Quantity of selected probiotic cultures in semi-hard cheese with low-cooking curd during the maturation process

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Abstract The work studies the survival of added selected probiotic bacteria *Lactobacillus acidophilus* (S1), *Lactobacillus casei* (S2), and *Lactobacillus plantarum*96 (S3) in semi-hard cheese with low-cooking curd during the maturation process. Cheeses were made according to the standard procedure (Polyfood SI 050 device). Probiotic lactobacilli strains *Lactobacillus acidophilus* (S1), *Lactobacillus casei* (S2), and *Lactobacillus plantarum*96 (S3) used in this study were added into the milk before the renneting process. The manufactured cheeses were matured for 6 months at the temperature of 10 °C. Cheese samples were taken for pH and titratable acidity measurements, lactobacilli enumeration, and chemical analysis at 30, 60, 90, 120, 150 and 180 days of maturation. At the end of the experiment (180 days) the cheese samples were analyzed also for the amount of lactic acid and protein contents. Initial numbers of lactobacilli inoculated into the milk (10^8 CFU mL⁻¹) decreased during the first 2 weeks of maturation and reached from $2.15 \cdot 10^7$ CFU g⁻¹ in S1 cheese to $4.32 \cdot 10^7$ CFU g⁻¹ in S3 cheese. The number of *Lactobacillus acidophilus* strain bacteria at the beginning of the maturation period was $2.47 \cdot 10^7$ CFU g⁻¹ and declined until day 120 of maturation to the number of $0.45 \cdot 10^6$ CFU g⁻¹. In the last

month of the experiment day 180 the viable cell numbers started to rise up to the final number of $0.41 \cdot 10^7$ CFU g⁻¹. The numbers of *Lactobacillus plantarum*96 varied around 10^8 CFU g⁻¹ during the whole period of the experiment. According to our results it was detected that in all experimental cheeses, the used probiotic lactobacilli reached the values above 10^6 CFU g⁻¹. Thus the legislated and therapeutic minimum limits set for the products containing probiotic bacteria for human diet were fulfilled.

Keywords Probiotic · Cheese · Technology · *Lactobacillus casei* · *Lactobacillus acidophilus* · *Lactobacillus plantarum*96

Introduction

Milk, dairy products and especially cheese play an important role in the diet of all population groups. Their daily consumption is recommended by health organizations and many authorities.

Cheeses produced in Slovakia are of a high quality and some of the cheese specialties that have a long lasting tradition here are also well known abroad (Herian 2005).

Cheese is characterized not only by the high content of essential nutrients such as proteins, fats, minerals, vitamins, etc. (Palo 2004), but it can also provide certain advantages as a carrier of probiotic microorganisms. However, one of the most important criteria of cheese evaluation as a probiotic food is whether the microorganisms are able to survive relatively long maturation periods taking at least 6 months.

The practical experiments have shown that to achieve any health effect 10^8 live probiotic bacteria must be consumed daily. That means that with a daily portion of 45 g (two slices) of cheese and the survival rate of 30 % there must be 6×10^6 of such bacteria in 1 g of cheese. That is the reason why there

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have been quite a lot of studies carried out focused on either probiotic bacteria survival in the process of cheese production or their effect on cheese quality.

The top priority of the contemporary cheese making industry in the world as well as in Slovakia is to provide healthy, safe and high-quality cheeses.

The objective of the present study was to produce semi-hard cheese with low-cooking curd with added *Lactobacillus acidophilus* (S1), *Lactobacillus casei* (S2) and *Lactobacillus plantarum*96 (S3), to study the survival of added bacteria during the maturation process, and to determine the effects on the chemical composition of the cheese.

