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Enhancement of biomass production and nutrition utilization by strain *Lactobacillus acidophilus* DGK derived from serial subculturing in an aerobic environment

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Successive subculturing under aerobic conditions for 14 days could be used to improve characteristics of a parent strain with a growth defect. Identification of the derived strain was based on the API 50 CHL test kit and partial sequencing of the *hsp60* gene. Results show that an aerotolerant strain, *Lactobacillus acidophilus* DGK, was created with improved nutrition utilization. Characteristics of shorter rod-shape, higher growth rate and higher level of lactic acid concentration were observed in the domesticated strain which produced higher biomass (4.54 g dry cell weight (DCW)/L) and viable counts (9.5 x 10^9 CFU/mL) compared to the original strain which were 1.06 g DCW/L and 1.61 x 10^9 CFU/mL, respectively under aerobic conditions. Utilization of carbon and nitrogen sources was significantly improved by the derived strain except the raffinose. These results indicate that serial subculturing is a plausible method for the generation of modified strains with enhancing nutrition utilization or improving various characteristics which were beneficial in industrial processes.

Key words: Biomass; serial subculture; *Lactobacillus acidophilus; hsp60* gene.

INTRODUCTION

Lactic acid bacteria (LAB) such as *Lactobacillus rhamnosus* GG (Wang et al., 2013; Soukoulis et al., 2014), *Lactobacillus bulgaricus* (Nagai et al., 2011; Ashraf and Shah, 2011), *Streptococcus thermophilus* (Iyer et al., 2010; Ashraf and Shah, 2011) or *Lactobacillus paracasei* 33 (Lin et al., 2014; Miao et al., 2014) have been extensively used in fermented milk or probiotic products for health benefit. Biomass production of these probiotics through fed-batch fermentation is important in industrial processes for cost reduction. During the technological processes for production of a probiotic product, factors such as medium formulation, stirring speed of blades, aeration rate, temperature and drying conditions may threaten the viability and activity of the bacteria which

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generally are microaerobic or anaerobic. Therefore, the development of effective strategies to improve certain characteristics, such as resistance to oxygen stress, bile salt and acid, which would allow adaptation to human gastrointestinal environments, and to survive the production process is of the utmost importance. These enhancements would increase cell growth and reduce the cost of commercialized probiotic products.

Most LAB strains lack catalase, cytochrome and heme-containing proteins (Chiancone and Ceci, 2010), which protect against oxidative stress or oxygen toxicity. Physiological adaptation to oxidative stress in aerobic microorganisms requires enzymatic defense against oxidative stress, examples of which include catalase, superoxide dismutase (SOD), glutathione reductase and peroxidase (Jänsch et al., 2011). NADH oxidase and iron-binding proteins were reported to correspond to oxygen tolerance or aerotolerance in Streptococcus mutants (Higuchi et al., 2000). Most of the studies on stress responses at physiological levels have focused on oxygen, temperature, acid and bile salt-adaptation processes (Sanz, 2007; Koponen et al., 2012). In one study, viability of the heat-adapted *Lactobacillus paracasei* was significantly enhanced to withstand heat stress during spray drying (Desmond et al., 2001). The approach to improve the survival of probiotic bacteria by stress adaptation has been based heavily on short exposures of viable cells to various sub-lethal stress factors to induce tolerance to subsequent lethal stress conditions (Saarela et al., 2004). The method of creating improved probiotic strains by acid stress adaptation treatments, resulting in improved stress tolerance and biological properties, has been reviewed previously (Sanz, 2007). Besides, cell length alternations and metabolic impacts derived from the stress reactions had been reported in lactobacilli (Serrazanetti et al., 2009; Krieger et al., 2013).

The strain Lactobacillus acidophilus GK isolated from healthy infant feces is sensitive to aerobic conditions. This impeded bacterial growth when incubated under aerobic conditions with a controlled pH and mild agitation at 60 to 150 rpm during fed-batch fermentation in preliminary experiments. This property has limited the production of L. acidophilus for probiotics. Prolonged exposure of LAB to preset conditions (such as acid) has been shown to be an effective strategy for generating derivative strains with a stable acid-resistant phenotype from acid-sensitive strains of species commonly found in the human gut (Andriantsoanirina et al., 2013). Therefore, the purpose of the present report was to develop L. acidophilus GK-derived strains with enhanced aerobic tolerance to adapt to the fermentation process and to compare the nutrition utilization between the domesticated strain and the parental strain. The results of this study will be important in the improvement of these strains' growth and functionality during aerobic fermentation, as well as to enable them to be stored as dried products for commercial use as probiotics.

MATERIALS AND METHODS

Microorganism, strain activation and medium composition

The LAB, *Lactobacillus acidophilus* GK, was provided by the Grape-King Company (Taiwan). This strain and its domesticated strain, *L. acidophilus* DGK, were cultured or subcultured in MRS broth supplemented with 0.05% cysteine (MRSC). Strains were maintained in a -80°C freezer, thawed at 37°C and incubated in MRSC broth at 37°C for 16 h or activated on an MRSC agar plate for 24 h. The medium compositions for carbon source or nitrogen source replacement are as follows: glucose, 10 g/L; yeast extract, 10 g/L; soy peptone (Scharlau), 10 g/L; sodium acetate, 5 g/L; ammonium citrate, 2 g/L; $MgSO_4.7H_2O$, 0.2 g/L; $MnSO_4.4H_2O$, 0.05 g/L and corn steep liquor 10 mL/L. Fermentation was performed in 10 mL of culture medium in a 50 mL Hinton flask with or without stirring at 150 rpm. The fresh culture (1%) was inoculated into fermentation medium and cultivated at 37°C for 24 h.

