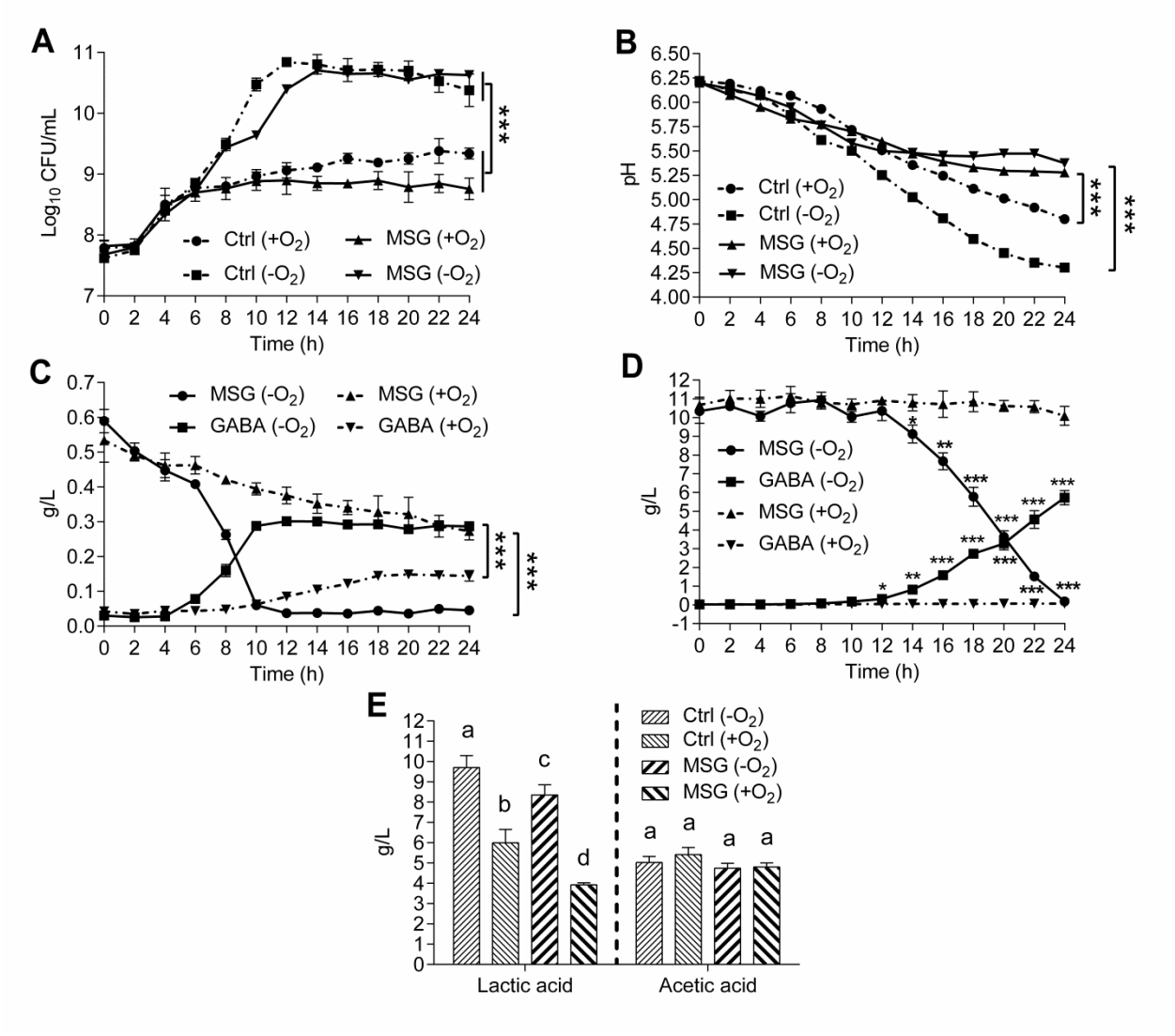
