The nutrient requirements of *Lactobacillus acidophilus* LA-5 and their application to fermented milk

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ABSTRACT

Lactobacillus acidophilus LA-5 is a suitable probiotic for food application, but because of its slow growth in milk, an increase in its efficiency is desired. To shorten the time required for fermentation, the nutrient requirements of *L. acidophilus* LA-5 were analyzed, including the patterns of consumption of amino acids, purines, pyrimidines, vitamins, and metal ions. The nutrients required by *L. acidophilus* LA-5 were Asn, Asp, Cys, Leu, Met, riboflavin, guanine, uracil, and Mn^{2+} , and when they were added to milk, the fermentation time of fermented milk prepared by *L. acidophilus* LA-5 alone was shortened by 9 h, with high viable cell counts that were maintained during storage of nutrient-supplemented fermented milk compared with the control. For fermented milk prepared by fermentation with *Streptococcus thermophilus*, *Lactobacillus delbrueckii* ssp. *bulgaricus*, and *L. acidophilus* LA-5, viable cell counts of *L. acidophilus* LA-5 increased 1.3-fold and were maintained during storage of nutrient-supplemented fermented milk compared with the control. Adding nutrients had no negative effect on the quality of the fermented milk. The results indicated that suitable nutrients enhanced the growth of *L. acidophilus* LA-5 and increased its viable cell counts in fermented milk prepared by *L. acidophilus* LA-5 alone and mixed starter culture, respectively.

Key words: *Lactobacillus acidophilus* LA-5, nutrient requirements, fermented milk, fermentation time, viable cell count

INTRODUCTION

Lactobacillus acidophilus is a commercial strain and probiotic that is widely used in the dairy industry to

obtain high-quality fermentation products (Batista et al., 2015; Felicio et al., 2016). *Lactobacillus acidophilus* LA-5 has the ability to reduce the serum cholesterol level, balance and stabilize the enteric microbiota, stimulate an immune response, improve lactose digestion, and potentially kill cancer cells (Lin and Chen, 2000; Sousa et al., 2008; Medellín-Peña and Griffiths, 2009). Fermented milk containing *L. acidophilus* LA-5 can protect against intestinal diseases by increasing beneficial bacteria and reducing potentially pathogenic bacteria (Savard et al., 2011). Moreover, the *L. acidophilus* LA-5 present in fermented milk was effective in reducing the *Streptococcus mutans* levels in saliva, and can also decrease the risk factors for acquiring nonalcoholic fatty liver disease (Nabavi et al., 2014; Bafna et al., 2018). Accordingly, fermented milk is an ideal carrier of *L. acidophilus* LA-5.

Many fermented dairy products on the Chinese market have been manufactured with a single probiotic strain such as Yakult (*Lactobacillus casei* Shirota), LAJOIE (*Lactobacillus paracasei* LPC-37), and Cherita (*Lactobacillus rhamnosus* GG ATCC 53103). However, the fermentation time required to manufacture fermented milk with a single probiotic strain is long (Soto et al., 2019). Quick production of fermented milk using *L. acidophilus* cannot occur because it also grows slowly in milk, which is unfavorable for the commercial development of fermented milk manufactured with a single probiotic strain. Therefore, to shorten the time required to produce fermented milk, preparation solely using *L. acidophilus* LA-5 should be considered. The survival of *Streptococcus thermophilus* and *Lactobacillus delbrueckii* ssp. *bulgaricus* during transit through the human digestive environment remained controversial (Ben-Yahia et al., 2012); several studies suggested that *S. thermophilus* and *L. delbrueckii* ssp. *bulgaricus* did not survive the passage of the human gastrointestinal tract (del Campo et al., 2005; Ballesta et al., 2008), whereas others showed the opposite (Mater et al., 2005; García-Hernández et al., 2012). Probiotics are often added to carry out co-fermentation with *S. thermophi-*

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lus and *L. delbrueckii* ssp. *bulgaricus*, including *L. acidophilus*; however, viable probiotic cell counts are often low in fermented milk (Talwalkar and Kailasapathy, 2003; Donkor et al., 2006). *Streptococcus thermophilus* and *Lactobacillus delbrueckii* ssp. *bulgaricus* grow faster than *L. acidophilus* during fermentation, and because *L. delbrueckii* ssp. *bulgaricus* produces lactic acid during fermentation and refrigerated storage, postacidification that causes loss of viability of *L. acidophilus* is an important factor to consider (Ng et al., 2011). Hence, increasing the viable cell counts and viability of *L. acidophilus* in fermented milk prepared by mixed starter culture should be considered.