Materials and methods

Semi-hard cheese with low-cooking curd production procedure Cheeses were made according to the standard procedure using the pre-set programme of the Polyfood SI 050 device (Italy). The cheeses were manufactured in the laboratories of the Institute of Microbiology and Gnotobiology, University of Veterinary Medicine and Pharmacy in Košice. Four hundred liters of antibiotic-free whole bovine milk were obtained from the Veterinary Medicine Research and Practical Farm of Košice University in Zemplínska Teplica. The milk was pasteurized at 75 °C for 30 s. After the milk was cooled to 40 °C, FD-DVS DCC-260 (CHr. Hansen, Denmark) starter culture containing *Lactococcus lactis* ssp. *cremoris*, *Lactococcus lactis* ssp. *lactis*, *Leuconostoc mesenteroides* ssp. *cremoris*, *Lactococcus lactis* ssp. *diacetylactis*, *Lactobacillus helveticus* and *Streptococcus thermophilus* (30 U/400 L), and CaCl₂ (40 mL/400 mL; 0.015–0.031 %) was added into the milk and fermented for 45 min. Then the milk was transferred to stainless-steel cheese vats, each containing 100 L of milk. The probiotic cheeses were inoculated individually with *Lactobacillus acidophilus* (LYOFAST LA 3) (S1), *Lactobacillus casei* (LYOFAST BGP 93) (S2) and *Lactobacillus plantarum*96 (BIOCENOL™LP96) (S3). Patented culture of *Lactobacillus plantarum*96 was isolated at the Institute of Microbiology and Gnotobiology, University of Veterinary Medicine and Pharmacy in Košice (S3). Cheese S4, without the addition of any probiotic culture, served as the control.

Opti-Ren 220 XL (Optiferm, Germany) containing *Rhizomucor miehei* was added to each cheese vat to a level sufficient to coagulate the milk in 45 min (20 mL 100 mL⁻¹). The coagulum was cut with a knife into small cubes (2.5×2.5×2.5 cm³) and the curds were low-cooked in whey for 5–40 min. Then the curds were transferred to plastic moulds (3.0 kg), and drained at 25 °C without pressure till to the next day. During the syneresis the cheeses were turned over at regular intervals every 4 h. Then the curds were labeled and left to dry for 28 h at 19 °C, and turned over again every 8 h. After 24 h the cheeses were salted (3 %). Then the cheeses were matured at the temperature

of 15 °C and were turned over every 8 h. The temperature was then adjusted to 10 °C. Next, the cheeses were coated with opti-cid P50 (Optiferm, Germany) containing *Streptomyces natalensis* and 2 weeks later the cheeses were waxed (Opti-Wax, Germany). The processed cheeses were matured for 6 months at the temperature of 10 °C.

Sampling Cheese samples were taken for pH and titratable acidity measurements, lactobacilli enumeration, and chemical analysis at 30, 60, 90, 120, 150 and 180 days of maturation. At the end of the experiment day 180 the cheese samples were also analyzed for the amount of lactic acid and protein content.

Microbiological analysis From each cheese group (S1, S2, S3, S4), 1 g cheese sample was transferred into a sterile bag under aseptic conditions and homogenized in 9 mL of sterile physiological solution for 3 min in homogenizer LT2 at 160 move/min. Serial dilutions were prepared by adding 1 to 9 mL sterile peptone water (0.1 g 100 mL⁻¹). Samples were tested for numbers of *Lactobacillus acidophilus*, *Lactobacillus casei* and *Lactobacillus plantarum*96 using standard methods (STN ISO 2006). The cheese samples for microbiological analysis were taken with sterile corkscrews from the center and peripheral parts of the cheese.

Compositional analysis Cheese samples were analyzed for fat content by Gerber's method, and sodium chloride (NaCl) content was analyzed using the Mohr method. Dry matter content was determined by drying 10 g of the samples at 105 °C until constant weight. Protein content was measured with a nitrogen analyzer (Kjeltec); a nitrogen to protein conversion factor of 6.38 was used. Titratable acidity was determined according to Soxhlet-Henkel and expressed as a °SH (Soxhlet-Henkel degrees). The pH of the cheese was measured by the pH meter (pH 340i/SET). Lactic acid content was measured with an isotachophoretic analyzer ZK-001 (Labeco, Slovakia). All analyses were performed in triplicate according to the standard methods suggested by AOAC (2005).