Adaptation of the aerotolerant strain from *L. acidophilus* GK

A fresh culture of *L. acidophilus* GK (1%) was subcultured into another MRSC medium every day. The culture containing 10 mL of medium in a Hinton flask was incubated at 37°C for 24 h at 150 rpm. After 14 days of successive subculturing and incubation in this manor, the final culture medium was diluted, spread onto an MRSC agar plate and incubated at 37°C for approximately 24 to 36 h.

Population analysis was carried out for further screening of the derived strains that exhibited higher biomass production through observing the colony size and smaller cell size through microscope examination. More than 400 larger colonies, which were also hypothesized to have a higher growth rate than the original strain, were selected, compared and cultivated under aerobic conditions (as above) with shaking at 150 rpm. After 16 h incubation, cell density was measured at 600 nm, and the size of the cells in fermented culture was observed by microscopy. The colonies with higher density and reduced size were isolated and preserved in 10% glycerol at -80°C for later use. A domesticated strain possessing the desired characteristics of aerotolerance, reduced size and high biomass production was selected from the isolated cultures through the above screening procedure and designated as *L. acidophilus* DGK.

Characteristics and identification of the domesticated strain *L. acidophilus* DGK

The differences in morphology, Gram-staining and acid/bile salt resistance were examined between the domesticated strain and the original one. For Gram-staining, a loop of fresh culture was spread onto a glass slice and then stained with crystal violet solution for 1 min. The slide was then washed, and iodine solution was added. Next, the slide was washed again with ethanol and stained with safranin for 30 s. After the stained slide air-dried, it was examined by microscopy. Cells with a deep blue color were identified as Gram-positive, while a red color indicated Gram-negative cells. To evaluate tolerance to acids and bile salts, 1 mL of fresh culture was mixed with 9 mL of phosphate-buffered saline (PBS) solution (pH 2 adjusted by HCl) and incubated at 37°C for 3 h with shaking at 80 rpm. Cell viability was determined by counting the number of colony forming units (CFU) after inoculation of serially diluted culture onto MRSC agar plates. The viable colony count for the control was obtained by serially diluting the cells with an acid-free PBS buffer control (pH 7.2) and counting the CFU on agar plates. Two-milliliter aliquots of the acid-treated samples (pH 2.0 PBS for 3 h) were centrifuged at 6000 rpm for 10 min to test for bile salt tolerance. The cell pellet was resuspended in pH 7.2 PBS and added to 10 mL of

MRSC broth containing 0.3% (W/V) of oxgall, and the cultures were incubated at 37°C for 24 h. Samples were taken at time intervals (3, 12 and 24 h) to determine CFU on MRSC agar plates. All cultures were performed in triplicate in 50 mL Erlenmeyer flasks containing 10 mL of the corresponding medium at 37°C for 24 h.

Biochemical tests were performed using an API 50 CHL test kit (BioMorieux Vitek, Inc., Hazelwood, MO, USA), according to the manufacturer's directions, to identify strains. A fresh culture (1 mL) was washed twice and resuspended in 1 mL of deionized water. The suspended culture was added to 5 mL of sterile water until the density matched the standard "1.0 McFarland Nephelometer" (PML Microbiologicals inc, USA). Two volumes of the diluted culture were poured into a 10 mL API 50 CHL cultivation medium for use as the seed culture. Each test well in the API 50 CHL was inoculated with 90 μL of the seed culture and incubated at 37°C for 24 or 48 h. The identification was carried out using the automated ATB Plus V2.6.8 analysis with the API 50 CHL version 5.0.

Amplification and sequencing of the partial *hsp60* gene

Oligonucleotide primers LB308F (TGAAGAAYGTNRYNGCYGG) and LB806RM (AANGTNCCVCGVATCTTGTT), previously described by Blaiotta et al. (2008), were used to amplify a 499-bp fragment internal to the *hsp60* gene. PCR amplifications were performed with a 25-μL total volume including 2 μL of the target DNA, 2 μL of each primer (25 μM), and 5.0 μL of 5 X Hot Start Mix II containing 0.75 U of Hot Start *Taq* DNA polymerase, 5 X reaction buffer, 10 mM $MgCl_2$ and 250 μM dNTP (Genemark, Taiwan). The PCR consisted of 35 cycles (20 s at 95°C, 30 s at 57°C and 30 s at 72°C) followed by a denaturing step at 95°C for 2 min and a final cycle at 72°C for 5 min. The PCR amplification fragments were resolved by agarose (2%, w/v) gel electrophoresis and visualized with SafeView DNA stain (Applied Biological Materials, Canada). After gel electrophoresis, the 499-bp PCR fragment was excised and purified by using a Micro-Elute DNA Clean/ Extraction kit (Genemark, Taiwan) and cloned by pOSI-T PCR cloning kit (Genemark, Taiwan). The recombinant pOSI-T plasmids were prepared and subjected to sequencing with the primer SP6 using an ABI Prism 377 DNA sequencer (Mission Biotech, Taipei, Taiwan). A search for DNA similarity was performed using the Blastn software (National Center for Biotechnology information, Bathesda, Maryland, USA).

