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Kefir: composition and evaluation of *in situ* antagonistic activity against *Staphylococcus aureus* and *Escherichia coli.*[1]

Kefir: composição e avaliação da atividade antagonista *in loco* frente a *Staphylococcus aureus* e *Escherichia coli.*

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ABSTRACT - The aim of this study was to investigate whether produced kefir meets the identity and quality standards for fermented milks, to check the possibility of assigning a nutrition declaration, and to evaluate the antagonistic activity of the fermented milk against *Staphylococcus aureus* and *Escherichia coli*. Two different formulations of kefir (Kefir 1 and Kefir 2) were prepared to determine the percentage composition, minerals, pH, total lactic acid bacteria, and antagonistic activity against *Staphylococcus aureus* and *Escherichia coli*. The results of the physicochemical evaluation indicated a statistically significant difference between the formulations, except for the percentage of lipids, Ca, K, Mg and Na. The formulations met the parameters of identity and quality in the fermented milks under evaluation. Possible nutrition declarations for Kefir 1 are 'source of proteins' and 'reduced calorie', and for Kefir 2, 'high protein content' and 'high zinc content'. The fermented milks showed significant antagonistic activity against the tested microorganisms (> 24 h), with no activity seen after this period. Further studies involving kefir are suggested, exploring its potential as a probiotic food, and its inclusion in the diet of the population.

Key words: Physicochemical characteristics. Probiotic potential. Fermented dairy product.

RESUMO - O objetivo do trabalho foi averiguar se o kefir produzido atende os padrões de identidade e qualidade de leites fermentados, verificar a possibilidade de atribuição de "declaração de propriedade nutricional" e avaliar a atividade antagonista do leite fermentado frente a *Staphylococcus aureus* e a *Escherichia coli*. Foram elaboradas duas formulações distintas de kefir (Kefir 1 e 2), onde foram determinadas a composição centesimal, minerais, pH, contagem de bactérias láticas totais e a atividade antagonista frente a *Staphylococcus aureus* e *Escherichia coli*. Os resultados da avaliação físico-química apontaram diferença estatística significativa entre as formulações, exceto para o percentual de lipídeos, Ca, K, Mg e Na. As formulações atenderam aos parâmetros de identidade e qualidade para leites fermentados avaliados. As alegações nutricionais possíveis para o Kefir 1 são "fonte de proteínas", "reduzido em calorias" e do Kefir 2, "alto conteúdo de proteínas" e "alto conteúdo de zinco". Os leites fermentados apresentaram atividade antagonista significativa frente aos micro-organismos testados (> 24 h), não sendo observada atividade após esse período. Mais estudos envolvendo o kefir são sugeridos, explorando suas potencialidades como alimento probiótico e a inserção na dieta da população.

Palavras-chave: Características físico-químicas. Potencial probiótico. Produto lácteo fermentado.

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INTRODUCTION

There are several types of fermented milk, such as yogurt, fermented or cultured milk, acidophilus milk, koumiss, curd and kefir, and their main distinguishing feature is the type of microorganism used in fermentation. Quality raw materials should be used in their production, and intrinsic and extrinsic factors related to the proper development of the microbial culture should be respected, in particular the composition of the medium, the temperature and the presence of oxygen (SAAD; CRUZ; FARIA, 2011).

Kefir is a fermented milk, the result of a complex and intriguing biological system, produced from kefir grains that display a symbiotic association of yeasts, lactic acid bacteria and acetic bacteria, surrounded by a gelatinous matrix referred to as 'kefiran'. Kefir production on an industrial scale takes place in countries such as Ireland, Turkey and Spain, where the main obstacle to an increase in production is the difficulty in standardizing the product due to the variable composition of the kefir grains. However, this type of fermented milk is widely consumed throughout the world, due to the homemade and traditional preparations that are associated with the functional properties accorded the food (MACHADO *et al*., 2012; MAGALHÃES *et al*., 2011; NAMBOU *et al*., 2014; WESCHENFELDER; CARVALHO; WIEST, 2010).

