Interference of different storage temperatures in the dynamics of probiotic *Bifidobacterium* spp. and *Streptococcus thermophilus* starter cultures in fermented milk

Interferência de diferentes temperaturas de armazenamento na dinâmica de **Bifidobacterium** *spp. probióticas e cultura starter* **Streptococcus thermophilus** *em leite fermentado*

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ABSTRACT: The variations of temperature during the cold chain can impair the quality of live foods, such as fermented milks. Probiotic bacteria are commonly added to food to provide the consumer with beneficial effects. Nevertheless, the concentration of probiotic in the end products should be elevated to ensure functionality. The aim of this study was to evaluate the viability of probiotic strains of bifidobacteria and starter strain of *Streptococcus thermophilus* in fermented milks at storage temperatures of 4 and 10°C, for a period of 28 days. Commercial cultures of *Bifidobacterium* spp. were added to milk fermented by *Streptococcus thermophillus* and stored for 28 days at 4 and 10°C. During this period, bifidobacteria and *S. thermophillus* cultures were monitored to check their behavior in the evaluated storage conditions. Viable bifidobacteria and *S. thermophillus* counts showed no significant variation during storage at 4 and 10°C (p < 0.05), indicating that both of these conditions are adequate for maintaining their initial concentrations. The results indicate that the storage conditions usually adopted in sale establishments of dairy products are suitable to maintain bifidobacteria and *S. thermophillus* cultures in fermented milk.

KEYWORDS: storage; probiotic; fermented milk; bifidobacteria.

RESUMO: As variações de temperatura que ocorrem durante a cadeia refrigerada da produção leiteira pode interferir na qualidade de alimentos bioativos, como leites fermentados. Bactérias probióticas são usualmente adicionadas a alimentos, visando a oferecer ao consumidor efeitos benéficos. O objetivo deste trabalho foi avaliar a viabilidade de culturas probióticas de bifidobactérias e de cultura starter de *Streptococcus thermophilus* em leites fermentados, armazenados nas temperaturas de 4 e 10°C por um período de 28 dias. Culturas comerciais de *Bifidobacterium* spp. foram adicionadas ao leite fermentado produzido com *Streptococcus thermophillus* e estocados por 28 dias a 4 e 10°C. Nesse período, as culturas de *Bifidobacterium* spp. e *Streptococcus thermophillus* foram monitoradas, com o objetivo de verificar seus comportamentos nas temperaturas de estocagem testadas. As contagens de ambos os micro-organismos não apresentaram variação significativa ao longo do período de estocagem a 4 e a 10°C (p < 0,05), indicando que as duas temperaturas testadas podem oferecer condições adequadas de conservação desse produto. Os resultados obtidos indicaram que as condições de conservação de alimentos usualmente adotadas para produtos lácteos em estabelecimentos comerciais são adequadas para manter as contagens de bifidobactérias e *Streptococcus thermophillus* em leites fermentados.

PALAVRAS-CHAVE: estocagem; probiótico; leite fermentado; bifidobactéria.

The cold storage of milk and dairy products, since the beginning of the production chain, has received great attention in several countries in the past years, including Brazil and other Latin American countries (Perin et al., 2012). In Brazil, the inclusion of this procedure in the dairy production is usually referred to as the "cold chain", and it is subjected to variations due to distinct characteristics of milk production: for raw milk, storage in dairy farms is allowed at temperatures ranging from 4 to 7ºC, when at industry arrival variations of up to 10ºC are permitted (Brasil, 2011). On retail sale, dairy products such as fermented milk are usually maintained at 10ºC (Brasil, 2000).

These variations in storage temperature can determine a deleterious result in dairy products. These conditions enable the growth of distinct spoilage and pathogenic microorganisms that are able to jeopardize the quality and safety of such products (Ammor; Mayo, 2007). Such variation in storage temperature has a particular concern for dairy products that are considered as "live foods", such as fermented milks: the temperature variation while storing these products can affect the viability and the concentrations of the added starter and probiotic cultures (Shah, 2000).

