Diversity and functional aspects of cheese ripening bacteria

Diversitet og funksjonalitet av bakterier viktige for modning av ost

Philosophiae Doctor (PhD) Thesis

Aleksandra Martinovic

Faculty of Chemistry, Biotechnology and Food Science

Norwegian University of Life Sciences

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ABSTRACT

Cheese traits can be seen in the context of metabolic activity of lactic acid bacteria (LAB) and their impact is important to obtain cheeses with specific characteristics. Accurate identification and characterisation of LAB is especially important when these are applied in the dairy industry. To identify LAB, various methods have been used, but the most accurate are the ones employing molecular biology techniques. Characterized strains of LAB can be used for the improvement of flavour, texture and aroma of cheeses. Many of the strains used nowadays in industrial production are originating from indigenous dairy products and niches. Therefore, it is extremely important to preserve this autochthonous diversity and scientifically support the production of traditional cheeses.

In the first part of this study the possibility of making traditional cheese with selected strains of indigenous lactic acid bacteria was explored. Most of the traditional dairy products of the South- Eastern Europe are produced from unpasteurized milk without addition of selected starter cultures. These artisanal food products contain an interesting biodiversity of indigenous microorganisms. In Montenegro, one of the most popular traditional cheeses is Njeguši cheese, belonging to the group of semi-hard cheeses. Most of the cheeses are produced from ewe's milk, but cow's milk is also widely used. One of the priorities of the food producers in Montenegro is to obtain a larger scale, standardized production of traditional food products, which are of good quality and considered safe for the consumers. Therefore, in this study indigenous strains of lactic acid bacteria isolated from various Montenegrin artisanal dairy products were tested as starters in Njeguši cheese. After their biochemical and molecular characterization, three isolates were selected and used as starter cultures in pilot plant experiments for the simulation of a possible semi industrial production of Njeguši cheese. When applying an adequate combination of added bacterial strains as starters, the flavour of Njeguši cheese, produced under pilot plant conditions, was a step in the right direction towards achieving the flavour comparable to that of the artisanal origin.

In the second part of the study the effects of two adjunct *Lactobacillus casei* strains on the lactobacilli population of low fat Cheddar cheese were described. The adjuncts were added at a low initial number and differed in their ability to utilize components of the milk fat globule

membrane (MFGM), which was controlled by addition of butter milk powder or skim milk powder. The most diverse microbial composition was revealed at the start and it became more uniform in the later stages of cheese ripening. The microorganisms present at the start influenced the lactobacilli population during ripening, but the adjuncts did not dominate the microbiota in the cheese. A higher content of MFGM components in the cheese seemed to influence the lactobacilli population and the composition of free amino acids during ripening. The low initial numbers of lactobacilli resulted in comparatively large distances between these cells in the fresh cheese. Electron micrographs of ripened cheese showed large clusters of clearly elongated lactobacilli.

Third part of the study, encompassed the analysis of the survival of selected bacteria in semi-hard experimental cheeses after exposure to human gastric and duodenal juices in an *ex vivo* model. Experimental cheeses (10 and 28 % fat) were supplemented with different strains of *Lactobacillus* sp. and *Propionibacterium* sp. and ripened for 7 and 70 weeks. After digestion, a greater numbers of the adjunct bacteria were recorded in the 7-wk cheeses compared to the well-matured cheeses (70 wk). The bacterial survival was strain dependent, and influenced by the fat content of the cheese. Lactobacilli showed better survival, especially in low-fat cheeses. The strains of propionibacteria also survived well during the digestion of the low-fat cheeses. The results confirmed that cheese can potentially be a good carrier matrix for bacteria to the intestine.

In addition, it has been shown that different strains present in cheese have different abilities to survive the conditions of the gastrointestinal tract. Younger cheese was indicated to be a better carrier, possibly because the bacteria present in those cheeses have had shorter exposure to the stress conditions occurring in cheese during prolonged maturation.

SAMMENDRAG

Ostens egenskaper kan sees i sammenheng med den metabolske aktiviteten til melkesyrebakterier (LAB). Nøyaktig identifisering og karakterisering av LAB er spesielt viktig når disse brukes i meieriindustrien. Det benyttes forskjellige metoder for å identifisere LAB, men de mest nøyaktige metodene anvender molekylærbiologiske teknikker. Karakteriserte stammer av LAB kan anvendes til forbedring av smak, tekstur og aroma i oster. Mange av stammene som brukes i industriell produksjon i dag stammer fra tradisjonelle meieriprodukter. Derfor er det ekstremt viktig å bevare dette mangfoldet, og vitenskapelig støtte opp under produksjon av tradisjonelle oster.

I den første delen av denne studien ble muligheten for å lage tradisjonell ost med utvalgte stammer av melkesyrebakterier isolert fra tradisjonelle meieriprodukter undersøkt. De fleste tradisjonelle meieriprodukter i sørøst-Europa er produsert av upasteurisert melk og uten tilsetning av utvalgte starterkulturer. Disse tradisjonelle matvarene inneholder et interessant biologisk mangfold av mikroorganismer som ikke er karakterisert. I Montenegro er Njeguši ost en av de mest populære tradisjonelle ostene. Njeguši ost tilhører gruppen av semi-harde oster. De fleste av disse ostene er produsert fra sauemelk, men kumelk er også mye brukt. En av prioriteringene for matprodusenter i Montenegro er å utvikle en standardisert, storskala produksjon av tradisjonelle matvarer av god kvalitet og som også er trygge for forbrukerne. I denne studien er derfor stammer av melkesyrebakterier isolert fra ulike montenegrinske, tradisjonelle meieriprodukter testet som syrekultur til produksjon av Njeguši ost. Etter biokjemisk og molekylær karakterisering av isolatene ble tre isolater valgt ut og brukt som syrekulturer i pilot forsøk for å simulere en mulig semi-industriell produksjon av Njeguši ost. Ved anvendelse av en egnet kombinasjon av bakteriestammer som syrekultur, ble ost med smak av Njeguši ost, produsert i pilot skala. Dette er et skritt i riktig retning med hensyn til å oppnå den"riktige" smaken sammenlignet med den opprinnelige tradisjonsosten.

I den andre delen av studien ble effekten av tilsats av 2 tilleggskulturer bestående av 2 *Lactobacillus casei* stammer på populasjonen av laktobasiller i en lav-fett Cheddarost beskrevet. Tillegggskulturene ble tilsatt i lavt antall og var forskjellige med hensyn til å utnytte komponenter av melkens fettkulemembran (MFGM), noe som ble regulert ved tilsetning av kjernemelkpulver eller skummetmelkpulver. Den mest varierte mikrobielle sammensetningen

ble funnet i tidlig fase, og den ble mer ensartet i senere stadier av ostens modning. Mikroorganismene tilstede i tidlig fase påvirket populasjonen av laktobasiller under modningen, men tilleggskulturene dominerte ikke bakteriefloraen i osten. Et høyere innhold av MFGM komponenter i osten så ut til å påvirke populasjonen av laktobasiller og sammensetningen av frie aminosyrer under modning. Det lave opprinnelige antallet av laktobasiller resulterte i relativt store avstander mellom disse bakteriecellene i fersk ost. Elektronmikroskopi av moden ost viste store klynger av tydelig langstrakte laktobasiller.

Tredje del av studiet omfattet analyse av overlevelse av utvalgte bakterier i semi-harde forsøksoster etter eksponering for human mage og tarm saft i en *ex vivo*-modell. Eksperimentelle oster (10 og 28% fett) ble tilsatt forskjellige stammer av *Lactobacillus* sp. og *Propionibacterium* sp. Under ysting og ble videre modnet i 7 og 70 uker. Etter fordøyelse, ble et større antall av de tilsatte bakteriene målt i 7-ukers ostene, sammenlignet med de godt modnede ostene (70 uker). Den bakterielle overlevelsen varierte blant ulike stammer, og ble påvirket av fettinnholdet i osten. Laktobasiller viste bedre overlevelse, spesielt i lav-fett oster. Propionsyrebakteriestammene overlevde også godt under fordøyelsen av lav-fett oster. Resultatene bekreftet at ost potensielt kan være en god bærermatriks for bakterier til tarmen. I tillegg er det vist at forskjellige stammer tilstede i osten har forskjellige evne til å overleve forholdene i mage-tarmkanalen. Det ble antydet at yngre ost kan være en bedre bærer, muligens fordi bakterier i disse ostene har hatt en kortere eksponering for stressbetingelsene som forekommer i ost under langvarig modning.

LIST OF PAPERS

PAPER I

Martinovic, A., Narvhus, J., Abrahamsen, R.K., Østlie, H.M, Skeie, S.B. Application of indigenous strains of lactic acid bacteria for semi-industrial production of autochthonous Montenegrin Njeguši cheese. *Manuscript*

PAPER II

Martinovic, A., Moe, K.M., Romeih, E., Aideh, B., Vogensen, F.K., Østlie, H., & Skeie, S. (2013). Growth of adjunct *Lactobacillus casei* in Cheddar cheese differing in milk fat globule membrane components. *International Dairy Journal, 31*, 70-82.

PAPER III

Martinovic, A., Brede, M.E., Vegarud, G.E., Østlie, H.M., Narvhus, J., & Skeie, S.B. (2016). Survival of lactic acid and propionibacteria in low- and full-fat Dutch-type cheese during human digestion Ex vivo. *Letters in Applied Microbiology, 62, 5,* 404-410.

Abbreviations

ARDRA: amplified ribosomal DNA restriction analysis

ARISA: automated ribosomal intergenic spacer analysis

BMP: butter milk powder

DNA: deoxyribonucleic acid

DVI: direct-to-vat inoculation cultures

DVS: direct vat set cultures

FISH: fluorescence in situ hybridization

FHL: facultative heterofermentative lactobacilli

ISH: in situ hybridization

LAB: lactic acid bacteria

MFGM: milk fat globule membrane

MLSA/MLST: multi locus sequence analysis/multi locus sequence typing

PAB: propionic acid bacteria

PEP: PTS: phosphoenolpyruvate phosphotransferase system

PFGE: pulsed field gel electrophoresis

PCR: polymerase chain reaction

RAPD: randomly amplified polymorphic DNA

Rep-PCR: repetitive element palindromic-PCR

RFLP: restriction fragment length polymorphism

Rep- PCR: repetitive element palindromic-PCR

RNA: ribonucleic acid

RT-PCR: reverse transcription PCR SMP

RTqPCR: reverse transcription quantitative PCR

SMRT: single-molecule real-time sequencing

SMP: skim milk powder

SSCP: single-strand conformation polymorphism

SSH: suppression subtractive hybridization

TGGE: temperature gradient gel electrophoresis

T-RFLP: terminal restriction fragment length polymorphism

1. AIM OF THE STUDY

Lactic acid bacteria (LAB) play a crucial role in cheese production and ripening, influencing all main characteristics of the cheese such are texture, sensory characteristic, aroma, etc. Therefore, it is particularly important to properly identify and characterize those strains that are used as a primary or secondary starter cultures as well as adjunct cultures in the cheese making. This can be done by the application of molecular methods.

Furthermore, the starter and adjunct cultures can be used for the improvement of the quality of reduced fat cheeses, influencing the textural characteristics, flavour and aroma of the ripened cheese.

Cheese can be a suitable carrier of beneficial strains of bacteria that can improve human health. It is as well very important to preserve biodiversity and richness of traditional cheese varieties that can be achieved via isolation and characterization of indigenous strains to be used in the cheese production.

In this research, the attempt was to address all of these aspects through the experimental set up with the aim of obtaining results that could represent a sound foundation of further research in the area.

The work is divided in the following research sections:

A) Application of selected strains of LAB in pilot plant production of the traditional Montenegrin Njeguši cheese. Three selected, previously identified and characterized strains of LAB (two belonging to *Lactococcus lactis* ssp. *lactis* and one belonging to *Lactobacillus* (*Lb.*) *plantarum*, isolated from indigenous cheeses of Montenegro, were used for semi-industrial production of Njeguši cheese (PAPER I).

B) Investigation of the development and dynamics of the lactobacilli population during ripening of low-fat Cheddar cheese differing in the content of milk fat globule membrane (MFGM) components, and with a low initial number of adjunct *Lb. casei* strains differing in their ability to utilize MFGM components added to the cheese milk (PAPER II).

C) Evaluation of *ex vivo* survival of selected microorganisms in cheese differing in fat

content and ripening time. Cheese supplemented with different adjunct lactic acid bacteria or propionic acid bacteria (PAB) were exposed to conditions of the human upper gastro intestinal tract (GIT) using simulated digestion with human gastric and duodenal juices (*ex vivo* digestion) (PAPER III).

2. BACKGROUND, MAIN RESULTS AND DISCUSSION

2.1. Say cheese

"Say cheese", calls the photographer in any part of the world when a photograph is to be taken. This is the extent to which the taste of cheese has influenced people such it brings a smile upon a mention (Weimer, 2007).

2.1.1. What is cheese

According to the Codex Alimentarius (Codex Alimentarius, FAO/WHO, Standard 283-1978, 2013), cheese is the fresh or ripened solid or semi-solid product in which the whey protein/casein ratio does not exceed that of milk, obtained by:

- Coagulating (wholly or partly) the following raw materials: milk, skimmed milk, partly skimmed milk, cream, whey cream, or buttermilk, through the action of rennet or other suitable coagulating agents, and by partially draining the whey resulting from such coagulation; or
- Processing techniques involving coagulation of milk and/or materials obtained from milk that give an end product which has similar physical, chemical and organoleptic characteristics as the product systemized under Classification of cheese.

2.1.2. The history of cheese production

The art of cheese production dates back to the ancient past and today there are more than 1000 different varieties of cheese. It is believed that cheese is originating from the area of today's Iraq, the „Fertile Crescent", between the Tigris and Euphrates rivers, 8.000 years ago. At the time of the „Agricultural Revolution", domestication of animals alights the man on the

nutrition quality of the milk and the modalities for its preservation. It is supposed that the first cheese was made accidentally by putting the milk into the pouch made from a sheep's stomach. The rennet of the pouch and high outer temperature caused the milk to coagulate. The specific, pleasant taste of the formed curd was the revelation and the new beginning of the cheese making era (Kindstedt, 2012).

Data on the production of milk and dairy products found in Egypt are at least from the year 4000 B.C. and Babylon (2000 B.C.). The athlete stadium in Jerusalem was named Tyroponeon (the valley of cheese makers). In ancient Greece, the cheese is mentioned in Homer's writings, whereas the art of cheese making was extremely important during the Roman Empire. Roman naturalist and writer, Pliny the Elder referrers to C*aseus Helveticus* ("Swiss cheese") in 23-79 B.C. Later on, the art of cheese making starts to be widespread throughout the Europe, especially in the monasteries where the famous cheese varieties have been developed (Wensledayle, Port du Salut, Formage de Tamie, Maroilles, Trapist, etc.). Certainly, most of the cheese varieties have been developed spontaneously, depending greatly on the local circumstances, such as the milk quality, natural microbiota and processing technologies applied.

By the beginning of XX century, cheese making is becoming more industrialized having the dominant cheese varieties produced worldwide. Over the last three decades a significant increase of the cheese production can be recorded globally. It grew from 11 mil tonnes produced in 1977 to over 18 mil tonnes in 2012 (PM Food & Dairy Consulting, 2014). The growth was driven by the application of innovation processing techniques, development of new products and market demands. Almost 75% of the world's cheese production is produced in Europe and Northern America. Europe (especially France, Ireland and Germany) are the most export oriented countries, while USA is the world's largest cheese producer, but with minor cheese exportion (PM Food & Dairy Consulting, 2014). Germany, UK and Italy imports the largest quantities of cheese in the world.

There are many attempts to classify cheeses. Usually, the cheeses are classified according to the moisture content. McSweeney, Ottogalli & Fox (2004) gave an overview of the diversity of cheese varieties (**Fig. 1**).

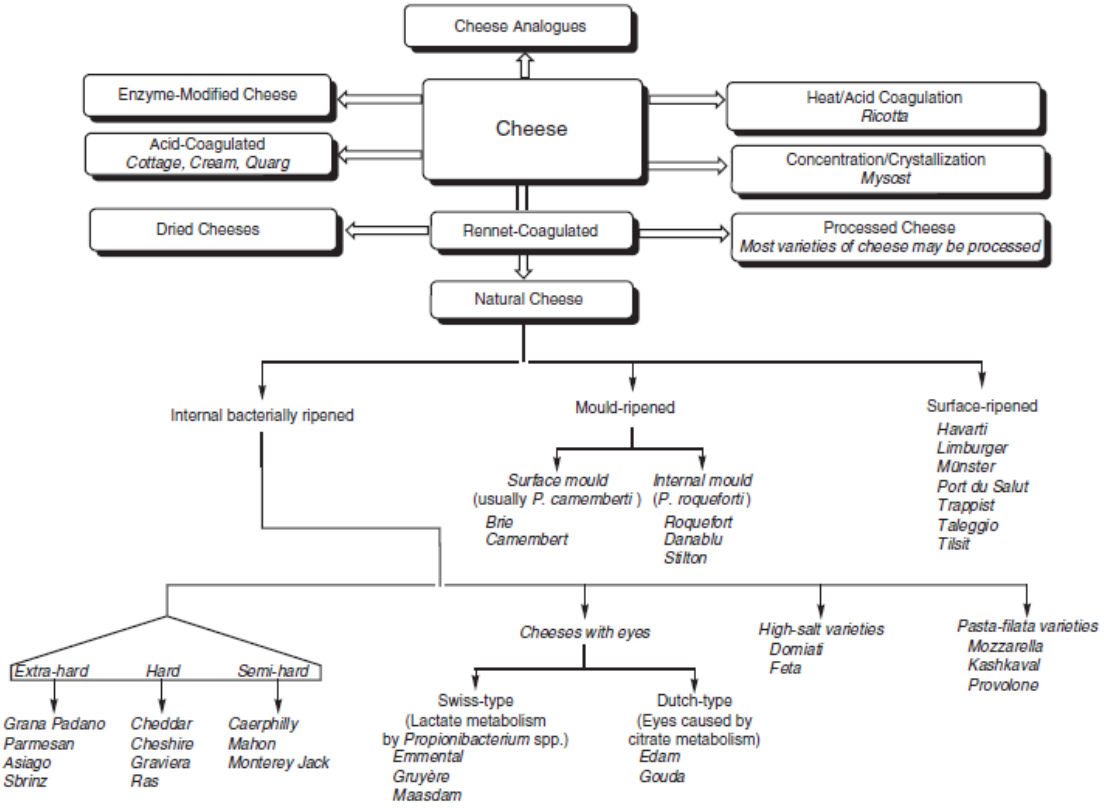


Figure 1. The diversity of cheese. Cheese varieties are classified into super-families based on the method of coagulation and further sub-divided based on the principal ripening agents and/or characteristic technology. Adapted from: McSweeney, Ottogalli & Fox, 2004.

There is no consensus on the number of cheese varieties in the world and different authors suggest the numbers varying from 400 up to 2000 varieties. Considering such a high number of different cheeses, it is quite demanding to make a reasonable classification of the cheese varieties.

In spite of the complexity in classification of cheese varieties, these can, according to McSweeney, Ottogalli & Fox (2004) be divided into:

- *Extra hard cheeses* (hard texture, ripened for a long period, aromatic flavour). The most important in this group are the Italian'Grana' types (Grana Padano, Granone Lodigiano, Parmigiano Reggiano), Asiago, Bagozzo, Bra, Formai de Mut; in addition, the 'Pecorino'

cheeses (Pecorino Romano, Pecorino Sardo, Pecorino Siciliano, Pecorino Toscano, Pecorino Pepato, Fiore Sardo), which are made from ewes' milk, , as are the Swiss varieties, Tete de Moine, Sbrinz, Sapsago, the Spanish cheeses, Cebrero, Pedroches and Manchego, the Greek cheeses, Kefalotiri and Gravera and Reggianito from South America. Njeguši cheese, produced in Montenegro, according to its traits also belongs to the group of hard cheeses;

- *Cheddar and Cheddar varieties* (cheese made of pasteurized milk, with the addition of mesophilic starters, and the application of a "cheddaring" process);
- *Cheeses with propionic acid fermentation* (presence of large cheese eyes, resulting from gas produced by propionic acid bacteria used in the cheese making). The best known varieties are Emmental, Maasdamer, Leerdamer and Jarlsberg.
- *Gouda type cheeses* (made from pasteurized cow's milk with a mesophilic LD-starter, ripening for 2-3 months, characterized with the catabolism of citrate to diacetyl and small eyes). The varieties also include Edam, Maribo, Danbo, Colonia, Norvegia, Svecia varieties;
- *Pasta-fillata cheeses* (the curd is heated to 55-60 °C, with smooth fibrous and sliceable texture. The best known are Mozzarella varieties with *Mozzarella di Bufala* (buffalo milk), *Mozzarella di vacca* (cows'milk; also called Fiordilatte, Scamorza or Provola), Caciocavallo, Cascaval, Kashkaval, Provolone, Kasseri and Kasar peyniri;
- *Cheese ripened in brine* such are Brinza, Beli Sir, Telemes, Kareish, Beyaz Peiniri;
- *Mould- ripened varieties* (surface mould ripened varietis such are Camemebert and Brie, and blue cheeses such are Cabrales, Gorgonzola, Danablu and Stilton);
- *Smear-ripened cheeses* (bacteria surface ripened cheeses, mostly made of ewes or goats milk);
- *Acid curd cheeses* such are Cottage or Quarg;
- *Whey cheeses,* etc.

2. 1. 3. Protection of the indigenous strains- treasuring of the national heritage

The characteristics and different varieties of traditional cheeses are highly influenced by the region of production, cultural heritage and the tradition of the population living in the specific area. These products show a strong link with the territory of origin, natural conditions and costumes of the local population.

Production of traditional cheeses in most of the cases involves utilization of raw, unpasteurized milk, resulting in a cheese texture and aroma highly influenced by the naturally occurring micro flora. The complex system of biochemical reactions in cheese is a result of metabolic activities of different microbial populations that change during the cheese ripening. Numerous external factors are also influencing the traits of traditional cheeses and therefore it is very difficult to control production process and maintain more standardized production. On the other hand, one of the possible solutions, and so far the most widespread in the process of cheese making, is to standardize production technology by using pasteurized milk, followed by the application of starter cultures. However, when applying commercial starter cultures the resulting cheese might lose its valuable authenticity. Isolates of the natural occurring microbiota and their application as starter or adjunct cultures in controlled cheese making processes may lead to preservation of the unique quality of traditional cheeses along with the more standardized production.

East Mediterranean area is featured with small precipitations, hot long summers and rocky terrain not suitable for crop growing. This highly influences the animal breeding systems in the area, being semi-extensive, with variable lactation periods lasting approximately 7 months as well as a variable quality of the milk used for cheese production (Boyazoglu & Morand-Fehr, 2001; Carić, 1993). Montenegro, although small in size, is rather rich in agro- and bio-diversity that results in, among other food products, different traditional dairy products: cheeses (soft, brined, semi-hard, pasta filata), fermented milks and specific full fat fermented cream ripened in an animal sack called ‘’skorup’’ (Dozet, Adžić, Stanišić & Živić, 1996).

Indigenous dairy products of Montenegro have not been studied to a great extent. First structured data were found in the works of Zdanovski (1947), Vukina (1949) and Rakočević (1950). These are followed by the research in the 1970-ties and 1980-ties which gave the insight into the structure and diversity of autochthonous dairy products of Montenegro (Adžić et al.,

1984; Dozet, Adžić, Stanišić & Ljumović, 1987; Dozet, Adžić, Stanišić & Živić, 1996; Dozet, Maćej & Jovanović, 2004).

Based on unique characteristics and specific features of indigenous cheeses Adžić, Dozet, Ljumović, Marković & Adžić (1997) propose a schematic overview of the most important dairy products of Montenegro that should as well be considered for nomination for Protection of Geographical Indications (PDO) (**Fig. 2**).

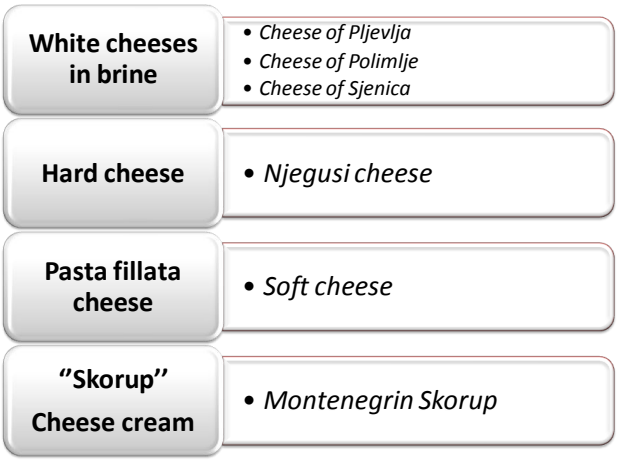


Figure 2. The most significant traditional Montenegrin dairy products

2.1.4. Njeguši cheese

One of the oldest traditional Montenegrin semi-hard cheeses- Njeguši cheese dates back to the age of Roman Empire where it was called ''*Caseus Doclestes*'' (Markeš, 1973) named after the state Doclea existing at the territory of today's Montenegro. Numerous writings evidence the quality and taste of this semi-hard cheese (Stieglitz, 1841).

Originally, Njeguši cheese is produced using ewe's, cow's or mixed milk, filtered through a cheese cloth and heated to the temperature of 32 to 35 °C. Rennet obtained from the lamb stomach is used for curdling occurring 30-60 minutes after rennet addition. The temperature is then increased to 40-45 °C to achieve an optimal whey separation. The cheese curd enclosed by a cloth is placed in a mould, loaded with a circuit or plate, and then pressed for about 5 to 12 hours before turning of the cheese and pressing for 24 hours. After pressing, the cheese is dry salted. The cheese is ripened at an average temperature of 16 °C and at the

humidity higher than 80 % for up to 25 days with occasional turning (**Fig. 3**). Sometimes, the cheese is smoked, dipped into olive oil or corn grains to obtain a more piquant taste.

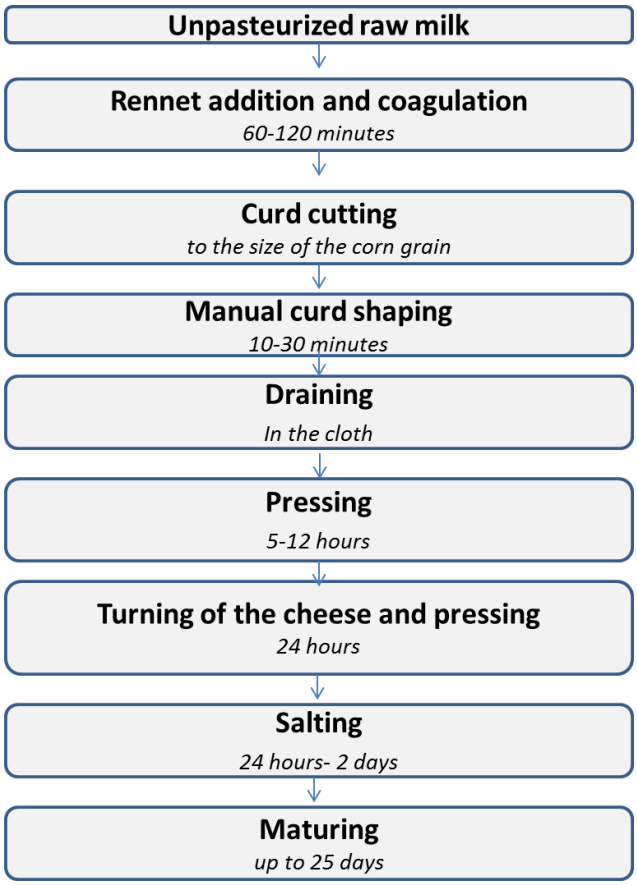


Figure 3. Flow diagram of production technology of Njeguši cheese

In the study performed by Dozet et al. (1996) on different Montenegrin households producing Njeguši cheese, the moisture content of the cheese ranged from 16.4 to 47.4 %, the fat in dry matter from 44.6 to 58.6, the total protein from 19.0 to 31.2 % and the percentage of salt from 1.1 to 1.2 %.

2.1.5. Application of indigenous strains in controlled production of traditional cheeses

Farmhouse fermented milk products are made using ancient technology developed in the country or region where this food is produced and can be considered as part of the country's cultural heritage. Traditional cheeses, as complex ecosystems, contain different types of microorganisms that are influencing cheese ripening and its sensory properties (Beresford &

Williams, 2004). Diversity of microorganisms in traditional cheeses, usually made from raw milk, depends on milk microbial diversity as well as on traditional practices applied in cheese making. Flavor of traditional raw milk cheeses is more intense when compared to those produced from pasteurized milk. This is due to the fact that diverse native microbiota expresses its metabolic activities during cheese ripening resulting in the richer flavor. The natural community of LAB, isolated from farmhouse fermented unpasteurized milk products represents a reservoir for obtaining strains with specific features, which could be used as starter cultures in the dairy industry (Marilley & Casey, 2004). Such strains may differ in many important properties, such as their rate and extent of acid production, sensitivity to bacteriophage infection, and ability to degrade casein.

Montenegro is a small country, but due its climate and geographical characteristics it has a high diversity of traditional food products, including dairy products (Dozet et al., 1996). In the various regions of the country, different traditional dairy foods are produced (such as soft, semi-hard, and pasta filata cheeses, fermented milks, etc.), which are influenced by climate conditions and type of the livestock breed in different areas (Mirecki, 2012). In order to achieve the successful application of LAB strains in industrial production, it is necessary to carry out accurate classification and identification and also to determine biochemical reactions of these bacteria when used as industrial starter cultures (Henriksen & Nilsson, 2001; Cogan et al., 2007; Escamilla-Hurtado et al., 1996).

A natural pool of lactic acid bacteria (LAB) may be used for standardization of the Njeguši cheese production process to obtain the desired quality and unique features of this traditional cheese. A collection of 100 LAB, isolated from indigenous dairy products of Montenegro (cheese, sour milk and ‘’skorup’’) has been made and these isolates have been characterized and identified using conventional microbiological as well as molecular techniques (Martinovic et al., 2005).

In the present study (**paper I**), bacterial strains isolated from indigenous dairy products of Montenegro were used. In the previous study, these strains were identified by 16S rDNA sequencing and discriminated using pulsed field gel electrophoresis (PFGE) (Martinovic et al., 2005). After their biochemical characterization (Martinovic et al., 2006) three strains were selected for use as starter cultures in pilot plant experiments of Njeguši cheese. The results of chemical, biochemical and sensory analyses indicated that experimental cheeses, made with

strain M (*L. lactis* ssp. *lactis* ABO19-3) and the combination of the strains M and A (*L. lactis* ssp. *lactis* ABO57-1), originally isolated from Njeguši cheese, showed the highest similarity with traditional Njeguši cheese. Strain M was able to synthesize the branched chain alcohols 2-methyl-1-propanol, 3-methyl-1-butanol and 2-methyl 1-butanol (Martinovic et al., 2006), which was also detected in cheeses where this strain was used as starter. The obtained results showed that methyl-ketones synthesis might be correlated with the synthesis of methyl-alcohols as they were identified in the same cheeses (**paper I**). Development of gross components and pH in the studied cheeses showed that these were not significantly influenced by the strain combinations used during cheese making, but were significantly dependent on the ripening time which was in accordance to the findings of other authors for Njeguši cheese (Adžić et al. 1997; Dozet, Maćej, & Jovanović, 2004; Mirecki et al., 2015). The salt content was at the optimum values for this type of cheese and as well was in accordance to findings of Mirecki et al. (2015).

In cheeses made with starter A (*Lactococcus lactis* ssp. *lactis*) (cheese A) the concentration of DL-pyro glutamic acid was increasing by the end of maturation period, while in cheeses made with strain M (*Lactococcus lactis* ssp. *lactis*) (cheeses A, AM and ALM) the highest concentration of this acid was recorded after 7 days of ripening (**paper I**). DL pyro-glutamic acid in cheese can be free or bound to N terminus of proteins (Lemieux & Simard 1992; Muchetti et al., 2000). It can be formed by enzyme-mediated cyclisation of glutamic acid by some strains of LAB (Mucchetti et al., 2002; Sforza et al., 2009).

Strain M was able to synthesize the branched-chain alcohols 2-methyl-1-propanol, 3-methyl-1-butanol and 2-methyl 1-butanol) (Martinovic et al., 2006). It has been shown in previous research that methyl aldehydes, responsible for the development of malty aroma in cheeses can positively contribute to the flavour development in some cheeses (Griffith & Hammond, 1989; Barbieri et al., 1994; Banks et al., 2001; Yvon & Rijnen, 2001; Thierry & Maillard, 2002).

2.1.6. Low fat cheeses

Increased awareness on ethology of chronic diseases associated with consumption of food with high fat content have resulted in higher demands towards low fat foods (Katsiari,

Voutsinas & Kondyli, 2002a; Kucukoner & Haque, 2006; Oliveira & Assumpcio, 2000; Katsiari, Voutsinas, Kondyli & Alichanidis, 2002b).

The dairy industry follows these trends as well, particularly in the cheese production with the attempts of improving the texture and functional properties of low fat cheeses (Nelson & Barbano, 2004; Koca & Metin, 2004; Madadlou, Khosroshahi & Mousavi, 2005; Kilcawley et al., 2007; Mohamed, 2015).

The great challenge for the dairy industry is to produce reduced fat cheese varieties that would be competitive to full fat varieties on the market (El Soda, 2014; Mohamed, 2015).

Altered processing parameters and fat reduction during low fat cheese production, results in a less acceptable flavour, texture and functional properties of cheese (Mohamed, 2015).

The flavour of the low fat cheese is impaired mostly by the deficiencies in fatty acids deriving from the milk fat (Urbach, 1997; Barlow et al., 1989; Banks, Brechany & Christie, 1989). Wijesunda & Watkins, (2000) have described that the flavour deficiencies in fat reduced Cheddar cheese are associated with the lower content of milk fat derived flavour compounds such as short to medium chain carboxylic acids, methyl ketones and β and σ-lactones. Furthermore, bitter off-flavours in low fat cheeses may be a result of reduced partitioning of hydrophobic bitter peptides in the fat phase (McSweeney, Nursten & Urbach, 1997).