These properties have been extensively studied, and their effects on promoting health may be related to the biological activity of the microorganisms present in the grains, and to the metabolites generated during the fermentation process, such as hydrogen peroxide, organic acids, diacetyl and bacteriocins, which show antagonistic activity against several pathogens and deteriorating microorganisms (GARROTE; ABRAHAM; ANTONI, 2000; MAGALHÃES *et al.*, 2011; OELSCHLAEGER, 2010). Foods like kefir are also an excellent source of nutrients, with proteins of high biological value, vitamins and minerals, one of the most important of which is calcium (ANTUNES *et al*., 2007a, 2013a; MAGALHÃES *et al*., 2011; WESCHENFELDER; WIEST; CARVALHO, 2009).

Kefir displays antimicrobial activity against pathogenic bacteria of interest in food. A large part of the studies to evaluate its behavior against pathogens isolate the microorganisms present in kefir grains or fermented milk, or even sterilize the food, with most of the tests being carried out *in vitro* (LEITE *et al*., 2013a; RIBEIRO, 2015; SANTOS *et al*., 2013; WENDLING; WESCHENFELDER, 2013; WESCHENFELDER; WIEST; CARVALHO, 2009). Consequently, the aim of this study was to analyze whether prepared kefir meets the principal parameters of identity and quality (established for fermented milks), to check whether a nutrition declaration can be assigned to the food, and to evaluate the *in loco* antagonistic activity of kefir-fermented milk formulations against the microorganisms of interest in food.

MATERIAL AND METHODS

Two formulations of kefir-fermented milk (Kefir 1 and Kefir 2) were prepared. The first from pasteurized whole milk and kefir grains (obtained from the Food Hygiene Laboratory of the Federal University of Rio Grande do Sul, Brazil) and the second, from pasteurized milk, powdered skimmed milk (12%) and kefir grains. The kefir grains were weighed (50g) and added to 500g of milk (5 °C ± 2 °C) in a sterilized glass container (ratio of 1:10), incubated in an aerobic medium in a BOD Incubation Chamber (model SP-500, SPLABOR) for 24 hours at 25 °C ± 2 °C and subsequently maintained at 5 °C ± 2 °C for a further 24 hours (second fermentation) (SANTOS *et al*., 2012; WESCHENFELDER *et al.*, 2011).

After fermentation, the fermented milk was separated from the kefir grains with the aid of a sterilized 12-mesh stainless steel sieve, to obtain the kefir formulations. The kefir grains retained in the sieve were again inoculated into another aliquot of the substrate (milk), repeating the above steps of the experiment. The kefir was stored in glass containers, each containing 180g, labelled and kept under refrigeration for 18 days (5 ºC ± 2 ºC). The microbiological and physicochemical tests were then immediately carried out.

Crude protein content was determined by Kjeldahl method. The moisture was analyzed by vacuum oven desiccation at 85 ºC, and the minerals or ashes were determined by incineration of the samples in a muffle furnace at 550 ºC. For the lipid fraction, the Gerber method was used for the pasteurized-milk samples, and ether-based Soxhlet extraction for the powdered milk and kefir; the pH of the fermented milk was also determined by pH meter (MP220, Mettler Toledo). The analyses were carried out following the protocols in Normative Instruction No. 68 of 12 December 2006 (BRAZIL, 2006). The value for total carbohydrates was determined by difference, where the value is equal to 100 - (% moisture + % proteins +% lipids + % ash). Calcium, sodium, magnesium, potassium and zinc were determined by flame atomic absorption spectrometry, where samples of the dairy products had previously been decomposed in a heated open block-digester (130 °C) using 10 mL of concentrated nitric acid (PEREIRA JUNIOR *et al*., 2009).

