Characterization of probiotic bacteria involved in fermented milk processing enriched with folic acid

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ABSTRACT

Yogurt products fermented with probiotic bacteria are a consumer trend and a challenge for functional food development. So far, limited research has focused on the behavior of the various probiotic strains used in milk fermentation. In the present study, we characterized folic acid production and the sensory and textural characteristics of yogurt products fermented with probiotic bacteria. Yogurt fermented with *Lactobacillus plantarum* had improved nutrient content and sensory and textural characteristics, but the presence of *L. plantarum* significantly impaired the growth and survival of *Lactobacillus delbrueckii* ssp. *bulgaricus* during refrigerated storage. Overall, *L. plantarum* was a good candidate for probiotic yogurt fermentation; further studies are needed to understand the major metabolite path of lactic acid bacteria in complex fermentation.

Key words: yogurt, probiotic strain, folic acid, complex fermentation

INTRODUCTION

Probiotics are defined by the United Nations Food and Agriculture Organization/World Health Organization as “live microorganisms (bacteria or yeasts) that when ingested or locally applied in sufficient numbers confer one or more specified demonstrated health benefits for the host” (FAO/WHO, 2001). Lactic acid bacteria (**LAB**) have potential probiotic use as food-grade fermentation starters (Sanders et al., 2013). As well, certain LAB strains can synthesize folates, a family of B-complex vitamins that function in 1-carbon metabolism to allow the de novo synthesis of amino acids and nucleosides (Rossi et al., 2011). Because of the perceived health benefits of probiotic strains, their use has expanded rapidly in health-based products.

Consumers nowadays have become more demanding of natural foods, and products created using environmentally friendly procedures are more attractive.

Probiotic-fermented milks possess many useful properties when they interact with host cells in the gastrointestinal tract, including anti-inflammatory activity (de Assis et al., 2016), antioxidant activity (Srivastava et al., 2015), antibacterial activity, and angiotensin I-converting-enzyme inhibitory activity (Abd El-Gawad, 2014; Rai et al., 2015). Folate deficiency is associated with many important diseases such as chronic heart failure, oxidative stress, and neural tube defects (Czeizel et al., 2013), but folic acid supplementation before and during early pregnancy (up to 12 wk of gestation) can prevent neural tube defects in offspring (Czeizel et al., 2011). Lactic acid bacteria strains with folate acid production are able to improve folate status and prevent folate deficiency in some deficient rodent models (Laiño et al., 2015). With probiotic supplementation, the interaction between adhesive probiotics and the host cells may trigger a signal cascade that leads to reductions in serum cholesterol and gastrointestinal infections, and improvements in lactose metabolism, inflammatory bowel disease, and immune system stimulation (Whelan and Quigley, 2013; Settanni and Moschetti, 2014).

However, no bacterial strain provides all of these potential benefits, not even probiotic strains. Folic acid concentrations in yogurts vary widely and are normally considered low because of inadequate selection of starter cultures and fermentation conditions. Traditionally, yogurt is fermented using *Lactobacillus delbrueckii* ssp. *bulgaricus* and *Streptococcus thermophilus* in the milk medium. These 2 strains establish a symbiotic relationship in milk and perform biochemical reactions that lead to a decrease in pH and the formation of a semi-solid texture and a distinctive yogurt flavor (Irigoyen et al., 2012). Promising probiotic strains, including *Bacillus*, *Enterococcus*, *Escherichia*, and *Propionibacterium* genera, and the yeast genus *Saccharomyces*, have been adopted in new yogurt products (Ceapa et al., 2013;

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Kanmani et al., 2013). However, from a technological point of view, the addition of different LAB strains to milk fermentation is a challenge. Texture, protein gel strength, and aroma can be affected by different kinds of probiotics in the milk fermentation process (Bouteille et al., 2013).

Yogurt is the most popular fermented dairy product, but limited research has focused on the behavior of the different probiotic strains during milk fermentation. The objectives of this study were to characterize the potential folic acid production of several species of lactobacilli (*Lactobacillus plantarum*, *Lactobacillus casei*, *Lactobacillus acidophilus*). We also evaluated texture parameters, post-acidification, and microorganism counts in probiotic yogurts during cold storage to determine the yogurt products' nutritional, textural, and sensory properties.

