

Development of Probiotic “*creamy requeijão*” Formulations Containing *Lactobacillus* Strains

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Abstract

Functional foods, with emphasis on probiotics, are products that, besides possessing adequate nutritional value, stimulate physiological and metabolic activities in the body. Thus, these benefits combined with greater awareness of the population in the search for food reeducation boost the consumption of this kind of food. “*Creamy requeijão*” is a dairy derivative that has a matrix with physicochemical characteristics suitable for its use as a potential carrier of probiotic microorganisms. Thus, this study aimed to the evaluation of probiotic “*creamy requeijão*” formulations containing, individually six *Lactobacillus* strains, as follows: *L. plantarum* ATCC 8014, *L. acidophilus* ATCC 4356, *L. delbrueckii* UFV H2B20, *L. fermentum* ATCC 9338, *L. casei* ATCC 7469 and *L. paracasei* SP11. The results revealed that during the storage of the formulations for a period of 65 days at 5 °C the cells remained viable at levels above 10^8 CFU.g⁻¹, which allows to classify these formulations as functional foods. In addition, the consumption of only 1 g per day of this food would be enough to attend the requirements of Brazilian legislation regarding the consumption of food with probiotic claims.

Keywords: functional foods, lactic acid bacteria, dairy products

1. Introduction

Probiotics are defined as "living microorganisms which, when consumed in adequate amounts, confer health benefits to the host by modulating the microbiota and preventing infection" (FAO, 2002). Besides promoting the maintenance of a healthy gastrointestinal tract microbiota, they confer resistance to colonization by pathogens, as well as help in the lactose digestion in intolerant individuals and promote activation of the immune system (Kechagia *et al.*, 2013; Kerry *et al.*, 2018). Dairy products are the main vehicles of probiotic microorganisms, being the pioneers in this category (Sanchez *et al.*, 2009). The global market for probiotics was valued at \$ 42.66 billion in 2016 and some projections indicate that this sector could reach \$ 64 billion in 2022 (www.marketsandmarkets.com).

Several functional foods can be found currently in the market. Fermented milks and yogurts are the most traditional, but the development of probiotic cheeses have been highlighted, because it is a matrix that presents higher fat content and higher pH. These characteristics favor the maintenance of microorganism integrity during food storage and in its passage through the gastrointestinal tract (Stanton *et al.*, 1998; Kechagia *et al.*, 2013). In this context, “*creamy requeijão*”, which is a typical Brazilian dairy product accepted by the population, has not been explored yet as a vehicle for probiotic microorganisms. According to the Pattern of Identity and Quality of “*creamy requeijão*” (PIQ), this food must present at least 55% of fat in the dry extract and the maximum of 65% of moisture (Brazil, 1997).

The probiotics microorganisms commonly found in commercial formulations comprise lactic acid species belonging to the genera *Lactobacillus*, *Bifidobacterium*, *Streptococcus* and *Enterococcus*, as well as yeasts such as *Saccharomyces boulardii* (Kerry *et al.*, 2018). In this scenario, the use of *Lactobacillus* species in the human diet is relevant since the beginning of the 20th century, being considered a promising group of microorganisms for the formulation of probiotic foods (Stiles & Holzapfel 1997; Singh *et al.*, 2011).

The National Agency of Sanitary Vigilance in Brazil recommends that, in order to ensure the beneficial effect of probiotics, a minimum of viable cells of these microorganisms should be consumed in the range of 10^8 to 10^9

CFU.g⁻¹ per day (Brazil, 2008). On the other hand, the International Association of Probiotics (2000) recommends that daily intake of these functional foods should be equal to or greater than 10⁷ CFU.g⁻¹. However, the Brazilian Normative Instruction No. 28/2018 does not establish maximum or minimum levels regarding the concentration of viable cells per gram in this sort of products (Brazil, 2018).

