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MICROBIOLOGICAL QUALITY OF SMEAR-RIPENED CHEESES STORED IN DIFFERENT TEMPERATURE REGIMES

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ABSTRACT

The purpose of this work was to study smear-ripened cheese, especially its microbiological quality. Samples were stored in different temperature conditions. The first group (A) of samples was stored in a refrigerator at 6 °C. The second group (B) of samples was stored at 6 °C for 21 days, next at -18 °C for 7 days and 7 days at 6 °C. The third group (C) of samples was stored at 6 °C before the date of minimum durability, next 7 weeks at -18 °C and after that at 6 °C for 7 days. I have observed lactic acid bacteria, *Brevibacterium linens*, coliforms, psychrotrophic organisms, *Escherichia coli***,** moulds, and yeast. The number of *Brevibacterium linens* was higher (p <0.05) at cooling/freezing for 1 week (log CFU.g^{-1}) than cooling (log CFU.g^{-1}) and cooling/freezing for 7 weeks after 35 days. A higher (p <0.05) number of psychrotrophic microorganisms was recorded at the end of the monitoring in samples stored in a refrigerator (A/49) in comparison with cheeses stored using cooling/freezing regime for one week (B/49). Among the samples stored at these temperature regimes, there were no statistically significant differences (p >0.05) in the numbers of LAB, coliforms, *E. coli*, moulds and yeast, neither at the end of the DMD nor at storage for 49 or 91 days, respectively.

Keywords: smear–ripened cheese; lactic acid bacteria; coliforms; moulds; yeast; *Brevibacterium linens*

INTRODUCTION

Cheese production is based on the use of both defined starter cultures and the presence of undefined indigenous microbial populations, including diverse yeasts and moulds, Gram-positive and Gram-negative bacteria **(Monnet et al., 2015; Adouard et al., 2015; Cotona et al., 2012)**. Smear-ripened cheeses belong to the group of acidic cheeses, characterized by distinctive to spicy fragrance and flavour typified by orange or golden yellow smear **(Teubner, 1998; Galaup et al., 2015)**. They gain these properties during aging especially through the action of proteolytic bacteria *Brevibacterium linens* **(Fox, 2004)**. This sebaceous micro flora facilitates typical ripening predominantly from the surface toward the centre **(Kadlec et al., 2012)** and contributes (apart from the influence of physical and chemical properties of milk, starter culture, and non-starter bacteria) to the development of the cheese characteristics **(Smit, 2003)**. In the cheese core, the dominant micro flora usually corresponds to lactic acid bacteria (LAB) species, while Gram-positive and catalase-positive bacteria, as well as yeasts, moulds and diverse Gram-negative bacteria *(Citrobacter spp., Enterobacter spp., Pseudomonas spp.)* constitute the subdominant micro flora **(Cotona et al., 2012)**. The presence of some Gram-negative bacteria is often used as a marker for hygiene conditions, as coliforms are indicative of faecal contamination, and they are also considered to be undesirable cheese contaminants **(Bockelmann et al., 2005)**. Contamination can occur not only during the processing of raw materials or food, including slicing, washing or cooling with contaminated water, contaminated tools, surface, or production worker, but also in packaging, transporting and distribution or through reservoir organisms, insects, soil and air **(Capozzi et al., 2009)**. The aim of this study was to perform microbiological analysis of smear-ripened cheese stored in three different temperature regimes and to monitor the development of cultural and contaminating microflora.

MATERIAL AND METHODOLOGY

We have analysed samples of smear-ripened cheeses, which were supplied by the manufacturer in consumer packaging weighing 100 g in cold boxes always on the day of production. Then we have stored the samples at the selected temperature conditions. For monitoring of microbiological indicators, we have chosen three types of storage regimes in accordance requirements of producer:

Type A**:** Storage in a refrigerator at 6 °C up to the date of minimum durability (DMD) and 14 days beyond this date. We have collected samples on the day of production (A/0), at the end of the DMD (A/35), and two weeks after the end of the DMD (A/49).

Type B**:** Storage of cheese after manufacture at cold storage temperature of 6 °C for 21 days. Cheeses were then frozen to a temperature of -18 °C for 7 days. For the following 7 days, they were again kept at cold storage temperature of 6 °C. We have collected samples on the day of production (B/0), at the end of the DMD (B/35), and two weeks after the end of the DMD (B/49).