Effects of carbohydrate and nitrogen sources on fermentation by the domesticated strain

The cultivation experiments were conducted using 50 mL Hinton flasks containing 10 mL of cultivation medium. The seed culture used as the inoculum was grown at 37°C for 16 h in a 10 mL test tube without shaking. The cultivation medium was inoculated with 1% (v/v) of the seed culture and incubated at 37°C for 24 h under either shaking (150 rpm) or static conditions. Various carbon and nitrogen sources were tested to examine the effects of medium compositions on cell growth and pH. Bacterial growth was measured as the absorbance of cell suspensions at 600 nm in a UV/VIS spectrophotometer (U-1800, Hitachi, Japan). The biomass was expressed as g/L of dry cells, based on a previously established regression between absorbance and cell dry weight. The supernatant was used for the analysis of glucose concentration. All of the experiments were performed in triplicate.

Analytical methods

The biomass of the fermented cultures was calculated by comparing the correlation curve between absorbance values of cell density (OD_{600nm}) and dry cell weight (g DCW/L). The pH was measured using a pH-meter SP-2300 (Suntex, Taiwan). The concentration of glucose in the supernatant was measured by the DNS method. Lactic acid was assayed by HPLC according to Andersson and Hedlund (1983).

Statistical analysis

Analyses of variance (ANOVAs) were performed on the data, and difference was considered significant at $p<0.05$.

RESULTS

Growth and characteristics of the domesticated strain *L. acidophilus* DGK

A fresh culture of *L. acidophilus* GK was successively subcultured into fresh MRSC medium every day and incubated with shaking (150 rpm), resulting in the adaptation of the strain to an aerobic environment. A population analysis of colonies selected after 14 days of culture was further applied to screen larger size of colonies, which might exhibit higher growth rate and biomass production. An aerotolerant strain was finally isolated and designated as *L. acidophilus* DGK. The appearance of the test tubes cultivating strains before and after adaptation was significantly different (Figure 1). *L. acidophilus* GK cells were concentrated at the bottom of the test tube, exhibiting the intrinsic characteristics of an anaerobic strain. In contrast, the domesticated strain, *L acidophilus* DGK, became aerotolerant, grew well and exhibited abundant biomass production, which would be beneficial for cell growth during fed-batch fermentation under agitation. Fermentation in bioreactors is generally performed under controlled conditions, which include monitoring temperature, pH, agitation and aeration rate. These conditions have been shown to affect LAB production significantly, with agitation being among the most important. The process of LAB biomass production in bioreactors occurs either by batch or fed-batch fermentation, in which stirring the medium is a critical step during the pH control and the feeding processes. In the present study, the growth of *L. acidophilus* GK was significantly reduced during pH control or feeding the concentrated medium due to agitation of the culture medium. The domesticated strain *L. acidophilus* DGK grew well in an aerobic environment and was suitable for cell proliferation during the fermentation process. NADH oxidase and superoxide dismutase (SOD) have been reported to play an important role in the regulation of aerobic metabolism in LAB (Higuchi et al., 2000). Therefore, it is possible that the aerotolerant strain *L. acidophilus* DGK might have higher activities of NADH oxidase or SOD when cultivated under aerobic conditions.

The cellular size of the domesticated strain was smaller than the parental strain, as shown in Figure 1. The cell density of the fermented culture by the domesticated strain (4.81 g DCW/L) was higher than that of the original

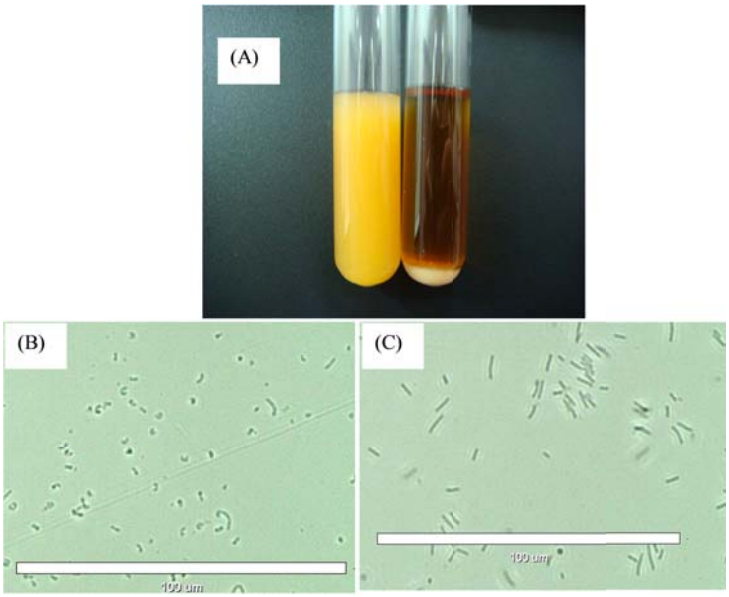

Figure 1. Appearance of cell cultures cultivated in MRSC for 16 h in test tube; left tube, the domesticated strain *L. acidophilus* DGK, right tube, the original strain *L. acidophilus* GK (A). Micrograph of the domesticated strain (B) and the original strain (C).