A count of the total lactic acid bacteria in the fermented milk (Kefir 1 and 2) was taken over 0, 2, 4, 7, 9, 11, 14, 16 and 18 days of storage at 5 ºC ± 2 ºC (BOD incubation chamber), with the pH monitored at the same time. Decimal dilutions of the fermented milk were carried out (BRAZIL, 2003), and then pour-plated to Man, Rogosa and Sharpe agar medium (MRS) and incubated at 36 ºC for 72 hours in an anaerobic medium.

The bacterial strains used to evaluate the *in situ* antagonistic activity consisted of two standard microorganisms of interest in food products, *Staphylococcus aureus* (ATCC 25923) and *Escherichia coli* (ATCC 11229). The bacterial inocula were reactivated in BHI (Brain Heart Infusion, OXOID) culture medium at 37°C for 24 hours in an aerobic medium, carrying out serial dilutions (up to 10^{-8}). To check the initial concentration of the inoculum under study, 0.l mL of the 10^{-6} and 10^{-7} dilutions were transferred to Petri dishes containing Baird-Parker Agar selective culture medium for *Staphylococcus aureus* (ATCC 25923) and Chromocult agar for *Escherichia coli* (ATCC 11229) and a count taken after 24 and 48 hours aerobic incubation at 37 °C.

Subsequently, five different population densities of *Staphylococcus aureus* (ATCC 25923), referred to in the study as population densities A, B, C, D and E (where A = highest tested population density and E = lowest tested population density), were incorporated into Formulations 1 and 2 of the fermented kefir milk at a ratio of 20:180. The contaminated formulations were maintained in a BOD incubation chamber at 5 ºC ± 2 ºC, and analyzed after 0 (equal to the initial population density), 24, 48 and 72 hours of storage, as per a technique adapted from Normative Ruling No. 62 of August 2003 (BRAZIL, 2003). The same procedure was carried out using five different population densities of *Escherichia coli* (ATCC 11229). Evaluation of *in situ* antagonistic activity against *Staphylococcus aureus* and *Escherichia coli* was based on counts of typical colonies of both standard inocula in the selective media. At the same time, blank samples of the different formulations of fermented kefir milk were submitted to microbiological analysis to confirm their initial innocuousness in relation to the bacteria under test.

All the analyses were carried out in triplicate, repeating each experiment three times. The results of the physicochemical analysis and total lactic acid bacteria counts were compared with the parameters of identity and quality established for fermented milks (BRAZIL, 2007). The results were also used to determine the caloric value and recommended daily intake according to the Collegiate Board of Directors Resolution No. 359/2003 (ANVISA, 2003a), 360/2003 (ANVISA, 2003b), 269/2005 (ANVISA, 2005) of the National Agency for Sanitary Surveillance (*Agência Nacional de Vigilância Sanitária*). RDC No. 54/2012 (ANVISA, 2012) was used to evaluate the nutrition declaration of the prepared fermented kefir milk formulations.

The data were submitted to analysis of variance (ANOVA) and Tukey's test ($p<0.05$) to compare mean values, using the SAS 9.0 software. The results were used to characterize and compare the formulations of fermented kefir milk and to verify the *in situ* antagonistic activity against microorganisms of interest in the tested foods, considering the different population densities and the different exposure times of the bacteria to Formulations 1 and 2 of the fermented kefir milk.

RESULTS AND DISCUSSION

The physicochemical, microbiological and sensory characteristics of the raw materials are fundamental for the production of quality dairy products, especially fermented milks, since they directly influence the fermentation process. The results of the analysis of the pasteurized and powdered milks used to produce the kefir are shown in Table 1.