MATERIALS AND METHODS

Strains and Cultures

We purchased *L. bulgaricus* (No. 1.1855) and *S. thermophilus* (No. 1.1854) from China General Microbiological Culture Collection Center. We stored *L. plantarum* (ATCC 14917), *L. acidophilus* (ATCC 4356), and *L. casei* (ATCC 15008) in our laboratory at -80°C in culture plus glycerol (20%, vol/vol) for further use. Raw milk was produced by Ningbo Dairy Group Co., Ltd., and had a fat content of 3.2%. All bacterial strains were cultured at 37°C in de Man, Rogosa, and Sharpe (MRS) medium before milk fermentation.

Fermentation Process

To prepare the mother culture, we inoculated heat-treated milk (90 to 95°C for 5 min) with 3% (wt/wt) *Lactobacillus* strains anaerobically at 37°C for 18 h, and maintained it at 4°C until milk fermentation (within 48 h). Milk fermentation took place at 43°C for 6 h and post-ripening at 4°C for 18 h before sensory evaluation and texture analysis. During acidification, pH decreased below 4.6. Milk gelation occurs at pH 5.2 to 5.4, when lactose converts to lactic acid in milk medium. The pH value of the yogurt was determined by inserting the electrode (Mettler Toledo, Shanghai, China) directly into the yogurt samples.

Folic Acid Production of Different Strains

We purchased a folic acid reference standard (CAS No. 59–30–3) from Sigma-Aldrich (Shanghai, China). We performed analysis of folic acid production from the

various *Lactobacillus* strains using HPLC on an Zorbax Eclipse XDB-C18 (4.6×250 mm, Agilent Technologies, Santa Clara, CA) at 280 nm. Mobile phase A consisted of 20% H_3PO_4 in water (vol/vol, pH 7.2), phase B consisted of 100% methanol (vol/vol), and the ratio of A to B was 90:10 under a flow rate of 1 mL/min.

Sensory Evaluation and Texture Analysis

Ten panelists were recruited for the sensory evaluation analysis. Panelists were asked to taste the samples in the order given and evaluate the appearance, taste, and aroma of the fermented milk. For each test, replicates were conducted to improve the power of analysis and detect true discriminators. All sensory attributes were evaluated using a scale of 1 to 9, where 1 was very low and 9 was very high.

Texture of the different kinds of yogurt were evaluated using a TAHD Plus Texture Analyzer (Stable Micro Systems, Godalming, UK) with a back extrusion rig and a 35-mm compression disk attached to an extension bar using 50-kg load cells (Ciron et al., 2010). Three replications were made at a pre-test 1.0 mm/s, test of 1.0 mm/s and post-test 10.0 mm/s at 30 mm above sample surface, penetrating to a depth of 30%. We used mean values to obtain a force-time curve.

Acidification Profile Determination

Production of acid was assessed by changes in pH of the yogurt during milk fermentation. Yogurt samples were weighed to 40.0 g and the pH measurements were performed using a Mettler Toledo M 220 pH meter (Mettler Toledo Instruments).

Microbial Counts and Stability

A 1-mL sample of yogurt was transferred to a screw-capped tube containing 9 mL of sterile saline water. Further dilutions were made from this original dilution, and the bacteria were counted after fermentation in duplicate by using MRS agar medium, which is selective for the *Lactobacillus* strains. The stability of the bacteria in the yogurt was assessed following analysis at 1, 7, 14, 21, and 28 d of refrigerated storage through microbial counts in selective medium.

Statistical Analysis

The experiment was repeated 3 times. Statistical analyses between groups were carried out using a 2-tailed Student's *t*-test with SPSS 13.0 software. Data are presented as means $\pm$ standard deviations. A *P*-

Figure 1. Folic acid profile of different fermented *Lactobacillus* strains during milk fermentation. (A) Folic acid analysis with HPLC, and (B) folic acid production in different strains.

value <0.05 or $P < 0.01$ was considered statistically significant. Means with the same letter were not significantly different by Duncan's test ($P > 0.05$).