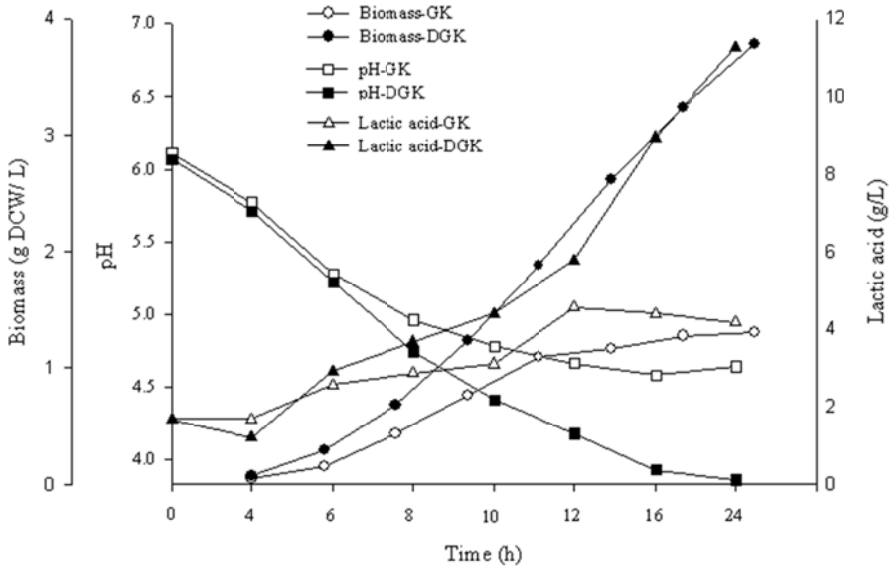

Figure 2. Cell density, pH, and lactate concentration of fermented culture by the domesticated strain *L. acidophilus* DGK or the original strain *L. acidophilus* GK in Hinton flasks with shaking (150 rpm). Fermentation conditions: 1% inoculum, 37 ℃, 24 h, 50 mL medium in 500 mL Hinton flask with shaking at 150 rpm. GK: the original strain *L. acidophilus* GK; DGK: the domesticated strain *L. acidophilus* DGK.

strain (2.60 g DCW/L) in MRSC medium under static conditions. These data are consistent with the fact that higher numbers of viable cells, based on the dried weight of cells, were observed in the domesticated strain com-pared to the original strain. These results may prove beneficial for industry as price of commercial products is based on the viable cell number per unit of weight.

Obvious differences in biomass, lactic acid concentration and pH value were observed during fermentation of the domesticated and original strains in an aerobic environment with agitation at 150 rpm (Figure 2). Biomass produced by the domesticated strain (3.79 g DCW/L) was significantly

Table 1. Analysis of the ability to metabolize partial carbohydrates from the API 50 CHL test of the domesticated strain *L. acidophilus* DGK and the original strain *L. acidophilus* GK*

Biochemical test	24 h		48 h	
	GK†	DGK	GK	DGK
Glycerol	-	-	+	+
D-sorbitol	+	-	+	+
D-sucrose	+	-	+	+
D-raffinose	+	-	+	-
Potassium gluconate	-	+	+	+

*Fermentation conditions: 37°C, 24 or 48 h. †GK, the original strain *L. acidophilus* GK; DGK, the domesticated strain *L. acidophilus* DGK.

higher than that of the original strain (1.31 g DCW/L) during the 24 h fermentation. In addition, a higher concentration of lactic acid (11.28 g/L) was obtained in the domesticated strain and was responsible for the lower pH value that was observed in this strain. More efficient glucose metabolism by the domesticated cells increased biomass, and lactic acid was the major metabolite.

Characteristics of the bacteria before and after domestication were examined. Gram-positive, rod-shape and catalase-negative results were found in both the domesticated cells and the original cells. Resistance to acid and bile salt was also similar (data not shown). Further identification of the strains was performed using the API 50 CHL test kit. The results verified that both strains were *L. acidophilus*. From the distinct results of the biochemical tests by the API 50 CHL kit (Table 1), sorbitol, sucrose and raffinose could not be well utilized by the domesticated strain in a 24 h culture; however, only D-raffinose was undigested in a 48 h culture. The inability to metabolize D-raffinose by the domesticated strain *L. acidophilus* DGK might induce cells to utilize glucose more efficiency and lead to more biomass and lactic acid production. For further confirmation of their similarity, two primers, LB308F and LB806RM, were used to amplify the heat shock protein (hsp) gene sequence. The comparison of the *hsp60* gene sequences of 499 bp between these two strains showed that 99.2% of the DNA sequence was the same (Figure 3). This result further verifies that these two strains are the same species by instinct. The comparative characterization of the domesticated strain of *L. acidophilus* with the original strain has allowed the identification of the phenotypic changes derived from aerotolerance acquisition relevant to probiotic applications.