Lactobacillus acidophilus is a fastidious organism with complex nutrient requirements. Its growth requires a variety of nutrients, including AA, vitamins, metal ions, buffers, and other compounds (Aller et al., 2014). It has been observed that milk lacking certain nutrients cannot support the rapid growth of *L. acidophilus*. The viability of *L. acidophilus* was improved in fermented milk supplemented with ascorbic acid and cysteine (Dave and Shah, 1997a,b). Adding onion juice to milk stimulated the growth of *L. acidophilus* because of the sugars, vitamins, and minerals present in the juice (Li et al., 2016). The aforementioned studies showed that suitable nutrients enhanced the growth and viability of *L. acidophilus*. However, few studies have investigated the nutrient requirements of *L. acidophilus* LA-5 and its ability to ferment milk alone and ferment milk in a mixed starter culture for the purpose of shortening the fermentation time and increasing its viable cell counts and viability.

The purpose of the present study is to determine the optimal method for reducing the fermentation time of milk prepared solely by *L. acidophilus* LA-5 and increase its viable cell counts and viability in fermented milk prepared by a combination of *S. thermophilus*, *L. delbrueckii* ssp. *bulgaricus*, and *L. acidophilus* LA-5, based on the nutritional requirements of *L. acidophilus* LA-5.

MATERIALS AND METHODS

Strain, Culture Conditions, and Fermentation Experiments

Lactobacillus acidophilus LA-5, *Streptococcus thermophilus* CHCC 6483, and *Lactobacillus delbrueckii* ssp. *bulgaricus*-12 were obtained from Chr. Hansen (Hørsholm, Denmark). *Lactobacillus acidophilus* LA-5 was activated and subcultured in de Man, Rogosa, and Sharpe (MRS) broth (HuanKai Microbial Sci. and Tech. Co. Ltd., Guangdong, China) at 37°C. Cells were harvested by centrifugation ($8,000 \times g$, 5 min, 4°C) and

washed twice with PBS buffer (pH 7.2). Then, the culture was fermented in a 10-L Biotech-7000 bioreactor (Shanghai Baoxing, Shanghai, China) containing 7 L of chemically defined medium (CDM) with 2% (vol/vol) inoculum. The CDM was prepared according to Hebert et al. (2004). The temperature and rotation speed were set at 37°C and 150 rpm, respectively. The growth rate was measured by spectrophotometric measurement at 600 nm. The cells were harvested at 0, 2.0, 4.5, 6.5, and 8.0 h by centrifugation ($8,000 \times g$, 5 min, 4°C). The pellet and supernatant were stored at -80°C for the next analysis. Batch fermentations were independently repeated 3 times.

Effects of Nutrients on the Growth of *L. acidophilus* LA-5

The relationship of CDM components and the growth of *L. acidophilus* LA-5 has been described by the single nutrient omission method (Vera Pingitore et al., 2009). Briefly, the nutrient was considered to be essential if its omission resulted in growth rates of less than half the maximum growth rate in the complete CDM. The nutrient was considered to be stimulatory when in its omission, the growth rate was between 50% and 80% of that observed in complete CDM. The nutrient was considered to be nonessential when in its omission, the growth rate was 80% (or more) of that obtained in the complete CDM.

Selection of Nutrients

Based on nutrient requirements, nutrients with a high consumption rate, AA with a high necessary rate, and essential and stimulatory nutrients were added to milk to conduct the preliminary single addition experiments with fermented milk. The aim of the preliminary single addition experiments was to select the nutrients that shorten the time required to prepare fermented milk using *L. acidophilus* LA-5. Accordingly, Asn, Asp, Leu, Met, riboflavin, guanine, uracil, and Mn^{2+} were selected, and the concentrations of these nutrients that were used are shown in Table 1.

