PAPER

LACTIC ACID BACTERIA MICROBIOTA OF "PIROT`S KASHKAVAL"

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ABSTRACT

"Pirot`s kashkaval" is an autochthonous dairy product belonging to pasta-filata cheeses. In order to determine the changes in the lactic acid bacteria microbiota during 60 days of ripening, 315 lactic acid bacteria strains were isolated from ewe`s and cow`s milk cheese and identified as *Enterococcus faecium, Pediococcus acidilactici, Pd. pentosaceus, Lactobacillus casei/rhamnosus, Lb. plantaum, Lb. casei, Lb. fermentum, Lb. paracesei, Lb. rhamnosus* and *Streptococcus macedonicus*. Enterococci were the most dominant isolated strains. In the final stages of ripening, the increase of the population of *Lb. casei* and *Pd. acidilactici* was observed for ewe`s and cow`s milk cheese, respectively.

Keywords: cow`s milk cheese, ewe`s milk cheese, molecular identification, lactic acid bacteria, "Pirot`s kashkaval",

1. INTRODUCTION

"Pirot`s kashkaval" cheese is an autochthonous dairy product produced in Pirot, Republic of Serbia and its surroundings by a specific technology. The production process, which differs this type of cheese from other produced in Serbia includes the cooking of partly fermented curd prior to ripening. This type of cheese has light to intensively yellow color, monolith, partially layered and elastically-plastic structure. The combination of cow's and ewe`s milk is, usually, being used for the production. The taste of "Pirot`s kashkaval" is mildly sour and piquant, specific and depends on the type of used milk (OSTOJIĆ *et al*., 2012).
The production is characteristic for the process of cooking of the sliced fermented curd for 5-8 min. at temperature of 72-75°C. The cheese curd is then salted, mixed, molded and additionally ripened for a few months (MANČIĆ and MANČIĆ, 2005). The cooking of curd in hot water has the significant influence on its microbiota. Thermal treatment of fermented curd is a way of pasteurization of cheese dough, which leads to certain biochemical and microbiological processes. During this process the significant drop in bacterial and yeast number occurs, so it can be very important in the cases when it's not possible to get good quality milk and when milk pasteurization is not applied (ALRUBAI, 1979).
The formation of sensory profile of cheeses is the result of metabolic activity of the present microbiota (BENITO de CARDENAS, *et al.*, 1990; MUSTAFA, 2006, DUAN *et al*., 2008). Products of glicolysis, proteolysis of casein and lipolysis of fats, as the main metabolic functions of lactic acid bacteria (LAB), have a great influence on cheese flavor.
Since starter cultures are not added during the production of "Pirot`s kashkaval", cheese flavor originates from metabolic products of autochthonous microbiota. The advantages of autochthonous microbiota are fast growth and development and production of specific sensory characteristics of the products. Furthermore, determination of the microbiota composition is an important step in analyzing traditional fermented dairy products and eventual devinition of autochthonous starter cultures. The aim of this work was the isolation and characterization of LAB present during the process of ripening of "Pirot`s kashkaval" unique in Serbia for the production process. For that purpose, 10 samples of cheese produced from cow`s and ewe`s milk were collected during 60 days of ripening and analysed.

2. MATERIALS AND METHODS

2.1. Cheese samples

"Pirot`s kashkaval" was produced by a traditional process from cow's and ewe`s milk. After the addition of rennet, the whey was separated, and the fresh curd was fermented for a few days. The fermented curd was sliced and cooked in hot water (72-75°C). After that, the curd was salted, molded and ripened in a ripening chamber. Sampling was performed during the ripening process of 10-15 days at 25°C and until the end of the two months ripening period at 10°C.
Samples of cow`s milk cheese (CC) and ewe`s milk cheese (EC), were collected during the process of ripening after 1 (CC1 and EC1), 5 (CC5 and EC5), 20 (CC20 and EC20), 30 (CC30 and EC30) and 60 (CC60 and EC60) days of ripening. The samples were then packed in

vacuum, transported and kept up to 3 days at + 5°C to the moment of microbiological analyses.

