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Potravinarstvo Slovak Journal of Food Sciences
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Slovak Journal of Food Sciences

Potravinarstvo Slovak Journal of Food Sciences
vol. 14, 2020, p. 1131-1136
https://doi.org/10.5219/1361
Received: 24 February 2020. Accepted: 6 September 2020.
Available online: 28 November 2020 at www.potravinarstvo.com

ISSN 1337-0960 (online)

INFLUENCE OF DIFFERENT STORAGE CONDITIONS ON THE OCCURRENCE OF ENTEROCOCCI IN SMEAR RIPENED CHEESES

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ABSTRACT

The number of enterococci was monitored in smear-ripened cheeses stored under different temperature regimes. Sampling and subsequent analyses were performed on the day of manufacture (A/0 = B/0 = C/0), at the end of BBD (A/35, B/35, C/35), two weeks after BBD (A/49, B/49), and eight weeks after BBD (C/91). No statistical difference (p >0.05) was found in the numbers of enterococci in cheeses stored under different temperature regimes until the Best-Before date or at the end of monitoring after 49 and 91 days respectively. At the beginning of storage (A/0, B/0, C/0), the numbers of enterococci in cheeses were 2.3 log CFU.g^{-1}. The highest number of enterococci was recorded after 49 days of storage at 6 °C at 5.4 log CFU.g^{-1}. During storage, there was an increase (p <0.05) in the numbers of enterococci in all types of temperature regimes. Enterococci content was influenced (p <0.05) by both the storage period and storage method (temperature regime).

Keywords: *Enterococcus* spp.; smear-ripened cheese; storage period and temperature

INTRODUCTION

Enterococcus sp. is a gram-positive bacterium found in various places: it is a commensal living in the digestive tract of animals, insects and humans **(Li et al., 2017)**, often found in fermented foods such as fermented dairy and meat products, in soil, water and plants **(Lebreton et al., 2014; Fuka et al., 2017)**. Enterococci are found in large quantities in traditional products made from raw milk, particularly in cheeses. Enterococci are associated with traditional European cheeses manufactured in Mediterranean countries, such as Greece, Italy, Spain and Portugal, from raw or pasteurized goats', ewes', water-buffalos', or bovine milk **(Moreno et al., 2006)**. Although their presence is generally considered to be due to inadequate hygiene conditions during processing **(Gelsomino et al., 2002)**, they can act as natural starter cultures in the production of various types of cheeses **(Giraffa, 2003)**. These are artisan cheeses produced in Southern Europe, e.g. Venaco cheese **(Casalta and Zennaro, 1997)**. Enterococci are found in cheeses made from both raw and pasteurized milk because they survive the pasteurization temperature **(İspirli et al., 2017)**. The resistance of enterococci to pasteurization temperatures and their ability to adapt to different substrates and different growth conditions not only leads to their discovery in foods made from raw materials (milk, meat) but also in foods obtained by the thermal process. They are therefore able to survive the conditions of food production. They can also contaminate finished products **(Hanchi et al., 2018)**. However, their incidence in traditional cheeses made from raw cow's milk of high microbiological quality is low **(Garabal et al., 2008)**, which is likely to result in the loss of some taste attributes.

In terms of pathogenicity and antibiotic resistance, as well as biogenic amine production, enterococci in food, of course, are not desirable microorganisms.

However, some enterococci are used in the industry **(Ogier and Serror, 2008)** because of their biochemical properties suitable for technological applications **(Centeno and Carballo, 2015)**. Some strains have been designed as complementary starting microbiota **(Franz et al., 2001; Moreno et al., 2003)**, *E. durans, E. faecalis* and *E. faecium* are also marketed as probiotic cultures **(Centeno and Carballo, 2015)**, show antimicrobial effects against alimentary pathogens, which is related to the presence of genes encoding enterotoxin production **(İspirli et al., 2017)**. For this reason, *Enterococcus* sp. is one of the most controversial genera belonging to lactic acid bacteria **(Lebreton et al., 2014)**. Enterococci can adapt to adverse environmental conditions. They are also known for their antibiotic resistance. The most important enterococcal diseases include urinary tract infections, nosocomial infections and superinfections, meningitis, bacterial endocarditis and bacteraemia **(Gardini et al., 2001; Arias and Murray, 2008)**.