According to the Food and Agriculture Organization (FAO) and the World Health Organization (WHO), probiotics are live organisms that, when administered at adequate amounts, have a beneficial effect on the health of the host. Once in the host's intestinal tract, these microorganisms cause changes in the intestinal microbiota in order to modulate the immune system, inhibit the adherence and multiplication of pathogens, and also prevent obesity and some types of cancer (Lourens-Hattingh; Viljoen, 2001). Ideally, probiotic cultures should present populations between 10^5 and 10^7 CFU.g^{-1} during the entire shelf life of the food, so that it can assure the desired beneficial effects for the consumer after ingestion (Ammor; Mayo, 2007; Davidson et al., 2000).

The present study aimed to investigate how usual variation in the storage temperature can interfere in the populations of starter and probiotic cultures in a fermented milk, during its shelf-life.

Four commercial cultures of lyophilized *Bifidobacterium* spp. were used: *B. animalis* Bb 12 (B1, Chr. Hansen A/S, Hørsholm, Denmark), *B. lactis* SAB 440A (B2, Clerici-Sacco Group, Cadorago, Italy), *B. lactis* BI-07 (B3, Danisco A/S, Copenhagen, Denmark) and *B. longum* LGM P-17500 (B4, Centro Sperimentale del Latte, Zelo Buon Persico, Italy). An additional culture of *S. thermophillus* (ST 066, Clerici-Sacco Group) was used to produce fermented milk, previously added to 3 liters of milk and stored at -18ºC until it was ready to be used.

Pasteurized nonfat milk was heated at 90ºC for five minutes and cooled to 39ºC, and then fermented for 4 hours by the addition of ST 066 (Clerici-Sacco Group) in a concentration of about 10^7 colony forming units per g (CFU/g). After fermentation, B1, B2, B3 and B4 were added in concentrations of about 10^7 CFU/g, in batches separated from the fermented milk, and distributed in sterile 200 mL flasks. The fermented milk samples were produced in three repetitions and stored at 4 and 10ºC for 28 days.

Immediately after the fermented milk was bottled and at seven-day intervals, a sample of each fermented milk batch was submitted to microbiological analysis. Aliquots of the samples were submitted to ten-fold dilution using NaCl 8.5 g/L, being two dilutions selected and pour-plated in duplicate using NNLP (MRS broth [Oxoid Ltd., Basingstoke, England] added to nalidixic acid, neomycin sulfate, lithium chloride and paramomycine sulfate) (Laroia; Martin, 1991; Shah, 2000) and M17 (Oxoid) culture media. NNLP plates were incubated at 37ºC for 48 hours under anaerobic conditions (GasPak EZ™ Gas Generating Container Systems, BD) to enumerate the *Bifidobacterium* spp., and M17 plates were incubated at 37ºC for 48 hours under aerobic conditions for *S. thermophillus* enumeration. After incubation, the colonies formed in both culture media were enumerated and the final results were expressed as CFU/g.

The obtained counts for bifidobacteria and *S. thermophillus* cultures were converted in $\log_{10}$, and compared considering different refrigeration temperatures and storage time, using the Analysis of Variance and the Tukey test ($p < 0.05$) to determine significant differences. All of the analyses were performed using STATISTICA Software 7.0 (StatSoft Inc., Tulsa, OK, USA).

The mean counts obtained for the different bifidobacteria and *S. thermophillus* cultures in fermented milk stored at 4 and 10ºC and in different storage periods are shown in Tables 1 and 2, respectively. No significant differences were found between the different populations observed in the assessed incubation temperatures and periods, indicating that the populations of cultures used remained stable during the study.

Considering that the initial populations of bifidobacteria cultures were about 10^7 CFU/g, typical of fermented milk with added probiotics (Zacarchenco; Massaguer-Roig, 2006), stocking at 4ºC allowed the concentration to be adequately preserved, and the same was true for the 10ºC temperature (Table 1). This temperature variation (4 to 10ºC) is usually observed in sale establishments, and, depending on the cultures that are present in different foods, it may lead to significant multiplication. Moreover, the 4ºC temperature is usually recommended to maintain microbial populations in milk and milk products to prevent their deterioration (Robinson, 2002). Nevertheless, Brazilian legislation allows fermented milk to be preserved at temperatures of 10ºC in sale establishments (Brasil, 2000). In a similar study, no significant changes were observed in the populations or in the culture viability of bifidobacteria added to yogurt (Vinderola et al., 2002) produced with different types of additives. In another study (Cruz et al., 2010), it was possible to observe that a commercial yogurt kept the count of bifidobacteria at 7 to 8 log for 84 days at 10ºC.