Effects of carbon sources on the growth of the domesticated strain

Carbon sources are the most important medium components for cell growth next to basic nutrients, such as amino acids, vitamins or trace elements. In the preliminary medium test, medium composed of glucose 10 g/L, yeast extract 10 g/L, soy peptone (Scharlau) 10 g/L, sodium acetate 5 g/L, ammonium citrate 2 g/L, $MgSO_4.7H_2O$ 0.2 g/L, $MnSO_4.4H_2O$ 0.05 g/L and corn steep liquor 10 mL/L was used to study biomass production under different carbon source replacements (glucose was replaced). The results shown in Table 2 indicate that biomass production of the domesticated strain was significantly higher than that of the original strain. Cell densities of the original strain (2.60 g DCW/L) or the domesticated strain (4.81 g DCW/L) in MRSC medium were higher than those in media substituted with different carbon sources. Before adaptation, maltose and galactose used as the carbon sources allowed greater biomass accumulation (2.02 g and 1.97 DCW/L, respectively) than the other carbon sources. However, after the adaptation, glucose as the carbon source led to the highest biomass production (4.44 g DCW/L), while sucrose, sorbitol and potassium gluconate as carbon sources produced less biomass. Glucose and lactose exhibited higher amounts of biomass production in the domesticated strain. Conversely, the domesticated stain with raffinose or maltose as the carbon source had reduced biomass production. The lower biomass production using raffinose as the carbon source was consistent with the result of the API test, which showed that the domesticated strain cannot metabolize raffinose. We observed that higher biomass correlated to a reduced pH.

Much lower biomass production was obtained when the original strain was incubated while shaking at 150 rpm at 37°C for 24 h (Table 3). The concentration of the cell density in the domesticated strain *L. acidophilus* DGK was significantly increased when cultivating while shaking, even with the different carbon sources, such as glucose, galactose, mannitol and lactose. These results indicate that the domesticated strain had been adapted to aerobic environments and exhibited higher efficiency of metabolism of the carbon sources with the exception of raffinose; although, biomass production was greater when the cultures were cultivated statically versus shaking.

Metabolic changes of carbohydrates have been detected in *Bifidobacterium* strains that have acquired bile tolerance (Sanchez et al., 2005). The aerotolerant derivative, *L. acidophilus* DGK, might display higher fermentative

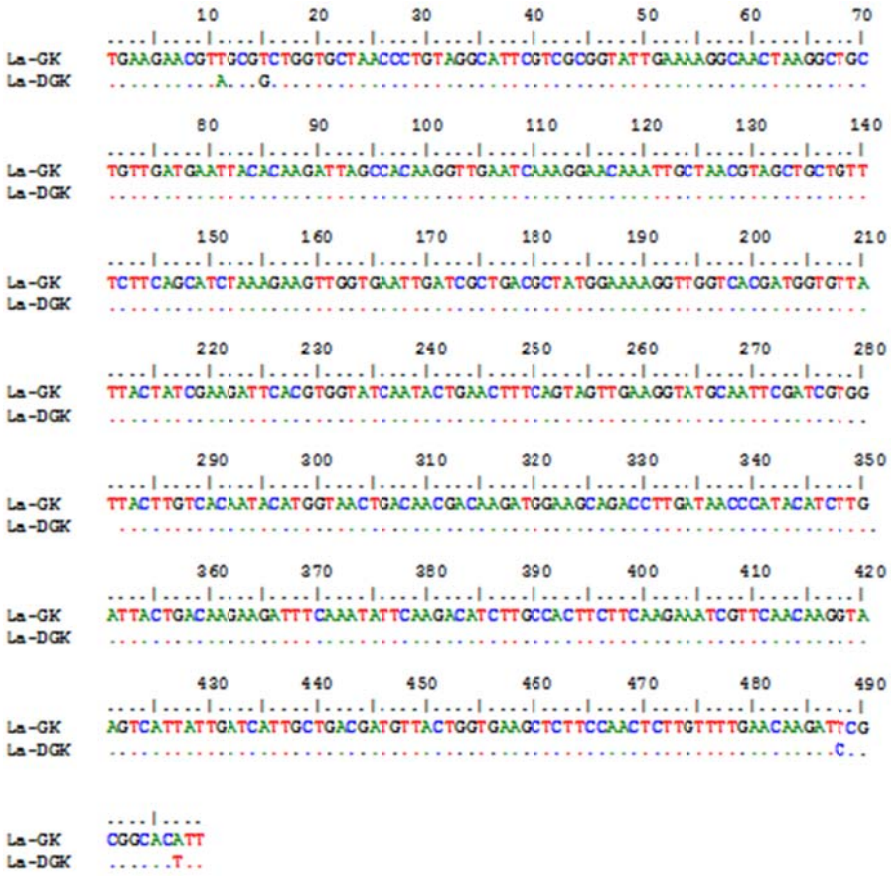

Figure 3. Partial DNA sequence of the hsp60 gene from the original strain *L. acidophilus* GK and the domesticated strain *L. acidophilus* DGK.

Table 2. Effects of carbon sources on biomass production of the original strain *L. acidophilus* GK and the domesticated strain *L. acidophilus* DGK grown statically

