The influence of ripening temperature on diversity of non-starter lactic acid bacteria in semi-hard cheeses

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INGA CIPROVICA1, ALLA MIKELSONE
1Latvia University of Agriculture Faculty of Food Technology
2 Liela street, Jelgava, LV 3001, LATVIA, phone/fax +37163022829,
e-mail: Inga.Cipovica@llu.lv

Abstract
The aim of the study was to evaluate impact of the selected ripening temperature on the growth rate of NSLAB in Latvian semi-hard Holandes and Krievijas cheeses.

The samples of Holandes and Krievijas cheeses have been analysed. Cheeses were produced at two cheese factories and ripened at laboratories of LUA for 60 days at 6 and 12 °C. Both ripening regimes help understand the influence of microflora on the quality of cheese. pH, water activity, CFU of Lactobacillus spp., identification of Lactobacillus species and isolation of DNA for confirmation of Lactobacillus species have been performed.

Ripening temperature influences the growth rate of NSLAB, but it is impossible to liberate cheese from their presence. The differences are observed in qualitative composition of cheese microflora. L.curvatus dominated during the rest of ripening time at 6 °C and L.plantarum 1 and L.paracasei were detected at 12°C in samples. This study reveals that the concentration of NSLAB differs between cheeses ripened at 6 °C and 12 °C at least by 1 log. Higher concentrations were found in cheeses ripened at 12 °C. These findings should be taking account, because any change has a significant impact on the sensory properties of cheese.

Key words: NSLAB, cheese, ripening, Lactobacillus spp.

Introduction
Raw milk is a natural growth medium for microorganisms. Composition and quality of microflora are variable. An integral part of milk microflora is non-starter lactic acid bacteria (NSLAB) (BERESFORD & al. [1]). Pasteurization is able to destroy essential microflora, enzymes and pathogens in milk. It should be noted that inactivation level of microorganisms depends on the amount of microorganisms, growth phase and other factors. Bactofugation, microfiltration, and application of food additives, cannot significantly decrease the proportion of Lactobacillus spp. and Leuconostoc spp. in milk (MIKELSONE & al. [2]). Defects caused by non-starter lactic acid bacteria are found in all dairy products, but the most representative they are in cheeses.

Early blowing caused by non-starter lactic acid bacteria is often mixed up with coliform bacteria. Non-starter lactic acid bacteria, mostly heterofermentative, produce diacetyl and acetoin, and high amounts of carbon dioxide (STACKEBRANDT & al. [3]). Carbon dioxide forms many small holes in cheese, and sometimes a significant gas pressure results in a sponge-like cheese texture. Identical defect is caused by coliform bacteria. This defect occurs during the beginning of cheese ripening while there is lactose in it. Technological methods to fight coliform bacteria differ as well.

The non-starter lactic acid bacteria could not be evaluated unambiguously. Individual strains of species are used for acceleration of cheese ripening, stimulation of protein hydrolysis and enhancement of concentration of free amino acids contributing to flavour and aroma of a well ripened cheese (TANOUS & al. [4]). It should be noted that the role of non-starter lactic acid bacteria in determination of cheese quality is still unclear. Besides there is
very little information available on diversity of non-starter lactic acid bacteria in Dutch type cheeses during ripening, especially in Latvian cheeses. The aim of the study was to evaluate impact of the selected ripening temperature on the growth rate of NSLAB in Latvian semi-hard Holandes and Krievijas cheeses.

Materials and methods

The influence of the ripening temperature on cheese quality during maturation was studied in two different semi-hard commercial Latvian cheeses (Holandes and Krievijas). Holandes and Krievijas cheeses are full fat semi-hard cheeses (45 and 50% fat in dry matter), they are made according to Dutch type cheese traditions using mesophilic starter cultures (Lactococcus lactis subsp. lactis, Lactococcus lactis subsp. cremoris, Lactococcus lactis subsp. lactis biovar. diacetylactis). Some technological peculiarities are existed during Holandes and Krievijas cheese production. Lactic acid fermentation process is enhanced during Krievijas cheese production by prolonged treatment of cheese grains and whey mixture and cheese pressing before brining. During Holandes cheese production the treatment of grains are provided without water addition before heating of cheese grains and whey mixture. Samples (n=18) of unripened Krievijas and Holandes cheeses from two manufacturers have been analyzed. Cheese samples after salting were delivered to the laboratories of Latvia University of Agriculture, where samples were ripened at 6 °C and 12 °C for 60 days at relative humidity 80-85%. Ripening temperature was chosen according to conclusions in research articles about variability of microflora (6 °C), and recommended ripening parameters (10-12 °C) in technology of Krievijas and Holandes cheeses. Both ripening regimes help better understand the influence of microflora on the quality of cheese, especially on sensory properties. Further in the article the following designations of analyzed cheeses are used Holandes I, Holandes II, Krievijas I and Krievijas II. Cheese samples were analyzed after salting immediately and then after 15, 30, 45 and 60 days of ripening. Different parameters are detected during the study.