The kefir formulations did not differ in relation to the percentage of lipids, since the powdered milk used in Formulation 2 was skimmed, but presented a statistically significant difference in relation to the other constituents of the percentage composition (Table 2). Both kefir formulations had more than 2.9% protein, meeting the parameter established by Brazilian legislation (a minimum of 2.9% milk protein) (BRAZIL, 2007), and can be classified as 'partially skimmed', which is the

Table 1 - Percentage and mineral composition (mg%) of the pasteurized and powdered milks used to prepare the fermented kefir milk

	Moisture	Proteins	Lipids	Carbohydrates	Minerals	Ca	K	Mg	Na	Zn
Milk	87.81 ± 0.03	3.70 ± 0.01	3.00 ± 0.10	4.65 ± 0.18	0.85 ± 0.29	72.42 ± 25.47	114.76 ± 27.38	23.92 ± 19.20	26.73 ± 5.54	0.52 ± 0.17
Powdered milk	1.81 ± 0.27	33.57 ± 0.49	0.68 ± 0.08	55.63 ± 0.94	8.31 ± 0.58	992.0 ± 58.19	1357.33 ± 29.74	73.67 ± 5.69	243.00 ± 21.28	4.73 ± 0.32

Table 2 - Percentage and mineral composition (mg%) of two formulations of fermented kefir milk

	Moisture	Proteins	Lipids	Carbohydrates	Minerals	Ca	K	Mg	Na	Zn
Kefir 1	88.01 ± 0.06 a	2.93 ± 0.12 a	2.64 ± 0.24 a	5.53 ± 0.11 a	0.89 ± 0.11 a	49.73 ± 1.20 a	112.00 ± 0.15 a	8.57 ± 0.33 a	22.60 ± 0.10 a	0.48 ± 0.02 a
Kefir 2	79.01 ± 0.07 b	6.94 ± 0.95 b	2.48 ± 0.22 a	9.96 ± 1.19 b	1.61 ± 0.20 b	66.00 ± 2.00 a	155.00 ± 3.23 a	11.00 ± 1.00 a	27.00 ± 1.00 a	2.10 ± 0.10 b

Similar lowercase letters in the same column indicate that there was no statistically significant difference ($p<0.05$) by Tukey's test

classification given when the fermented milk has from 0.6 to 2.9% lipids (BRAZIL, 2007).

As it is a food of animal origin, it is important to note that the intake of a portion of the prepared fermented milks (200g) corresponds to 8% and 18% of the RDI (recommended daily intake) for proteins in Formulations 1 and 2 respectively (ANVISA, 2003a, 2003b). Formulation 1 can therefore receive the attribute 'source of proteins', and Formulation 2, 'high protein content' (ANVISA, 2003b, 2012). The kefir prepared by Magalhães *et al*. (2011) from grains of family origin after 24 hours fermentation, can also receive the attribute 'source of proteins', highlighting the importance, from the nutritional point of view, of fermented kefir milk in the diet of the population.

The consumption of one portion of Kefir 1 corresponds to 115.2 Kcal or 5.8% of the RDI, and Kefir 2, to 179.44 Kcal or 8.9% of the recommended daily value, based on a diet of 2000 Kcal (ANVISA, 2003b); the attribute 'reduced calorie' or 'light' can be used for Formulation 1 when compared to Formulation 2 (ANVISA, 2012). This difference can be explained by the use of powdered milk in Formulation 2. Thus, dairy products produced with a high level of solids, give a higher energy value and contribute with aspects related to the texture, syneresis and sensoriality of the final product (LIMA; ALMEIDA; GIGANTE, 2006).

Determining the composition of minerals present in fermented milk is fundamental to evaluating the nutritional impact of the food (TURKER; KIZILKAYA; CEVIK, 2013). Considering the results, the two formulations of kefir can receive the attribute 'low sodium content', since they had less than 80mg of sodium, based on a portion of 200g (ANVISA, 2003a; ANVISA 2012). Whereas for the calcium content, neither of the formulations can receive the attribute 'source of calcium', as neither reached the minimum of 15% of the RDI of the mineral per portion, which for adults is 1000 mg, according to RDC No. 269/2005 (ANVISA, 2005). The same evaluation applies to magnesium, where Formulation 2 (which had a larger amount of the mineral), reached 8.5% of the RDI per portion. For zinc, Formulation 2 can receive the attribute 'high zinc content', since it had 60% of the RDI for adults for this mineral (ANVISA, 2005, 2012). Zinc is considered a fundamental element for the processes of cell growth, differentiation and division, with an effect on taste and appetite. It should also be emphasized that the recommended daily intake of each mineral depends on factors such as age, sex and nutritional status, as well as the bioavailability of the mineral.

