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STUDY ON DYNAMICS OF MICROFLORA GROWTH IN PROBIOTIC RENNET CHEESE MODELS

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The production of probiotic cheeses is increasing. Probiotics cheeses always need the application of both traditional mesophilic starter cultures and probiotic strains of lactic acid bacteria (LAB). The manufacture of probiotic cheese only with probiotics is not possible because of their weak growth in milk and the lack of proteolytic activity. The aim of this work was to determine the dynamics of growth of LAB in four probiotic rennet cheese matrices depending on ripening temperature (6°C, 10°C, and 14°C for 8 weeks) and probiotic strains used (*Lb. casei* Defensis DN-114001, *Bif. animalis* subsp. *lactis* Bb-12, *Lb. acidophilus* La-5). The present work demonstrates that there are probiotic cultures capable to survive in cheese matrices at a level meeting the therapeutic minimum requirements. The number of lactococci was above 7 log CFU/g, aside from ripening temperature. It was not observed an inhibitory effect of probiotic strain on mesophilic starter culture, independently on the probiotic one used. The growth of *Lb. casei* strain Defensis DN-114001 was better at 14°C than at 6°C, whereas the population number of *Bif. animalis* subsp. *lactis* Bb-12 decreased during the ripening of cheese models, aside from the temperature. Probiotic strain *Lb. acidophilus* La-5 was characterised by much better viability than *Bif. animalis* subsp. *lactis* Bb-12. The reduction of pH value has been observed during the maturation of cheese samples.

INTRODUCTION

Various types of cheeses are produced using different lactic acid bacteria types and several stages according to principles that have been worked out by years of experimentation. Each type of cheeses has its specific production formula. Lactococci, lactobacilli and streptococci are among the starter cultures commonly used in cheesemaking. In general, lactic acid bacteria play an important role in the production and ripening of rennet cheese, contributing to their organoleptic, biochemical, and physical properties [Hui, 1993; Grattepanche *et al.,* 2008]. Lactic acid, carbon dioxide, diacetyl, aldehydes, ketones, alcohols, esters, and organic acids are produced by lactic acid bacteria (LAB) or their enzymes during the fermentation process and the ripening period [Dinakar & Mistry, 1994; Francis, 2000; Bamforth, 2005].

All kinds of cheese can also be an excellent carrier of some health-promoting (probiotic) strains of lactic acid bacteria such as *Lactobacillus* sp. and *Bifidobacterium* sp. [Stanton *et al.,* 1998; Ross *et al.,* 2002]. Probiotics are defined as living microorganisms, which upon ingestion in certain numbers have beneficial effects on human health beyond inherent general nutrition. Although probiotic strains of LAB are beneficial to human health, the manufacture of probiotic cheese should have minimum changes when compared to traditional products.

Ample researches indicate that cheese is a viable carrier for probiotic strains of LAB [Boylston *et al.,* 2004]. The viability of bacteria cells is one of the most important factors determining the health-promoting value of probiotic cheese. Probiotic strains of LAB have to survive during the cheesemaking, the ripening period of cheeses, as well as in the gastrointestinal tract after cheese consumption by humans, which involves passage through the stomach and small intestine. Consequently, probiotic cultures should possess the ability to tolerate conditions such as low temperature, high sodium chloride concentrate, low pH and bile, and be viable and healthy in the food product at the time of consumption [Gomes *et al.,* 1998; Daigle *et al.,* 1999; Vinderola *et al.,* 2000]. The sensitivity of some probiotics to acidity and low pH limits the viability of these bacteria. In case of probiotic cheese, probiotic strains of LAB should not only retain viability during the relatively long ripening time of 6-24 months, but also be capable of tolerating the conditions encountered in the GIT. Having a higher pH than the more traditional fermented foods, cheeses may provide a more stable milieu to support the long-term survival of probiotic organisms [Stanton *et al.*, 1998]. Additionally, the production of probiotic cheeses using only probiotic strains of LAB is not possible because of their weak growth in milk and the absence of proteolytic activity. Probiotic cheeses always need the application of traditional mesophilic starter cultures.

The aim of this work was to determine the dynamics of growth of lactic acid bacteria (LAB) in probiotic rennet cheese matrices depending on ripening temperature and probiotic strains used.