It is also well known that textural defects in the low fat cheese are a consequence of the influence of fat on the microstructure, texture and functionality of the cheese resulting in increased firmness, rubberness, hardness and dryness of the cheese (Olson & Johnson, 1990; Guinee, Auty & Fenelon, 2000). In low fat cheeses, the breakdown of casein is often insufficient, which results in calcium retention in the curd and higher firmness of the cheese. The yield of the low fat cheeses is lower when compared to the full fat cheeses (Mohamed, 2015).

In order to improve the quality of the low fat cheeses different procedures are applied in the dairy industry such are improvement trough processing techniques, application of adjunct cultures and utilization of additives or novel methods for fat removal (Collins, McSweeney & Wilkinson, 2003; Kilcawley et al., 2007; Liu, Xu & Guo, 2008).

Adjunct cultures are also used to increase functionality of the low fat cheeses. Experiments with low-fat Feta-type cheese resulted in development of a flavour similar to the full-fat variant when a commercially available adjunct culture CR-213 (Chr. Hansen's Laboratorium, Copenhagen, Denmark) was used in the production process (Katsiari et al.,

2002b). It has been shown that homofermentative and facultative heterofermentative lactobacilli may contribute to the development of desirable flavour in reduced-fat Cheddar cheese due to their metabolic activities (Fox et al., 1996; Fox, Mc Sweeney & Lynch, 1998; Lynch et al., 1999; El Soda, Madkor & Tong, 2000a,b; Beresford, Fitzsimons, Brennan & Cogan, 2001). In low-fat cheese, it has been assumed that the nonstarter lactic acid bacteria (NSLAB) microbiota develops differently compared to the microbiota of full-fat cheeses (Laloy, Vuillemard, El Soda & Simard, 1996). In the present research, the diversity of NSLAB was highest at the beginning of ripening, while towards the end it was more uniform.

The milk fat globule membrane (MFGM) contains different glycoconjugates in the form of glycoproteins and glycolipids (Mather, 2000; Liu et al., 2005; Moe, Faye, Abrahamsen, Østlie & Skeie, 2012). Oligosaccharides from the glycoproteins of MFGM can be a potential energy source for the cheese-ripening microbiota, since the monosaccharides from the glycoconjugates and amino acids created in the proteolysis can be a significant energy source for NSLAB (Martley & Crow, 1993; Crow et al., 1995; Hynes, Ogier & Delacroix-Buchet, 2001; Di Cagno et al., 2003; Moe et al., 2012). Low-fat cheese has a lower content of MFGM, which is a possible energy source for NSLAB.

Buttermilk contains lactose, caseins, minerals and serum proteins and is rich in water-soluble MFGM components (Rombaud, Van Camp, & Dewettinck, 2006; Vanderghem et al., 2010). This membrane contains polar lipids and specific membrane proteins ensuring structural integrity and stability of the milk fat (Vanderghem et al., 2010; Danthine et al., 2000; Dewettinck et al., 2008; Jiménez-Flores & Brisson, 2008; Singh, 2006). Therefore, buttermilk has a big potential for the application in the food industry (Vanderghem et al., 2010).

Addition of buttermilk, which is rich in MFGM components (Morin, Pouliot & Britten, 2008) to the low-fat cheese, may increase the content of possible energy sources for the cheese microbiota. It has been demonstrated that some lactobacilli adjuncts isolated from cheese are able to grow and survive for an extended period in a medium with MFGM as the only added carbohydrate source (Moe et al., 2012). In **paper II**, effects of two adjunct *Lb. casei* strains on the lactobacilli population of low fat Cheddar cheese were described. The adjuncts, differing in their ability to utilize MFGM components were added during Cheddar cheese making using either butter milk powder or skim milk powder to control their access to MFGM components. The diversity of lactobacilli in the experimental cheeses was becoming more uniform by the end

of maturation time, while resulting electron micrographs of the cheese matrix confirmed that lactobacilli were distributed in clusters embedded in the protein matrix. This research revealed that adjuncts and MFGM components influenced the development of microbiota of the cheeses as well as synthesis of branched chain amino acid Leu in the cheese during ripening. The production of diacetyl and acetoin was highest in the cheeses without added adjuncts. Previous research show that in Cheddar cheese citrate is decreasing rather slowly and enzymatic activity of NSLAB flora is causing its decrease at late stages of ripening (Thomas, 1987a; Singh, Drake & Cadwallader, 2003). Diacetyl and acetoin may be synthesized through transamination of Asp and Asn leading to formation of oxaloacetate which by spontaneous decarboxylation can be metabolized to diacetyl by some lactobacilli (Kieronczyk et al., 2004; Skeie, Kieronczyk, Næs & Østlie, 2008). The present research (**paper II**) showed that cheeses without added adjuncts had reduced contents of Asp and Asn, and it was assumed that indigenous NSLAB flora in these cheeses caused this degradation, resulting in increased levels of diacetyl and acetoin. Ribose can be used as a carbon source for mesophilic lactobacilli in the later stages of ripening (Thomas, 1987b), while lysed lactococcal cells may release ribose from RNA and *N*-acetylgluconsamin from degraded cell walls (Østlie, Vegarud, & Langsrud, 1995; Adamberg et al., 2005). In the present study, 97% of the isolates were ribose positive indicating that a possible source of carbon for mesophilic lactobacilli may be connected to the fact that they possess some glycosidehydrolase activity and can utilize sugars from glycoproteins of the MFGM as an energy source (Williams & Banks, 1997*;* Fox, McSweeney & Lynch, 1998).

In the present study the highest levels of 3-methyl butanal, originating from Leu, were measured starting from 6 weeks of ripening in cheeses supplemented with BMP. In these cheeses also the highest amounts of Leu were recorded. Aldehydes in cheese, originating from transamination of branched chain amino acids, as well as from imides that may be decarboxylated to the corresponding aldehydes (McSweeney & Sousa, 2000; Kieronzcyk, Skeie, Olsen & Langsrud, 2001; Marilley & Casey, 2004*,*). Supplementation of the cheese with buttermilk components seemed to facilitate better growth of microorganisms able to degrade these branched chain amino acids.

Production of low fat cheeses supplemented with adjuncts and suitable energy sources for their growth may significantly influence the sensory parameters of these cheeses that can be further explored for the application in the cheese making industry. Taking into consideration

these findings, along with those of other authors, it can be seen that the production of low fat cheeses supplemented with adjuncts and suitable energy sources for their growth may significantly improve the sensory parameters of these cheeses, fulfilling the market requirements at the same time.

2.2. THE WORLD OF LAB

Lactic Acid Bacteria (LAB) as one of the biggest and most widespread bacterial groups share common morphological, metabolic and physiological properties. There is no general definition of the name of Lactic Acid Bacteria. Typical LAB is Gram positive, catalase negative, without cytochromes, anaerobic but aero tolerant, strictly fermentative producing lactic acid as the main end product of the sugar fermentation (Salminen & Von Wright, 1998; Von Wright & Axelsson, 2012). These bacteria can be found in various food products (dairy, meat, vegetables, fruits, cereals, etc.), and are the part of the normal microbiota of the mouth, digestive system and vagina of mammals. Their metabolic activities are important in the production of numerous food products, especially in the dairy industry. Physiological activity of LAB is featured by the production of lactic acid, leading to the decrease of pH, proteolytic activity, ability for exopolysaccharide synthesis, production of aroma compounds, bacteriocine production and their probiotic activity.

Lactic acid bacteria constitutes a group of Gram positive, non-spore forming, non-respiring coccobacilli, bacilli and cocci with a DNA base composition of less than 53 mol % G+C and producing lactic acid as the main metabolite during carbohydrate fermentation. Current taxonomic classification of LAB shows that they belong to the phylum *Firmicutes*, class *Bacilli* and order *Lactobacillales*. The different families include *Aerococcaceae, Carnobacteriaceae, Enterococcaceae, Lacobacillaceae, Leuconostocaceae* and *Streptococcaceae* (Von Wright & Axelsson, 2012). There are 12 common genera related to the food products: *Aerococcus, Carnobacterium, Enterococcus, Tetrageonococcus, Vagococcus, Lactobacillus, Pediococcus, Leuconostoc, Oenococcus, Weissella, Lactococcus* and *Streptococcus* (Von Wright & Axelsson, 2012). Genus *Lactobacillus, Leuconostoc, Lactococcus, Pediococcus* and *Streptococcus* make the sole heart of the group (Von Wright & Axelsson, 2012).

The first pure culture of LAB was isolated by J. Lister in 1873 which he named *Bacterium lactis* (most probably what we now know as *L. lactis*). Significant achievement in the classification of this bacterial group was done by Henneberg (1904), when the connection between milk acidification and the bacteria producing lactic acid become obvious. Later in time, when classification was done based on morphology, glucose fermentation patterns, growth on different temperatures and sugar utilization (Orla-Jensen, 1919) the genera *Lactobacillus*, *Leuconostoc*, *Pediococcus* and *Streptococcus* were characterized. Orla Jensen (1919) classified LAB into the genera *Lactobacillus*, *Leuconostoc*, *Pediococcus* and *Streptococcus.*

At that time, solely the phenotypic characteristics could be examined, but later on, detailed research based on macromolecules such as nucleic acids was established.

Method of partial or total 16S rRNA gene sequencing is the most widespread method for studies of the bacterial phylogeny and taxonomy. This gene is present in all bacteria. Some regions of the 16S rRNA gene are highly conserved throughout the bacterial evolution allowing the comparison between distant related organisms, while other regions are variable allowing the comparison of closely related organisms (Janda & Abbott, 2007; Petti, 2007). The 16S rRNA gene sequencing method is commonly used for reliable identification and confirmation of bacteria offering information on the degree of similarity of sequences between different genera and species (Gonzales et al., 2007; Abosereh, El Ghani, Gomaa & Foua, 2016). The technique itself is based on the similarity percentage, meaning that two organisms are closely related if the 16S rRNA gene sequences have identity values higher than 97% (Adimpong et al., 2012).

However, this technique also has certain limitations due to the quality of the isolated DNA, impurities/inhibitors present, amount of the isolated DNA, utilization of different primer sets or incomplete sequences (Temmerman, Huys & Swings, 2004; Vetrovsky & Baldrian, 2013). Closely related species where 16S rRNA sequencing can provide information only up to the genus level due to the high similarity of 16 S rRNA gene sequences among certain species exists. This was the case in the recently diverged LAB species *Lb. plantarum*, *Lb. paraplantarum* and *Lb. pentosus* (Felis & Dellaglio, 2007; Pot & Tsakalidou, 2009; Ueda, Nomoto, Yoshida & Osawa, 2014).

First genome sequences of LAB were discovered at the beginning of this century, by sequencing of genomes of *L. lactis* spp. *lactis* IL1403 (Bolotin et al., 2001), *Lb. plantarum* WCFS1 (Kleerebezem et al., 2003) and *Bifidobacterium longum* NCC2705 (Schell et al., 2002)

that doesn't belong to the group of LAB, but is often considered in the same context. Since then, the number of sequenced LAB genomes has grown significantly resulting in wide pool of available genomic data (Douillard & de Vos, 2014).

Previously, the classification of LAB was done on the basis of their metabolic activities, cell morphology, growth temperature and the protein patterns in the cell wall and in the cell (William & Sandler, 1971; Decallone et al., 1991; Tsakalidou et al., 1994; Gatti, Fornasari & Neviani, 1997; Morelli, 2001). These methods however have biased reproducibility, while the potential of the genome sequencing is not fully detectable since the gene expression highly depends on the environmental conditions (Mohania et al., 2008). **Fig. 4** shows the major phylogenetic groups of LAB based on 16 S rRNA data.

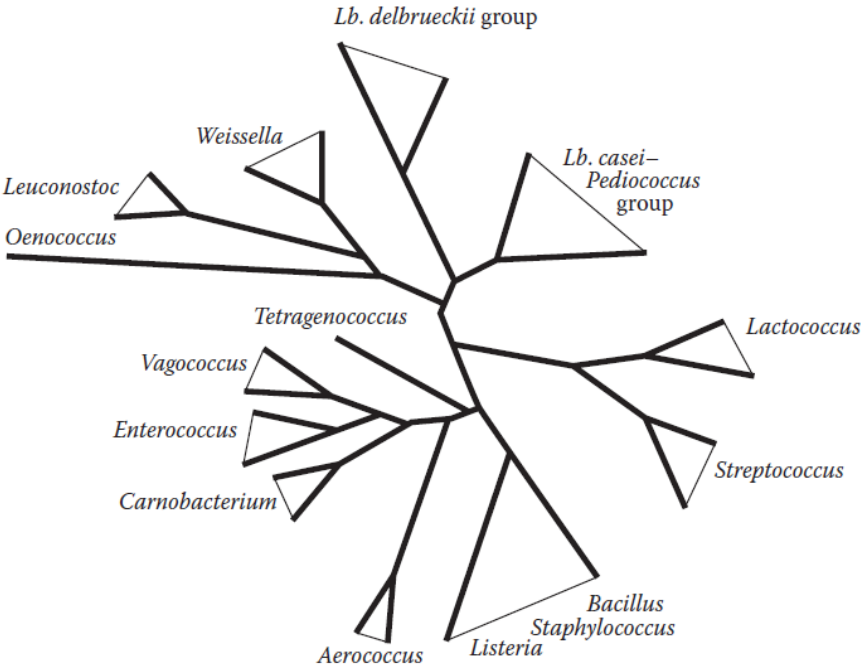


Figure 4. Schematic, unrooted phylogenetic tree of the LAB, including some aerobic and facultatively anaerobic *Firmicutes*. Note: Evolutionary distances are approximate**.** Adapted from Von Wright & Axelsson (2012).

The genus *Bifidobacterium,* was previously generally considered together with LAB belong to the Gram-positive bacteria with a high G+C content but it belongs to *Actinobacteria*, and the genus *Propionibacterium*, present in some cheese varieties, has never been classified as a LAB, but belongs to *Actinobacteria* (Ludwig & Klenk, 2001).

2.2.1. Methods to study cheese microbiota

Nowadays, numerous phenotypic and genotypic methods for identification have been developed. Depending on the methodology used, all methods can be divided into culture-dependent or culture-independent methods. **Fig. 5.** gives an overview of the existing culture-dependent and culture-independent methods applied in dairy research.

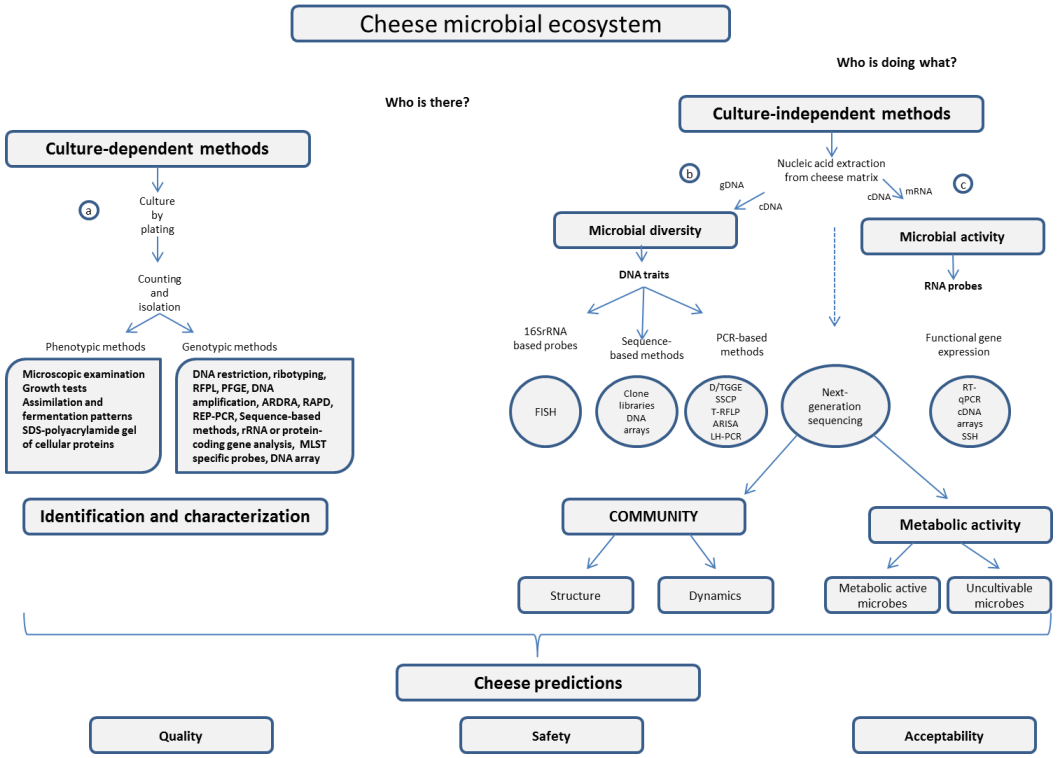


Figure 5. Flow diagram of the culture-dependent and culture-independent methods to study the community structure and activity of cheese microbiota. a Culture-dependent methods. b, c Culture-independent methods for microbial diversity (b) and microbial activity (c). RFLP restriction fragment length polymorphism, ribotyping, PFGE pulsed-field gel electrophoresis, ARDRA amplified ribosomal DNA restriction analysis, RAPD randomly amplified polymorphic DNA, REP-PCR repetitive extragenic palindromic PCR, ARISA automated rDNA internal spacer analysis, DGGE denaturing gradient gel electrophoresis, FISH fluorescence in situ hybridization, ISH in situ hybridization, LH-PCR length heterogeneity polymerase chain reaction, qPCR quantitative PCR, RT-PCR reverse transcription PCR, RTqPCR reverse transcription quantitative PCR, SSCP single-strand conformation polymorphism, SSH suppression subtractive hybridization, TGGE temperature gradient gel electrophoresis, T-RFLP terminal restriction fragment length polymorphism (Ndoye, Andriamahery, LaPointe & Roy, 2011).

2.2.2. Culture- dependent methods for identification of cheese microbiota

The culture-dependent methods are limited to bacteria isolated from the given sample after plate enrichment and they are divided into traditional and molecular methods.

Traditional methods include phenotypic methods. Phenotypic tests include morphological characterization of isolates by microscopy, as well as physiological ones by determination of the growth characteristics of isolates. Biochemical tests designed for determination of carbohydrate fermentations (API tests), can be used for preliminary identification of LAB to genus or species level.

Molecular biology based methods represent support to the conventional phenotypic/biochemical methods, featured by short time for the analysis, good repeatability and reliability. Macromolecules (DNA and RNA) document the evolutionary history and are used for decades now to determine the relatedness of microorganisms. For identification of unknown isolates and evaluation of their evolutionary relatedness, 16S rRNA comparative sequence analysis is often used, developed in 1987 by Woese (Woese, 1987). DNA-DNA hybridization is a very useful tool for the determination of the relatedness between the strains (Stackebrandt & Gobel, 1994).

In most cases it is necessary to discriminate different strains within the same species of LAB. There are numerous molecular techniques dealing with strain identification. The most commonly used strain typing methods are shown in **Fig. 6.**

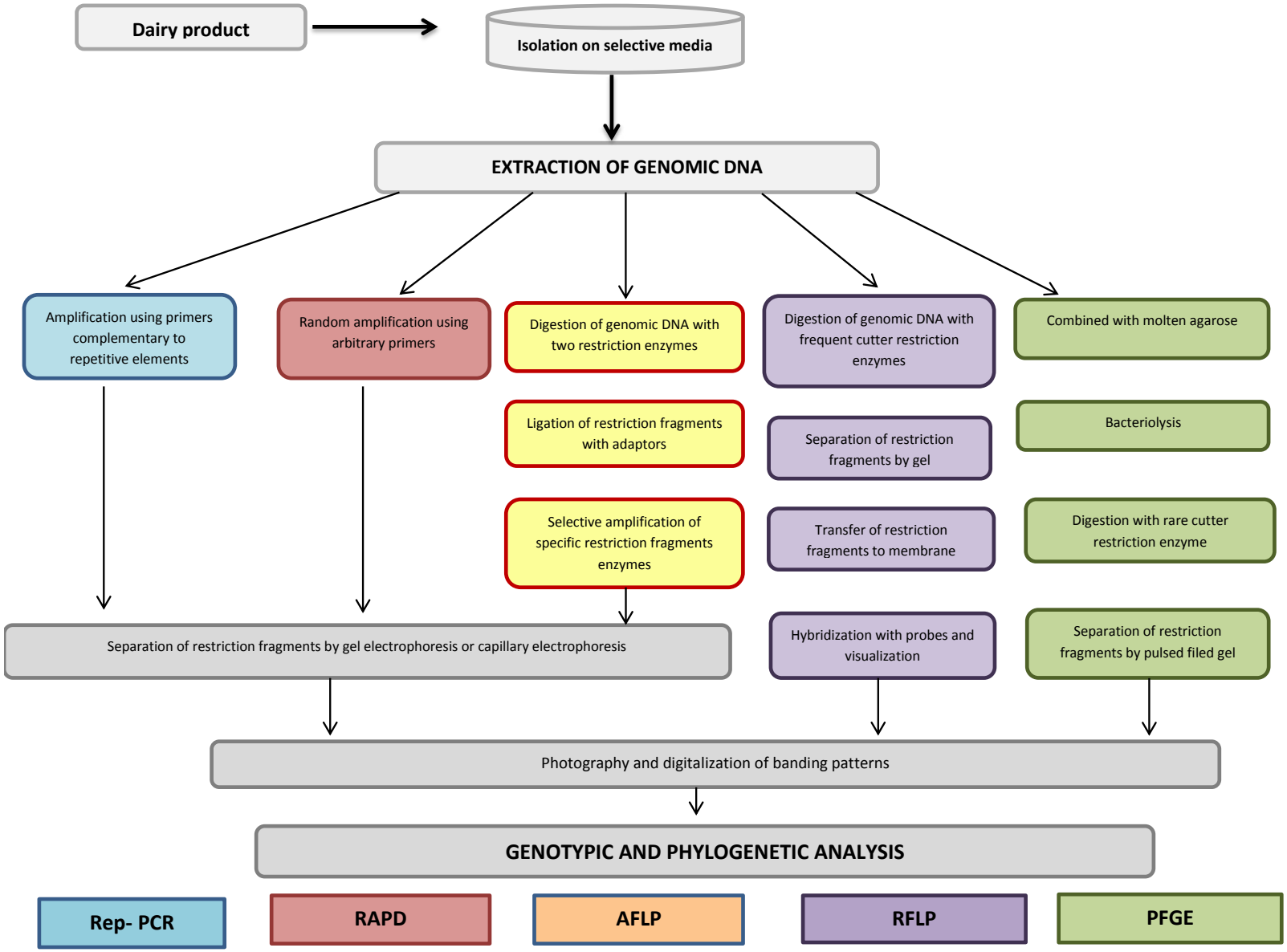


Figure 6. Flow chart of DNA banding pattern-based genotyping methods for identification of bacterial strains.Created based on information given by Li, Raoult & Fournier (2009).

One of these is the pulse field gel electrophoresis (PFGE) that involves digestion of the genomic DNA with rare-cutting restriction enzymes, which are separated in the alternating, pulsed electric field (Klein et al., 1998; Vancanneyt et al., 2006; Njeru et al., 2010). The obtained fingerprint profiles represent the whole genome and are mainly used for discrimination of evolutionary close strains (Holzapfel, Haberer, Geisen, Bjorkroth & Shillinger, 2001; Delgado & Mayo, 2004).

AFLP (amplified fragment length polymorphism) is a PCR- based method widely used for strain identification. Initially the DNA is hydrolyzed by two restriction frequent cutting enzymes, followed by PCR with two different primers that correspond to the adapters ligated to the restriction fragments. The application products are separated on denaturating polyacrylamide gels with 50 to 100 restriction fragments amplified and detected. The AFLP technique is applied in the analysis of complex populations of LAB (Busconi, Reggi & Fogher, 2008; Scheirlinck et al., 2009).

RAPD *(*random amplification of polymorphic DNA) technique is based on annealing of the short arbitrary primers to the multiple random target sequences. Resulting patterns are useful for strain discrimination of LAB (Oh-Sik, 2002; Spano et al., 2002).

RFLP (restriction fragment length polymorphism) technique employs the digestion of the complete chromosomal DNA allowing strain discrimination. The technique is widely used for species and strain identification (Deveau & Moineau, 2003; Mainville Robert, Lee & Farnworth, 2005; Claisse, Renouf & Lonvaud-Funel, 2007; Mohania et al., 2008).

Molecular typing techniques, such as repetitive element sequence-based PCR (rep-PCR) offer a high discriminatory power for identification of LAB (De Urraza et al., 2000; Berthier, Beuvier, Dasen & Grappin, 2001; Ben Amor, Vaughan & De Vos, 2007; Singh, Pawas, Singh & Heller, 2009). It is used for determination of the phylogenetic relationships between species and strains, having high differentiation power. The method is easily applicable in all types of research laboratories, and it is based on the amplification of short repetitive sequence elements in the chromosome (Versalovic, Schenider, Bruijn & Lupski, 1994; Randazzo, Caggia & Neviani, 2009). The technique has been used for monitoring the dynamics of starter bacteria in Cheddar cheese (Dasen et al., 2003) as well as for the identification and detection of lactobacilli and *Bifidobacterium* species (Gevers, Huys & Swings, 2001; Krizova, Spanova & Rittich, 2008). The

application of the $(GTG)_5$ primer has shown to give the highest discriminatory power (Gevers, Huys & Swings, 2001; Kostinek et al., 2005; Svec, Drab & Sedlacek, 2005; Ouadghiri, Amar, Vancanneyt & Swings, 2005).

Molecular ribotyping is the technique which recognizes ribosomal genes trough hybridization of the chromosomal DNA patterns with 23S and 16S rRNA gene probes employing Southern blotting. This results in higher reproducibility and accuracy of the method for strain identification (Grimont & Grimont, 1986; Charteris, Kelly, Morelli & Collins, 1997). The discriminatory power is similar to that of PFGE, when lactobacilli or bifidobacteria were analysed (Tynkkynen et al., 1999; Mättö et al., 2004). However, ribotyping – although robust - is a very labourous and cost intensive technique.

MLSA/MLST (Multi Locus Sequence Analysis/Multi Locus Sequence Typing) is another technique, which offers sequencing of several gene loci, usually of housekeeping genes, such as *atpD*, *gyrB*, *recA*, *rpoB*, *rpoD,* often combined with conserved 16S and 23S rRNA genes (Maiden et al., 1998; Hanage, Fraser & Spratt, 2006; Bennasar, Mulet, Lalucat & García-Valdés, 2010). Application of MLSA has for example been described for lactic acid bacteria populations in sourdough (De Vuyst & Vancanneyt, 2007).

The most contemporary methods such as "next generation" sequencing techniques, the "454 sequencing" and the "Solexa/Illumina", "SOLiD", Life Sciences Ion-Torrent and Pacific Biosciences Single-molecule real-time sequencing (SMRT) sequencing can provide sequence data of the whole genomes in less than one day (Margulies et al., 2005; Hudson, 2008; Loman et al., 2012ab; Kelleher et al., 2015). Application offers a huge pool of information containing billions of small sequence reads providing sequencing of a large number of isolates in a short time.

With the ongoing development of genomics, complete genome sequences are available for many of the species and strains found in starter cultures, enabling a deeper insight into the metabolic properties of the organisms (Dellaglio et al., 2005; Pfeiler & Klaenhammer, 2007). Transcriptomics enables the creation of a realistic insight into the cell metabolism during its growth in milk (Pedersen, Iversen, Sorensen & Johansen, 2005; Smeianov et al., 2007).

In the present research, different conventional, biochemical and molecular techniques were applied for identification of unknown LAB species and their discrimination at the strain level (**paper II**). In total, 100 isolates were chosen on the basis of their cultivation and

morphological differences and presumptive lactobacilli were characterized on the basis of their carbohydrate fermentation patterns. To ensure accurate identification at the species level, the 16S rRNA sequence analysis was used. Discrimination of the FHL at strain level was done by rep-PCR analysis, according to a method adapted from Versalovic, Schneider, de Brujin & Lupski (1994).

Phenotypic characterization showed that all of the isolates were Gram positive and catalase negative bacilli and their CO_2 production was at the level of homofermentative organisms. The results obtained by API 50 CHL showed that 97 out of 100 isolates tested in this study were ribose positive. These data also revealed a higher diversity of the isolates at the beginning of the ripening, while the population composition became more uniform by the end of maturation (24 weeks of ripening). Most of the isolates were identified as *Lb. casei/Lb. paracasei*. The results of API testing of the selected isolates were in accordance with the chemical and microbial characterization of the cheeses indicating a clear grouping according to the adjuncts in the intermediate phase of ripening (4-10 weeks), whereas after 24 weeks the differences between the experimental factors were less apparent.

Rep-PCR showed some isolates from the same maturation time clustered together in separate clusters. The numbers of clusters with at least 80% similarity decreased during the ripening period. This indicates a change in microbial composition of the studied cheeses over the maturation period, while none of the investigated isolates clustered with more than 80 % similarity with the adjuncts *Lb. casei* INF448 or INF 456. Previous studies of semi-hard cheeses also show that mesophilic lactobacilli predominate in the later stages of cheese ripening (Berthier & Ehrlich, 1998; Crow, Curry & Hayes, 2001; Østlie, Eliassen, Florvaag, & Skeie, 2004). Certain strains of *Lb. casei/Lb. paracasei* appeared to be common in all cheeses and they most probably originate from the cheese milk or dairy environment (Fitzsimons, Cogan, Condon & Beresford, 1999; Antonsson, Ardö & Molin, 2001; Antonsson, Molin & Ardö, 2003). The most uniform microbiota was observed at the end of ripening with *Lb. casei/Lb. paracasei* representing the most abundant *Lb.* species regardless of experimental factors used. As shown by other authors (Beresford, Fitzsimons, Brennan & Cogan, 2001; Banks & Williams, 2004) the NSLAB population of Cheddar cheeses is dominated by *Lb. casei/Lb. paracasei.*

2.2.3. Culture independent methods for identification of cheese microbiota

Culture independent methods are based on total DNA/RNA isolation from the sample and it offers the possibility of studying the complete microbial diversity in a single step. Monitoring of the diversity and dynamics of the cheese microbiota during cheese ripening is essential in contemporary research. Culture-independent molecular biology techniques offer a high spectrum of possibilities for determination of not only different species and strains, but also the metabolic activities of microorganisms in the course of cheese ripening (Jany & Barbier, 2008). In this type of analyses it is not necessary to grow bacteria on culture media but nucleic acids (DNA or RNA) are isolated directly from the given sample. This shortens the time necessary for the analysis and provides a complete insight into the complexity of microbial communities in cheese, avoiding possible errors that may occur when a cultivation step is involved.

Common techniques used for studying the cheese ecosystem are denaturating gradient gel electrophoresis (DGGE), (Randazzo, Torriani, Akkermans, de Vos, & Vaughan, 2002), single-strand conformation polymorphism (SSCP) (Duthoit, Godon & Montel, 2003), fluorescent *in situ* hybridization (FISH) (Ercolini, Hill & Dodd, 2003), length heterogeneity-PCR (LH-PCR) (Lazzi et al., 2004), quantitative real-time PCR (qPCR) (Friedrich & Lenke, 2006), and terminal-restriction fragment length polymorphism (T-RFLP) (Arteau, Labrie & Roy, 2010).
Furthermore, next generation sequencing, e.g. pyrosequencing (Margulies et al., 2005; Humblot & Guyot, 2009; Roh et al., 2010; Jung et al., 2011) are applied to study the diversity and dynamics of food fermentations. More recently, next generation sequencing has been used for the investigation of microbial composition of different cheeses (Quigley et al., 2012; Lusk, 2012; Alegria et al., 2012; Ercolini, De Filippis, La Storia & Iacono, 2012; Masoud, 2012; Planý et al., 2016; Porecllato & Skeie, 2016).

2.3. ROLE OF THE LAB IN THE DAIRY INDUSTRY

Milk, as a growth medium, is featured with high nutrition value and it represents a suitable environment for the development of different microorganisms. Raw milk, kept at room temperature is susceptible for spoilage, and after a few days it becomes sour due to the activities

of LAB. The bacteria from spontaneous fermented milk can be transferred to milk and make a foundation for controlled acidification.

The cheese making process strongly relates to the activity of LAB. These bacteria are initiating the production of lactic acid, facilitating the curd formation and are called starter bacteria (Parente & Cogan, 2004). Starter cultures are contributing to the acid formation, texture and flavour development in cheese. Since LAB are naturally occurring in the raw milk, over the time cheese makers developed different modalities for the inoculation of the cheese milk with starter cultures. At the beginning of the commercial cheese production, the application of starter cultures was based on using the liquid cultures, but later in time freeze-dried culture technology was developed in the form of direct vat set (DVS) or direct-to-vat inoculation (DVI) cultures (Høier et al., 2010).

Generally, starter cultures can be divided in *primary* cultures initiating the production of lactic acid from lactose early in the cheese production and *secondary starters* that cause biochemical changes in the cheese during ripening (Parente & Cogan, 2004).

The most common LAB species used as primary starter cultures are shown in Tab. 1.

Table 1. LAB species in various culture types and typical product applications. Adapted from Høier et al., 2010.

Culture types	Species	Product application
Mesophillic		
O type		
	L. lactis subsp. *lactis*	Cheddar cheese
	L. lactis subsp. *cremoris*	Feta cheese, Cottage cheese
LD type		
	L.. lactis subsp. *lactis*	Gouda cheese
	L.. lactis subsp. *cremoris*	Tilsiter cheese
	L. lactis subsp. *lactis* biovar. *diacetylactis*	Soft cheeses with mould
	Leuconostoc mesenteroides subsp. *cremoris*	
Thermophilic		
Streptococcus type	*Streptococcus thermophilus*	Mozzarella cheese, Stabilized Brie, Swiss-type cheese
Yoghurt type	*S. thermophilus*	Mozzarella cheese
	Lb. delbrueckii subsp. *bulgaricus*	Pizza cheese
Lactobacillus type	*Lb. helveticus*	Swiss-type cheese
	Lb. delbrueckii subsp. *lactis*	Grana cheese
Mixed types		
	L.. lactis subsp. *lactis* *L.. lactis* subsp. *cremoris* *S. thermophilus*	Cheddar cheese
	L.. lactis subsp. *lactis* *L.. lactis* subsp. *cremoris* *S. thermophilus*	Feta cheese White brine cheeses

Taking into account the growth temperature, primary starter cultures are classified as mesophilic (optimum growth temperature ranging from 20-30^{o}C) and thermophilic bacteria (optimal growth temperatures 40-45^{o}C) (ref). Mesophilic cultures can be divided into LD (containing citrate-fermenting bacteria producing aroma and CO_2) and O cultures (containing acid-producing strains without the ability for gas production) (Høier et al., 2010).

