THE CONTENT OF NITRATES, NITRITES AND IMPORTANT GROUPS OF MICROORGANISMS IN EDAM-TYPE CHEESES

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ABSTRACT. The content of nitrates, nitrites and important groups of microorganisms in Edam-type cheeses. *The objective of the research work was to determine the content of nitrates, nitrites and important groups of microorganisms in Eesti and Pühajärve cheeses, and to study their changes and interaction during the process of ripening.*

The content of nitrates and nitrites of cheeses was determined according to the Nordic Standard NMKL No. 165, 2000 by using liquid chromatograph. For microbiological analyses the respective IDF and EV standard methods were applied.

After pressing, the fresh Eesti cheese contained 55 mg $NaNO_3$ /kg on the average. During ripening for 3–4 weeks, the content of nitrates decreased by 10 mg/kg, maintaining the average level of 45 mg $NaNO_3$/kg in fully ripened cheeses.

After pressing, the average nitrate content of fresh Pühajärve cheeses was 69.8 mg $NaNO_3$/kg. During the average ripening period of 14 days, similarly to that of Eesti cheese the content of nitrates decreased by 10 mg/kg. Thus, the average level of nitrates 59.8 mg $NaNO_3$ / kg (range 37–88 mg/kg) in ripe Pühajärve cheeses exceeded the EU limits (50mg/kg) by 9.8 mg/kg.

The content of nitrites in all the studied cheese batches of both Eesti and Pühajärve cheeses was <5 mg $NaNO_2$ / kg, i.e. in compliance with the norms set by the EU.

The average total bacterial count of Eesti cheese after pressing was 480 million cfu/g, count of lactococci, lactobacilli and that of spores of anaerobic bacteria 360 million cfu/g, 3800 cfu/g and 31.4 cfu/g, respectively. In the course of ripening, both the total bacterial count and content of lactococci dropped, retaining the level of 7.8 million cfu/g and 3.1 million cfu/g, respectively. The count of lactobacilli and spores of anaerobic bacteria increased, reaching in ripened cheeses the average of 4.7 million cfu/g and 229 cfu/g, respectively.

The average total bacterial count of Pühajärve cheese after pressing was 98 million cfu/g, the count of lactococci, lactobacilli, and spores of anaerobic bacteria was 75 million cfu/, 14 thousand cfu/g and 170 cfu/g, respectively. During ripening the total bacterial count and count of lactococci decreased, ranging between 9.9 million cfu and 8.2 million cfu per gram, on the average, whereas the count of lactobacilli and spores of anaerobic bacteria increased up to 12 million and 201 cfu per gram, respectively.

Statistical analysis of the obtained data (Spearman correlation) revealed that higher activity and content of starter lactococci inhibited the development of anaerobic bacteria at the ripening stage ($P<0.01$). The nitrate content of cheeses also suppressed the development of anaerobic bacteria and lactobacilli, whereas it did not affect the reproduction of lactococci of starter cultures ($P<0.05$).

Keywords: *nitrates, nitrites, total count of bacteria, lactococci, lactobacilli, spores of anaerobic bacteria, Edam-type cheese.*

Introduction

The content of nitrates and nitrites in milk and dairy products had not received much attention until the late 70s and early 80s of the past century.

Nitrates and nitrites are water-soluble salts of nitric acid (HNO_3) and nitrous acid (HNO_2). In nature, nitrates and nitrites are produced in the course of nitrification process. Nitrates in soil, ground and surface water derive mainly from intensive application of nitrogenous fertilizers. From soil they easily drain into water-bodies, assimilate into growing plants, and consequently, into feeds or human foods. With food, the nitrates and nitrites not used for biosynthesis of plant organs, get into human and/or animal organism, where they readily form nitrosamines, known as carcinogenic compounds.

Milk is contaminated with nitrates and nitrites by means of secretory or postsecretory processes, whereas nitrates and nitrites are generated either exo- or endogenically.

The organism of mammals is capable of removing hazardous substances from milk that may endanger first nutrition of their offspring. Only a thousandth of assimilated nitrates is transfered from feed to milk (Bielak, Barbarasz, 1982), whereas the carry-over of nitrites is extremely low, as they are almost entirely reduced in rumen within 2–3 hours. The reported average content of nitrates in cow milk was 1–5 mg/l, and even in case of acute nitrate toxicity it did not exceed 15 mg/l (Harding, Gregson, 1978; Küünal, Sell, 1990).

