Evaluation of the effect of supplementing fermented milk with quinoa flour on probiotic activity

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ABSTRACT

In this work, we investigated the effect of supplementing fermented milk with quinoa flour as an option to increase probiotic activity during fermented milk production and storage. Fermented milk products were produced with increasing concentrations of quinoa flour (0, 1, 2, or 3 g/100 g) and submitted to the following analyses at 1, 14, and 28 d of refrigerated storage: post-acidification, bacterial viability, resistance of probiotics to simulated gastrointestinal (GI) conditions, and adhesion of probiotics to Caco-2 cells in vitro. The kinetics of acidification were measured during the fermentation process. The time to reach maximum acidification rate, time to reach pH 5.0, and time to reach pH 4.6 (end of fermentation) were similar for all treatments. Adding quinoa flour had no effect on fermentation time; however, it did contribute to postacidification of the fermented milk during storage. Quinoa flour did not affect counts of *Bifidobacterium animalis* ssp. *lactis* BB-12 or *Lactobacillus acidophilus* La-5 during storage, it did not protect the probiotic strains during simulated GI transit, and it did not have a positive effect on the adhesion of probiotic bacteria to Caco-2 cells in vitro. Additionally, the adhesion of strains to Caco-2 cells decreased during refrigerated storage of fermented milk. Although the addition of up to 3% quinoa flour had a neutral effect on probiotic activity, its incorporation to fermented milk can be recommended because it is an ingredient with high nutritive value, which may increase the appeal of the product to consumers.

Key words: functional food, fermented dairy product, probiotic, gastrointestinal resistance, Caco-2 adhesion

INTRODUCTION

The increase in consumer knowledge about the effects of food on health has led to an increase in the

functional foods market. Functional foods not only provide nutrients but also contain biologically active components that can improve health and well-being, reducing the risk of disease by beneficially affecting one or more target functions in the body (Mohammadi and Mortazavian, 2011). Food fermented by probiotic bacteria belongs to this category of functional foods.

Probiotics are live microorganisms that confer benefits to the host when consumed in adequate amounts (FAO/WHO, 2002). Reports indicate that probiotics exert beneficial effects on the immune system and the gut, reduce side effects associated with antibiotic use, reduce symptoms associated with irritable bowel syndrome, help alleviate lactose intolerance, and have antimicrobial and anticancer properties (Fontana et al., 2013). Dairy products such as yogurt, fermented milk, and cheese are the most important foods in the current probiotic food industry (Madhu et al., 2012). It is commonly accepted that probiotics must be present at a minimum level, ranging from 10^6 to 10^9 cfu/mL of the product, to be beneficial. The best-known probiotic microorganisms include strains within the *Lactobacillus* and *Bifidobacterium* genera (Vasiljevic and Shah, 2008).

Many ingredients can be added to fermented milk products to enhance their nutritional value and stimulate probiotic growth and activity during cold storage. These additives can keep the population of probiotics above the minimum recommended dose and meet consumer needs for new products. In addition, these ingredients may also help probiotics survive passage through the gastrointestinal (GI) tract by increasing their resistance to gastric and enteric juices (Hernandez-Hernandez et al., 2012; Bedani et al., 2013).

The potential of quinoa to provide the above-mentioned benefits is still unknown. Quinoa (*Chenopodium quinoa* Willd.) is a pseudocereal originally from the Andean region, which is home to one of the oldest cultures in the American continent. This grain is still grown in Peru, Bolivia, Chile, Ecuador, Colombia, and Argentina and has recently been introduced into Europe, North America, Africa, and Asia (Bhargava et al.,

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2006). Compared with other cereals, quinoa has higher protein content (16 g/100 g) with a balanced amino acid composition and greater amounts of lysine (5.8 g/100 g) and methionine (2.4 to 5.1 g/100 g). Quinoa is also rich in dietary fiber, nutrients, and a wide range of vitamins. Its antioxidant capacity is associated with its content of phenolic compounds, such as α -tocopherol (vitamin E; Fischer et al., 2013). Quinoa flour contains approximately 7 g/100 g of dietary fiber and is rich in unsaturated FA (Hager et al., 2012).

Considering that quinoa flour is a source of growth factors and potential prebiotic components, we investigated the effects of supplementing milk with 1 to 3% quinoa flour on kinetics of acidification during fermentation and changes in postacidification, viable counts of *Streptococcus thermophilus* and probiotic strains (*Bifidobacterium animalis* ssp. *lactis* BB-12 and *Lactobacillus acidophilus* La-5), resistance of probiotics to simulated GI conditions, and in vitro adhesion of probiotics to intestinal cells during 28 d of refrigerated storage in fermented milk.

MATERIALS AND METHODS

Culture and Ingredients

Skim milk powder (Nestlé, Araçatuba, SP, Brazil), ABT-4 culture (Chr. Hansen, Valinhos, SP, Brazil), which is composed of *Streptococcus thermophilus*, *Lactobacillus acidophilus* La-5, and *Bifidobacterium animalis* ssp. *lactis* BB-12, and commercial quinoa flour (Sun Products do Brasil Ind. Com. Ltda., Jaraguá do Sul, SC, Brazil) were used in this study. According to the manufacturer, the quinoa flour was composed of (/100 g) 74.3 g of carbohydrates, 13.5 g of protein, 4.9 g of fat, and 5.1 g of dietary fiber.