2.2. Isolation and determination of LAB number

Cheese samples (10 g) were transferred aseptically to 90 mL of 2% (w/v) sodium citrate solution (t=45°C) and homogenized for 30 minutes. The isolation of LAB was performed by serial dilution method. A volume of 1 mL of appropriate dilution was transferred into a Petri dishes and layered with MRS (Torlak, Belgrade, Serbia), M17 (Merck, Darmstadt, Germany) and MSE agar (tripton 10 g L^{-1}, gelatine 2.5 g L^{-1}, yeast extract 5 g L^{-1}, sucrose 100 g L^{-1}, glucose 5 g L^{-1}, sodium citrate 1 g L^{-1}, sodium azide 0.075 g L^{-1} and agar 13 g L^{-1}) for determination of presumptive lactobacilli, lactococci and leuconostocs, respectively. After solidification, second layer of medium was poured in order to achieve micro-aerophilic conditions preferable for the growth of LAB. Plates with MRS and M17 agar were incubated for 48 h at 30 and 45°C in order to determine both mesophilic and thermophilic LAB strains, while the MSE agar plates were incubated 48h at 30°C. Determination of total number of mesophilic bacteria was performed on nutrition agar (NA) plates (Torlak, Belgrade, Serbia) (48 h, 30°C). The determination of number of bacteria was performed in triplicate and the values are presented as the mean value.
After the incubation and enumeration, at least 30 LAB colonies were selected from each sample and purified. Preliminary characterization of the isolates was done by Gram staining and catalase test. Bacterial cultures were stocked in liquid medium (MRS and M17 broth), with addition of 20% (v/v) glycerol at the temperature of -20°C until further analysis.

2.3. Molecular identification of isolates

The extraction of total DNA, PCR amplification with (GTG)5 primer and electrophoresis were done by already described method (NIKOLIĆ *et al.*, 2008). Sequencing of 16S rRNA genes was performed by multiplying of fragments by U968 (5′-AACGCGAAGAACCTTAC-3′) and L1401 (5′-AACGCGAAGAACCTTAC-3′) primers (RANDAZZO *et al.*, 2002) and using Taq DNA polymerases. Reaction was carried out in PCR System 2700 (Applied Biosystems) with the following parameters: starting denaturation of DNA during 5 min at 94°C, 30 cycles of 30 s at 94°C, 30 s at 55°C, 30 s at 72°C and the extension of incomplete products for 7 min at 72°C. Electrophoresis of multiplied products was done at 1% (w/v) agar gel with the addition of ethidium bromide. Multiplied fragments were purified using QIAquick PCR Purification KIT/250 (QIAGEN GmbH, Hilden, Germany), while their sequencing was done in Macrogen in Seoul, South Korea. BLAST algorithm (www.ncbi.nlm.nih.gov/BLAST) was used for determining the most similar sequence from NCBI base.

3. RESULTS AND DISCUSSION

3.1. Determination of number of bacteria

The changes of total mesophilic bacteria and LAB number determined at MRS, M17 and MSE agar plates in analyzed ewe`s and cow`s milk cheese samples are shown in Figs. 1 and 2, respectively. During the first 5 days of ripening, a slight increase of total number of

mesophilic LAB determined at MRS agar plates was noticed, from 7.7 to 7.8 log CFU g^{-1}, after which the number decreases to 6.4 log CFU g^{-1} (Fig. 1).