Nevertheless, all of the starters are originating from indigenous strains, gradually used and adapted to the industrial production (Limsowtin, Powell & Parente, 1996; Beresford, Fitzsimons, Brennan & Cogan, 2001; Parente & Cogan, 2004). These are still used in some of the European dairies but are nowadays to great extent replaced by the commercial starters produced by big companies, which are mostly a mixture of different undefined strains, e.g. mixed-strain starters (Parente & Cogan, 2004).

Other microorganisms e.g. *Propionibacterium freundreicii, Brevibacterium linens, Debaryomyces hansenii, Geotrichum candidum, Penicillium roqueforti, Penicillium camemberti* do not have the acidification ability, but influence organoleptic and biochemical properties of cheese and belong to the group of secondary starters (Parente & Cogan, 2004).

It is very important to properly characterize the single strains before their utilization as starter cultures. The selection criteria are related to different metabolic activities, primarily acidification ability of the strains, resistance to bacteriophages, plasmid profiles, etc. Tab. 2 outlines selection criteria for LAB cheese cultures.

Table 2. Screening criteria for LAB for cheese cultures. Adapted from: Parente & Cogan, 2004.

Basic screening criteria	**Specific selected criteria**	**Production criteria**
Acidification rate	Acidification rate in the specific cheese-making profiles	Ease of production with high cell densities and activity
Phage sensitivity	Proteinase and peptidase activity	Ease of concentration
DNA and plasmid profiles	Texture properties	Stability during freezing and drying
Species identification	Strain interaction	Storage stability
Favour and off-flavour production in milk	Sugar fermentation profile	
Antibiotic resistance	Flavour screening in model cheese systems	

2.3.1. NSLAB and adjunct cultures

Stated earlier, secondary microorganisms play a significant role during cheese ripening. Nonstarter Lactic Acid Bacteria (NSLAB) are still an uncontrolled factor in the modern cheese production industry and may cause positive, as well as negative effects in the cheese products (Crow, Curry & Hayes, 2001; Coolbear et al., 2008; Montel et al., 2014; Gobbetti, De Angelis, Di Cagno, Mancini & Fox, 2015).

Secondary microbiota is most extensively studied in Cheddar cheese represented by mesophilic lactobacilli and pediococci (Peterson & Marshall, 1990; Singh, Drake & Cadwallader, 2003; Hassan, Mona, Abd El Gawad & Enab, 2013). This group of bacteria is named Non Starter Lactic Acid Bacteria. Nonstarter lactobacilli are a widespread group among NSLAB species in most of the cheese varieties (Beresford, Fitzsimons, Brennan & Cogan, 2001; Beresford & Williams, 2004). Most commonly found representatives of this group are *Lb. paracasei*, *Lb. plantarum*, *Lb. rhamnosus, Lb. casei* and *Lb. curvatus* depending on type and age of the cheese (Williams & Banks, 1997; Fitzsimons, Cogan, Condon & Beresfrod, 2001; Settanni & Moschetti, 2010; Solieri, Bianchi & Giudici, 2012). Those lactobacilli belong to the group of facultative heterofermentative lactobacilli (FHL) (Kandler & Weis, 1986). FHL were found in more than 50 cheese varieties (Østlie et al., 2016), while the most extensive studies have been conducted on Cheddar cheese (Singh, Drake & Cadwallader, 2003).

Although mesophilic lactobacilli are found in the raw milk and dairy environment, they are overgrown by acidifying microorganisms primarily *Lactococcus* spp. However, they are often present in cheese as a secondary microbiota. This is especially the case with the raw milk cheeses, but mesophilic lactobacilli are also found in cheeses manufactured using modern technologies and pasteurized milk. During cheese maturation the numbers of starter bacteria is decreasing from 10^9 to 10^7 cfu g^{-1}, while nonstarter lactobacilli are present in low numbers, 10^2 to 10^4 cfu g^{-1} at the beginning of the ripening process, but reaches levels of 10^6 to 10^8 cfu g^{-1} after a few weeks of ripening (Shakeel Ur Rehman, Waldron & Fox, 2004; Gobbetti, De Angelis, Di Cagno, Mancini & Fox, 2015).

NSLAB in cheese have the ability to grow under highly selective conditions, under which the lactose is utilized by starter bacteria, the pH value is in the range from 4.9 to 5.3, temperature from 15 to 31 ^oC, moisture 50 %, salt content 4-6% and with low oxygen availability. NSLAB

are able to utilize other components as growth substrates besides the lactose such as lactate, citrate, glycerol, amino sugars, amino acids and other metabolites (Peterson & Marshall, 1990). They possess a wide spectrum of hydrolytic enzymes and have an efficient lipolysis and proteolysis (Khalid & Marth, 1990; Williams & Banks, 1997; Gobbetti, De Angelis, Di Cagno, Mancini & Fox, 2015).

Adjunct cultures are the selected strains of cheese related microorganisms that are added to the cheese milk to improve the development of the cheese sensory quality (El Soda, Madkor & Tong, 2000ab; Tarakci & Tuncturk, 2008). There are commercially available adjunct cultures on the market, designed for improving the quality of the full fat and low fat cheeses (Tarakci & Tuncturk, 2008).

Non-starter lactic acid bacteria, especially FHL, are used as adjuncts to contribute to the development of desirable cheese flavour by replacing the indigenous NSLAB microbiota, which results in more controlled cheese production (Fox, McSweeney & Lynch, 1998; Hynes, Bergamini, Suarez & Zalazar, 2003; Cogan et al., 2007; El Soda, Madkor & Tong, 2008). An important feature of FHL is their recorded peptidase activity, including their ability to hydrolyse bitter peptides along with the release of amino acids (Gagnaire, Thierry & Léonil, 2001; Chamba & Irlinguer, 2004). Catabolism of amino acids by FHL, which is strain dependent, results in the synthesis of α-ketoglutarate, and through their production of various aroma compounds they may be very valuable as adjunct cultures (Rijnen, Courtin, Gripon & Yvon, 2000, Tanous et al., 2002; Chamba & Irlinger, 2004).

2.3.2. LAB and their relation to the flavour development in cheese

When growing in milk, the major energy source for LAB is lactose, which is phosphorylated during its transport across the cytoplasmic membrane involving phosphoenolpyruvate phosphotransferase system (PEP-PTS), or is transported with the permease system without phosphorylation. Accumulated inside the cell, the phosphorylated lactose is hydrolysed to glucose and galactose-6-phospate (Walstra, Wouters & Geurts, 2006). This PEP-PTS is characteristic for lactococci. Inside the cell the unphosphorylated lactose is hydrolysed to glucose, while galactose is being further phosphorylated to glucose-6-phosphate (Thompson, Brower & Farrell, 1987; Walstra, Wouters & Geurts, 2006, Høier et al., 2010).

Table 3. *Lactate production by various LAB. Adapted from: Høier et al., 2010*

Organism	Transport system	Pathway	Main fermentation products (Moles per mole lactose fermented)	Lactate isomer
Lactococcus spp.	PEP-PTS	Homo-fermentative	4 lactate	L
Streptococcus spp.	Permease	Homo-fermentative	2 or 4 lactate[a]	L
Group I *Lactobacillus* spp.	Permease	Homo-fermentative	2 or 4 lactate	D or LD
Group II and III *Lbactobacillus* spp.	Permease	Hetero-fermentative	Lactate[b] + ethanol + acetate+ $2CO_2$[c]	D and/or L
Leuconostoc spp.	Permease	Hetero-fermentative	2lactate + 2ethanol + $2CO_2$	D

[a]2 moles of lactate if galactose is stoichiometrically secreted, and 4 moles of lactate if galactose is fully metabolized.

[b] The stoichiometry of the fermentation products depends on the species and growth conditions.

[c] Additional ATP and acetate are produced under aerobic conditions.

In lactococci, streptococci and group I *Lb.* spp., glucose-6-phosphate is further metabolized via Embden-Meyerhof (EM) pathway resulting in lactic acid synthesis characteristic for homo-fermentative lactic acid fermentation (Walstra, Wouters & Geurts, 2006; Høier et al., 2010) **(Tab. 3)**. In hetero-fermentative LAB, glucose is fermented via the phosphoketolase pathway where the end products besides lactic acid are CO_2, ethanol or acetic acid. Acetic acid is produced under aerobic conditions from acetyl phosphate, together with extra ATP. This fermentation pattern can be found in *Leuconostoc* spp., and group II and III of *Lb.* spp. (Høier et al., 2010). Under aerobic conditions, in groups II and III of *Lb.* spp,. one extra molecule of ATP and one extra mole of acetate is produced. Since most of the lactose in cheese is lost with the whey, the contribution of lactate to the cheese flavour is actually its influence to the decrease of pH, reflecting to the growth of secondary flora and activity of ripening enzymes (McSweeney, 2004). In Swiss-type cheeses, lactate is metabolized to propionate, acetate, H_2O and CO_2 by *Propionibacterium freidenreichii* spp. *shermanii,* causing the formation of the characteristic large eyes (McSweeney, 2004).

Pyruvate has a central role in synthesis of short chain components essential for the flavour formation in dairy products such as diacetyl, acetoin, acetaldehyde, acetate and ethanol (Cogan & Hill, 1993; Escamilla-Hurtado, Tomasini-Campocosio, Valde´s-Martı´nez & Soriano-Santos, 1996; Henriksen & Nilsson, 2001; Syu, 2001; Melchiorsen et al., 2002).

Many LABs have limited capacity of amino acids synthesis from inorganic nitrogen (Von Wright, & Axelsson, 2012). Dairy LAB are mainly able to utilize peptides and amino acids created through primary protein degradation by external proteins. In cheese making, a very important feature of LAB metabolism is their ability to degrade the protein fractions resulting in textural changes in the cheese during maturation and flavour development. The extracellular membrane serine proteinase in *L. lactis* spp. (lactocepin- PrtP), together with several intracellular peptidases, influences the hydrolysis of casein in cheese (Walstra, Wouters & Geurts, 2006; Liu et al., 2010). There are different classification types of lactocepins depending on their ability for hydrolysis of caseins (Visser, 1993; Exterkate, Alting & Bruinenberg, 1993; Broadbent et al., 2006). Since lactocepin is plasmid encoded, the proteolytic activity is highly dependent on the presence of this plasmid or not (Høyer et al., 2010; Kok & Venema, 1988; Kunji et al., 1996; Siezen, 1999), unlike the proteinases of *S. thermophilus*, *Lb. delbrueckii* subsp. *bulgaricus* and *Lb. helveticus* that are chromosomally encoded (Gilbert et al. 1996; Pederson, Mileski, Weimer & Steele, 1999; Fernandez-Espla, Garault, Monnet & Rul, 2000).

When too much of the hydrophobic peptides are created via the activity of proteinases, bitterness can occur in cheese (Lemieux & Simard, 1991, Lortal & Chapot-Chartier, 2005).

As a result of proteolytic and peptidolytic activities numerous peptides and free amino acids are created in the cheese matrix (Christensen, Dudley, Pederson & Steele, 1999; Broadbent & Steele, 2007). These amino acids can be further catabolized by cheese microbiota, via different pathways, to different flavor compounds (Yvon & Rijnen, 2001).

The first step of conversion of amino acids to α-keto acids is transamination. Aminotransferase activity of bacteria in cheese is leading to the oxidation of amino acids to keto acids (Ardö, 2001). The process of transamination requires the presence of α-ketoglutarate as amino acceptor and its amount might directly influence the reaction (Høier et al., 2010).

Created keto acids are further catabolized by α- keto acid decarboxylases to aldehydes or by hydroxyl acid dehydrogenases to hydroxy acids (Christensen, Dudley, Pederson & Steele, 1999). In the latter phases aldehydes can be converted to alcohols or carboxylic acids by corresponding dehydrogenases.

NSLAB can contribute both to the development of the desired flavour but can also have some negative effects to taste and texture of the cheese (McSweeney et al., 1993; Fox, Mc Sweeney & Lynch, 1998; Thomas & Crow, 1983; Johnson, Nelson & Johnson, 1990; Taylor,

Keefe, Windham & Howell, 1982*)*. Defects caused by the colonisation of cheese by NSLAB include abnormal flavour profiles, gas slits and lactate crystal formation (Crow et al., 2002; Gobbetti et al., 2015). On the other hand, NSLAB are essential for the development of the diversity and typical features of many cheese varieties (Coolbear et al., 2008; Montel et al., 2014; Gobbetti et al., 2015).

Aromatic branched chain amino acids and methionine are the most important flavour compounds in cheeses. Their conversion can result in the synthesis of components such as phenyl ethanol, phenyl acetaldehyde, indole, skatole influencing the development of cheese flavour. Christensen, Dudley, Pederson & Steele (1999) and Gao, Oh & Steele (1997) have discovered that tryptophan metabolites in *L. lactis* spp. *lactis* are strain dependent meaning that the selection of starter bacteria can be a powerful tool for avoiding the creation of undesirable flavour.

In the present study (**paper I**), cheeses M and AM had the highest concentrations of volatile sulphur-containing compounds. Such compounds originate from methionine and include methanethiol, dimethyl sulphide and dimethyl trisulphide. (Urbach, 1995; Bruinenberg, Roo & Limsowtin,1997). It has been observed that non-industrial strains have a wider pool of amino-acid converting enzymes (Smit, Smit & Engels, 2005).

The novel techniques, such as transcriptomics and proteomics, deepen the knowledge of the metabolic activities of LAB bacteria and can be used to predict the circumstances under which some of the metabolites are synthetized (Neves, Pool, Kok, Kuipers & Santos, 2005; Pfeiler & Klaenhammer, 2007).

2.4. CHEESE AS A CARRIER

The microbiome of the human gut represents a specific ecosystem, having complex metabolic activities that, to great extent, influence physiology and susceptibility to inflammation and diseases of every individual (Fujimura, Slusher, Cabana & Lynch, 2010; Brown, De Coffe, Molca & Gibson, 2012; Pfefferele & Renz, 2014). The composition of the microbial community in the human gut is highly variable, depending on age, dietary habits and the genetics of the specific individual (Lozupone, Stombaugh, Gordon, Jansson, & Knight, 2012; Cotillard et al., 2013; Schloissnig et al., 2013).

Most of the human adult microbiota lives in the gut. Only in the human colon does microbial cell density exceed 10^{11} cells/g content, being equivalent to 1–2 kg of body weight (Walker, Duncan, Louis & Flint, 2014). It has been estimated that the human gut microbiome accounts for more than 5 million different genes (Human Microbiome Project Consortium, 2012a). Over 1,000 different species colonize the human gut (Human Microbiome Project Consortium, 2012b). Most of these belong to the phyla of *Firmicutes*, *Bacteroidetes* and *Actinobacteria*, while *Proteobacteria*, *Fusobacteria*, *Cyanobacteria* and *Verrucomicrobia* are less present (Qin, Li & Raes, 2010). Remarkably, given this high inter-individual variability in the gut microbiota composition, a core gut microbiome, shared by healthy adults, has been identified, which suggests that it plays a role in the maintenance of health status (Turnbaugh & Gordon, 2009). This core microbiome is proven to have a influence in polysaccharide digestion, immune system development, defense against infections, synthesis of vitamins, fat storage, angiogenesis regulation, and behavior development (Sekirov, Russell & Antunes, 2010; Qin, Li & Raes, 2010; Cryan & O'Mahony, 2011; Flint et al., 2012). Interestingly, genes encoded by the human core gut microbiome encode proteins required for host survival, but they are not present in the human genome, this finding led to the definition of the gut microbiome as "our forgotten organ" (O'Hara & Shanahan, 2006).

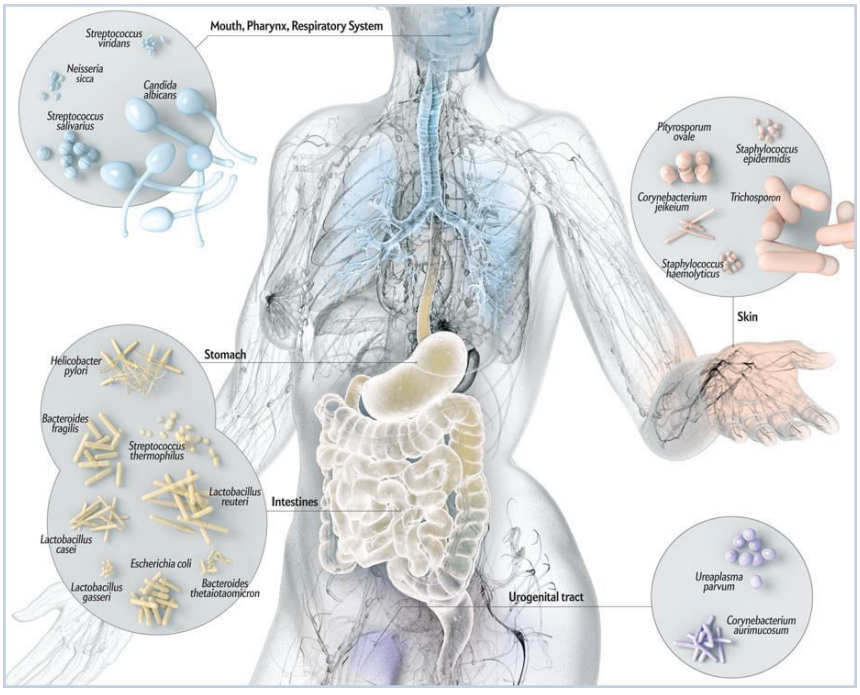


Figure 7. Diagram showing the diversity of human microbiome

Adapted from: Human microbiome project (2012a)

Changes of the normal balance of the gut microbiota may result in the occurrence of changes in energy harvest from foods, numerous physiological disorders and diseases (Lozupone et al., 2012; Cotillard et al. 2013, Schloissnig et al. 2013).

Resilience of the normal physiological status of the microbial population in the human GIT can be achieved via introduction of exogenous microorganisms (Lozupone et al., 2012; Cotillard et al. 2013).

2.4.1. Improvement of the gut health via addition of a suitable microbiota

Recent research is focusing on improvement of the gut health by maintaining the microbial balance in the GIT via addition of a suitable microbiota (Cotillard et al., 2013; Smid & Lacroix, 2013). Complex relations in the microbial ecosystem of the human gut emphasize the significance of the interactions between the host and the existing microbiome, as well as the interactions between the existing microbiome of the human gut and bacterial cells introduced via food carriers (Eckburg et al., 2005; Schloissnig et al., 2013). The main prerequisite for the efficient incorporation of the exogenous microorganisms into the existing microbiota is its survival and adaptation to diverse and severe GIT conditions. Food microorganisms most probably contribute to the maintenance and recovery of the balance in the gut microflora. Therefore, the consumption of fermented foods could support preservation of a healthy physiological state of the gut microbiota and the improvement of its resilience. Different fermented foods, especially fermented dairy products, may act as carriers, and may preserve the viability of the introduced bacteria, thus enabling them to survive the severe conditions in the GIT and potentially attain a synergism with the existing gut microbiota. It is well known that fermented dairy products in general are good carriers for living bacteria (Sharp, McMahon & Broadbent, 2008; Karimi, Mortazavian, Gomez & Da Cruz, 2011; Pitino et al., 2012). Cheese, in particular might be a good carrier of bacteria, as it has a firmer texture, higher pH, and a better buffer capacity in comparison to fermented milks and yogurts (da Cruz et al., 2009). In the research conducted by Sumeri et al. (2012) on 22 weeks old, open texture cheeses it was shown that cheese, due to its numerous beneficial properties, was a better choice for administration of

viable beneficial bacteria compared to fermented milk products. The results obtained in this research support these findings.

To evaluate the survival of the cheese microbiota during human gastrointestinal digestion it is also necessary to have insight into the metabolic activities of these bacteria when exposed to factors in the cheese environment such as low pH, lack of available carbohydrates, low O_2, low redox potential, low a_w, and processes of proteolysis and/or lipolysis. Furthermore, the cheese bacteria need to be able to survive the hostile environment of the stomach during their passage through the gastro intestinal tract (Jacobsen et al., 1999).

Therefore, in order to determine the contribution of food-related bacteria to the microbiota of the intestine, extensive studies should be undertaken, such as testing of their survival through the digestive tract (Ekmekcioglu, 2002). The food bacteria have primarily been exposed to *in vitro* tests for the purpose of testing their ability to survive the severe conditions of the digestive tract, exposure to acidic conditions and the activity of bile salts (Borchers et al. 2009).

Most of the dairy products found on the market having such potential are fermented milks and yogurts. These products contain large numbers of bacteria and may also be supplemented with strains having potential probiotic properties. Among LAB, the genus *Lactobacillus* has been the most studied for this type of applications, but also other lactic acid bacteria and bifidobacteria have been applied (Kimoto et al., 1999; Guarner et al., 2005; Mather, Langella, Corthier & Flores, 2005; Kimoto-Nira et al., 2007; Lee, 2007).

In vitro digestion models are usually performed with commercial digestive enzymes of animal origin (Bordenave, Sannier, Ricart & Piot, 2000; Hernández-Ledesma, Quirós, Amigo & Recio, 2007; Kim et al., 2007; Schmelzer et al., 2007,Escudero, Sentandreu & Toldra, 2010). However, it has been proved that human digestive juices contain enzymes and cofactors that differ from purified animal enzymes and therefore are more reliable for application in *ex vivo* digestion models (Chiang, Sanchez-Chiang, Mills & Tang, 1976; Whitcomb & Lowe, 2007; Ulleberg et al., 2011).

The survival rate of food bacteria when exposed to the severe conditions of the digestive tract is usually tested by in vitro tests (Borchers, Selmi, Meyers Keen & Gershwin, 2009). When bacteria are subjected to standard *in vitro* testing, the results of such tests usually indicate only their tolerance to the digestive conditions, without taking into account other stress factors that

also affect their survival in the GIT (Faye, Tamburello, Vegarud & Skeie, 2012). Therefore, recent experiments have shown that a more suitable option is to use digestion model systems that are potentially more valid when addressing survival of different bacteria in the human GIT, since in such models, bacteria are better exposed to the complex conditions in the upper part of the human digestive tract (Faye, Tamburello, Vegarud & Skeie, 2012; Sumeri et al., 2012; Adouar et al., 2016).

In the present study (**paper III**) young and aged cheeses differing in fat content and supplemented with different culture combinations of starter and adjunct bacteria, were tested for survival of the cheese microbiota after exposure to an *ex vivo* model digestion. Human gastrointestinal enzymes were used in order to simulate realistic conditions in the GIT, since commercial non-human enzymes differ in various characteristics such as the content and types of amylases, lipases, bile salts, cell mucus, etc. (Ulleberg et al. 2011). Survival of lactobacilli during digestion was higher in the low fat cheeses (10% fat) when compared to full fat cheeses (28% fat). The low fat cheeses had higher moisture content, and thereby a higher a_w value, most probably creating better conditions for bacterial growth and survival. The obtained results also suggest that the propionic acid bacteria (PAB) are less exposed to stress conditions in younger cheeses than in more aged cheeses.

NSLAB increase during cheese maturation since these is only slightly inhibited by the conditions in cheese (Beresford, Fitzsimons, Brennan & Cogan, 2001; Banks & Williams 2004; Beresford & Williams 2004). Lactobacilli are able to survive for over 3 years in cheese at a storage temperature of 10°C (Beresford & Williams 2004), and are able to use other energy sources beside lactose (Martinovic et al., 2013). In the present research (**paper III**) a good survival of lactobacilli was observed during digestion in cheese ripened for 7 wk. However, as the stress conditions in the more aged cheeses were higher this most probably influenced the decrease in numbers of lactobacilli during ripening (70 wk cheeses. Revealed findings on the survival during digestion of *Lb. paracasei* INF 448 and *Lb. paracasei* INF 456 in 7 wk old cheese are in agreement with findings obtained for these same strains, when tested under similar conditions during ex vivo digestion of fermented milk (Faye, Tamburello, Vegarud, & Skeie, 2012). The numbers of PAB used in the present research increased to around log 9 cfu g^{-1} cheese throughout the first 7 wk of maturation (Porcellato et al., 2013). In Swiss type cheese where PAB are used as adjunct cultures, high scalding temperatures of the curd do not impair the growth of

PAB and these can be found in high numbers ranging from 10^8 -10^9 cfu/g in the cheese a couple of weeks after cheese making (Beresford et al., 2001; Fröhlich-Wyder et al., 2002; Rossi et al., 2000). PAB can also tolerate pH of the cheese between 5 to 6 and the salt concentration in the moisture phase lower than 5% (Walstra, Wouters & Geurts, 2006; Poonam et al., 2012). Other findings also confirm that PAB from commercially available calcium-enriched orange juice exposed to simulated gastric and small intestinal juice models have shown that they can survive severe conditions in the GIT (Huang & Adams, 2004; Soumalianen, Sigvart- Mattila, Mâttö & Tynkkynen, 2008).

In the present study, facultative heterofermentative *Lb. paracasei* ssp. *paracasei* INF 448 and *Lb. paracasei* ssp. *paracasei* INF 456 showed a good survival under acidic conditions during digestion, but with approx. 2-3 log higher bacteria numbers after digestion of cheese ripened for 7 wk compared to 70 wk. It has been shown that heterofermentative lactobacilli show greater acid and salt tolerability than homofermentative lactobacilli (Charteris et al., 1998; Alander et al., 1999; Fox, McSweeney, Cogan, Guinee, 2004). In cheese, lactobacilli develop as large clusters of cells trapped in the cheese matrix (**Paper III**). In the present study, the microenvironment around such clusters regulates the metabolic activity and nutrient supply of the bacteria. It has been shown that the fat content influenced the survival during digestion of lactobacilli in 7 week old cheeses supplemented with *Lb. rhamnosus GG* and *Lb. paracasei* ssp. *paracasei* INF 448. Furthermore, within the 10 % fat cheeses the numbers of lactobacilli remained more stable during digestion, indicating that these could enter duodenum in substantial numbers.

This study demonstrated that cheese can function as a vehicle for delivery of a variety of food related microorganisms to the intestine and that the younger cheese was a better carrier matrix of the cheese bacteria. Besides the age of the cheese, the survival rate was influenced by the fat content of the cheese. Most of the lactobacilli and all the PAB survived well during digestion of the low-fat cheeses, but some less well in the full fat cheese. This study also showed the ability of cheese lactobacilli and PAB to survive the severe conditions of GIT.

CONCLUDING REMARKS AND FUTURE PERSPECTIVES

The dairy industry highly depends on the application of LAB. Their diversity and vast metabolic activities will be a constant source of new challenges for the science enlightening the different roads to be applied in cheese making.

- Traditional cheeses, as a reservoir of new strains of LAB, are the pool for the future research and for preservation of cultural heritage of different nations. Montenegro, as a Mediterranean country has still unexplored potentials of "wild" microbiota that should be preserved and their possible applications in diary industry should be explored. Present research showed that indigenous strains isolated form artisanal Montenegrin products can find their applications as starter cultures in the dairy industry. In the future, the research in this area should be focused on comprehensive studies of the diversity of indigenous LAB from Montenegrin dairy products, their metabolic properties and preservation for their possible future applications in dairy industry.

- Recent health concerns have revealed the need for decreasing the proportion of the full fat cheeses in the daily diet of the modern human. Having in mind that the flavor of the low fat cheeses is often considered as not as favorable as the one of the full fat cheeses, tis research attempted to explore the possibilities of applying different strains of NSALB during the production of low fat cheeses. It has been discovered that the diversity of NSALB is increasing during maturation, while at the same time the flavor of the cheese is positively influenced by their metabolic activities. The addition of SMP to the cheese milk, coupled with different combinations of adjunct cultures and innovations in cheese technology can further contribute to the development of low cheeses with improved flavor characteristics. The importance of the application of modern molecular- biology techniques can emphasize the reasoning of adjunct supplementation in these cheeses.

- Cheese, due to its properties can act as a good protective matrix for the delivery of different beneficial microorganisms into GIT of humans. Present research showed that different strains and species have different ability to survive GIT conditions when added

to the cheeses with different fat content. *Ex vivo* digestion has shown to be a reliable and yet etic, technique for the simulation of the GIT conditions. Future research should focus on application of this model for studying survival rate of different LAB strains when added to the cheese during manufacture as it can have numerous beneficial effects not only for the dairy industry, but also to the field of medicine.

REFERENCES

Abosereh, N.A., El Ghani, S.A., Gomaa, R. S. & Foua, M. T. (2016). Molecular identification of potential probiotic lactic acid bacteria strains isolated from Egyptian traditional fermented dairy products. *Biotechnology, 15*, 35-43.

Adamberg, K., Antonsson, M., Vogensen, F. K., Nielsen, E. W., Kask, S., Moller, P. L., & Ardo, Y. (2005). Fermentation of carbohydrates from cheese sources by non-starter lactic acid bacteria isolated from semi-hard Danish cheese. *International Dairy Journal, 15*, 873–882.

Adimpong, D.B., Nielsen, D.S., Sorensen, K.I. Derkx, P.M.F. & Jespersen, L. (2012). Genotypic characterization and safety assessment of lactic acid bacteria from indigenous African fermented food products. *BMC Microbiology, 12,1*, 75–86.

Adouar, N., Magne, L., Cattenoz, T., Guillemin, H., Foligné, B., Picque, D., & Bonnarme, P. (2016). Survival of cheese-ripening microorganisms in a dynamic simulator of the gastrointestinal tract. *Food Microbiology*, *53*, 30-40.

Adžić, N., Dozet, N., Ljumović, M., Marković, M. & Adžić, Z. (1997). Autohtoni mlječni proizvodi Crne Gore koje treba zaštititi po osnovu porijekla (Indigenous dairy products of Montenegro that should be protected on the basis of origin). *Poljoprivreda i šumarstvo, 43*, 3, 127-134.

Adžić, N., Ljumović, M., Petrović, S., Tomović, M., Dozet, N., Stanišić, M. & Perović, M. (1984). Kvalitet mlijeka za proizvodnju njeguškog sira (Quality of milk used for the production of Njeguši cheese). *Mljekarstvo, 34*, *3*, 76-81.

Alander, M., Satokari, R., Korpela, R., Saxelin, M., Vilpponen-Salmela, T., Mattila-Sandholm, T. & von Wright, A. (1999). Persistence of colonization of human colonic mucosa by a probiotic strain, *Lactobacillus rhamnosus* GG, after oral consumption. *Applied and Environmental Microbiology, 65*, *1*, 351-354.

Alegria, A., Szczesny, P., Mayo, B., Bardowski, J. & Kowalczyk, M. (2012). Biodiversity in Oscypek, a traditional Polish cheese, determined by culture-dependent and –independent approaches. *Applied and Environmental Microbiology, 78*, 1890–1898.

Antonsson, M., Ardö, Y. & Molin, G. (2001). A comparison between the microflora of Herrgård cheese from three different dairies. *International Dairy Journal, 11*, 285–291.

Antonsson, M., Molin, G. & Ardö, Y. (2003).*Lactobacillus* strains isolated from Danbo cheese as adjunct cultures in a cheese model system. *International Journal of Food Microbiology, 85*, 159-169.

Arteau, M., Labrie, S. & Roy, D. (2010). Terminal restriction fragment polymorphism and automated ribosomal intergenic spacer analysis profiling of fungal communities in Camembert cheese. *International Dairy Journal*, *20*, 545–554.

Ardö, Y. (2001) Cheese ripening. General mechanisms and specific cheese varieties. *Bulletin of the International Dairy Federation*, *369*, 7–12.

Ardö, Y. (2006). Flavour formation by amino acid catabolism. *Biotechnology Advances, 24,* 2, 238-242.

Ayad, E. H. E., Verheul A., Wouters J.T.M. & Smit G. (2000). Application of wild starter cultures for flavour development in pilot plant cheese making. *International Dairy Journal, 10*, 169–179.

Banks, J.M., Brechany, Y.E. & Christie, W.W. (1989). The production of low fat Cheddar cheese. *International Journal of the Society of Dairy Technology*, *42*, 6–9.

Banks, J., Yvon, M., Gripon, J. C., de la Fuente, M. A., Brechany, E. Y., Williams, A. & Muir, D. D. (2001). Enhancement of amino acid catabolism in Cheddar cheese using α-ketoglutarate: amino acid degradation in relation to volatile compounds and aroma character. *International Dairy Journal*, *11,* 235–243.

Banks, J. M. & Williams, A. G. (2004). The role of nonstarter lactic acid bacteria in Cheddar cheese ripening. *International Journal of Dairy Technology, 57*, 145-152.

Barbieri, G., Bolzoni, I., Careri, M., Manglia, A., Parolari, G., Spagonoli, S. & Virgili, R. (1994). Study of the volatile fraction of parmesan cheese. *Journal of Agricultural and Food Chemistry, 42,* 1170–1176.

Barlow, I., Lloyd, G.T., Ramshaw, E.H., Miller, A J., McCabe, G.P. & McCabe, L. (1989). Correlations and changes in flavour and chemical parameters of Cheddar cheeses during maturation. *Australian Journal of Dairy Technology*, *44*, 7–18.

Ben Amor, K., Vaughan, E. E. & DeVos, W. M. (2007). Advanced molecular tools for the identification of lactic acid bacteria. *Journal of Nutrition, 137*, 741-747.

Bennasar, A., Mulet, M., Lalucat, J. & García-Valdés, E. (2010). Pseudo MLSA: a database for multigenic sequence analysis of *Pseudomonas* species. *BMC Microbiology, 10*, 118.

Beresford, T.P., Fitzsimons, N.A., Brennan, N.L. & Cogan, T.M. (2001). Recent advances in cheese microbiology. *International Dairy Journal, 11*, 259-274.

Beresford, T. & Williams, A. (2004). The microbiology of cheese ripening. In: *Cheese: Chemistry, Physics and Microbiology* ed. Fox, P.F., McSweeney, P.L.H., Cogan, T.M. and Guinee, T.P. pp 287-318, Elsevier Academic Press, London.