Type C: Storage of cheese at cold storage temperature of 6 °C throughout the DMD (35 days). The samples were then kept frozen at -18 °C for 7 weeks. Subsequently the samples were unfrozen and stored at cold storage temperature of 6 °C for 7 days. We have collected samples at the end of the DMD (C/35) and 8 weeks after the end of the DMD (C/91).

We have monitored the following microbiological indicators: lactic acid bacteria (according to **ISO 15214:1998),** cultivation on MRS agar (Noack, France) at 30 °C, the counting of colonies was carried out after 72 hours. We have determined psychrotrophic microorganisms according to **ISO 17410:2001** on PCA growth medium (Noack, France); cultivation was carried out at 6.5 °C for 10 days. We have determined moulds and yeasts according to **ISO 21527-1:2008** on the DRBC growth medium (Noack, France); cultivation was carried out at 25 °C for 3 to 5 days. We have determined coliform microorganisms according to **ISO 4832:2010** on VRBL growth medium (Noack, France), at 37 °C. We have carried out counting of colonies after 48 hours. We have cultivated *Escherichia coli* on COLIFORM agar (Merck, Germany) for 48 hours at 37 °C. We have performed determination of *Brevibacterium linens* on agar M17 (Noack, France), cultivation was at 30 °C for 48 hours. During the experiment, we have successively analysed 5 batches of cheese. For one analysis we have always used 3 consumer packages. We have carried out analyses in the microbiology laboratory of the Department of Food Technology at the Mendel University in Brno from March to July 2011, according to **ISO 7218: 2007**.

From each 100 g package we have cut off 10 g of sample using a sterile scalpel. In order to get a representative sample, the sector passed through all of the segments. The sample included both the centre and edge of the cheese. After adding of 90 mL saline, we have homogenized the sample and diluted it to the desired decimal dilution.

We have performed statistical analysis using Statistica CZ, version 10. The program calculated basic statistical characteristics, such as mean and standard error of the mean. For comparison of groups, it has used simple analysis of variance. We have also used regression analysis.

RESULTS AND DISCUSSION

The aim of the experiment was to monitor the number of different groups of microorganisms during storage under different temperature conditions and to compare these numbers at the end of minimum durability and at the end of storage. The microorganisms included coliforms, psychrorotrophs, moulds, yeast, *Escherichia coli*, *Brevibacterium linens***,** and lactic acid bacteria.

Lactic acid bacteria (LAB)

At the beginning of the monitoring (A, B, C/0), the number of LAB was 8.6 log CFU.g^{-1}. Number of LAB (Figure 1) at the end of the minimum durability after 35 days of storage in regime A was 8.3 log CFU.g^{-1}. In the storage regime B, the number of LAB was 8.7 log CFU.g^{-1} and in regime C it was 8.3 log CFU.g^{-1}. At the end of storage, the number of LAB for cheeses stored in regime A after 49 days was 8.9 log CFU.g^{-1}. In regime B, it was 8.7 log CFU.g^{-1} and in storage regime C after 91 days, it was 8.8 log CFU.g^{-1}. Among the samples stored at these temperature regimes, there were no statistically significant differences (p >0.05) among the number of LAB both at the end of the DMD and the storage for 49 and 91 days respectively. Lactic acid bacteria used in manufacturing of smear-ripened cheese belong to the group of mesophilic bacteria, whose temperature minimum is between 5 °C and 15 °C. It is interesting, that the difference between the appropriate minimum temperature and the reasonable optimum temperature is about 22 °C. On the other hand, the difference between the optimum temperature and the maximum temperature is about 6.5 °C. This indicates that microorganisms tolerate suboptimal temperatures better than hyperoptimal ones. As stated by **Görner and Valík (2004)**, freezing is not the cause of death of all cells and this may be the cause for permanently higher numbers of LAB in samples stored in various temperature regimes. **Komprda et al., (2012)** also found the same number of lactic acid bacteria (8.2 log CFU.g^{-1}) at the end of the DMD in the smear-ripened cheeses stored at 5 °C. Likewise, according to **Tan et al., (2008)**, the numbers of LAB in smear-ripened cheeses range between 8 and 9 log CFU.g^{-1}.

