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MICROBIOLOGICAL STUDY OF TRADITIONAL CHEESE PRODUCED IN RUGOVA REGION OF KOSOVO

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Abstract

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Total mesophilic bacteria, anaerobic bacteria, lactic acid bacteria, enterobacteria as well as yeast and moulds were investigated during the production of the traditional Rugova cheese without using any starter culture. This type of cheese has been produced and consumed by the local population in the Rugova region for centuries, and is important for their economy. Samples from different stages of production (raw milk, curd and cheese during maturation on day 1, 8 and 14) were collected from local farms in the Rugova region, Kosovo, during winter. After sample collection and their serial dilution, samples were plate on different media and incubated at the appropriate temperature. According to identical morphology and physiology the number of 52 isolates obtained from de Man, Rogosa, and Shape (MRS), and M17 agar was reduced to ten Gram-positive and catalase negative strains. These strains were subjected to API 50 CHL fermentation profiling. There was a variety of microbial groups detected in all stages of cheese production, with the dominant microbial groups being *Lactococcus* (from 6.44 log cfu/ml in milk to 8.39 log cfu/gr in cheese D14) and *Lactobacillus* (from 5.79 log cfu/ml in milk to 7.58 log cfu/gr in cheese D14). *Enterobacter* and yeast and moulds were found in lower amounts compared to the other microbes in all stages. The level of *Leuconostoc* was approximately the same as *Lactobacillus.* Almost all microbial groups were low in curd and started to increase from day one of cheese manufacturing. Three of the ten isolates were identified as *Lactococcus lactis ssp. lactis*, four as *Lactobacillus plantarum (2 isolates)* and *L. curvatus* (2 isolates). One isolate of each of *Lactobacillus paracasei ssp. paracasei*, *L. brevis* and *L. pentosus* were identified. These results confirm the competitiveness of high numbers of LAB during fermentation of this cheese.

Key words: traditional cheese; Rugova region; microbial activity; LAB

Introduction

Rugova is a very important region in Kosovo, with a high potential for development of mountain tourism. It is located north-west of the city of Peja and was designated a national park in 2013. For centuries, people of this region and other regions of Kosovo and Balkans (Thraco-Illyrian) have consumed white cheese produced by cow's, sheep's, goat's milk or a combination of milks. In the past, they used the stomach of small ruminants to isolate rennet enzymes to coagulate the milk, the product was then fermented and matured by the natural microflora present in milk. Today, they use commercial rennet enzyme for milk coagulation, whereas fermentation and maturation of cheese occur via the activity of normal microflora. These cheeses have an unique shape and sensorial characteristics that may come from local ambient conditions particular to a certain region, includ-

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ing species and animal nutrition, as well as the milk used for cheese production, all of which are dependent on the production practices. A diagram of the manufacturing and ripening stages of traditional Rugova cheese are shown in Figure 1.

It is known that the natural microflora of milk is responsible for the fermentation and maturation of cheese produced by traditional methods (Randazzo et al., 2002; Alegria et al., 2012). Previously, it has been shown that lactic acid bacteria (LAB) are the predominant microorganism in most fermented foods (Gulitz et al., 2013; Vehapi and Kurteshi, 2013; Sudun et al., 2013; Abegaz, 2014; Guetouache et al., 2015). They ferment lactose to lactate and are the dominant population in bovine, goat, sheep and buffalo milk prior to pasteurisation. The most common LAB genera in milk include *Lactococcus*, *Lactobacillus*, *Leuconostoc*, *Streptococcus and Enterococcus* (Quigle et al., 2013). Although there is information about food security in artisanal cheese in Kosova market and for kaçkavall cheese in Balkan, there is a lack of information available about the microbial diversity and interactions between microorganisms present in white cheese produced by traditional methods (Caric, 1993; Vehapi and Kurteshi, 2013). For small dairy farmers, artisanal cheeses are value added products, with a viable source of income beyond what they can expect from selling milk.

Raw milk can contain a diverse bacterial population that may have positive and negative effects in fermented products such as cheese. Positive effects include the role of LAB in food safety (Alegria et al., 2010) of products in general, especially in milk fermented products, also as a probiotic in the human and animal gut. Microorganisms can have negative effects, causing spoilage (e.g *Pseudomonas*, *Clostridium*, *Bacillus* and other spore-forming microbes), as well as disease (e.g. *Listeria, Salmonella, Escherichia coli, Campylobacter* and mycotoxin-producing fungi), (Quigle et al., 2013).