Berthier, F. & Ehrlich, S.D. (1998). Rapid species identification within two groups of closely related lactobacilli using PCR primers that target 16S/23S rRNA spacer region. *FEMS Microbiology Letters, 161*, 97-106.

Berthier, F., Beuvier, E., Dasen, A. & Grappin, R. (2001). Origin and diversity of mesophilic lactobacilli in Comte cheese, as revealed by PCR with repetitive and species-specific primers. *International Dairy Journal, 11*, 293-305.

Bolotin A., Wincker P., Mauger S., Jaillon O., Malarme K. & Weissenbach J. (2001). The complete genome sequence of the lactic acid bacterium *Lactococcus lactis* ssp. *lactis* IL1403. *Genome Research,11*, 731–753.

Borchers, A.T., Selmi, C., Meyers, F.J., Keen, C.L. & Gershwin, M.E. (2009). Probiotics and immunity. *Journal of Gastroenterology, 44*, 1, 26-46.

Bordenave, S., Sannier, F., Ricart, G. & Piot, J. M. (2000). Characterization of a goat whey peptic hydrolysate produced by an ultrafiltration membrane enzymic reactor. *Journal of Dairy Research, 67*, 551- 559.

Boyazoglu, J. & Morand-Fehr, P. (2001). Mediterranean dairy sheep and goat products and their quality. A critical review. *Small Ruminant Research, 10*, 1–11.

Broadbent, J., Rodrıguez, B., Joseph, P., Smith, E. & Steele, J. (2006). Conversion of *Lactococcus lactis* cell envelope proteinase specificity by partial allele exchange. *Journal of Applied Microbiology*, *100*, 1307–1317.

Broadbent, J. R. & J. L. Steele. (2007). Biochemistry of cheese flavor development: insight from genomic studies of lactic acid bacteria, pp. 177-192. In K. R. Caldwaller, M. A. Drake, and R. J. McGorrin (eds.), *Flavor of Dairy Products*. Amer. Chem. Soc., Washington, DC.

Broome, M.C., Powel, I. B. & Limsowtin, G. K. Y. (2003). Starter cultures: Specific properties. In *Encyclopedia of Dairy Sciences*. Vol I ed. Regisnki, H. Fuquay, J.W. & Fox, P. F. 269 – 275. London: Academic Press.

Brown, K., De Coffe, D., Molcan, E. & Gibson, D.L. (2012). Diet-induced dysbiosis of the intestinal microbiota and the effects on immunity and disease. *Nutrients, 4,* 1095-1119.

Bruinenberg, P.G., de Roo, G. & Limsowtin, G.K.Y. (1997). Purification and characterisation of cystathioninec-lyase from *Lactococcus lactis* subsp. cremoris SK11: possible role in flavor compound formation during cheese maturation. *Applied and Environmental Microbiology 63*, 561-566.

Busconi, M., Reggi, S. & Fogher, C. (2008). Evaluation of biodiversity of lactic acid bacteria microbiota in the calf intestinal tracts. *Antonie Van Leeuwenhoek, 94*, 145-155.

Caric, M. (1993). Ripened Cheese Varieties Native to the Balkan Countries, in: Fox P.F. (Ed.), *Cheese. Chemistry, Physics and Microbiology*, Vol. 2, 2nd edn., Chapman & Hall, London, UK, 1993, pp. 263–279.

Chamba J. F. & Irlinger, F. (2004). "Secondary and adjunct cultures," in *Cheese: Chemistry, Physics and Microbiology*, Vol. 1 *General Aspects*, 3rd Edn., eds Fox P. F., McSweeney P. L. H., Cogan T. M., Guinee T. P., editors. (London: Elsevier Academic Press), 191–206.

Charteris, W.P., Kelly, P.M., Morelli, L. & Collins, J.K. (1997). Selective detection, enumeration and identification of potentially probiotic *Lactobacillus* and *Bifidobacterium* species in mixed bacterial populations. *International Journal of Food Microbiology*, *35*, 1–27.

Chiang, L., Sanchez-Chiang, L., Mills, J.N. & Tang, J. (1967). Purification and properties of porcine gastricsin. *Journal Biological Chemistry*, *242*, 3098–3102.

Christensen, J.E., Dudley, E.G., Pederson, J.A. & Steele, J.L. (1999). Peptidases and amino acid catabolism in lactic acid bacteria. *Antonie Van Leeuwenhoek*, *76*, 217-246.

Claisse, O., Renouf, V. & Lonvaud-Funel, A. (2007). Differentiation of wine lactic acid bacteria species based on RFLP analysis of a partial sequence of rpoB gene. *Journal Microbiology Methods*, *69*, 387– 90.

Codex Alimentarius, FAO/WHO. (2013). Codex general standard for cheese. Standard 283-1978, Formerly CODEX STAN A-6-1973. Adopted in 1973. Revision 1999, Amendments 2006, 2008, 2010, 2013.

Cogan, T. M., Beresford, T. P., Steele, J., Broadbent, J., Shah, N. P. & Ustunol, Z. (2007). Invited review: Advances in starter cultures and cultured foods. *Journal of Dairy Science, 90*, 4005-4021.

Cogan, T.M. & Hill, C. (1993). Cheese starter cultures. *In Cheese: Chemistry, Physics and Microbiology*. Fox, P.F. (ed.) Chapman & Hall, London. Vol 1: General Aspects, 2nd edn., pp.: 193–256.

Collins, Y.F., McSweeney, P.L.H. & Wilkinson, M.G. (2003). Lipolysis and free fatty acid catabolism in cheese: a review of current knowledge. *International Dairy Journal*, *13*, 841-866.

Cotillard, A., Kennedy, S.P., Kong, L.C., Prifti, E., Pons, N., Le Chatelier, E., Almeida, M., Quinquis, B., Levenez, F., Galleron, N., Gougis, S., Rizkalla, S., Batto, J.M., Renault, P. ANR MicroObes consortium, Doré, J., Zucker, J.D., Clément, K. & Ehrlich, S.D. (2013). Dietary intervention impact on gut microbial gene richness. *Nature, 500*, 585-588.

Coolbear, T., Crow, V., Harnett, J., Harvey, S., Holland, R. & Martley, F. (2008). Developments in cheese microbiology in New Zealand—use of starter and non-starter lactic acid bacteria and their enzymes in determining flavor. *International Dairy Journal*, *18*, 705–713.

Crow, V. L., Coolbear, T., Gopal, P. K., Martley, F. G., McKay, L. & Reipe, H. (1995) The role of autolysis of lactic acid bacteria in the ripening of cheese. *International Dairy Journal*, *5*, 855–875.

Crow, V., Curry, B. & Hayes, M. (2001). The ecology of non-starter lactic acid bacteria (NSLAB) and their use as adjuncts in New Zealand Cheddar. *International Dairy Journal*, *11*, 275–283.

Crow, V., Curry, B., Christison, M., Hellier, K., Holland, R. & Liu, S. Q. (2002). Raw milk flora and NSLAB as adjuncts. *Australian Journal of Dairy Technology*, *57*, 99-105.

Cryan, J. F. & O'Mahony, S. M. (2011). The microbiome-gut-brain axis: from bowel to behavior *Neurogastroenterology and Motility*, *23*, 187–192.

Curioni, P. M. G. & Bosset, J. O. (2002). Key odorants in various cheese types as determined by gas chromatography – olfactometry. *International Dairy Journal, 12,* 959-984.

da Cruz, A.G., Buriti, F.C.A., de Souza, C.H.B., Faria, J.A.F. & Saad, S.M.I. (2009). Probiotic cheese: health benefits, technological and stability aspects. *Trends Food Sci Technol 20*, 344-354.

Danthine, S., Blecker, C., Paquot, M., Innocente, N. & Deroanne, C. (2000). Evolution des connaissances sur la membrane du globule gras du lait: synthèse bibliographique. *Le Lait, 80*, 209-222.

Dasen, A., Berthier, F., Grappin, R., Williams, A. G. & Banks, J. (2003). Genotypic and phenotypic characterization of the dynamics of the lactic acid bacterial population of adjunct-containing Cheddar cheese manufactured from raw and microfiltered pasteurised milk. *Journal of Applied Microbiology, 94,* 595–607.

De Vuyst, L. & Vancanneyt, M. (2007). Biodiversity and identification of sourdough lactic acid bacteria. *Food Microbiology, 24*, 120-127.

Decallone, J., Delmee, M., Wauthoz, P., El lioul, M. & Lambert, R. (1991). A rapid procedure for the identification of lactic acid bacteria based on the gas chromatographic analysis of cellular fatty acids. *Journal of Food Protection*, *54*, 217–24.

Delgado, S. & Mayo, B. (2004). Phenotypic and genetic diversity of *Lactococcus lactis* and *Enterococcus* spp. strains isolated from Northern Spain starter-free farmhouse cheeses. *International Journal of Food Microbiology*, *90*, 309-319.

Dellaglio, F., Cleenwerck, I., Felis, G. E., Engelbeen, K., Janssens, D. & Marzotto, M. (2005). Description of *Gluconacetobacter swingsii* sp. nov. and *Gluconacetobacter rhaeticus* sp. nov., isolated from Italian apple fruit. *International Journal Systematic and Evolutionary Microbiology*, *55*, 2365–2370.

Deveau, H. & Moineau, S. (2003). Use of RFLP to characterize *Lactococcus lactis* strains producing exopolysaccharides. *Journal of Dairy Science*, 86, 1472– 5

De Urazza, P.J., Gomez-Zavaglia, A., Lozano, M.E., Romanowski, V. & de Antoni, G. L. (2000). DNA fingerprinting of thermophilic lactic acid bacteria using repetitive sequence-based polymerase chain reaction. *Journal of Dairy Research, 67*, 381-392.

Dewettinck, K., Rombaud, R., Thienpont, N., Trung Le, T., Messens, K. & Van Camp, J. (2008). Nutritional and technological aspects of milk fat globule membrane material. *International Dairy Journal, 18*, 436-57.

Di Cagno, R., De Angelis, M., Corsetti, A., Lavermicocca, P., Arnoult, P., Tossut, P., Gallo, G. & Gobbetti, M. (2003). Interaction between sourdough lactic acid bacteria and exogenous enzymes: Effects on the microbial kinetics of acidification and dough textural properties. *Food Microbiology*, *20*, 67–75.

Dozet, N., Adžić, N., Stanišić, M. & Ljumović, M. (1987). Kvalitet i tehnologija autohtonih mliječnih proizvoda Crne Grore (Quality and technology of autochthonous dairy products of Montenegro). *Zbornik radova Poljoprivrednog instituta Titograd*.

Dozet, N., Adžić, N., Stanišić, M. & Živić, N. (1996). Autohtoni mlječni proizvodi (Indigenous dairy products). *Poljoprivredni institut Podgorica*, SILMIR-Beograd.

Dozet, N., Maćej, O. & Jovanović, S. (2004). Autohtoni mliječni proizvodi osnova za razvoj specifičnih, originalnih mliječnih prerađevina u savremenim uslovima (Autochthonous dairy products as the basis for the development of specific original dairy products in the contemporary conditions). *Biotechnology in Animal Husbandry, 20*, 31-48.

Douillard, F.P. & de Vos, W.M. (2014). Functional genomics of lactic acid bacteria: from food to health. *Microbial Cell Factories, 13*, 1, S8.

Duthoit, F., Godon, J.J. & Montel, M.C. (2003). Bacterial community dynamics during production of registered designation of origin Saler cheese as evaluated by 16S rRNA gene single-strand conformation polymorphism analysis. *Applied and Environmental Microbiology*, *69*, 3840–3848.

El-Soda, M., Madkor, S. A. & Tong, P. S. (2000a). Evaluation of commercial adjuncts for use in cheese ripening: Comparison between attenuated and not attenuated lactobacilli. *Milchwissenschaft*, *55*, 260-293.

El-Soda, M., Madkor, S. A., & Tong, P. S. (2000b). Adjuncts cultures: recent developments and potential significance to the cheese industry. *Journal of Dairy Science*, *83*, 609-619.

El Soda, M., Madkor, S. M. & Tong, P. S. (2008). Adjunct cultures: recent developments and potential significance to the cheese industry. *Journal of Dairy Science, 83*, 609–619.

El Soda, M. (2014). Production of low fat Cheddar cheese made using exopolysaccharide-producing cultures and selected ripening cultures. *Advances in Microbiology, 4*, 986-995.

Eckburg, P.B., Bik, E.M., Bernstein, C.N., Purdom, E., Dethlefsen, L., Sargent, M., Gill, S.R., Nelson, K.E. & Relman, D.A. (2005). Diversity of the Human Intestinal Microbial Flora. *Science 30*, 1635–1638.

Ekmekcioglu, C. (2002). A physiological approach for preparing and conducting intestinal bioavailability studies using experimental systems. *Food Chemistry*, *76*, 225–230.

Ercolini, D., Hill, P.J. & Dodd, C.E.R. (2003). Bacterial community structure and location in Stilton cheese. *Applied and Environmental Microbiology, 69, 3540–3548.*

Ercolini, D., De Filippis, F., La Storia, A. & Iacono, M. (2012). "Remake" by high-throughput sequencing of the microbiota involved in the production of water buffalo mozzarella cheese. *Applied and Environmental Microbiology*, *78*, 8142-8145.

Escamilla-Hurtado, M. L., Tomasini-Campocosio, A., Valde´s-Martı´nez, S. & Soriano-Santos, J. (1996). Diacetyl formation by lactic bacteria. *Review of Latinoamerican Microbiology, 38*, 129–137.

Escudero, E., Sentandreu, M.A. & Toldra, F. (2010). Characterization of peptides released by in vitro digestion of pork meat. *Journal of Agricultural and Food Chemistry*, *58*, 5160–5165.

Exterkate, F.A., Alting, A.C. & Bruinenberg, P.G. (1993). Diversity of cell envelope proteinase specificity among strains of *Lactococcus lactis* and its relationship to charge characteristics of the substrate-binding region. *Applied and Environmental Microbiology*, *59*, 3640–3647.

Faye, T., Tamburello, A., Vegarud, G.E. & Skeie, S. (2012). Survival of lactic acid bacteria from fermented milks in an in vitrodigestion model exploiting sequential incubation in human gastric and duodenum juice. *Journal of Dairy Science, 95*, 558-566.

Felis, G.E. & Dellaglio, F. (2007). Taxonomy of lactobacilli and bifidobacteria. *Current Issues Intestinal Microbiology, 8*, 44-61.

Fernandez-Espla, M. D., Garault, P., Monnet, V. & Rul, F. (2000). *Streptococcus thermophilus* cell wall-anchored proteinase: release, purification, and biochemical and genetic characterization. *Applied and Environmental Microbiology*, *66*, 4772–4778.

Fitzsimons, N. A., Cogan, T.M., Condon, S. & Beresford, T. (2001). Spatial and temporal distribution of non-starter lactic acid bacteria in Cheddar cheese. *Journal of Applied Microbiology*, *90*, 600–606.

Flint, H. J., Scott, K.P., Duncan, S.H., Louis, P. & Forano, E. (2012). Microbial degradation of complex carbohydrates in the gut. *Gut Microbes*, 289–306.

Fox, P. F., Wallace, J. M., Morgan, S., Lynch, C.M., Niland, E.J. & Tobin, J. (1996). Acceleration of cheese ripening. *Antonie Leeuwenhoek, 70*, 271-297.

Fox, P. F., McSweeney, P. L. H., & Lynch, C.M. (1998).Significance on non-starter lactic acid bacteria in Cheddar cheese. *Australian Journal of Dairy Technology, 53*, 83-89.

Friedrich, U. & Lenke, J. (2006). Improved enumeration of lactic acid bacteria in mesophilic starter cultures by using multiplex quantitative real-time PCR and flow cytometry-fluorescence in situ hybridization. *Applied and Environmental Microbiology*, 72, 4163–4171.

Fröhlich-Wyder, M.T., Bachmann, H.P. & Gerard Casey, M. (2002) Interaction between propionibacteria and starter /non-starter lactic acid bacteria in Swiss-type cheeses. *Lait 82*, 1-15.

Fujimura, K.E., Slusher, N.A., Cabana, M.D. & Lynch, S. (2010). Role of the gut microbiota in defining human health. *Expert Review of Anti Infective Therapy, 8*, 435–454.

Gagnaire, V., Thierry, A. & Léonil, J. P. (2001). *Propionibacteria* and facultatively heterofermentative *lactobacilli* weakly contribute to secondary proteolysis of Emmental cheese. *Le Lait*, *81*, 339-353.

Gatti, M., Fornasari, E. & Neviani, E. (1997). Cell-wall protein profiles of dairy thermophilic lactobacilli. *Letters in Applied Microbiology*, *25*, 345–348.

Gao, S., Oh, D.H. & Steele, J.L. (1997). Aromatic amino acid catabolism by lactococci. *Le Lait*, *77*, 371-381.

Gevers, D., Huys, G. & Swings, J. (2001). Applicability of rep-PCR fingerprinting for identification of *Lactobacillus* species. *FEMS Microbiology Letters*, *205*, 31-36.

Gilbert, C., Atlan, D., Blanc, B., Portailer, R., Germond, J.E., Lapierre, L. & Mollet, B. (1996). A new cell surface proteinase: Sequencing and analysis of the prtB gene from *Lactobacillus delbrueckii* subsp. *bulgaricus*. *Journal of Bacteriology, 178*, 3059-3065.

Gobbetti, M., De Angelis, M., Di Cagno, R., Mancini, L. & Fox, P.F. (2015). Pros and cons for using non-starter lactic acid bacteria (NSLAB) as secondary/adjunct starters for cheese ripening. *Trends in Food Science & Technology 45,* 167-178.

Gonzalez, L., Sandoval, H., Sacristan, N., Castro, J.M., Fresno, J.M. & Tornadijo, M.E. (2007). Identification of lactic acid bacteria isolated from Genestoso cheese throughout ripening and study of their antimicrobial activity. *Food Control, 18*, 716-722.

Griffith, R. & Hammond, E. G. (1989). Generation of Swiss cheese flavor components by the reaction of amino acids with carbonyl compounds. *Journal of Dairy Science*, *72*, 604–613.

Grimont, F. & Grimont, P.D.A. (1986). Ribosomal ribonucleic acid gene restriction as potential taxonomic tools. *Annales de'l Institut Pasteur Microbiologie*, *137B*, 165 – 175.

Guarner, F., Perdigon, G., Corthier, G., Salminen, S., Koletzko, B., & Morelli, L. (2005). Should yoghurt cultures be considered probiotic? *British Journal of Nutrition*, *93*, 783–786.

Guinee, T.P., Auty, M.A.E. & Fenelon, M.A. (2000). The effect of fat content on the rheology, microstructure and heat-induced functional characteristics of Cheddar cheese. *International Dairy Journal 10*, 277-288.

Hanage, W.P., Fraser, C. & Spratt, B.G. (2006). Sequences, sequence clusters and bacterial species. *Philosophical Transactions of the Royal Society B: Biological Sciences 361*, 1917-1927.

Hassan, F.A.M., Abd El- Gawad, M.A.M. & Enab, A.K. (2013). Flavour Compounds in Cheese (Review*). Research on Precision Instrument and Machinery*, *2*, 2, 15-29.

Henneberg, W. (1904). Zur kenntnis der milchsaurebakterien der Brennerei-Maische, der mich des bieres, der presshefe, der melasse, des sauerkohls, der sauren gurken und des sauerteoges; sowie einige bemerkungen uber die milchsaurebakterien des menschlichen magens. *Zentralbl Bakteriol Parasitenkd*, Abt. II 11, 154–170.

Henriksen, C.M. & Nilsson, D. (2001). Redirection of pyruvate catabolism in *Lactococcus lactis* by selection of mutants with additional growth requirements. *Applied Microbiology and Biotechnology*, *56*,767– 775.

Hernández-Ledesma, B., Quirós, A., Amigo, L. & Recio, I. (2007). Identification of bioactive peptides after digestion of human milk and infant formula with pepsin and pancreatin. *International Dairy Journal*, *7*, 42-49.

Holzapfel, W.H., Haberer, P., Geisen, R., Bjorkroth, J. & Schillinger, U. (2001). Taxonomy and important features of probiotic microorganisms in food and nutrition. *American Journal of Clinical Microbiology*, *73*, 365– 373.

Høier, E., Janzen, T., Rattray, F., Sorensen, K., Borsting, M.W. & Brockmann, E. (2010). The production, application and action of lactic cheese starter cultures. 2010, *In: Technology*

of Cheesemaking (eds. B.A. Law, A.Y. Tamime). Wiley- Blackwell, Chichester, 166–192.

Huang, Y. & Adams, M.C. (2004). In vitro assesment of the upper gastrointestinal tolerance of potential probiotic dairy propionibacteria. *International Journal of Food Microbiology, 91,* 3, 253-260.

Hudson, M.E. (2008). Sequencing breakthroughs for genomic ecology and evolutionary biology. *Molecular Ecology Resources, 8*, 3-17.

Human Microbiome Project Consortium. (2012a). A framework for human microbiome research. *Nature*, *486*, 215–221.

Human Microbiome Project Consortium. (2012b). Structure, function and diversity of the healthy human microbiome. *Nature*, *486*, 207–214.

Humblot, C. & Guyot, J.P. (2009). Pyrosequencing of tagged 16S rRNA gene amplicons for rapid deciphering of the microbiomes of fermented foods such as pearl millet slurries. *Applied and Environmental Microbiology*, *75*, 4354–4361.

Hynes, E., Ogier, J.C. & Delacroix-Buchet, A. (2001). Proteolysis during ripening of miniature Washed curd cheeses manufactured with different strains of starter bacteria and a *Lactobacillus plantarum* adjunct culture. *International Dairy Journal*, *11*, 587-597.

Hynes, E., Bergamini, C,V., Suarez, V. & Zalazar, C.A. (2003). Proteolysis on Reggianito cheeses manufactured with natural whey cultures and selected strains of *Lactobacillus helveticus*. *Journal of Dairy Science, 86,* 3831–3840.

Janda, M.J. & Abbott, S. L. (2007). 16S rRNA Gene Sequencing for Bacterial Identification in the Diagnostic Laboratory: Pluses, Perils, and Pitfalls. *Journal of Clinical Microbiology, 45, 9,* 2761-2764.

Jany, J.L. & Barbier, G. *(2008).* Culture-independent methods for identifying microbial communities in cheese. *Food Microbiology, 25*, 839–848.

Jacobsen, C.N., Rosenfeldt Nielsen, V., Hayford, A.E., Moller, P.L., Michaelsen, K.F., Paerregaard, A., Sandstrom, B., Tvede, M., & Jakobsen, M. (1999). Screening of probiotic activities of forty-seven strains of *Lactobacillus* spp. by in vitro techniques and evaluation of the colonization ability of five selected strains in humans. *Applied and Environmental Microbiology*, *65*, 4949-4956.

Jiménez-Flores, R. & Brisson, G. (2008). The milk fat globule membrane as an ingredient: why, how, when? *Dairy Science Technology*, *88*, 5-18.

Johnson, E.A., Nelson, J.H. & Johnson, M. (1990). Microbiological safety of cheese made from heat-treated milk. *Journal of Food Protection*, 53, 519–540.

Jung, J.Y., Lee, S. H., Kim, J.M., Park, M. S., Bae, J. W., Hahn, Y., Madsen, E.L. & Jeon, C.O. (2011). Metagenomic analysis of kimchi, a traditional Korean fermented food. *Applied and Environmental Microbiology*, *77*, 2264–2274.

Kandler, O. & Weiss, N. (1986). Microbiology of mesu, a traditional fermented bamboo shoot product, In: *Bergey's Manual of Systematic Bacteriol*. Sneath PHA, Mair NS, Sharpe ME, Holt JG (Eds). Baltimore: W illiams, W ilkins 2: 1209 -1234.

Katsiari, M.C., Voutsinas, L.P. & Kondyli, E. (2002a). Improvement of sensory quality of low-fat Kefalogravieratype cheese with commercial adjunct cultures. *International Dairy Journal 12*, 757-764.

Katsiari, M.C., Voutsinas, L.P., Kondyli, E. & Alichanidis, E. (2002b). Flavour enhancement of low-fat Feta-type cheese using a commercial adjunct culture. *Food Chemistry 79*, 193-198.

Karimi, R., Mortazavian, A., Gomes, M. & Da Cruz, A. (2011). Viability of probiotic microorganisms in cheese during production and storage: A review. *Dairy Science and Technology, 91*, 283–308.

Khalid, N.M. & Marth, E.H. (1990). Lactobacilli – their enzymes and role in ripening and spoilage of cheese: a review, *Journal of Dairy Science*, *73*, 2669–2684.

Kelleher, P., Murphy, J., Mahony, J. & van Sinderen, D. (2015). Next-generation sequencing as an approach to dairy starter selection. *Dairy Science and Technology, 95*, 545-568.

Kleerebezem, M., Boekhorst, J., van Kranenburg, R., Molenaar, D., Kuipers, O.P., Leer, R., Tarchini, R., Peters, S.A., Sandbrink, H.M., Fiers, M.W.E.J., Stiekema, W., Lankhorst, R. M. K., Bron, P.A., Hoffer, S.M., Nierop Groot, M.N., Kerkhoven, R., de Vries, M., Ursing, B., de Vos, W. & Siezen, R. J. (2003). Complete genome sequence of *Lactobacillus plantarum* WCFS1. *PNAS, 100*, 4, 1990-1995.

Kieronczyk, A., Skeie, S., Olsen, K. & Langsrud, T. (2001). Metabolism of amino acids by resting cells of non-starter lactobacilli in relation to flavor development in cheese.*International Dairy Journal, 11*, 217-224.

Kilcawley, K., O'Connell, P., Hickey, D. K., Sheehan, E. M., Beresford, T. & McSweeney, P. L. H. (2007). Influence of composition on the biochemical and sensory characteristics of commercial Cheddar cheese of variable quality and fat content. *International Journal of Dairy Technology, 60*, 81-88.

Kim, S.B., Ki, K.S., Khan, M.A., Lee, W.S., Lee, H.J., Ahn, B.S. & Kim, H.S. (2007). Peptic and tryptic hydrolysis of native and heated whey protein to reduce its antigenicity. *Journal of Dairy Science*, *90*, 4043–4050.

Kimoto, H., Kurisaki, J., Tsuji, T.M., Ohmomo, S. & Okamoto, T. (1999). Lactococci as probiotic strains: adhesion to human enterocyte-like Caco-2 cells and tolerance to low pH and bile. *Letters in Applied Microbiology*, *29*, 313–316.

Kimoto-Nira, H., Mizumachi, K., Nomura, M., Kobayashi, M., Fujita, Y., Okamoto, T., Suzuki, I., Tsuji, N.M., Kurisaki, J.I. & Ohmomo, S. (2007). *Lactococcus* sp. as potential probiotic lactic acid bacteria. *Japan Agricultural Research Quarterly*, *41*, *3*, 181-189.

Kindsted, P. (2012). Cheese and Culture: A History of Cheese and its Place in Western Civilization. *Chelsea Green Publishing Co.*, ISBN-10: 1603584110.

Klein, G., Pack, A., Bonaparte, C. & Reuter. G. (1998). Taxonomy and physiology of probiotic lactic acid bacteria. *International Journal of Food Microbiology, 41*, 103-125.

Koca, N. & Metin, M. (2004). Textural, melting and sensory properties of low-fat fresh Kashar cheeses produced by using fat replacers. *International Dairy Journal*, *14*, 365–373.

Kok, J. & Venema, G. (1988). Genetics of proteinases of lactic acid bacteria. *Biochemie*, *70*, 475-488.

Kostinek, M., Specht, I., Edward, V.A., Schillinger, U., Hertel, C., Holzapfel, W.H. & Franz, C.M.P. (2005). Diversity and technological properties of predominant lactic acid bacteria from fermented cassava used for the preparation of Gari, a traditional African food. *Systematic and Applied Microbiology*, *28*, 6, 527–540.

Krizova, J., Spanova, A. & Rittich, B. (2008). RAPD and Rep PCR fingerprinting for characterization of *Bifidobacterium* species. *Folia Microbiologica*, *53*, 2, 99-104.

Kucukoner, E. & Haque, Z.U. (2006). Physicochemical properties of low-fat and full-fat Cheddar cheeses. *International Journal of Dairy Technology*, *59*, 166-170.

Kunji, E. R. S., Mierau, I., Hagting, A., Poolman, B. & Konings, W. N. (1996). The proteolytic systems of lactic acid bacteria. *Antonie Van Leeuwenhoek*, *70*, 187–221.

Laloy, E., Vuillemard, J.C., El Soda, M. & Simard, R.E. (1996). Influence of the fat content of Cheddar cheese on retention and localization of starters. *International Dairy Journal, 6*, 729-740.

Lazzi, C., Rossetti, L., Zago, M., Neviani, E. & Giraffa, G. (2004). Evaluation of bacterial communities belonging to natural whey starters for Grana Padano cheese by length heterogeneity-PCR. *Journal of Applied Microbiology, 96, 481–490.*

Law, B. A. (2001). Controlled and accelerated cheese ripening: The research base for new technologies. *International Dairy Journal, 11,* 383–398.

Lee, M. R. (2007). Understanding Ca equilibrium in cheese during ripening and its effects on functional properties of cheese. Ph. D. thesis, University of Wisconsin-Madison, USA.

Lemieux, L. & Simard, R.E. (1991). Bitter flavour in dairy products. 1. A review of the factors likely to influence its development, mainly in cheese manufacture. *Le Lait*, *71*, 599-636.

Limsowtin, G.K.Y., Powell, I.B. & Parente, E. (1996). Types of starters. In: Cogan, T.M., Accolas, J.P (Eds.), *Dairy Starter Cultures*. VCH Publishers, Inc., New York, 101-129.

Li, W., Raoult, D. & Fournier, P.E. (2009). Bacterial strain typing in the genomic era. *FEMS Microbiology Reviews*, *33*, 892–916.

Liu, J., DeYoung, S. M., Zhang, M. Zhang, M., Cheng, A. & Saltiel A. R. (2005). Changes in integrin expression during adipocyte differentiation. *Cell Metabolism*, *2*, 165–177.

Liu, H., Xu, X. M. & Guo, S. D. (2008). Comparison of full-fat and low-fat cheese analogues with or without pectin gel through microstructure, texture, rheology, thermal and sensory analysis. *International Journal of Food Science and Technology, 43*, 1581-1592.

Liu, M., Bayjanov, J., Renckens, B., Nauta, A. & Siezen, R (2010). The proteolytic system of lactic acid bacteria revisited: a genomic comparison. *BMC Genomics 11*, 1, 36.

Loman, N.J., Constantinidou, C., Chan, J.Z.M., Halachev, M., Sergeant, M., Penn, C.W., Robinson, E.R. & Pallen, M.J. (2012a). High-throughput bacterial genome sequencing: an embarrassment of choice, a world of opportunity. *Nature Reviews in Microbiology, 10*, 9, 599–606.

Loman, N.J., Misra, R.V, Dallman, T.J., Constantinidou, C., Gharbia, S.E., Wain, J. & Pallen, M.J. (2012b). Performance comparison of benchtop high-throughput sequencing platforms. *Nature in Biotechnology, 30,* 5, 434–439.

Lortal, S. & Chapot-Chartier, M. P. (2005). Role, mechanism and control of lactic acid bacteria lysis in cheese. *International Dairy Journal*, *15*, 857–871

Lozupone, C.A., Stombaugh, J.I., Gordon, J.I., Jansson, J.K. & Knight, R. (2012). Diversity, stability and resilience of the human gut microbiota. *Nature, 489*, 220-30.

Ludwig, W. & Klenk, H.P. (2001). Overview: a phylogenetic backbone and taxonomic framework for procaryotic systematics. In *Bergey's Manual of Systematic Bacteriology*, 2nd edn, pp. 49-65. Edited by D. R. Boone, R. W. Castenholz & G. M. Garrity. New York : Springer.

Lusk, T. S., Ottesen, A. R., White, J. R., Allard, M. W., Brown, E. W. & Kase, J. A. (2012). Characterization of microflora in Latin-style cheeses by next-generation sequencing technology. *BMC Microbiology, 12*, 254.

Lynch, C. M., Muir, D.D., Banks, J.M., Mc Sweeney, P. L. H. & Fox, P.F. (1999). Influence of adjunct cultures of *Lactobacillus paracasei* ssp. *paracasei* or *Lactobacillus plantarum* on Cheddar cheese ripening. *Journal of Dairy Science, 82*, 1618- 1628.

Madadlou, A., Khosroshahi, A. & Mousavi, M.E. (2005). Rheology, microstructure, and functionality of low-fat Iranian white cheese made with different concentrations of rennet. *Journal of Dairy Science*, *88*, 3052–62.

Mainville, I., Robert, N., Lee, B. & Farnworth, E.R. (2005). Polyphasic characterization of the lactic acid bacteria in kefir. *Systematic and Applied Microbiology*, *29*, 59–68.

Mather, I. H. (2000). A review and proposed nomenclature for major proteins of the milk fat globule membrane. *Journal of Dairy Science*, *83*, 203-247.

Margulies, M., Egholm, M., Altman, W.E., Attiya, S., Bader, J.S., Bemben, L.A., Berka, J., Braverman, M.S., Chen, Y.J., Chen, Z., et al. (2005). Genome sequencing in microfabricated high-density picolitre reactors. *Nature, 437,* 376–380.

Markeš, M. (1973). O porijeklu, proizvodnji i klasifikaciji sireva koji se proizvode u Jugoslaviji (About the origins, production and classification of the cheeses produced in Yugoslavia). *Mljekarstvo, 23*,10, 228-233.

Marilley, L. & Casey, M. G. (2004). Flavours of cheese products: Metabolic pathways, analytical tools and identification of producing strains. *International Journal of Food Microbiology, 90*,139–159.

Martinović, A., Radulović, Z., Wind, A., Janzen, T. & Obradović, D. (2005) Isolation ad characterization of bacterial flora from farmhouse fermented milk products of Serbia and Montenegro. *Acta Veterinaria, 55*, 307-318.

Martinovic, A., Abrahamsen, R.K. & Obradovic, D. (2006). Biochemical activities of selected strains of Lactic Acid Bacteria. *Biotechnology in Animal Husbandry, 22,* 139-151.

Martinovic, A., Moe, K.M., Romeih, E., Aideh, B., Vogensen, F.K., Østlie, H. & Skeie, S. (2013). Growth of adjunct *Lactobacillus casei* in Cheddar cheese differing in milk fat globule membrane components. *International Dairy Journal, 31*, 70-82.