Studies involving the incorporation of probiotic microorganisms into different food categories have been developed with emphasis on goat milk ice cream supplemented with *L. acidophilus*, and *B. lactis* (Akalin *et al.*, 2018), chocolates containing *L. paracasei* and *L. acidophilus* containing prebiotic carbohydrate such as inulin (Konar *et al.*, 2018); Minas cheese containing *L. acidophilus* LA 14, and *B. longum* BL 05 (Lollo *et al.*, 2015) and cream cheese containing *B. animalis* subsp. *lactis* DSM 10140, and *L. reuteri* DSM 20016 (Speranza *et al.*, 2017). In these studies, the authors considered the mentioned foods as suitable matrices for incorporation and maintenance of probiotic species.

Considering the characteristics of “creamy requeijão” as a vehicle for probiotic microorganisms, based on the consumer's acceptance of this product and the consumer interest regarding functional foods, the present study aimed to evaluate the development of a probiotic “creamy requeijão” formulation containing different *Lactobacillus* species, considering the cell maintenance when the product was stored for 65 days / 5 °C, as well as its physicochemical composition.

2. Material and Methods

2.1 Micro-organisms and Inoculum Preparation

Six *Lactobacillus* strains were evaluated, consisting of: *L. plantarum* ATCC 8014, *L. acidophilus* ATCC 4356, *L. delbrueckii* UFV H2B20, *L. fermentum* ATCC 9338, *L. casei* ATCC 7469 and *L. paracasei* SP11 (Nestlé), maintained at -20 °C in glycerol 20% (v/v). The activation of these strains was performed by inoculating 2 mL of the stock cultures in 198 mL of Man Rogosa & Sharpe - MRS medium (De Man, Rogosa & Sharpe, 1960) previously sterilized, followed by incubation for 24 h at 37 °C. Subsequently, successive replications were performed in order to obtain a higher inoculum volume, followed by concentration of the cells so that a high amount of *Lactobacillus* could be reached in a small volume of inoculum. The cells were then suspended in 50 mL of reconstituted skim milk (10%) and transferred to two sterile Erlenmeyer flasks, followed by incubation at 37 °C for 2 h, following methodology proposed by Drunkler (2009), with adaptations.

2.2 Preparation of Probiotic “creamy requeijão” Formulations

The probiotic “creamy requeijão” was prepared by taking 9 L of pasteurized milk (3% fat) heated to 82 °C and added with 85% food grade lactic acid (Purac), diluted (1:10), with slow stirring until coagulation was observed. Afterwards, the clotted milk was cooled at room temperature (23 ± 2 °C), and the curd was separated and pressed in a properly hygienized nylon sieve. Butter cream was then added to this curd, in amount equivalent to 50% of its weight, followed by manual homogenization and heating to 85 °C. After, sodium chloride (1.3% w/w) sodium citrate and polyphosphates (1% (w/w), diluted in water at 80 °C, were added, followed by homogenization for approximately 5 min and cooling at 50 °C.

The obtained product was then characterized regarding its physicochemical properties, and a fraction of it was used to incorporate the *Lactobacillus* strains, and then evaluated regarding its microbiological aspects, and cell viability.

One portion of the product (control) was packed while still hot in a properly sterilized polypropylene pot, sealed with PVC film (Wyda Pratic), and stored at 5 ± 1 °C. The remaining product was cooled to 50 °C and portioned in 6 fractions. Each fraction was inoculated, individually, with *Lactobacillus* strains according to methodology described by Drunkler (2009), in order to get an initial cell concentration of approximately 10¹⁰ to 10¹³ CFU.g⁻¹ followed by cooling and stored at 5 ± 1 °C. Samples of each one were collected after 7, 30, 45 and 65 days for characterization.

2.3 Analytical Methods

2.3.1 Determination of Cellular Viability

The population of viable cells (CFU.g⁻¹) in the samples of potential probiotic “creamy requeijão”, was determined by pour plate method, as described by Silva, Junqueira & Silveira (2001).

2.3.2 Determination of pH, Titratable Acidity, and Lipid Content

The pH was determined by the electroanalytical method in digital pH meter (GEHAKA® PG1800) employing 2 g of sample at room temperature (23,0 ± 2,0 °C) diluted in 20 mL of water. The titratable acidity, expressed as lactic acid (%), and the lipid content was determined according to Zenebon *et al* (2008).