Statistical analysis The statistical method of processing and evaluation of the individual experiments results was used (MS Excel 2007). ANOVA parameter test and induction statistics methods pair *t*-test for testing means of related parameters. Correlation phi coefficient was used to assess the dependence of the relationship between the two nominal variables (SPSS, GaphPad Prism 5).

Results

The composition of the S1, S2, S3 and S4 cheeses (data are not shown) were within the range accepted for semi-hard

cheese with low-cooking curd for which dry matter is minimum 52–60 % (w/w), salt in dry matter is maximum 30 % (w/w), and fat in dry matter is 30–60 %.

There were no significant differences ($p < 0.05$) among experimental cheeses S1, S2, S3 and control (S4) for levels of dry matter, salt in dry matter and protein and lactic acid content. The content of lactic acid in cheeses of the S1 group increased during the maturation period up to $1.94 \text{ g } 100 \text{ g}^{-1}$ cheese. In the S2 group of experimental cheeses the final value of lactic acid was $1.57 \text{ g } 100 \text{ g}^{-1}$ cheese. The lowest value of lactic acid was in the S3 experimental group of cheeses ($1.46 \text{ g } 100 \text{ g}^{-1}$ containing *Lactobacillus plantarum*96). The mean value of the protein content at 180 days of the maturation process in each group of cheese samples was in 22.12 % (S1), 23.54 % (S2), and 21.73 % (S3, S4), respectively.

The numbers of lactobacilli used in this study of the probiotic cheeses during the experiment are shown in Table 1 and Figs. 1, 2, and 3.

Initial numbers of lactobacilli inoculated into the milk (10^8 CFU mL^{-1}) decreased during the first 2 weeks of maturation and reached from $2.15 \times 10^7 \text{ CFU g}^{-1}$ in S1 cheese to $4.32 \times 10^7 \text{ CFU g}^{-1}$ in S3 cheese. The viable cell numbers of S1 and S2 tested probiotic lactobacilli strains began to decrease after 30 days of maturation (Fig. 1).

The number of *Lactobacillus acidophilus* strain bacteria at the beginning of the maturation period was $2.47 \times 10^7 \text{ CFU g}^{-1}$ and declined until the 120 days of maturation to the number of $0.45 \times 10^6 \text{ CFU g}^{-1}$. On the 180 days the viable cell numbers started to rise up to the final number of $0.41 \times 10^7 \text{ CFU g}^{-1}$. The difference between the beginning and final number of *Lactobacillus acidophilus* was $2.06 \times 10^7 \text{ CFU g}^{-1}$ ($p < 0.0005$).

The viable cell numbers of *Lactobacillus casei* also began to decrease after the beginning of the maturation process, but then, from 120th day of maturation the *Lactobacillus casei* number increased until the end of the maturation period, and it did not decrease below 10^8 CFU g^{-1} in S2 cheeses. Similar growth of *Lactobacillus plantarum*96 in experimental cheese S3 was observed (Fig. 1). The numbers of *Lactobacillus plantarum*96 varied at 10^8 CFU g^{-1} during the whole period of the experiment.

Figure 2 shows the comparison of the individual probiotic lactobacilli growth in the central parts of the experimentally produced cheeses. The numbers of lactobacilli in the central part of the cheese samples during the maturation period decreased until 120 days, and from day 150 of the experiment lactobacilli started to grow rapidly ($p < 0.01$) until the mean values of $0.79 \times 10^8 \text{ CFU g}^{-1}$.

Comparing the peripheral parts of samples taken from experimentally produced cheeses, a statistically significant difference of $p < 0.05$ was found among the probiotic lactobacilli used.

Figure 3 shows that numbers of individual probiotic lactobacilli at the beginning of the maturation period was the same in comparison to the samples taken from the central part of the cheese. The numbers of individual lactobacilli strains in the peripheral part of the cheese decreased in 120–150 days, however, not below the level of 10^6 CFU g^{-1} . In the next maturation period the numbers of used lactobacilli strains moderately increased up to the mean value of $0.83 \times 10^7 \text{ CFU g}^{-1}$. The number of *Lactobacillus plantarum*96 and *Lactobacillus casei* strains grew rapidly from day 120 to the final mean value of $0.51 \times 10^8 \text{ CFU g}^{-1}$.