The lethal dose of $NaNO_3$ for adult humans has been estimated to be about 8 g/kg body weight (Heechen, Blüthgen, 1991). The toxicity of nitrites ($NaNO_2$ and KNO_2) is approximately four times higher. The major toxic effect of nitrites derives from development of methaemoglobinema, *i.e.* haemoglobin is transformed into methaemoglobin, which reduces the oxygen-carrying capacity of the blood. As the methaemoglobin content in human blood exceeds 70–90%, the condition can be fatal (Deeb, Sloan, 1975).

Nitrates are supplemented to bulk milk in cheesemaking to prevent late gas defects. The dose ranges from 2 to 20 g nitrate/100 kg milk. Due to this, nitrate amounts ranging from 0.2 to 104 mg/kg can be found in cheese. According to the data obtained by Heechen *et al.* (1991), supplementation of 15 g nitrate to 100 kg bulk milk increased the nitrate content in cheese up to 56 ppm. During ripening it decreased to 30 ppm/kg, which is below the permissible standard of 50 ppm/kg.

Munksgaard *et al*. (1987) reported, that the nitrate content dropped by 6–20% already within the first 24 hours of cheese ripening. Most of the nitrates were lost into fresh brine. During the following 4-week ripening the content of nitrates was still lowering. The reduction of nitrates slowed down gradually, and ended up at the level which constituted 15% of the initial nitrate supplementation. It was found that 11–51% of the supplemented nitrates were not associated with cheese proteins, whereas ammonia constituted 4–9% and nitrates 0.3-10%. Furthermore, it was observed that 4.2% of nitrates were reduced into gaseous $N_{2,}$ and 5.2–29.7% into gaseous N_2O, which both released from cheese (Munksgaard *et al*., 1987).

Microorganisms play a significant role in natural oxidation and reduction processes of nitrates and nitrites. However, there is only fragmentary information available regarding the effect of cheese microbe populations, comprising technology- and contamination-induced bacteria, on the nitrate and nitrite content transformation dynamics.

The objective of the present research work was to determine the content of nitrates, nitrites and important groups of microorganisms in Edam-type cheeses, and to find out their potential interaction.

Material and Methods

To study the content of nitrates and nitrites in Edam-type cheeses, the two most popular brands, Eesti cheese and Pühajärve cheese, both in high demand among the consumers in the Estonian market, were chosen.

The investigations were carried out from February to May 2003, and from March to May 2004. Cheese samples (0.7–1.0 kg) were taken after pressing and after ripening from the production batches of cheese industry. The samples were wrapped in plastic film, chilled to the temperature 0...+4 °C, and isothermally transported to the laboratory. Besides the content of nitrates and nitrites, the microbiological data of the cheese samples, such as total count of bacteria, the count of lactococci, lactobacilli, and spores of anaerobic bacteria were determined.

Nitrates and nitrites were examined in 5 samples of Eesti and 5 samples of Pühajärve cheese, and the microbiological data in 6 samples of Pühajärve and 8 samples of Eesti cheese.

The samples were homogenized, nitrates and nitrites were extracted by acetonitril-containing hot water. The samples were filtered and the filtrate was transferred into the column Alltech C_{18} and analytical column Allsep Anion Column. The nitrates and nitrites were determined by using liquid chromatograph Shimadzu LC 10 AS and UV detector Shimadzu SPD-10A operated at 215 nm. The smallest amount of nitrates determined was 10 mg NO_3^-/kg and 5mg NO_2^-/kg. The above method has been validated by the Swedish National Food Administratin and acknowledged as Nordic Standard NMKL No. 165, 2000 (Pentchuk *et al*., 1986; Merino *et al*., 2000).

The samples for microbiological analyses were prepared according to EVS 633:1994 and EVS 639:1994 standards. The total bacterial count was determined according to the IDF Standard 100A:1987 method, using LAB 115 culture medium. Mesophilic lactococci were identified at 25 °C in culture medium M17 (LAB 92) (Collins *et al*., 1995), mesophilic lactobacilli were determined at 30 °C in culture medium LAB 93 (culture medium MRS, IDF Standard 117A:1988).

To detect spores of anaerobic bacteria, the respective one-tenth dilutions of cheeses were heated in water bath for 10 min at 80 °C, then rapidly cooled down to 30 °C and inoculated into Petri dishes a´ 1 cm^3. The inoculations, made in culture medium (*Reinforced clostridial agar*), were cultivated at 30 °C in plastic sacks in household gas atmosphere, i.e. in anaerobic conditions. All the formed colonies were counted, whereas it was calculated that there was one viable spore per each colony (LAB M, 1995).