Turker, Kizilkaya and Cevik (2013), when evaluating the mineral composition of kefir produced with both cow's milk and goat's milk, found higher values for minerals when compared to the present study; the kefir produced with goat's milk presenting greater amounts of calcium, phosphorus, potassium, sodium and magnesium, and the kefir produced with cow's milk presenting greater amounts of copper, iron and zinc. It should be noted that the cattle feed, number of lactations, time of year and industrial processing influence the mineral composition of the raw material used to prepare the fermented milks.

The addition of powdered milk to Formulation 2 did not influence the pH stability of the kefir formulations during storage, the same occurred with Formulation 1 (with no added powdered milk), where pH stability was also maintained throughout storage (Table 3).

Incorporating more substrate to the raw material to be fermented can effect an increase in the buffering capacity of the food, delaying the fall in pH and preventing significant changes during storage of the fermented milks. This change in the intrinsic factor of the food has a positive influence on the survival of microbial cultures

Table 3 - Mean values for pH in two formulations of fermented kefir milk evaluated over 18 days storage at 5 °C ± 2 °C

Time (days)	0	2	4	7	9	11	14	16	18
Kefir 1	3.97 ± 0.24 a	3.98 ± 0.24 a	4.00 ± 0.14 a	4.13 ± 0.18 a	4.07 ± 0.10 a	4.26 ± 0.01 a	4.31 ± 0.01 a	4.29 ± 0.01 a	4.27 ± 0.04 a
Kefir 2	4.58 ± 0.08 a	4.50 ± 0.21 a	4.42 ± 0.05 a	4.42 ± 0.05 a	4.35 ± 0.01 a	4.37 ± 0.05 a	4.30 ± 0.21 a	4.32 ± 0.47 a	4.28 ± 0.18 a

Similar lowercase letters on the same line indicate that there was no statistically significant difference ($p<0.05$) by Tukey's test

throughout storage, including probiotics, in addition to making the product more palatable (CUNHA *et al*., 2008; RANADHEERA; BAINES; ADAMS, 2009).

The total lactic acid bacteria count of the two kefir formulations (Table 4), although showing a statistically significant difference during storage, maintained the minimum value recommended for fermented kefir milks, which is 10^7 CFU/g (BRAZIL, 2007). The higher values in Formulation 2 can be explained by the chemical composition of the substrate used in the fermentation, and by the less acidic pH of Formulation 2 during the first hours of storage (Table 3). Kefir grains present a very large diversity of microorganisms such as yeasts, lactic acid bacteria and acetic bacteria, which influences the microbiological composition of the fermented milk (LEITE *et al*., 2013b; MAGALHÃES *et al*., 2011; NAMBOU *et al*., 2014). Despite being produced with kefir grains from different sources, total lactic acid bacteria counts in kefir are generally greater than 10^7 CFU/g (ANSELMO *et al*., 2010; RIBEIRO, 2015).

Neither Kefir 1 nor Kefir 2 displayed growth in *Escherichia coli* or *Staphylococcus aureus* in the blank samples, demonstrating the high quality of the raw material and manufacturing process of the fermented milk. For antagonistic activity, it was seen that kefir Formulations 1 and 2 presented statistically similar behavior when faced with different population densities of *Staphylococcus aureus* (ATCC 25923), (except Kefir 2 - population density E - 72 hours) and *Escherichia coli* (ATCC 11229), as can be seen in Tables 5 and 6.

Taking into account that population density E for *Staphylococcus aureus* and *Escherichia coli* was 10^4 CFU/g (time 0), it can be said that the *Escherichia coli* was more sensitive than the *Staphylococcus aureus* (ATCC 25923), since the concentration after 24 hours was $3.23x10^2$ CFU/g and $1.47x10^3$ CFU/g respectively (Kefir 1). This behavior was also seen in Formulation 2, at population densities D and E.