RESULTS

Folic Acid Production Detection of Different Strains

We evaluated the folic acid production of 5 LAB strains in yogurt products. Folic acid production was different among the strains: *L. plantarum* had the highest folic acid production, at 63.23 μg/mL, and *L. casei* and *L. acidophilus* came in at 45.41 and 42.78 μg/mL, respectively (Figure 1).

Sensory and Textual Characteristics of Yogurt Products

The developed product showed good nutritional, textural, and sensory characteristics after probiotic fermentation. We found some differences in the sensory and textural characteristics of the yogurt products with

the different LAB starters. Yogurt fermented with *S. thermophilus*, *L. bulgaricus*, and *L. plantarum* had high scores: the lowest fat content was 1.93% and the highest sensory evaluation score was 8.9 (Figure 2A). We found a better texture profile with *L. plantarum* than with the other probiotics using texture analyzer equipment (Figure 2B). The ratio of *L. plantarum* in the fermentation culture also had an effect on the quality of the probiotic yogurt product. When the ratio of *S. thermophilus* to *L. bulgaricus* to *L. plantarum* was 1:1:4, the quality profile was higher than with other microbe ratios (Figure 3).

Microbiological Viable Counts of Bacteria in Yogurt Products

The total number of strains increased in probiotic yogurt groups fermented with *L. plantarum* (Figure 4A). As well, the microbiological viable counts of *L. plantarum* changed with the ratio of the LAB strains, and *L. plantarum* increased progressively to become the dominant strain in groups d and e (up to 3.5 E9 cfu/mL).

DISCUSSION

Although folic acid is present in infant formula milk powder, its concentration is low, ranging from 20 to 200 $\mu\text{g}/100\text{ g}$. The folate bio-enriched fermented milk was able to increase plasma folate concentrations and

decrease homocysteine levels (Laiño et al., 2015). To increase folic acid concentrations in milk, microbiological fermentation is a promising alternative. In our research, *L. plantarum* had the highest folic acid production; other strains had low folic acid production or none. This may have been because these strains lacked the genes

Figure 2. Sensory and textural characteristics of yogurt products with different starters. (A) pH, fat, and sensory evaluation of fermented milks with different starters; (B) texture analysis (firmness, consistency, and cohesiveness) of yogurt products with different starters. Group a = control, *Streptococcus thermophilus*:*Lactobacillus delbrueckii* ssp. *bulgaricus* = 1:1; group b = *Strep. thermophilus*:*L. bulgaricus*:*Lactobacillus plantarum* = 1:1:1; group c = *Strep. thermophilus*:*L. bulgaricus*:*Lactobacillus acidophilus* = 1:1:1; and group d = *Strep. thermophilus*:*L. bulgaricus*:*Lactobacillus casei* = 1:1:1. Error bars represent SD. * $P < 0.05$; ** $P < 0.01$.

Figure 3. Sensory evaluation, firmness, and fat content of yogurt products fermented with probiotic *Lactobacillus plantarum*. The ratios on the x-axis indicate the proportions of *Streptococcus thermophilus*:*Lactobacillus delbrueckii* ssp. *bulgaricus*:*Lactobacillus plantarum* (from 1:1:1 to 1:1:5). Error bars represent SD. Means with different letters (a,b) were significantly different by Duncan's test ($P < 0.05$).

for the 6-hydroxymethyl-7,8-dihydropterin pyrophosphate (DHPPP) de novo biosynthetic pathway, which is essential for the biosynthesis of folic acid (Capozzi et al., 2012). Some researchers have also found that fermentation time had an effect on folic acid levels in cultures of LAB (Lin and Young, 2000).

Unlike traditional milk products fermented with *L. bulgaricus* and *S. thermophilus*, adding probiotic strains to milk fermentation may give yogurt products new characteristics. Analysis of the texture profile of yogurt offers the advantages of reduced test time and quantification of structure. Screening the various strains in this research, we found that *L. plantarum* was a better choice for probiotic yogurt products, with better sensory and textural characteristics than the other strains. As well, functional genomics studies have explored how *L. plantarum* responds to various environmental stresses from a molecular perspective, and multifunctional activity of bioactive peptides can be obtained from milk products fermented with *L. plantarum* (Aguilar-Toalá et al., 2017). Both the cohesiveness and consistency value of products fermented with *L. plantarum* were higher than those for other probiotic candidates in our study. The improvement in these properties was because of changes in the proportion of bacterial strains in the yogurt; it was revealing that when the ratio of *S. thermophilus* to *L. bulgaricus* to *L. plantarum* was 1:1:4,

we obtained a high textural score. The pH value of this kind of fermentation product was also influenced by the *L. plantarum* ratio in the starter (data not shown), a finding that has been shown in other fermented products, such as buffalo milk curd (Bhanu Jaseja, 2016).

