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Original scientific paper

THE NUMBER AND COMPOSITION OF YEASTS AND MOULDS DEVELOPING IN BEATEN CHEESE PRODUCED IN INDUSTRIAL CAPACITY

Vesna Levkov, Daniela Beličovska, Elena Eftimova

Institute of Animal Science Skopje, Ss. Cyril and Methodius University in Skopje, Blvd. Ilinden 92a, Skopje, N. Macedonia
vesna.levkov.1@istoc.ukim.mk

A b s t r a c t: The changes in the number end composition of yeasts and moulds during beaten cheese production and ripening were studied. The cheese was prepared in dairy in western part of North Macedonia by using raw cows' milk and without adding starter culture. The obtained results indicate the presence of yeasts in all stages of cheese preparation. The number of yeasts in milk was in range of 3.62–4.25 log CFU·ml^{-1} and has increased during dry ripening reaching maximum value of 5.91 log CFU·g^{-1} in the 3rd day of ripening during the 3rd producing process. The beaten cheese ripening in brine results in decreasing yeasts number reaching value of 3.60 log CFU·g^{-1}. The yeasts isolates were determinate as *Kluyveromyces marxianus, Debaryomyces hansenii, Kluyveromyces lactis, Pichia polymorpha, Pichia membranifaciens, Trichosporon pululans, Torulopsis* sp., *Candida* sp. The moulds were not noticed in all stages during beaten cheese making and ripening. They were continuously present during dry ripening till the 15th day of brining. Isolated species were determined as *Aspergillus niger, A. oryzae, Mucor mucedo, M. pusilus, Penicillium* sp., *Trichotecium* sp., and *Trichoderma viride.*

Key words: beaten cheese, yeasts, moulds, composition and dynamics

БРОЈ И СОСТАВ НА КВАСЦИ И МУВЛИ КОИ СЕ РАЗВИВААТ ВО БИЕНО СИРЕЊЕ ПРОИЗВЕДЕНО ИНДУСТРИСКИ

А п с т р а к т: Во трудот се презентирани резултатите од истражувањата на составот и бројот на квасци и мувли во текот на производството на биено сирење. Сирењето произведено од сурово кравјо млеко без додавање стартер-култури, во млекарница во западниот дел на Македонија. Добиените резултати укажуваат на присуство на квасци во сите фази од подготовката на сирењето. Нивната застапеност во млекото беше во границите од 3,62–4,25 log CFU·ml^{-1} и се зголемуваше во текот на сувото зреење на сирењето. Максималната вредност од 5,91 log CFU·g^{-1} беше забележана во фазата на трет ден суво зреење во третиот производен процес. Во фазите на зреење на биеното сирење во саламура забележително е намалување на бројот на квасците достигнувајќи вредност од 3,60 log CFU·g^{-1}. Изолираните квасци беа детерминирани како *Kluyveromyces marxianus, Debaryomyces hansenii, Kluyveromyces lactis, Pichia polymorpha, Pichia membranifaciens, Trichosporon pululans, Torulopsis* sp., *Candida* sp. Што се однесува до мувлите, тие не беа забележани во сите фази на изработка и зреење. Нивно континуирано присуство во биеното сирење беше забележано од фазата на сувото зреење на сирењето сè до петнаесеттиот ден од зреењето во саламура. Изолираните видови мувли беа детерминирани како *Aspergillus niger, A. oryzae, Mucor mucedo, M. pusilus, Penicillium* sp., *Trichotecium* sp., и *Trichoderma viride.*

