RATE OF POTENTIALLY PATHOGENIC BACTERIA IN FRESH SOFT CHEESES MADE ON SMALL DAIRY FARMS IN SERBIA

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ABSTRACT

This study investigated the rate of potentially pathogenic bacteria in fresh soft cheeses made on small dairy farms in Serbia. This type of cheese is made on individual farms from cow, sheep and goat milk. White fresh cheeses from mountain villages of Serbia are economically important for these areas. The purpose of this study was to investigate the micro flora of white cheeses with special emphasis on the interaction between the potentially pathogenic bacteria and starter lactic acid bacteria involved in the fermentation of the cheeses depending on their geographical origin. The most frequently isolated pathogenic microorganisms were *Enterococci* (log count 7.95), *Micrococaceae* (log count 8.66), yeasts (log count 5.48) and moulds (log count 1.87). Moulds were more frequently present in samples taken during summer and winter. The most frequently isolated moulds were: *Penicilllium* spp. 35%, *Alternaria* spp. 11% and *Cladosporium* spp. 17% during summer, and *Penicillium* spp. 23% and *Rhizopus* spp. 15.4% during autumn. The most frequently isolated lactic acid bacteria were *Lactobacillus casei* ssp. *casei*.

Key words: *soft cheeses, farm manufactured, Serbia mountain.*

1. INTRODUCTION

Various kinds of fresh soft cheese are the most popular cheeses among consumers in Serbia. They are made on smallholders' dairy farms from cow, sheep and goat milk and consumed in fresh form. White fresh cheeses from mountain villages of Serbia are also economically important for these areas. The traditional method of production involves renneting, curd formation, fermentation and final preparation for markets. Due to the individual approach to manufacture, the technological and sensory properties of the final product differ from one farm to the other Parvathey Seema Nair, Puthuvallil Kumaran Surendran (2004)

The aim of this work was to investigate the interaction between the potentially pathogenic bacteria and the starter lactic acid bacteria and its impact on the quality of the final product in particular on the defect of the late fermentation.

From 1970 through 1990 in the USA, outbreaks of *Salmonella* (Luca Settani,Giancarlo Mochetti, 2010, Maria Cousta, Marios Mataragas,Panagiotis Skandamis, Eleftherios H.,Drosinos 2010, Weerkamp et al., 1996), *Listeria monocytogenes* (Sharpe, 1979; Samson and Van Reenen-Hoekstra, 1988;), *Escherichia coli* (Cristina M.B.S. Pintado, Maria A.S.S.Ferreira, Isabela Sousa 2010., Brooks J.C., Martinez B., Stratton J.,Bianchini A., Krokstrom,R.Hutkins 2012), Group C *Streptococcus* and *Brucella melitensis* infections (Sharpe, 1979;) were associated with the consumption of cheese. A few simple errors were identified in the outbreaks reviewed in these series: use of raw milk to prepare fresh or soft cheese, improper pasteurization of milk (Official Analytic Chemists, 1990, Axelson L.T 1993) and postpasteurisation contamination (Altekruse S.F, Timbo B.B, Mowbray J.C, Bean N.H, Potter M.E. 1998). Strict sanitary requirements for cheese manufacture codified by the FDA in 1950 addressed hazards identified in these outbreaks. These requirements include the use of pasteurised milk for the preparation of soft or fresh cheese and safe manufacturing practices in production and processing (Paswey M 1964, Altekruse S.F, Timbo B.B, Mowbray J.C, Bean N.H, Potter M.E.1998).

2. MATERIALS AND METHODS

Cheese manufacture and sampling

Whole or skimmed raw milk was coagulated at 28-32ºC by adding rennet. 1.5-2 hours later the resulting curd was cut into approximately equal parts (cubes 4-5 cm across) and left at room temperature (approximately 15-18ºC). The curd was then placed in perforated plastic or wooden molds, 12x12x7 cm in size which allowed the whey to drain. After 20 minutes dry salt was added on the surface of the curd, 4 kg of pressure was applied onto the molds and whey was allowed to drain for another 40-60 minutes. Ripening of the cheese was performed in well-aerated and relatively humid rooms at the temperature of around 18ºC for 8-10 hours. The cheese squares were left untouched for 10-15 minutes and then they were put in salt for 6-12 hours at 10-15^{0}C.