In relation to *S. thermophillus*, some types of fermented milk with specific added cultures of bifidobacteria ranged during different storage periods and temperatures, but at no significant level (Table 2). Oliveira et al. (2002) described a similar behavior for *S. thermophillus* cultures in fermented

Table 1. Mean counts of bifidobacteria cultures from fermented milk (prepared with *Streptococcus thermophilus*) stored at 4 and 10°C for 28 days, obtained by NNLP culture media by conventional plating.

Culture	Storage temperature	Days of Incubation					Statistics
		0	7	14	21	28	
B1	4	6.20	6.38	6.16	6.21	6.32	$F_{(4,10)} = 0.08, p = 0.987$
	10	6,20	6,95	5,90	6,35	6,06	$F_{(4,10)} = 0.74, p = 0.588$
		$F_{(1,4)} = 0.00, p = 1.000$	$F_{(1,4)} = 0.63, p = 0.473$	$F_{(1,4)} = 0.49, p = 0.523$	$F_{(1,4)} = 0.11, p = 0.759$	$F_{(1,4)} = 0.14, p = 0.724$	
B2	4	5.70	5.56	4.99	5.05	5.28	$F_{(4,10)} = 0.85, p = 0.527$
	10	5.70	5.26	5.26	4.98	5.07	$F_{(4,10)} = 0.85, p = 0.526$
		$F_{(1,4)} = 0.00, p = 1.000$	$F_{(1,4)} = 0.14, p = 0.734$	$F_{(1,4)} = 0.20, p = 0.680$	$F_{(1,4)} = 0.08, p = 0.792$	$F_{(1,4)} = 0.98, p = 0.378$	
B3	4	6.87	6.84	6.86	6.75	6.42	$F_{(4,10)} = 0.23, p = 0.916$
	10	6.87	6.75	6.55	6.75	6.27	$F_{(4,10)} = 0.43, p = 0.786$
		$F_{(1,4)} = 0.00, p = 1.000$	$F_{(1,4)} = 1.95, p = 0.235$	$F_{(1,4)} = 5.88, p = 0.072$	$F_{(1,4)} = 0.00, p = 0.996$	$F_{(1,4)} = 0.01, p = 0.930$	
B4	4	7.09	6.90	6.93	6.61	7.35	$F_{(4,10)} = 1.85, p = 0.195$
	10	7.09	6.94	6.63	6.34	7.30	$F_{(4,10)} = 0.87, p = 0.514$
		$F_{(1,4)} = 0.00, p = 1.000$	$F_{(1,4)} = 0.02, p = 0.905$	$F_{(1,4)} = 0.31, p = 0.609$	$F_{(1,4)} = 0.21, p = 0.673$	$F_{(1,4)} = 0.01, p = 0.912$	
all	4	6.47	6.50	6.24	6.15	6.34	$F_{(4,38)} = 0.37, p = 0.829$
	10	6.47	6.47	6.09	6.21	6.16	$F_{(4,38)} = 0.49, p = 0.745$
		$F_{(1,114)} = 0.00, p = 1.000$	$F_{(1,114)} = 0.00, p = 0.949$	$F_{(1,114)} = 0.19, p = 0.671$	$F_{(1,114)} = 0.02, p = 0.880$	$F_{(1,114)} = 0.14, p = 0.711$	

*All statistics presented: Analysis of Variance (ANOVA). Mean counts with distinct lower case indicate significant differences in a same row (Tukey test, p < 0.05). Mean counts with distinct upper case indicate significant differences in the same column (Tukey test, p < 0.05); nc = no counts recorded; F = ANOVA test; df = degrees of freedom; p = level of significance.