Analysis of AA, Vitamins, Purines, Pyrimidines, and Ions

The whole-cell AA composition and the amounts of each AA that were consumed were determined by an amino acid analyzer (Acquity UPLC, Waters Corp., Milford, MA). Thiamine, riboflavin, nicotinic acid, Capantothenate, pyridoxal, folic acid, cyanocobalamin, biotin, and inositol were measured by RP-HPLC with an UV detector (Heudi et al., 2005). The presence of

p-aminobenzoic acid was determined by HPLC (Okdeh et al., 2003). Concentrations of adenine, guanine, xanthine, thymine, and uracil were measured by liquid chromatography-tandem MS according to Stentoft et al. (2014). The concentrations of K^+ , Na^+ , Mg^{2+} , Fe^{2+} , and Mn^{2+} were determined using an ion exchange method (Pohl and Prusisz, 2007).

Preparation of Fermented Milk

The culture media MRS (for *L. acidophilus* and *L. delbrueckii* ssp. *bulgaricus*) and M17 (for *S. thermophilus*) were used to activate strains at 37°C. Strains were inoculated and propagated 2 times in MRS and M17 until strain activity was stable. Mother culture was prepared with sterilized skim milk that had 5% inoculation amount of the activated strains. The mother cultures of *S. thermophilus* and *L. delbrueckii* ssp. *bulgaricus* were cultivated at 43°C, and *L. acidophilus* was cultivated at 37°C. They were then stored at 4°C until the curd was firm. For fermented milk prepared using only *L. acidophilus* LA-5, milk was heated to 95°C for 5 min and cooled to 37°C, and then inoculated with 3% (vol/vol) of the mother culture of *L. acidophilus* LA-5 (with viable cell counts 2.0×10^6 cfu/mL) and incubated at 37°C until the pH of the milk reached 4.5. For fer-

mented milk prepared using mixed starter culture, milk was heated to 95°C for 5 min and cooled to 43°C, and then inoculated with 3% (vol/vol) of mother culture of *S. thermophilus*, *L. delbrueckii* ssp. *bulgaricus*, and *L. acidophilus* LA-5 in a 1:1:1 ratio (with viable cell counts 2.0×10^6 cfu/mL for each strain), and incubated at 43°C until the pH of the milk reached 4.5. When fermentation was completed, all samples were quickly cooled in an ice bath and then stored at 4°C.

According to the nutrients selected, fermented milk was prepared with Asn, Asp, Cys, Leu, Met, riboflavin, guanine, uracil, and Mn^{2+} (Table 1). Fermented milk that had not been supplemented with nutrients was used as a control. Batch fermentations were repeated 3 independent times.

Microbiological, Physicochemical, Sensory, and Textural Analyses of Fermented Milk

Lactobacillus acidophilus LA-5, *Streptococcus thermophilus* CHCC 6483, and *Lactobacillus delbrueckii* ssp. *bulgaricus*-12 were enumerated in MRS-maltose agar (pH 6.4), M17 agar (pH 7.0), and MRS-glucose agar (pH 4.58), respectively (Ashraf and Shah, 2011). Sample pH values were measured at room temperature with a combined glass electrode attached to a Jenway

Table 1. Nutrients added to milk to assess effects on fermentation

Nutrient	Milk (g/L)	Consumption (g/L)	Requirement (g/L)	Addition (g/L)
Asn	— ¹	0.1253	0.0871	0.1253
Asp	—	0.1253	0.0871	0.1253
Cys	—	0.0089	0.0507	0.0507
Gln	0.05126	0.1024	0.1071	0.1071
Glu	0.05126	0.1024	0.1071	0.1071
Gly	0.000419	0.0309	0.0416	0.0416
Ile	0.000164	0.0411	0.0434	0.0434
Leu	0.000961	0.0634	0.0668	0.0668
Lys	0.003115	0.0336	0.0627	0.0627
Met	0.000211	0.0201	0.0520	0.0520
Phe	0.000148	0.0356	0.0466	0.0466
Pro	—	0.0182	0.0179	0.0182
Thr	0.000285	0.0531	0.0259	0.0531
Tyr	0.001305	0.0509	0.0427	0.0509
Val	0.003751	0.0280	0.0459	0.0459
Ala	—	0.0491	0.0973	0.0973
Arg	0.000551	0.0312	0.0971	0.0971
Guanine	—	0.00455	—	0.00455
Adenine	0.002728	0.00461	—	0.00461
Xanthine	—	0.00277	—	0.00277
Uracil	—	0.00523	—	0.00523
Thymine	—	0.00424	—	0.00424
Thiamine	0.00064	0.000264	—	0.000264
Riboflavin	0.00175	0.000485	—	0.000485
Ca-pantothenate	0.00346	0.000241	—	0.000241
Mg^{2+}	0.11	0.064	—	0.064
Fe^{2+}	—	0.00985	—	0.00985
Mn^{2+}	—	0.00213	—	0.00213

¹— = undetected.