Fresh cheeses have about 80% relative humidity and 23-65% fat in semi solid matter. Farm manufactured cheeses are not standardized.

Carbohydrate fermentation patterns of the strains were determined using API 50 CH system (BioMerieux), while homophermentative coccal-shaped organisms of doubtful classification were tested using API 20 STREP system (BioMerieux) (Association of Official Analytic Chemists, 1990).
Fresh cheese samples were taken from the market and microbiological analyses were performed within the following 24 h (Axelson, 1993., Beuchat L.R 1992).

Isolation of pathogenic microorganisms, moulds and yeasts
Cheese samples were taken aseptically. 20 g of each sample was homogenised in 180 mL of 2% (w/v) sodium citrate solution. Subsequently, serial 10 fold dilutions were prepared from this homogenisate using 0.1% (w/v) pepton water. MRS agar(Merck), M17 agar (Merck) and Rogosa agar at the pH of 5.7 were used for the isolation of lactic acid bacteria. Slantez-Bartley agar (Merck) was used for *Enterococci*, Violet Red Bile agar (Merck) for coliforms and Baird Parker agar (Merck) for *Staphylococcus aureus.* The presence of these species was confirmed by the coagulase test.
YGC agar (Merck), Czapek, PDA and Sabouroud agar were used for moulds and yeasts identification and count (Cristina M.B.S. Pintado, Maria A.S.S.Ferreira, Isabela Sousa (2010).
For the isolation of moulds the following media was used: potato dextrosa agar (PDA) at pH 5.6 which was prepared as described in the bacteriological Analytical Manual and supplement with filter sterilized chloramphenicol and chlortetracycline immediately before use. Other media were also used: sabouroud dextrosa agar and Czapek agar prepared as described in the Official Method of Analysis. Three dilutions were made which were transferred to sterilized plates (1 mL per plate approximately) both from samples and from three peptone diluents. Triplicate Sabouroud agar pour plating were made of appropriate dilutions as the percentage of samples contaminated with mould are listed in Table 1. At the end of the specified incubation period, the plates were analysed for fungal population (CFU per gram). Growing colonies were first identified macroscopically and then microscopically. First, a particular mould was isolated in order to obtain a pure culture and then the color of the colony and changes in the medium surface texture (described as loose or compact, plane, wrinkled or buckled, velvety, matted, floccose, hairy, ropy, gelatinous, etc.), odor, any changes in the submerged hyphen and spores (color, shape, septation, size, etc.) were noted. Any moulds growing on the surface of the Sabouroud medium were transferred to a select medium, like PDA or Czapek agar, which were selective for a certain species of moulds. This information was used to place the species in the correct Class and Order, while further characteristics were used to determine the Family and then Genus and Species. Identification of moulds was carried out according to their micromorphological properties using mould determination keys (International Dairy Federation1996, Altekruse S.F, Timbo B.B, Mowbray J.C, Bean N.H, Potter M.E. 1998, Beuchat LR 1992 ,Booth C 1971).
pH values were tested in 167 samples of raw milk from cheese producing households compared to the chemical parametre of milk pH usin a pH-meter. Additionally, soluble nitrogen (% of total nitrogen) and non-protein nitrogen (% of total nitrogen), levels of NaCl and fat contents of the cheese samples were recorded.

3. RESULTS AND DISCUSSION

Pathogenic microorganisms were isolated from fresh cheeses prepared from unpasteurised milk and from samples of cheeses prepared from pasteurised milk. The results indicated that coliform bacteria and *E. coli* were isolated in fresh white cheeses manufactured from unpasterised milk (Austwick, 1975; Booth, 1971; Beuchat, 1992 Brooks J.C., Martinez B., Stratton J.,Bianchini A., Krokstrom,R.Hutkins 2012). *Staphylococcus aureus* numbers were below the threshold level of detection of 10 cfu/g^{-1}. The count of mould and yeasts were high in some samples. Yeast levels were found between 10^3 to 10^6 /g in various chesses (Luca Settani,Giancarlo Mochetti 2010)
The results of mean log counts of microbial groups in fresh cheese samples are presented in le 1.