Клучни зборови: биено сирење: квасци; мувли: состав и динамика

INTRODUCTION

Cheeses are dynamic environment containing unique microorganisms (Beresford, 2001) that are changed in numbers and composition during their ripening. These changes occur as a result of nutrients and environmental condition modifications during cheese processing and ripening (Wiliams et al., 2002). The ripening process represent a wide range of biochemical reactions (Gardini et al., 2006)

in which, according to Beresford et al. (2001), the microorganisms that take part are mainly divided into two groups: starter lactic acid bacteria and secondary microorganisms. Yeasts are usually part of secondary microorganisms, but moulds are undesirable in cheese unless according to the production technologies are intentionally added. The adverse conditions that developed during ripening process like reduction of moisture content, decrease in pH, high salt concentration, low storage temperature, doesn't have negative impact on yeasts and moulds developing (Lopandic et al., 2006). They are able to ferment and assimilate the lactose, as well as organic acids produced by the lactic acid bacteria (LAB). According to Ferreira and Viljoen (2003) they have certain proteolytic and lipolytic abilities Yeast proteases and lipases participate in the formation of some aromatic components in cheese, but yeasts if present in large quantity can cause spoilage of cheese because they participate in the formation of excess gas, unpleasant smell, as well as softening and changing cheese color (Gardini et al., 2006). Moulds can be used as a starter culture in the production of certain types of cheese such as Roquefort, Blue, Stilton, Gorgonzola, Camembert, Brie, etc. Their role is to participate in the creation precursors of aroma components in cheeses which contribute to the improvement and emphasis on their taste and smell as well as modification of their structure (Wouters et al., 2002). The presence of moulds in the cheese is considered undesirable because they reduce its nutritional value and can be toxic due to the ability to produce mycotoxins which present a risk to human health (Hymery et al., 2014). During the production process of cheese, the additional input of moulds is caused by cheese production tools, air, shelves and walls in the facilities where cheese is made, ripened and kept (Basílico et al., 2002).

Beaten cheese is produced almost on the entire territory of North Macedonia and can be produced in diaries mostly from cows' milk by adding starter cultures, yet on the market a traditional types of beaten cheese are also found produced from raw cow's and sheeps' milk. It is semi-hard cheese with yellowish color and holes of different sizes and patterns that ripens and is kept in brine. The high concentration of salt is specific for this kind of cheese. The production technology itself is also characteristics because the curd is breaking in small pieces by a special device. This process is called "beating", thus providing the name of the cheese itself – beaten cheese (Sulejmani et al., 2014a). Although the studied beaten cheese was produced in industrial capacity, raw unpasteurized milk was used without adding starter cultures. Some of the tools used for manufacturing were similar to the traditional ones and were made of wood. All this in combination with production technology, characteristics of the geographical region and animal nutrition can have influence on taste and aroma of the produced cheese, but also on the number and composition of studied yeasts and moulds (Micari et al., 2007). The study of beaten cheese, the traditional one, was mostly performed by Sulejmani et al. (2014a, 2014b). Levkov et al. (2014, 2017, 2019) focused on physicochemical characteristics, proteolysis and volatile cheese components, and the microbiological characteristics of cheese, in particular, characteristics of lactic acid bacteria, but also yeasts and moulds were subject of investigation. More detailed data related to the presence of yeasts and moulds in beaten cows' cheese prepared in industrial capacity are not available. The aim of this research has been to determine the changes in number of yeasts and moulds during cheese production and ripening, as well as to isolate the yeasts and moulds species from different cheese production stages, and to identify them based on their morphological and physiological characteristics.

MATERIALS AND METHODS

Cheese making technology

The technology of cheese making is following: collected milk in dairy is preheated to a temperature of 34–37˚C and pour into a cheese tub. Then the commercial rennet is added into milk by constant mixing with wooden tool. The milk renneting last 40–45 minutes and the curd is crushed into small pieces with wooden tool. The curd is then left to rest for 20–30 minutes and the separated whey is removed from the tub. The curd is crushed with wooden tool again and warm water (60-75˚C) is poured over the curd. The water temperature was not constant and was different in different cheese making trials. After 30 minutes the water is removed from the tub and the curd is cut to a larger pieces and put in cheese clots. To remove the rest of the whey the curd is pressed using 300 kg press. The curd in cheese cloth is then hanged and left to drain for additional 24 hours on temperature of 30˚C. After that the cheese is left for dry ripening for five days. The cheese is then cut into pieces (7-8 cm wide) and salt with coarse salt. After 24 hours the cheese is put in 22% brine and left to ripe 45 days.