Milk Base and Inoculum Preparation

Four different milk bases were prepared: milk supplemented with quinoa flour at concentrations of 1 (**FMQ1**), 2 (**FMQ2**), and 3 (**FMQ3**) g/100 g and milk without quinoa flour (control, **PMC**). The skim milk powder (Nestlé) was reconstituted in water to 13 g/100 g of total solids. All the milk bases were heated at 90°C for 10 min in a water bath, transferred to 500-mL sterile Schott flasks inside a laminar flow chamber, and stored at 4°C for 24 h before use.

The ABT-4 preculture was suspended, in amounts recommended by the manufacturer, in 50 mL of sterile milk and activated at 42°C for 30 min. The initial colony counts after milk inoculation were approximately 10^6 cfu/mL for *L. acidophilus* La-5 and 10^7 cfu/mL for *S. thermophilus* and *B. animalis* ssp. *lactis* BB-12.

Fermented Milk Production and Kinetic Parameters

Before fermentation, milk (500 mL) was heated to 42°C and 2 mL of the preculture was added. After inoculation, the flasks were transferred to a water bath connected to a CINAC system (Cynétique d'acidification, Alliance Instruments, Frepillon, France) that continuously measures pH, allowing evaluation of the acidification rate throughout the fermentation process. Batch fermentations were performed in 3 independent trials at 42°C until the pH reached 4.6. From the collected data, the acidification rate ($V_{\max}$) was calculated as the change in pH over time (dpH/dt) and expressed as 10^{-3} pH units/min. During the incubation period, the following kinetic parameters were also calculated: (1) time (h) at which $V_{\max}$ was reached ($t_{V_{\max}}$); (2) time (h) to reach pH 5.0 ($t_{\text{pH}5.0}$); and (3) time (h) to reach pH 4.6 (i.e., time to complete the fermentation; $t_{\text{pH}4.6}$).

At the end of fermentation, the fermented milk was cooled to 15°C in an ice bath, and the clot was broken up with a stainless steel perforated disk with up and down movements for approximately 1 min. The product was packed into 80-mL sterile plastic cups and stored at 4°C for 28 d.

Postacidification and Titratable Acidity

The postacidification of the fermented milk was measured at 1, 14, and 28 d of storage at 4°C by measuring the pH in triplicate using a pH meter (model PG1800, Gehaka, São Paulo, Brazil). Acidity, expressed as percent of lactic acid per 100 g, was determined in triplicate for each trial by titration with 0.1 N NaOH solution (AOAC International, 2005).

Microbiological Analyses

Bacterial cell counts for each treatment were done in duplicate at 1, 14, and 28 d of storage. Colony counts for *Strep. thermophilus*, *L. acidophilus* La-5, and *B. animalis* ssp. *lactis* BB-12 were performed using M17 agar (Himedia, Mumbai, India), de Man, Rogosa, and Sharpe (MRS) agar (Acumedia, Lansing, MI) with added bile solution (Sigma-Aldrich, St. Louis, MO; MRS-bile), and MRS agar (Acumedia) with added lithium chloride (0.2 g/100 g) and sodium propionate (0.3 g/100 g; Sigma-Aldrich; MRS-LP), respectively. The M17, MRS-bile, and MRS-LP media were prepared according to IDF (1997), IDF (1995), and Vinderola and Reinheimer (1999), respectively. *Streptococcus thermophilus* and *L. acidophilus* La-5 plates were incubated at 37°C for 48 h under aerobic conditions; *B. animalis* ssp. *lactis* BB-12 plates were incubated at 37°C for 72 h under anaerobic conditions provided by Anaerobac (Probac, São Paulo,

Brazil). The results were expressed as $\log_{10}$ cfu/mL of fermented milk.

Probiotic Survival Under Conditions Simulating the GI Tract

The survival of probiotic bacteria in fermented milk submitted to simulated GI conditions was evaluated according to the method described by Buriti et al. (2010), with modifications, after 1, 14 and 28 d of refrigerated storage. Briefly, the fermented milk samples were diluted in 0.5% NaCl, and 10 mL was transferred to 3 sterile flasks. The pH was adjusted to 2.0–2.2 with 0.5 *N* HCl, and pepsin (3 g/L, from porcine stomach mucosa, Sigma-Aldrich) and lipase (0.9 mg/L, Amano lipase F-AP15 from *Rhizopus oryzae*, Aldrich Chemical Company, Milwaukee, WI) solutions were added to the samples. The samples were then incubated at 37°C for 2 h (gastric phase). Next, the sample pH was increased to 4.3–5.2 using an alkaline solution containing bile (10 g/L, bovine bile, Sigma-Aldrich) and pancreatin (1 g/L, pancreatin from porcine pancreas, Sigma-Aldrich), and the samples were reincubated at 37°C for 2 h (enteric phase 1). Finally, the pH was increased to 7.0–7.3 using the same alkaline solution, the bile and pancreatin concentrations were adjusted (10 and 1 g/L, respectively), and samples were incubated again at 37°C for 2 h (enteric phase 2). Aliquots of 1 mL were collected from duplicate samples at the beginning of the assay and after the end of the gastric and both enteric phases to assess GI transit tolerance of probiotic bacteria. The media and growth conditions used were the same as described in the Microbiological Analyses section. The experiment was performed in duplicate.