Table 1. Mean log counts of microbial groups in Fresch cheeses samples

Microorganism	**Samples of Fresh white cheeses (log count)**
Coliform bacteria	8.33
Enterococci	7.95
Micrococacceae	8.66
Staphylococcus aureus	nf
Yeasts	5.48
Moulds	1.87

Isolation of lactic acid bacteria was carried out by picking of 7-9 colonies per cheese sample and per batch, from each of the M17, Rogosa and MRS agar plates. It was observed that lactobacilli, mainly *Lactobacillus casei* ssp. *casei* predominated in all of the cheese samples (Table 2) (48.27%). *Lactobacillus casei* ssp. *casei* is also one of the most frequently isolated lactid acid bacteria in other cheese varieties and was considered to play an important role in some kinds of cheeses throughout ripening as well as in fresh cheeses. *Lactobacillus plantarum* was found to be the second predominating *Lactobacillus species* (6.89%) as was observed in other cheeses varieties and was followed by *Lb. casei* ssp. *rhamnosus* (6.03%).

Some authors (Weerkamp et al., 1996; Altekruse et al., 1998) have shown that certain species of the genus *Enterococcus*, apart from having important caseinolitic and lipolytic effects, stimulate the production of acid by some lactococci and favor development and gas production by lactic acid bacteria.

It is important to point out that the Micrococcaceae family include certain microorganisms which could pose a potential danger to the health of the consumer (above all *Staphylococcus aureus* coagulasa positive producers of toxins). From this point of view, the low counts of Micrococcaceae are a cause of relief.

The mould and yeasts counts undergo an increase of log unit during coagulation and whey drainage and continue to increase to a maximum level in two week-old cheeses. We have not been able to find any reference which would allow us to compare our data to those of other similar cheeses. The metabolic activity of the yeasts which consume lactic acid results in a pH increase and decrease in titrable acidity. It is also necessary to note that in our case, the samples were taken from the inside of the cheeses and the yeasts and molds are most likely to appear on the surface even in those cheeses which are showing a very low superficial microbial growth.

Lactic acid bacteria isolated from this variety of cheeses seems to play a fundamental role in the development of their organoleptic characteristics. LAB has an additional role in the development of the organoleptic characteristics of this cheese.

The results of the spread and development of mould in fresh cheeses samples were presented in Table 3.

Table 3. Spread and development of mould in fresh soft cheese during the year in the mountain region of Serbia.

Genus of moulds	**Season of the year**			
	Winter %	**Spring %**	**Summer %**	**Autumn %**
Absidia	nf	nf	2,2	7,7
Alternaria	6,2	nf	11	7,7
Aspergillus	nf	nf	6,5	6,0
Cladosporium	28	nf	17	7,7
Fusarium	nf	55	4,4	7,7
Geotrichum	15	15	10	nf
Mucor	21	nf	11	7
Penicillium	28	30	35	23
Rhizopus	nf	nf	nf	15,4
Scopulariopsis	nf	nf	2,2	7,7
Trichoderma	nf	nf	nf	7,4

Legend: nf- not found

Most frequently isolated genus of moulds were: *Absidia, Alternaria, Aspergillus, Cladosporium, Fusarium, Geotrichum, Mucor, Penicillium, Rhizopus, Scopulariopsis* and *Trichoderma*. Most frequently isolated moulds in summer and winter were: in Summer *Penicilllium* spp. 35%, *Alternaria* spp. 11% and *Cladosporium* spp. 17% and in Autumn *Penicillium* spp. 23% and *Rhizopus* spp 15.4%.

The results of species of lactic acid bacteria isolated during the ripening period of fresh soft cheeses from Stara Planina were presented in Table 2.