Martinovic, A., Brede, M.E., Vegarud, G.E., Østlie, H.M., Narvhus, J. & Skeie, S.B. (2016). Survival of lactic acid and propionibacteria in low- and full-fat Dutch-type cheese during human digestion Ex vivo. *Letters in Applied Microbiology, 62, 5,* 404-410.

Martley, F. G. & Crow, V. L. (1993). Interactions between non-starter microorganisms during cheese manufacture and ripening. *International Dairy Journal*, *3*, 461–483.

Masoud, W., Vogensen, F. K., Lillevang, S., Abu Al-Soud, W., Sorensen, S. J. & Jakobsen, M. (2012). The fate of indigenous microbiota, starter cultures*, Escherichia coli, Listeria innocua* and *Staphylococcus aureus* in Danish raw milk and cheeses determined by pyrosequencing and quantitative real time (qRT)-PCR. *Journal of Food Microbiology, 153*, 192–202.

Mater, D. D., Langella, P., Corthier, G. & Flores, M. J. (2005). Evidence of vancomycin resistance gene transfer between enterococci of human origin in the gut of mice harbouring human microbiota. *Journal of Antimicrobial Chemotherapy*, *56,* 975–978.

Mättö, J., Malinen, E., Suihko, M.-L., Alander, M., Palva, A. & Saarela, M. (2004). Genetic heterogeneity and functional properties of intestinal bifidobacteria. *Journal of Applied Microbiology*, *97*, 459-462.

Maiden, M.C. (1998). Horizontal genetic exchange, evolution, and spread of antibiotic resistance in bacteria. *Clinical infectious disease, 1,* 12-20.

McSweeney, P.L.H., Fox, P.F., Lucey, J.A., Jordan, K.N. & Cogan, T.M. (1993). Contribution of the indigenous microflora to the maturation of Cheddar cheese. *International Dairy Journal*, *3*, 613-634.

McSweeney, P.L.H., Nursten, H.E. & Urbach G. (1997). Flavours and off-flavours in milk and dairy products. in: Fox P.F. (Ed.), *Advanced Dairy Chemistry*, Chapman and Hall, London, UK, 2nd ed., vol. 3, pp. 403–468.

McSweeney, P.L.H. & Sousa, M.J. (2000). Biochemical pathways for the production of flavour compounds in cheese during ripening. *Le Lait, 80*, 293–324.

McSweeney, P.L.H. (2004). Biochemistry of cheese ripening: introduction and overview. In Cheese: Chemistry, Physics and Microbiology, Vol 1: General Aspects, 3rd edn, pp 347–360. Fox P F, McSweeney P L H, Cogan T M and Guinee T P, eds. London: Elsevier.

McSweeney, P.L.H., Ottogali, G. & Fox, P.F. (2004). Diversity of cheese varieties: An overview. In Cheese: Chemistry, Physics and Microbiology, Vol 1: General Aspects, 3rd edn, pp 347– 360. Fox P F, McSweeney P L H, Cogan T M and Guinee T P, eds. London: Elsevier.

Melichiorsen, C.R., Jokumsen, K.V., Villadsen, J., Israelsen, H. & Arnau, J. (2002). The level of pyruvate-formate lyase controls the shift from homolactic to mixed-acid product formation in Lactococcus lactis. *Applied Microbiology and Biotechnology, 58, 338-344.*

Mirecki, S. (2012). Protection of traditional dairy products in Montenegro. *Agriculture and Forestry*, *57,* 2, 27-42.

Mirecki, S., Popović, N., Antunac, N., Mikulec, N. & Plavljanić, D. (2015). *Production* technology and some quality parameters of Njeguši cheese. *Mljekarstvo*, *65*, 4, 280-286.

Moe, K.M., Faye, T., Abrahamsen, R.K., Østlie, H.M. & Skeie, S. (2012). Growth and survival of cheese ripening bacteria on milk fat globule membrane isolated from bovine milk and its monosaccharides. *International Dairy Journal, 25,* 29-35.

Mohamed, A. G. (2015). Low-fat cheese: a modern demand. *International Journal of Dairy Science*, *10,6*, 249-265.

Mohania, D., Nagpal, R., Kumar, M., Bhardwaj, A., Yadav, M., Jain, S., Marotta, F., Singh, V., Parkash, O. & Yadav, H. (2008). Molecular approaches for identification and characterization of lactic acid bacteria. *Journal of Digestive Diseases, 9,* 190–198.

Montel, M. C., Buchin, S., Mallet, A., Delbes-Paus, C., Vuitton, D., Desmasures, N. & Berthier, F. (2014). Traditional cheeses: rich and diverse microbiota with associated benefits. *International Journal of Food Microbiology, 177*, 136–154.

Morin, P., Pouliot, Y. & Britten, M. (2008). Effect of buttermilk made from creams with different heat treatment histories on properties of rennet gels and model cheeses. *Journal of Dairy Science*, *91*, 871-882.

Morelli, L. (2001). Taxonomy and physiology of lactic acid bacteria, effects and function on nutrition. Report of a joint FAO/WHO expert consultation on evaluation on health and nutritional properties of probiotics in food including powder milk with lactic acid bacteria. Food and Agricultural Organization of the United Nations, New York, NY. Available at: ftp://ftp.fao.org/es/esn/food/.

Mucchetti, G., Locci, F., Gatti, M., Neviani, E., Addeo, F., Dossena, A. & Marchelli, R. (2000). Pyroglutamic acid in cheese: presence, origin and correlation with ripening time of Grana Padano cheese. *Journal of Dairy Science*, *83,* 659-665.

Mucchetti, G., Locci, F., Massara, P., Vitale, R. & Neviani, E. (2002). Production of pyroglutamic acid by thermophilic lactic acid bacteria in hard-cooked mini-cheeses. *Journal of Dairy Science, 85,* 2489–2496.

Narvhus, J. A., Østeraas, K., Mutukumira, T. & Abrahamsen, R. K. (1998). Production of fermented milk using a malty compound-producing strain of *Lactococcus lactis* subsp. *lactis* biovar. *diacetylactis* isolated from Zimbabwean naturally fermented milk. *International Journal of Food Microbiology , 41*, 73-80.

Nelson, B.K. & Barbano, D.M. (2004). Reduced-fat cheddar cheese manufactured using a novel fat removal process. *Journal of Dairy Science*, *87*, 841–53.

Neves, A.R., Pool, W.A., Kok, J., Kuipers, O.P. & Santos, H. (2005). Overview on sugar metabolism and its control in *Lactococcus lactis*– the input from in vivo NMR. *FEMS Microbiology Reviews*, *29,* 531–554.

Ndoye, B., Andriamahery Rasolofo, E., LaPointe, G. & Roy, D. (2011). A review of the molecular approaches to investigate the diversity and activity of cheese microbiota. *Dairy Science and Technology*, *91*, 495–524.

Njeru, P.N., Rösch, N., Ghadimi, D., Geis, A., Bockelmann, W., de Vrese, M., Schrezenmeir, J. & Heller, K.J. (2010). Identification and characterization of lactobacilli isolated from Kimere, a spontaneously fermented pearl millet dough from Mbeere, Kenya (East Africa). *Beneficial Microbes, 1, 3*, 243-52.

O'Hara, A.M. & Shanahan, F. (2006). The gut flora as a forgotten organ. *EMBO Report, 7,* 688–693.

Olson, N.F. & Johnson, M.E. (1990). Light cheese products: Characteristics and economics. *Food Technology*, *44*, 93-96.

Orla-Jensen, S. (1919). The lactic acid bacteria. E. Munksgaard,Kobenhavn.

Oh-Sik, K. (2002). Characterization of isolated *Lactobacillus* spp. and classification by RAPD-PCR analysis. *Journal of Microbiology*, *38*, 137– 44.

Ouadghiri, M., Amar, M., Vancanneyt, M. & Swings, J. (2005). Biodiversity of lactic acid bacteria in Moroccan soft white cheese (Jben), *FEMS Microbiology Letters, 251*, 267-271.

Østlie, H.M., Vegarud, G.E. & Langsrud, T. (1995). Autolysis of lactococci: detection of lytic enzymes by polyacrylamide gel electrophoresis and characterization in buffer systems. *Applied and Environmental Microbiology, 61*, 3598-3603.

Østlie, H. M., Eliassen, L., Florvaag, A. & Skeie, S. (2004). Phenotypic and PCR-based characterization of the microflora in Norvegia cheese during ripening. *International Journal of Food Microbiology, 94*, 287–299.

Østlie, H.M., Kraggerud, H., Longva, A.B. & Abrahamsen, R.K. (2016). Characterisation of the microflora during ripening of a Norwegian semi-hard cheese with adjunct culture of propionic acid bacteria. *International Dairy Journal, 54,* 43-49.

Pfeiler, E.A. & Klaenhammer, T.R. (2009). Role of transporter proteins in bile tolerance of *Lactobacillus acidophilus*. *Applied and Environmental Microbiology, 75*, 6013-6016.

Pfefferle, P.I. & Renz, H. (2014). The mucosal microbiome in shaping health and disease. *F1000Prime Reports, 6*, 1-9.

Pedersen, M. B., Iversen, S. L., Sorensen, K. I. & Johansen, E. (2005). The long and winding road from the research laboratory to industrial applications of lactic acid bacteria. *FEMS Microbiology Reviews*, *29*, 611- 624.

Pederson, J., Mileski, G., Weimer, B. & Steele, J. (1999). Genetic characterization of a cell envelope associated proteinase from *Lactobacillus helveticus* CNRZ32. *Journal of Bacteriology*, *181*, 4592–4597.

Parente E. & Cogan T.M. (2004). Starter Cultures: General Aspects. In Cheese: *Chemistry, Physics and Microbiology*, Vol 1 General Aspects, 3rd edn Fox P F, McSweeney P L H, Cogan T M and Guinee T P, eds Elsevier, London.

Peterson, S.D. & Marshall R.T. (1990). Nonstarter lactobacilli in Cheddar cheese: a review. *Journal of Dairy Science*, *73*, 1395-1410.

Petti, C. A. (2007). Detection and identification of microorganisms by gene amplification and sequencing. *Clinical Infectious Diseases, 44*, 1108-1114.

Pitino, I., Randazzo, C.L., Cross, K.L., Parker,M.L., Bisignano, C., Wickham, M.S.J., Mandalari, G. & Caggia, C. (2012). Survival of *Lactobacillus rhamnosus* strains inoculated in cheese matrix during simulated human digestion. *Food Microbiology, 31*, 57-63.

Planý, M., Kuchta, T., Šoltýs, K., Szemes, T., Pangallo, D. & Siekel, P. (2016). Metagenomic analysis of Slovak Bryndza cheese using next-generation 16S rDNA amplicon sequencing. *Nova Biotechnologica et Chimica, 15, 1,* 23-34.

PM Food & Dairy Consulting. (2014). World Cheese Market 2000- 2020. PM Food & Dairy Consulting, 2014.

Poonam, Pophaly, S.D., Tomar, S.K. & Singh, R (2012). Multifaceted attributes of dairy propionibacteria: a review. *World Journal of Microbiological Biotechnology, 28*, 3081-3095.

Porcellato, D., Østlie, H., Brede, M.E., Martinovic, A. & Skeie, S. (2013). Dynamic of adjunct non starter lactic acid bacteria and propionic acid bacteria in low-fat and full-fat cheese. *International Dairy Journal, 33*, 164-111.

Porcellato, D., & Skeie, S. B. (2016). Bacterial dynamics and functional analysis of microbial metagenomes during ripening of Dutch-type cheese. *International Dairy Journal*, 61, 182-188.

Pot, B. & Tsakalidou, E. (2009). Taxonomy and metabolism of *Lactobacillus*. In: *Lactobacillus* Molecular Biology: From Genomics to Probiotics, Ljungh, A. and T. Wadstrom (Eds.). Chapter 2, *Caister Academic Press, Norfolk, UK., ISBN-13: 9781904455417*, 3-58.

Qin, J., Li, R. & Raes, J. (2010). A human gut microbial gene catalogue established by metagenomic Sequencing. *Nature*, *464*, 59–65.

Quigley, L., O'Sullivan, O., Beresford, T. P., Ross, R. P., Fitzgerald, G. F. & Cotter, P. D. (2012). High-throughput sequencing for detection of subpopulations of bacteria not

previously associated with artisanal cheeses. *Applied and Environmental Microbiology, 78,* 5717–5723.

Randazzo, C.L., Torriani, S., Akkermans, A.L.D., de Vos, W.M. & Vaughan, E.E. (2002). Diversity, dynamics, and activity of bacterial communities during production of an artisanal Sicilian cheese as evaluated by 16S rRNA analysis. *Applied and Environmental Microbiology, 68*, 1882–1892.

Randazzo, C.I., Caggia, C. & Neviani E. (2009). Application of molecular approaches to study lactis acid bacteria in artisanal cheese. *Journal of Microbiological Methods*, *78*, 1-9.

Rakočević, M. (1950). Njeguški sir (Njeguši cheese). *Stočarstvo*, 6.

Rijnen, L., Courtin, P., Gripon, J.C. & Yvon, M. (2000). Expression of a heterologous glutamate dehydrogenase gene in *Lactococcus lactis* highly improves the conversion of amino acids to aroma compounds *Applied and Environmental Microbiology, 66*, 1354– 1359.

Roh, S.W., Kim, K.H., Nam, Y.D., Chang, H.W., Park, E.J. & Bae, J.W. (2010). Investigation of archaeal and bacterial diversity in fermented seafood using barcoded pyrosequencing. *ISME Journal, 4,*1*,* 1-16.

Rombaud, R., Van Camp, J. & Dewettinck, K. (2006). Phospho- and sphingolipid distribution during processing of milk, butter and whey. *International Journal of Food Science and Technology*, *41*, 435-443.

Rossi, F., Dellaglio, F., Peluso, M. & Torriani, S. (2000). Dairy propionibacteria: occurrence-resistance to technological stresses and antagonistic properties (cheesemaking). *Industry Aliment, 39*, 553-557.

Salminen S. & Von Wright A. (1998). Lactic Acid Bacteria: Microbiology and Functional Aspects, 2nd edn, Eds Marcel Dekker, New York

Schloissnig, S., Arumugam, M., Sunagawa, S., Mitreva, M., Tap, J., Zhu, A.,Waller, A., Mende, D.R., Kultima, J.R., Martin, J., Kota, K., Sunyaev, S.R., Weinstock, G.M. & Bork, P. (2013). Genomic variation landscape of the human gut microbiome. *Nature, 493*, 45-50.

Scheirlinck, I., Van der Meulen, R., De Vuyst, L., Vandamme, P. & Huys, G. (2009). Molecular source tracking of predominant lactic acid bacteria in traditional Belgian sourdoughs and their production environments. *Journal of Applied Microbiology, 106,* 4*,* 1081-92.

Schell, M.A., Karmirantzou, M., Snel, B., Vilanova, D., Berger, B., Pessi, G., Zwahlen, M. C., Desiere, F., Bork, P., Delley, M., Pridmore, R.D. & Arigoni, F. (2002). The genome

sequence of *Bifidobacterium longum* reflects its adaptation to the human gastrointestinal tract. *PNAS, 99, 22,* 14422-14427.

Schmelzer, C.E.H., Schöps, R., Reynell, L., Ulbrich-Hofmann, R., Neubert, R.H.H. & Raith, K. (2007). Peptic digestion of beta-casein. Time course and fate of possible bioactive peptides. *Jornal of Chromatography*, *1166*, 108–115.

Sforza, S., Cavorta, V., Galaverna, G., Dossena, A. & Marchelli, R. (2009). Accumulation of non-proteolytic aminoacyl derivatives in Parmigiano-Reggiano cheese during ripening. *International Dairy Journal*, *19*, 582–587.

Shakeel Ur Rehman, Waldron, D. & Fox, P. F. (2004). Effect of modifying lactose concentration in cheese curd on proteolysis and in quality of Cheddar cheese. *International Dairy Journal*, *14*, 591–597.

Sharp, M., McMahon, D.J. & Broadbent, J. (2008). Comparative evaluation of yogurt and low fat Cheddar cheeses delivery media for probiotic *Lactobacillus casei*. *Journal of Food Science, 73*, 375-377.

Sekirov,I., Russell, S.L., Antunes, L.C.M. & Finlay, B.B. (2010). Gut microbiota in health and disease *Physiological Reviews*, *90*, 859–904.

Settanni, L. & Moschetti, G. (2010). Non-starter lactic acid bacteria used to improve cheese quality and provide health benefits. *Food Microbiology, 27*, 691–697.

Siezen, R.J. (1999). Multidomain, cell envelope proteinases of lactic acid bacteria. *Antonie van Leeuwenhoek*, *76*, 139-155.

Singh, T.K., Drake, M.A. & Cadwallader, K.R. (2003). Flavor of Cheddar Cheese: A Chemical and Sensory Perspective. *Comprehensive reviews in food science and food safety, 2,* 166-189.

Singh, H. (2006). The milk fat globule membrane-A biophysical system for food applications. *Current Opinion in Colloid Interface Science*, *11*,154-163.

Singh, S., Pawas, G., Singh, R. & Heller, K.J. (2009). Application of molecular identification tools for *Lactobacillus*, with a focus on discrimination between closely related species: A review. *LWT-Food Science and Technology, 42*, 448-457.

Skeie, S., Lindberg, C. & Narvhus, J. (2001) Development of amino acids and organic acids in Norvegia, influence from milk treatment and adjunct *Lactobacillus*. *International Dairy Journal, 11*, 399-411.

Skeie, S., Kieronczyk, A., Næs, R.M. & Østlie, H. (2008). *Lactobacillu*s adjuncts in cheese: Their influence on the degradation of citrate and serine during ripening of a washed curd cheese. *International Dairy Journal, 18,* 158-168.

Smeianov, V.V., Wechter, P., Broadbent, J.R., Hughes, J.E., Rodriguez, B.T., Christensen, T.K., Ardo, Y. & Steele, J.L. (2007). Comparative high-density microarray analysis of gene expression during growth of *Lactobacillus helveticus* in milk versus rich culture medium. *Applied and Environmental Microbiology*, *73*, 2661-2672.

Smid, E.J. & Lacroix, C. (2013). Microbe- microbe interactions in mixed culture food fermentations. *Current Opinion in Biotechnology, 24*,148-154.

Smit, G., Smit, B. A. & Engels, W. J. M. (2005). Flavour formation by lactic acid bacteria and biochemical flavour profiling of cheese products. *FEMS Microbiology Reviews 29*, 591-610.

Stackebrandt, E. & Gobel, B.M. (1994). Taxonomic note: a place for DNA-DNA re-association and 16S rRNA sequence analysis in the present species definition in bacteriology. *International Journal Systematic Bacteriology*, *44*, 846-849.

Stieglitz, H. W. A. (2008). Ein Besuch auf Montenegro. Reprint from 1841. *Bibliolife, 1*, 212.

Solieri, L., Bianchi, A. & Giudici, P. (2012). Inventory of nonstarter lactic acid bacteria from ripened Parmigiano Reggiano cheese as assessed by a culture dependent multiphasic approach. *Systematic and Applied Microbiology, 35*, 270– 277.

Soumalianen, T., Sigvart- Mattila, P., Mâttö, J. & Tynkkynen, S. (2008). In vitro and in vivo gastrointestinal survival, antibiotic susceptibility and genetic identification of *Propionibacterium freundreichii* ssp. *shermanii* JS. *Interantional Dairy Journal, 18,* 271-278.

Spano, G., Beneduce, L., Tarantino, D., Zapparoli, G. & Massa, S. (2002). Characterization of *Lactobacillus plantarum* from wine must by PCR species-specific and RAPD-PCR. *Letters in Applied Microbiology*, *35*, 370 –37 4.

Svec, P., Drab V. & Sedlacek I. (2005). Ribotyping of *Lactobacillus casei* group strains isolated from dairy products. *Folia Microbiologica*, *50*, 223–228.

Sumeri, I., Adamberg, K., Uusna, R., Sarand, I. & Paalme, T. (2012). Survival of cheese bacteria in gastrointestinal tract simulator. *International Dairy Journal, 25*, 36-41.

Syu, M.J. (2001). Biological production of 2,3-butanediol. *Applied Microbiology and Biotechnology, 55*, 10– 18.

Tanous, C., Kieronczyk, A., Helinck, S., Chambellon, E. & Yvon, M. (2002). Glutamate dehydrogenase activity: a major criterion for the selection of flavour-producing lactic acid bacteria strains. *Antonie Van Leeuwenhoek, 82*, 271–278.

Taylor, S.L., Keefe, T.J., Windham, E.S. & Howell, J.F. (1982). Outbreak of histamine poisoning associated with consumption of Swiss cheese. *Journal of Food Protection, 45*, 455-457.

Tarakci, Z. & Tuncturk, Y. (2008). The effect of adjunct cultures on some chemical and biochemical properties of white-brined cheese. *Journal of Food Biochemistry*, *32*, 4, 490-505.

Temmerman, R., Huys, G. & Swings, J. (2004). Identification of lactic acid bacteria: Culture-dependent and culture-independent methods. *Trends in Food Science and Technology*, *15*, 348-359.

Thierry, A. & Maillard, M. B. (2002). Production of cheese flavour compounds derived from amino acid catabolism by *Propionibacterium freudenreichii. Le Lait*, *82,* 17–32.

Thomas, T. D. & Crow, V. L. (1983). Mechanism of (d)-lactic acid formation in Cheddar cheese. *New Zealand Journal of Dairy Science and Technology*, *18*, 131–141.

Thomas, T.D. (1987a). Acetate production from lactate and citrate by non-starter bacteria in Cheddar cheese.*New Zealand Journal of Dairy Science and Technology*, *22*, 25-38.

Thomas, T. D. (1987b). Cannibalism among bacteria found in cheese. *New Zealand Journal of Dairy Science and Technology*, *22*, 215-219.

Thompson, M.P., Browcr, D.P. & Farrell, H.M. (1987). Absence of detectable calmodd in cow's milk by a modified gel electrophoresis method. *Journal of Dairy Science*, *70*, 11-34.

Tsakalidou, E., Manolopoulou, E., Kabaraki, E., Zoidou, E., Pot., B., Kersters, K. & Kalantzopoulos, G. (1994). The combined use of whole-cell protein extracts for the identification (SDS-PAGE) and enzyme activity screening of lactic acid bacteria isolated from traditional Greek dairy products. *Systematic and Applied Microbiology*, *17*, 444-458.

Turnbaugh, P.J. & Gordon, J.I. (2009). The core gut microbiome, energy balance and obesity. *Journal of Physiology*, *587*, 4153–4158.

Tynkkynen, S., Satokari, R., Saarela, M., Mattila-Sandholm, T. & Saxelin, M. (1999). Comparison of ribotyping, randomly amplified polymorphic DNA Analysis, and pulsed-field gel electrophoresis in typing of *Lactobacillus rhamnosus* and *L. casei* strains. *Applied and Environmental Microbiology, 65*, 3908–3914.

Ueda, S., Nomoto, R., Yoshida, K.I. & Osawa, R. (2014). Comparison of three tannases cloned from closely related *Lactobacillus* species: *L. plantarum, L. paraplantarum* and *L. pentosus*. *BMC Microbiology*, *14*, 87.

Ulleberg, E.K., Comi, I., Holm, H., Herud, E.B., Jacobsen, M. & Vegarud, G. (2011). Human gastrointestinal juices intended for use in in vitro digestion models. *Food Digestion*, *2*, 52–61.

Urbach, G. (1995). Contribution of lactic acid bacteria to flavour compound formation in dairy products. *International Dairy Journal, 5*, 877-903.

Urbach, G. (1997). The flavour of milk and dairy products: II. Cheese: Contribution of volatile compounds. *International Journal of Dairy Technology, 50*, 79-89.

Vancanneyt, M., Huys, G., Lefebvre, K., Vankerckhoven, V., Goossens, H. & Swings, J. (2006). Intraspecific genotypic characterization of *Lactobacillus rhamnosus* strains intended for probiotic use and isolates of human origin. *Applied and Environmental Microbiology, 72,* 5376-83.

Vanderghem, C., Bodson, P., Danthine, S., Paquot, M. & Blecker, C. (2010). Milk fat globule membrane and butter-milks: from composition to valorization. *Biotechnology, Agronomy, Society and Environment, 14*, 485-500.

Versalovic, J., Schneider, M., de Bruijn, F.J. & Lupski, J.R. (1994). Genomic fingerprinting of bacteria using repetitive sequence based PCR (rep-PCR). *Methods of Cellular Molecular Biology, 5*, 25-40.

Vetrovsky, T. & Baldrian, P. (2013). The variability of the 16S rRNA gene in bacterial genomes and its consequences for bacterial community analyses. *PLoS One, 8,* 57923.

Visser, S. (1993). Proteolytic enzymes and their relation to cheese ripening and flavour: an overview. *Journal of Dairy Science*, *76*, 329-350.

Von Wright, A. & Axelsson, L. (2012). Lactic acid bacteria: an introduction. In: Lactic Acid Bacteria, Microbial and Functional Aspects, 4th Edition (Lahtinen, S., Ouwehand, A.C., Salminen, S. and Von Wright, A., Eds.), pp. 1-16. CRC Press, Boca Raton, FL.

Vukina, R. (1949). Poizvodnja durmitorskog sira (The production of Durmitor cheese). *Stocarstvo*, 11-12.

Walker, A.W., Duncan, S.H., Louis, P. & Flint, H.J. (2014). Phylogeny, culturing, and metagenomics of the human gut microbiota. *Trends in Microbiology, 22, 5,* 267-274.

Walstra P., Wouters J.T.M. & Geurts T.J. (2006). *Dairy Science and Technology*. 2nd Ed. Taylor and Francis Group, Boca Raton-London.

Weimer, B. (2007). Improving the flavour of cheese. *Woodhead Publishing, 2007*, ISBN: 9781845690076.

Whitcomb, D.C. & Lowe, M.E. (2007). Human pancreatic digestive enzymes. *Digestive Diseases and Sciences, 52*, 1–17.

Wijesunda, C. & Watkins, P. (2000). Milk fat is essential for the development of cheddar cheese flavour. *Australian Journal of Dairy Technology*, *55*, 86-91.

William, R.A.D. & Sandler, S.A. (1971). Electrophoresis of glucose-6-phosphate dehydrogenase, cell wall composition and the taxonomy of heterofermentative lactobacilli. *Journal of General Microbiology*, *65*, 351–358.

Williams, A. G. & Banks, J. M. (1997). Proteolytic and other hydrolytic enzyme activities in nonstarter lactic acid bacteria (NSLAB) isolated from Cheddar cheese manufactured in the United Kingdom. *International Dairy Journal, 7*, 763–774.

Woese, C. R. (1987). Bacterial evolution. *Microbiology Reviews*, *51*, 221-271.

Yvon, M. & Rijnen, L. (2001). Cheese flavour formation by amino acid catabolism. *International Dairy Journal, 11,* 185–201.

Zdanovski, N. (1947). Ovčije mljekarstvo (Sheep dairy), Zagreb.

Paper I

Application of indigenous strains of lactic acid bacteria for semi-industrial production of autochthonous Montenegrin Njeguši cheese

Aleksandra Martinovic[ab], Judith Narvhus[b], Roger K. Abrahamsen[b], Hilde Marit Østlie[b], Siv Borghild Skeie[b]

[a]*University of Donja Gorica, Faculty of Food Technology, Food Safety and Ecology, Donja Gorica bb., Podgorica, Montenegro*

[b]*Norwegian University of Life Sciences, Department of Chemistry, Biotechnology and Food Science, P.O. Box 5003, N-1432 Aas, Norway*

Corresponding author, Tel.:+382 69 737 403

E-mail address: aleksandra.martinovic@udg.edu.me (A. Martinovic)

Abstract

Most of the traditional dairy products of the South-Eastern Europe are produced from unpasteurized milk without addition of selected starter cultures. These artisanal food products contain an interesting biodiversity of indigenous microbiota. In Montenegro, one of the most popular traditional cheeses is Njeguši cheese, belonging to the group of semi-hard cheeses. Most of the cheeses are produced from ewe's milk, but cow's milk is also widely used. In Montenegro, there are no existing legal regulations defining the specificities of the production or the raw material used for the production of autochthonous food products, and therefore the producers make the decision about what type of milk they use for cheese production. One of the priorities of the food producers in Montenegro is to obtain a larger scale, standardized production of traditional food products, which are of good quality and considered safe for the consumers. Therefore, in this study, indigenous strains of lactic acid bacteria isolated from various Montenegrin artisanal dairy products were tested as starter cultures for the production of Njeguši cheese. Three isolates were selected and used as starter cultures in pilot plant experiments for the production of Njeguši cheese. When applying an adequate combination of added bacterial strains as starters, the flavour of Njeguši cheese, produced under pilot plant conditions, was comparable to that of the artisanal origin.

Key words: Lactic acid bacteria, Njeguši cheese, traditional cheese production

1. Introduction

Farmhouse fermented milk products are made using ancient technology developed in the country or region where this food is produced and can be considered as part of the country's cultural heritage. The natural community of lactic acid bacteria (LAB) isolated from farmhouse fermented milk products represents a reservoir for obtaining strains with specific features, which could be used as starter cultures in the dairy industry (Marilley and Casey 2004). Such strains may differ in many important properties, such as their rate and extent of acid production, sensitivity to bacteriophage infection, and ability to degrade casein. Montenegro is a small country, but due to its climate and geographical characteristics, it has a high diversity of traditional food products, including dairy products (Dozet et al. 1996). In the various regions of the country, different traditional dairy foods are produced (such as soft, semi-hard, and pasta filata cheeses, fermented milks, etc.), which are influenced by climate conditions and type of the livestock breed in the different areas (Mirecki 2012).

One of the most appreciated and best known traditional Montenegrin cheeses is Njeguši cheese, dating back to the time of the Roman Empire when Montenegro was a part of Doclea, and the cheese was distributed to Rome under the name “Caseus Doclestes” (Markeš 1973). Its production was recorded later in the travelogue of Heinrich Stieglitz when describing the hospitality of the people from Njeguši as well as the extremely good taste of the cheese that he was served (Steiglitz 1841).

Njeguši cheese belongs to the group of full fat semi-hard cheeses, produced originally from ewe's milk. Nowadays, the main raw material for its production is cow’s milk. The cheese microbiota originates from the raw milk and from the environment. The rind and the inner part of this cheese is yellowish. The cheese is rather compact and elastic and has a homogenous structure.

71 The taste is acidic with an aroma of lactic acid. In the production of Njeguši cheese, the cooled
72 raw milk is filtered through a cheese cloth before heating to approximately 35 °C. After the
73 addition of natural rennet, the cheese coagulum (formed after 30-60 minutes) is cut to the size of
74 sweet corn kernels and the temperature is increased to 45 °C in order to achieve optimal whey
75 separation from the curd particles. The curd is then transferred to wooden or metal moulds, and
76 pressed for 24 hours with turning of the cheese after 12 h of pressing. The curd is pressed, using a
77 wooden circular board and then a stone is put on top for extra weight. After pressing, the cheese is
78 dry salted for two days. Salt is added 2-3 times per day. The cheese is ripened on wooden shelves
79 in stone cellars, at approximately 16 °C and with 83-88 % relative humidity (RH) for 4 weeks.
80 However, the ripening can be prolonged in order to obtain a more piquant taste, or the cheese can
81 be stored in corn or olive oil.

82 The methods of the cheese making are artisanal, and traditionally every phase of
83 manufacture is done manually, contributing to a variable quality. In order to satisfy consumer
84 demands the producer should provide a final product with consistent quality and an attractive
85 flavour and microbial safety. The flavour of the cheese results from the presence of various
86 components released through the enzymatic reactions caused by enzymes originating from the
87 milk and rennet as well as from the metabolic activity of the microbiota of the cheese.

88 The metabolic activity of LAB has always been of significance for the production and
89 conservation of different products such as fermented milks, cheeses, meat, vegetables and wine.
90 Today, the modern dairy industry aims to standardize the production process of fermented
91 products to ensure a more uniform quality of the product and to ensure its microbiological safety.
92 Therefore, active starter cultures of LAB are usually applied during the production process. Most

of the cultures available on the commercial market originate from natural isolates of LAB that have proven to contribute to high quality products.

In order to achieve the successful application of LAB strains in industrial production, it is necessary to carry out accurate classification and identification, and also to determine biochemical reactions of these bacteria when used as industrial starter cultures (Cogan et al. 2007; Escamilla-Hurtado et al. 1996; Henriksen and Nilsson 2001).

The objective of this study was the application of selected strains of LAB, isolated from indigenous dairy products of Montenegro, for the pilot plant production of Njeguši cheese. The strains were previously investigated for their biochemical characteristics (Martinovic et al. 2005, Martinovic et al. 2006) with the aim of assessing their potential for use as starter cultures in cheese production. Cheeses produced with different starter combinations were analysed over the initial ripening period of 21 days for the microbiological composition, chemical composition and volatile compounds. The possible application of the selected strains would most probably lead to a more controlled production of the Njeguši cheese, resulting in an improved and standardized quality.

1. Materials and methods

Origin of the strains and growth conditions

Bacterial strains were previously isolated from indigenous dairy products of Montenegro, identified by 16S rDNA sequencing and discriminated using Pulsed Field Gel Electrophoresis (PFGE) (Martinovic et al. 2005). The strains were biochemical characterized to be used as starter cultures for a controlled pilot plant production of Njeguši cheese (Martinovic et al. 2006). Strains

were selected according to their acidification ability, their production of volatile compounds, organic acids and their carbohydrate degradation patterns (Martinovic et al. 2006). Three strains were selected for cheese making trials from a collection of 23 strains isolated from autochthonous fermented milk products of Montenegro. The three strains were: ABO57-1 and ABO19-3, identified as *Lactococcus lactis* ssp. *lactis* (marked as strains A and M respectively) and ABO19-2 identified as *Lactobacillus plantarum* (marked as L strain)). In addition, a commercial DL-starter (CH-N 22, Christian Hansen, Hørsholm, Denmark) featured with high acidification activity (marked with C) was also used. The strains were kept in MRS medium, supplemented with 15 % glycerol (v/v) at -80 °C, sub-cultured three times in M17 broth (Merck, Darmstadt, Germany) for lactococci and MRS broth (Merck, Darmstadt, Germany) for *Lactobacillus* at 30 °C overnight. These starters were then inoculated to 100 mL of UHT milk (1.5 % fat) (TINE, Norway) and incubated at 30 °C for 24 hours before inoculation in the cheese milk.