The dilution liquid, used in microbiological analyses, was prepared according to the IDF 100A:1987 methods.

Results and Discussion

The preliminary studies of the nitrate and nitrite content of cheeses sold in the Estonian market, were accomplished under the permission the Estonian Health Protection Inspectorate, using the results of their analyses performed in 2001 and 2002 (Table 1).

Table 1. The content of nitrates and nitrites in cheeses sold in Estonian market (unpublished data of the Estonian Health Protection Inspectorate)

Name of cheese (date of making)	Producer	Nitrites ($NaNO_2$) mg/kg	Nitrates ($NaNO_3$) mg/kg
Caciotta cheese	Estonia	<5	93
Eesti cheese (11.11.01)	Estonia	<5	43
Lahe cheese (14.12.01)	Estonia	<5	<5
Marta cheese (08.01.02)	Estonia	21	<5
Dutch head of cheese (23.11.01)	Estonia	<5	<5
Eesti cheese	Estonia	<5	17
Dutch cheese	Estonia	<5	75
Smoked cheese with juniper	Estonia	<5	9
Smoked cheese (23.11.01)	Latvia	<5	17
Vene cheese (18.11.01)	Estonia	<5	40
Saare Atleet	Estonia	<5	50
Cheese Palanga	Lithuania	<5	116
Saaremaa cheese	Lithuania	<5	84
Caraway cheese	Estonia	<5	45

It was established that the content of nitrates in cheeses ranged between <5–116 $NaNO_3$ mg/kg. Cheeses manufactured in Estonia had a nitrate content mostly below 50 mg/kg, in several cases even below 5 mg/kg, while the cheeses produced in Lithuania contained 84–116 mg/kg of nitrogen, which exceeded the EU standards approximately 1.5–2 times. The content of nitrites did not range as much, showing the level below 5 mg/kg, with the exception of Marta cheese, which contained 21 mg/kg nitrites (Table 1).

Further to the above investigations, a more detailed study of the content of nitrates and nitrites of well-known and best selling cheese brands in Estonia was initiated. Two cheese brands were chosen: Eesti cheese, the techology of which has been improved by Estonian cheesemakers, and Pühajärve cheese, an Edam-type cheese with one of the shortest, only 14-day manufacture time.

After pressing the fresh Eesti cheese contained 55 mg $NaNO_3$/kg on the average (range 50–59 mg/kg) (Table 2; Figure 1). During ripening, which usually lasted for 3–4 weeks, the content of nitrates decreased by 10 mg, maintaining the average level of 45 mg $NaNO_3$/kg.

While in fresh cheeses the nitrate content exceeded the limits set by the EU norms (50 $NaNO_3$ mg/kg), it dropped and remained below the level of 50 mg/kg in all the studied cheese lots in the course of ripening. During the ripening period, reduction of nitrates into nitrites and other nitro-compounds takes place. Similar results were also obtained by Hanza *et al*., (2004), Luf (2002), Kyriakidis *et al*., (1997).

The nitrite content of Eesti cheese samples, taken after pressing and after ripening, was <5mg $NaNO_2$/kg in all the studied batches of cheese.

After pressing, the average nitrate content of fresh Pühajärve cheese was 69.8 mg $NaNO_3$/kg (range 49–90 mg/kg) (Table 2, Figure 2). During the mean ripening period of 14 days, the content of nitrates decreased to 59.8 mg $NaNO_3$/ kg, ranging between 37 and 88 mg/kg.

Similarly to the fresh Eesti cheese, the nitrate content of Pühajärve cheese also exceeded the current EU permitted limits of 50 mg/kg, whereas in Pühajärve cheese it was 14.8 mg/kg higher on the average. Although during ripening the content of nitrates dropped by 10 mg $NaNO_3$/kg on the average, there was still a 14.8 mg/kg difference with Eesti cheese. Due to the high content of nitrates in fresh Pühajärve cheese, a higher nitrate content was also observed in fully ripened cheeses, exceeding the allowable limit of 50 mg/kg by 9.8 mg/kg on the average (Table 2).