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MATERIALS AND METHODS

Bacterial strains and culture conditions

The probiotic *Lactobacillus* strains used in this study were obtained from Chr. Hansen Poland (*Lb. acidophilus* La-5) or were isolated from a commercial milk-based drink (*Lb. casei* Defensis DN-114001). The probiotic *Bifidobacterium animalis* subsp. *lactis* strain Bb-12 and a traditional cheese starter culture R-603 (consisting in *Lactococcus lactis* subsp. *lactis*) were also obtained from Chr. Hansen Poland. Starter cultures obtained from Chr. Hansen Poland were stored at -18°C and before the experiments were dissolved in sterilized milk. The biomass of *Lb. casei* Defensis DN-114001 was routinely cultured in MRS broth (Merck, Germany) at 37°C for 12-14 h and centrifuged (12,000×g, 7 min, 4°C) for culture broth removal.

Cheese manufacture

Laboratory-scale cheesemaking trials were performed with a liquid blended homogeneous concentrate consisting of non-fat milk powder (157 g/L of sterilized distilled water, 30%-fat cream (158 g/L), citric buffer (1.75 g/L), and NaCl (4 g/L). The concentrate was inoculated with commercial and probiotic strains cultures (5 mL of solution of 5 g of starter culture dissolved in 50 mL of sterilized milk), incubated (at 30°C/30 min), coagulated (Standard Premium 225, Chr. Hansen, 1 mL/L, 35 min after starter addition), incubated (at 31°C/40 min), than the curd was cut and cooked (at 36°C/25 min and next at 41°C/50 min), and submitted to different ripening conditions (at 6°C, 10°C, and 14°C for 8 weeks).

The experiments involved the production of four models of probiotic rennet cheeses under laboratory conditions differing in the probiotic strains applied. "A" model contained only the mesophilic starter culture R-603, "B" model contained the R-603 culture and a probiotic strain *Lb. casei* Defensis DN-114001, "C" model contained the R-603 culture and *Bifidobacterium animalis* subsp. *lactis* Bb-12, and "D" model contained the R-603 culture and a probiotic strain *Lb. acidophilus* La-5.

Microbiological analysis of cheeses

The number of LAB (lactococci, lactobacilli, and bifidobacteria separately) and pH value have been measured in raw cheese samples as well as after 2, 4, 6, and 8 weeks of ripening period. The number of lactococci was measured using a traditional plate method and M17 agar (Merck, Germany). Plates with inoculum have been incubated aerobically at 30°C/72 h. The number of lactobacilli or bifidobacteria was measured using MRS agar acidified to pH 4.8 (BioMerieux, France) and incubated anaerobically at 37°C/72 h [Shah, 2000; Tharmaraj & Shah, 2003; Phillips *et al.,* 2006; Antunes *et al.,* 2007].

Statistical assessment

Laboratory-scale cheesemaking was performed in two independent repetitions. The mean values of microbial counts were analysed statistically and compared using ANOVA and Tukey's test ($p<0.05$), (Statgraphics Centurion XV).

RESULTS AND DISCUSSION

Industrial cheesemaking involves the application of starter cultures containing *L. lactis* subsp. *lactis*, *L. lactis* subsp. *cremoris*, *Leuconostoc cremoris* and/or *L. lactis, Leuconostoc,* and *L. lactis* subsp. *diacetylactis* [Hui, 1993]. The amount and type of starter may vary significantly depending on the type of cheese and characteristics desired. The starter organisms reach about 9 log CFU/g in curd with a pH of about 5.7. In general, the population of mesophilic starter organisms may reach in excess of 8-9 log CFU/g in curd or cheese and does not vary during the ripening period of cheeses [Francis, 2000].

In the present work, cheese model "A" contained only mesophilic R-603 starter culture at the initial number 7.1--7.8 log CFU/g depending on the trial (Table 1). During eight weeks of ripening at 6°C, the population number of lactococci increased until 8.7 log CFU/g at the end of the 8th week. The quickest growth of lactococci has been observed at the beginning of ripening period, before the 2nd week of ripening. A similar dynamic rate of growth of lactococci has been found in cheese models "A" ripened at 10°C and 14°C. The statistical analysis confirmed that the applied parameters of temperature of cheese ripening did not influence the number of lactococci ($p=0.3099$). The initial pH values of cheese models "A" ripened at 6°C, 10°C, and 14°C were 6.50, 6.46, and 6.42 respectively. The changes of pH values in these cheese models were influenced by ripening temperature ($p=0.0001$) and time ($p=0.0001$). A stronger decrease of pH value has been observed in cheese models ripened at 10°C and 14°C than at 6°C (Table 1).

TABLE 1. Changes of microflora population and pH of cheese model A (means and standard deviations of 2 determinations).