Adhesion to Caco-2 Cells

The Caco-2 cell line BCRJ 0059 (Rio de Janeiro Cell Bank, Rio de Janeiro, Brazil) was routinely cultured (passages 29–31) in Dulbecco's modified Eagle's minimum (DMEM; Sigma Aldrich) supplemented with 20% heat-inactivated fetal bovine serum (Cultilab, Campinas, SP, Brazil), a mixture of penicillin (100 IU/mL) and streptomycin (100 µg/mL, Sigma Aldrich), and 1% nonessential amino acid solution (Sigma Aldrich), at 37°C, in an atmosphere of 5% CO₂. The adhesion assay was performed as described by Ranadheera et al. (2012), with modifications. Briefly, Caco-2 cells were seeded at a concentration of 10⁵ cells/well into 24-well tissue culture plates (Nest Biotechnology Co. Ltda., Wuri, China) and incubated at 37°C in an atmosphere of 5% CO₂ (Thermo Electrone, Sunnyvale, CA) until a confluent monolayer had formed (15–17 d). One day before the adhesion assays, the medium was replaced

with the same medium but without antibiotics. Before adhesion, the monolayer was washed once with PBS (pH 7.4) to remove all traces of the medium. An aliquot of 1 mL of each fermented milk sample was transferred to postconfluent monolayers of Caco-2 cells in 24-well tissue culture plates and incubated at 37°C in a 5% CO₂ atmosphere for 2 h. Cells were then washed at least 3 times with PBS to remove nonadherent bacteria and detached from each well by the addition of 1 mL of Triton X-100 (0.5% vol/vol; Sigma Aldrich). The suspension (1 mL) from each well was then transferred to a tube containing 9 mL of peptone water, serially diluted, and plated on the appropriate media, in duplicate, to determine adhesion ability. Percentage adhesion was calculated by determining the viable bacterial counts of the fermented milk before the adhesion assay and viable bacterial counts adhered to the cell layers from each type of fermented milk. The experiment was performed in duplicate.

Statistical Analyses

Results were analyzed using one-way ANOVA. The treatments were compared within the same storage period, and the samples of the same treatment were compared over storage time. These analyses were carried out using STAT software version 2.0 (UNESP, Jaboticabal, São Paulo, Brazil). Mean values were compared using the Tukey test at $P \leq 0.05$.

RESULTS AND DISCUSSION

Kinetic Parameters of Acidification

The acidification profiles of the fermented milk control and supplemented with quinoa flour (1–3%) were evaluated using the parameters $V_{\max}$, $t_{V_{\max}}$, pH in $V_{\max}$ ($\text{pH}_{V_{\max}}$), $t_{\text{pH}5.0}$, and $t_{\text{pH}4.6}$ (Table 1).

The $V_{\max}$ was significantly ($P \leq 0.05$) higher in the fermented milk control without quinoa flour and in FMQ1. The $\text{pH}_{V_{\max}}$ was also significantly different ($P \leq 0.05$) among treatments. The buffering capacity of the fermented milk product is directly proportional to its TS content, and a higher TS content can result in higher kinetic parameters and longer fermentation (Varghese and Mishra, 2008). We found that the addition of greater concentrations of quinoa flour (2 and 3 g/100 g) led to lower $V_{\max}$ values. Therefore, the addition of this ingredient decreased the ability of the ABT-4 microorganisms to acidify the milk. An inverse relationship between $V_{\max}$ and $t_{V_{\max}}$ was observed: a lower $V_{\max}$ corresponded to a longer time required to reach $V_{\max}$ ($t_{V_{\max}}$). However, the results for $t_{V_{\max}}$, $t_{\text{pH}5.0}$, and $t_{\text{pH}4.6}$ were statistically ($P > 0.05$) equal for all

Table 1. Kinetic parameters (mean $\pm$ SD) of acidification of the fermented milk control and fermented milk supplemented with quinoa flour at different concentrations

Treatment ¹	Kinetic parameter ²				
	$V_{\max}$ (10^{-3} pH units/min)	$t_{V_{\max}}$ (h)	pH _{$V_{\max}$}	$t_{\text{pH}5.0}$ (h)	$t_{\text{pH}4.6}$ (h)
FMC	23.40 $\pm$ 0.84 ^a	2.08 $\pm$ 0.11 ^a	5.73 $\pm$ 0.03 ^a	2.97 $\pm$ 0.28 ^a	4.54 $\pm$ 0.52 ^a
FMQ1	20.08 $\pm$ 1.57 ^{ab}	2.15 $\pm$ 0.21 ^a	5.59 $\pm$ 0.03 ^b	2.92 $\pm$ 0.35 ^a	4.42 $\pm$ 0.49 ^a
FMQ2	18.17 $\pm$ 0.15 ^b	2.17 $\pm$ 0.19 ^a	5.57 $\pm$ 0.01 ^{bc}	2.95 $\pm$ 0.30 ^a	4.53 $\pm$ 0.57 ^a
FMQ3	17.27 $\pm$ 0.06 ^b	2.20 $\pm$ 0.14 ^a	5.46 $\pm$ 0.05 ^c	2.90 $\pm$ 0.18 ^a	4.54 $\pm$ 0.37 ^a

^{a-c}Different letters within a column denote significant differences ($P \leq 0.05$) among treatments.