Medium (control group)	Original strain *L. acidophilus* GK		Domesticated strain *L. acidophilus* DGK	
	Biomass (g DCW/L)	pH	Biomass (g DCW/L)	pH
MRSC	2.60 ± 0.04^{a†}	3.83 ± 0.01^{f}	4.81 ± 0.04^{a}	3.75 ± 0.01^{i}
Carbon source*				
Glucose	1.30 ± 0.05^{e}	4.15 ± 0.01^{c}	4.44 ± 0.14^{b}	3.80 ± 0.01^{h}
Fructose	1.48 ± 0.02^{d}	4.15 ± 0.01^{c}	2.93 ± 0.06^{e}	3.99 ± 0.01^{f}
Galactose	1.97 ± 0.05^{b}	4.12 ± 0.01^{c}	3.74 ± 0.23^{d}	4.00 ± 0.01^{f}
D-Mannitol	1.25 ± 0.04^{e}	5.00 ± 0.01^{b}	2.66 ± 0.09^{f}	4.60 ± 0.01^{e}
Lactose	1.82 ± 0.05^{c}	4.07 ± 0.02^{d}	4.09 ± 0.05^{c}	3.92 ± 0.01^{g}
Sucrose	0.80 ± 0.13^{g}	5.22 ± 0.02^{a}	1.13 ± 0.03^{i}	5.34 ± 0.01^{a}
D-Raffinose	1.71 ± 0.17^{c}	4.01 ± 0.01^{e}	1.13 ± 0.02^{i}	5.31 ± 0.02^{a}
D-Sorbitol	0.96 ± 0.04^{f}	5.20 ± 0.01^{a}	1.25 ± 0.02^{h}	5.06 ± 0.01^{c}
Potassium gluconate	0.88 ± 0.01^{f}	5.26 ± 0.01^{a}	1.17 ± 0.04^{i}	5.30 ± 0.01^{a}
Glycerol	1.30 ± 0.05^{e}	4.06 ± 0.02^{d}	1.30 ± 0.07^{h}	5.11 ± 0.01^{b}
Maltose	2.02 ± 0.09^{b}	4.08 ± 0.01^{d}	1.58 ± 0.02^{g}	4.90 ± 0.01^{d}

*Composition of the basal medium: Yeast extract, 10 g/L; Soy peptone, 10 g/L; Sodium acetate, 5 g/L; Ammonium citrate, 2 g/L; $MgSO_4 \cdot 7H_2O$, 0.2 g/L; $MnSO_4 \cdot 4H_2O$, 0.05 g/L; Corn steep liquor 10 ml l^{-1}. Fermentation conditions: 1% inoculum, 37°C, 24 h, at 10 mL medium/50 mL Hinton flask without shaking. Each value represents the mean ± SD from the experiment conducted in triplicate. †Data in the same column with different letters are significantly different at $p<0.05$.

Table 3. Effects of carbon sources on the biomass of the original strain *L. acidophilus* GK and the domesticated strain *L. acidophilus* DGK grown while shaking at 150 rpm

Medium (control group)	Original strain *L. acidophilus* GK		Domesticated strain *L. acidophilus* DGK	
	Biomass (g DCW/L)	pH	Biomass (g DCW/L)	pH
MRSC	1.06 ± 0.04^{a†}	4.85 ± 0.02^{g}	4.54 ± 0.07^{a}	3.96 ± 0.04^{g}
Carbon source*				
Glucose	0.89 ± 0.03^{c}	5.12 ± 0.01^{c}	3.85 ± 0.12^{c}	4.06 ± 0.02^{f}
Fructose	0.97 ± 0.12^{b}	5.12 ± 0.02^{c}	2.06 ± 0.03^{d}	4.23 ± 0.01^{e}
Galactose	0.66 ± 0.01^{e}	5.41 ± 0.02^{a}	4.26 ± 0.29^{b}	4.06 ± 0.01^{e}
D-Mannitol	0.78 ± 0.03^{d}	5.35 ± 0.17^{b}	3.81 ± 0.05^{c}	3.89 ± 0.12^{h}
Lactose	0.98 ± 0.03^{b}	5.10 ± 0.01^{cd}	3.93 ± 0.28^{c}	4.06 ± 0.01^{f}
Sucrose	0.97 ± 0.01^{b}	5.03 ± 0.03^{e}	0.89 ± 0.02^{g}	5.49 ± 0.01^{c}
D-Raffinose	0.93 ± 0.01^{b}	5.09 ± 0.03^{d}	0.82 ± 0.02^{g}	5.57 ± 0.01^{b}
D-Sorbitol	0.77 ± 0.02^{d}	5.34 ± 0.01^{b}	0.97 ± 0.03^{gf}	5.47 ± 0.01^{c}
Potassium gluconate	0.74 ± 0.01^{d}	5.42 ± 0.02^{a}	1.15 ± 0.03^{ef}	5.85 ± 0.02^{a}
Glycerol	0.75 ± 0.02^{d}	5.35 ± 0.02^{b}	0.80 ± 0.03^{g}	5.49 ± 0.01^{c}
Maltose	0.91 ± 0.02^{bc}	4.90 ± 0.01^{f}	1.29 ± 0.10^{e}	5.34 ± 0.01^{d}

*Composition of the basal medium is the same as Table 2. †Data in the same column with different letters are significantly different at $p<0.05$.

ability to carbohydrates and increased enzymatic activities, such as glucosidase, which could favor carbohydrates metabolism. The derived strain might improve carbohydrate hydrolysis activity and be more suitable for application in the production process of probiotic cells.

Effects of nitrogen sources on the growth of the domesticated strain

A basal medium (PM) consisting of glucose (20 g/L), yeast extract (10 g/L, sodium acetate (5 g/L) and $MnSO_4 \cdot 4H_2O$ (0.05 g/L) was used to study the supplementation of different nitrogen sources (10 g/L) under static or shaking fermentation (Tables 4 and 5). The results presented in Table 4 show that, in the original strain under static conditions, higher biomass was obtained when corn steep liquor (CSL) was the nitrogen source versus other nitrogen sources. CSL is an important nitrogen alternative to peptone or yeast extract. CSL contains amino acids and vitamins that may be beneficial for the growth of LAB. Biomass production was significantly enhanced in the domesticated strain *L. acidophilus* DGK compared to the parental strain. Among the nitrogen sources examined in the present study, beef extract, peptone, ammonium citrate and monosodium glutamate (MSG) produced a higher amount of cells in the static conditions. Cheaper organic nitrogen sources, such as MSG and CSL, and inorganic nitrogen sources, such as ammonium citrate, can be used as nitrogen alternatives to reduce production costs.