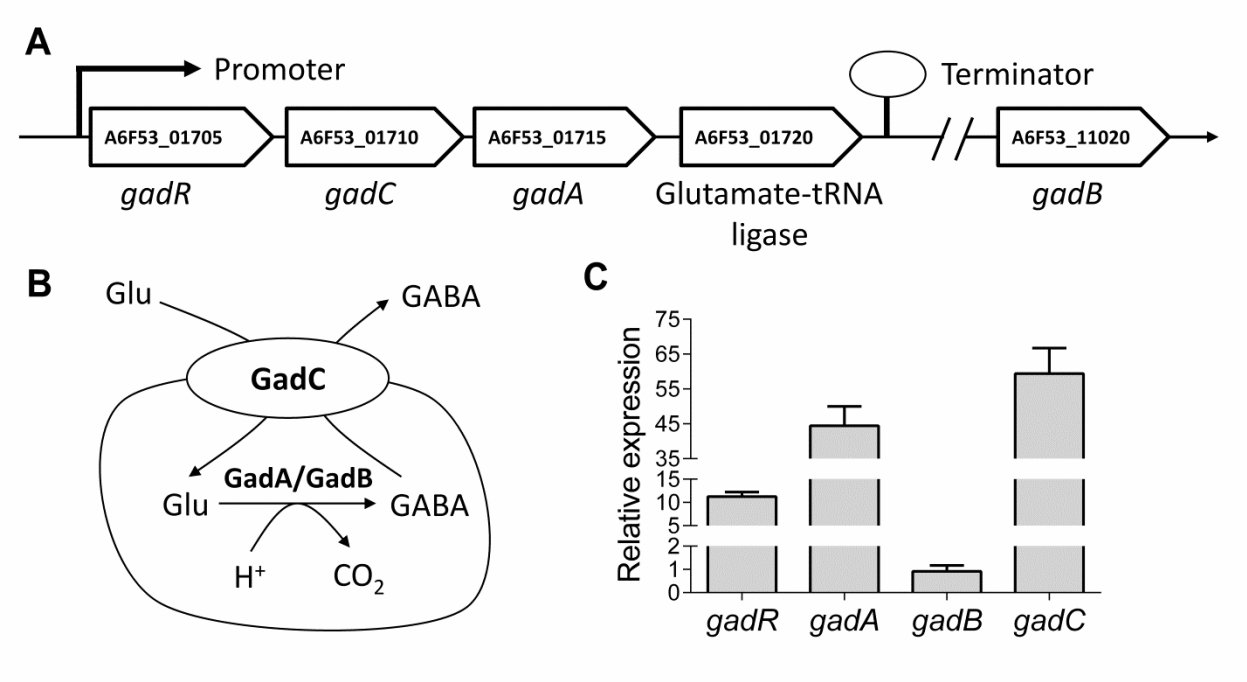