Although cheese was produced in dairy the technology of production is semi-industrial and more like traditional ones.

Cheese sampling

The analyses were made on milk and beaten cheese produced from raw cows' milk in a dairy in western region of North Macedonia. The samples were taken within three consecutive cheese production processes (repetitions). Samples of milk, curd, curd after draining, cheese during dry ripening and dry salting and after 10, 30 and 45 days of brining were taken aseptically and kept under refrigerated condition till the analytical process. Yeasts and moulds analyses were performed within 24 hours after sampling.

Determination of number and composition of yeasts and moulds

Aliquot of 10 ml of milk or 10 g of curd and cheese samples were taken. Serials of decimal dilutions of milk were made with a sterile physiological solution. Cheese samples were homogenized with 90 ml of sterile 2% sodium-citrate solution and then decimal dilutions with sterile physiological solution were made. Yeast extract glukose chloramphenicol agar (YGC agar) was inoculated with prepared samples and incubated under aerobic conditions at 25˚C for 5 days. Isolates of yeasts and moulds were obtained by random picking from YGC agar plates and were purified by consecutive subculturing on YGC agar. Purified isolates were kept on Malt Extract Agar and subcultured in the interval of one month. Determination of yeasts was made according to their morphological characteristics (appearance and color of colonies, appearance of cells under microscope and their size), the way of reproduction, and capability of spore formation as well as their physiological abilities such as fermentation and assimilation of glucose, galactose, lactose maltose and sucrose ability to form pellicle, ring or sediment. Identification was made according to the Lodder & Kreger-van Rij, (1952) and Kreger-van Rij (1984).

Morphological characteristics of moulds (appearance and color of colonies) and morphology of the vegetative mycelium were analyzed by slide observation under the microscope Nikon E 80. Determination of moulds was performed according to the "A Manual of Soil Ffungi" (Gilman, 1957) and "Practical Mycology Mmanual for Identification of Fungi" (Funder**,** 1953)

RESULTS AND DISCUSSION

The analyses of beaten cheese have shown the presence of yeasts throughout the entire production stages in three different production processes. Their presence was first ascertained in the milk from which the cheeses were made (Figure 1), in values ranging from 3.62–4.25 log CFU·ml^{-1}. As it was expected their number has been increased as a result of yeast entrapment in curd. During the period of dry ripening the number of yeasts has increased reaching their maximum value of 5.91 log CFU·g^{-1} in the 3rd day of ripening during the 3rd producing process. The pH value of cheeses during this stage of production was in the range of 4.90–5.27 thereby confirming their ability to survive adverse condition created during cheese ripening. During cheese brining the number of yeasts starts to decrease reaching value of 3.60 log CFU·g^{-1} in cheese from the 3rd production process. Gardini et al. (2006) indicates that high concentrations of NaCl can influence the metabolytic activities of yeasts as well as species succession. The results are similar in comparison to Levkov et al. (2019) who have studied the occurrence of yeasts and moulds in traditional beaten cheese made of sheeps' milk.

The results of this study are also in accordance with the studies of Özdemir et al. (2010), Kołakowski et al. (2012), Padilla et al. (2014), but the number of yeasts in the milk for the production of beaten cheese is higher than that compared to the milk used to produce Pecorino Crotonese cheese (1.07 log CFU·g^{-1} (Gardini et al., 2006). The presence of yeasts and moulds at the beginning of ripening of Karin Kaymagi cheese was 3.60–5.54 log CFU·g^{-1} while in the final product was 4.81–6.53 log CFU·g^{-1}. Peščić-Mikulec & Jovanović (2005) detected high proportion of yeasts in Serbian white traditional cheese as well. In the Italian sheep cheese Fiore Sardo, Di Cagno et al. (2003) found the presence of the yeasts amounting to 4 CFU·g^{-1}. The increase in the number of yeasts during the cheese ripening (Figure 1) can be influenced by higher temperature (25°C) of the room where cheese was ripened and kept. Yeasts can be additionally introduced into milk and further in cheese throughout wooden tools or cheese clots because they are widely spread in the premises where cheese is prepared or ripened, on the production surfaces, in the ripening equipment, even in the air (Cardoso et al., 2015). But the brine where cheese is kept and ripened can be also a source of yeasts and moulds in cheese (Kołakowski et al., 2012). The higher number of yeasts especially in the initial stage of