Table 5 shows the effect of different nitrogen sources on biomass production under shaking conditions. When comparing the biomass produced by the domesticated strain from Table 4 and Table 5, we found that higher biomass was obtained in shaking conditions, compared to static conditions, when beef extract, malt extract, soy peptone, yeast peptone and tryptone were used as the nitrogen sources. In contrast, ammonium citrate as the nitrogen source produced more biomass in the static situation. Thus, the improvement of biomass production depended on both the nitrogen source and the cultivation environment. Medium with urea as the nitrogen source in both the static and shaking conditions produced the lowest biomass (0.81 g DCW/L), indicating that this inorganic compound was not suitable for the growth of LAB.

DISCUSSION

In the present study, it was shown that characteristics of LAB could be modified through an adaptation strategy by successively cultivating strains under specific conditions and screening the stable strains. Some papers have described the modification of strains by adapting them in extreme environments and have revealed difficulties for maintaining their stabilities. In this study, we isolated the stable strain *L. acidophilus* DGK not only by successive subculture for 14 days in aerobic conditions but also through population analysis of over 400 colonies. The aerotolerant strain had increased biomass production in aerobic environments and possessed an improved efficiency of nutrient utilization as well as production of lactic acid. This modified strain might be more adaptable to the stirring process of pH control and the feeding strategy used during fed-batch fermentation. The identification of the domesticated strain by API 50 CHL and hsp60 gene sequence analysis confirmed that the modified strain was derived from the starting strain and had improved growth characteristics and nutrition

Table 4. Effects of nitrogen sources on the biomass production of the original strain *L. acidophilus* GK and the domesticated strain *L. acidophilus* DGK grown statically.

Medium (control group)	Original strain *L. acidophilus* GK		Domesticated strain *L. acidophilus* DGK	
	Biomass (g DCW/L)	pH	Biomass (g DCW/L)	pH
MRSC	2.56 ± 0.03^{a†}	3.76 ± 0.01^{k}	04.59 ± 0.35^{a}	3.67 ± 0.02^{e}
PM*	0.06 ± 0.01^{i}	5.55 ± 0.01^{f}	02.54 ± 0.05^{g}	3.68 ± 0.01^{ih}
Nitrogen source to PM*				
Beef extract	0.11 ± 0.01^{d}	5.46 ± 0.02^{f}	2.98 ± 0.11^{ef}	3.74 ± 0.01^{ef}
Malt extract	0.10 ± 0.01^{de}	5.39 ± 0.02^{g}	1.18 ± 0.04^{c}	3.68 ± 0.02^{h}
Corn steep liquor	1.04 ± 0.02^{b}	4.19 ± 0.04^{j}	3.32 ± 0.23^{c}	3.90 ± 0.01^{h}
Peptone	0.09 ± 0.01^{efg}	5.54 ± 0.03^{d}	3.31 ± 0.07^{c}	3.71 ± 0.01^{fg}
Soy peptone	0.09 ± 0.01^{ef}	5.40 ± 0.02^{g}	1.42 ± 0.05^{j}	3.92 ± 0.01^{c}
Yeast peptone	0.10 ± 0.01^{de}	5.50 ± 0.02^{e}	1.46 ± 0.06^{j}	3.71 ± 0.01^{g}
Tryptone	0.10 ± 0.01^{de}	5.38 ± 0.01^{g}	1.48 ± 0.03^{j}	3.68 ± 0.01^{h}
Ammonium citrate	0.07 ± 0.01^{hi}	5.80 ± 0.01^{b}	3.86 ± 0.03^{b}	3.99 ± 0.01^{b}
Ammonium chloride	0.09 ± 0.01^{ef}	5.30 ± 0.01^{ih}	2.19 ± 0.02^{h}	3.56 ± 0.01^{h}
Ammonium nitrate	0.08 ± 0.01^{hfg}	5.54 ± 0.02^{ih}	1.98 ± 0.03^{i}	3.66 ± 0.01^{ih}
Ammonium sulfate	0.08 ± 0.01^{hfe}	5.34 ± 0.01^{h}	2.29 ± 0.03^{g}	3.61 ± 0.01^{j}
Ammonium phosphate	0.13 ± 0.01^{c}	5.72 ± 0.01^{c}	2.72 ± 0.10^{h}	3.71 ± 0.01^{g}
Urea	0.04 ± 0.01^{j}	5.84 ± 0.04^{a}	0.81 ± 0.08^{k}	5.06 ± 0.04^{a}
MSG	0.07 ± 0.01^{hig}	5.56 ± 0.01^{d}	13.71 ± 0.05^{d}	3.82 ± 0.01^{d}
H_2O	0.07 ± 0.01^{hi}	5.53 ± 0.04^{de}	1.48 ± 0.03^{g}	3.64 ± 0.02^{i}

*Composition of basal medium (PM): Glucose, 20 g/L; Yeast extract, 10 g/L; Sodium acetate, 5 g/L; $MnSO_4 \cdot 4H_2O$, 0.05 g/L; nitrogen source added: 10 g/L. †Data in the same column with different letters are significantly different at $p<0.05$.