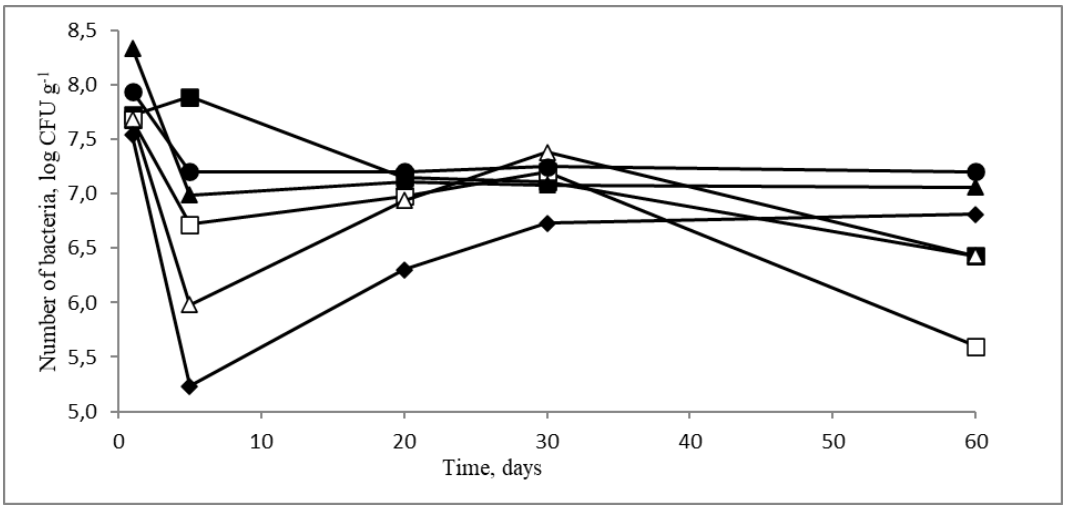

Figure 1. The change of number of bacteria during ripening of Pirot cheese produced from ewe`s milk at MRS (■), M17 (▲), MSE (♦) and NA (●) plates incubated at 30 (full symbols) and 45 °C (empty symbols).

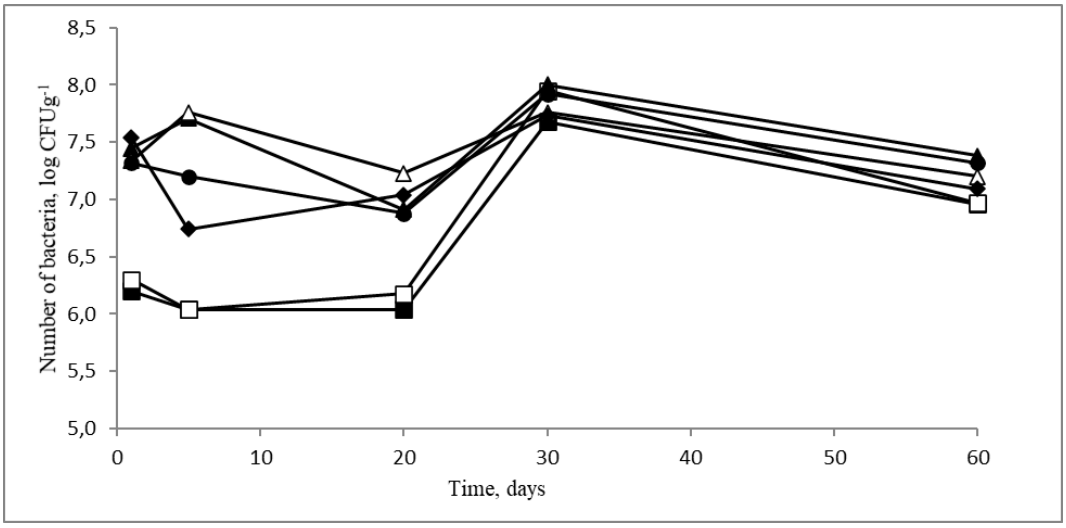

Figure 2. Change of the number of bacteria during ripening of Pirot cheese produced from cow`s milk at MRS (■), M17 (▲), MSE (♦) and NA (●) plates, incubated at 30°C (full symbols) and 45°C (empty symbols).

The number of microorganisms on M17, MSE and nutrition agar plates decreased in the first 5 days of ripening. Thus, number of mesophilic LAB at M17 agar plates decreased from 8.3 to 6.9 log CFU g^{-1} during the first 5 days, after which it was constant with the value of 7.1 log CFU g^{-1}. The number of thermophilic LAB was lower compared to the number of mesophilic LAB, regardless the used medium. Also, number of thermophilic bacteria decreased continually after the 30th day of ripening. The total number of mesophilic bacteria incubated at nutrition agar plates in first 5 days decreased from 7.9 to