Brevibacterium linens

The determined quantity of *Brevibacterium linens* at the beginning of storage (A, B, C/0) was 8.6 log CFU.g^{-1}. At the end of the DMD after 35 days of storage (Figure 2) in storage regime A, the detected value was 7.8 log CFU.g^{-1}. In the storage regime B, the value was 8.3 log CFU.g^{-1} and in the storage regime C, the value was 7.8 log CFU.g^{-1} of *Brevibacterium linens.* At the end of storage in the regime A after 49 days, the recorded value was 8.4 log CFU.g^{-1}. In the storage regime B, the value was 8.1 log CFU.g^{-1} and under the conditions of regime C after 91 days, it was 7.8 log CFU.g^{-1} of the bacteria *Brevibacterium linens.* Statistically significant differences (p <0.05) were found in the numbers of *Brevibacterium linens* at the end of the DMD (after 35 days) and at the end of monitoring after 49 and 91 days, respectively. Higher (p <0.05) counts of *Brevibacterium linens* have occurred at the end of the DMD in the storage regimen B (B/35), when compared to other storage methods. At the end of monitoring after 49 and 91 days, respectively, I have found higher number (p <0.05) of bacteria in samples stored in the refrigerator (A/49), compared to the samples stored in the regime C (C/91). *Brevibacterium linens* may assert itself on the surface of the cheese after the present lactic acid is metabolized and neutralized by yeast and cocci and the surface pH rises to values of 5.7 to 6 **(Kadlec et al., 2012, Mounier et al., 2008)**. **Eliskases-Lechner and Ginzinger (1995)** found after 6 weeks of aging in refrigerated conditions *Brevibacterium linens* at the surface of the cheese to be 9 log CFU.cm^{-2}. During freezing, the concentration of water useful for microrganisms decreases and their growth is partly or completely inhibited **(Görner and Valík, 2004)**. However, freezing only brings the activity of microorganisms to a standstill, rather than killing them **(Šilhánková, 2008)**. This fact may cause a lower number of *Brevibacterium linens* at the end of storage after long-term freezing. Storing in a refrigerator leads to multiplication of bacteria due to a suboptimal temperature, but in the other temperature regimes, it leads to stabilization of the number of *Brevibacterium linens.* Therefore, a short-term freezing should not influence the number *Brevibacterium linens.*

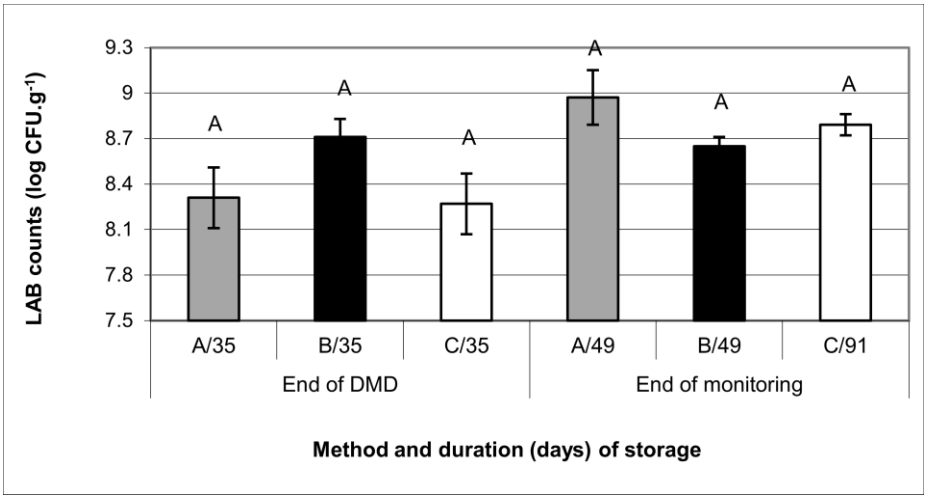

Figure 1 Number of LAB (log CFU.g^{-1}) in the smear-ripened cheese stored for 35, 49, and 91 days respectively, under different temperature regimes: cooling (A), cooling/freezing 1 week (B), cooling/freezing 7 weeks (C), n = 15.