Table 2. Mean counts of *Streptococcus thermophilus* from fermented milk (added to bifidobacteria cultures) stored at 4 and 10°C for 28 days, obtained by M17 culture media by conventional plating.

Added bifidobacteria	Storage temperature	Days of Incubation					Statistics
		0	7	14	21	28	
B1	4	7.85	8.88	7.71	8.47	7.80	F(4,10) = 0.36, p = 0.831
	10	7.85	9.03	8.92	8.42	7.90	F(4,10) = 1.36, p = 0.320
		F(1,4) = 0.00, p = 1.000	F(1,4) = 0.02, p = 0.889	F(1,4) = 0.79, p = 0.425	F(1,4) = 0.01, p = 0.926	F(1,4) = 0.02, p = 0.889	
B2	4	8.50	8.57	8.88	8.43	8.32	F(4,10) = 0.19, p = 0.940
	10	8.50	8.55	8.85	9.16	8.62	F(4,10) = 0.52, p = 0.725
		F(1,4) = 0.00, p = 1.000	F(1,4) = 0.09, p = 0.745	F(1,4) = 0.01, p = 0.937	F(1,4) = 1.10, p = 0.353	F(1,4) = 0.23, p = 0.657	
B3	4	7.86	8.34	8.48	6.27	8.14	F(4,10) = 1.42, p = 0.298
	10	7.86	8.72	7.65	7.08	7.72	F(4,10) = 0.79, p = 0.558
		F(1,4) = 0.00, p = 1.000	F(1,4) = 0.10, p = 0.768	F(1,4) = 2.46, p = 0.192	F(1,4) = 3.20, p = 0.148	F(1,4) = 0.06, p = 0.816	
B4	4	8.14	8.25	8.24	7.38	7.90	F(4,10) = 0.26, p = 0.896
	10	8.14	8.32	8.43	7.18	8.75	F(4,10) = 0.89, p = 0.507
		F(1,4) = 0.00, p = 1.000	F(1,4) = 0.00, p = 0.952	F(1,4) = 0.18, p = 0.692	F(1,4) = 0.05, p = 0.834	F(1,4) = 0.35, p = 0.594	
all	4	8.07	8.51	8.33	7.64	8.06	F(4,38) = 0.99, p = 0.424
	10	8.07	8.65	8.47	7.96	8.20	F(4,38) = 1.04, p = 0.395
		F(1,114) = 0.00, p = 1.000	F(1,114) = 0.10, p = 0.758	F(1,114) = 0.12, p = 0.729	F(1,114) = 0.46, p = 0.502	F(1,114) = 0.07, p = 0.790	

*All statistics presented: Analysis of Variance (ANOVA). Mean counts with distinct lower case indicate significant differences in a same row (Tukey test, p < 0.05). Mean counts with distinct upper case indicate significant differences in a same column (Tukey test, p < 0.05); nc = no counts recorded; F = ANOVA test; df = degrees of freedom; p = level of significance.

dairy products containing probiotic cultures, whereas significant interferences were observed only in products fermented by *Lactobacillus* after storage at 4ºC for 21 days.

The viability of bifidobacteria cultures in fermented products during storage may be influenced by certain factors, such as post-acidification, oxygen content and presence of antimicrobial compounds (SHAH, 2001). Considering that only the *S.*

thermophillus culture was used for fermentation, and that its population remained stable during the entire storage period and at the different temperatures under assay (Table 2), acidity was most likely controlled, therefore, it did not interfere with the viability of the added probiotic culture (Table 1). By using a type of cheese with *S. thermophilus* as starter culture to deliver probiotic strains, BERGAMINI et al. (2010)

showed that the *B. lactis* population maintained more than 10^7 CFU.g^{-1} throughout the 60 days of ripening at 12°C, even if with some statistical difference.

The results obtained lead to the conclusion that the storage conditions assessed, usually observed in dairy industries and sale establishments, allowed the adequate preservation of populations of commercial bifidobacteria cultures added to fermented milk produced with *S. thermophillus*.

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