A statistical significance was found with a 95 % confidence interval ($p < 0.05$) between the central and peripheral parts of the experimentally produced cheeses with the selected probiotic cultures growth. Comparing the central and peripheral parts of cheese samples on day 180 of the experiment, statistical significance was found at the level of $p < 0.05$, whereas the numbers of the *Lactobacillus casei* strain compared to the *Lactobacillus acidophilus* and *Lactobacillus plantarum*96 strains were considerably higher ($p < 0.01$) in the central parts than in the peripheral parts of the experimentally produced cheese.

Discussion

Bacteria of dairy fermentation mainly of the *Lactobacillus* genus create, apart from the known substances, a lot of presently unidentified substances which are effective against

Table 1 The mean number of probiotic lactobacilli used in the experimentally manufactured semi-hard cheese with low-cooking curd during the maturation period and their correlation dependence

Probiotic bacteria	Logarithm of the population/CFU g ⁻¹						Correlation dependence
	Days of maturation process						
	30	60	90	120	150	180	
<i>Lactobacillus acidophilus</i>	7.39	6.91	6.59	5.65	5.51	6.61	0.0001**
<i>Lactobacillus casei</i>	7.67	7.38	7.47	7.00	7.17	7.71	0.0086*
<i>Lactobacillus plantarum</i> 96	6.51	7.38	7.32	7.07	7.07	7.39	0.0001**

CFU colony-forming unit

* $p = 0.01$, ** $p = 0.1$

Fig. 1 Comparison of the growth of probiotic lactobacilli in manufactured semi-hard cheese with low-cooking curd during the maturation period

harmful microorganisms. They have a protective influence in food storage which can be used clinically.

Start cultures are a part of useful microorganisms and their enzymes carry out important biochemical changes during the production process. These cultures are intentionally introduced into milk in the process of cheese production. Based on the research of Görner and Valík (2004) mesophilic bacterial cultures of the fermentation such as *Lactococcus lactis* spp. *lactis*, *Lactococcus lactis* spp. *cremoris* a *Leuconostoc mesenteroides* spp. *cremoris* are mainly used in the cheese production with low-cooking curd. In the cheese with the low-cooking curd, apart from the basic mesophilic culture, there are other bacterial strains of the dairy fermentation.

In order to use probiotic bacteria with proven health benefits in the manufacture of dairy products, sometimes the process has to be modified and adapted for the strains, due to their high sensitivity. According to Cruz et al. (2009) there are two options for the addition of probiotic bacteria during cheese processing which can directly affect the survival rate of these microorganisms: probiotic bacteria can be added before the fermentation (together with the starter culture), or after it.

As it is stated by Walstra et al. (1993) *Lactobacillus* spp. could be used as an additional culture or non-starter lactic acid bacteria. In all kinds and types of cheeses matured longer than 14 days various mesophilic lactobacilli grow and develop (*Lactobacillus plantarum*, *Lactobacillus brevis*, *Lactobacillus*

casei and others). Their numbers reach the values of 10^6 to 10^8 CFU g^{-1} of cheese (Albenzio et al. 2004).

The numbers of the *Lactobacillus acidophilus* cultures in the experimental cheese samples varied at the beginning of maturation at approximately 10^7 CFU g^{-1} and these counts remained stable up until the end of the maturation period (180 days). Naylor and Sharpe (1958) evaluated semi-hard cheese, where the count of the genus *Lactobacillus* in 1 g of cheese varied at approximately 10^0 – 10^4 after 10 days of storage. On the 20th day of storage the numbers of the lactobacilli increased up to the values of 10^6 – 10^7 g^{-1} and on the 60th day their counts reached the values of 10^7 – 10^8 for a gram of cheese.

Similarly, Johnston et al. (2002) states the beginning numbers of *Lactobacillus* bacteria in 1 mL of the cow milk at 10^4 – 10^6 and in pasteurized milk at 10^2 – 10^4 . These counts of *Lactobacillus* bacteria grew rapidly in the case of cheese produced from cow milk up to the values of 10^6 – 10^7 g^{-1} and in cheese made from pasteurized milk, the counts of *Lactobacillus* spp. reached the value of 10^5 – 10^6 g^{-1} .