Temperature of cheese ripening (°C)	Time of cheese ripening (days)				
	0	2	4	6	8
	Number of lactococci (log CFU/g)				
6	7.8±0.01	8.7±0.01	8.2±0.01	8.5±0.01	8.7±0.01
10	7.1±0.01	8.7±0.01	8.6±0.23	9.0±0.01	8.6±0.03
14	7.5±0.11	8.2±0.59	8.1±0.22	9.0±0.01	8.2±0.01
	Acidity, pH				
6	6.50±0.021	5.82±0.007	5.75±0.014	5.54±0.007	5.65±0.028
10	6.46±0.010	5.53±0.200	5.41±0.040	5.11±0.020	5.10±0.040
14	6.42±0.010	5.32±0.020	5.09±0.010	4.89±0.020	5.16±0.090

Table 2 shows the changes of microflora population and pH of cheese model "B" containing the R-603 culture and a probiotic strain *Lb. casei* Defensis DN-114001. The probiotic strain *Lb. casei* was growing better during the ripening at 14°C than at 6°C (the influence of temperature of ripening was statistically significant, p=0.0001). The initial number of *Lb. casei* was 7.6-7.8 log CFU/g (Table 2) and increased insignificantly during eight weeks of ripening at 14°C to 8.9 log CFU/g. In cheese model ripened at 6°C the population number of the probiotic strain *Lb. casei* Defensis DN-114001 decreased insignificantly to 7.2 log CFU/g. The reduction of pH value has been observed in all studied cheese samples "B". The greatest reduction in pH value was observed in "B" model (cheese with *Lb. casei* addition) ripened at 14°C, in which the pH value decreased from initial 6.4 to 4.5 after eight weeks of ripening (Table 2).

The population of *Bif. animalis* subsp. *lactis* Bb-12 decreased considerably during the ripening of cheese models aside from the temperature (Table 3, p=0.0001) and the increase of ripening temperature from 6°C to 14°C did not improve the viability of the probiotic strain studied (p=0.0773). After eight weeks of ripening the number of bifidobacteria was at a level of 5.9-6.3 log CFU/g. It meant that the applied probiotic strain of *Bif. animalis* subsp. *lactis* was not suitable for the production of probiotic cheese. The reduction of pH has been observed in all studied cheese samples "C" from the initial value of 6.37-6.40 to the final value of 4.91-5.46 (Table 3).

Probiotic strain *Lb. acidophilus* La-5 studied in the present work was characterized by much better viability than the probiotic strain of *Bif. animalis* subsp. *lactis*. Its initial population number was 7.3-7.5 log CFU/g, and during the whole ripening period it was decreasing insignificantly to 6.7-7.2 log CFU/g (Table 4). The population number of *Lb. acidophilus* La-5 did not depend on the applied ripening temperature nor time (p=0.2014 and p=0.1839, respectively). The reduction of pH value has been observed in all studied cheese samples "D", and the changes of pH values were influenced by ripening temperature (Table 4).

Recommendations for the minimum suggested level for probiotic strains of lactic acid bacteria in food to attain this viability are quite variable. In general, the food industry has applied the recommended level of 6.0 log CFU/g at the time of consumption for probiotic strains of *Lb. acidophilus* and other LAB [Boylston *et al.,* 2004]. Dinakar & Mistry [1994]

TABLE 2. Changes of microflora population and pH of cheese model B (means and standard deviations of 2 determinations).

Temperature of cheese ripening (°C)	Time of cheese ripening (days)				
	0	2	4	6	8
	Number of lactococci (log CFU/g)				
6	7.8±0.02	8.9±0.01	8.5±0.01	8.8±0.01	8.9±0.01
14	8.0±0.01	9.2±0.02	9.1±0.01	9.1±0.65	9.2±0.01
	Number of *Lb. casei* (log CFU/g)				
6	7.6±0.15	6.6±0.01	6.9±0.01	7.1±0.03	7.2±0.01
14	7.8±0.01	9.1±0.06	8.8±0.01	9.1±0.01	8.9±0.01
	Acidity, pH				
6	6.40±0.049	5.56±0.002	5.36±0.007	5.20±0.007	5.34±0.007
14	6.34±0.020	5.01±0.010	4.62±0.020	4.23±0.030	4.45±0.080

nd – not detected

TABLE 3. Changes of microflora population and pH of cheese model C (means and standard deviations of 2 determinations).