cheese maturing is probably as a result of the interaction with lactic acid bacteria, which reaches a maximum level in this production period (8.17 log CFU·g^{-1}. Yeasts can have a positive effect on the ripening process by stimulating the growth of the lactic acid bacteria because of their ability to synthesize vitamins and amino acids (Fereirra and Viljoen 2003). According to the authors, lactic acid bacteria with their metabolism enable the creation of organic acids used by the yeasts.

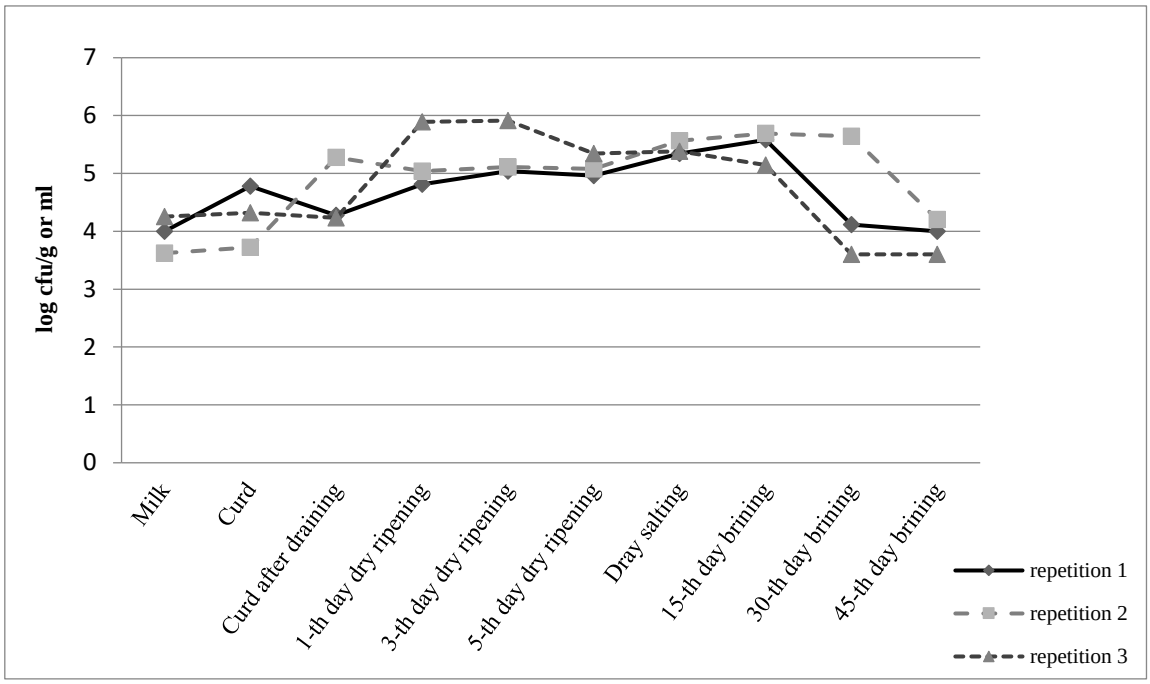

Fig. 1. Growth of yeasts during beaten cheese production and ripening

From three different production processes of beaten cheese a total of 30 isolates of yeasts were recorded. This isolates were determinate as *Kluyveromyces marxianus* 10%, *Debaryomyces hansenii* 2.6%, *Kluyveromyces lactis* 10%, *Pichia polimorpha* 10%, *Pichia membranifaciens* 10%, *Trichosporon pululans* 15.4%, *Torulopsis sp.*, 26%, *Candida crusei* 2.6%, *Candida sp.*10%.