¹Treatments: FMC = fermented milk control, without quinoa flour; FMQ1, FMQ2, and FMQ3 = fermented milk supplemented with 1, 2, and 3% quinoa flour, respectively; $n = 3$.

² $V_{\max}$ = maximum acidification rate; $t_{V_{\max}}$ = time to reach $V_{\max}$; pH _{$V_{\max}$} = pH in $V_{\max}$; $t_{\text{pH}5.0}$ = time to reach pH 5.0; $t_{\text{pH}4.6}$ = time to reach pH 4.6 (end of fermentation).

treatments (Table 1). The addition of quinoa flour had no effect ($P > 0.05$) on the fermentation time of products, which is good from an industrial standpoint.

Postacidification and Titratable Acidity

Throughout the study, significant differences ($P \leq 0.05$) in pH reduction and titratable acidity increase were observed for all products (Table 2). However, acid production was low at 28 d, and the products had acidities typical of commercial products. Consumption of residual lactose and production of lactic acid occur during the storage of fermented milk as a result of the metabolic activity of lactic acid bacteria (i.e., post-acidification; Oliveira et al., 2009).

Fermented milk with quinoa flour exhibited significantly lower titratable acidity values ($P \leq 0.05$) than the control treatment at all time points, and the pH value was significantly different ($P \leq 0.05$) between treatments only at 28 d of storage, when the fermented milk

control showed the highest pH and samples fermented with 2 and 3 g/100 g of quinoa flour had the lowest pH values. The pH decreased from 0.28 to 0.38 units during 28 d of storage, but the reduction was greater in samples supplemented with quinoa flour than in the control sample, and the acidity increased from 0.24 to 0.27% in fermented milk during storage. Likewise, Zare et al. (2013) found that fermented milk supplemented with 1, 2, and 3 g/100 g of pea flour exhibit a greater reduction in pH during storage compared with the control product. However, other studies reported that the addition of fiber (apple, wheat, bamboo, inulin, orange, lemon, date, and wheat bran) did not affect the pH and acidity of yogurts (Staffolo et al., 2004; Sendra et al., 2008; Hashim et al., 2009). Thus, postacidification seems to depend on the type of starter culture and the ingredients in the milk base.

We observed the largest decrease in acidity and increase in pH between d 1 and 14 because the microorganisms have high metabolic activity at higher

Table 2. Titratable acidity and postacidification (pH; mean $\pm$ SD) of fermented milk control and fermented milk supplemented with quinoa flour during refrigerated storage

Parameter	Treatment ¹	Storage time (d)		
		1	14	28
Titratable acidity	FMC	0.82 $\pm$ 0.04 ^{B,c}	1.03 $\pm$ 0.01 ^{A,b}	1.06 $\pm$ 0.01 ^{A,b}
	FMQ1	0.87 $\pm$ 0.04 ^{C,b}	1.08 $\pm$ 0.01 ^{B,a}	1.14 $\pm$ 0.04 ^{A,a}
	FMQ2	0.88 $\pm$ 0.01 ^{C,b}	1.09 $\pm$ 0.01 ^{B,a}	1.14 $\pm$ 0.04 ^{A,a}
	FMQ3	0.94 $\pm$ 0.04 ^{C,a}	1.09 $\pm$ 0.01 ^{B,a}	1.14 $\pm$ 0.03 ^{A,a}
pH	FMC	4.59 $\pm$ 0.03 ^{A,a}	4.31 $\pm$ 0.01 ^{B,a}	4.31 $\pm$ 0.03 ^{B,a}
	FMQ1	4.63 $\pm$ 0.03 ^{A,a}	4.30 $\pm$ 0.06 ^{B,a}	4.27 $\pm$ 0.05 ^{B,ab}
	FMQ2	4.64 $\pm$ 0.02 ^{A,a}	4.28 $\pm$ 0.04 ^{B,a}	4.25 $\pm$ 0.03 ^{B,b}
	FMQ3	4.63 $\pm$ 0.03 ^{A,a}	4.29 $\pm$ 0.06 ^{B,a}	4.25 $\pm$ 0.01 ^{B,b}

^{A-C}Different uppercase letters in a row denote significant differences for the same treatment during storage period ($P \leq 0.05$).

^{a-c}Different lowercase letters in a column denote significant differences among treatments ($P \leq 0.05$).

¹Treatments: FMC = fermented milk control, without quinoa flour; FMQ1, FMQ2, and FMQ3 = fermented milk supplemented with 1, 2, or 3% quinoa flour, respectively; $n = 3$.

pH, which occurs during the beginning of the storage, which is in agreement with a previous study (Beal et al., 1999).

Viability of Microorganisms

The inoculation rates of *Strep. thermophilus* and probiotic strains in milk before fermentation showed no significant difference ($P > 0.05$) between treatments (data not shown). During the storage period, the population of *L. acidophilus* La-5 ranged from 7.04 to 7.94 $\log_{10}$ cfu/mL (Table 3), showing a reduction from 0.17 to 0.43 $\log_{10}$ cfu/mL at the end of storage. The population of *B. animalis* ssp. *lactis* BB-12 ranged from 7.61 to 8.49 $\log_{10}$ cfu/mL during 28 d of storage (Table 3), decreasing from 0.10 to 0.49 $\log_{10}$ cfu/mL. Despite the variations observed in the probiotic populations, the minimum therapeutic dose (between 10^6 and 10^9 cfu/mL in the product; Vasiljevic and Shah, 2008) was achieved in all treatments until d 28.