Temperature of cheese ripening (°C)	Time of cheese ripening (days)				
	0	2	4	6	8
	Number of lactococci (log CFU/g)				
6	7.9±0.04	8.7±0.01	8.7±0.01	8.7±0.03	8.8±0.04
10	7.1±0.01	8.2±0.08	6.5±0.01	9.1±0.01	8.7±0.01
14	7.6±0.01	8.7±0.01	8.9±0.12	8.5±0.01	8.2±0.01
	Number of *Bif. aminalis* subsp. *lactis* (log CFU/g)				
6	7.0±0.01	6.3±0.15	6.2±0.11	6.0±0.01	6.3±0.01
10	7.5±0.01	6.4±0.02	6.2±0.04	6.2±0.02	6.0±0.15
14	7.7±0.38	6.7±0.01	6.5±0.01	6.3±0.21	5.9±0.02
	Acidity, pH				
6	6.40±0.042	5.63±0.028	5.49±0.001	5.39±0.007	5.46±0.014
10	6.37±0.010	5.51±0.120	5.29±0.110	5.01±0.020	5.00±0.010
14	6.39±0.010	5.23±0.010	5.08±0.030	4.62±0.010	4.91±0.010

TABLE 4. Changes of microflora population and pH of cheese model D (means and standard deviations of 2 determinations).

Temperature of cheese ripening (°C)	Time of cheese ripening (days)				
	0	2	4	6	8
	Number of lactococci (log CFU/g)				
6	8.1±0.02	8.9±0.01	8.7±0.01	9.2±0.03	9.0±0.01
10	7.4±0.01	7.8±0.01	7.8±0.01	7.7±0.26	7.9±0.01
14	7.5±0.03	8.7±0.01	8.8±0.01	8.6±0.01	8.3±0.01
	Number of *Lb. acidophilus* (log CFU/g)				
6	7.4±0.03	7.3±0.01	7.1±0.01	7.6±0.01	7.2±0.01
10	7.3±0.01	6.8±0.20	6.9±0.01	6.7±0.01	6.8±0.02
14	7.5±0.01	7.3±0.02	7.0±0.41	7.1±0.02	6.7±0.03
	Acidity, pH				
6	6.37±0.057	5.59±0.007	5.45±0.042	5.34±0.028	5.38±0.014
10	6.39±0.010	5.41±0.230	5.25±0.240	4.95±0.070	4.94±0.110
14	6.40±0.010	5.21±0.010	5.02±0.001	4.73±0.010	4.76±0.010

proved that bifidobacteria could remain viable and multiply their number in cheese during these 24 weeks. Bifidobacteria studied by Dinakar & Mistry [1994] did not affect the flavour intensity, texture, or appearance of the cheese compared with that of the control. Lactic acid content increased in cheeses during ripening, but differences between treatments were minor. Acetic acid and ethanol, common metabolites of bifidobacteria, were not detected during ripening [Dinakar & Mistry, 1994].

Gomes *et al.* [1995] studied the possibility of using a starter entirely composed of *Bifidobacterium* sp. strain Bo and *Lb. acidophilus* strain Ki for the manufacture of Gouda cheese. *Lb. acidophilus* strain Ki was able to grow and produce acid in the cheese. The population number of *Lb. acidophilus* strain Ki observed in the cheese was at a level of 8 log CFU/g (an increase of one log cycle or more), provided that the scalding temperature was 38°C. *Bifidobacterium* sp. strain Bo showed no growth under any of the conditions chosen. The numbers of *Bifidobacterium* sp. strain Bo present in the cheese were at a level of 9 log CFU/g. The acetic acid values found demonstrated that *Bifidobacterium* sp. strain Bo actively contributed to the acidification of the cheese, a factor which probably also accounts for the significant difference in the sensory quality of the cheese. After 1 week, *Bifidobacterium* sp. strain Bo reached average levels of 9 log CFU/g. During the whole storage period studied, the average population number of *Lb. acidophilus* strain Ki decreased by two log cycles to 7 log CFU/g, whereas that of *Bifidobacterium* sp. strain Bo decreased by less than one log cycle to 8 log CFU/g.

Mc Brearty *et al.* [2001] studied the application of different strains of commercial probiotic bifidobacteria in Cheddar cheese production. Two Cheddar cheeses were manufactured at a pilot scale and inoculated with mesophilic cheese starters as well as with *Bif. lactis* Bb-12 strain (at a level of 7 log CFU/mL of cheesemilk) and *Bif. longum* BB-536 strain (at a level of 6 log CFU/mL of cheesemilk). The number of *Bif. lactis* increased to 8 log CFU/g after 1 day of ripening at 8°C, and maintained at this level throughout the next 6 months of ripening. While the number of *Bif. longum* reached the level of 6 log CFU/g after 1 day of ripening, and then was reduced to 5 log CFU/g, following six months of ripening.