The aim of this study was to analyse the microbial community, their ecology and activity in traditional Rugova cheese produced by traditional methods. These types of cheese are manufactured from raw milk produced by local cows (Busha, Simmental), however, there is an absence of standardisation in such food production and they do not possess European commission traditional product status as PDO-protected designation of origin or PGI-protected geographical indication (European commission, 2006). This study will provide useful information about the diversity, dynamics and activity of microbial population during the production of traditional Rugova cheese.

Material and Methods

Sample collection and culture conditions

Samples were collected in the Rugova region from three local farms during winter. Milk, curd, fresh cheese (1 day), cheese after 8 and 14 days of ripening were sampled aseptically and kept refrigerated until analysis in the laboratory.

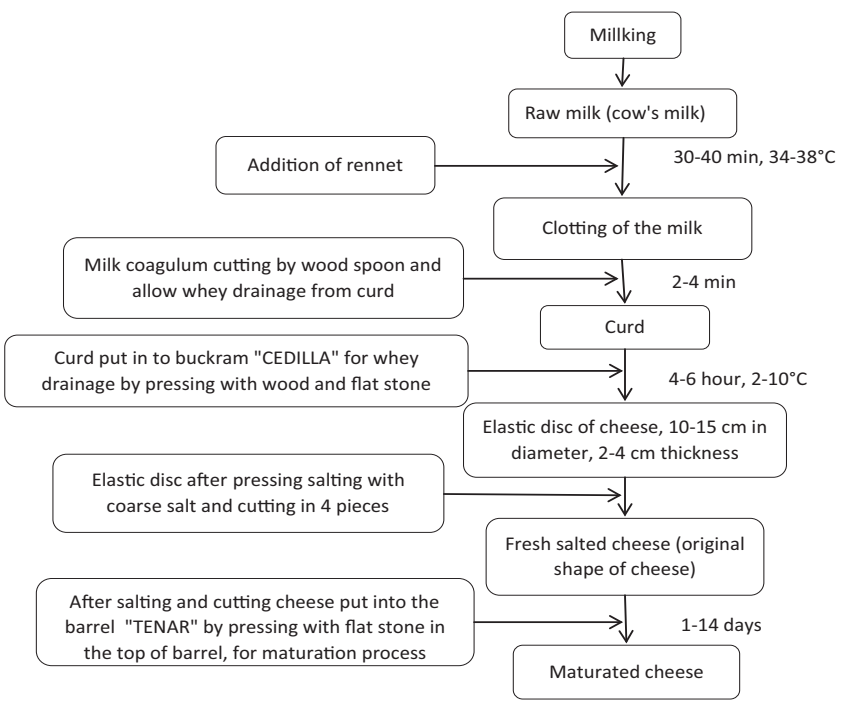

Fig. 1. Scheme of manufacturing and ripening of traditional Rugova cheese

Microbial analyses were performed by culturing in certain media. Ten-gram samples of curd, fresh cheese, cheese after 8 and 14 days ripening were mixed with 90 ml of buffered peptone water (BPW) and homogenised for approximately 5 min with a Stomacher Lab Blender 400 (VWR). Raw milk and homogenised samples were serially diluted in Pepton solution and all samples were plated for bacterial enumeration according to the pour plate method. Briefly, 1 ml aliquots of the diluted samples were inoculated directly into the molten media. Yeast and moulds were plated by the spread method, with 0.1 ml aliquots of the diluted samples were plated in yeast extract-glucose-chloramphenicol agar (YGCA, Biomerieux).

Aerobic mesophilic bacteria were cultured on milk plate count agar (MPCA; Liofilchem, Italy) and counted after 72 h of incubation at 30°C. Total anaerobic bacteria were grown on brain hard infusion agar (BHIA; Biolife, Italy) and counted after 72 h incubation at 37°C in anaerobic conditions. *Lactococci* were cultured on M17 agar (Biolife, Italy) and colony counts were performed after 48 h of incubation at 30°C. For *Lactobacillus* enumeration, colonies were grown on de Man, Rogosa, and Shape agar (MRSA; Biolife, Italy) and counted after 72 h of culture in aerobic conditions at 37°C. *Leuconostoc* were cultured in MSE agar medium (Biolife, Milan, Italy) and counted after incubation for 4 days at 22°C. *Eneterobacteria* were grown on MacConkey agar (MCA; Biolife, Italy) and were counted after 48 h of incubation at 37°C. Yeast and moulds grown on YGC-agar were counted after 5 days of incubation at 22°C.

