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Isolation and characterization of Lactobacillus microflora from homemade white sheep's milk cheeses during ripening

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Abstract. *The Lactobacillus microflora of white cheese made from sheep's milk was studied during the maturation period of 10 to 100 days in the basement at a temperature of 10-12°C. Eight samples from different stages of natural white cheese ripening were taken for testing. A total of 28 strains were isolated and identified as Lactobacillus spp. based on their growth, gram-stain activity, catalase and oxidase. Their affiliation to this genus was confirmed by PCR with genus specific primers, 16S ribosomal RNA. The results show that in the early stages of cheese ripening (5th-6th, 10-39 day) the most commonly found species are: Lactobacillus plantarum and Lactobacillus curvatus, while in later stages of the cheese ripening (7th-10th, 40-80 day), the following species are found: Lactobacillus plantarum and Lactobacillus paracasei subsp. Paracasei.*

Keywords: cheese, *Lactobacillus plantarum, Lactobacillus curvatus, Lactobacillus paracasei subsp. Paracasei*, ripening

Introduction

White cheese enjoys a very high status in local cuisines and is an important part of the traditional food in our country and in the Balkans. It is made from sheep, goat, cow, buffalo milk as well as mixed milk. Milk from different animals is processed according to the same technology of parameters and technological operations are made with its specific features. The production of white cheese from cow's milk in the last decade in North Macedonia takes more than 70% of the total cheese production, because the production of cow's milk is the highest. Regardless, white sheep's milk cheese remains in the first place as the original brine white cheese that is in great demand on the world market (Alichanidis and Polychroniadou, 2008; Fox et al., 2017). Sheep's milk contains large amounts of casein, fat and mineral salts. Lactose is less present in it compared to cow's milk (Park et al., 2007; Raynal-Ljutovac et al., 2008). This feature determines its specific technological properties. Raw sheep milk is comparatively with the highest content of microorganisms, due to the fact that its production has relatively lower hygienic-sanitary conditions. Sheep's milk is stored, under the same other conditions, for longer time until the initial acidity, thanks to its chemical composition, and mostly to proteins and salts. However, the microflora during the processing of milk into cheese and during its maturation, if not inactivated by pasteurization, actively develops and leads to unpleasant consequences (Fox et al., 2017). The basic technological requirements of sheep's milk intended for the production of white cheese are the following: normal content of casein, fat and dry fat-free substance, total microbial count, no inhibitory substances, for having good biological properties (Robinson and Wilbey, 1998). Sheep milk intended for the production of white cheese should have the following quality indicators: acidity from 8.8 to 12.0 SH, pH 6.5-6.8, protein not less than 5.2%, fat content not less than 4%; and dry non-oily substances 9.5%, density 1.034 to 1.042 not to contain mechanical impurities, antibiotics and other inhibitory substances, to have normal taste, smell and color (Regulation EC, No 853/2004).

Microorganisms play a significant role in the development of well-balanced aroma/flavor and texture in cheese and therefore give cheese a dynamic nature. Cheese contains a complex microflora including bacteria, yeast and molds. Cheese microflora is divided into two basic groups, namely starter culture and secondary flora. Both groups basically contribute to the formation of desired aroma/flavor and textural characteristic in cheese. Cheese starter ferments milk sugar (lactose) to yield lactic acid and other organic acids which reduce the milk. *Lactococcus lactis*, *Streptococcus thermophiles*, *Lactobacillus helveticus* and *Lactobacillus delbrueckii* are the most common lactic acid bacteria used in the manufacture of cheese (Bresford et al., 2001; Pintado and Gomes da Cruz, 2014).

Lactic acid bacteria are necessary in the production of cheese, their biochemical activity and enzymes in milk are

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important for the formation of aroma, taste and texture of cheese (Smit et al., 2005; Settanni and Moschetti, 2010). The lactic acid bacteria in raw milk cheeses are very diverse on generic level as well as in different species within the same genus (Jans et al., 2012; Alfonzo et al., 2013). To preserve the unique and typical characteristics of traditional cheeses and their microbial diversity, it is necessary to study their microbiological characteristics (Giannino et al., 2009; Kamimura et al., 2019).