7.2 log CFU g^{-1}, and that value maintained to the end of ripening. The number of mesophilic LAB determined on MSE agar plates decreased in first 5 days from 7.5 to 5.2 log CFU g^{-1} and after that it increased to 6.8 log CFU g^{-1} (Fig. 1).
The number of bacteria in cow`s milk cheese samples is shown in Fig. 2. The increase of the LAB number at the beginning of fermentation (to 5th day) was only noticed on M17 agar plates for both mesophilic (from 7.5 to 7.7 log CFU g^{-1}) and thermophilic bacteria (from 7.3 to 7.8 log CFU g^{-1}). In the following 15 days, number of mesophilic LAB decreased to 6.9 log CFU g^{-1}, and thermophilic to 7.2 log CFUg^{-1}. At the end of fermentation, the number of LAB on M17 agar plates was 7.3 log CFU g^{-1}. The number of LAB on MRS agar in the first 20 days decreased for both bacterial groups reaching the value of cca 6.0 log CFU g^{-1}. Mesophilic and thermophilic LAB on MRS agar plates reached maximum on the 30th day of ripening (7.7 log CFU g^{-1}), afterwards a slight decrease was observed (Fig. 2).
High initial number of LAB in both cheese types can be explained by the process of fermentation, which took place before curd cooking. The fermented curd was thermally processed by immersion in hot water before sampling, but it can be assumed that heat stressed cells remained viable. The decrease of the LAB number was observed, regardless the type of milk used for making cheese, in the period from the 1st to the 5th day of ripening due to the adaptation of the microbiota to the ripening conditions. This decrease is in accordance with the literature results (ALRUBAI, 1979; GOBBETTI *et al*., 1997; BARUZZI *et al.*, 2002), which indicate that cooking of curd has a great influence on the microbiota and lead to a significant decrease of total number of bacteria in the first stage of ripening. The differences in the LAB number of cow`s and ewe`s milk cheese are probably the result of the different microbiota composition in these two cheese types. The stagnation of LAB number during the ripening of cow`s milk cheese lasts longer (30 days, Fig. 1) in relation to the samples of ewe`s milk cheese (20 days, Fig. 2). After that, the constant number of bacteria was achieved in the range from 6.5 to 7.4 log CFU g^{-1}, for both type of cheese. At the end of fermentation, the lowest number was observed for thermophilic LAB on MRS agar plates. This can be explained by the ripening conditions (temperature 10°C) which favors the growth of mesophilic bacteria. The domination of mesophilic LAB in the later stages of pasta filata cheese ripening has already been reported by SUCCI *et al*. (2016).
Determined LAB number in analyzed samples is in accordance with earlier researches for "Pirot`s kashkaval" (MIJAČEVIĆ *et al.*, 2005a; MIJAČEVIĆ *et al.*, 2005), Italian cheese Pugliese, Silano and Molise, produced from pasteurized cow milk (GOBBETTI *et al.*, 2002; COPPOLA *et al.*, 2003; PIRAINO *et al.*, 2005), Mozzarella from cow milk (de CANDIA *et al.*, 2007) and Taleggio cheese from Lombardi in Italy (GOBBETTI *et al.*, 1997).

3.2. Identification of lactic acid bacteria isolates

From 5 different samples of Pirot`s ewe`s milk cheese 173 LAB isolates were obtained. The identification was performed by (GTG)5-PCR fingerprinting and 16S rRNA gene sequencing. According to the (GTG)5-PCR fingerprinting low level of diversity was observed among the isolates and representative fingerprints are shown in Fig. 3. Most of the isolates belong to cocci (130), while 43 belong to bacilli. Isolates were identified as the representatives of *Enterococcus faecium, Pediococcus acidilactici, Pediococcus pentosaceus, Lactobacillus casei/rhamnosus, Lactobacillus plantaum, Lactobacillus casei, Lactobacillus fermentum, Lactobacillus paracesei* and *Streptococcus macedonicus*. Among them, the most dominant were *En. faecium* (50%) and *Pd. acidilactici* (16%), while the least isolated strains were *Lb. fermentum* (1%) and *Lb. paracasei* (0.5%). During 60 days of ripening of Pirot`s