Identification of isolates

Plates with 30 to 300 colonies were selected for LAB isolation. One to six colonies were randomly selected by the media to control their purity, by streaking three or four times on nutrient agar (NA). From the 52 isolates obtained from MRS and M17 agar, after morphological and physiological tests, the number was reduced to 10 Gram positive and catalase negative. All 10 strains were subjected to API 50 CHL fermentation profiling and carbohydrate fermentation patterns were determined using the API 50 CHL test kit (API System bioMerieux, Marcy l'Etoile, France), according to Turchi et al. (2005).

Statistical analysis

All experimental data are presented as average ±SD. Plate counts were log transformed before statistical analysis. Statistical significance was determined using ANOVA and differences between means compared by Tukey's test. The results were considered significant if $P < 0.05$.

Results

Evaluation of microbiota present in traditional Rugova cheese samples, during production and ripening is summarised in Table 1. The initial counts of microbial group (MPCA) in raw milk were 7.18 log cfu/ml, with a decrease in curd and increase during ripening (8.37 log cfu/gr). Total mesophilic bacteria on day 14 significantly ($p < 0.05$) increased in comparison to milk, curd and day 1 cheese. Mesophilic bacteria in anaerobiosis reached a maximum countin cheese (day 8 of fermentation), slowly decreasing in the final product. The high cell counts of *Lactococci* were similar to the counts obtained for total mesophilic bacteria, suggesting that they are the dominant microbes in Rugova cheese.

Significant differences were observed on day 14 compared with other stages (milk, curd, cheese day 1, cheese day 8), also between day 8 cheese compared with milk and curd. The number of *Lactobacillus* colonies was high in all samples but compared with *Lactococcus* they were 1 logarithmic unit lower. The *Enterobacter* population were present in the range from 4.83 to 6.22 log cfu/ml/gr. No significant changes were observed between stages, except on day 8 and 14 compared with curd. *Leuconostoc* bacteria were present in the range from 5.71 to 7.47 log cfu/ml/gr. Finally, yeast and moulds in milk and curd were low, increased in cheese (4.73 log cfu/ml/gr), but remained low in the final product, with no significant differences detected between the stages.

A total of 10 isolates were randomly selected for phenotypic and biochemical characterisation. The incubation conditions

Table 1

Enumeration of distinct microbial groups during production of traditional Rugova cheese (average values ± Standard deviation, log_{10} cfu /ml/gr)

Samples	MPCA (Total mesophilic bacteria)	BHIA (Total anaerobic bacteria)	LM17A (*Lactococcus* spp.)	MRSA (*Lactobacillus* spp.)	MCA (*Enterobacteria* spp.)	MSE (*Leuconostoc* spp.)	YGCA (Yeast and molds)
Milk	7.18±0.46	5.80±0.72	6.44±0.41	5.79±0.89	4.83±0.60	5.71±0.86	4.00±0.0
Curd	5.91±0.68	5.21±0.28	5.81±0.37	5.00±0.61	4.10±0.51	5.77±1.05	4.00±0.0
[a]Cheese D1	6.57±0.09	6.49±0.1	6.58±0.07	6.43±0.15	5.50±0.29	5.69±0.20	4.43±0.31
[b]Cheese D8	7.74±0.15	8.28±0.77	7.31±0.30	6.49±0.33	6.04±0.70	6.25±0.83	4.73±0.02
[c]Cheese D14	8.37±0.21	7.95±0.60	8.39±0.31	7.58±0.11	6.22±0.58	7.47±0.06	4.67±0.19

a – cheese day 1, b – cheese day 8 and c – cheese day 14

Table 2
Lactic acid bacteria isolated from milk and cheese during production of traditional Rugova cheese