Kasimoglu *et al.* [2004] investigated the survival of *Lb. acidophilus* during brining and ripening of Turkish white cheese. Two types of white cheeses, traditional cheese (made with only *L. lactis* subsp. *lactis* and *L. lactis* subsp. *cremoris*) and probiotic cheese (made with *L. lactis* subsp. *lactis, L. lactis* subsp. *cremoris* and *Lb. acidophilus* 593 N strain) were produced and ripened in a vacuum pack or in brine at 4°C for 90 days. *Lb. acidophilus* cells survived in probiotic cheeses to numbers over 7 log CFU/g. In opposite, Phillips *et al.* [2006] demonstrated that the viability of *Lb. acidophilus* strains in Cheddar cheese was not satisfactory. Two applied *Lb. acidophilus* strains grew poorly with their counts decreasing to 3 log CFU/g after 32 weeks. In comparison, the count of *Bifidobacterium* sp. remained at high numbers in cheese, *i.e.* at >7 log CFU/g after 32 weeks of ripening. Similarly, the counts of *Lb. casei* (>7 log CFU/g), *Lb. paracasei* (>7 log CFU/g), and *Lb. rhamnosus* (>8 log CFU/g) strains survived well.

Ong *et al.* [2006] and Ong & Shah [2008] studied the survival of probiotic bacteria in Cheddar cheeses manufactured with a combination of starter lactococci, *Lactobacillus acidophilus* 4962, *Lb. casei* 279, and *Bifidobacterium longum* 1941. All probiotic adjuncts survived the manufacturing process and maintained their viability of >7.5 log CFU/g at the end of ripening [Ong *et al*., 2006]. The counts of *Lb. acidophilus* in probiotic cheeses remained at >log 6 CFU/g after 24 weeks of ripening at 4, 8 or 12°C [Ong & Shah, 2008].

Summarizing, having a higher pH than the more traditional probiotic foods, rennet cheeses should provide a more stable milieu to support the long-term survival of probiotic organisms. The present work demonstrated that the application of an inadequate mixture of mesophilic starter cultures and probiotic strains, as well as improper ripening temperature could reduce the population of probiotic microflora to the level below the therapeutic minimum. The results obtained indicate that not all studied probiotic strains are suitable for the production of probiotic rennet cheese, and that the correct ripening temperature should be selected.

To perform health-promoting functions, the probiotic bacteria must not inhibit mesophilic starter culture and be viable at the time of consumption and maintain their viability throughout the gastrointestinal tract [Boylston *et al.,* 2004]. Vinderola *et al.* [2002] demonstrated many interactions among LAB starter and probiotic strains in probiotic dairy products. For example, *Lb. delbrueckii* subsp. *bulgaricus* strains inhibited *S. thermophilus* strains, whilst *Lb. acidophilus* strains were inhibited by *Lb. casei* or *Bifidobacterium* strains.

In the present work, the inhibitory effect of probiotic strains on the mesophilic starter culture has not been observed in all cheese models (Tables 2-4). Independently from ripening temperature (p=0.5819), the final number of lactococci in the 8th week of ripening was above 7 log CFU/g, and was many times above 9 log CFU/g. The changes of pH values were significantly influenced by ripening temperature and time (p=0.0001 both). With the highest ripening temperature, the lowest pH values were measured.

The results of the present work are consistent with findings of Ong *et al.* [2006], who studied the viability of starter lactococci and probiotic bacteria in probiotic Cheddar cheeses. The number of lactococci in probiotic cheeses decreased by 1-2 log cycles, but their count was not significantly different in comparison to control cheeses (produced with only starter lactococci).

Consumers are interested in buying probiotic cheese containing live probiotic cells but cheese producers need to acquire scientific information in order to choose the best growing starter cultures to their products. After the comparison of results obtained with those reported in scientific publications, the present work demonstrated that there were probiotic cultures capable to survive in cheese matrices at a level meeting the therapeutic minimum requirements.

CONCLUSIONS

1. The count of starter lactococci is not influenced by the application of *Bif. animalis* subsp. *lactis* strain Bb-12, *Lb. casei* Defensis DN-114001 or *Lb. acidophilus* La-5 culture.

2. *Bif. animalis* subsp. *lactis* strain Bb-12 culture is not suitable for the production of probiotic cheese ripened at the applied temperature.

3. Probiotic strain *Lb. casei* Defensis DN-114001 grows better in cheese ripening at 14°C than at 6°C.

4. Strain *Lb. acidophilus* La-5 is characterised by better viability than *Bif. animalis* subsp. *lactis* Bb-12 and its population number decreases at the applied ripening temperature.

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