cow`s milk cheese 142 LAB isolates were isolated and identified as *En. faecium* (44.4% of isolates), *Pd. acidilactici* (50%), *Pd. pentosaceus* (0.7%), *Lb. plantarum* (1.4%), *Lactobacillus rhamnosus* (0.7%) and *Lb. casei* (2.8%).
The presence of different LAB strains is important in cheese ripening due to their unique metabolism products (COGAN, 2000). Dominancy of particular LAB strain is directly dependent of milk type, animal breeding, and way of feeding, type and quality of pasture, altitude, and production process, cheese storage conditions and many other (TOPISIROVIĆ *et al.*, 2006). The presence and prevalence of concrete LAB strains in analysed cheese samples is mainly affected by LAB heat resistance during the curd cooking. Among LAB strains isolated from Pirot`s cheese samples, the representatives of genera *Enterococcus* and *Pediococcus* dominate, while in a smaller percentage *Lactobacillus* spp. and *Streptococcus* sp. were present. The obtained results are in accordance with literature data according to which LAB isolated from cheese belong to genera *Enterococcus, Pediococcus, Lactobacillus, Streptococcus* and *Lactococcus* (FLEET, 1999; POZNANSKI *et al.*, 2004; MENG *et al.*, 2018; VANDERA *et al.*, 2019).

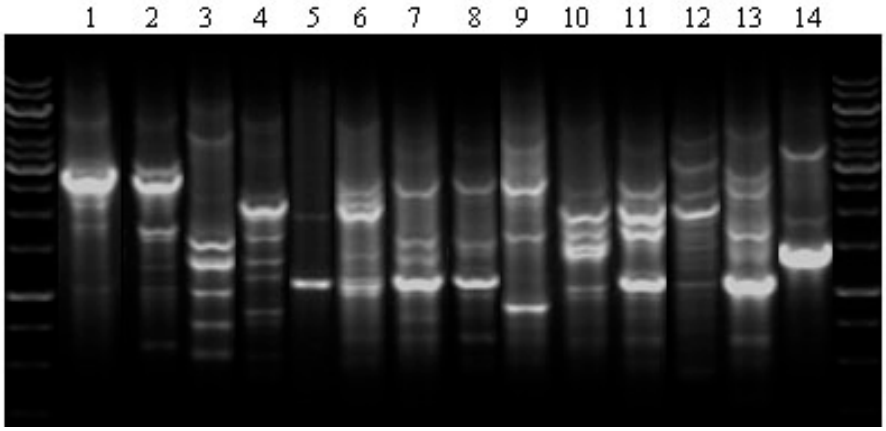

Figure 3. Representative (GTG)5-PCR fingerprints of LAB strains isolated from Pirot cow`s and ewe`s milk cheese: *En. faecium* (1,2), *Pd. acidilactici* (3), *Pd. pentosaceus* (4), *Lb.plantarum* (5), *Lb. casei* (6,7,8), *Lb. fermentum* (9), *Lb. paracesei* (10,11), *Lb. rhamnosus* (12), *Lb. casei/rhamnosus* (13) and *St. macedonicus* (14).

3.3. Microbiota dynamics in "Pirot`s kashkaval"

The results of monitoring changes in microbiota during ripening of cheese made from ewe`s milk are presented on Fig. 4. After 24 hours (sample EC1), the most frequently isolated was *En. faecium* (60%), while *St. macedonicus* (36%) and *Pd. acidilactici* (4%) were isolated as well. In sample after 5 days of fermentation (EC5) the population of *En. faecium* increased (85%), as well as pediococci (9%), while streptococci were not isolated. The percentage of enterococci decreased significantly after 20 days (EC20), while the percentage of pediococci (*Pd. acidilactici* – 47% and *Pd. pentosaceus* – 18%) and lactobacilli increased (*Lb. casei/rhamnosus* 6% and *Lb. plantarum* 9%). In the sample after 60 days of fermentation (EC60) *En. faecium* (55%) was dominant, while *Lb. casei* (20%), *Pd. acidilactici* (14%) and *Lb. casei/rhamnosus* (3%) were also isolated. *Lb. fermentum* (5%) and *Lb. paracasei* (3%) were identified only in this sample.
The change of microbiota during 60 days cow`s milk cheese ripening is shown in Fig. 5.