Determination of the pH was performed to cheese samples, according to LVS ISO 5546:2010 ‘Caseins and caseinates – determination of the pH’, using pH-meter “3520 pH Meter” (JENWAY, Barloworld Scientific Ltd., Essex, UK).

Determination of water activity was performed for cheese samples using “Meter AquaLab LITE” (Decagon Inc, USA). Water activity was measured with accuracy ± 0,015. The calibration of equipment was performed by 0.5M KCl (Lot 932375, Decagon).

Determination of Lactobacillus spp. was performed in analysed samples, according to LVS ISO 15214:1998, using MRS agar media (Scharlau, Spain). Media was prepared according to LVS CEN ISO/TS 11133-1:2009. Sample dilutions were performed according to ISO 6887-5:2010. Colony forming units of lactic acid bacteria were determined by ISO 15214:1998. The chosen parameters for cultivation of lactic acid bacteria in MRS agar were 72 hours at 37 °C, taking as a basis regimes recommended in the literature (COEFURET & al. [5]).

Identification of Lactobacillus spp. was performed taking by API 50 CHL (BioMerieux, France). APILAB Plus version 4.0 was used for identification of species.

Isolation of DNA was performed for most frequently identified representatives of Lactobacillus spp. - L. plantarum 1 and L. curvatus using PowerSoil DNA Isolation Kit (MO BIO Laboratories Inc.).

Polymerase chain reaction analysis was performed for confirmation of the isolated Lactobacillus species. The obtained sequences were analyzed at Staden Package 1.6.0. release (http://staden.sourceforge.net/) and compared to sequences available in the data base BLAST (www.ncbi.nlm.nih.gov ).
Data analysis was performed by using StatistiXL and Microsoft Excel programs. The single factor analysis of variance, Tukey’s test and correlation analysis were used.

**Results**

Information on the variability of species under the influence of temperature and ripening time in Krievijas cheeses is given in Table 1.

**Table 1. The changes of *Lactobacillus* spp. during Krievijas cheese ripening**

<table>
<thead>
<tr>
<th>Ripening time, days</th>
<th>at 6°C</th>
<th>at 12°C</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Krievijas I</td>
<td>Krievijas II</td>
</tr>
<tr>
<td>Unripened</td>
<td><em>Lc. lactis</em> subsp.<em>lactis</em> 1, <em>L. curvatus</em></td>
<td><em>Lc. lactis</em> subsp.<em>lactis</em> 1, <em>Lc. lactis</em> subsp.<em>lactis</em> 2, <em>L. curvatus</em></td>
</tr>
<tr>
<td>15 days</td>
<td><em>Lc. lactis</em> subsp.<em>lactis</em> 2, <em>L. curvatus</em></td>
<td><em>L. curvatus</em></td>
</tr>
<tr>
<td>30 days</td>
<td><em>L. curvatus</em></td>
<td><em>L. helveticus</em></td>
</tr>
<tr>
<td>45 days</td>
<td></td>
<td><em>L. paracasei</em> subsp.<em>paracasei</em> JCM 8133</td>
</tr>
<tr>
<td>60 days</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

In the beginning of Krievijas cheese ripening at 6 °C the presence of starter flora was detected and the prevalence of *L. curvatus* was observed. The level of residual lactose in fresh curd is usually low but nevertheless some is likely to be present when the non-starter lactic acid bacteria population is becoming established in cheese. The subsequent increase in the non-starter lactic acid bacteria population is likely to occur after the lactose has been utilised (see Fig.1. and Fig.2.) From a sample ripened for 45 days at 6 °C *L. helveticus* was isolated. Williams and Banks (WILLIAMS & al. [6]) reported on isolation of *L. helveticus* from Cheddar cheese ripened for 6 – 9 months, considering this species as non-starter lactic acid bacteria. During ripening of Krievijas cheese I and II at 12 °C, the presence of *L. plantarum* 1 was detected.