Santos *et al*. (2013) tested the inhibition capacity of kefir produced from three artisanal preparations of kefir grains, and found a reduction of at least 30% against *Staphylococcus aureus* (ATCC 6538), *Escherichia coli* (ATCC 11229), *Salmonella typhi* (ATCC 6539), *Listeria monocytogenes* (ATCC 15313) e *Bacillus cereus* (RIBO 1222-173-S4), with the greatest inhibition against *Bacillus cereus*. Under the conditions of the experiment, the pH of 6.05 was not responsible for the inhibition, and it is suggested by the authors that other substances present

Table 4 - Total lactic acid bacteria counts in two formulations of fermented kefir milk evaluated over 18 days storage at 5 ºC ± 2 ºC expressed in CFU/g

Time (days)	0	2	4	7	9	11	14	16	18
Kefir 1	2.3×10^8 a	4.4×10^7 b	6.4×10^7 ab	4.7×10^7 b	3.1×10^7 b	7.5×10^7 ab	9.1×10^7 ab	4.1×10^7 b	5.1×10^7 b
Kefir 2	5.2×10^8 a	4.8×10^8 a	4.2×10^8 ab	3.2×10^8 b	5.9×10^7 c	7.8×10^7 c	9.2×10^7 c	5.6×10^7 c	5.6×10^7 c

Similar lowercase letters on the same line indicate that there was no statistically significant difference ($p<0.05$) by Tukey's test

Table 5 - *Staphylococcus aureus* counts (ATCC 25923) after *in situ* confrontation with two formulations of fermented kefir milk, expressed in CFU/g

	Time (hours)	Different population densities of Staphylococcus aureus (ATCC 25923) (A > B > C > D > E)				
		A	B	C	D	E
Kefir 1	0	3.90×10^8 a	3.90×10^7 a	3.90×10^6 a	3.90×10^5 a	3.90×10^4 a
	24	1.83×10^6 b	1.73×10^5 b	2.20×10^4 b	2.97×10^3 b	1.47×10^3 b
	48	1.37×10^6 b	3.40×10^5 b	5.10×10^4 b	5.00×10^3 b	5.67×10^2 b
	72	3.57×10^6 b	3.33×10^5 b	2.60×10^4 b	5.43×10^3 b	4.40×10^2 b
Kefir 2	0	3.90×10^8 a	3.90×10^7 a	3.90×10^6 a	3.90×10^5 a	3.90×10^4 a
	24	2.57×10^6 b	2.20×10^5 b	4.50×10^4 b	2.80×10^4 b	5.23×10^3 b
	48	4.57×10^6 b	1.57×10^5 b	5.53×10^4 b	1.27×10^4 b	1.47×10^3 bc
	72	1.10×10^6 b	5.63×10^5 b	5.37×10^4 b	2.43×10^4 b	4.20×10^2 c

Similar lowercase letters in the same column (considering each kefir formulation separately) indicate that there was no statistically significant difference ($p<0.05$) by Tukey's test; Log-transformation was applied to population densities for statistical analysis

Table 6 - *Escherichia coli* counts (ATCC 11229) after *in situ* comparison with two formulations of fermented kefir milk, expressed in CFU/g