For both probiotic bacteria, the largest decreases at the end of storage were obtained for the treatment without quinoa flour. Thus, supplementing milk with quinoa flour resulted in a significantly ($P \leq 0.05$) higher population of *L. acidophilus* La-5 and *B. animalis* ssp. *lactis* BB-12 compared with the control treatment at 28 d of storage. However, these changes were of little microbiological significance, because they were $<1 \log_{10}$ cfu/mL. Nevertheless, the addition of quinoa flour at the highest concentration (3 g/100 g) showed a tendency toward stimulation of *B. animalis* ssp. *lactis* BB-12 during storage. The population of this strain

was 0.87 $\log_{10}$ cfu/mL greater than in the fermented milk control. These results are similar to those previously observed for fermented milk with lentils, pea, and orange fiber (Sendra et al., 2008; Agil et al., 2013; Zare et al., 2013).

The improvement in growth of this probiotic strain in the FMQ3 treatment could be related to the compounds present in this ingredient. Quinoa flour is characterized by high carbohydrate and fiber contents and is relatively high in linoleic acid (52.68 g/100 g of total FA content), an amount that is 26% and 37% of the dietary reference intake for males and females, respectively. It is also rich in oleic (23.93 g/100 g of total FA content) and α -linolenic (4.60 g/100 g of total FA content) acids. Folate, potassium, phosphorous, magnesium, and calcium contents are also high (180 μ g/100 g, 553.8, 441.6, 229.9, and 49.8 mg/100 g, respectively). Additionally, quinoa flour is high in iron and zinc (5.4 and 3.3 mg/100 g respectively; Hager et al., 2012). Because probiotic microorganisms require high nutrient levels, these compounds benefit the growth of probiotics during storage of fermented milk. However, further studies are necessary to elucidate the specific components that are involved in improving the growth and survival of probiotics in products supplemented with quinoa flour.

In addition, we observed a positive correlation between the population of probiotic strains ($r = 0.87$ and $r = 0.95$ for La-5 and BB-12, respectively) and the pH value of the samples on d 1 of storage. However, at the end of storage, a negative correlation was observed between these parameters ($r = -0.94$ and $r = -0.96$, for La-5 and BB-12, respectively). These data are in

Table 3. Counts ($\log_{10}$ cfu/mL; mean $\pm$ SD) of *Streptococcus thermophilus*, *Lactobacillus acidophilus* La-5, and *Bifidobacterium animalis* ssp. *lactis* BB-12 in fermented milk during refrigerated storage

Microorganism	Treatment ¹	Storage time (d)		
		1	14	28
<i>L. acidophilus</i> La-5	FMC	7.47 $\pm$ 0.03 ^{A,b}	7.43 $\pm$ 0.11 ^{A,b}	7.04 $\pm$ 0.02 ^{B,b}
	FMQ1	7.73 $\pm$ 0.19 ^{AB,a}	7.86 $\pm$ 0.09 ^{A,a}	7.56 $\pm$ 0.05 ^{B,a}
	FMQ2	7.71 $\pm$ 0.12 ^{B,ab}	7.94 $\pm$ 0.06 ^{A,a}	7.51 $\pm$ 0.10 ^{C,a}
	FMQ3	7.83 $\pm$ 0.09 ^{A,a}	7.91 $\pm$ 0.08 ^{A,a}	7.63 $\pm$ 0.08 ^{B,a}
<i>B. animalis</i> ssp. <i>lactis</i> BB-12	FMC	8.10 $\pm$ 0.27 ^{A,b}	7.93 $\pm$ 0.09 ^{AB,b}	7.61 $\pm$ 0.06 ^{B,c}
	FMQ1	8.46 $\pm$ 0.06 ^{A,a}	8.47 $\pm$ 0.12 ^{A,a}	8.36 $\pm$ 0.04 ^{A,b}
	FMQ2	8.44 $\pm$ 0.07 ^{A,a}	8.47 $\pm$ 0.02 ^{A,a}	8.40 $\pm$ 0.05 ^{A,ab}
	FMQ3	8.49 $\pm$ 0.05 ^{A,a}	8.45 $\pm$ 0.06 ^{A,a}	8.48 $\pm$ 0.05 ^{A,a}
<i>Strep. thermophilus</i>	FMC	9.41 $\pm$ 0.23 ^{A,a}	9.49 $\pm$ 0.09 ^{A,a}	9.28 $\pm$ 0.09 ^{A,a}
	FMQ1	9.43 $\pm$ 0.02 ^{AB,a}	9.53 $\pm$ 0.09 ^{A,a}	9.30 $\pm$ 0.12 ^{B,a}
	FMQ2	9.42 $\pm$ 0.13 ^{A,a}	9.31 $\pm$ 0.06 ^{A,b}	9.25 $\pm$ 0.07 ^{A,a}
	FMQ3	9.58 $\pm$ 0.26 ^{A,a}	9.29 $\pm$ 0.03 ^{AB,b}	9.23 $\pm$ 0.06 ^{B,a}

^{A-C}Different uppercase letters in a row denote significant differences for the same treatment during storage period ($P \leq 0.05$).