It is noticeable that the genus *Torulopsis* and *Trichosporon* are dominant in studied cheese, especially the species *Trichosporon pullulans*. It is noticeable that yeasts are present in all stages of processing and ripening which indicates their importance of cheese structure, taste and aroma development. Representatives of the genera *Torulopsis, Trichosporon, Candida* and *Kluyveromyces* were found in milk, curd and in cheese during dry ripening. The representatives of *Debariomyces, Trichosporon* and *Pichia* were isolated from the stages of slating and brine ripening. The species *Trichosporon pullulans,* and *P. polimorpha,* were also isolated from the brine. The investigations of traditional ewe's milk beaten cheese by Levkov et al. (2019) are similar with the results of this study.

The species such as *K. lactis, D. hansenii, S. cerevisiae* are capable of fermenting lactose and galactose, and can also utilize more carbon and nitrogen sources and produce alcoholic, acidic and cheesy flavor (Gardini et al 2006). The influence of yeasts on the ripening of beaten cheese should be additionally determined by examining the proteolytic and lipolytic abilities of the isolated species. Studies by Wojtatowicz et al. (2001), Gardini et al. (2006), Ouadghri et al. (2014) show that yeasts have different abilities to participate in cheese ripening due to differences in their ability to break down cheese proteins and fats which depends on the activity of the intra and the extracellular enzymes. It is thought that species with extracellular proteolytic enzymes contribute to proteolysis of cheese (Gardini et al 2006).

Unlike yeasts the moulds were not noticed in all stages during beaten cheese making and ripening. Initially the milk used for cheese making in 3-th repetition contains 3.65 log CFU·ml^{-1} moulds (Figure 2). The curd form all three repetitions contains mould in quantum of 3.0-3.87 log CFU·g^{-1}.In

the subsequent stages of cheese ripening (dry ripening till 15-th day of brining) the moulds were continuously present. The cheese from repetition 3 in the last stages of ripening didn't contain moulds, unlike cheeses from the first and second repetition where the number of moulds were in the range of 3.47–4.50 log CFU·g^{-1}. It is important to emphasize that no visible appearance of moulds was observed in all stages of cheese ripening and making. All this results differ from study of Levkov et al. (2019) who stated much lower presence of these microorganisms in traditional beaten cheese.