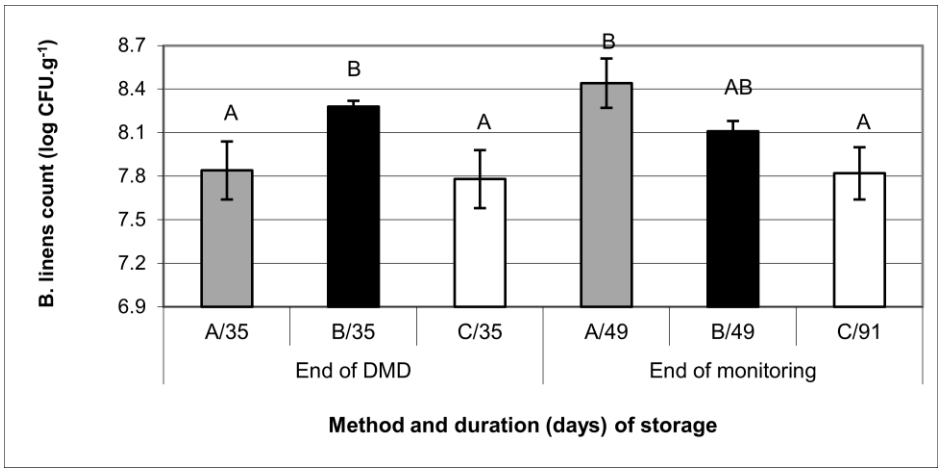

Figure 2 Number of *Brevibacterium linens* (log CFU.g^{-1}) in the smear-ripened cheeses stored for 35, 49, and 91 days respectively, under different temperature regimes: cooling (A), cooling/freezing 1 week (B), cooling/freezing 7 weeks (C). Averages labelled with different letters were statistically different (p <0.05) within a given factor, n = 15.

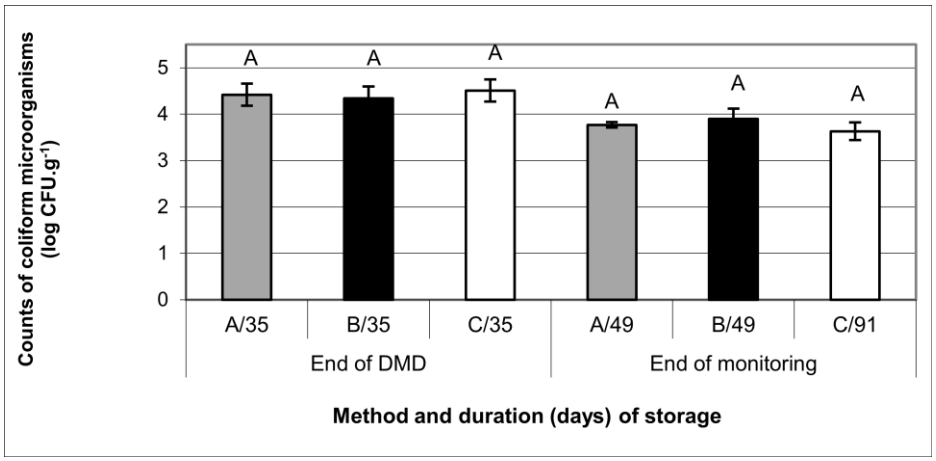

Figure 3 Number of coliform microorganisms (log CFU.g^{-1}) in smear-ripened cheeses stored for 35, 49, and 91 days respectively, under different temperature regimes: cooling (A), cooling/freezing 1 week (B), cooling/freezing 7 weeks (C), n = 15.

Coliform microorganisms

In the analysed cheeses stored at different temperature conditions, the counts of coliform microorganisms were comparable (p >0.05) both at the end of the DMD and at the end of monitoring (Figure 3). At the beginning of storage (A, B, C/0), the count of coliforms detected in cheeses was 4.7 log CFU.g $^{-1}$. When stored in the regime A after 35 days, I have found 4.4 log CFU.g^{-1} of coliform microorganisms. In the storage regime B, it was 4.3 log CFU.g^{-1} and under the temperature conditions of regime C, it was 4.5 log CFU.g^{-1}. At the end of the monitoring, the number of coliform microorganisms in the storage regime A, after 49 days, was 3.8 log CFU.g^{-1}.

In the storage regime B, it was 3.9 log CFU.g^{-1} and in the regime C, it was 3.6 log CFU.g^{-1}. **Dolci et al., (2009)** found on the surface of smear-ripened cheeses 5-6 log CFU.cm^{-2} of coliform microorganisms. **Maher et al., (2000)** stated an increase in the number of coliforms within 42 days of ripening of these cheeses. **Doležalová et al., (2013)** have found lower counts of coliform bacteria in smear-ripened cheeses stored at temperatures up to 8 °C, namely 4.6 log CFU.g^{-1}, which corresponds with our results.