^{a-c}Different lowercase letters in a column denote significant differences among treatments ($P \leq 0.05$).

¹Treatments: FMC = fermented milk control, without quinoa flour; FMQ1, FMQ2, and FMQ3 = fermented milk supplemented with 1, 2, or 3% quinoa flour, respectively; n = 3.

Table 4. Probiotic survival (viable count; log₁₀ cfu/mL; mean ± SD) of *Lactobacillus acidophilus* La-5 and *Bifidobacterium animalis* ssp. *lactis* BB-12 in fermented milk during refrigerated storage, before (t0) and during exposure to in vitro simulated gastric conditions for 120 min (t120, pH 2.0–2.2), and enteric conditions for 240 min (t240, pH 4.3–5.2) and 360 min (t360, pH 7.0–7.3)

Strain	Treatment ¹	Day	Exposure to simulated gastrointestinal conditions			
			t0	t120	t240	t360
La-5	FMC	1	7.45 ± 0.07 ^{A,b}	4.76 ± 0.08 ^{B,c}	4.16 ± 0.08 ^{D,b}	4.36 ± 0.07 ^{C,c}
	FMQ1		7.78 ± 0.16 ^{A,a}	5.20 ± 0.04 ^{B,b}	4.79 ± 0.04 ^{C,a}	4.57 ± 0.02 ^{D,b}
	FMQ2		7.84 ± 0.13 ^{A,a}	5.70 ± 0.06 ^{B,a}	4.87 ± 0.06 ^{C,a}	4.77 ± 0.04 ^{C,a}
	FMQ3	14	7.93 ± 0.04 ^{A,a}	5.80 ± 0.17 ^{B,a}	4.96 ± 0.16 ^{C,a}	4.84 ± 0.01 ^{C,a}
	FMC		7.32 ± 0.13 ^{A,b}	4.47 ± 0.04 ^{B,b}	3.23 ± 0.08 ^{C,c}	3.43 ± 0.13 ^{C,c}
	FMQ1		7.77 ± 0.12 ^{A,a}	5.27 ± 0.08 ^{B,a}	4.38 ± 0.22 ^{C,b}	4.12 ± 0.27 ^{D,b}
	FMQ2	28	7.99 ± 0.06 ^{A,a}	5.07 ± 0.23 ^{B,a}	4.68 ± 0.10 ^{C,a}	4.60 ± 0.03 ^{C,a}
	FMQ3		7.79 ± 0.17 ^{A,a}	5.30 ± 0.07 ^{B,a}	4.44 ± 0.19 ^{C,ab}	4.69 ± 0.09 ^{C,a}
	FMC		7.08 ± 0.07 ^{A,b}	2.77 ± 0.25 ^{B,c}	2.53 ± 0.14 ^{B,b}	2.52 ± 0.05 ^{B,c}
	FMQ1		7.66 ± 0.06 ^{A,a}	3.91 ± 0.22 ^{B,ab}	3.07 ± 0.06 ^{C,a}	2.82 ± 0.14 ^{D,b}
	FMQ2		7.48 ± 0.06 ^{A,a}	3.79 ± 0.12 ^{B,b}	3.04 ± 0.10 ^{C,a}	3.19 ± 0.02 ^{C,a}
	FMQ3		7.70 ± 0.03 ^{A,a}	4.10 ± 0.29 ^{B,a}	3.21 ± 0.09 ^{C,a}	3.23 ± 0.02 ^{C,a}
BB-12	FMC	1	8.00 ± 0.03 ^{A,c}	7.97 ± 0.06 ^{A,c}	7.76 ± 0.08 ^{B,c}	7.66 ± 0.05 ^{B,c}
	FMQ1		8.33 ± 0.03 ^{A,b}	8.20 ± 0.10 ^{A,b}	7.92 ± 0.13 ^{B,b}	7.92 ± 0.13 ^{B,b}
	FMQ2		8.54 ± 0.81 ^{A,a}	8.47 ± 0.03 ^{A,a}	7.94 ± 0.02 ^{B,b}	7.94 ± 0.04 ^{B,b}
	FMQ3	14	8.52 ± 0.09 ^{A,a}	8.40 ± 0.04 ^{A,a}	8.07 ± 0.03 ^{C,a}	8.20 ± 0.09 ^{B,a}
	FMC		8.06 ± 0.08 ^{A,b}	8.03 ± 0.03 ^{A,b}	7.87 ± 0.06 ^{B,b}	7.79 ± 0.06 ^{B,b}
	FMQ1		8.37 ± 0.13 ^{A,a}	8.38 ± 0.03 ^{A,a}	8.03 ± 0.05 ^{B,a}	8.03 ± 0.07 ^{B,a}
	FMQ2	28	8.47 ± 0.06 ^{A,a}	8.42 ± 0.03 ^{A,a}	7.97 ± 0.09 ^{C,ab}	8.17 ± 0.06 ^{B,a}
	FMQ3		8.46 ± 0.06 ^{A,a}	8.34 ± 0.04 ^{A,a}	8.03 ± 0.02 ^{B,a}	8.14 ± 0.17 ^{B,a}
	FMC		7.78 ± 0.07 ^{A,b}	7.74 ± 0.15 ^{A,b}	7.56 ± 0.06 ^{B,b}	7.18 ± 0.19 ^{C,b}
	FMQ1		8.41 ± 0.07 ^{A,a}	8.26 ± 0.04 ^{A,a}	7.91 ± 0.07 ^{B,a}	8.02 ± 0.01 ^{B,a}
	FMQ2		8.36 ± 0.10 ^{A,a}	8.27 ± 0.08 ^{A,a}	7.84 ± 0.03 ^{C,a}	8.01 ± 0.01 ^{B,a}
	FMQ3		8.49 ± 0.08 ^{A,a}	8.31 ± 0.13 ^{B,a}	7.87 ± 0.00 ^{C,a}	8.05 ± 0.07 ^{D,a}