The presence of lactobacilli in home-made cheese can be used to obtain a collection of new isolates that can be further investigated to obtain potential probiotics that will be used as starter cultures to determine the type of fermentation that takes place in the cheese and to reduce the number of rotting microorganisms eg *E. coli* and to improve the nutritional value of the cheese. According to Blaya et al. (2018), microbial community modeling can contribute to improving the efficiency and reduce the cost of food processes such as cheese ripening. In order to maintain the production of high-quality traditional cheeses, it is necessary to study the microbial diversity, especially the attendant lactic acid bacteria as essential for cheese production.

Material and methods

Preparation of the white cheese

The cheese for our examination was prepared in a household using traditional cheese-making procedure: 25 kg of white cheese is obtained from 100 liters of sheep milk with a pH of 6.6, protein not less than 5.2% and fat content not less than 6.5%.

The stages of cheese preparing were as follows:

1st stage: 100 liters of sheep's milk were heated to 72°C for a few minutes and then cooled to 30°C;

2st stage: after cooling the milk, a starter culture prepared in a traditional way was added;

3st stage: the cheese is cut into 8 cm pieces, placed in plastic bins and salted with sea salt of 150-200 g;

4th - 12th stages: white cheese is placed in a basement to mature from the 10th to the 100th day in air temperature of 10-12°C.

Samples collection

The samples (n=8) were taken from the fifth, sixth, seventh, eighth, ninth, tenth, eleventh and twelfth stages of the white cheese preparation (Table 1).

Table 1. Stages of samples collected of white cheese

Stages of white cheese maturation							
5th	6th	7th	8th	9th	10th	11th	12th
From 10 to 20 day	From 20 to 30 day	From 40 to 50 day	From 50 to 60 day	From 60 to 70 day	From 70 to 80 day	From 80 to 90 day	From 90 to 100 day
Days of sampling for examination							
Sample 1 day 10	Sample 2 day 26	Sample 3 day39	Sample 4 day 53	Sample 5 day 64	Sample 6 day 80	Sample 7 day 83	Sample 8 day 94

Isolation of genus Lactobacillus

Isolation of lactobacilli strains from home-made white sheep's milk cheeses was performed following classical microbiological approaches. MRS broth and MRS agar medium (MRS broth, Merck, KGaA, 64271 Darmstadt Germany) suitable for isolating lactobacilli strains were used. After pre-enrichment of the culture in 0.5% skim milk (Merck, KGaA, Darmstadt Germany) for 16-18h and a series of dilutions, the culture was cultured on agar MRS medium. From each Petri dish with MRS agar medium, colonies from all samples were randomly selected and the selected strains were subsequently cultured in 42°C MRS broth.

DNA Isolation

Chromosomal DNA from a single-colony inoculated @, GenElute TM, bacterial genomic DNA kit (Sigma Aldrich Co.) was isolated on 24 isolates obtained from raw sausage. From the resulting DNA, 20 μm sections were made and stored at -20°C for several months until needed.

The genus-specific PCR primers

After isolating the DNA of the isolates for gender PCR, we used the following primers Lact71R / Lab0667F (1) and LactoF / R (2) in a reaction volume of 20 μl. The marker we used for molecular weight is 100 bp.

Table 2. PCR primers specific for the genus Lactobacillus

Lab0677F Lact71R	5-'CTCCATGTGTAGCGGTG-3' 5'-TCAAAACTAAACAAAGTTTC-3	Moura et al., 2007
LactoF LactoR	5'-TGGAAACAGRTGCTAATACCG-3' 5'-GTCCATTGTGGAAGATTCCC-3'	Byun et al., 2004

Electrophoresis 1% agarose gel

PCR fragments were separated by electrophoresis in 1% agarose gels in Tris-borate-EDTA (TBE) buffer in Mini Rapide Unit (Cleaver Scienfic Ltd.) at constant 70V for 35 min. The gels were visualized with ethidium bromide staining on a UV transilluminator.

Sequencing of the 16S rRNA genes.

The sequencing of the samples was performed on the DNA genetic analyzer ABI PRISM®310 (PE Applied Biosystems).

Results and discussion

Twenty-eight strains are isolated from 8 stages of home-made white cheese maturation (stages 5th to 12th) and are characterized phenotypically. From the fifth to the seventh phase of the cheese ripening process, 10 isolates are isolated and the remaining 18 isolates are isolated from the eighth to the tenth phase. After pre-enrichment of the culture in 0.5%

skim milk, the isolates were grown anaerobically on a selective nutrient medium for lactobacilli MRS-agar and the temperature used to cultivate them was 42°C (Chou and Weimer, 1999; Ashraf and Shah, 2011). After inoculating the isolates on the selective nutrient medium, white glossy colonies were obtained which were additionally tested by classical microbiological tests. Catalase and oxidase negative rod shaped bacteria indicate that the strains are related to the genera Lactobacillus. To confirm that the genus Lactobacillus was isolated, DNA of all 28 isolates was used and PCR was used with primers specific for the genus Lactobacillus (Heilig et al., 2002; Gevers et al., 2003).