Isolate (s)	Source	Medium	Gram reaksion	Catalas test	Morphology	Identification of species[a]
FA39	Cheese	MRS	+	–	Rods	*Lactobacillus curvatus*
FA40	Milk	MRS	+	–	Rods	*Lactobacillus paracasei* ssp.*paracasei* 1
FA1	Milk	MRS	+	–	Rods	*Lactobacillus plantarum* 1
FA13	Cheese	MRS	+	–	Rods	*Lactobacillus plantarum* 1
FA15	Cheese	MRS	+	–	Rods	*Lactobacillus brevis*
FA17	Cheese	M17	+	–	Cocci in pairs	*Lactococcus lactis* ssp. *lactis*
FA38	Cheese	MRS	+	–	Rods	*Lactobacillus pentosus*
FA29	Cheese	MRS	+	–	Rods	*Lactobacillus curvatus*
FA42	Milk	M17	+	–	Cocci in pairs	*Lactococcus lactis* ssp. *lactis*
FA9	Cheese	M17	+	–	Cocci in pairs	*Lactococcus lactis* ssp. *lactis*

a – identification of species were made by bioMerieux software

and types of media used for selection are summarised in Table 2. From the 10 isolates, 3 were identified as cocci and 7 rods. With regard to the cocci, all isolates were identified as *Lactococcus lactis ssp. lactis*, with an agreement between count results. Two isolates were identified as *Lactobacillus plantarum 1* and two as *Lactobacillus curvatus*. There was one isolate of each of *Lactobacillus paracasei ssp. paracasei 1, Lactobacillus pentosus* and *Lactobacillus brevis*. Three species were homofermentative, producing only lactic acid and seven were heterofermentative, producing lactic acid as well as other acids and alcohols.

Discussion

To the best of our knowledge, this is the first report of the microbial population of Rugova cheese produced by traditional methods. The high concentration of LAB in this traditional cheese is in agreement with other studies which reported high numbers of such bacteria in traditional cheeses of different countries (Hassanzadazar and Ehsani, 2013).

Overall, the colony counts indicated that *Lactococcus* were predominate microbes during maturation of cheese, which is similar to that reported for other cheese samples (Randazzo et al., 2002; Mikulec and Jovanovic, 2005; Alegria et al., 2012). Furthermore, the counts of lactobacilli in the final Rugova cheese product were approximately the same (average number 7.58 log cfu/ml/gr) as other traditional dairy products (Cheriguene et al., 2007). In a previous investigation of traditional cheese, all microbial groups reached their maximal counts in curd (Mikulec and Jovanovic, 2005). In this study, there were low concentrations of almost all microbial groups in curd, which increased in cheese. Yeast and moulds were low in milk and curd samples, slowly increasing in day 8 cheese, but remaining low in the final product, which is in agreement with other authors (Alegria et al., 2012; Mikulec and Jovanovic, 2005). Enterobacterial counts were not very high in milk but increased slowly during the production of cheese, similar to previous reports (Randazzo et al., 2002).

All isolates identified in the present work were found in different cheeses during production and maturation investigated by other authors (Randazzo et al., 2002; Cheriguene et al., 2007; Alegria et al., 2012). Except for the role of LAB in fermentation and maturation of cheese, most of them have antibacterial activity against other pathogenic bacteria, and also can be used as a probiotic for animal and human nutrition. *Lactobacillus curvaturs* present in traditional Rugova cheese is an important species. This species previously isolated from Azerbaijani homemade cheese, has been shown to have antimicrobial and antifungal activity, with a bacteriostatic mode of action (Ahmadova et al., 2013). *Lactococcus lactis ssp. lactis* is known as a nisine Z producer that has antibacterial effects against *Clostridium tyrobutiricum*, thus has the potential to be used for preservation of food products (Hurst et al., 1993; Rilla et al., 2003; Topisirovic et al., 2006). Biosurfactant isolated from *Lactobacillus paracasei ssp. paracasei* has been investigated for its antimicrobial and antiadhesive properties (Gudina et al., 2010). Others species identified in this study (*Lactobacillus plantarum, L. pentosus and L. brevis*), present in cheese microbiota, also known for their antibacterial activity and as probiotics present in the gastrointestinal tract of animals and humans (Zago et al., 2011; Tajabadi et al., 2013; Waki et al., 2014).

Conclusions

The results obtained in this study regarding the presence of natural microflora in traditional Rugova cheese provide future perspectives for further research on other LAB, which can be used as a starter culture for cheese production. The dominance of LAB during fermentation of this cheese could decrease pathogenic bacteria due to acid environment caused by their production of lactic acid or bacteriocins. Further studies are ongoing to determine the presence of other species of bacteria

that have crucial effects in fermentation and maturation process, and in cheese quality and safety. Nonetheless, LAB, especially *Lactococcus spp*. found during the production and maturation of Rugova cheese, may have a potential role as a starter culture for commercial cheese production.

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