3510 pH meter (Keison Products, Chelmsford, UK). The titratable acidity and water-retaining capability were determined according to Jia et al. (2016). Textural properties were analyzed by compression tests with the Texture Analyzer TA-Plus (Lloyd Instruments, Bognor Regis, UK) as described by Mudgil et al. (2018). The sensory properties of the fermented milk were evaluated according to Ahtesh et al. (2018). Sensory properties of the fermented milk were assessed by 20 trained panelists recruited from staff members and students from the Northeast Agricultural University. Samples in 30-mL white plastic cups coded with 1 digit at room temperature were presented to each panelist. Water and crackers were given to panelists for palate cleansing between samples, allowing 15-min breaks between sessions. Panelists were advised not to swallow the product. Each panelist evaluated 4 samples for flavor, bitterness, texture, appearance, and acceptance, using a 10-point hedonic scale (1 = dislike extremely to 10 = like extremely).

Statistical Analysis

The data were analyzed with ANOVA using Statistica 9.2 software (StatSoft Inc., Tulsa, OK). The comparison between means was determined by using the Tukey's significant difference test ($P < 0.05$). All data were presented as the mean or mean $\pm$ standard deviation.

RESULTS

Nutrient Consumption Profiles

As shown in Figure 1a-c, the AA consumed in the greatest amounts were Asp + Asn (0.94 mmol/L), followed by Glu + Gln (0.69 mmol/L), Ala (0.55 mmol/L), and Leu (0.48 mmol/L); the AA consumed in the smallest amount was Phe (0.03 mmol/L). The remainder of the consumption amounts ranged from 0.06 to 0.44 mmol/L. For the consumption rate of AA, the highest rate was for Asp + Asn (88.97%), followed by Lys (81.98%), Leu (77.24%), Tyr (69.69%), Thr (68.94%), Glu + Gln (66.99%), and Ala (66.85%). The lowest consumption rate was for Phe (17.54%), and the remainder of the consumption rates ranged from 22.63 to 56.45%.

As shown in Figure 1d, for purines and pyrimidines, the largest consumption amount was for uracil (0.0463 mmol/L), followed by adenine (0.0341 mmol/L) and thymine (0.0336 mmol/L). The smallest consumption amount was for xanthine (0.0182 mmol/L), followed by guanine (0.0301 mmol/L). The highest and lowest

consumption rates were uracil at 65.90% and xanthine at 40.85%. The consumption rates of guanine, thymine, and adenine were 53.15%, 50.05%, and 49.94%, respectively.

As shown in Figure 1e-f, the largest consumption amount of vitamins was for *p*-aminobenzoic acid (0.0085 mmol/L), followed by biotin (0.0060 mmol/L), inositol (0.0043 mmol/L), and pyridoxal (0.0021 mmol/L). The least consumed vitamin was cyanocobalamin (0.00008 mmol/L). The remainder of the consumption amounts of vitamins ranged from 0.0003 to 0.0013 mmol/L. The highest consumption rate was for riboflavin (61.70%), followed by thiamine (29.46%), Ca-pantothenate (25.96%), and folic acid (19.61%). The lowest consumption rate was for *p*-aminobenzoic acid (14.90%). The remainder of the consumption rates of vitamins ranged from 17.48 to 19.52%.

As shown in Figure 1g-h, the largest amount of minerals consumed was for Na^+ (10.36 mmol/L), with a consumption rate of 18.90%, followed by K^+ (4.22 mmol/L) and Mg^{2+} (0.531 mmol/L) at consumption rates of 16.07% and 38.32%, respectively. The consumption of Mn^{2+} was 0.141 mmol/L, but its consumption rate was the highest (91.02%). The least consumed ion was Fe^{2+} (0.0354 mmol/L) at a consumption rate of 58.28%.