Some consumers reject fat-enriched yogurt (10 g/100 g) more vigorously because they are aware of the high energy content of fat and have learned to avoid such products. Design a product with strong sensory properties and low energy content from fat will be important from a nutritionist's viewpoint (Hoppert et al., 2014).

We know that *L. plantarum* is a good source of esterase enzymes, because lipolytic and esterase activity have been described in *L. plantarum* WCFS1, which contained Lp_3505 esterase (Esteban-Torres et al., 2015). With microbial growth during fermentation and enhanced lipid hydrolysis in yogurt, we observed a sharp decrease in fat content (from 2% to 1.5%) in the *L. plantarum* yogurt in our study. Milk inoculation with *L. plantarum* could significantly reduce bitterness caused by proteinase (Gomez et al., 1996), also a reason why *L. plantarum* had a greater influence on the sensory characteristics of yogurt products. Where the relative proportion of *L. plantarum* was lower compared with other groups (group c), we observed a slight up trend of fat content (1.99%). However, the downtrend of fat content was significant when the ratio of *L. plantarum*

Figure 4. Microbiological viable count of bacteria in different yogurt products. (A) Total microbiological viable count of the 3 lactic acid bacteria; (B) microbiological viable count of each strain in the different yogurt products. (a) *Streptococcus thermophilus*:*Lactobacillus delbrueckii* ssp. *bulgaricus*:*Lactobacillus plantarum* = 1:1:1; (b) *Strep. thermophilus*:*L. bulgaricus*:*L. plantarum* = 1:1:2; (c) *Strep. thermophilus*:*L. bulgaricus*:*L. plantarum* = 1:1:3; (d) *Strep. thermophilus*:*L. bulgaricus*:*L. plantarum* = 1:1:4; (e) *Strep. thermophilus*:*L. bulgaricus*:*L. plantarum* = 1:1:5. Error bars represent SD. ** $P < 0.01$.

went from 1:1:3 to 1:1:5; *L. plantarum* may possess lipase activity during milk fermentation.

Some results have also shown that *L. plantarum* WCFS1 significantly impaired the growth and survival of *L. bulgaricus* in yogurt during refrigerated storage (Settachaimongkon et al., 2016), consistent with the results of the present study that when the ratio of *S. thermophilus* to *L. bulgaricus* to *L. plantarum* was 1:1:4 or 1:1:5, the microbiological viable count increased with the *L. plantarum* counts in the yogurt throughout the refrigerated storage period (Table 1).

Table 1. Microbiological viable count (cfu/mL) of yogurt bacteria during refrigerated storage

Days	<i>Lactobacillus delbrueckii</i> ssp. <i>bulgaricus</i>	<i>Streptococcus thermophilus</i>	<i>Lactobacillus plantarum</i>
1	1.52×10^9	1.18×10^9	2.54×10^9
7	1.64×10^9	1.21×10^9	2.85×10^9
14	1.32×10^9	1.03×10^9	2.21×10^9
21	1.05×10^9	1.22×10^9	1.53×10^9
28	0.83×10^9	1.01×10^9	1.21×10^9

CONCLUSIONS

We found that new yogurt products fermented with the probiotic strain *L. plantarum* had higher nutrient content and better sensory and textural characteristics, and they were suitable for healthy consumption. The presence of *L. plantarum* significantly impaired the growth and survival of *L. bulgaricus* during refrigerated storage. However, the product's acidification profile was slightly influenced by the addition of this strain. Further studies are needed to provide insight into the major metabolite path of LAB in complex fermentation, which is responsible for significant pH changes in yogurt products.

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