Escherichia coli

I have not found any statistically significant differences (p >0.05) among the numbers of *E. coli* in cheeses stored in various temperature regimes at the end of the DMD or at the end of monitoring after 49 and 91 days respectively (Figure 4). At the start of storage (A, B, C/0), the number of *E. coli* was 1.0 log CFU.g^{-1}. The number of colonies in storage regime A after 35 days was 1.2 log CFU.g^{-1}. When stored at the temperature regime B, the count was 1.7 log CFU.g^{-1} and under the conditions of regime C, it was 1.0 log CFU.g^{-1}. At the end of the monitoring in the regime A after 49 days, the count of *E. coli* was 1.8 log CFU.g^{-1}. In the storage regime B, I found it to be 0.9 log CFU.g^{-1} and under the conditions of regime C, after 91 days, it was 1.8 log CFU.g^{-1}. In comparison to our data, **Maher et al., (2000)** detected much larger numbers of *E. coli*. After 42 days of curing, in smear-ripened cheeses they found 4.0 log CFU.g^{-1} of these bacteria. According to **Görner and Valík (2004)**, breeding of *Escherichia coli* does not take place at temperatures below 5 °C. This is consistent with our results as well as with those from many other studies. According to **Frazier and Westhoff (1988)**, the growth rate of microorganisms decreases after reaching this minimum temperature. However, a slow metabolic activity can proceed, which may cause a tendency of slow growth of bacteria in the storage conditions of the regime A. It also explains the tendency to increase the number of bacteria in the regime C that occurred after defrosting the sample and its subsequent storage at 6 °C. **Heredia et al., (2009)** have also confirmed that *Escherichia coli* can reliably survive temperature of -20 °C in a freezer. **O'Brien et al., (2009)** state the minimum temperature for the growth of these pathogens to be approximately 7 °C. In cheese, *E. coli* is used as an indicator for assessing post-pasteurisation contamination. Its presence may indicate inadequate pasteurisation, poor hygiene conditions during processing, or post-processing contamination **(O'Brien et al., 2009)**.

Psychrotrophic microorganisms

Their number was at the beginning of monitoring (A, B, C/0) 7.9 log CFU.g^{-1}. At the end of the DMD after 35 days, the number of psychrotrophic microorganisms in regime A was 7.9 log CFU.g^{-1}. In the storage regime B, it was 8.3 log CFU.g^{-1}. For the samples stored under the regime C 7.9 log CFU.g^{-1} (Figure 5). There were no statistically significant differences (p >0.05) among the samples in the number of psychrotrophic microorganisms. At the end of monitoring after 49 days in the regime A, I have detected 8.8 log CFU.g^{-1} of them. In the storage regime B, the count was 8.4 log CFU.g^{-1}. In the storage regime C after 91 days, I have detected 8.5 log CFU.g^{-1} of psychrotrophic microorganisms. I have recorded a higher (p <0.05) number of psychrotrophic microorganisms at the end of the monitoring, in samples stored in the refrigerator (A/49) in comparison with cheeses stored by using cooling/freezing method for one week (B /49). Decreasing temperature of the environment significantly extends the generation time and the lag phase of psychrotrophic microorganisms. According to **Gorner and Valík (2004)** these microorganisms grow in acidic cheeses at temperatures higher than 5 °C, which explains the increase in the number of microorganisms after 49 days at conditions in a cooler. Microbial cells do not tolerate repeated freezing and thawing, but devitalization in this manner is not reliable. If thawing follows a short time after the freezing, there is an increase in the number of these bacteria and under these conditions they can survive in the long run **(Žiška and Martinková, 1990)**.

Moulds

At the beginning of storage (A, B, C/0), the number of moulds was 7.5 log CFU.g^{-1}. After 35 days (Figure 6), in the storage regime A, I have found 7.6 log CFU.g^{-1} of moulds. In storage regime B, I have found 7.1 log CFU.g^{-1} of moulds and in the storage regime C their number was 7.4 log CFU.g^{-1}. I have found no statistically significant differences (p >0.05) in the number of moulds among the samples stored in different storage regimes. At the end of monitoring of samples stored at the regime A after 35 days, we have detected 7.9 log CFU.g^{-1} of moulds. In the storage regime B, the count was 7.8 log CFU.g^{-1} and in samples stored in the regime C after 91 days it was 7.6 log CFU.g^{-1}. At the end of monitoring, after 49 and 91 days respectively, I could not demonstrate any statistically significant differences (p >0.05) in the numbers of moulds among samples stored at different temperature regimes. The reason for the high numbers of moulds, even in frozen specimens, may be the fact that some species can be extremely psychrotrophic. They stop their growth at temperatures as low as -20 °C to -30 °C when all water freezes out in the food **(Görner and Valík, 2004)**. According to **Sørhaug (2011)**, moulds are commonly present not only in the air, but also occur on the production equipment and can degrade various types of cheeses. Using the cooler temperatures, however, reduces the risk of mycotoxins, which could adversely affect the health of consumers.