Table 5. Effects of nitrogen sources on biomass production of the original strain *L. acidophilus* GK and the domesticated strain *L. acidophilus* DGK grown while shaking at 150 rpm

Medium (control group)	Original strain *L. acidophilus* GK		Domesticated strain *L. acidophilus* DGK	
	Biomass (g DCW/L)	pH	Biomass (g DCW/L)	pH
MRSC	0.97 ± 0.16^{a†}	4.06 ± 0.01^{l}	4.79 ± 0.11^{a}	3.90 ± 0.01^{c}
PM*	0.07 ± 0.01^{e}	5.55 ± 0.01^{de}	3.10 ± 0.02^{e}	3.69 ± 0.01^{h}
Nitrogen source to PM*				
Beef extract	0.11 ± 0.01^{b}	5.53 ± 0.01^{de}	3.62 ± 0.04^{b}	3.74 ± 0.01^{g}
Malt extract	0.11 ± 0.01^{b}	5.43 ± 0.02^{fh}	2.88 ± 0.12^{ef}	3.69 ± 0.01^{h}
Corn steep liquor	0.96 ± 0.06^{a}	4.85 ± 0.01^{k}	3.39 ± 0.04^{c}	3.74 ± 0.01^{g}
Peptone	0.09 ± 0.01^{cde}	5.61 ± 0.02^{d}	3.64 ± 0.08^{b}	3.81 ± 0.01^{e}
Soy peptone	0.09 ± 0.01^{cde}	5.60 ± 0.13^{d}	3.62 ± 0.02^{b}	3.78 ± 0.01^{f}
Yeast peptone	0.11 ± 0.01^{cd}	5.45 ± 0.02^{ghi}	3.54 ± 0.11^{b}	3.76 ± 0.01^{fg}
Tryptone	0.10 ± 0.01^{d}	5.47 ± 0.02^{fgh}	3.57 ± 0.05^{b}	3.74 ± 0.01^{g}
Ammonium citrate	0.07 ± 0.01^{e}	5.83 ± 0.01^{b}	2.02 ± 0.10^{i}	4.16 ± 0.01^{b}
Ammonium chloride	0.08 ± 0.01^{cde}	5.42 ± 0.01^{hi}	2.63 ± 0.10^{h}	3.59 ± 0.01^{k}
Ammonium nitrate	0.08 ± 0.01^{cde}	5.40 ± 0.01^{ij}	1.95 ± 0.04^{i}	3.77 ± 0.04^{f}
Ammonium sulfate	0.07 ± 0.01^{e}	5.34 ± 0.05^{j}	2.75 ± 0.02^{g}	3.63 ± 0.01^{j}
Ammonium phosphate	0.13 ± 0.01^{b}	5.73 ± 0.01^{c}	2.96 ± 0.05^{e}	3.84 ± 0.01^{d}
Urea	0.04 ± 0.01^{f}	5.93 ± 0.03^{a}	0.73 ± 0.07^{j}	5.14 ± 0.01^{a}
MSG	0.08 ± 0.01^{de}	5.55 ± 0.02D^{e}	3.27 ± 0.09^{d}	3.93 ± 0.01^{c}
H_2O	0.08 ± 0.01^{e}	5.50 ± 0.02^{efg}	2.81 ± 0.03^{fg}	3.65 ± 0.01^{i}

*Composition of basal medium (PM) is the same as Table 4. †Data in the same column with different letters are significantly different at $p<0.05$.

utilization. This change may be due to gene modification during the adaptation process. Stability of biomass production by this modified strain was verified by repeated cultivation and analysis. The process of domestication and population analysis for screening for the enhancement of desired characteristics might be applicable to other probiotics in the industrial production of LAB starter or chemical-producing strains.

An aerotolerant probiotic strain, *L. acidophilus* DGK, displaying improved biomass production and nutrient utilization might be due to gene modification. It would be particularly worthwhile to further study the proteins induced in the DGK strain during aerobic stress, to identify those proteins that are important for increased tolerance to oxygen stress. It is possible that this adaptation could enhance the biomass production during industrial fermentation and might also lengthen the viability of this probiotic strain during storage at room temperature. The application of prolonged exposures to aerobic conditions has been particularly useful to generate stable and highly aerotolerant *L. acidophilus* strains from naturally aerobically sensitive strains. This treatment also intro-duced phenotypic changes that could lead to strains with improved biological properties, although each derivative strain requires further individual evaluation.

The ability to metabolize raffinose was inhibited in the domesticated strain, as was shown by the API assay and carbon source replacement experiments. Glucose, galactose and lactose were efficiently utilized by the domesticated strain in both the static and shaking conditions.

The cell growth of the modified strain was significantly improved by the addition of various nitrogen sources either under static or shaking conditions. The effect of these additions was more significant in the shaking conditions than in the static conditions. By comparing the efficiency of carbon and nitrogen source replacement by the domesticated strain, we conclude that both the fermentation environments and the carbon and nitrogen sources highly impact biomass production. The meta-bolism and utilization of nutrients by the modified strain varied not only based on the growth environment but also on the varieties of nutrients available to the strain.

Conflict of interests

The authors have not declared any conflict of interest.

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