Tabel 2. The content of nitrates in Eesti and Pühajärve cheeses after presssing and ripening

Seg No	Content, mg/kg			
	Eesti cheese		Pühajärve cheese	
	after pressing	after ripening	after pressing	after ripening
1	59	44	49	37
2	57	45	90	88
3	51	51	84	66
4	50	48	67	67
5	58	37	59	41
Mean	55	45	69.8	59.8

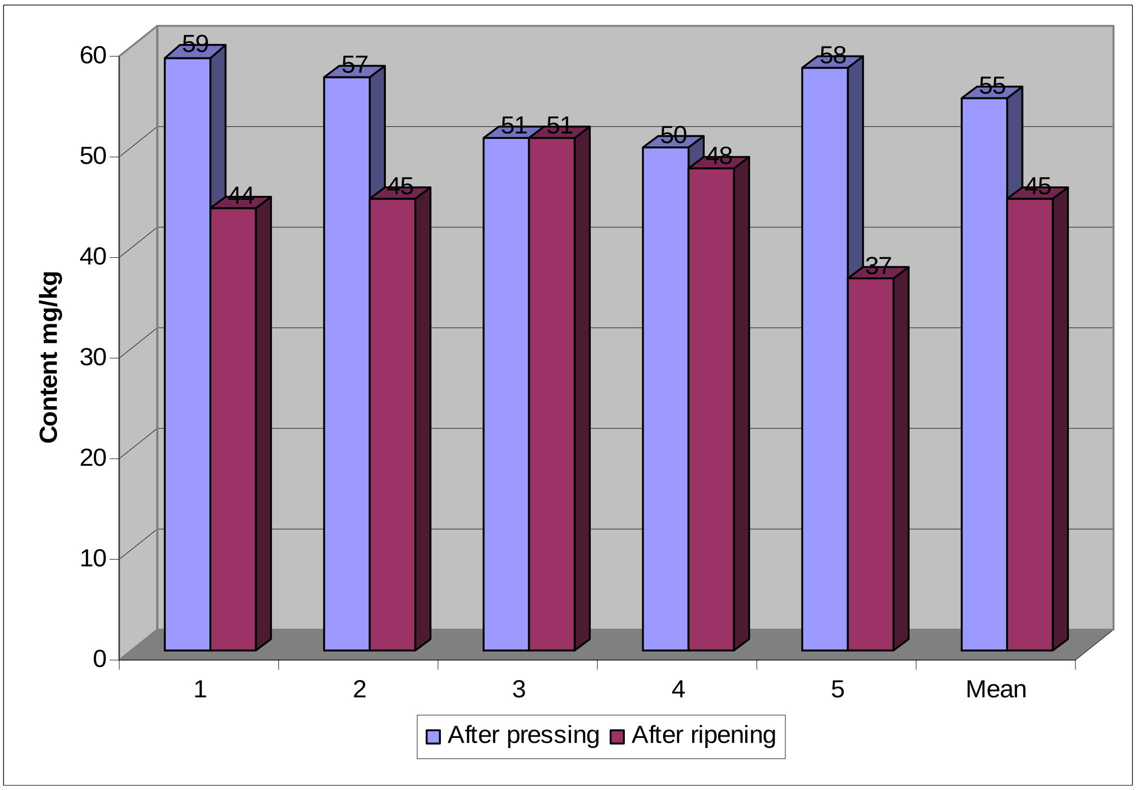

Figure 1. The content of nitrates in Eesti cheese after pressing and after ripening