Scientific hypotheses

Hypothesis 1: Enterococci survive the freezing process and their number does not change significantly during the freezing of cheeses.

Hypothesis 2: The duration of the storage of cheeses affects their safety in terms of enterococcal content.

MATERIAL AND METHODOLOGY

The smear ripening cheese for testing was delivered as small rounds (diameter 45 mm, height 10 mm), where a 100 g package contained 5 pieces of these round portions. Before shipment, the cheeses ripened for 7 days, while the best before date (BBD) on the package was 28 days. A total of 5 batches of the product were analyzed. Each batch of the samples was made from different pasteurized milk. Analyzed smear-ripened cheese is produced from fat-free sour curd. To the curd was added water, neutralization salts and *Candida valida* and bacteria *B. linens*. After the formation of cheese by a drying process, which allows for the reproduction of oxidizing yeasts *Torulopsis* spp. and *Candida* spp., which creates a suitable environment for the onset of action of proteolytic bacteria *B. linens* that during maturation forms on the surface of cheeses which are golden yellow to orange sebum in color.

The samples were supplied by the cheese manufacturer and were divided into three groups designated as (A), (B) and (C) then stored in different temperature regimes. Sampling and subsequent analyses were performed on the day of manufacture (A/0 = B/0 = C/0), at the end of BBD (A/35, B/35, C/35), two weeks after BBD (A/49, B/49), and eight weeks after BBD (C/91).

Samples in the temperature regime (A) were stored at 6 °C after production for the entire BBD (7 days before and 28 days after shipment), i.e. 35 days. These cheeses were then stored for 14 days after the BBD expired at 6 °C, i.e. 49 days in total (6 °C/35 days → 6 °C /14 days). Samples in the temperature regime (B) were stored at 6 °C after production for 28 days (7 days before and 21 days after shipment), i.e. 28 days, then they were frozen and stored at -18 °C for 7 days. Subsequently, after BBD (35 days), the cheeses were stored at 6 °C for 14 days, i.e. 49 days in total (6 °C/28 days → -18 °C/7 days → 6 °C/ 14 days). Samples in the temperature regime (C) were stored at 6 °C after production for the entire BBD (7 days before and 28 days after shipment), i.e. 35 days. They were then frozen and stored for 49 days (7 weeks) at -18 °C. After 49 days of freezing, the cheeses were stored at 6 °C for 7 days, i.e. 91 days in total (6 °C/35 days → -18 °C/ 49 days → 6 °C/7 days).

The analyses were carried out in the laboratory of the Institute of Food Technology, Faculty of AgriSciences of Mendel University in Brno according to **STN EN ISO 7218:2007 (2008)**. For one analysis, 3 consumer packages were always used. 10 g of each 100 g package of cheese was taken with a sterile scalpel by cutting a section through the sample through all the wheels; the sample contained the center and the edge of the cheese **(Figure1)**.

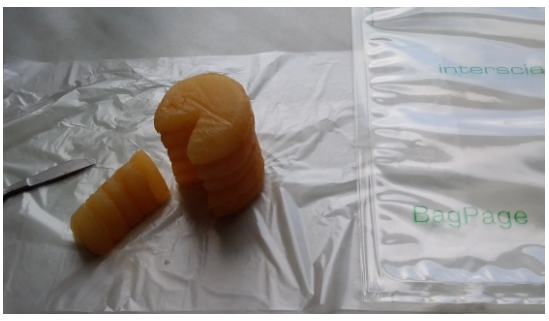

Figure 1 Sample preparation (smear ripened cheese).

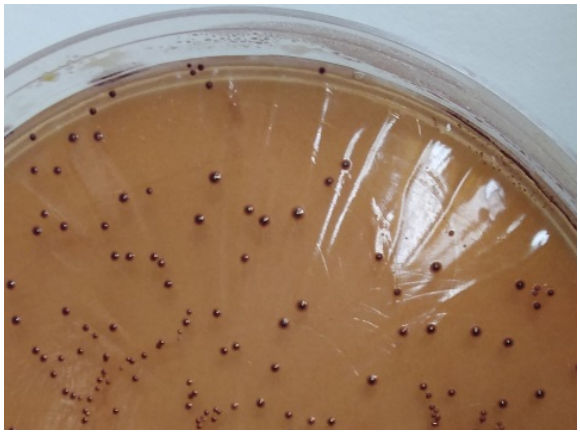

Figure 2 *Enterococcus* spp. in Slanetz-Bartley agar (isolated from smear ripened cheese).