Cheese-making procedure

The cheese-making procedure was designed as an imitation of the traditional production process, except that pasteurized milk was used with the inoculation of different combinations of the LAB strains as starters. Six different combinations of LAB strains were used in two repetitions of the cheese-making as shown in Tab.1.

150 L of cheese milk was pasteurized (72 °C, 15 s) and cooled to 35 °C before transfer to stainless steel cheese vats. After starter addition, the milk was mixed for 5 minutes and supplemented with 15 mL (10 mL 100 L^{-1} milk) of rennet (Animal Rennet, Naturen, Chr. Hansen, Hørsholm, Denmark). The milk was left to coagulate for 30 min, and then the curd was cut. Following resting for 30 min, the curd was stirred for 45 min.

During the last 20 min of stirring, the temperature was increased to 36 °C. The curd (approximately 1.5 kg) was then pre-pressed for 15 min in a pre-pressing vat and then cut into cubes (15 x 15 cm), put into round stainless steel moulds and pressed for 24 h at 1.5 bar.

The cheese was dry-salted (99,8 % fine salt, Akzo, Nobel, Amersfoort, Netherlands) on the first and second day after pressing. The cheeses were ripened at 18 °C at a relative humidity (RH) of 87 %. The cheese was turned twice a day for the first three days, and thereafter once a day. The cheeses were not covered with the plastic or waxed since the traditional procedure was followed. Cheese was analysed after 1, 7, 14 and 21 days of ripening. The cheeses are further denoted according to the starter strain used, i.e. cheese with starter strain A is cheese A.

Microbiological analyses

The presence of coliform bacteria was investigated in the cheese milk after pasteurization, after addition of starter cultures, in the drained whey, in the pre-pressed cheese and in one day old cheeses. Cheese samples (10 g) were added to 90 ml sterile 2% w/ v sodium citrate solution and homogenized for 2 min in an Omni mixer (Omni International, Waterbury, CT, USA). Serial dilutions in Ringer's solution were plated on suitable media for enumeration of microorganisms. Coliform bacteria were enumerated on VRB agar (Merck, Germany) by plating the dilutions (10^{-1} to 10^{-3}) of 1 day old cheeses and incubation at 37 °C for 24 h. Lactic acid bacteria were enumerated in 1, 7, 14 and 21 day-old cheeses by plating on M17 agar (Merck) for lactococci and incubating at 30 °C for 48 h and MRS agar (Merck) (De Man et al. 1960) for lactobacilli incubated at 30 °C for 72 h.

Chemical analyses

Samples for chemical analysis were taken from 1, 7, 14 and 21 day-old cheeses. Sampling was done according to IDF-standard 50C (1995). The dry matter (DM) (IDF standard 4A 1982), pH (Skeie et al. 2001) fat (IDF standard 105 2008), protein (IDF Standard 20 A 1986) and salt (IDF standard 88A 1988) content were analyzed at each sampling time.

Sugars and organic acids were analyzed using High Performance Liquid Chromatography (HPLC) according to the method used by Narvhus et al. (1998). Volatile compounds were determined by headspace gas chromatography (HSGC) according to the method described by Narvhus et al. (1998). As extraction rates for individual components from the cheese matrix have not been determined, quantification is presented as the integrated peak area g^{-1} cheese, which allows a semi-quantitative comparison between cheese samples.

Sensory analyses of the cheeses

Mature experimental cheeses were evaluated by four Norwegian cheese judges scoring six cheese attributes: appearance, colour, consistency, firmness, odour and flavour according to the method of Ritz et al., 1991. According to results obtained, cheeses were classified into different quality categories.

Statistical analysis of the cheeses

Significant differences ($P<0.05$) between treatment factor (starter culture) and age were found by using the Proc Mixed procedure with repeated measurements using SAS Enterprise Guide 4 (SAS Institute Inc., Cary, USA). Replicate block was treated as a random factor and a variance components covariance model was used when analyzing the data. Statistical analyses

185 were carried out on the gross composition data using SAS Enterprise Guide 4 (SAS Institute Inc.,
186 NC USA). A general linear model (GLM) was used to perform the analysis of variance (ANOVA)
187 using the starter combinations and replicate blocks as experimental factors.

188 Principal component analysis (PCA) and partial least squares regression (PLS2) were
189 performed using UNSCRAMBLER 7.01 (Camo ASA, Oslo). The data sets were weighted by
190 dividing each response variable by the standard deviation of that variable. Full cross validation
191 was used for validating the data set. Samples shown to be outliers were removed from further
192 analyses.

193

194 **2. Results**

195 *Microbial population in cheeses*

196 The number of lactococci in the cheeses ranged from 7.25 to 8.26 log cfu g^{-1} after 1 d of
197 ripening (Fig. 1-a). Maximum numbers were detected in 14 day old cheeses. Maximum number
198 of presumptive lactobacilli was recorded in 7 d old cheese (Fig. 1-b). The development of the
199 number of lactobacilli in cheese A was significantly different from the development of the
200 numbers recorded in cheeses C and AM. In cheese AM the number of presumptive lactobacilli
201 decreased rapidly after 14 d of ripening in comparison to other cheeses. The growth of coliform
202 bacteria in cheese was slight or absent at all of the samplings points.

203

204 *Chemical composition of cheese*

205 The development of pH and gross composition of the experimental cheeses during the 21
206 d ripening period is shown in Tables 2 and 3. The DM, fat in dry matter (FDM) content and pH
207 were not significantly influenced by the strain combinations used during cheese making, but

were significantly dependent on the ripening time ($P<0.001$). Average DM content at the beginning of ripening was 50.5 %, while at the end of ripening period it was 69.4 % (Tab. 2). The pH value ranged in average from 5.0 at the beginning of ripening to 4,7 at the end of ripening period (Tab. 2). The protein content (results not shown) increased uniformly from initial 17.2±0.64 % in the fresh cheese to 28.5±1.53 % at the end of the investigated ripening period ($P<0.001$). Soluble nitrogen (SN/TN; Tab. 3) in cheeses was not significantly influenced by starter type ($P >0.05$) but by age ($P<0.001$) and in average it increased from 3.7±1.73 at start to 7.8±1.02 at the end of ripening.

The lactose concentration decreased from the 1st until the 7th day of ripening ($P<0.001$). The lactose concentration ranged from an average of 16.51mmol kg^{-1} at 1d of ripening to 4.6 mmol kg^{-1}- at the end of ripening period (Fig. 2a). The obtained results showed that starter combination had a significant influence on the galactose content in the studied cheeses ($P<0.001$) (Fig. 2b). Galactose started to decrease after 7 days of ripening in cheeses containing *L. plantarum* (cheeses AL and AML), whereas in cheeses made with only the lactococci starters (cheeses A and M) the concentration of galactose was relatively stable throughout ripening. The lowest galactose concentrations throughout ripening were recorded in cheese C (control with commercial DL starter) with 0.34 mmol kg^{-1}kg after 7 days of maturation (Fig 2b). Glucose was not detected in any of the cheeses, irrespective of sampling time.

Principal component analysis (PCA) (Fig 3) showed that the cheeses were clearly separated in groups according to the starter strain(s) used (Fig 3). The content of lactose, orotic acid, and α-ketoglutaric acid at the left and succinic acid at the right were responsible for the separation along PCA loadings plot (Fig 3) with the oldest cheeses placed to the right, but also with a separation due to the starter strain used with the young cheeses added *Lb. plantarum* (L)

more to the left (Fig 3). Along PC2, the content of formic acid in the upper part and DL-pyroglutamic acid and galactose in the lower part were responsible for the separation along PC2 and these compounds caused the separation of cheeses depending on the strain used in the cheese making (Fig 3). The separation revealed three different groupings: cheese C; cheeses M, AM, ALM; and cheese A and AL (marked with rings in the score plot, Fig. 3). Cheese (C) made with a commercial starter showed a clear separation from the other cheeses.

The concentration of lactic acid increased ($P<0.001$) from the first to the seventh day of ripening in all of the cheeses (Fig. 4a). The development of formic acid was different in control cheese C when compared to the other cheeses (Fig. 4b), with the highest values at the beginning of ripening, a drop at 7 days and then again increase by the end of ripening period.

The concentration of lactic acid increased ($P<0.001$) from the first to the seventh day of ripening in all of the cheeses (Fig. 4a). The development of formic acid was different in control cheese C when compared to the other cheeses (Fig. 4b), with the highest values at the beginning of ripening, a drop at 7 days and then again increase by the end of ripening period.

An increase in the concentration of DL- pyroglutamic acid during the first 14 d of ripening was followed by a decrease in concentration in all of the cheeses ($P<0.001$), except cheese M. The concentration of the DL-pyroglutamic acid was significantly influenced by the type of starter used ($P<0.001$). In cheeses with A starter (cheese A)) the concentration of DL-pyroglutamic acid was increasing by the end of the maturation period. In cheeses M and AML the highest concentration was recorded after 7 days of ripening, while in cheese C the highest concentration was found after 14 days of ripening (Fig. 5a). Succinic acid was significantly higher ($P<0.001$) in cheese C than in the other cheeses during the whole ripening period and increased during the experimental maturation period in all cheeses (Fig. 5b).

254 Volatile compounds were analyzed at each sampling time and the distribution was
255 evaluated by PCA. The PCA plot (Fig. 6) showed that PC1 explained the variance in the cheese
256 samples due to age of the cheese by 31 % and it can be seen that cheese M at the beginning of the
257 ripening period (dotted circle), had a higher concentration of methyl aldehydes and alcohols
258 ($P<0.01$) compared to the other experimental cheeses. Cheese C and A ripened for more than 14 d
259 grouped together based on the amounts of ethanol (Fig 6, dashed circle).
260
261 *Sensory analysis*
262 The results of the sensory analysis of the experimental cheeses are shown in Tab. 4. All
263 of the cheeses were judged to be of good quality, based on criteria such as appearance, taste and
264 flavour and texture. In the different cheeses, variations in aroma were noticed depending on
265 starter cultures used. In cheeses M and AM, the typical Njeguši cheese aroma was recorded.
266 This might be related to the higher concentrations of methyl aldehydes observed in these
267 cheeses, also shown in the PCA plot (Fig. 6).
268 The sensory properties of the cheeses changed in accordance with changes in aroma.
269 Saltiness increased during ripening along with the achievement of a more intense piquant cheese
270 taste. On the basis of the sensory analysis it was determined that cheeses M and AM had the
271 characteristic taste and appearance of Njeguši cheese with favourable sensory characteristics,
272 especially for cheese M. Cheese made using the commercial DL starter (cheese C) was
273 characterized with a neutral taste, while cheese A was more acidic.
274
275
276

3. Discussion

The DM and FDM content increased, as expected, during ripening and reached high values that are in accordance with the findings of other authors for this indigenous hard cheese. The protein content was correlated with the increase in DM, but was higher after 21 d compared to the results from previous investigations of Njeguši cheese (Adžić et al. 1997; Dozet et al. 2004; Mirecki et al. 2015). Differences in the cheese making technique, in the relative humidity during ripening and thereby some differences in the DM of the cheeses might be the reason for this observation. The salt content in the ripened cheese was in the optimum range for this group of hard cheeses (Mirecki et al. 2015).

In the cheeses to which *Lb. plantarum* was added, the numbers of presumptive lactobacilli remained relatively high throughout the investigated ripening time (> 6 log cfu g^{-1}) The growth of presumptive lactobacilli in cheese C, A, M and AM might be a result of the development of NSLAB in the cheese.

Degradation of lactose to lactate is essential for cheese production. Lactose is usually metabolised by the glycolytic pathway in the early stages of the cheese ripening, resulting in a decrease in pH. Most of the metabolism of lactose by LAB was recorded during the first 7 days of maturation, when the DM content was not that high, and the developments of lactic acid and SN/TN had the highest increase.

A relatively high content of formic acid was found in cheeses added *L. plantarum* (L), cheeses M and AM as well as in control cheese (C) made with commercial starter indicating that the synthesis of formic acid is strain dependent. Most of the microbial metabolic activity was recorded during the first 7 days of maturation, when the DM content was not that high, during this

299 period the lactose content decreased, and the developments of lactic acid and SN/TN had the
300 highest increase.

301 The content of DL-pyroglutamic acid was influenced by the strain combination used for
302 the cheese making, and developed differently in cheese with the A strain versus cheeses with the
303 M strain. In cheeses made with A (*Lc. lactis* subsp *lactis*) strain the synthesis of DL-
304 pyroglutamic acid increased over the maturation period. DL-pyroglutamic acid in cheese is
305 found as a free acid or bound to the N terminus of proteins and peptides (Lemieux and Simard
306 1992; Mucchetti et al. 2000). Furthermore, it has been reported that it is formed by some LAB
307 trough enzyme-mediated cyclisation of glutamic acid (Mucchetti et al. 2002; Sforza et al. 2009).

308 The obtained results showed that in the young cheese samples containing *Lb. plantarum,*
309 the concentration of α-ketoglutaric acid was significantly higher than in young cheeses without
310 lactobacilli addition. It is known that some lactobacilli strains have glutamate dehydrogenase
311 activity, therefore influencing the sensory attributes of the cheese (Williams et al. 2006).

312 It has been observed that non-industrial strains of LAB may have a greater selection of
313 amino-acid converting enzymes (Smit et al. 2005). Ayad et al. (2000) showed that lactococci
314 isolated from natural niches were able to produce rather unusual flavour components and/or
315 flavour profiles. A malty compound-producing strain of *L. lactis* ssp. *lactic* biovar. *diacetylactis*
316 was for instance isolated by Narvhus et al. (1998) from Zimbabwean naturally fermented milk.

317 Branched chain aldehydes originate from α-keto acids and 2- methyl propanal, 2-methyl
318 butanal and 3-methyl butanal are formed from valine, isoleucine and leucine, respectively (Ardö
319 2006). These amino acids metabolites are responsible for the occurrence of malty flavor in
320 cheeses (Sheldon et.al. 1971), often associated with an undesirable flavor in dairy products
321 (Morgan 1976; Ayad et al. 2000). Griffith and Hammond (1989), on the other hand, emphasize

322 that malty aroma associated with aldehydes, originating from the branched-chain amino acids
323 valine, leucine and isoleucine, could,in lower concentrations, contribute to a pleasant taste in
324 cheese. This is confirmed in other research where these compounds are recognized as key flavor
325 components in some indigenous cheeses (Bosset and Gauch 1993) and are produced by
326 indigenousstrains of LAB (Mauriello et al. 2001; Weerkamp et al. 1996; Ayad et al. 1999).
327 Banks et al. (2001) also reported that the aroma of Cheddar cheese could be improved trough
328 intensified production of the branched-chain amino acids. 3-methyl butanal and 2-methyl
329 butanal are for instance identified as flavour compounds in Cheddar and Emmental cheeses
330 (Curioni and Bosset, 2002). Furthermore, 2- and 3-methyl aldehydes are a major part of the
331 volatile fraction of several cheeses such as Cheddar, Camembert, Emmental, and Parmesan
332 (Barbieri et al. 1994; Yvon and Rijnen 2001; Thierry and Maillard 2002). When balanced with
333 other volatiles it is assumed that they contribute to the overall desirable cheese flavor. In this
334 research, the highest concentration of 2- and 3-methyl aldehyde was found in younger cheeses.
335 Strain M (*Lc. lactis* ssp. *lactis*) has been identified to synthetize these compounds to greater
336 extent when compared to the other strains used in this research (Martinovic et al. 2006). The
337 malty flavor could actually be sensed even during cheese making in the batches added strain M.
338 This strain was initially isolated from Njeguši cheese, and sensory analysis in this research
339 showed that cheese M have had the properties most similar to indigenous Njeguši cheese. In the
340 research performed by Ayad et al. (2001), formation of the malty aroma compounds 3-
341 methylbutanal, 2-methylbutanal and 2-methylpropanal has been achieved in milk cultures by
342 combining two lactococci strains, one producing free amino acids and the other degrading
343 leucine and this may also affect the flavor of cheese made with these strains.

Aldehydes can be reduced to primary alcohols or even further oxidized to acids (Ardö 2006). Strain M was able to synthesise the branched-chain alcohols 2-methyl-1-propanol, 3-methyl-1-butanol and 2-methyl 1-butanol (Martinovic et al., 2006), which was also detected in cheeses where this strain was used as starter (cheeses M and AM). These compounds may contribute to a positive flavour of cheese (Ayad et al. 2000). The obtained results showed that methyl-ketone synthesis might be correlated with the synthesis of methyl-alcohols as they were identified in the same cheeses. In addition, the PCA plot of the volatile compounds of the experimental cheeses showed that they were correlated.

Conclusion

The diverse pool of indigenous strains should be preserved with the aim of their possible application in the dairy industry. The metabolic activity of the microbiota isolated from traditional cheeses should therefore be explored

This research attempted to make a foundation for controlled production of Njeguši cheese, using some of the previously identified indigenous strains of LAB as starter cultures. The obtained results indicate that the indigenous strain M belonging to the *Lc. lactis* ssp. *lactis* is a promising candidate to be used as a starter culture for industrial production of Njeguši cheese in Montenegro.

References

Adžić, N., Dozet, N., Ljumović, M., Marković, M. & Adžić, Z. (1997). Autohtoni mlječni proizvodi Crne Gore koje treba zaštititi po osnovu porijekla (Indigenous dairy products of Montenegro that should be protected on the basis of origin). *Poljoprivreda i šumarstvo 43, 3:* 127-134.

Adžić, N., Ljumović, M., Petrović, S., Tomović, M., Dozet, N., Stanišić, M. & Perović, M. (1984). Kvalitet mlijeka za proizvodnju njeguškog sira (Quality of milk used for the production of Njeguši cheese). *Mljekarstvo, 34, 3*: 76-81.

Ardö, Y. (2006). Flavour formation by amino acid catabolism. *Biotechnology Advances, 24, 2*: 238-242.

Ayad, E. H. E., Verheul, A., de Jong, C., Wouters, J. T. M. & Smit, G. (1999). Flavour forming abilities and amino acid requirements of *Lactococcus lactis* strains isolated from artisanal and non-dairy origin. *International Dairy Journal, 9***:** 725–735.

Ayad, H. E., Verheul, A., Wouters, J. T. M. & Smit, G. (2000). Application of wild starter cultures for flavour developmnet in pilot plant cheese making. *International Dairy Journal, 9:* 725-735.

Ayad, E. H. E., Verheul, A., Engels, W. J. M., Wouters, J. T. M. & Smit, G. (2001). Enhanced flavour formation by combination of selected lactococci from industrial and artisanal origin with focus on completion of a metabolic pathway. *Journal of Applied Microbiology, 90:* 59-67.

Banks, J., Yvon, M., Gripon, J. C., de la Fuente, M. A., Brechany, E. Y., Williams, A. & Muir, D. D. (2001). Enhancement of amino acid catabolism in Cheddar cheese using α-

390 ketoglutarate: amino acid degradation in relation to volatile compounds and aroma
391 character. *International Dairy Journal, 11:* 235–243.
392 Barbieri, G., Bolzoni, I., Careri, M., Manglia, A., Parolari, G., Spagonoli, S. & Virgili, R.
393 (1994). Study of the volatile fraction of parmesan cheese. *Journal of Agricultural and*
394 *Food Chemistry, 42:* 1170–1176.
395 Bosset, J. O. & Gauch, R. (1993). Comparison of the volatile flavour compounds of six
396 European 'AOC' cheeses by using a new dynamic headspace GCMS method.
397 *International Dairy Journal, 3:* 359– 377.
398 Budinich, M., Diaz-Muñiz, I., Cai, H., Rankin, S. A., Broadbent, J. R. & Steele, J. L. (2011).
399 Growth of *Lactobacillus paracasei* ATCC 334 in a cheese model system: a biochemical
400 approach. *Journal of Dairy Science, 94:* 5263–5277.
401 Cogan, T. M., Beresford, T. P., Steele, J., Broadbent, J., Shah, N. P. & Ustunol, Z. (2007).
402 Invited review: Advances in starter cultures and cultured foods. *Journal of Dairy*
403 *Science, 90:* 4005-4021.
404 Curioni, P. M. G. & Bosset, J. O. (2002). Key odorants in various cheese types as determined
405 by as chromatography – olfactometry. *International Dairy Journal, 12:* 959-984.
406 De Man, J. C., Rogosa, M. & Sharpe, M. E. (1960). A medium for the cultivation of lactobacilli.
407 *Journal of Applied Bacteriology, 23:* 130–135.
408 Dozet, N., Adžić, N., Stanišić, M. & Živić, N. (1996). Autohtoni mlječni proizvodi (Indigenous
409 dairy products). *Poljoprivredni institut Podgorica*, SILMIR-Beograd.
410 Dozet, N., Maćej, O. & Jovanović, S. (2004). Autohtoni mliječni proizvodi osnova za razvoj
411 specifičnih, originalnih mliječnih prerađevina u savremenim uslovima (Autochthonous

412 dairy products as the basis for the development of specific original dairy products in the
413 contemporary conditions). *Biotechnology in Animal Husbandry, 20, 3-4:* 31-48.
414 Griffith, R. & Hammond, E. G. (1989). Generation of Swiss cheese flavor components by the
415 reaction of amino acids with carbonyl compounds. *Journal of Dairy Science, 72:* 604–
416 613.
417 Henriksen, C. M. & Nilsson, D. (2001). Redirection of pyruvate catabolism in *Lactococcus*
418 *lactis* by selection of mutants with additional growth requirements. *Applied*
419 *Microbiology and Biotechnology, 56:* 767– 775.
420 IDF. (1982). Cheese and processed cheese: Determination of the total solids content. *IDF*
421 *Standard* 4a. Brussels, Belgium: *International Dairy Federation.*
422 IDF. (1986). Milk: Determination of Nitrogen Content (Kjeldahl Method) and Calculation of
423 Crude Protein Content, *IDF standard* 20a**.** Brussels, Belgium: *International Dairy*
424 *Federation.*
425 IDF. (1988). Chesse and chesse products, determination of chloride content, potentiometric
426 titration method. *IDF standard* 88a. Brussels, Belgium: *International Dairy Federation.*
427 IDF. (1995). Milk and milk products: Guidance on sampling. *IDF standard* 50c**.** Brussels,
428 Belgium: *International Dairy Federation.*
429 IDF. (2008). Milk- Determination of fat content- Gerber butyrometers. *IDF Standard* 105*: 2008*.
430 Brussels, Belgium: *International Dairy Federation.*
431 Lemieux, L. & Simard, R. E. (1992). Bitter flavour in dairy products. II. A review of bitter
432 peptides from caseins: their formation, isolation and identification, structure masking
433 and inhibition. *Le Lait, 72:* 335–382.
434 Marilley, L. & Casey, M. G. (2004). Flavours of cheese products: Metabolic pathways,

435 analytical tools and identification of producing strains. *International Journal of Food*
436 *Microbiology, 90:* 139–159.
437 Markeš, M. (1973). O porijeklu, proizvodnji i klasifikaciji sireva koji se proizvode u Jugoslaviji
438 (About the origins, production and classification of the cheeses produced in Yugoslavia).
439 *Mljekarstvo, 23, 10:* 228-233.
440 Martinović, A., Radulović, Z., Wind, A., Janzen, T. & Obradović, D. (2005). Isolation ad
441 characterization of bacterial flora from farmhouse fermented milk products of Serbia and
442 Montenegro. *Acta Veterinaria, 55:* 307-318.
443 Martinovic, A., Abrahamsen, R. K. & Obradovic, D. (2006). Biochemical activities of selected
444 strains of Lactic Acid Bacteria. *Biotechnology in Animal Husbandry, 22, 1-2:* 139-151.
445 Mauriello, G., Moio, L., Moschetti, G., Piombino, P., Addeo, F. & Coppola, S. (2001).
446 Characterization of lactic acid bacteria strains on the basis of neutral volatile compounds
447 produced in whey. *Journal of Applied Microbiology, 90:* 928– 942.
448 Mirecki, S. (2012). Protection of traditional dairy products in Montenegro. *Agriculture and*
449 *Forestry, 57, 2:* 27-42.
450 Mirecki, S., Popović, N., Antunac, N., Mikulec, N. & Plavljanić, D. (2015)**.** Production
451 technology and some quality parameters of Njeguši cheese. *Mljekarstvo, 65, 4:* 280-286.
452 Morgan, M. E. (1976). The chemistry of some microbially induced flavor defects in milk and
453 dairy foods. *Biotechnology in Bioengineering, 18:* 953– 965.
454 Mucchetti, G., Locci, F., Gatti, M., Neviani, E., Addeo, F., Dossena, A. & Marchelli, R. (2000).
455 Pyroglutamic acid in cheese: presence, origin and correlation with ripening time of
456 Grana Padano cheese. *Journal of Dairy Science, 83:* 659-665.

457 Mucchetti, G., Locci, F., Massara, P., Vitale, R. & Neviani, E. (2002). Production of
458 pyroglutamic acid by thermophilic lactic acid bacteria in hard-cooked mini-cheeses.
459 *Journal of Dairy Science, 85:* 2489–2496.
460 Narvhus, J. A., Østeraas, K., Mutukumira, T. & Abrahamsen, R. K. (1998). Production of
461 fermented milk using a malty compound-producing strain of *Lactococcus lactis* subsp.
462 *lactis* biovar. *diacetylactis* isolated from Zimbabwean naturally fermented milk.
463 *International Journal of Food Microbiology, 41:* 73-80.
464 Sforza, S., Cavorta, V., Galaverna, G., Dossena, A. & Marchelli, R. (2009). Accumulation of
465 non-proteolytic aminoacyl derivatives in Parmigiano-Reggiano cheese during ripening.
466 *International Dairy Journal*, *19:* 582–587.
467 Sheldon, R. M., Lindsay, R. C., Libby, L. M. & Morgan, M. E. (1971). Chemical nature of
468 malty flavour and aroma produced by *Streptococcus lactis* var. *maltigenes*. *Applied*
469 *Microbiology, 22:* 263-266.
470 Skeie, S., Kieronczyk, A., Næs, R. M. & Østlie, H. (2008). Lactobacillus adjuncts in cheese:
471 Their influence on the degradation of citrate and serine during ripening of a washed curd
472 cheese. *International Dairy Journal, 18:* 158-168.
473 Smit, G., Smit, B. A. & Engels, W. J. M. (2005). Flavour formation by lactic acid bacteria and
474 biochemical flavour profiling of cheese products. *FEMS Microbiology Reviews, 29:*
475 591–610.
476 Stieglitz, H. W. A. (2008). Ein Besuch auf Montenegro. Reprint from 1841 *Bibliolife, 1:* 212.
477 Thierry, A. & Maillard, M. B. (2002). Production of cheese flavour compounds derived from
478 amino acid catabolism by *Propionibacterium freudenreichii. Le Lait, 82:* 17–32.

Weerkamp, A. H., Klijn, N., Neeter, R. & Smit, G. (1996). Properties of mesophilic lactic acid bacteria from raw milk and naturally fermented raw milk products. *Netherlands Milk and Dairy Journal, 50:* 319–332.

Williams, A. G., Withers, S. E., Brechany, E. Y. & Banks, J. M. (2006). Glutamate dehydrogenase activity in lactobacilli and the use of glutamate dehydrogenase-producing adjunct Lactobacillus spp. cultures in the manufacture of cheddar cheese. *Journal of Applied Microbiology, 101, 5:* 1062-1075.

Yvon, M. & Rijnen, L. (2001). Cheese flavour formation by amino acid catabolism. *International Dairy Journal, 11:* 185–201.

Table 1. Starter combinations used for the cheese making experiment.

Cheese	Starter combinations	Inoculation (%)
C	DL- starter (CH-N 22, Christian Hansen)	0,5
M	*Lactococcus lactis* ssp. *lactis* (ABO19-3)	0,5
A	*Lactococcus lactis* ssp. *lactis* (ABO57-1)	0,5
AM	*Lactococcus lactis* ssp. *lactis* (ABO57-1 and ABO19-3)	0,25 each
AL	*Lactococcus lactis* ssp. *lactis* (ABO57-1) and *Lactobacillus plantarum* (ABO19-2) (L)	0,41 of A and 0,01 of L
AML	*Lactococcus lactis* ssp. *lactis* (ABO57-1 and ABO19-3) and *Lactobacillus plantarum* (ABO19-2)	0,24 of A, 0,24 of M and 0,02 of L

Table 2. Development of pH, dry matter (DM) and fat in dry matter (FDM) (presented as mean values and SD) during ripening of experimental cheeses. All responses was significantly different by age ($p<0.001$), whereas no significant differences were found by culture ($p>0.05$).

Culture*	pH				DM (%)				FDM			
	1d	7d	14d	21d	1d	7d	14d	21d	1d	7d	14d	21d
C	4.9±0.01	4.7±0.01	4.8±0.18	4.7±0.00	51.0± 0.38	61.2±1.27	68.0±0.40	70.4±1.21	53.0±0.19	51.1±2.17	50.9±0.58	54.4±1.99
M	5.0±0.05	4.7±0.16	4.8±0.20	4.7±0.01	51.0±0.92	60.5±1.02	66.2±1.14	70.2±0.41	52.3±1.12	53.4±1.86	49.9±1.01	52.7±0.26
M	4.9±0.02	4.7±0.06	4.7±0.11	4.5±0.20	50.0±0.62	58.9±2.91	65.0±0.30	69.6±0.72	54.3±0.69	51.8±1.30	50.6±0.02	54.5±2.81
A	5.0±0.06	4.8±0.11	4.8±0.06	4.8±0.13	52.2±0.78	60.7±0.23	66.4±0.17	68.7±2.76	53.8±1.17	53.2±3.30	50.9±0.01	53.7±0.63
AL	5.0±0.06	4.9±0.23	4.7±0.09	4.7±0.14	47.9±1.02	61.4±0.46	67.1±0.51	69.8±0.66	56.3±1.06	52.9±1.41	51.2±0.85	53.0±0.04
AML	4.9±0.04	4.7±0.08	4.7±0.09	4.6±0.04	50.7± 0.71	59.9±0.37	65.8±2.40	68.2±3.69	50.3±5.47	55.1±0.91	50.6±0.14	53.0±2.89

*C- DL- starter (CH-N 22, Christian Hansen)
M- Lactococcus lactis ssp. *lactis* (ABO19-3)
A-Lactococcus lactis ssp. *lactis* (ABO57-1)
AM-Lactococcus lactis ssp. *lactis* (ABO57-1 and ABO19-3)
AL-Lactococcus lactis ssp. *lactis* (ABO57-1) and *Lactobacillus plantarum* (ABO19-2) (L)
AML-Lactococcus lactis ssp. *lactis* (ABO57-1 and ABO19-3) and *Lactobacillus plantarum* (ABO19-2)

Table 3. Development of salt and soluble nitrogen/total nitrogen content (SN/TN) (presented as mean values and SD) during ripening of experimental cheeses.

Culture*	Salt (%)				SN/TN			
	1d	7d	14d	21d	1d	7d	14d	21d
C	0.1±0.05	3.0±0.59	3.5±0.91	4.0±0.48	2.8±0.75	3.6±2.43	7.6±0.05	7.7±0.56
M	0.1±0.02	1.9±1.36	2.6±2.15	2.4±1.46	4.3±1.70	9.5±0.40	6.7±0.88	8.3±1.16
AM	0.1±0.02	2.1±0.64	2.3±1.05	3.2±1.50	4.0±2.33	5.0±4.01	7.8±0.49	9.6±1.48
A	0.1±0.01	1.8±0.57	3.0±1.76	2.7±1.07	3.9±2.44	7.4±0.20	7.0±1.45	7.2±1.08
AL	0.0±0.03	2.3±2.36	3.1±1.57	3.3±1.27	4.8±3.09	6.7±0.08	8.1±1.19	7.8±1.68
AML	0.0±0.01	2.7±1.86	3.6±0.70	3.9±0.59	2.9±0.07	6.0±2.40	6.8±0.58	6.3±0.16

*C- DL- starter (CH-N 22, Christian Hansen)
M- Lactococcus lactis ssp. *lactis* (ABO19-3)
A-Lactococcus lactis ssp. *lactis* (ABO57-1)
AM-Lactococcus lactis ssp. *lactis* (ABO57-1 and ABO19-3)
AL-Lactococcus lactis ssp. *lactis* (ABO57-1) and *Lactobacillus plantarum* (ABO19-2) (L)
AML-Lactococcus lactis ssp. *lactis* (ABO57-1 and ABO19-3) and *Lactobacillus plantarum* (ABO19-2)

Table 4**.** Sensory evaluation of the studied cheeses

Quality category	Scores	Cheese*
Excellent	18.2 - 19.6	A, M
Good	15.5 -17.1	AM, AML
Mediocre	13.5 -14.5	AL
Acceptable	11.2 - 13.1	C
Not acceptable	< 11.2	-

*C- DL- starter (CH-N 22, Christian Hansen)
M- Lactococcus lactis ssp. *lactis* (ABO19-3)
A-Lactococcus lactis ssp. *lactis* (ABO57-1)
AM-Lactococcus lactis ssp. *lactis* (ABO57-1 and ABO19-3)
AL-Lactococcus lactis ssp. *lactis* (ABO57-1) and *Lactobacillus plantarum* (ABO19-2) (L)
AML-Lactococcus lactis ssp. *lactis* (ABO57-1 and ABO19-3) and *Lactobacillus plantarum* (ABO19-2)

534
535 **LEGENDS TO FIGURES**

536

537 **Fig. 1.** Numbers of *Lactococci* (a) and *Lactobacilli* (b) during 21 day ripening of
538 experimental cheeses with different strain combinations: cheese C (DL culture CH-N 22) (○),
539 cheese M (strain M- *Lactococcus lactis* ssp. *lactis)* (□), cheese AM (strains A and M-
540 *Lactococcus lactis* ssp. *lactis)* (■), cheese A (strain A- *Lactococcus lactis* ssp. *lactis)* (Δ),
541 cheese AL (strains A- *Lactococcus lactis* ssp. *lactis* and L- *Lactobacillus plantarum)* (▲),
542 cheese AML (strains A and M- *Lactococcus lactis* ssp. *lactis* and strain L- *Lactobacillus*
543 *plantarum*) (♦).