Necessary AA Profiles

According to the concentrations of AA in the whole-cell hydrolysates, the required amounts of AA were analyzed. The ratio of the requirement of necessary AA during different periods to all required amounts of AA in the fermentation process is presented in Figure 2. The proportion of Glu + Gln was 10.71%, and was higher than the amount of other AA demanded for the growth of *L. acidophilus* LA-5. The Ala (9.73%), Arg (9.71%), and Asp + Asn (8.70%) were next, followed by Pro (7.19%), Leu (6.68%), and Lys (6.28%). The required amounts of Met, Cys, Phe, Val, Tyr, Ile, Gly, Ser, His, and Thr ranged from 2.59 to 5.20% of all AA required.

Effects of Nutrients on the Growth of *L. acidophilus* LA-5

As Figure 3 shows, Cys, Glu, Ile, Leu, Lys, Met, Phe, Thr, Try, Val, Arg, and glucose are essential nutrients for the growth of *L. acidophilus* LA-5. The Gly, Ca-pantothenate, and Mn^{2+} were stimulatory nutrients that stimulated the growth of *L. acidophilus* LA-5. Others were nonessential nutrients that had no obvious effects on the growth of *L. acidophilus* LA-5.

Figure 1. Residual concentrations of (a, b, c) AA, (d) purines and pyrimidines, (e, f) vitamins, and (g, h) minerals at different culture times ($t_0 = 0$ h, $t_1 = 2.0$ h, $t_2 = 4.5$ h, $t_3 = 6.5$ h, $t_4 = 8.0$ h). (a) The growth curve of *Lactobacillus acidophilus* LA-5. The results are expressed as the mean $\pm$ SD of 3 independent experiments. OD₆₀₀ = optical density at 600 nm.

Selection of the Nutrients

According to the present results, some nutrients that are lacking in milk were necessary for *L. acidophilus* LA-5 (Table 1). Based on the consumption patterns of nutrients and their concentrations in milk, AA with higher consumption rates (more than 50%) should be added to milk, whereas the consumption rates of purines, pyrimidine, vitamins, and ions were greater than 20%. Therefore, Asp, Asn, Lys, Leu, Tyr, Thr, Glu, Gln, Ala, Ile, guanine, adenine, xanthine, uracil, thymine, thiamine, riboflavin, Ca-pantothenate, Mg^{2+} , Fe^{2+} , and Mn^{2+} were supplied to milk for the preliminary single addition experiments. According to the necessary AA profiles and their concentrations in milk, AA with higher requirement ratios (close to or more than 8%) should also be added, and therefore, Arg and Pro were added to milk. In addition, essential nutrients and growth-stimulating nutrients, namely, Cys, Met, Phe, Val, and Gly, were also added for the preliminary single-addition experiments. Figure 4a shows the nutrients that promoted the fermentation process. The fermentation time in single nutrient-supplemented fermented milk was shortened by at least 2 h compared with that of the control sample. According to the results, Asn, Asp, Cys, Leu, Met, riboflavin, guanine, uracil, and Mn^{2+} were added to milk together, and concentrations of these nutrients are shown in Table 1.

Effects of Nutrients on the Properties of Fermented Milk

When fermented milk was prepared solely by *L. acidophilus* LA-5, compared with the control, the fermentation time of the milk supplemented with the nutrients was reduced approximately 9 h (Figure 4a-b). The viable cell counts of *L. acidophilus* LA-5 in fermented milk prepared solely by *L. acidophilus* LA-5 during storage are shown in Figure 4c. At the end of the fermentation process, the viable cell counts of fermented milk supplemented with the nutrients reached 2.17×10^9 cfu/mL, which was approximately 1.8-fold that of the control. Nutrient-supplemented fermented milk prepared solely by *L. acidophilus* LA-5 contained significantly higher levels of *L. acidophilus* LA-5 than the control sample ($P < 0.05$). Hence, the nutrients had positive effects on the growth and maintenance of *L. acidophilus* LA-5 viability. At the end of storage, the pH value decreased by 0.4 to 0.5 units, and similar trends in pH decline were observed in all of the samples (Figure 5a). For fermented milk prepared by mixed starter culture, there were no significant differences in fermentation time (approximately 5 h) between the