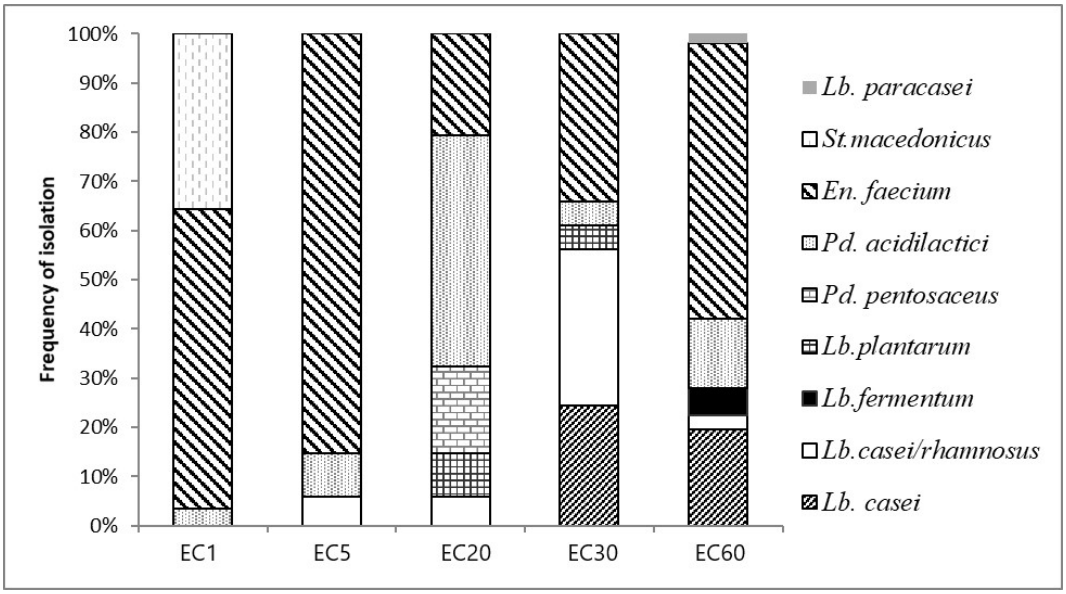

Figure 4. Frequency of isolation of LAB strains after 1 (EC1), 5 (EC5), 20 (EC20), 30 (EC30) and 60 (EC60) days of ripening of Pirot ewe`s milk cheese.