Enterococci were determined on the Slanetz-Bartley agar (soil composition according to **STN EN ISO 7899-2 (2001)**, aerobically grown at 37 °C for 48 h **(Figure 2)**. Confirmation was made by growth on bile esculin agar (composition according to **STN EN ISO 7899-2 (2001)**; incubation at 44 °C for 24 h) and the catalase test (negative).

Statistical evaluation was performed in the Statistica Statsoft program (version 12) and Microsoft Excel 2010. Basic statistical characteristics, such as mean and standard deviation of the mean were calculated.

To compare the *Enterococcus* spp. content during the storage period within the given temperature regime, a simple scattering analysis method (ANOVA) including the Duncan post-hoc test was used. Normality was tested by the Shapiro-Wilk test.

The proportion of factors (temperature and storage period including interactions) on the total variability of BA content in cheese was calculated using the general linear model (two-way ANOVA with interactions).

RESULTS AND DISCUSSION

Influence of storage temperature on the number of enterococci

No statistical difference (p >0.05) was found in the number of enterococci in cheeses stored under different temperature regimes until the Best-Before date or at the end of monitoring after 49 and 91 days **(Figure 3)** respectively. Enterococcal counts were 4.7 log CFU.g^{-1} when stored under the regime (A) until BB date after 35 days of ripening, 4.8 log CFU.g^{-1} when stored under temperature regime (B) and 4.7 log CFU.g^{-1} under the regime (C). At the end of monitoring, 5.4 log CFU.g^{-1} of enterococci was detected in cheeses after storage for 49 days under the temperature regime (A), 5.2 log CFU.g^{-1} when stored under the regime (B) and 5.0 log CFU.g^{-1} under the regime (C) after 91 days.

Enterococci are highly resistant to environmental conditions, grow over a wide temperature range (25 to 45 °C), tolerate pH from 4.8 to 11.0, the salt content of

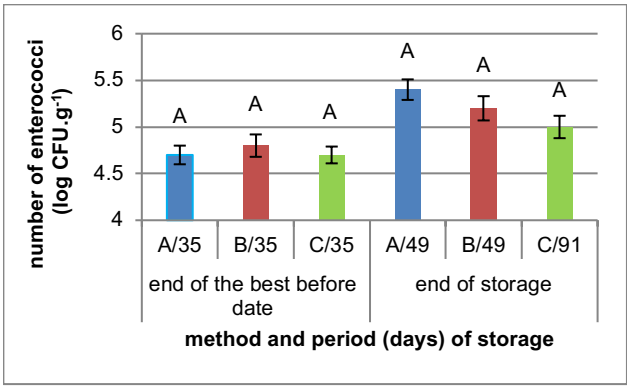

Figure 3 Comparison of the the number of enterococci (log CFU.g^{-1}) in smear ripened cheeses stored in different temperature regimes (A, B, C) and analysed at the end of the best before date after 35 days of ripening and at the end of storage after 49 or 91 days of ripening. Note: A/35: storage at 6 °C/35 days, B/35: Storage at 6 °C/28 days and at -18 °C/7 days, C/35: storage at 6 °C/35 days) (A: storage at 6 °C/35 days and at 6 °C/14 days, B: storage at 6 °C/28 days and at -18 °C/7 days and at 6 °C/14 days, C: storage at 6 °C/35 days and at -18 °C/49 days and at 6 °C/7 days; n = 15.

6.5 percent, bile presence of 40 percent and survive at 60 °C for 30 minutes **(Domig, Mayer and Kneifel, 2003)**. **López-Díaz et al. (2000)** indicated a wider range of growth temperatures from as low as 10 °C.

Due to their high resistance, they survive and tolerate temperature changes well, as shown in **Figure 3**. **Bockelmann et al. (2005)** reported that the usual number of enterococci on the surface of smear-ripened cheeses is <6 log CFU. cm^{-2}. Higher counts of enterococci compared to our study, >8 log CFU. cm^{-2}, were also detected by **Amato et al. (2012)** for fully ripe smear-ripened cheeses when stored at 4 to 8 °C. **Komprda et al. (2012)** also recorded higher counts compared to our study (depending on the season) until the Best Before date (42 days after production) when stored at 5 °C, namely 6 to 7 log CFU.g^{-1}, and 5.2 to 6.4 log CFU.g^{-1} after 66 days after production, wherein the lower value corresponds to the counts of enterococci that we found in our experiment at the end of the monitoring under the regimes (A/49), (B/49) and (C/91). Also, **Fontana et al. (2010)** reported higher counts of enterococci in smear-ripened cheese compared to our study, namely 7.2 log CFU.cm^{-2}. According to **Schneller, Good and Jenny (1997)**, the distribution of enterococci in cheese may be inhomogeneous.