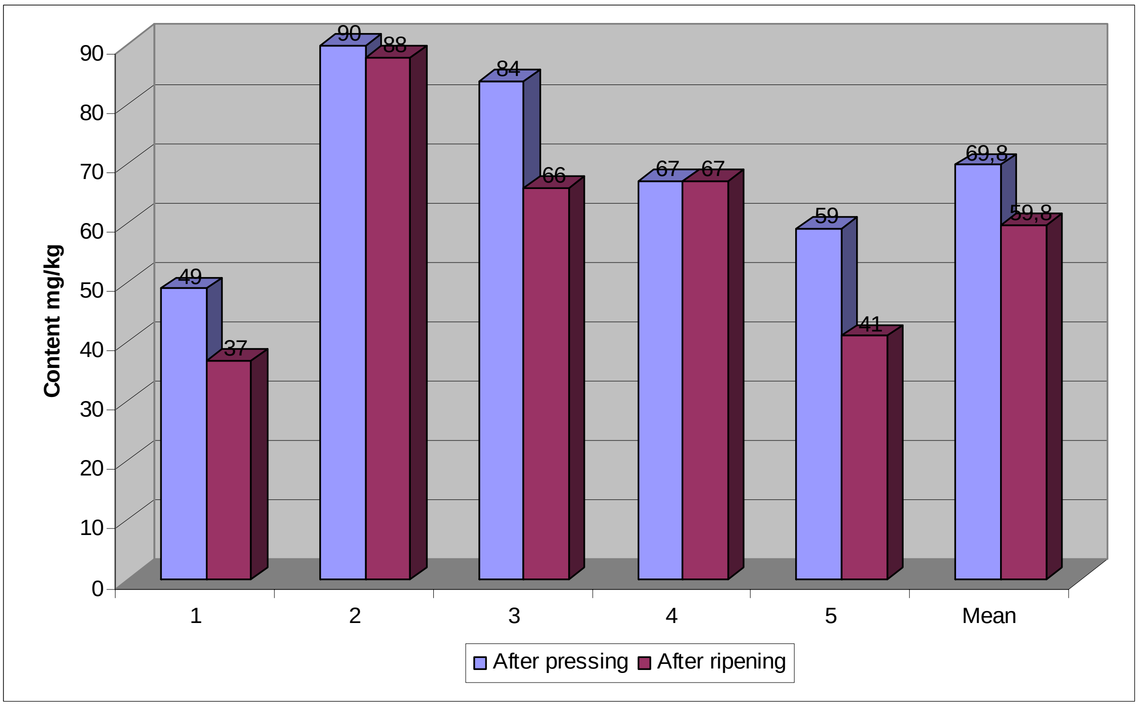

Figure 2. The content of nitrates in Pühajärve cheese after pressing and after ripening

Similarly to that of Eesti cheese, after pressing and after ripening the content of nitrites of Pühajärve cheese was also, <5 mg $NaNO_2$/kg in all the studied cheese batches.

As a diverse population of microorganisms, composed of different groups of bacteria along with respective enzymatic activity, is an important contributor to cheese ripening, experiments were conducted to study the microbial count of fresh and ripe cheese as well as to evaluate its correlation with the content of nitrates and nitrites.

The average total bacterial count of Eesti cheese (fresh cheeses) after pressing was 480 million cfu/g, count of lactococci 360 million cfu/g, lactobacilli 3800 cfu/g and that of spores of anaerobic bacteria 31.4 cfu/g (Table 3).

Table 3. The content of microorganisms in Eesti cheese after pressing

Seg No	Count of microorganisms, cfu/g			
	Total count of bacteria ($x10^6$)	Lactococci ($x10^6$)	Lactobacilli ($x10^3$)	Spores of anaerobic bacteria
1	250	190	5,0	48
2	400	300	5,0	31
3	590	560	3,9	45
4	450	100	4,2	24
5	640	480	3,3	21
6	660	630	4,3	27
7	560	160	2,9	26
8	310	460	2,1	29
Mean	480	360	3,8	31

In the course of ripening, both the total bacterial count and content of lactococci dropped, retaining the level of 7.8 million cfu/g and 3.1 million cfu/g, respectively (Table 4). While the total count of bacteria and count of lactococci decreased during ripening, the content of lactobacilli and spores of anaerobic bacteria increased, reaching the average of 4.7 million and 229 cfu/g, respectively (Table 4).

Table 4. The content of microorganisms in Eesti cheese after ripening

Seg No	Content of microorganisms, cfu/g			
	Total count of bacteria ($x10^6$)	Lactococci ($x10^6$)	Lactobacilli ($x10^6$)	Spores of anaerobic bacteria
1	5,4	1,7	2,1	309
2	6,1	4,2	3,5	173
3	5,7	1,8	5,2	345
4	8,5	2,6	6,5	295
5	1,3	2,9	4,8	175
6	1,0	4,6	6,0	165
7	7,3	3,9	5,4	180
8	6,8	3,6	3,9	190
Mean	7,8	3,1	4,7	229

The average total bacterial count of Pühajärve cheese after pressing (fresh cheeses) was 98 million cfu/g, the count of lactococci, lactobacilli and spores of anaerobic bacteria 75 million cfu/g, 14 thousand cfu/g and 170 cfu/g, respectively (Table 5).