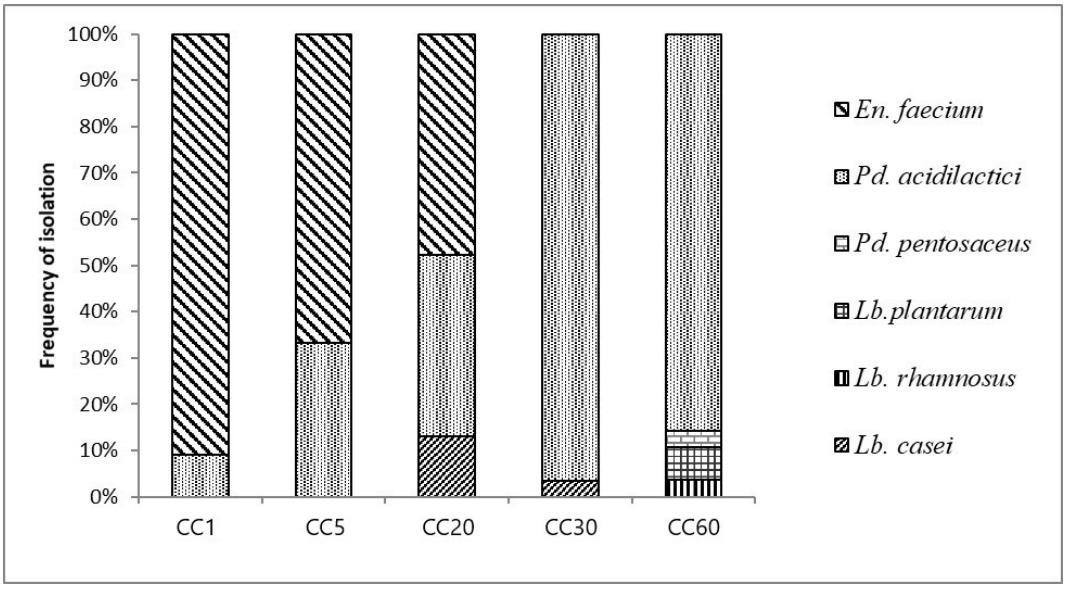

Figure 5. Frequency of isolation of LAB strains after 1 (CC1), 5 (CC5), 20 (CC20), 30 (CC30) and 60 (CC60) days of ripening of Pirot cow`s milk cheese.

At the beginning of ripening, in the sample CC1, two strains of LAB where isolated: *En. faecium* with 91% and *Pd. acidilactici,* 9%. The population of *En. faecium* decreased (66%), while the population of *Pd. acidilactici* (33%) increased in the sample CC5. After 20 days of ripening (CC20) *Pd. acidilactici* was isolated in the frequency of 39%. On the other hand, the presence of *En. faecium* was on the previous level, 47%. In this sample, *Lb. casei* was isolated with 13% of population. To the end of ripening, *Pd. acidilactici* was dominant with 97% in the sample CC30 and 85% in the sample CC60. Also, another representative of

pediococci, *Pd. pentosaceus*, was isolated, with 4% of total population of CC60. Lactobacilli were isolated in low frequency in the samples after 30 days (*Lb. casei* - 3%) and 60 days (*Lb. rhamnosus* - 4% and *Lb. plantarum* - 7%) of ripening.

Generally, enterococci were the most numerous genera in Pirot`s cheese, thus *En. faecium* made 50% of total identified microbiota in ewe`s milk cheese and 44% in cow`s milk cheese. This strain was identified in all phases of ripening in ewe`s milk cheese, and the highest number was observed at the beginning of ripening. In the cow`s milk cheese samples, *En. faecium* dominated in the first stage of ripening, while it was not isolated after 30 days. Enterococci often present the considerable part of LAB microbiota of many traditionally produced types of cheese (FONTECHA *et al*., 1990; COGAN *et al*., 1997; MOREA *et al*., 1999; DOMINGOS-LOPES *et al*., 2017; VANDERA *et al*., 2019). The presence of enterococci (especially *En. faecalis* and *En. faecium*) is characteristic for cheese made of ewe`s or goat's milk. As well, these two species have a very important role in lipolysis, proteolysis and production of diacetyl in cheese (GIRAFFA, 2002). The prevalence of enterococci in analysed samples are probably the result of the use of raw milk for the cheese production. The number of enterococci can be up to 60000 times higher in cheese produced from raw milk than from pasteurized milk (PAPPA *et al*., 2019).

Pediococci often take part in autochthonous microbiota of cheese and have a significant impact on ripening of many cheese types (BHOWMIK and MART, 1990). *Pd. acidilactici* was isolated from all analyzed cheese samples. In ewe`s milk cheese the highest percent of isolation was in the sample after 20 days of ripening, while in cow`s milk cheese it was dominant in the final stage of ripening. *Pd. acidilactici* and *Pd. pentocaseus* were identified in cheese produced in the Mediterranean (POZNANSKI *et al*., 2004; MOREA *et al*., 2007; AYDEMIR *et al*., 2015; De PASQUALE *et al*., 2019), while in ripe Italian cheese Parmigiano Reggiano they are predominant (GOBBETTI *et al*., 2002). On the other hand, in the analysis of Pirot`s cow`s milk cheese (OSTOJIĆ, 2012), this bacterium was not identified.