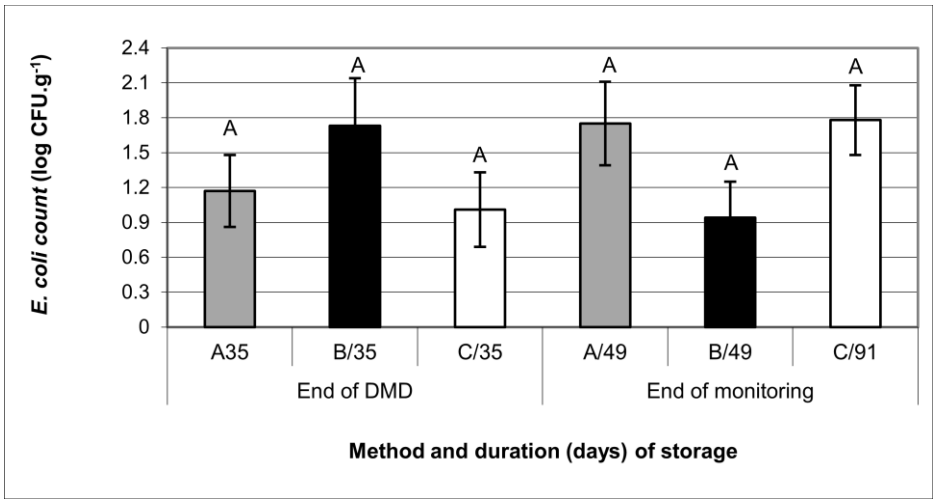

Figure 4 Number of *E. coli* (log CFU.g^{-1}) in smear-ripened cheeses stored for 35, 49, and 91 days respectively, under different temperature regimes: cooling (A), cooling/freezing 1 week (B), cooling/freezing 7 weeks (C), n = 15.

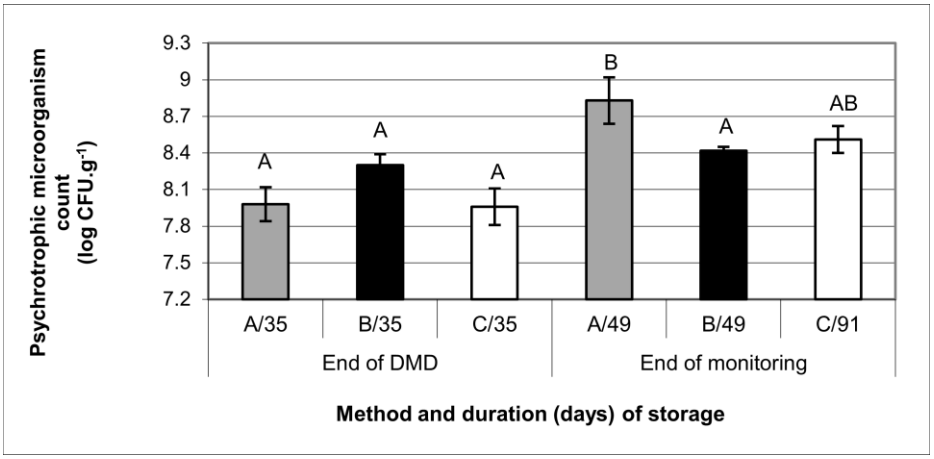

Figure 5 Number of psychrotrophic microorganisms (log CFU.g^{-1}) in smear-ripened cheeses stored for 35, 49, and 91 days respectively, under different temperature regimes: cooling (A), cooling/freezing 1 week (B), cooling/freezing 7 weeks (C). Averages labelled with different letters were statistically different (p <0.05) within a given factor, n = 15.