2.3.3 Determination of Total Dry Extract (TDE) and Fat Content in Dry Extract (FDE)

The TDE and FDE content in the “*creamy requeijão*” was determined by drying 3 g of the samples in a microwave oven (Brastemp mod. BMP 40E SAAB), according to the methodology described by Van Dender (2000).

2.4 Microbiological Analyzes

Microbiological characterization of the samples was performed, in relation to the presence of total and thermotolerant coliforms, *Staphylococcus spp* and *Salmonella*, according to Silva (2001).

3. Results and Discussion

3.1 Characterization of “*cream requeijão*” Regarding the Cellular Viability

The results showed in Figure 1 demonstrate that the populations of the strains *L. plantarum* ATCC 8014, *L. delbrueckii* UFV H2B20 and *L. fermentum* ATCC 9338 decreased 3,0 log cycles when the respective formulations were stored at 5 °C for 65 days. On the other hand, formulations containing the strains *L. casei* ATCC 7469, *L. paracasei* SP11 and *L. acidophilus* ATCC4356 showed a decrease of only one cycle under the same conditions.

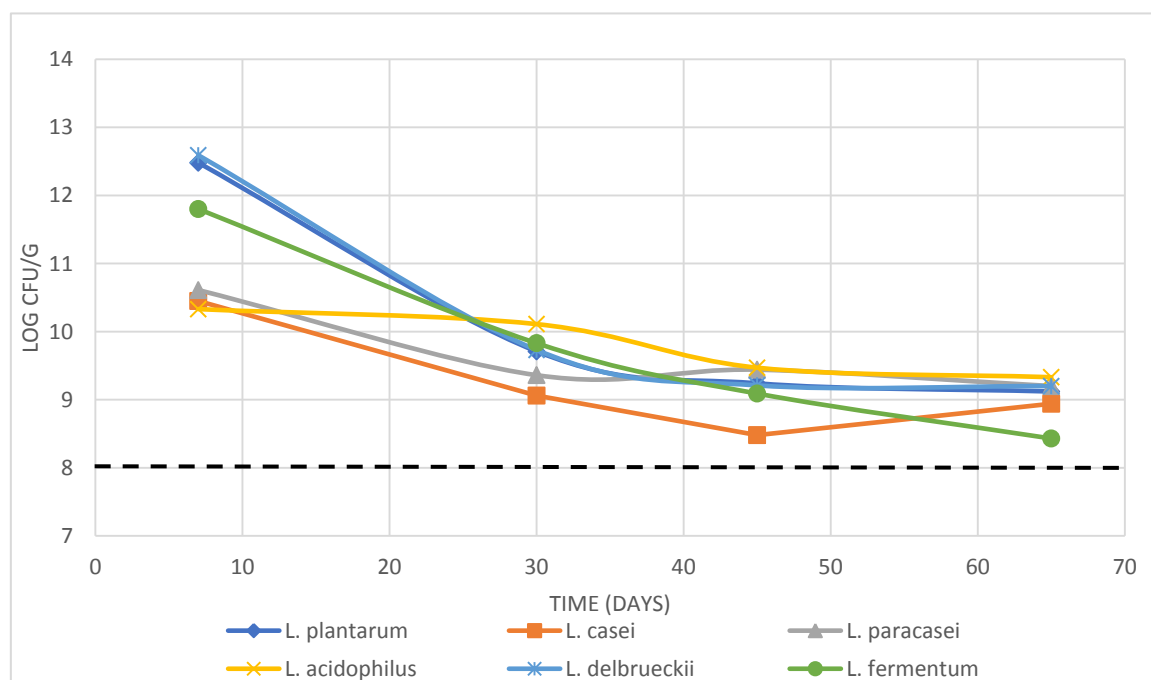


Figure 1. Profile of the *Lactobacillus* strains viability in “*creamy requeijão*” stored for 65 days at 5 °C. Dashed line corresponds to the minimum amount of probiotic cells to be consumed according to Brazilian law

According to Saad, Cruz & Faria (2011) there are factors that may interfere in the survival of probiotic strains, including the quality of the raw material (considering that milk may contain residues of antibiotics and pesticides and its pH, lipid and salt content), as well as the strain used and storage conditions, such as temperature and oxygen level in the medium.