The other LAB strains identified in Pirot`s cheese belonged to lactobacilli (*Lb. plantarum, Lb. casei/rhamnosus, Lb. rhamnosus, Lb. casei* and *Lb. paracasei*) and streptococci (*St. macedonicus*). Lactobacilli represent dominant population of many types of cheese where they have a significant effect on formation of aroma compounds (BERESFORD *et al*., 2001; WOUTERS *et al*., 2002; De PASQUALE *et al*., 2019). *Lb. plantarum* was isolated from Italian and Argentinean cheese (ZAGO *et al*., 2011) and *Lb. fermentum* was isolated from Caciocavallo Pugliese (MOREA *et al*., 2007). *Lb. rhamnosus* was identified in cheese Parmigiano Reggiano (COPPOLA *et al*., 2005; SUCCI *et al*., 2005; De DEA LINDNER *et al*., 2008; BOVE *et al*., 2011) and Irish Cheddar cheese (MLALAZI *et al*., 2011). *Lb. casei* and *Lb. paracasei* were isolated and identified in Spanish cheese Cabrales (BELÉN-FLÓREZ *et al*., 2006), Turkish cheese Kasar (AYDEMIR *et al*., 2015) and Italian cheese Montasio (MARINO *et al*., 2003). These lactobacilli can have good probiotic potential and possibility to produce different bacteriocins (MLALAZI *et al*., 2011; ZAGO *et al*., 2011; MILIĆEVIĆ *et al*., 2014). The largest diversity of LAB strains in the analysed samples was observed after two months ripening period. High diversity of LAB strains and significant changes in microbiota composition has been reported in various types of pasta filata cheeses as the result of different processing (curd cooking and stretching) and ripening conditions (GOBBETTI *et al*., 2018). Additionally, greater diversity of *Lactobacillus* strains has been noticed in the later stage of ripening, in correlation with the results of SANT`ANNA *et al*. (2019).

In Pirot`s ewe`s milk cheese the population of *St. macedonicus* was also detected. This bacteria was first identified in Greek cheese Kasseri (TSAKALIDOU *et al*., 1998; GEORGALAKI *et al*., 2000) and was also isolated from Italian types of cheese from raw

milk: Asiago, Montasio, Monte Veronese, Morlacco, Spressa, Fontina, Ragusano, Mozzarella (LOMBARDI *et al.*, 2004), Nostrano di Primiero (POZNANSKI *et al.*, 2004), Toma piemontese (ZEPPA *et al.*, 2004) as well as from Pirot cow`s milk cheese (OSTOJIĆ *et al.*, 2012). This streptococcus has good acidification, proteolytic and bacteriocin characteristics, thus some authors claim it as multi-functional and very suitable for the production of dairy products (De VUYST and TSAKALIDOU, 2008).
Microbiological profile of LAB population similar to LAB population of Pirot`s cheese was reported for traditionally made types of cheese in the region of the Mediterranean: Caciovallo Molise (COPPOLA *et al.*, 2003), Caciocavallo Pugliese (MOREA *et al.*, 2007), Montasio (MARINO *et al.*, 2003), Toma piemontese (ZEPPA *et al.*, 2004), Nostrano si Primiero (POZNANSKI *et al.*, 2004) and Kasar (AYDEMIR *et al.*, 2015).

4. CONCLUSIONS

"Pirot`s kashkaval" is an autochthonous product traditionally made in Serbia. In order to continue and promote the production of this cheese, is very important to understand the composition and the changes in microbiota which occurs during ripening. Since LAB are being recognized as the most important in cheese ripening, the isolation and identification of LAB from "Pirot`s kashkaval" is of primary importance. LAB microbiota isolated during ripening of "Pirot`s kashkaval" made of cow`s and ewe`s milk was constituted of the genera *Enterococcus, Pediococcus, Lactobacillus* and *Streptococcus*. The most dominant species, in both types of cheese, was *En. faecium*. During the ripening of cow`s milk cheese, the population and diversity of lactobacilli increased. On the other hand, ripening of ewe`s milk cheese was characterized by the increase of pediococci population. Understanding of the microbiological changes which occurred during ripening of "Pirot`s kashkaval" can contribute to the definition of starter cultures suitable for industrial production of "Pirot`s kashkaval".

AKNOLEDGMENTS

This work was performed at the University of Nis, Faculty of Technology in Leskovac.

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