Fig. 2. Wu et al.



484

485 **Fig. 3.** Wu et al.

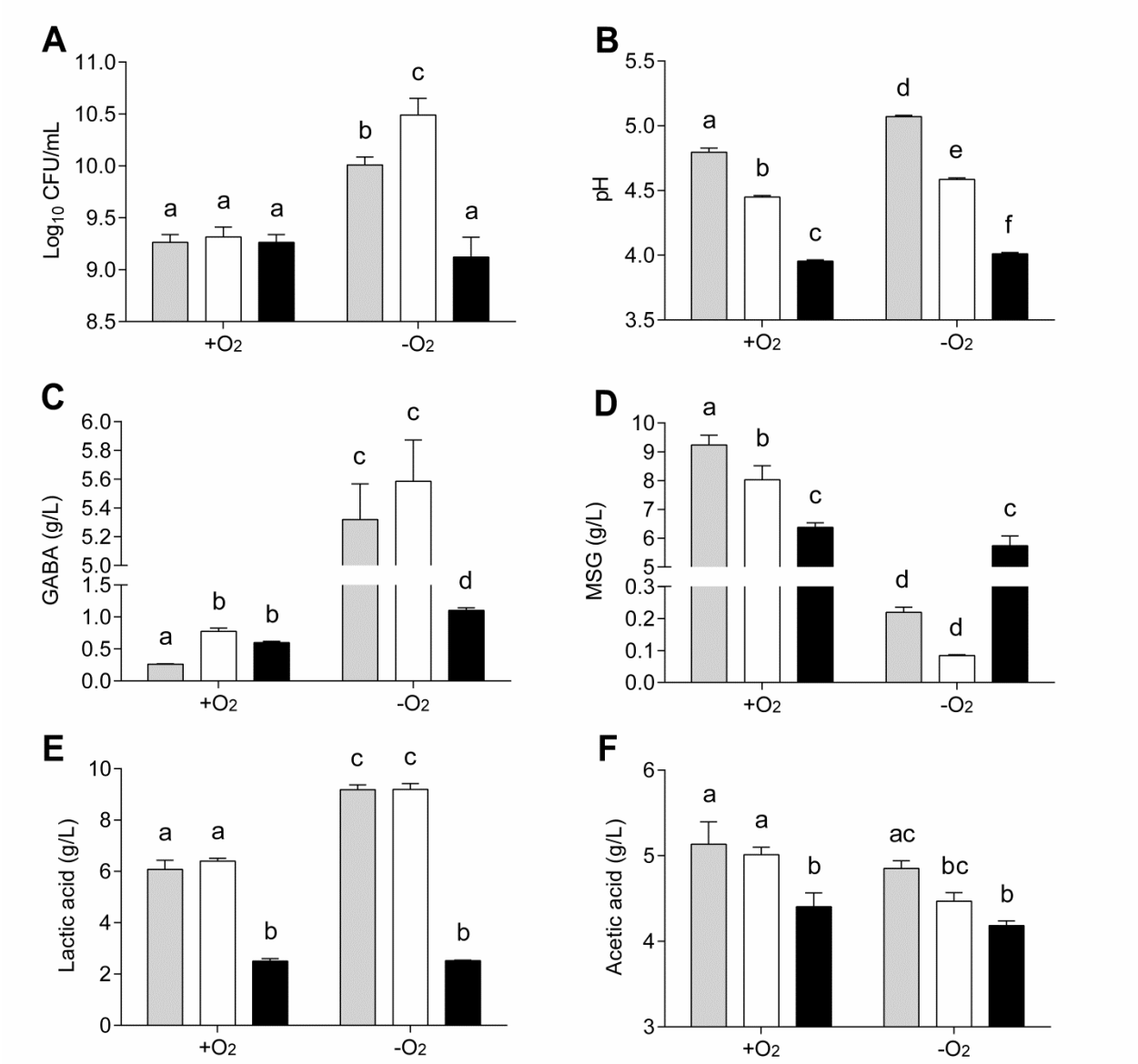
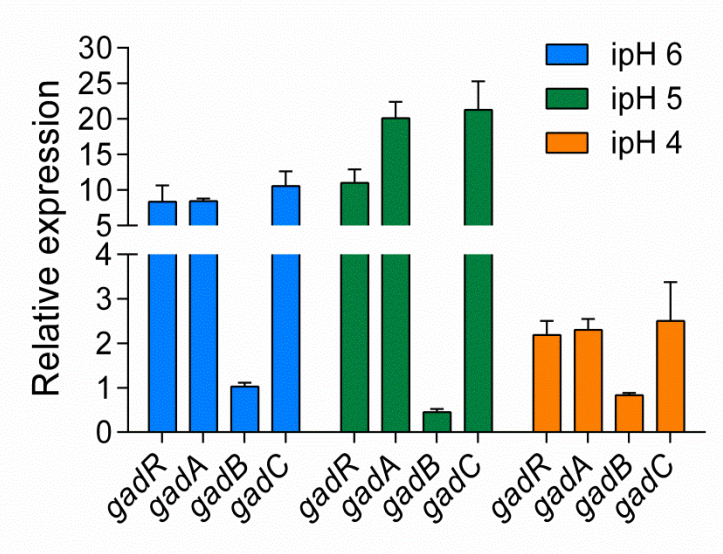
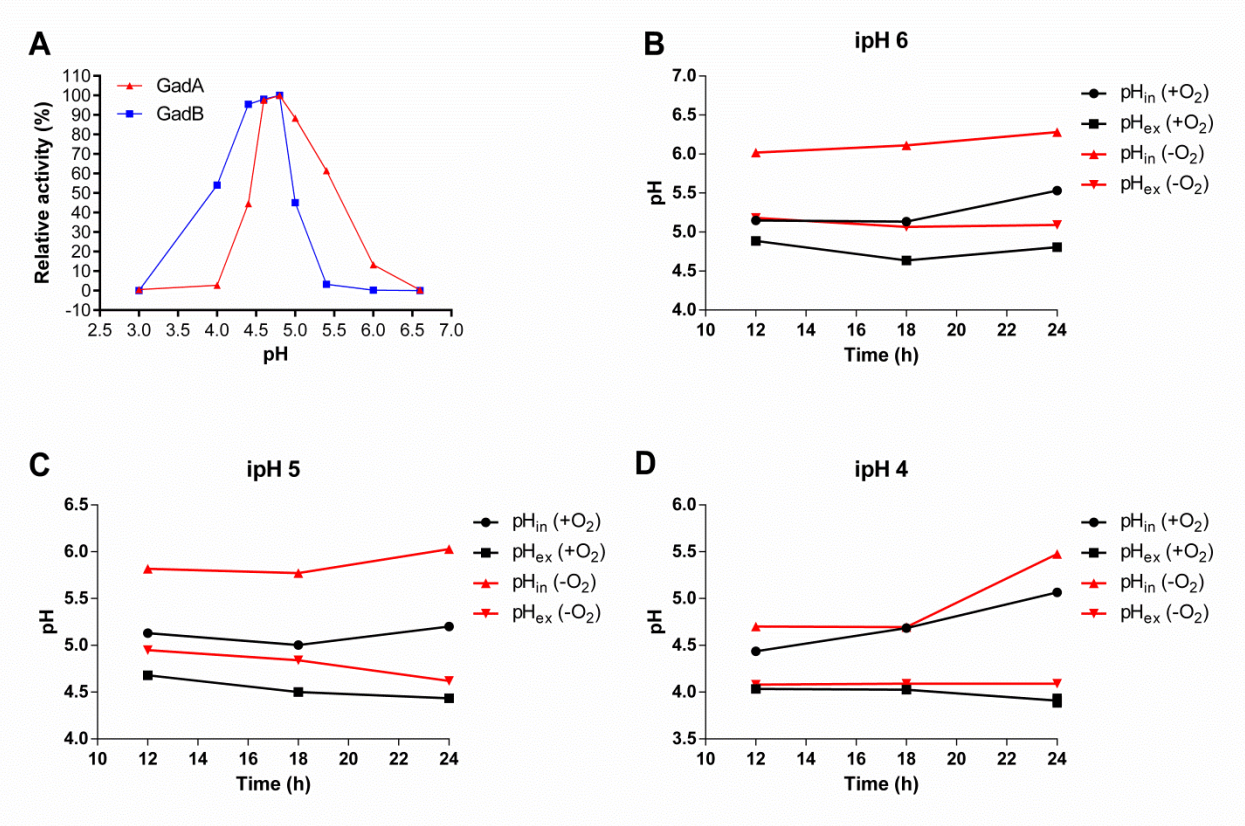

A
Log10 CFU/mL
11.0
10.5
10.0
9.5
9.0
8.5
a a a b c a
+O2 -O2
B
pH
5.5
5.0
4.5
4.0
3.5
a b c d e f
+O2 -O2
C
GABA (g/L)
6.0
5.8
5.6
5.4
5.2
5.0
1.5
1.0
0.5
0.0
a b b c c d
+O2 -O2
D
MSG (g/L)
10
9
8
7
6
5
0.3
0.2
0.1
0.0
a b c d d c
+O2 -O2
E
Lactic acid (g/L)
10
8
6
4
2
0
a a b c c b
+O2 -O2
F
Acetic acid (g/L)
6
5
4
3
a a b ac bc b
+O2 -O2

Fig. 4. Wu et al.



488

489 **Fig. 5.** Wu et al.



490

491 **Fig. 6.** Wu et al.