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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.

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

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production of GABA from *Lb. brevis* (Li et al., 2010a; Li et al., 2010b; 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 °C 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 °C 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™ GO Microplate Spectrophotometer (Thermo Scientific) at 37 °C 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 °C 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 °C 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 °C and 100 rpm). Samples were collected every 2 h 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 °C 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*, *gadA*, *gadB* and *gadC* in *Lb. brevis* 145) listed in Table 1 was quantified by RT-qPCR assay. Reference gene, *tuf* in *Lb. brevis* (Table 1), was used to normalize the expression of target genes. The efficacy of qPCR amplification using each pair of primers was in the range of 90–110% and non-specific amplification products including primer dimers were not detected by melt curve analysis and agarose gel electrophoresis (data not shown). The comparative critical threshold method ($2^{-\Delta\Delta Ct}$) was used to calculate the relative