	Time (hours)	Different population densities of Escherichia coli (ATCC 11229) (A > B > C > D > E)				
		A	B	C	D	E
Kefir 1	0	4.47×10^8 a	4.47×10^7 a	4.47×10^6 a	4.47×10^5 a	4.47×10^4 a
	24	2.13×10^6 b	2.63×10^5 b	5.67×10^4 b	3.83×10^3 b	3.23×10^2 b
	48	1.77×10^6 b	1.50×10^5 b	3.47×10^4 b	3.83×10^3 b	2.00×10^2 b
	72	1.70×10^6 b	2.17×10^5 b	1.27×10^4 b	4.33×10^3 b	1.93×10^2 b
Kefir 2	0	4.47×10^8 a	4.47×10^7 a	4.47×10^6 a	4.47×10^5 a	4.47×10^4 a
	24	2.60×10^6 b	2.80×10^5 b	3.67×10^4 b	4.37×10^3 b	4.40×10^2 b
	48	1.53×10^6 b	2.40×10^5 b	2.80×10^4 b	3.53×10^3 b	3.37×10^2 b
	72	1.40×10^6 b	1.20×10^5 b	2.60×10^4 b	1.40×10^3 b	1.83×10^2 b

Similar lowercase letters in the same column (considering each kefir formulation separately) indicate that there was no statistically significant difference ($p<0.05$) by Tukey's test; Log-transformation was applied to population densities for statistical analysis

in the fermented milk, such as hydrogen peroxide and bacteriocins, may be associated with the verified inhibition capacity. The diverse microbiota present in kefir grains may also have influenced the inhibition capacity of the fermented milk against the pathogens.

In the study by Anselmo *et al*. (2010), strains of *Bacilus cereus* and *Clostridium perfringes* were inoculated (10^6 CFU/g) into kefir of Italian and Peruvian origin, and kept at 4 °C for 30 days. Under the conditions of that experiment, it took at least 12 days for total reduction of the confronted microbial load, *Clostridium perfringes* being the most sensitive. The pH of the fermented milks remained between 3.6 and 4.1, showing that pathogenic bacteria can survive for a significant time at a low pH and at refrigerated temperatures.

In the present study, antagonistic activity was significant during 0 to 24 hours exposure; however, total reduction of the inoculated microbial load was not seen. From 24 to 72 hours there was no significant reduction, with the number of CFU/g of pathogenic bacteria seen to stabilize in the two kefir formulations, irrespective of the confronted population density.

Considering Table 5, the antagonistic activity of kefir Formulations 1 and 2 can be considered a protection factor when confronted with population densities C (3.90×10^6 CFU/g) and D (3.90×10^5 CFU / g) of *Staphylococcus aureus* from 0 to 24 hours, since at these pathogen concentrations, the fermented milk was able to reduce the concentration to values below 10^5 CFU/g (the amount necessary for toxin production).

Dias *et al*. (2012) contaminated milk samples with *Escherichia coli* O157:H7, *Salmonella* Typhimurium and Enteritidis, *Staphylococcus aureus* and *Listeria monocytogenes*, subsequently producing kefir from the contaminated raw material. An analysis was carried out after 0, 6, 12, 24, 48 and 72 hours fermentation; *Salmonella* Typhimurium and Enteritidis survived for 24 hours fermentation and the other confronted bacteria were still present after 72 hours fermentation.

Even though, for the above-mentioned authors, the concentration of the confronted *Staphylococcus aureus* was lower (10^3 CFU/g), the survival of the microorganism after 72 hours fermentation in the fermented milk reinforces the importance of quality raw materials and of hygiene when preparing fermented milks. This includes those foods where antimicrobial factors have already been found, since the initial microbial load of the pathogen is directly related to the final microbial load after confrontation with the fermented milk. Therefore, if the microorganisms under test were to contaminate kefir in a factory, for example, they might survive in sufficient number and for a long enough time to cause damage to health, with the initial microbial load being a determining factor.

CONCLUSION

The kefir formulations showed significant antagonistic activity against *Staphylococcus aureus* (ATCC 25923) and *Escherichia coli* (ATCC 11229) after 24 hours exposure, with no antagonistic activity seen between 24 and 72 hours confrontation. For the nutrition declaration, and considering a portion of 200g, Kefir 1 can be considered a 'source of proteins' and 'reduced calorie' or 'light', whereas Kefir 2 is a fermented milk with 'high protein content' and 'high zinc content', and appears to be an interesting food from the nutritional point of view.

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