544 **Fig. 2.** Development of lactose (a) and galactose (b) during ripening of experimental
545 cheeses with different strain combinations: cheese C (DL culture CH-N 22) (○), cheese M
546 (strain M- *Lactococcus lactis* ssp. *lactis)* (□), cheese AM (strains A and M- *Lactococcus lactis*
547 ssp. *lactis)* (■), cheese A (strain A- *Lactococcus lactis* ssp. *lactis)* (Δ), cheese AL (strains A-
548 *Lactococcus lactis* ssp. *lactis* and L- *Lactobacillus plantarum)* (▲), cheese AML (strains A and
549 M- *Lactococcus lactis* ssp. *lactis* and strain L- *Lactobacillus plantarum*) (♦).

550 **Fig. 3.** Principal component analysis (PCA) with loadings (a) and scores (b) of the
551 sugars and organic acids in the studied cheeses. Thirty-one percent and sixteen percent of the
552 variation was explained by the first two components. Each sample is marked accordingly:
553 culture (C, M, A, AM, AL and AML), sampling day (1, 7, 14 and 21) and replicate (a, b).
554 Cultures: cheese C (DL culture CH-N 22), cheese M (strain M- *Lactococcus lactis* ssp. *lactis)*,

555 cheese AM (strains A and M- *Lactococcus lactis* ssp. *lactis)*, cheese A (strain A- *Lactococcus*
556 *lactis* ssp. *lactis)*, cheese AL (strains A- *Lactococcus lactis* ssp. *lactis* and L- *Lactobacillus*
557 *plantarum*), cheese AML (strains A and M- *Lactococcus lactis* ssp. *lactis* and strain L-
558 *Lactobacillus plantarum*). The solid circle marks the control cheeses, the dotted circle the
559 cheeses with culture M and the circle with dashed lines the cheese with culture A.
560
561 **Fig. 4.** Development of lactic acid (a) and formic acid (b) in experimental cheeses during
562 ripening: Cheese C (DL culture CH-N 22) (○), cheese M (strain M- *Lactococcus lactis* ssp.
563 *lactis)* (□), cheese AM (strains A and M- *Lactococcus lactis* ssp. *lactis*) (■), cheese A (strain
564 A- *Lactococcus lactis* ssp. *lactis)* (Δ), cheese AL (strains A- *Lactococcus lactis* ssp. *lactis* and
565 L- *Lactobacillus plantarum)* (▲), cheese AML (strains A and M- *Lactococcus lactis* ssp. *lactis*
566 and strain L- *Lactobacillus plantarum*) (♦).
567 **Fig. 5.** Development of DL-pyroglutamic acid (a) and succinic acid (b) in experimental
568 cheeses during ripening: Cheese C (DL culture CH-N 22) (○), cheese M (strain M- *Lactococcus*
569 *lactis* ssp. *lactis*) (□), cheese AM (strains A and M- *Lactococcus lactis* ssp. *lactis*) (■), cheese
570 A (strain A- *Lactococcus lactis* ssp. *lactis*) (Δ), cheese AL (strains A- *Lactococcus lactis* ssp.
571 *lactis* and L- *Lactobacillus plantarum*) (▲), cheese AML (strains A and M- *Lactococcus lactis*
572 ssp. *lactis* and strain L- *Lactobacillus plantarum*) (♦).
573 .
574

Fig. 6. Principal component analysis (PCA) with loadings (a) and scores (b) of the volatiles components in experimental cheeses. Each sample is marked accordingly: culture (C, M, A, AM, AL and AML), sampling day (1, 7, 14 and 21) and replicate (a, b). Samples marking: cheese C (DL culture CH-N 22), cheese M (strain M- *Lactococcus lactis* ssp. *lactis*), cheese AM (strains A and M- *Lactococcus lactis* ssp. *lactis)*, cheese A (strain A- *Lactococcus lactis* ssp. *lactis)*, cheese AL (strains A- *Lactococcus lactis* ssp. *lactis* and L- *Lactobacillus plantarum*), cheese AML (strains A and M- *Lactococcus lactis* ssp. *lactis* and strain L- *Lactobacillus plantarum*). The solid circle marks the control cheeses; the dotted circle the 1 day old samples and the circle with dashed lines the ripened cheeses (age 14 and 21 days of ripening).

Figure 1.

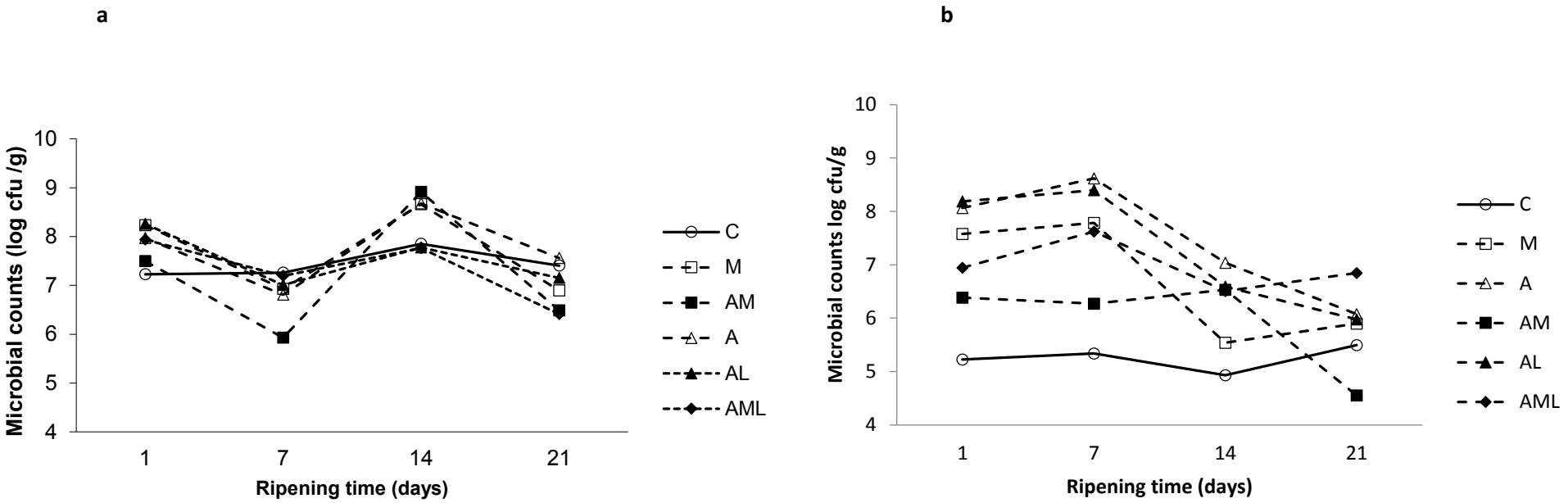

a
Microbial counts (log cfu /g)
10
9
8
7
6
5
4
1
7
14
21
Ripening time (days)
C
M
AM
A
AL
AML
b
Microbial counts log cfu/g
10
9
8
7
6
5
4
1
7
14
21
Ripening time (days)
C
M
A
AM
AL
AML

Figure 2.

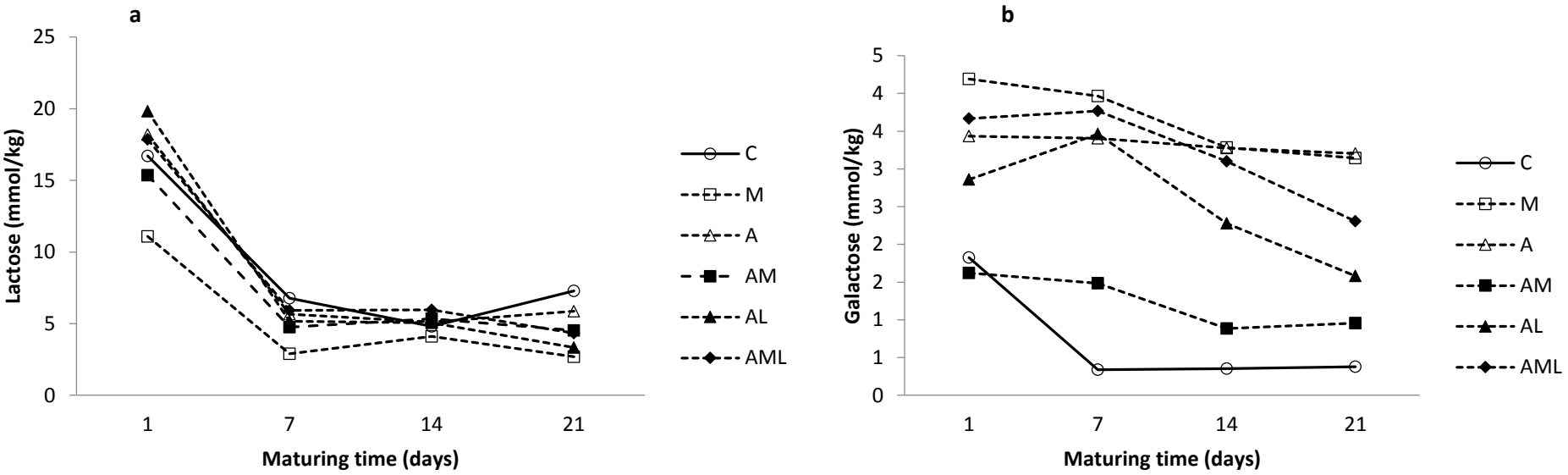
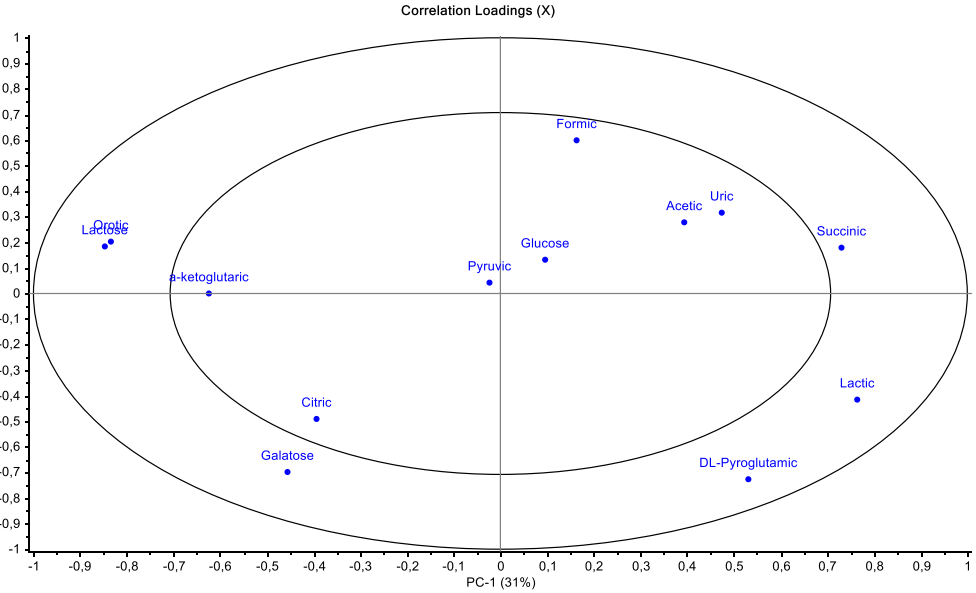

a
Lactose (mmol/kg)
25
20
15
10
5
0
1
7
14
21
Maturing time (days)
C
M
A
AM
AL
AML
b
Galactose (mmol/kg)
5
4
4
3
3
2
2
1
1
0
1
7
14
21
Maturing time (days)
C
M
A
AM
AL
AML

Figure 3


Correlation Loadings (X)
Formic
Acetic
Uric
Succinic
Glucose
Pyruvic
a-ketoglutaric
Citric
Galatose
DL-Pyroglutamic
Lactic
PC-1 (31%)

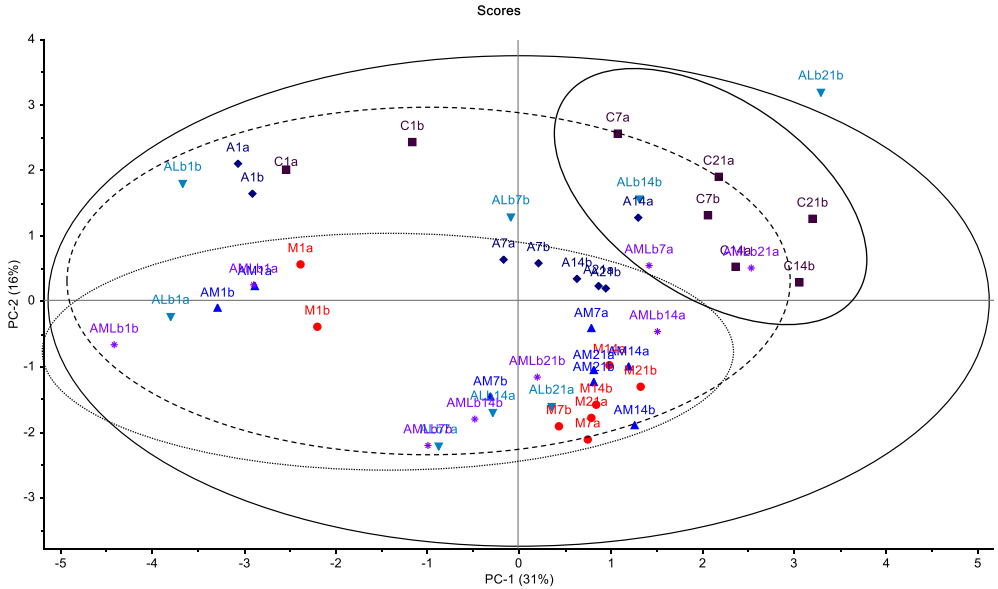

Scores
PC-1 (31%)
PC-2 (16%)

Figure 4.

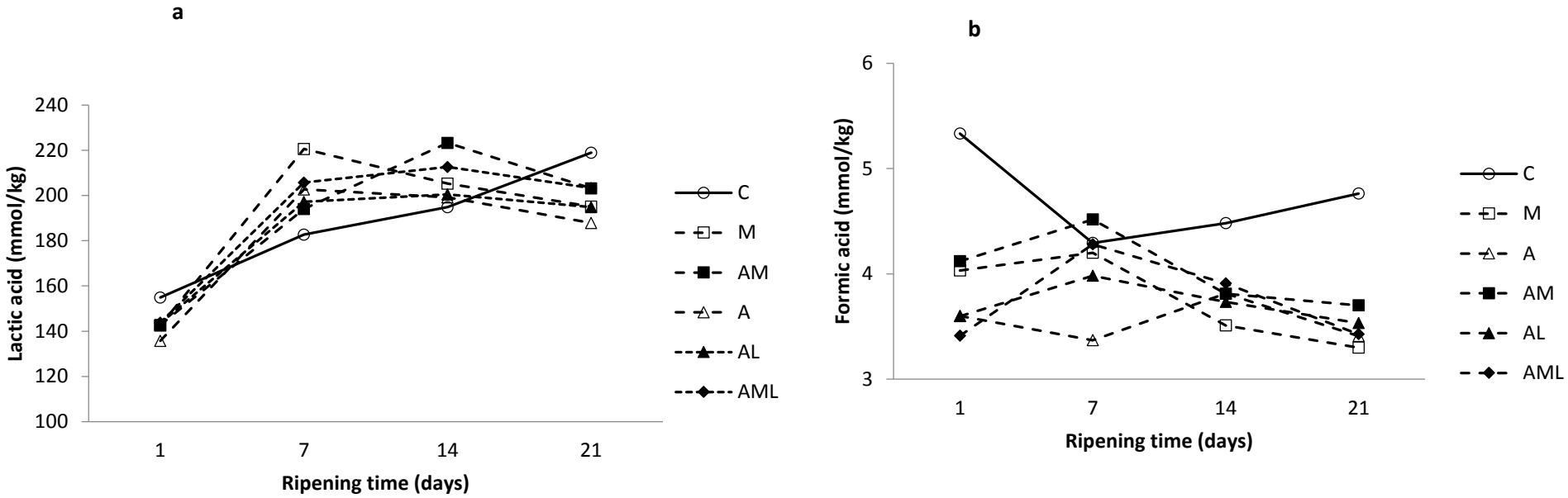

a
Lactic acid (mmol/kg)
240
220
200
180
160
140
120
100
1
7
14
21
Ripening time (days)
C
M
AM
A
AL
AML
b
Formic acid (mmol/kg)
6
5
4
3
1
7
14
21
Ripening time (days)
C
M
A
AM
AL
AML

Figure 5.

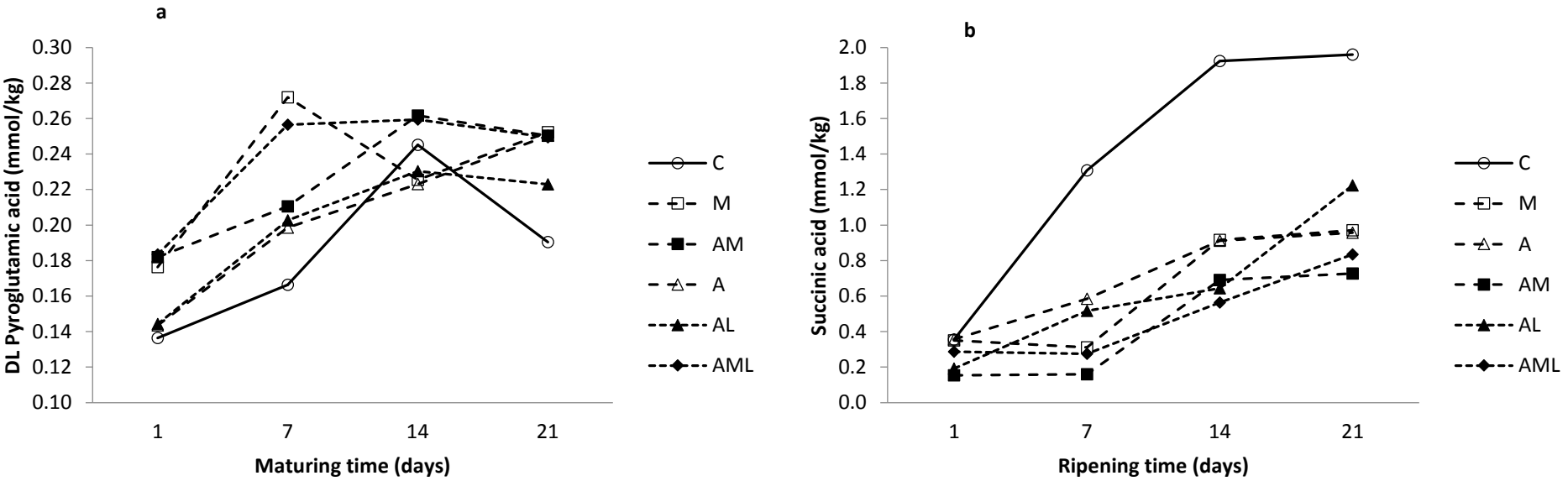
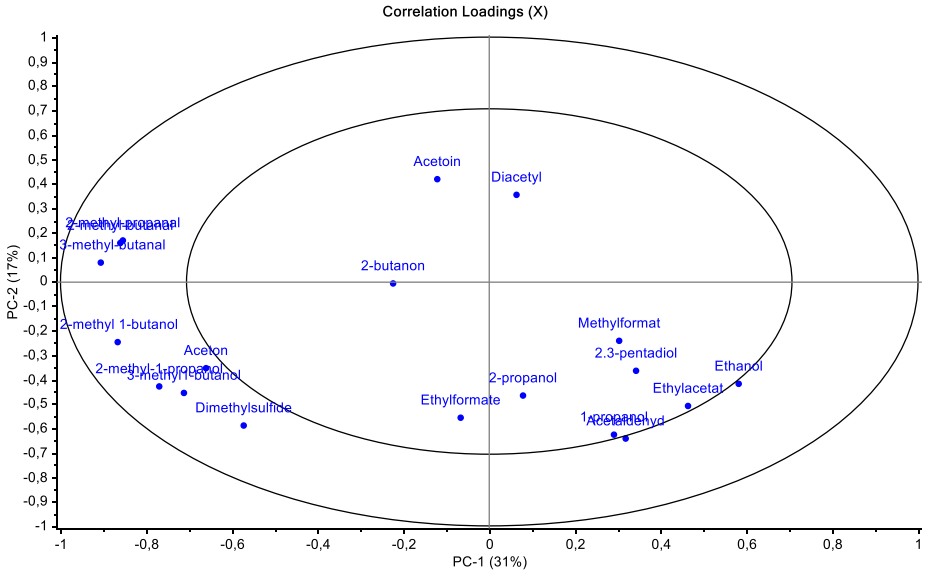
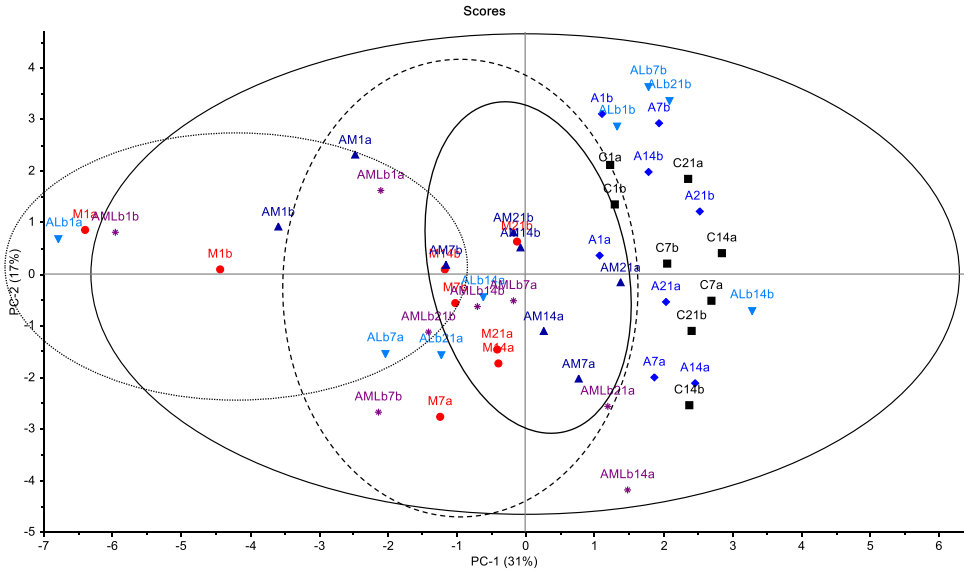

a
DL Pyroglutamic acid (mmol/kg)
0.30
0.28
0.26
0.24
0.22
0.20
0.18
0.16
0.14
0.12
0.10
1
7
14
21
Maturing time (days)
C
M
AM
A
AL
AML
b
Succinic acid (mmol/kg)
2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
1
7
14
21
Ripening time (days)
C
M
A
AM
AL
AML

Figure 6.


Correlation Loadings (X)
PC-2 (17%)
PC-1 (31%)
Acetoin
Diacetyl
2-butanon
2-methyl-propanal
3-methyl-butanal
2-methyl 1-butanol
Aceton
2-methyl-1-propanol
3-methyl-1-butanol
Dimethylsulfide
Ethylformate
2-propanol
Methylformat
2.3-pentadiol
Ethylacetat
Ethanol
1-propanol
Acetaldehyd


Scores
PC-2 (17%)
PC-1 (31%)
ALb7b
ALb21b
A1b
ALb1b
A7b
AM1a
AMLb1a
C1a
A14b
C21a
C1b
A21b
ALb1a
M1a
AMLb1b
AM1b
AM21b
AM14b
C14a
M1b
AM7b
A1a
C7b
AM21a
A21a
C7a
ALb14b
ALb14a
AMLb7a
AMLb21b
AM14a
C21b
ALb7a
ALb21a
M21a
M14a
AM7a
A7a
A14a
AMLb21a
C14b
AMLb7b
M7a
AMLb14a

Paper II

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Growth of adjunct *Lactobacillus casei* in Cheddar cheese differing in milk fat globule membrane components

Aleksandra Martinovic [a], Kim Marius Moe [a], Ehab Romeih [c], Bashir Aideh [b], Finn K. Vogensen [b], Hilde Østlie [a], Siv Skeie [a,*]

[a] Norwegian University of Life Sciences, Department of Chemistry, Biotechnology and Food Science, P.O. Box 5003, N-1432 Aas, Norway
[b] University of Copenhagen, Faculty of Sciences, Department of Food Science, Rolighedsvej 30, DK-1958 Frederiksberg C, Denmark
[c] Cairo University, Faculty of Agriculture, Department of Dairy Science, Giza 12613, Egypt

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ABSTRACT

The effect of two adjunct *Lactobacillus casei* strains on the lactobacilli population of low-fat Cheddar cheese is described. The adjuncts, added at a low initial number, differed in their ability to utilise components of the milk fat globule membrane (MFGM); these were controlled by addition of butter milk powder or skim milk powder. The most diverse microbial composition was revealed at the start of cheese ripening and became more uniform in the later stages. The microorganisms present at the start influenced the lactobacilli population during ripening, but the adjuncts did not dominate the microflora in the cheese. A higher content of MFGM components in the cheese seemed to influence the lactobacilli population and the composition of free amino acids during ripening. The low initial numbers of lactobacilli resulted in comparatively large distances of separation between these cells in fresh cheese; electron micrographs of ripened cheese showed large clusters of clearly elongated lactobacilli.

1. Introduction

Non-starter lactic acid bacteria (NSLAB) in cheese originate from raw milk or from the production plant environment (Beresford, Fitzsimons, Brennan, & Cogan, 2001; Cogan et al., 2007). The population of NSLAB isolated from Cheddar cheese made from pasteurised milk is dominated by *Lactobacillus casei*/*paracasei* (Beresford et al., 2001; Fox, McSweeney, & Lynch, 1998). Certain NSLAB, especially mesophilic facultative heterofermentative lactobacilli, are used as adjuncts to contribute to the development of desirable cheese flavour by replacing the indigenous NSLAB microflora, which results in more controlled cheese production (Cogan et al., 2007; El Soda, Madkor, & Tong, 2008; Fox et al., 1998; Hynes, Bergamini, Suarez, & Zalazar, 2003). The growth substrates for the NSLAB microflora in cheese are not fully known, but it has been hypothesised that mesophilic lactobacilli are able to utilise, in addition to residual carbohydrates, citrate and amino acids, the components of degraded cell walls and RNA from lysed cells, as well as the monosaccharide moieties of the glycoconjugates in the milk fat globule membrane (MFGM; Adamberg et al., 2005; Fox et al., 1998; Laloy, Vuillemard, El Soda, & Simard, 1996; Moe, Faye, Abrahamsen, Østlie, & Skeie, 2012; Østlie, Vegarud, & Langsrud, 1995; Williams & Banks, 1997).

Numerous studies have been carried out to improve the overall quality of low-fat cheese (Collins, McSweeney, & Wilkinson, 2003; Kilcawley et al., 2007; Liu, Xu, & Guo, 2008) that, generally, is inferior compared with the full-fat alternative. It has been shown that homofermentative and facultative heterofermentative lactobacilli may contribute to the development of desirable flavour in reduced-fat Cheddar cheese due to their metabolic activities (Beresford et al., 2001; El Soda, Madkor, & Tong, 2000; Fox et al., 1996, 1998; Lynch, Muir, Banks, McSweeney, & Fox, 1999). In low-fat cheese, it has been assumed that the NSLAB microflora develops differently from the microflora of full-fat cheeses (Laloy et al., 1996). Low-fat cheese has a lower content of MFGM, which is a possible energy source for NSLAB. Addition of buttermilk, which is rich in MFGM components (Morin, Pouliot, & Britten, 2008), to low-fat cheese may increase the content of possible energy sources for the cheese microflora. We have recently shown that some lactobacilli adjuncts isolated from cheese are able to grow and survive for an extended period in a medium with MFGM isolate as the only added carbohydrate source (Moe et al., 2012). The MFGM is mainly composed of phospholipids, sphingolipids, glycoproteins and other minor compounds (Morin et al., 2008).

Most of the experimental cheeses made with adjuncts use inoculations of greater than log 4 cfu mL^{-1} (Broadbent, Houck,

* Corresponding author. Tel.: +47 64 96 58 44.
E-mail address: siv.skeie@umb.no (S. Skeie).

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Johnson, & Oberg, 2003; Lynch, McSweeney, Fox, Cogan, & Drinan, 1996; Puchades, Lemieux, & Simard, 1989; Skeie et al., 2008a; Skeie, Kieronczyk, Næs, & Østlie, 2008b) to suppress or reduce the growth of the indigenous NSLAB flora and affect the cheese microflora. However, if the adjunct is added at a level mimicking the number of NSLAB usually found in cheese milk (1–2 log cfu mL^{-1}), a dynamic growth situation will presumably develop with the indigenous NSLAB flora. To our knowledge, experiments utilising low inoculation levels of adjunct *Lb. casei*/*paracasei* strains have not been published.

To assess the diversity of the microbial population in cheese, molecular based 16S rRNA sequencing has been used for classification at the species level (Berthier & Ehrlich, 1998; Coeuret, Dubernet, Bernardieau, Gueguen, & Vernoux, 2003). In addition, polymerase chain reaction (PCR) amplification of repetitive bacterial DNA elements (rep-PCR) has been proven to be a reliable technique for typing of different bacteria at the strain level, and has been applied for studying LAB communities of different food products, including cheese (Ben Amor, Vaughan & De Vos, 2007; Berthier, Beuvier, Dasen, & Grappin, 2001; De Urraza, Gomez-Zavaglia, Lozano, Romanowski, & Antoni, 2000; Singh, Pawas, Singh, & Heller, 2009).

The objective of this study was to investigate the development and dynamics of the lactobacilli population during ripening of low-fat Cheddar cheese differing in the content of MFGM components, and with a low initial number of adjunct *Lb. casei* strains differing in their ability to utilise MFGM components added to the cheese milk.

2. Materials and methods

2.1. Experimental design

Low-fat Cheddar cheese was made with two experimental factors in three replicate blocks (cheesemaking days: A, B and C). Factor 1: MFGM composition, was achieved by adding either butter milk powder (BMP) or skim milk powder (SMP) to the cheese milk. In cheese milk with SMP addition, cream was added to standardise the fat and protein content to be comparable with that of the BMP cheese milk. Factor 2: adjunct, was achieved using two adjunct cultures, *Lb. casei* INF 448 and *Lb. casei* INF 456, (INF, culture collection of Department of Chemistry, Biotechnology and Food Science, University of Life Sciences, Aas, Norway) that differed in their ability to utilise MFGM components (Moe et al., 2012); both were added at a level of log 2 cfu mL^{-1}.

Lb. casei INF 448 and *Lb. casei* INF 456 were previously described as *Lb. paracasei* INF 448 and *Lb. paracasei* INF 456 (Moe et al., 2012; Østlie, Eliassen, Florvaag, & Skeie, 2004; Skeie et al., 2008b) according to species specific PCR-identification. The reclassification is based on the observation that the type strain *Lb. casei* ATCC (American Type Culture Collection) 393^T is most probably a contamination and not the original Orla-Jensen strain #7 as claimed by the ATCC (Larsen, Aideh, Kilstrup, Michelsen, & Vogensen, 2008).

The different vats were denoted: SMP (cheese milk with SMP, cream and without adjunct), BMP (cheese milk with BMP and without adjunct), SMP + 448 (cheese milk with SMP, cream and adjunct *Lb. casei* INF 448), BMP + 448 (cheese milk with BMP and adjunct *Lb. casei* INF 448), SMP + 456 (cheese milk with SMP, cream and adjunct *Lb. casei* INF 456) and BMP + 456 (cheese milk with BMP and adjunct *Lb. casei* INF 456).

Analysis of variance (ANOVA) was carried out with SAS software (SAS Institute Inc., Cary, NC, USA) using a general linear model (GLM) procedure with replicate block, MFGM components and adjuncts as classification variables at each ripening step. Differences between the means of the experimental factors were found by Tukey's honest significant difference (HSD) test for pair-wise comparison of the means.

2.2. Production of skim milk powder and butter milk powder

The SMP and BMP were produced from 900 L of whole milk from the university farm at the Norwegian University of Life Sciences, as described by Romeih, Moe, and Skeie (2012). The BMP had 96.6% dry matter (DM) and 9.8% fat; the SMP had 97.1% DM and 2.26% fat. According to Morin et al. (2008), the phospholipid content is 8.5 times higher in sweet pasteurised buttermilk than in skimmed milk.

2.3. Cheese milk

Raw milk was obtained from a local farm in Cork, Ireland, and was separated (45 °C) and standardised to 0.5% fat using a table top milk separator (Clair, Milky; Althofen, Austria), before pasteurisation (72 °C, 15 s). The six cheesemaking vats, containing 20 L of milk each, were mixed and supplemented with different amounts of BMP, SMP and cream according to the procedure described by Romeih et al. (2012). The vats of cheese milk were stored at 4 °C for 17 h to ensure that the proteins of the powders were fully hydrated before further cheesemaking.

2.4. Cheesemaking procedures

The cheese milk was heated to 30 °C, and 1% single strain starter culture (*Lactococcus lactis* subsp. *lactis* ML-8), cultured for 24 h in 200 mL reconstituted skim milk (10%, w/v); was added to each of the vats. The adjunct cultures, grown in De Man, Rogosa and Sharpe (MRS) broth at 30 °C for 24 h and further diluted in Ringer's solution, were added 15 min after the addition of the starter culture. To the treatment vats with adjunct, 10 mL of the adjunct culture was added to achieve log 2 cfu mL^{-1} of adjuncts in the cheese milk. Cheddar cheese was made as described by Romeih et al. (2012).

2.5. Cheese analyses

Cheese sampling was carried out as described in IDF (1995) standard 50c after 24 h (time 0) and after 2, 4, 6, 10 and 24 weeks of ripening. Microbial counts (Skeie, Lindberg, & Narvhus, 2001), pH (Skeie et al., 2001) and dry matter (IDF, 1982) were measured immediately after sampling. Presumptive lactococci were enumerated on M17 agar (Oxoid, Basingstoke, Hampshire, England) after aerobic incubation at 30 °C for 2 days and presumptive lactobacilli on Rogosa agar (Oxoid) after anaerobic incubation at 30 °C for 4 days.