Figure 2. Necessary AA at different culture times ($t_1 = 2.0$ h, $t_2 = 4.5$ h, $t_3 = 6.5$ h, $t_4 = 8.0$ h). The results are expressed as the mean $\pm$ SD of 3 independent experiments.

control and nutrient-supplemented fermented milk, and the pH value decreased by 0.3 to 0.4 units during storage (Figure 5a-b). The viable cell counts of *L. acidophilus* LA-5 in fermented milk prepared by mixed starter culture during storage are shown in Figure 4d. Nutrient-supplemented fermented milk prepared by mixed starter culture contained significantly higher levels of *L. acidophilus* LA-5 than the control sample ($P < 0.05$), the viable cell counts of *L. acidophilus* LA-5 in nutrient-supplemented fermented milk prepared by mixed starter culture were approximately 2.3-fold that of the control when fermentation was completed. At the end of the storage period, the survival of *L. acidophilus* LA-5 in the nutrient-supplemented fermented milk prepared by mixed starter culture was 2-fold that of the control. However, there was not a significant difference ($P > 0.05$) in the viable cell counts of *S. thermophilus* and *L. delbrueckii* ssp. *bulgaricus* (Figure 4e-f). As Figure 5c, Table 2, Figure 5d, and Table 3 show, no differences were observed in the textural and sensory properties between the control and nutrient-supplemented fermented milk.

DISCUSSION

A shortage of nutrients is the major limiting factor for the growth of lactic acid bacteria (Youssef et al., 2005). In the current study, small select nutrients enhanced the growth of *L. acidophilus* LA-5 in milk. Accordingly, improving the nutrient-limiting conditions might be an effective way to accelerate the fermentation process.

Every species of *Lactobacillus* has distinctive growth requirements for essential energy, and carbon and nitrogen sources (Ummadi and Curic-Bawden, 2010). However, the ability of *Lactobacillus* to synthesize some nutrients such as AA is limited. Therefore, an exogenous source of nutrients is required for the most optimal growth (Foucaud et al., 2001). Because *L. acidophilus* LA-5 is an important commercial starter strain, it is necessary to determine its nutrient requirements so that it can be efficiently utilized to ferment specific foods. The present results indicated that nutrients with a high consumption rate, AA with a high necessary rate, and growth-stimulating and essential nutrients should be considered in specific fermented foods manufactured with *L. acidophilus* LA-5 so that its viable cell counts are increased and fermentation time is shortened.

The Asn, Asp, Cys, Leu, Met, riboflavin, guanine, uracil, and Mn^{2+} reduced the fermentation time and increased viable cell counts of *L. acidophilus* LA-5 in fermented milk. The genome of *L. acidophilus* NCFM is closely related to *L. acidophilus* LA-5, and the results showed that *L. acidophilus* has the ability to synthesize Cys, Asp, Asn, Met, and guanine but not Leu, riboflavin, or uracil (Altermann et al., 2005). The Cys, Asp, and guanine were synthesized through a de novo pathway, Met was synthesized by Ser and Cys, Asn could be produced via Asp, and all involved compli-

cated synthesis pathways. Additionally, *L. acidophilus* was auxotrophic for guanine and uracil because they could not reduce ribonucleotide to the corresponding deoxyribonucleotide for DNA synthesis (Kilstrup et al., 2005). The supplied substrates for synthesizing nutrients were deficient in milk, or the rates of synthesis of these nutrients could not meet the growth requirements of *L. acidophilus* LA-5. Additionally, *L. acidophilus* LA-5 obtained AA by hydrolyzing milk protein, which did not support good growth. Hence, these nutrients were important to enhance the growth of *L. acidophilus* LA-5 in milk. If the supplied nutrients were absent or insufficient, the high level of viable cell counts and high survival rate of *L. acidophilus* LA-5 were not achieved.

The Cys increased the viable cell counts of *L. acidophilus* in milk by acting as one of the main redox potential donors, and increasing the redox potential during storage (Soto et al., 2019). According to Solval et al. (2019), Asp and Leu were the main free AA present in egg white hydrolysates, higher yields of cell biomass and production of lactic acid was observed in *L. acidophilus* NRRL B-4495 when they grew in a medium containing egg white hydrolysates. The Leu enhanced the growth of *L. acidophilus*, which was essential for *L. acidophilus* throughout the fermentation period in both ovine and milk (Gomes and Malcata, 1998). Riboflavin enhanced the growth of *L. acidophilus* ATCC 314, with

Figure 3. Effect of nutrient omission on the growth rate of *Lactobacillus acidophilus* LA-5. The growth rate is expressed as a percentage and is based on the growth rate when a nutrient was omitted from the chemically defined medium (CDM) compared with that in the CDM. The results are expressed as the mean $\pm$ SD of 3 independent experiments.