Freezing is not the cause of the devitalization of all cells **(Görner and Valík, 2004)**. As reported by **Kadlec, Melzoch and Voldřich (2012)**, during freezing enzymatic reactions proceed slowly. Because they are mesophilic bacteria, according to **Šilhánková (2008)**, when the temperature falls below the optimal growth level, the rate of propagation drops sharply and eventually stops, although the sensitivity to cold shock varies among different bacteria. The devitalizing effects of low temperatures are, according to **Drdák et al. (1996)**, more efficient in repeated freezing and thawing, but this is not the case with bacterial spores that may survive under these conditions.

Possible sources of contamination of cheeses with enterococci are the raw material (milk), production workers, production equipment, water taps, saline solutions, tanks; however, their origin in cheeses is sometimes unclear **(İspirli et al., 2017)**. **Greifová et al. (2003)** reported that the decisive enterococci contamination of milk comes from the milking equipment and plant feed. In raw milk, enterococci are clear indicators of inadequate decontamination of equipment and machinery. As thermoresistant bacteria, some species of enterococci survive required pasteurization temperature, therefore they are a normal part of pasteurized milk.

Influence of the storage period on the number of enterococci

At the beginning of storage (A/0, B/0, C/0), the numbers of enterococci in cheeses were 2.3 log CFU.g^{-1}. At the end of storage, 5.4 log CFU.g^{-1} of enterococci was detected in cheeses under (A) temperature regime, 5.2 log CFU.g^{-1} when stored under (B) regime and 5.0 log CFU.g^{-1} when stored under (C) regime conditions. During storage, there was an increase (p <0.05) in the number of enterococci in all types of temperature regimes **(Figure 4)**.

Increasing enterococcal numbers during ripening was described by **Novella-Rodríguez et al. (2004)** for goat cheeses made from raw milk, **Roig-Sagués et al. (2002)** for Spanish cheeses, **Maher and Murphy (2000)**, **Macedo et al. (2004)** and **Martuscelli et al. (2005)** for smear-ripened cheeses. Counts of enterococci increased during 60 days of ripening of cheeses made from pasteurized milk in an experiment by **Martuscelli et al. (2005)** from 3.3 ±0.2 to 8.8 ±0.3 log CFU.g^{-1}, which are much higher values compared to our study. According to **Komprda et al. (2012)**, at 5 °C enterococci counts did not change significantly in smear-ripened cheeses (p >0.05); at

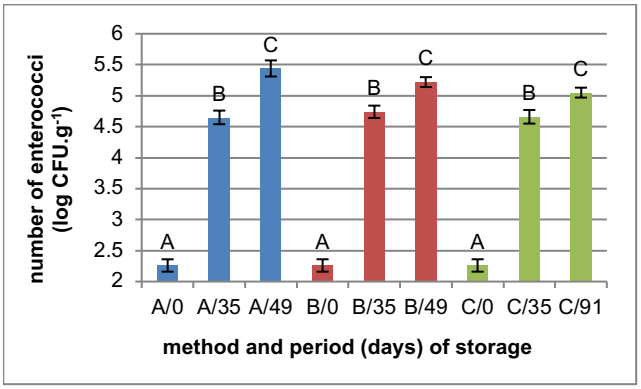

Figure 4 Comparison of the the number of enterococci (log CFU.g^{-1}) in smear ripened cheeses stored in different temperature regimes (A, B, C) and analysed on production day (0), at the end of the best before date after 35 days of ripening. Note: A/35: storage at 6 °C/35 days, B/35: storage at 6 °C/28 days and at -18 °C /7 days, C/35: storage at 6 °C/35 days and at the end of storage after 49 or 91 days of ripening: A: storage at 6 °C/35 days and 6 °C/14 days, B: storage at 6 °C/28 days and at -18 °C/ 7 days and 6 °C/14 days, C: storage at 6 °C/35 days and at -18 °C/49 days and at 6 C/7 days. Averages marked with different letters are statistically different within a given factor (storage period) (p <0.05); n = 15.