DNA fragment sequencing of most frequently identified *L. plantarum* 1 and *L. curvatus* revealed that nucleotide sequence of *L. curvatus* of ripened Krievijas cheese II for 60 days at 6 °C conforms to *L. paracasei* subsp.*paracasei* JCM 8133, but in Krievijas cheese II ripened for 60 days at 12 °C – to *L. paracasei* MH55. In their turn, isolated *L. plantarum* 1 from Krievijas cheese II ripened for 45 and 60 days at 12 °C conforms to *L. plantarum* S4 and *L. plantarum* DSPV 354T, respectively.

API 50 CHL system applied for identification of *Lactobacillus* phenotypically showed satisfactory results when determining genus of microorganisms. Tynkkynen and co-authors (TYNKKYEN & al. [7]) build on evidence that system precision is considerably lower when identifying microorganisms up to species. This might be due to the fact that the system was initially developed for identification of *Lactobacillus* genus for medical needs (COEURET & al. [5]) as a supplementary system for atypical fermentation models (ARHNÉ & al.[8]; CHAMBA [9]; COEURET & al. [5]). Some authors (MUYANJA [10], TEMMERMAN & al. [11]) concluded that phenotypic methods have limitation in terms of
reproducibility, low taxonomic resolution and very often allow identification only at the genus level.

Variability of species under the influence of temperature and ripening time in Holandes cheese samples is given in Table 2.

L. *paracasei* subsp. *paracasei* domination was observed in in Holandes cheese I. From the sample ripened at 6 °C, *L. curvatus* was isolated as well, but from the sample ripened at 12 °C - *L. rhamnosus*. In Holandes cheese II domination of *L. paracasei* subsp. *paracasei* was observed until the 45th day of ripening at 6 °C, and prevalence of *L. plantarum* 1 from the 30th until the 60th day of ripening at 12 °C. According to Copolla (COPOLLA & al. [12]) and Fitzsimons (FITZSIMONS [13]) observation, most frequently *L. casei*, *L. paracasei*, *L. plantarum*, *L. rhamnosus* and *L. curvatus* are isolated from cheeses.

According to Fitzsimons (FITZSIMONS & al. [14]), Williams (Williams & al. [15] and their co-authors findings, the dominating non-starter lactic acid bacteria species usually change during the ripening, and are represented by one *Lactobacillus* species at the end of ripening. In the opinion of these authors, *L. paracasei*, *L. plantarum* and *L. brevis* dominate in unripened cheeses, but *L. paracasei* – in ripened cheeses.

Diversity of microflora and its growth intensity in analyzed cheeses depend on water activity, salt content, pH, ripening temperature and oxidation-reduction potential. Therefore the dynamics of the colony forming units of non-starter lactic acid bacteria under the influence of some factors is evaluated.

Quantity of microflora is characterized by the count of lactic acid bacteria colony forming units in experimental Krievijas (Fig. 1) and Holandes (Fig. 2) cheeses.

Non-starter lactic acid bacteria growth rate during ripening of Krievijas cheese is temperature-dependent but ripening temperature has little influence on the final numbers of lactobacilli in analyzed cheese. Shakel-Ur-Rehman and co-authors (SHAKEL-UR-REHMAN & al. [16]) reported that in cheese ripened at temperature 1 °C, the non-starter lactic acid bacteria population was 3 log cycles lower than in a cheese ripened at 8 °C. Other authors have similar

### Table 2. The changes of *Lactobacillus* spp. during Holandes cheese ripening

<table>
<thead>
<tr>
<th>Ripening time, days</th>
<th>Holandes I at 6°C</th>
<th>Holandes II at 6°C</th>
<th>Holandes I at 12°C</th>
<th>Holandes II at 12°C</th>
</tr>
</thead>
<tbody>
<tr>
<td>45 days</td>
<td><em>L. paracasei</em> subsp. <em>paracasei</em> 1, <em>L. curvatus</em></td>
<td><em>L. paracasei</em> subsp. <em>paracasei</em> 3, <em>L. curvatus</em></td>
<td><em>L. paracasei</em> subsp. <em>paracasei</em> 1</td>
<td><em>L. plantarum</em> 1</td>
</tr>
<tr>
<td>60 days</td>
<td><em>L. paracasei</em> subsp. <em>paracasei</em> 2</td>
<td><em>L. curvatus</em></td>
<td><em>L. paracasei</em> subsp. <em>paracasei</em> 1, <em>L. rhamnosus</em></td>
<td><em>L. paracasei</em> subsp. <em>paracasei</em> 1, <em>L. rhamnosus</em></td>
</tr>
</tbody>
</table>
opinions that ripening temperature influences considerably the growth of non-starter lactic acid bacteria in cheese (FOLKERTSMA & al. [17]; FENELON & al. [18]). More rapid growth rate of non starter lactic acid bacteria in Krievijas cheese at 12ºC is explained by temperature closer to optimum of the mesophilic bacteria activity.