In the case of the experimentally made cheese containing the *Lactobacillus casei* probiotic culture, the numbers at the beginning of maturation varied at approximately 10^7 CFU g^{-1} and from the 3rd month the values reached the level of 10^8 CFU g^{-1} . Naylor and Sharpe (1958) concluded that the basic maturing cheese microflora at the beginning of the maturation period

Fig. 2 Comparison of the growth of probiotic lactobacilli in the central part of manufactured semi-hard cheese with low-cooking curd during the maturation period

Fig. 3 Comparison of the growth of probiotic lactobacilli in the peripheral parts of manufactured semi-hard cheese with low-cooking curd during the maturation period

consists of homofermentative *Lactobacillus plantarum* and *Lactobacillus casei* cultures. However, during the maturation process (after 60 days of storing) the basic microflora of the cheese was partially replaced by heterofermentative kinds mainly *Lactobacillus brevis* which survived in cheese up until the end of the storage period (180 days).

Lactobacillus casei can be used as an additional probiotic culture, because it is acid resistant and survives in the matured cheese at high counts (Antonsson et al. 2002). These statements correlate with our findings that the *Lactobacillus casei* bacterial culture retains the values of 10^8 CFU g⁻¹. Testing the numbers of the *Lactobacillus casei* bacteria in Cheddar type of cheese, Fox and Mcsweeney (1998) stated that these microorganisms retained the values of 10^7 CFU g⁻¹ for a gram of cheese. This is also confirmed by Ong et al. (2006) who obtained *Lactobacillus casei* count in Cheddar cheese in the value of 10^9 CFU g⁻¹.

Ong et al. (2006) added combinations of *Lactobacillus acidophilus*, *L. casei* and *Bifidobacterium longum*; and *L. acidophilus*, *L. paracasei* and *B. lactis* to Cheddar cheese. In this case, cheese was produced following a standard procedure in which milk, after being standardized, was heat-treated to 31 °C before the inoculation of the cheese starter culture and probiotic bacteria. All probiotic adjuncts survived the manufacturing process and maintained their viability until the end of the ripening process.

The numbers of the *Lactobacillus plantarum*96 bacteria varied at the beginning of the maturation period at about 10^6 CFU g⁻¹. During 30 and 60 days of maturation process the count of *Lactobacillus plantarum*96 increased to 10^8 CFU g⁻¹. The similar results were recorded by Songisepp et al. (2004) in semi-hard probiotic cheese containing *Lactobacillus casei* and *Lactobacillus plantarum*. In the control group of cheeses as well as in the probiotic group of cheeses containing *Lactobacillus plantarum*, the count of bacteria varied at approximately 10^8 CFU g⁻¹, whereas in the cheese containing *Lactobacillus casei* the number of bacteria was mildly lower than 10^7 CFU g⁻¹. During the maturation process the count of both cultures used was stabilized at the value of 10^7 CFU g⁻¹ (after 66 days of storage).

Comparing the samples from the peripheral parts of cheese with the central samples of the individual probiotic pieces of cheese, the mean number of individual probiotic lactobacilli in the peripheral parts of cheese was higher at the beginning of the maturation process. We assume that the reason for this difference would be the irregular spread of the probiotic cultures in the matured cheese. It seems to us that the differences in the counts of the individual probiotic lactobacilli might have been caused by different requirements of the microorganisms based on the inner environmental conditions in the central and peripheral parts of the experimentally produced cheese which could have affected their reproduction.

Conclusions

According to our results, it was detected that in all experimental cheeses the used probiotic lactobacilli reached the values above 10^6 CFU g⁻¹. Thus the legislative and therapeutic minimum limits set for the products containing probiotic bacteria for human diet was fulfilled. The results of this experiment showed that probiotic lactobacilli strains survive in cheese during long maturation period (180 days). This results in increasing the quality and nutritional value of probiotic cheeses and should be attractive for consumers and producers of cheese.

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