^{A–D}Different uppercase letters in a row denote significant differences ($P \leq 0.05$) among sampling period of the in vitro assay at each day of storage for the same treatment.

^{a–c}Different lowercase letters in a column for each probiotic strain denote significant differences ($P \leq 0.05$) among treatments at each sampling day.

¹Treatments: FMC = fermented milk control, without quinoa flour; FMQ1, FMQ2, and FMQ3 = fermented milk supplemented with 1, 2, or 3% quinoa flour, respectively; n = 3.

agreement with other studies showing that the acidification during storage does not necessarily correspond to kinetics of microbial growth (Seo et al., 2009; Zare et al., 2013).

The population of *Strep. thermophilus* ranged from 9.23 to 9.58 log₁₀ cfu/mL during storage of the products (Table 3). The variations found in the population, both over time and among treatments, were minimal. Several studies have also demonstrated the stability of the population of *Strep. thermophilus* during food product storage (Marafon et al., 2011; Bedani et al., 2013).

Probiotic Resistance to Simulated GI Conditions

We observed a significant reduction ($P \leq 0.05$) in the population of probiotic bacteria during passage through the simulated conditions of the GI tract. The protective effect of quinoa flour on the resistance of probiotic strains was not significant from a microbiological standpoint, because the difference between the probiotic counts in control and in supplemented fermented milk at the end of the in vitro assay was <1

log₁₀ cfu/mL. The population reduction after the assay was influenced by the storage period (Table 4).

Probiotic strains must reach the intestine in sufficient concentrations to exert their beneficial effects. Therefore, an ideal carrier must be suitable for human consumption, ensure the viability of the strains during the manufacturing time and shelf-life of the product, and protect the probiotics during passage through the gastrointestinal tract so that they can reach the colon (Possemiers et al., 2010).

Recent studies have reported that food matrix and dietary content structures may interact with probiotic strains, protecting them during transit through the GI tract (Lavermicocca, 2006; Valerio et al., 2006). The presence of quinoa flour may have provided some protection for the survival of probiotics during the simulated passage through the GI tract by acting as a protective cover against gastric and enteric juices. Therefore, it is possible that flour components (protein, lipids, and fiber) improved probiotic tolerance to simulated GI conditions. Nevertheless, this effect was only pronounced for *L. acidophilus* La-5 against gastric juice

action on d 28 of storage and in the enteric phase of the in vitro assay on d 14 of storage.

We observed that the viability of *L. acidophilus* La-5 at the end of the in vitro assay decreased by 3 log units on d 1 for all treatments, by 4 log units for the FMC treatment and by 3 log units for the other treatments on d 14, and 4 log units for all treatments on d 28. The gastric phase showed a greater deleterious effect on *L. acidophilus* La-5 cells than the enteric phase. Generally, the acid tolerance of lactic acid bacteria depends on the pH profile of their H⁺-ATPase enzyme and on their cytoplasmic membrane, which varies by species as well as according to exogenous conditions, including the growth medium and incubation conditions (Madureira et al., 2011).

Bifidobacterium animalis ssp. *lactis* BB-12 was more resistant than *L. acidophilus* La-5 to the GI conditions, and counts of all treatments decreased by <1 log unit at the end of the assay at all storage times. Several studies have reported that *B. animalis* ssp. *lactis* is highly resistant to oxygen and gastrointestinal stress (Andriantsoanirina et al., 2013). Some strains of *Bifidobacterium* spp. are thought to have the ability to adapt to acid stress situations, and the behavior of this microorganism during exposure to low pH may be strain dependent (Mainville et al., 2005; Mättö et al., 2006; Madureira et al., 2011).

Generally, bile salts are accumulated in the bacterial cytoplasm and rapidly dissociated, promoting proton accumulation. However, in our study, *B. animalis* ssp. *lactis* BB-12 showed enhanced resistance to bile. The high survival of this strain during the enteric phase can be ascribed to its ability to increase the activity of the membrane-bound F₀F₁-ATPase enzyme that pumps protons from the cytoplasm to the extracellular environment. When the cells are previously exposed to

acidic conditions, F₀F₁-ATPase enzyme is overproduced and better control of the intracellular pH is observed (Sanchez et al., 2013). Therefore, the acidic conditions in the gastric phase of the in vitro assay could have led to greater bacterial tolerance to bile during the enteric phase.