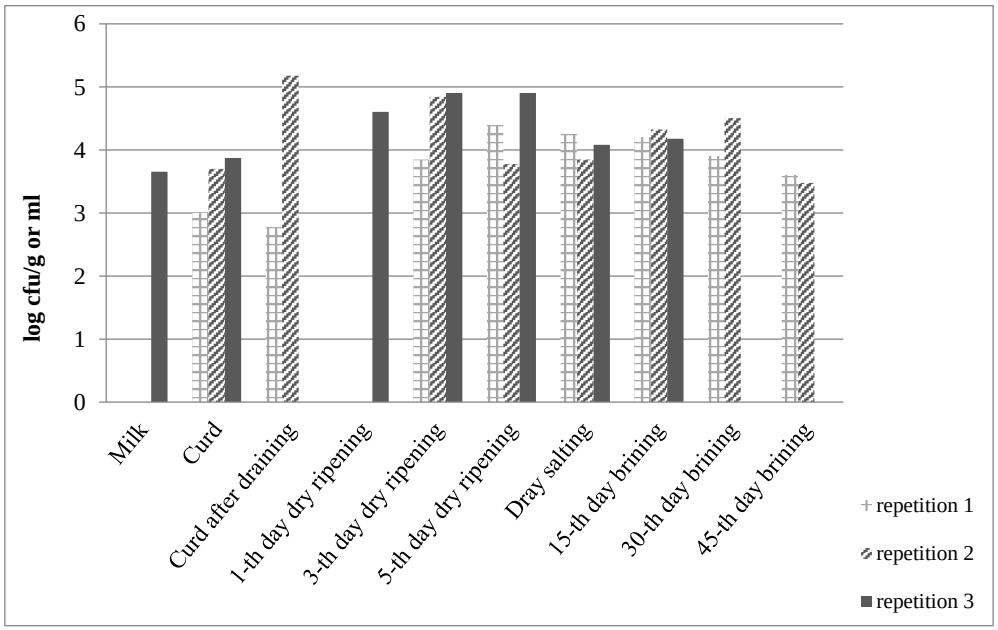

Fig. 2. Growth of moulds during beaten cheese production and ripening

From the studied cheese 11 mould isolates were recorded. They were determined as *Aspergillus niger* 9.9%, *A. oryzae* 9.9%, *Mucor mucedo* 18.1%, *M. pusilus* 9.9%, *Penicillium* sp., 18.1%, *Trichotecium* sp. 18.1%, *Trichoderma viride* 9.9%. The mould species isolated from studied beaten cheese are different compared to the mould species determined in traditional one (Levkov et al. 2019). This is expected since the moulds enter the milk and cheese during milking process or cheese preparing. The milk tanks, wooden tools for breaking milk coagulum, the walls of the premises, the wooden inventory in the premises for production and ripening of cheese, are natural habitats of moulds and their spores (Banjara et al., 2015), and can easily spread into the milk and cheese through the air.

The presence of moulds during ripening and salting indicates their adaptability to low pH, high concentration of salt and high concentration of fat (Fox et al., 2000; Basílico et al., 2001). The number of fungi in both variants of beaten cheese was similar to the number of fungi in the Karin Kaymagi cheese (Özdemir et al., 2010). In the traditional Montasio cheese, the moulds were present during the whole cheese production process (Marino et al., 2003). The authors believe that they inhabit the walls and shelves where cheese is kept. Their proper and regular cleaning can cause mould removal and growth control. Freitas & Malcata (2000) in the Idiazábal cheese found the presence of mould of the genus of *Penicillium, Cephalosporium, Aspergillus, Geotrichum, Pullularia, Mucor, Candida, Acremonium.* In the Roncal cheese, existing moulds belonged to the following nine genera: *Penicillium, Cephalosporium, Aspergillus, Geotrichum, Pullularia, Mucor, Paecilomyces, Candida, Acremonium*. Kure & Skaar (2000) isolated and identified visible moulds of two types of Norwegian semi-hard cheese Jarlsberg and Norvegia. In both cheeses, the most commonly isolated species was *P. roqueforti* ssp. *roqueforti*. In addition, 69.8% of the isolates belonged to the genus *Penicillium*, as follows: *P. solitum, P. commune, P. palitans*. Of the two types of cheese, other types of fungi belonging to the following genus were also isolated: *Alternaria, Aureobasidium, Cladosporium, Epicoccum, Mucor, Geotrichum, Phoma* and *Ulocladium.*

Isolated species of *Aspergillus and Penicillium*, are known producers of mycotoxins that can be dangerous for human health. Cheese contamination with mycotoxins is most commonly due to the use of contaminated milk for cheese preparation or less frequently as a result of the growth of fungi that produce mycotoxins (Hymery et al,, 2014; Banjara et al., 2015). Although mycotoxogenic moulds are isolated from studied beaten cheese, they do not pose a threat to the health of consumers, as the synthesis of mycotoxins in moulds differs, that is, not every species is capable to produce those (Hymery et al., 2014). However, it is necessary to take greater care in reference to hygiene in the locations where milking takes place, as well as in places where milk is processed and produced into cheese. Regular cleaning and sanitation is also required with appropriate devices for such purposes.

CONCLUSION

The presence of yeasts in beaten cheese in all stages of its production and ripening indicate their significant role in the formation of its taste and aroma. The formation of precursors of aromatic compounds might be a result of activity of *Trichosporon pullulans, Debariomyces hansenii, Kluyveromyces lactis*. The number and composition of the yeasts present in examined cheeses can also be affected by the additional contamination from the equipment and the environment in which cheese is produced. However, further researches need to be conducted in order to determine the proteolytic and lipolytic properties of the isolated species. Moulds, on the other hand, are result of cheese contamination from the environment where it is produced and ripened. In order to maintain good quality of cheese, regular hygiene and sanitation of the equipment, devices and premises needs to be done.

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