Table 5. The content of microorganisms in Pühajärve cheese after pressing

Seg No	Content of microorganisms, cfu/g			
	Total count of bacteria ($x10^6$)	Lactococci ($x10^6$)	Lactobacilli ($x10^3$)	Spores of anaerobic bacteria
1	115	82	14	218
2	89	58	19	164
3	82	66	15	168
4	90	72	12	100
5	96	88	11	150
6	114	85	14	220
Mean	98	75	14	170

During ripening, the total bacterial count and count of lactococci decreased, ranging between 9.9 million cfu and 8.2 million cfu per gram on the average (Table 6). At the same time the content of lactobacilli and spores of anaerobic bacteria increased to 12 million and 201 cfu per gram, respectively.

Table 6. The content of microorganisms in Pühajärve cheese after ripening

Seg No	Content of microorganisms, cfu/g			
	Total count of bacteria ($x10^6$)	Lactococci ($x10^6$)	Lactobacilli ($x10^6$)	Spores of anaerobic bacteria
1	9.8	10.0	8.5	241
2	13.0	8.1	13.0	168
3	7.9	7.7	10.0	173
4	9.9	7.8	18.0	300
5	9.8	7.1	13.0	180
6	9.0	8.2	9.3	145
Mean	9.9	8.2	12.0	201

Although no considerable differences were observed between Eesti and Pühajärve cheese regarding the development of microorganisms in general, significant dissimilarities were found between single groups of microorganisms (Figure 3).

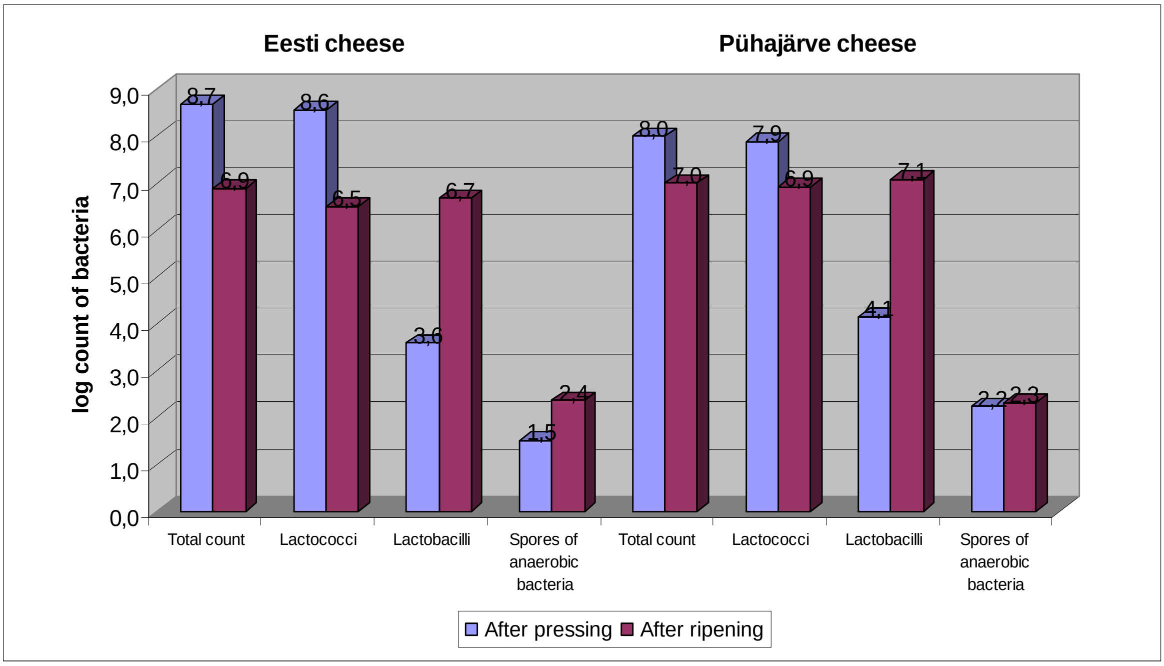

Figure 3. The content of microorganisms in Eesti and Pühajärve cheeses after pressing and after ripening

Before ripening, Eesti cheese contained 3–4 times less lactobacilli and 4–6 times less spores of anaerobic bacteria than Pühajärve cheese. During ripening stage, the content of lactobacilli as well as spores of anaerobic bacteria increased in both brands of cheese. While Pühajärve cheese still had a 2–3 fold higher lactobacilli content, the spores of anaerobic bacteria developed considerably faster in Eesti cheese, exceeding the average content of sporogenes in Pühajärve cheese already 1–2 times after ripening.