For both strains tested in all treatments, we observed little variation in viable cell numbers during the enteric phase, which can be explained by the neutral pH prevailing during that period. In vivo, if the cells reached this point, they would most likely adhere to the intestinal epithelium and begin to exert their beneficial biological activities.

Adhesion of Probiotic Bacteria to Intestinal Cells In Vitro

We observed that on the first day of analysis, the adhesion of *L. acidophilus* La-5 was greater ($P \leq 0.05$) in the FMQ2 treatment, whereas *B. animalis* ssp. *lactis* BB-12 showed greater adhesion in the FMC treatment. However, on d 28, we found no differences ($P > 0.05$) among the treatments for both strains (Table 5). Compared with the initial number of cells ($7-8 \log_{10}$ cfu/mL), the proportion of each probiotic strain that adhered to the cell line used for the assay was relatively low for all fermented milk samples ($3-5 \log_{10}$ cfu/mL), as observed in other studies using the same strains (Fernández de Palencia et al., 2008; Ranadheera et al., 2012).

The in vitro tests showed that, besides being dependent on the matrix composition, adhesion is also strain specific. *Lactobacillus acidophilus* La-5 showed lower rates of adherence to Caco-2 cells than did *B. animalis* ssp. *lactis* BB-12 at the beginning of storage. Certain probiotic strains may exhibit greater adhesion capacity due to specific physiological and biochemical

Table 5. Adhesion (%; mean $\pm$ SD) of *Lactobacillus acidophilus* La-5 and *Bifidobacterium animalis* ssp. *lactis* BB-12 to Caco-2 cells during refrigerated storage

Microorganism	Treatment ¹	Storage time (d)	
		1	28
<i>L. acidophilus</i> La-5	FMC	0.02 $\pm$ 0.00 ^{A,b}	0.01 $\pm$ 0.00 ^{A,a}
	FMQ1	0.04 $\pm$ 0.01 ^{A,b}	0.01 $\pm$ 0.00 ^{B,a}
	FMQ2	0.05 $\pm$ 0.02 ^{A,a}	0.01 $\pm$ 0.00 ^{B,a}
	FMQ3	0.03 $\pm$ 0.01 ^{A,ab}	0.01 $\pm$ 0.00 ^{B,a}
<i>B. animalis</i> ssp. <i>lactis</i> BB-12	FMC	0.13 $\pm$ 0.03 ^{A,a}	0.01 $\pm$ 0.00 ^{B,a}
	FMQ1	0.04 $\pm$ 0.00 ^{A,c}	0.01 $\pm$ 0.00 ^{B,a}
	FMQ2	0.08 $\pm$ 0.02 ^{A,b}	0.01 $\pm$ 0.00 ^{B,a}
	FMQ3	0.06 $\pm$ 0.01 ^{A,bc}	0.01 $\pm$ 0.00 ^{B,a}

^{A,B}Different uppercase letters in a row denote significant differences for the same treatment during storage period ($P \leq 0.05$).

^{a-c}Different lowercase letters in a column for each microorganism denote significant differences among treatments ($P \leq 0.05$).

¹Treatments: FMC = fermented milk control, without quinoa flour; FMQ1, FMQ2, and FMQ3 = fermented milk supplemented with 1, 2, or 3% quinoa flour, respectively; n = 3.

characteristics, such as the presence of structures on the surface of bacterial cells that bind to intestinal mucus (Sanchez et al., 2008). Low adherence capacity to intestinal cells can result in reduced time to excretion of probiotics in feces (Saxelin et al., 2010). The storage time of fermented milk reduced ($P \leq 0.05$) the ability of probiotic organisms to adhere to Caco-2 cells. This could be related to the low pH in the samples at the end of storage. Pretreatment at pH 3.0 significantly reduced the adhesion of *Enterococcus faecium* strains to IPEC-J2 cells (Marcinákova et al., 2010). When evaluating the adhesion of *Lactobacillus rhamnosus* GG to Caco-2 cells, Deepika et al. (2011) also reported a reduction in bacterial adhesion during storage in food models simulating yogurt and ice cream, and they concluded that storage time affects bacterial adherence more than other factors such as fat and sugar contents. In that study, a reduction in the hydrophobicity of probiotic cells over time also occurred and was linked to decreased adhesion to Caco-2 cells.

Several studies have determined the adhesion of probiotics or potentially probiotic strains to several cell lines (Argyri et al., 2013; Ryu and Chang, 2013). However, few studies have been conducted to determine the effect of food or specific components on the adhesion properties of bacterial cells. Even though we did not observe an effect of quinoa flour on probiotic adhesion, the properties of bacterial cell surfaces and, consequently, their adherence to the intestinal mucosa may be influenced by the ingredients or by the type of food matrix carrier (Deepika et al., 2011; Ranadheera et al., 2012). Further studies involving greater amounts of quinoa flour could be carried out to verify whether it affects the probiotic resistance to GI conditions and adhesion to the epithelial cells.

CONCLUSIONS

Addition of up to 3% quinoa flour to fermented milk had no effect on fermentation kinetics or on probiotic activity through the 28-d storage time. Nevertheless, the addition of quinoa flour to fermented milk is advantageous because it is an ingredient with high nutritive properties, which may increase the appeal of the product to consumers.

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