Table 2. Species of lactic acid bacteria isolated during ripening period of fresh soft white cheeses from Stara Planina in Serbia

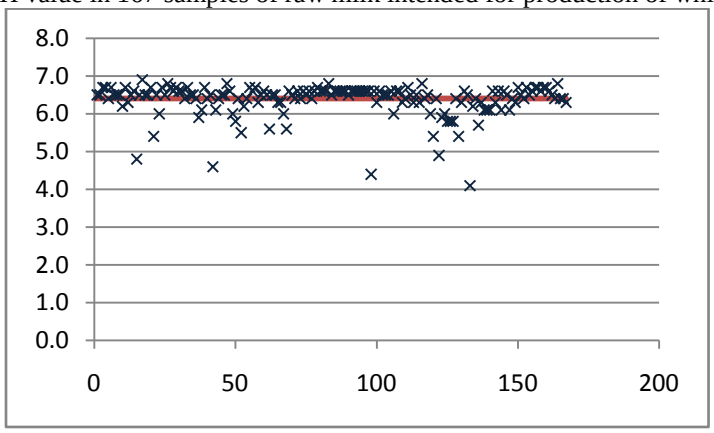
Species/genus	No. of isolates	%
Lactobacillus casei ssp. *casei*	**56**	**48,27**
Lb. plantarum	**8**	**6,89**
Lb. casei ssp. *rhamnosus*	**7**	**6,03**
Lb. lactis ssp. *lactis*	**15**	**12,93**
Lb. curvatus	**10**	**8,62**
Lb. coryniformis	**12**	**10,34**
Leuconostoc mesenteroides ssp. *dextranicum*	**5**	**4,31**
Enterococcus faecium	**3**	**2,58**
Total	116	

Identification of isolates was performed according to the criteria of Berge's manual of determinative bacteriology and the methods and criteria of Sharpe (Raper KB, Stolk C, Hadlok R 1976. Robert A. Samson, Ellen S. van Reenen-Hoekstra, 1988). The ripening of these cheeses is very short (one day).

Physicochemical analyses

Soluble nitrogen (% of total nitrogen) values of the fresh cheeses were found to be 9.2 -12.8. On the other hand non-protein nitrogen (% of total nitrogen) contents of the samples were 3.3 and 7.5. The values were found to be lower than the ones observed in the literature. The pH was measured with a pH-meter for milk samples. (Samson RA, Stolk C, Hadlok R 1976). The value of pH was very similar to that observed for some type of cheeses. Equally, the levels of NaCl were variable. The cause of this variation is probably the modification of the salting method. With respect to the NaCl content the levels increased gradually to 2.5%. As seen from the Figure 1 of variation of pH of samples of raw milk intended for production white slice of cheese are quite variable.

Figure 1. Determine a pH value in 167 samples of raw milk intended for production of white brined cheese in brine



4. CONCLUSION

High incidence of pathogenic bacteria indicated variability in hygienic conditions of the production of this type of cheese. Most frequently moulds were isolated in summer and winter and these were: in Summer *Penicilllium* spp. 35%, *Alternaria* spp. 11% and *Cladosporium* spp. 17%; and in Autumn *Penicillium* spp. 23% and *Rhizopus* spp 15.4% (Brooks J.C., Martinez B., Stratton J.,Bianchini A., Krokstrom,R.Hutkins (2012).

Most frequently isolated pathogenic microorganisms were Enterococci (log count 7, 95 and Micrococaceae (Log count 8, 66) then yeasts (log count 5.48) and moulds (log count 1.87) (Maria Cousta, Marios Mataragas,Panagiotis Skandamis, Eleftherios H.,Drosinos 2010):) .

High incidence of *Lb. casei* ssp. *ramnosum*, *Lb. casei* ssp. *Plantarum*, *Lb. lactis* ssp. *lactis*, *Lb. casei* ssp. *casei* in fresh cheese samples indicated that they probably play an important role in the manufacture of fresh soft cheese from Stara planina in Serbia. (Parvathey Seema Nair, Puthuvallil Kumaran Surendran 2004) It is assumed that the role of *Lb. casei* ssp. *casei* is important as natural mountain flora of these cheeses.

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