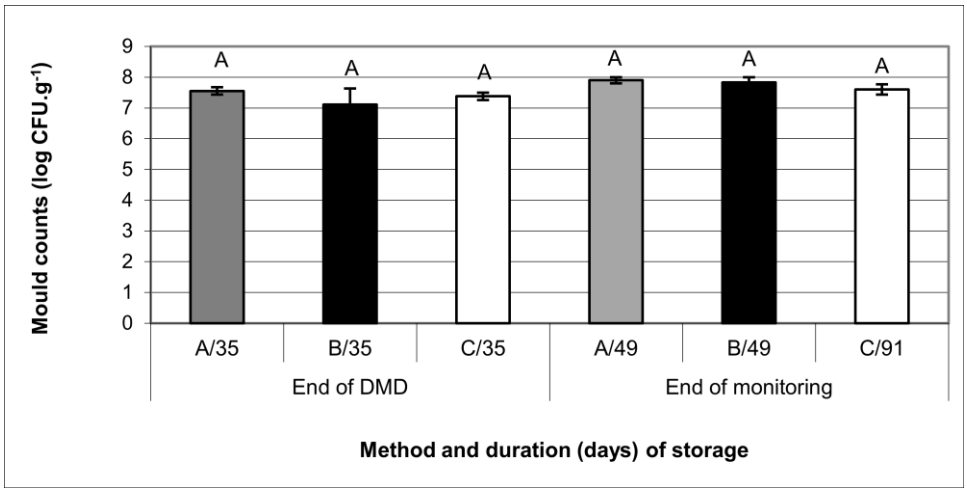

Figure 6 Number of moulds (log CFU.g^{-1}) in smear-ripened cheeses stored for 35, 49, and 91 days respectively, under different temperature regimes: cooling (A), cooling/freezing 1 week (B), cooling/freezing 7 weeks (C), n = 15.

Moulds can easily contaminate the cheese production site and the product itself. They grow very easily on cheese because they can overcome conditions unfavourable to other microorganisms, such as low temperatures, reduced water activity, high salt concentrations, low pH, and lack of carbohydrates **(Bachmann et al., 2005)**.

Yeast

At the beginning of monitoring (A, B, C/0), their count was 7.9 log CFU.g^{-1}. Both at the end of the DMD and at the end of storage, we could not detect any statistical difference in the number of yeasts (p >0.05). In samples stored in the temperature regime A after 35 days, I have detected 7.9 log CFU.g^{-1} of yeast. In the samples of the temperature regime B, the yeast count was 8.1 log CFU.g^{-1}. In the samples in the temperature regime C, the yeast count was 8.0 log CFU.g^{-1}. In samples analysed at the end of the monitoring, after 49 days in the regime A, I have detected 8.1 log CFU.g^{-1} of yeast. In the regime B, the count was 8.3 log CFU.g^{-1} and after 91 days in regime C, I found 8.1 log CFU.g^{-1} of yeast (Figure 7). The number of yeasts in the samples examined at the end of the monitoring after 49 and 91 days respectively, I have not found any statistical difference (p >0.05) among the storage regimes temperatures. Higher yeast counts are caused by their addition to the sebaceous cultures. This also agrees with the findings by **Eliskases-Lechner and Ginzinger (1995)**, who investigated the occurrence of yeasts in soft, smear-ripened cheeses. They found that after 21 days, the yeast count was 7 log CFU.g^{-1}. **Dolci et al., (2009)** reported the surface yeast counts to be 6 log CFU.cm^{-2}. Likewise **Doležalová et al., (2013)** reported the number of yeasts and moulds up to 7 log CFU.g^{-1}. According to **Görner and Valík (2004)** the yeast counts during maturation range between 4 and 9 log CFU.cm^{-2}. In an experiment by **Wyder and Puhan (1999)** yeast counts during maturation reached 8 log CFU.g^{-1}. **Carminati (1999)** reported 7.7 log CFU.g^{-1} after 40 days of storage. **Irlinger and Mounier (2009)** recorded similar yeast counts. At the beginning of ripening, the yeast count in their experiment was 7 log CFU.cm^{-2}, while in the middle of the ripening time it increased to 7.7 log CFU.cm^{-2}.