![Graph showing colony forming units (CFU) of non-starter lactic acid bacteria in Krievijas cheeses during ripening.](image1)

**Figure 1.** The dynamics of colony forming units of non-starter lactic acid bacteria in Krievijas cheeses during ripening

Similar situation was observed in Holandes cheeses. Changes in the count of colony forming units show that their increase depends on temperature, composition of the starter, availability of nutrients and other factors. Although the population size remains relatively stable in Holandes cheese samples from 45th day to the end of ripening, the population is not statistic but is in a dynamic state as the balance of the species and the strains change.

pH is one of the most important factors during cheese ripening, enhancing activity of enzymes and regulating the growth of microorganisms. Due to technology peculiarities of Krievijas cheese, the pH after pressing rises up to 5.2 – 5.3 creating characteristic acidity of Krievijas cheese, which corresponds to the results of Krievijas cheeses I and II (Fig. 3), respectively, pH 5.25 and 5.22.

![Graph showing colony forming units (CFU) of non-starter lactic acid bacteria in Holandes cheeses during ripening.](image2)

**Figure 2.** The dynamics of colony forming units of non-starter lactic acid bacteria in Holandes cheeses during ripening
Increase of pH at the beginning of Krievijas cheese ripening must be explained by application of lactic acid in further chemical processes and degradation of protein and fat. Dispersion analysis revealed significant differences (p<0.05) between Krievijas cheeses ripened at 6 and 12 °C.

Lactic acid is converted into lactic acid during growth of lactic acid bacteria in Holandes cheese, which causes a decrease in pH during beginning of ripening. Lactococcus strains produce only lactic acid as long as they are growing and lactose is available. These activities contribute to afresh acidic flavour of the cheeses, and the lactic acid produced will only be further metabolised into different flavour compounds with NSLAB present. These metabolic products increase pH in cheese during later stages of ripening.

In order to make sure about the influence of pH on the water activity, correlation analysis was performed. The obtained results reveal a strong negative linear correlation (Krievijas cheeses \( r=0.87 \); Holandes cheeses \( r=86 \)) between the parameters under the study. This supports opinion of several authors (FOX & al. [19]; FOLKERTSMA & al. [17]; ASTON & al. [21]) that during cheese ripening while the amount of water-soluble nitrogen compounds and the application of lactic acid increase, the rise of the pH value and reduction of water activity take place. Water activity in Krievijas cheeses during ripening varied from 0.994 up to 0.960, but in Holandes cheeses from 0.995 up to 0.971.
In order to control the rate of cheese ripening and the growth dynamics of mesophilic non-starter lactic acid bacteria, some researchers have suggested decreasing of ripening temperature. Decreased ripening temperature slows down the growth rate of mesophilic non-starter lactic acid bacteria, but it is impossible to liberate cheese from their presence. Also this study reveals that the population of non–starter lactic acid bacteria differs between cheeses ripened at 6 and 12 °C at least by 1 log. Higher concentrations were found in cheeses ripened at 12 °C. These findings should be taking into account, because the temperature at ripening is subordinated to the rate of biochemical processes in manufacture of a particular cheese variety. Any change has a significant impact on the whole complex of the sensory properties of cheese.

Conclusions

Identified *Lactobacillus* spp. well-adapted to variable parameters of cheese ripening, and their population and growth rate are dependant on diversity of substrate in cheeses. The prevalence of non-starter lactic acid bacteria species in cheeses varies during ripening and at the end of ripening were represented by one species, more often *L. curvatus*, *L. paracasei* subsp. *paracasei* or *L. plantarum*. Diversity of lactic acid bacteria species in Krievijas and Holandes cheeses depends on selected ripening temperature and time. Representatives of *Lactobacillus* genus and its colony forming units differs between same variety cheeses manufactured at different plants. The close correlation was determined between changes of pH and aw in analyzed Krievijas and Holandes cheeses. This indicates intensity of biochemical and microbiological processes during ripening.

References