The results in Figure 1 show that all 28 newly isolated white cheese isolates are amplified, confirming that the isolates belong to the genus Lactobacillus.

The results of 16S rRNA analysis in Figures 2 and 3 show that the species *Lactobacillus plantarum* was the largest - 70% and *Lactobacillus curvatus* - 30% was most common in the early stages (5th-7th) of cheese ripening, while the following species are found in other later stages (8th-10th) of cheese ripening: *Lactobacillus plantarum* - 62% and *Lactobacillus paracasei subsp. Paracasei* - 38%. The obtained results show that from the beginning of maturation of white cheese from 10 to 39 days and from 40 to 80 days of maturation there are different types of lactobacilli.

Studies by other authors show that *Lactobacillus plantarum* is the dominant white cheese ripening process (Todorov and Franco, 2010; Papadopoulou et al., 2018).

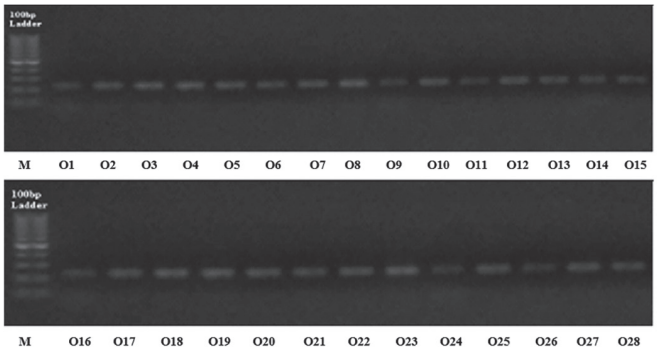

Figure 1. 1% agarose gel determination of Lactobacillus genus belonging to genus-specific PCR primers

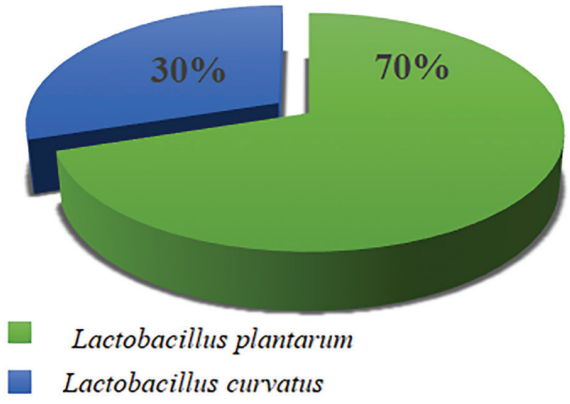

Figure 2. Results of the analysis of the 16S rRNA sequence in the early stages of cheese

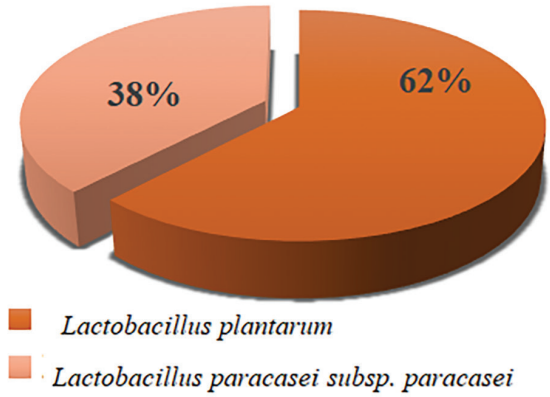

Figure 3. Results of the analysis of the 16S rRNA sequence later stages of cheese ripening

Conclusion

From our conducted examination it was found that: (i) from the fifth to the twelfth phase (10th to the 100th day) of white cheese maturation in the early stages of cheese ripening (5th-6th, 10-39 day), two types of lactobacilli have been discovered - *Lactobacillus plantarum* and *Lactobacillus curvatus,* while in other later stages of cheese maturation (7th-10th, 40-80 day) another species of lactobacilli was discovered *Lactobacillus paracasei subsp. Paracasei*; (ii) the isolates obtained from our test with additional new tests can be further used as starting cultures.

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