The results show that at the end of the experiment the number of LAB or *Brevibacterium linens* did not decrease**,** which means that after thawing the process of ripening continued, while maintaining the sensory quality of the product **(Jarošová and Cwiková, 2014)**. In connection with it the number of coliform microorganisms decreased. *B. linens* namely produce antimicrobial substances that inhibit the growth of many food-poisoning bacteria as well as several yeasts and moulds. Regarding *E. coli*, the Commission Regulation (EC) No 2073 (2005) gives as a criterion for processing cheese made from heat-treated milk in 5 samples number up to 2 log CFU.g^{-1}, wherein two of the five samples may contain 3 log CFU.g^{-1}. These values were not achieved even after 91 days of storage. Although *E. coli* survives cooling plant temperatures, subsequent low temperature storage (6 °C) and high number of cultural micro flora prevented the increase of the number of the bacteria. *Escherichia coli* are used as an indicator of direct or indirect faecal contamination of food, and therefore the possible presence of enteric pathogens. Our figures therefore point to a strict adherence to hygiene throughout the production process. Yeast counts were comparable in all types of storage regimes, both at the end of the DMD and at the end of monitoring and matched the values reported in other studies. Relatively high number of yeast is due to the fact that they are added as culture microorganisms. Coexisting yeasts and bacteria often reach high population densities, typically between 8 and 10 log CFU.g^{-1} of cheese at the time of consumption. The high number of moulds is related to the fact that *G. candidum* although taxonomically a yeast, is usually automatically assigned to moulds because of its appearance. However, white moulds, which were found during our experiment on smear-ripened cheeses, are still generally considered to be undesirable contaminants. The issue of moulds in smear-ripened cheeses is still little explored. One possible way to minimize contamination by moulds (excluding high-quality raw materials and adherence to good manufacturing and hygienic practices in the production) may be filtering the air in the indoor

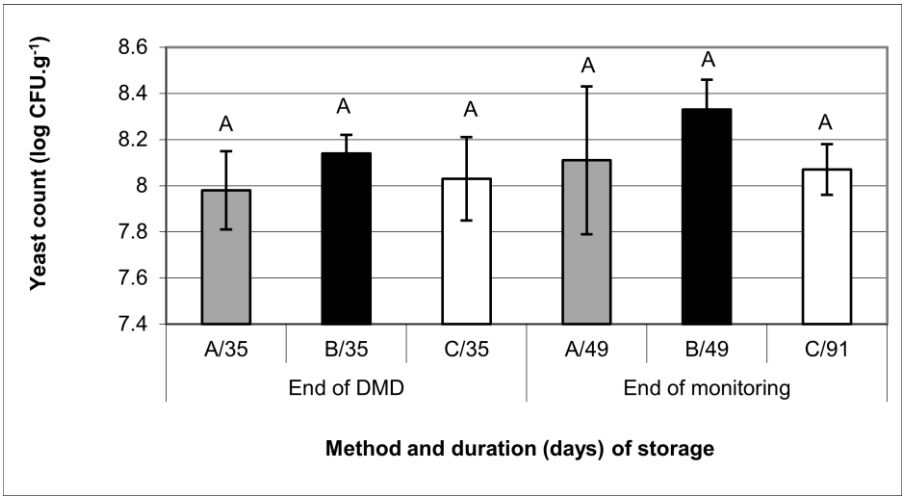

Figure 7 Number of yeasts (log CFU.g^{-1}) in smear-ripened cheeses stored for 35, 49, and 91 days respectively, under different temperature regimes: cooling (A), cooling/freezing 1 week (B), cooling/freezing 7 weeks (C), n = 15.

facility.

The gained results will be used by the manufacturers of smear-ripened cheeses to find possible ways to extend the DMD of cheeses without compromising their quality or breaching of safety.

CONCLUSION

Smear-ripened cheeses in addition to cultural microflora also contain undesirable microorganisms. They contribute not only to spoilage, but may also include dangerous pathogens that are able to cause foodborne illness. One of the easiest ways to reduce potential risk to a minimum and maintain the required quality is to keep these products at the appropriate temperature. This reduces the growth of undesirable microorganisms, while allowing for the maturation and development of the sensory properties of cheese.

The aim of our study was to assess whether the different temperature regimes will maintain the development of cultural micro flora and will continue maturing and how the selected temperature regime will affect the number of contaminating microorganisms.

During the storage (49 days), the number of LAB, *Brevibacterium linens*, *E. coli*, and yeasts remained unchanged (p >0.05). The number of coliform microorganisms decreased (p <0.001), while the number of psychrotrophic microorganisms and moulds increased (p <0.001).

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