Figure 4. (a) Changes in the pH of samples after nutrients were added that promoted fermentation by *Lactobacillus acidophilus* LA-5, and (b) the time it takes for *L. acidophilus* LA-5 to ferment nutrient-added milk. Formulation represents milk that was supplemented with 9 selected nutrients. (c) Changes in the viable cell counts of *L. acidophilus* LA-5 in control and nutrient-supplemented fermented milk prepared solely by *L. acidophilus* LA-5 during storage at 4°C. (d, e, f) Changes in the viable cell counts of *L. acidophilus* LA-5, *Streptococcus thermophilus*, and *Lactobacillus delbrueckii* ssp. *bulgaricus* in control and nutrient-supplemented fermented milk prepared by mixed starter culture during storage at 4°C, respectively. The results are expressed as the mean or the mean $\pm$ SD of 3 independent experiments. Symbols with different letters differ significantly ($P < 0.05$).

Figure 5. (a) Changes in the pH of fermented milk prepared solely by *Lactobacillus acidophilus* LA-5 and fermented milk prepared by mixed starter culture samples during storage at 4°C. ●, ■ = fermented milk and control, respectively, prepared solely with *L. acidophilus* LA-5; ▼, ▲ = fermented milk and control prepared with mixed starter culture. (b) Changes in the pH of fermented milk prepared by mixed starter culture during fermentation. (c) Graphic representation of the mean sensory evaluation by quantitative descriptive analysis of control and nutrient-supplemented fermented milk prepared solely by *L. acidophilus* LA-5. (d) Graphic representation of the mean sensory evaluation by quantitative descriptive analysis of control and nutrient-supplemented fermented milk prepared by mixed starter culture. The results are expressed as the mean or the mean $\pm$ SD of 3 independent experiments.

a 26% increase in soy milk (Ewe et al., 2010). Guanine significantly stimulated the growth of *L. johnsonii* NCC 533, a member of the acidophilus group of intestinal lactobacilli, in whole and skim milk, which may be due to it serving as a deoxyribonucleotide substrate for bacterial growth (Elli et al., 1999). According to Lv et al. (2017), uracil was important for the growth of *L. acidophilus* KLDS 1.0738. The Mn^{2+} enhanced the growth of *L. acidophilus* by acting as enzyme cofactor (Fouad et al., 2010). For *L. acidophilus* LA-5, the physiological functions and requirements of these nutrients may be the main reasons for promoting the fermenta-

tion of fermented milk. The result indicated that they are likely to promote the metabolism and growth of *L. acidophilus* LA-5 as a stimulating factor.

The levels of dissolved oxygen, hydrogen peroxide, and storage conditions could result in reduced viability of probiotics (Talwalkar and Kailasapathy, 2003; Donkor et al., 2006). Generally, *L. acidophilus* does not possess a sufficient scavenging mechanism, and therefore, the intracellular accumulation of toxic oxygenic metabolites such as superoxide anion and hydroxyl radical could lead to its death (Talwalkar and Kailasapathy, 2003). Onions are a source of sulfur compounds, vita-

Table 2. Properties of the fermented milk prepared by *Lactobacillus acidophilus* LA-5 alone after production (mean $\pm$ SD)

Sample	Fermentation time (h)	Viable cell counts ($\times 10^9$ cfu/mL)	Titratable acidity ($^{\circ}$ T)	Water-retaining capability (%)	Hardness (g)	Adhesiveness (g)	Springiness (g)	Cohesiveness (g)
Control	24 $\pm$ 1.78	1.21 $\pm$ 0.26	76 $\pm$ 6.54	33.64 $\pm$ 0.02	23.45 $\pm$ 0.69	-85 $\pm$ 5.27	928 $\pm$ 32.68	723 $\pm$ 29.65
Nutrient-supplemented fermented milk	15 $\pm$ 1.45	2.17 $\pm$ 0.16	75 $\pm$ 6.65	36.45 $\pm$ 0.02	25.15 $\pm$ 0.65	-90 $\pm$ 5.95	935 $\pm$ 34.78	734 $\pm$ 27.41