The results obtained in the present study are similar to those reported by Speranza *et al* (2017) in their research with cream cheese formulations supplemented with *Bifidobacterium animalis* subsp. *yactis* DSM 10140 and *L. reuteri* DSM 20016, stored for 28 days at 4 °C. These results demonstrate the potential of dairy products as a matrix for carrying probiotic microorganisms.

On the other hand, lower results were reported by Konar *et al* (2018) studying sugarless white chocolate as a matrix, inoculated with a *L. paracasei* strain, showing that the initial cell concentration was 10^9 CFU.g⁻¹, and after 60 days storage at room temperature, the population decreased to 10^6 CFU.g⁻¹. The authors pointed out that this result should be due to the low water activity in the matrix studied. Similarly, Teixeira (2012), studying “*creamy requeijão*” containing *L. acidophilus* and *B. bifidum*, reported that these strains were maintained at

stable viability levels after storage for 15 days at 10 °C. After this period there was a decrease in the *Lactobacillus* population, equivalent to two log cycles, reaching a cell population of 4.0×10^7 CFU.g⁻¹, and 2.0×10^8 CFU.g⁻¹ of *B. bifidum*. The incorporation of *Bifidobacterium animalis* subsp. *Lactis* Bb-12 and prebiotic compounds (inulin and oligofructose) in “creamy requeijão” formulations were also evaluated by Drunkler, (2009), and the results showed that the cells remained viable at about 10^6 CFU.g⁻¹ for 60 days storage.

However, regarding the daily intake of probiotic foods, it should be emphasized that is necessary to consume 10^{6-7} CFU. g⁻¹, which is contained in 100 g of the product, according to the National Agency of Sanitary Vigilance recommendation (Brazil, 2008). Therefore, it becomes unfeasible, considering that the average “creamy requeijão” consumption by the population is 30g per day. In this context, it is worth mentioning that in only 1 g of the different formulations evaluated in the present work was verified a viable cell population greater than 10^8 CFU after 65 days of storage at 5 °C. This result demonstrates the technical feasibility of using “creamy requeijão” as a vehicle of the probiotic strains.

“Creamy requeijão” is a product of high acceptance in the different social classes in Brazil, so it can easily be inserted into the daily diet of the population, with an additional benefit of having the probiotics in its formulation. Therefore, it can contribute to the gastrointestinal health of consumers, and presents potential to be marketed, due to the benefits on daily consumption of functional foods.

3.2 Physicochemical Characterizations of the “creamy requeijão” Formulations

As showed in Table 1, the pH values in the probiotic “creamy requeijão” (6.20 ± 0.10 to 6.70 ± 0.13) were lower than the control formulation (6.44 ± 0.17 to 6.73 ± 0.18), after the storage for 65 days at 5 °C.

Table 1. pH values and standard deviation of the probiotic “creamy requeijão” formulations evaluated during 65 days at 5 °C

Time (days)	Formulations						
	Control	LP	LC	LI	LA	LD	LF
7	6.65±0.18	6.40±0.16	6.44±0.19	6.40±0.16	6.41±0.17	6.20±0.10	6.22±0.19
30	6.73±0.18	6.49±0.17	6.30±0.17	6.42±0.18	6.39±0.15	6.22±0.15	6.40±0.19
45	6.44±0.17	6.51±0.19	6.39±0.10	6.70±0.13	6.46±0.18	6.32±0.16	6.52±0.17
65	6.51±0.19	6.58±0.13	6.44±0.12	6.69±0.15	6.54±0.11	6.41±0.19	6.69±0.14

LP = *Lactobacillus plantarum* ATCC 8014; LC= *Lactobacillus casei* ATCC 7469; LI= *Lactobacillus paracasei* SP11; LA= *Lactobacillus acidophilus* ATCC 4356; LD= *Lactobacillus delbrueckii* UFV H2B20; LF= *Lactobacillus fermentum* ATCC 9338.