Statistical analysis of the obtained data (*Spearman correlation*) revealed that higher activity and content of starter lactococci inhibited the development of anaerobic bacteria at the ripening stage ($P<0.01$). The nitrate content of cheeses also suppressed the development of anaerobic bacteria and lactobacilli, whereas it did not affect the reproduction of lactococci of starter cultures ($P<0.05$).

Conclusions

The objective of the research work was to determine the content of nitrates, nitrites and important groups of microorganisms in Eesti and Pühajärve cheeses, and to study their changes and interaction during the process of ripening.

Five samples of both, Eesti cheese and Pühajärve cheese, were examined for nitrates and nitrites, whereas 8 samples of Eesti cheese and 6 samples of Pühajärve cheese were microbiologically tested.

The content of nitrates and nitrites of cheeses was determined according to the Nordic Standard NMKL No. 165, 2000 by using liquid chromatograph. For microbiological analyses the respective IDF and EV standard methods were applied.

Analysis of the data from 2001 and 2002, provided by the Estonian Health Protection Inspectorate, revealed that nitrate content in cheese sold in Estonia varied within the range of 25–116 mg $NaNO_3$/kg. In cheese produced in Estonia the nitrate content was mostly below 50 mg/kg, while it was 1.5–2 times higher in cheese manufactured in Lithuania. The content of nitrites of the same cheeses, however, did not vary as much, mostly showing the level below 5 mg $NaNO_2$/kg. An exception was Marta cheese, which contained 21 mg/kg nitrites.

After pressing, the fresh Eesti cheese contained 55 mg $NaNO_3$/kg on the average. During ripening for 3–4 weeks, the content of nitrates decreased by 10 mg/kg, maintaining the average level of 45 mg $NaNO_3$/kg in fully ripened cheeses.

After pressing, the average nitrate content of fresh Pühajärve cheeses was 69.8 mg $NaNO_3$/kg. During the average ripening period of 14 days, similarly to that of Eesti cheese the content of nitrates decreased by 10 mg/kg. Thus, the average level of nitrates 59.8 mg $NaNO_3$/ kg (range 37-88 mg/kg) in ripe Pühajärve cheeses exceeded the EU limits (50mg/kg) by 9.8 mg/kg.

The content of nitrites in all the studied cheese batches of both Eesti and Pühajärve cheeses was <5 mg $NaNO_2$/kg, *i.e.* in compliance with the norms set by the EU.

The average total bacterial count of Eesti cheese after pressing was 480 million cfu/g, count of lactococci, lactobacilli and that of spores of anaerobic bacteria 360 million cfu/g, 3800 cfu/g and 31.4 cfu/g, respectively. In the course of ripening, both the total bacterial count and content of lactococci dropped, retaining the level of 7.8 million cfu/g and 3.1 million cfu/g, respectively. The count of lactobacilli and spores of anaerobic bacteria increased, reaching in ripened cheeses the average of 4.7 million cfu/g and 229 cfu/g, respectively.

The average total bacterial count of Pühajärve cheese after pressing was 98 million cfu/g, the count of lactococci, lactobacilli, and spores of anaerobic bacteria was 75 million cfu/g, 14 thousand cfu/g and 170 cfu/g, respectively. During ripening the total bacterial count and count of lactococci decreased, ranging between 9.9 million cfu and 8.2 million cfu per gram, on the average, whereas the count of lactobacilli and spores of anaerobic bacteria increased up to 12 million and 201 cfu per gram, respectively.

No significant differences were observed between Eesti and Pühajärve cheese, regarding the development of microorganisms in general.

It appeared, however, that in Eesti cheese the higher development activity of spores of anaerobic bacteria was probably due to the lower nitrate content of cheese.

Statistical analysis of the obtained data (*Spearman correlation*) revealed that higher activity and content of starter lactococci inhibited the development of anaerobic bacteria at the ripening stage ($P<0.01$). The nitrate content of cheeses also suppressed the development of anaerobic bacteria and lactobacilli, whereas it did not affect the reproduction of lactococci of starter cultures ($P<0.05$).

In cheesemaking, active starter cultures should be used to elevate the levels of lactococci in cheese. At the same time the milk with the lowest count of spores of anaerobic bacteria should be chosen to reduce supplementation of nitrates and keep the content of nitrates as low as possible in ripened cheese.

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