Table 3. Properties of the fermented milk prepared by mixed starter culture after production (mean $\pm$ SD)

Sample	Fermentation time (h)	Viable cell counts ¹ ($\times 10^7$ cfu/mL)	Titratable acidity ($^{\circ}$ T)	Water-retaining capability (%)	Hardness (g)	Adhesiveness (g)	Springiness (g)	Cohesiveness (g)
Control	5.2 $\pm$ 0.16	1.25 $\pm$ 0.23	75 $\pm$ 5.65	42.39 $\pm$ 0.02	46.22 $\pm$ 0.79	-140 $\pm$ 4.47	983 $\pm$ 37.18	809 $\pm$ 26.75
Nutrient-supplemented fermented milk	5.3 $\pm$ 0.15	2.93 $\pm$ 0.16	72 $\pm$ 6.25	42.45 $\pm$ 0.02	45.05 $\pm$ 0.63	-134 $\pm$ 5.54	979 $\pm$ 38.48	804 $\pm$ 29.64

¹Viable cell counts are the counts of *Lactobacillus acidophilus* LA-5.

mins, and minerals, from which these compounds might scavenge hydrogen peroxide during the fermentation of *L. acidophilus* NCFM (Li et al., 2016). It has been reported that Cys and Met had the ability to scavenge hydrogen peroxide (Zhou and Elias, 2014). Riboflavin showed the significant suppression against hydrogen peroxide scavenging (Nabavi et al., 2009). According to Sharma et al. (2013), Mn²⁺-metabolite complexes could protect essential enzymes from oxidative damage. For *L. acidophilus* LA-5, maintaining high viable cell counts and viability might be attributed to additional nutrients that are able to scavenge hydrogen peroxide and protect *L. acidophilus* during storage of fermented milk. The nutrient supplementation did not affect the viable cell counts of *S. thermophilus* and *L. delbrueckii* ssp. *bulgaricus* in this study. It has been observed that the growth of *S. thermophilus* and *L. delbrueckii* ssp. *bulgaricus* was suppressed when 250 or 500 mg/L of Cys was supplied (Dave and Shah, 1997b).

The present results indicate that suitable concentrations of available nutrients in fermented milk prepared by mixed starter culture were necessary to promote the growth of *L. acidophilus* LA-5 without affecting the growth of other strains. Additionally, no obvious difference was observed in the quality between the control and nutrient-supplemented fermented milk, which was consistent with similar results obtained by Sun et al. (2019).

Production costs of fermented milk mainly includes the cost of raw milk, cost of auxiliary materials, costs of fuel and power, labor costs, undepreciated cost of the asset, management cost, and packing cost. The present results showed that the fermentation time of fermented milk prepared solely by *L. acidophilus* LA-5 was shortened by 9 h by addition of nutrients. Compared with the production costs of fermented milk supplemented without the nutrients, cost of auxiliary materials increases 0.48%, and fuel and power costs, labor costs, undepreciated cost of the asset, and management cost decrease 34.4% in fermented milk supplemented with the nutrients. Detailed information about the formula for the cost of the proposed technology can be found in Supplemental File S1 (<https://doi.org/10.3168/jds.2020-18953>; Shafaei and Brunn, 1999).

CONCLUSIONS

Supplementation with Asn, Asp, Cys, Leu, Met, riboflavin, guanine, uracil, and Mn²⁺ shortened the fermentation time of milk by 9 h. The viable cell counts and viability of *L. acidophilus* LA-5 were approximately 1.8-fold and 1.4-fold that of the control, respectively, in fermented milk prepared only with *L. acidophilus*

LA-5. Furthermore, the viable cell counts and viability of *L. acidophilus* LA-5 were approximately 2.3-fold and 2-fold that of the control, respectively, in nutrient-supplemented fermented milk prepared by mixed starter culture containing *L. acidophilus* LA-5. The present research provides a reference model for regulating the growth of probiotics in fermented milk prepared by a single probiotic strain and in fermented milk prepared by a mixed starter culture containing the probiotic strain, based on the nutrient requirements of probiotics.

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