Drunkler (2009) reported that the pH decreased was not significantly altered ($p > 0.05$) in symbiotic “creamy requeijão” formulations in relation to control formulation after 60 days of storage at 5 °C. This observation was also verified in the present work, where the pH values after 65 days of the control formulation (6.51 ± 0.19) and the respective probiotic formulations remained between 6.41 ± 0.19 to 6.69 ± 0.15 .

The results in Table 2 show that the lactic acid content was higher in the formulations containing the *Lactobacillus* strains, ranging from 0.43% to 0.30% after 7 days at 5 °C and 0.28% at 0.24% after 65 days in relation to the control formulation (0.17% after 7 days at 5 °C and 0.12% after 65 days). This observation might be due to the production of lactic acid that occurred during the strains preactivation step in reconstituted milk at 10% at 37 °C for 2 h, prior to the inoculation in the curd.

Table 2. Values of lactic acid contents and standard deviation in the different probiotic “creamy requeijão” formulations stored for 65 days at 5 °C

Time (days)	Formulations						
	Control	LP	LC	LI	LA	LD	LF
7	0.17±0.07	0.35±0.08	0.30±0.09	0.38±0.08	0.30±0.08	0.43±0.09	0.43±0.09
30	0.12±0.09	0.23±0.09	0.30±0.09	0.32±0.09	0.29±0.07	0.23±0.08	0.27±0.09
45	0.14±0.09	0.26±0.08	0.29±0.08	0.33±0.09	0.29±0.09	0.26±0.09	0.28±0.08
65	0.12±0.08	0.25±0.09	0.28±0.09	0.24±0.08	0.28±0.09	0.28±0.09	0.24±0.09

LP = *Lactobacillus plantarum* ATCC 8014; LC= *Lactobacillus casei* ATCC 7469; LI= *Lactobacillus paracasei* SP11; LA= *Lactobacillus acidophilus* ATCC 4356; LD= *Lactobacillus delbrueckii* UFV H2B20; LF= *Lactobacillus fermentum* ATCC 9338.

3.4 Moisture, Total Dry Extract and Fat in Dry Extract in Probiotic “cream requeijão”

The results shown in Table 3 demonstrate that the content of fat in the dry extract, and the humidity of the “cream requeijão” formulations attend the minimum standard described in the Technical Regulation - Administrative Rule 359 (Brazil, 1997). These values consist of fat content in the dry extract (FDE) should be higher than 55% and the maximum Total Dry Extract (TDE) content of 65%.

Table 3. Physicochemical characteristics of probiotic “creamy requeijão” formulations after 65 days at 5 °C

Formulation	Moisture (%w/w)	TDE (%w/w)	FDE (%w/w)
Control	60.00	40.00	67.22
<i>L. acidophilus</i> ATCC 4356	62.58	37.42	65.52
<i>L. plantarum</i> ATCC 8014	62.00	38.00	63.81
<i>L. paracasei</i> SP11	64.15	35.85	65.52
<i>L. casei</i> ATCC 7469	64.95	35.05	66.30
<i>L. delbrueckii</i> UFV H2B20	64.03	35.97	66.49
<i>L. fermentum</i> ATCC 9338	63.45	36.55	65.82

TDE = Total dry extract; FDE = Fat in dry extract

3.5 Microbiological Characterization of the Probiotic “creamy requeijão”

The microbiological characterization of the different “creamy requeijão” formulations, stored for 65 days at 5 °C, reveals that all formulations are in agreement with the microbiological standards established in Technical Regulation - Administrative Rule 359 (Brazil, 1997).

4. Conclusions

In the present study, it has been demonstrated that “creamy requeijão” is a suitable vehicle for carrying strains of probiotic microorganisms without changing the characteristics of this dairy food. The formulations evaluated had a population above 10^8 CFU g⁻¹ of viable cells after 65 days at 5 °C. In this context, it is emphasized that the consumption of only 1 g of these formulations is enough to attend the daily intake requirements of products with probiotic claim, according to Brazilian law.

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