the end of ripening, the counts of enterococci were 6.1 log CFU.g^{-1}, which is more than 1 log higher than our study recorded. Although the number of enterococci in cheeses may be 1 log CFU.g^{-1}, values reaching up to 7 log CFU.g^{-1} have been found **(Pircher et al., 2007)**. Based on the experiment, **Rea et al. (2004)** concluded that the increase in enterococcal numbers in the first 6 hours (from 5 log to 7 log CFU.g^{-1}) is due to the water loss during cheese processing, which appears to be the cause of the apparent increase in enterococcal numbers. The survival of enterococci in cheese during ripening is due to a wide range of growth temperatures, tolerance to high temperatures, salt and acid concentrations **(Šilhánková, 2008)**. In contrast to enterobacteria, enterococci show a higher resistance to antagonist bacteria, which are represented by starter microorganisms **(Macedo et al., 2004)**. An increase in the number of enterococci during the ripening of smear-ripened cheeses was also described by **Calasso et al. (2016)**; after 30 days of ripening their number increased to 4.5 ±0.2 log CFU.g^{-1}, which is comparable to our experiment. Also **Schneller, Good and Jenny (1997)** noted an increase in the number of enterococci during cheese ripening. Levels of enterococci in different cheese curds range from 10^4 to 10^6 CFU g^{-1} and in the fully ripened cheeses from 10^5 to 10^7 CFU g^{-1} **(Moreno et al., 2006)**.

Enterococci are widespread in nature and get into raw milk and dairy products during production, especially from the equipment and also from the water where basic hygiene conditions are not observed **(Ogier and Serror, 2008; Li et al., 2017)**. Enterococci are commonly found in raw milk with different microbiota reported in different countries, reflecting local practices and levels of hygiene **(Čanigová et al., 2016)**. We are, however, not able to fully explain the origin of enterococci in cheeses, as the cheeses were made from pasteurized milk. Thus, enterococci originating from the gastrointestinal tract of a dairy cow should be devitalized by heat treatment. The same conclusions were reached by **Gelsomino et al. (2002)**, who found that dairy cow manure is not a source of enterococci, even though the same enterococcal strains were found in milk, cheese and dairy equipment as in dairy cow manure. On the contrary, **Görner and Valík (2004)** reported that Kielwein did not find a link between the species composition of enterococci in dairy cow manure and the milking equipment in 1997 and concluded that enterococci cannot be considered as indicators of faecal contamination but as indicators of inadequate sanitation.

This study also evaluated the contribution of the method and time of storage to the variability in the number of enterococci. Analyses carried out (two-way ANOVA with interactions) show that both the storage period and the storage method (temperature regime; **Table 1**) influenced the enterococcal content.

Table 1 Influence of individual factors (storage method, storage period) on the enterococci content (initial measured values in log CFU.g^{-1}) in cheeses.

Indicator	% explained variability			
	Storage method	Storage period	Interaction	Error
Enterococci	7*	82*	7*	4

Note: * p <0.05.

According to **Giraffa (2003)**, it is difficult to answer the question of whether the presence of enterococci in food is safe. It is necessary to carry out a more detailed identification of the individual strains present in cheeses and to evaluate the pathogenicity of these strains **(Franz, Holzapfel and Stiles, 1999)**. Due to the variety of *Enterococcus* species and their importance in food, the detection and enumeration of enterococci have become an important concern in current research activities **(Hamad, Selim and Yassin, 2019)**.

CONCLUSION

The hypothesis that enterococci survive the freezing process and their numbers do not change significantly can be confirmed. No statistical difference (p >0.05) was found in the numbers of enterococci in cheeses stored under different temperature regimes until the Best-Before date or at the end of monitoring after 49 and 91 days, respectively.

The hypothesis that the duration of the storage of cheeses affects them in terms of enterococcal content can be confirmed. During storage, their numbers increased (p <0.05). However, it should be noted that the view of enterococci is not unambiguous. On the one hand, they are used as cheese-making cultures in some countries, and on the other hand, they can cause health problems in immunocompromised individuals.

From the viewpoint of pathogenicity and resistance to antibiotics, as well as the production of biogenic amines, enterococci in foods are not desirable microorganisms.

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