Volatile compounds were determined by headspace gas chromatography (HSGC) according to the method of Narvhus, Østeraas, Mutukumira, and Abrahamsen (1998) with modifications as described by Skeie et al. (2008b). As extraction rates for individual components from the cheese matrix have not been determined, quantification is presented as the peak area g^{-1} cheese, which is comparable among the present cheese samples.

Amino acids were analysed using HPLC with *o*-phthaldialdehyde (OPA) and fluorenylmethyl chloroformate (FMOC) derivatisation according to a modified method previously described by Bütikofer and Ardö (1999). To 1.5 g of cheese 15.0 mL of 0.1 M HCl was added. The 0.1 M HCL contained 0.4 μmol mL^{-1} of L-norvalin (Sigma, St. Louis, MO, USA) and 0.4 μmol mL^{-1} of piperidine-4-carboxylic acid (PICA; Fluka, St. Louis, MO, USA) as internal standards. The sample was homogenised by an Ultra-Turrax (Pro Scientific Inc, Monroe, CT, USA) for 5 min at 20,000 rpm, sonicated for 30 min (Branson, Soest, The Netherlands), centrifuged (40 min, 4 °C, ~3000 × *g*; Beckman J2-MC, GMI Inc., MN, USA) and 1.0 mL of the supernatant was added to 1.0 mL of 4% trichloracetic acid

(Merck, Darmstadt, Germany) before mixing on a Vortex-Genie 2 (Aldrich, St. Louis, MO, USA) and placed on ice for 30 min before further analysis. After centrifugation (5 min, 5 °C, ~11,148 × g; Eppendorf 5415 D, Hamburg, Germany), the samples were filtered with a 0.2 μm MFS-13 mm CA filter (Advantec, Dublin, CA, USA) and analysed directly or stored in the freezer (−20 °C) until analysis. The separation of the amino acids was carried out using a Perkin Elmer series 410 pump (Perkin Elmer, Waltham, MA, USA), an Agilent Technologies 1200 series autosampler (Agilent Technologies, Waldbronn, Germany), a Perkin Elmer 200 column oven and an Agilent Technologies 1200 series thermostat. The system was driven by an EZChrom Elite (Agilent Technologies). An XTerra RP 18 column with 150 × 4.6 mm (Waters, MA, USA) was used and separations were carried out at 42 °C.

2.6. Cheese electron micrographs

From the centre of the cheese blocks ripened for 24 weeks, small cubic samples (approximately 3 × 3 × 3 mm) were prepared as described by Liu et al. (2008) with the following modifications: by the end of the CO_2 sample drying step, some of the dried cubic samples from each treatment were gently cut from the centre into two pieces using a fine scalpel. Samples were then mounted on aluminium scanning electron microscope (SEM) stubs, followed by gold coating in a Sputter Coater Polaron SC 7640 (Quorum Technologies Ltd, East Sussex, UK). A high vacuum Zeiss SEM EVO-50-EP (Carl Zeiss SMT Ltd., Cambridge, UK) was used to examine the samples at 5 kV and a magnification of 7500×.

2.7. Bacterial isolates

2.7.1. Morphological and physiological characterisation of the isolates

From Rogosa agar plates, approximately 16 colonies were randomly picked from each of the six cheeses at each time point of cheese ripening, from each replicate block. The isolates were transferred to 1.0 mL MRS broth (MRS, Merck) (De Man, Rogosa, & Sharpe, 1960) and grown at 30 °C for 24 h. All the isolates were subsequently purified three times on MRS agar and a total of 993 isolates were stored at −80 °C in 1.5 mL aliquots of MRS medium supplemented with 15% (v/v) glycerol. Working cultures were provided by transferring frozen cultures into MRS broth and incubation overnight at 30 °C.

Isolates were analysed by observing the morphology using phase contrast microscopy, Gram reaction and catalase reaction (3% H_2O_2). Carbon dioxide production was determined using an infrared gas analyser (ADC 225 Mk3, Analytical development, Hoddesdon, Hertfordshire, UK) by the method of Narvhus, Hulbækdal, Baugerød, and Abrahamsen (1991) with modifications as described by Østlie, Helland, and Narvhus (2003) after 20 h incubation at 30 °C in MRS broth.

In total, 100 isolates are further described in this paper, and these were chosen on the basis of their cultivation and morphological differences (colony morphology, Gram staining, catalase reaction, exopolysaccharide and CO_2 production). These isolates are further described in this paper by their isolation number and the cheese from which they were isolated; isolation number (powder + adjunct − weeks of ripening), e.g., 23 (SMP + 456 − 0). Presumptive lactobacilli were characterised on the basis of their carbohydrate fermentation patterns using the API 50 CHL system (BioMérieux, Marcy l'Etoile, France). Analysis of the obtained fermentation profiles was done using the APILAB Plus version 4.0 program (BioMérieux). Principal component analysis of API 50 CHL results was made by using The Unscrambler Client 9.5 (CAMO Process AS, Oslo, Norway).

2.7.2. 16S rRNA sequence analysis

The universal primers 5′-GAGTTTGATCCTGGCTCAG-3′ and 5′-AGAAAGGAGGTGATCCAGCC-3′ (*Escherichia coli* positions 9–27 and 1544–1525 respectively) were used for amplification of an approximately 1540 bp DNA fragment of the 16S rRNA gene. The primers were synthesised by Invitrogen Ltd. (Paisley, Scotland). PCR reactions were carried out in a 50 μL reaction mixture containing 2 μL of each 20 pmol primer, 5 μL 10 × PCR buffer, 1 μL 10 mM dNTP, 4 μL 25 mM $MgCl_2$, 2 μL DNA template and 0.5 μL 5 U μL^{-1} Taq Polymerase (Applied Biosystems, Carlsbad, CA, USA). The PCR reaction was carried out in a DNA-Thermal Cycler (Perkin Elmer Cetus, Waltham, MA, USA) using the following program: one cycle of denaturation at 97 °C for 3 min; 34 cycles consisting of denaturation at 94 °C for 15 s, primer annealing at 54 °C for 15 s, elongation at 72 °C for 1 min; a final extension step at 72 °C for 10 min.

The PCR products were purified using E.Z.N.A.™ Cycle-Pure Kit (Omega Biotek, Norcross, GA, USA) according to the procedure recommended by the supplier. Sequencing was done using a Big-Dye v3.1 terminator cycle sequencing kit, the primers 5′-CAGCMGCCGCGGTAATWC-3′, 5′-TAACACATGCAAGTCGAACG-3′ and 5′-ACGGGCGGTGTGTRC-3′ (*E. coli* positions 519–536, 50–70 and 1406–1392, respectively) and the sequencing device ABI Prism 377 DNA (Applied Biosystems). The PCR reactions were carried out using the following program: one cycle of denaturation at 96 °C for 10 s; 25 cycles consisting of denaturation at 96 °C for 10 s, primer annealing at 50 °C for 5 s, polymerisation and ddNTPs incorporation at 60 °C for 4 min. Sequences were edited using BioEdit software (Abbott, CA, USA) and analysed using BLAST (basic local alignment search tool; http://blast.ncbi.nlm.nih.gov/Blast.cgi).

2.7.3. Repetitive sequence based PCR analysis of facultative heterofermentative lactobacilli isolates

The rep-PCR method was adapted from the method described by Versalovic, Schneider, de Brujin and Lupski (1994) and was used for the confirmation of NSLAB strain identity. Bacterial isolates from MRS agar plates were incubated in MRS broth for 24 h at 30 °C. In addition, the adjuncts used in this study and the strain *Lb. casei* 7R1, previously described as *Lb. paracasei* 7R1 in Christiansen, Waagner Nielsen, Vogensen, Brogren, and Ardö (2006), were also analysed to compare the rep-PCR profiles. The rep-PCR protocol was as described by Christiansen et al. (2006), using the rep-PCR primers REP1R-Dt: (5′-III NCG NCG NCA TCN GGC-3′), and REP2R-Dt: (5′-NCG NCT TAT CNG GGC CTA C-3′).

Banding patterns of rep-PCR products were normalised using the *Lb. casei* 7R1 profile and clustered by Bionumerics 4.5 (Applied Maths, Saint-Martens-Latem, Belgium) and the dendrogram was constructed on the basis of Dice's Coefficient of similarity with the un-weighted pair group method with arithmetic averages clustering algorithm (UPGMA).

3. Results

3.1. Cheese composition

The development of the gross composition during ripening is shown in Table 1. The BMP significantly reduced the content of dry matter (and protein, results not shown) in the cheese compared with the cheese with added SMP by ~1%, and decreased the pH by ~0.05 ($P < 0.05$). Cheeses with adjuncts had a significantly lower pH after 24 weeks of ripening than cheeses without adjuncts. The different replicate blocks were significantly different ($P < 0.05$) with regards to both pH, dry matter (DM), some free amino acids (Tables 2 and 3, shown by large standard deviations (SD)) and some volatile compounds (results not shown) and this may explain the large standard deviation found within the experimental factors for

Table 1
Development of pH and gross composition during 24 weeks of ripening and significant effects of the experimental factors.[a]

Factor[b]	pH			DM (%)			FDM (%) at 6 w	Salt (%) at 6 w	SM (%) at 6 w
	24 h	6 w	24 w	24 h	6 w	24 w			
SMP	5.16 ± 0.02	5.24 ± 0.16	5.39 ± 0.11	53.5 ± 0.8	53.0 ± 1.5	52.6 ± 1.0	13.8 ± 0.7	1.7 ± 0.3	3.5
BMP	5.13 ± 0.03	5.19 ± 0.16	5.31 ± 0.14	52.6 ± 0.7	52.1 ± 0.5	51.5 ± 0.6	13.4 ± 0.2	1.8 ± 0.2	3.7
SMP + 448	5.19 ± 0.03	5.24 ± 0.17	5.32 ± 0.11	53.5 ± 0.3	52.5 ± 0.9	52.4 ± 0.6	14.0 ± 1.0	1.8 ± 0.0	3.7
BMP + 448	5.14 ± 0.01	5.16 ± 0.15	5.26 ± 0.14	52.4 ± 1.3	51.3 ± 0.9	51.0 ± 1.0	13.7 ± 0.2	1.6 ± 0.3	3.3
SMP + 456	5.15 ± 0.03	5.24 ± 0.12	5.30 ± 0.12	53.4 ± 1.0	52.7 ± 0.8	52.3 ± 0.8	13.9 ± 0.9	1.5 ± 0.1	3.2
BMP + 456	5.18 ± 0.01	5.23 ± 0.13	5.28 ± 0.13	52.8 ± 0.2	51.9 ± 0.6	51.5 ± 1.1	13.5 ± 0.2	1.6 ± 0.4	3.3
Effects (significant differences within each experimental factor)									
MFGM components (SMP, BMP)	ns	0.05	0.001	0.01	0.01	0.001	ns	ns	ns
Adjunct (0, 448, 456)	ns	ns	0.001	ns	ns	ns	ns	ns	ns
Tukey			0 > 448,456						
Rep block (A, B, C)	ns	0.001	0.001	0.05	0.001	0.001	ns	0.01	0.01

[a] Abbreviations are: DM, dry matter; FDM, fat in dry matter; SM, salt-in moisture, all per kg of cheese; ns: not significant. Significance level tested at $P = 0.05$; ns, not significant; Tukey's significance test for differences between means within the experimental factor of adjunct type.
[b] SMP, skim milk powder; BMP, butter milk powder; +448, adjunct addition of *Lb. casei* INF 448, +456, adjunct addition of *Lb. casei* INF 456.

some responses. Replicate block C had a 1% higher moisture content than replicate block A and B (Table 1, shown by large SD), resulting in higher levels of most volatile compounds and free amino acids, most probably due to an increased microbial activity although the enumerated numbers did not differ between the replicate blocks. Salt and fat were measured at 6 weeks of ripening and the cheeses had a similar salt content regardless of treatment.

3.2. Microbial development

At the start of ripening, the cheeses had up to log 9.6 cfu g^{-1} of presumptive lactococci as counted on M17 agar. From 4 weeks on, these numbers were reduced, and after 24 weeks the numbers were around log 6.9 cfu g^{-1} (results not shown). In the early phases of ripening (6 weeks) the numbers of lactococci were slightly but significantly ($P < 0.05$) higher in cheeses with adjunct *Lb. casei* INF 456, than in all the other cheeses.

The growth of lactobacilli as enumerated on Rogosa agar (Fig. 1) showed significant differences in the growth between cheeses with or without added adjuncts ($P < 0.001$). Generally, the number of lactobacilli increased during 0–10 weeks of ripening in all the experimental cheeses. Presumptive lactobacilli in cheeses with added adjuncts reached log 8 cfu g^{-1} after 10 weeks of ripening. In cheeses without adjuncts the number of lactobacilli were 3–4 log cfu g^{-1} lower from 0 to 10 weeks of ripening and were still increasing up to 24 weeks of ripening.

When comparing the cheeses without adjuncts (cheeses with added only SMP or BMP), significant ($P < 0.001$) differences were found between the replicate blocks at the start of ripening until 6 weeks of age (results not shown). Cheeses from replicate block A showed no growth on Rogosa agar after 24 h, but had log 1.4 and 3.7 cfu g^{-1} after 2 weeks and 6 weeks of ripening, respectively. Cheese from replicate block B had log 1.7 cfu g^{-1} after 24 h and log 3.1 and 5.7 cfu g^{-1} on Rogosa agar after 2 weeks and 6 weeks, respectively. However, even though replicate block C had a higher moisture content than replicate block A and B, no growth were seen on Rogosa agar until 6 weeks of ripening in cheese from replicate block C, and even at that stage, the numbers were low at log 1.48 cfu g^{-1}.

3.3. Electron micrographs of matured cheese

As shown in the electron micrographs in Fig. 2, the starter lactococci (black arrows) were displayed in the form of clusters immersed and dispersed uniformly throughout the protein matrix after 24 weeks of ripening. The cavities of fat globules, which were removed during sample preparation, can be seen as smooth surface concave areas, spherical in shape (white arrows). Most of the lactococcal cells seem to be associated to these areas. The adjunct lactobacilli were very difficult to find and when found, they appeared in the shape of a huge cluster of long bacilli cells embedded in the protein matrix (Fig. 2b).

Table 2
The content of free amino acids (μmol g^{-1}) significantly influenced by experimental factors in the cheeses after 10 weeks of ripening.[a]

Factor[b]	Thr	Arg	GABA	Met	Ile	Leu	Lys	FAA
SMP	0.76 ± 0.05	0.74 ± 0.06	0.40 ± 0.05	0.64 ± 0.06	0.37 ± 0.06	4.22 ± 0.12	2.45 ± 0.35	31.95 ± 1.41
BMP	0.75 ± 0.01	0.78 ± 0.04	0.49 ± 0.08	0.63 ± 0.03	0.37 ± 0.04	4.39 ± 0.19	2.22 ± 0.17	31.73 ± 1.11
SMP + 448	0.71 ± 0.10	0.70 ± 0.04	0.45 ± 0.13	0.62 ± 0.05	0.34 ± 0.02	4.25 ± 0.26	2.20 ± 0.10	31.40 ± 1.23
BMP + 448	0.68 ± 0.03	0.68 ± 0.03	0.63 ± 0.13	0.56 ± 0.02	0.32 ± 0.02	4.29 ± 0.20	2.06 ± 0.05	30.53 ± 0.43
SMP + 456	0.76 ± 0.10	0.60 ± 0.10	0.49 ± 0.06	0.62 ± 0.04	0.35 ± 0.05	4.22 ± 0.28	2.30 ± 0.02	31.20 ± 1.84
BMP + 456	0.81 ± 0.10	0.62 ± 0.10	0.55 ± 0.08	0.61 ± 0.03	0.34 ± 0.03	4.39 ± 0.36	2.21 ± 0.18	32.09 ± 1.92
Effects (significant differences within each experimental factor)								
MFGM components (SMP, BMP)	ns	ns	0.01	0.05	ns	0.04	0.03	ns
Adjunct (0, 448, 456)	0.009	0.0017	ns	0.02	0.02	ns	ns	ns
Tukey	456 > 448	0 > 456		0 > 448				
Rep block (A, B, C)	0.01	ns	ns	0.0008	0.0003	0.0002	0.03	0.0013

[a] Abbreviations are: GABA, gamma aminobutyric acid; FAA, free amino acids. Significance level tested at $P = 0.05$; ns, not significant; Tukey's significance test for differences between means within the experimental factor of adjunct type.
[b] SMP, skim milk powder; BMP, butter milk powder; +448, adjunct addition of *Lb. casei* INF 448, +456, adjunct addition of *Lb. casei* INF 456.

Table 3
The content of free amino acids (μmol g^{-1} cheese) significantly influenced by experimental factors in the cheeses after 24 weeks of ripening.[a]

Factor[b]	Asp	Glu	Asn	Ser	Thr	FAA
SMP	1.22 ± 0.09	8.41 ± 1.02	3.33 ± 0.54	1.44 ± 0.28	1.28 ± 0.18	46.41 ± 5.85
BMP	1.33 ± 0.17	8.68 ± 0.41	3.39 ± 0.31	1.36 ± 0.18	1.28 ± 0.09	47.42 ± 2.94
SMP + 448	1.75 ± 0.65	8.82 ± 0.95	3.52 ± 0.39	1.48 ± 0.07	1.25 ± 0.08	48.03 ± 4.54
BMP + 448	1.75 ± 0.64	9.85 ± 2.05	3.93 ± 0.82	1.71 ± 0.34	1.37 ± 0.26	54.44 ± 11.39
SMP + 456	1.60 ± 0.57	9.98 ± 1.76	4.13 ± 0.69	1.71 ± 0.18	1.53 ± 0.21	54.21 ± 9.72
BMP + 456	1.65 ± 0.66	10.50 ± 2.26	4.29 ± 0.79	1.78 ± 0.15	1.59 ± 0.24	56.32 ± 11.66
Effects (significant differences within each experimental factor)						
MFGM components (BMP, SMP)	ns	ns	ns	ns	ns	ns
Adjunct (0, 448, 456)	0.05	0.05	0.01	0.05	0.02	ns
Tukey	0 < 448,456	ns	0 < 456	ns	456 > 0,448	
Rep block (A, B, C)	0.0003	0.01	0.007	ns	ns	0.01

[a] Abbreviation: FAA, free amino acids. Significance level tested at $P = 0.05$; ns, not significant; Tukey's significance test for differences between means within the experimental factor of adjunct type.
[b] SMP, skim milk powder; BMP, butter milk powder; +448, adjunct addition of *Lb. casei* INF 448, +456, adjunct addition of *Lb. casei* INF 456.

3.4. Development of flavour compounds

The concentration of diacetyl (results not shown) and acetoin (Fig. 3) decreased until six weeks of ripening in all cheeses, but increased from 6 to 10 weeks of ripening and then again decreased until 24 weeks of ripening. The measured levels of acetoin in the 24 h old cheeses were two times higher ($P < 0.05$) in the cheeses without adjuncts compared with cheeses with added adjuncts.

Production of 3-methyl butanal was 1.2 times higher in cheeses with added BMP compared with cheeses with added SMP throughout ripening (Fig. 4). The differences were significant after 6 and 10 weeks of ripening ($P < 0.05$). After 10 weeks of ripening the contents of 3-methyl butanal was significantly higher ($P < 0.05$) in cheeses with BMP and adjunct *Lb. casei* INF 456 than in cheeses with SMP and the adjunct *Lb. casei* INF 448.

3.5. Amino acid composition

Free amino acids (FAAs) were analysed after 10 and 24 weeks, and the distribution of FAAs was evaluated by principal component analysis (PCA). The PCA showed that Tyr and Trp were not significant for the distribution of the samples, and were therefore omitted from further statistical analysis. The PCA plot (Fig. 5) showed that PC1 explained the variance in cheese samples due to age and replicate block, and PC2 explained the variance in cheeses due to the experimental treatments. The cheeses made in replicate block C had significantly ($P < 0.01$) higher levels of FAAs after 24 weeks of ripening, and this is also reflected in the PCA (Fig. 5a). However, the relationship between the experimental factors within each replicate block was fairly similar both after both 10 and 24 weeks (Fig. 5a). The cheeses with adjuncts in replicate block C had a higher

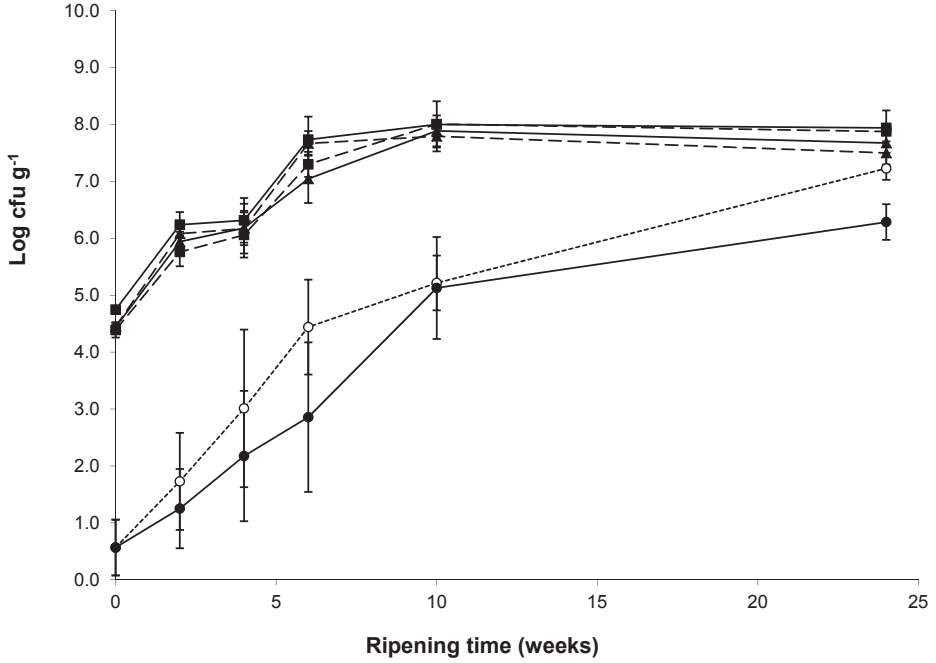

Fig. 1. Development of lactobacilli as enumerated on Rogosa agar (log cfu g^{-1}) during cheese ripening ($n = 3$). Each data point represents mean ± standard deviation; —○— butter milk powder (BMP), —■— BMP448, —▲— BMP456, - - -○- - - SMP, - - -■- - - SMP448, - - -▲- - - SMP456. Numerals indicate the culture adjunct.

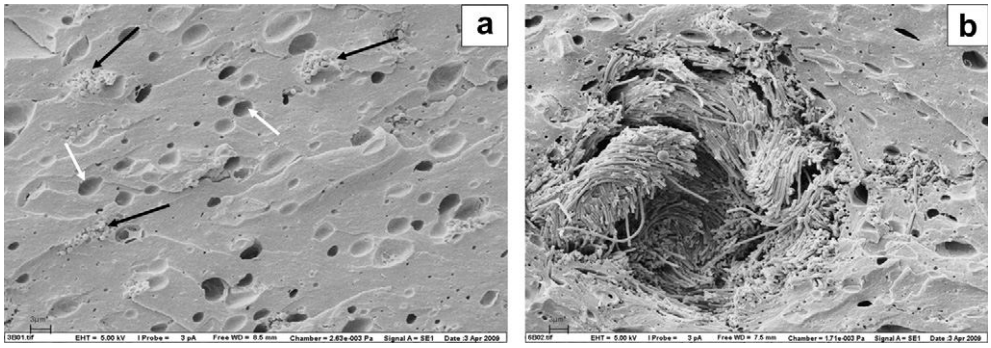

Fig. 2. Electron micrographs (5000×) of low-fat Cheddar cheese ripened for 24 weeks. a) Cheese matrix with lactococci evenly distributed (black arrows) and smooth surface concavity areas originally occupied by fat globules (white arrows) and b) cheese matrix with lactobacilli cluster.

content of FAAs, whereas no such clear difference could be seen in replicate blocks A and B. The content of gamma-aminobutyric acid (GABA), His, Arg, Glu and Lys was responsible for the separation along PC2, and some of them were also significantly affected by the treatment factors.

The FAAs significantly ($P < 0.05$) affected by the experimental factors during cheese ripening are shown in Table 2 (after 10 weeks) and Table 3 (after 24 weeks). Cheeses with BMP had a significantly ($P < 0.01$) higher content of GABA and significantly ($P < 0.03$) lower levels of Lys than cheeses with SMP after 10 weeks of ripening. Cheeses with added *Lb. casei* 456 had significantly lower levels of Arg after 10 weeks of ripening and higher levels of Glu after 24 weeks of ripening than the cheeses without adjunct and cheeses added *Lb. casei* 448. When replicate block C was omitted from the dataset, ANOVA revealed that cheeses with BMP had significantly higher levels of Leu after 10 weeks and 24 weeks of ripening than cheeses with added SMP (results not shown). Cheeses with adjuncts had significantly lower contents of Ile than the cheese without adjunct after 10 weeks of ripening. Cheeses added *Lb. casei* INF 456 had higher levels of Thr than the cheeses without adjuncts and cheeses with *Lb. casei* 448, both after 10 and 24 weeks. After 24 weeks of ripening the cheeses without adjuncts had lower levels of Asp, Asn and Ser than the cheeses with adjuncts.

3.6. Phenotypic characterisation

Phenotypic characterisation showed that all of the isolates (a total of 993) were Gram positive and catalase negative bacilli showing colony morphology on MRS agar ranging from large round, white colonies to small, opalescent colourless colonies. The CO_2 production was at the level of homofermentative organisms (lower than 1000 mg kg^{-1}) ranging from 98 mg kg^{-1} to 261 mg kg^{-1}.

The results obtained by API 50 CHL showed that 97 out of 100 isolates tested in this study were ribose positive. Among the isolates from 24-h cheeses, the API 50 CHL results revealed the highest phenotypic diversity (results not shown), whereas the isolates from the ripened cheeses had a more similar fermentation pattern. From the PCA (Fig. 6) the isolates from 4- (25 isolates) and 24- (18 isolates) week old cheeses were clustered on the basis of their sugar fermentation pattern, whereas the isolates from 24-h cheese seemed to be randomly distributed (results not shown). A clear clustering of the isolates was observed after four weeks of ripening with respect to the adjuncts as well as to the powder addition (MFGM content; Fig. 6a). Isolates from cheeses with added *Lb. casei* INF 448 formed one cluster, whereas cheeses with added *Lb. casei* INF 456 formed a second cluster. A third cluster was formed with

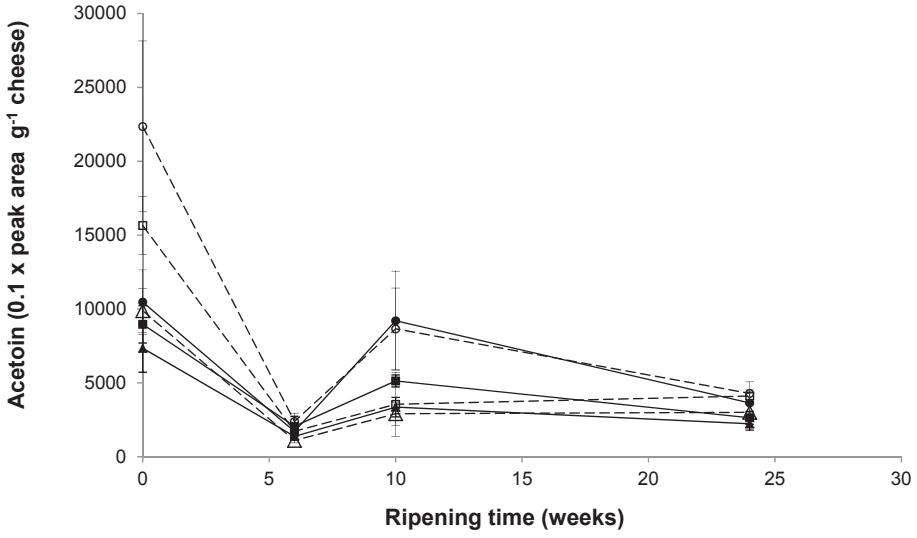

Fig. 3. Development of acetoin (shown as 0.1% of the peak area per gram of cheese) during the ripening of cheese ($n = 3$). Each data point represents mean ± standard deviation; —○— butter milk powder (BMP), —■— BMP448, —▲— BMP456, - - -○- - - SMP, - - -■- - - SMP448, - - -▲- - - SMP456. Numerals indicate the culture adjunct.

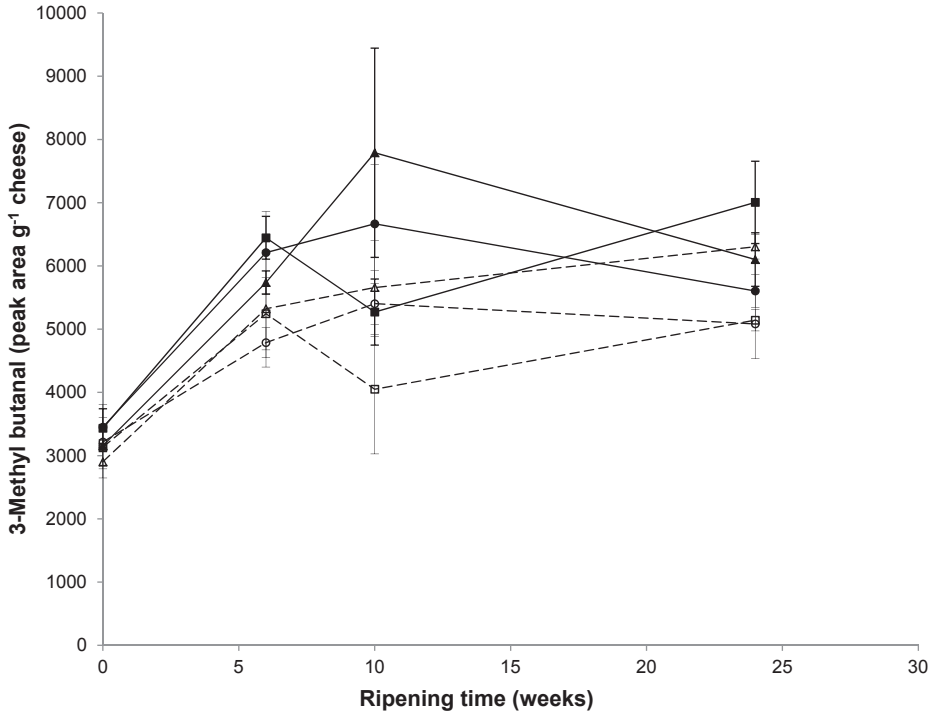

Fig. 4. Development of 3-methyl butanal (area per gram of cheese) during cheese ripening ($n = 3$). Each data point represents mean ± standard deviation. —○— butter milk powder (BMP), —■— BMP448, —▲— BMP456, - - -○- - - SMP, - - -■- - - SMP448, - - -▲- - - SMP456. Numerals indicate the culture adjunct.

the isolates from the cheeses without added adjunct (only SMP or BMP). The differences between isolates diminished throughout the maturation period and the 24-week old cheese isolates (Fig. 6b) made two distinct clusters with no relationship to the experimental design.

3.7. Species identification by 16S rRNA sequence analysis

The highest microbial diversity was observed in cheeses at start of maturation (24 h of ripening), and the population composition became more uniform by the end of maturation (24 weeks of ripening). Most of the isolates were identified as *Lb. casei*/*Lb. paracasei*. At the start of ripening (time 0), 27 out of 32 analysed isolates from all cheeses were identified as *Lb. casei*/*Lb. paracasei*, four of the tested isolates were identified as *Lactobacillus brevis* (isolates 5 (SMP + 448 − 0), 13 (BMP + 448 − 0), 23 (SMP + 456 − 0) and 24 (SMP + 456 − 0)), whereas one belonged to *Lactobacillus rhamnosus* (17 (SMP + 456 + 0)).

In the 10 week old cheeses, 24 out of 25 tested isolates belonged to *Lb. casei*/*Lb. paracasei* and only one isolate was characterised as *Lactobacillus curvatus* (66 (BMP − 10)). All of the 42 tested isolates from 4 to 24 week old cheeses were identified as *Lb. casei*/*Lb. paracasei*.

3.8. Rep-PCR typing

Rep-PCR gave bands in the range of 0.3–8 kbp, which made it possible to cluster the 100 isolates. The REP1R-Dt and REP2R-Dt primer set generated fingerprints containing between 10 and 22 visualised PCR products. The analysis of the generated banding patterns is shown as a dendrogram (Fig. 7a–d).

It can be seen that some isolates from the same maturation time clustered together in separate clusters. The numbers of clusters with at least 80% similarity decreased during the ripening period. This indicates a change in microbial composition of the studied cheeses over the maturation period. However, none of the investigated isolates clustered with more than 80% similarity with the adjuncts *Lb. casei* INF 448 or INF 456.

Most of the isolates from the 24-h old cheese (Fig. 7a) grouped together with at least 40% similarity, showing little grouping on the basis of the experimental factors. Clustering with at least 80% similarity was observed in ten small clusters. The clustering of the isolates was rather random and seemed not to correlate with the adjunct addition; however, some clustering was correlated with the MFGM composition of the cheeses. In the 4 week old cheeses (Fig. 7b), clustering based on the different experimental treatments was observed in five clusters with at least 80% similarity. The first cluster comprised isolates from cheese with added *Lb. casei* INF 456 (isolates 52, 53, 54, 55, 56 and 57) with the exception of one isolate from the vat supplemented with *Lb. casei* INF 448 (isolate 51). This cluster also had two sub-groups due to the MFGM content of the cheese. The second cluster was grouped according to MFGM content, but seemed to be random regarding the adjuncts. The isolates of the second and third cluster had a random clustering and mostly consisted of isolates from the cheese vats without added adjuncts or the vats supplemented with the adjunct strain *Lb. casei* INF 448. The last two clusters were grouped according to the adjunct addition; they all came from vats added *Lb. casei* INF 448.

Clustering of the isolates from the 10 week old cheeses (Fig. 7c) showed three different clusters with at least 80% similarity, and also showing sub-clusters within the clusters. The first group (isolates 60, 61, 62, 64 and 65) represents the isolates from the cheese vats without added adjuncts and one isolate supplemented with *Lb. casei* INF 448 (isolate 67). The second group (isolates 68, 69, 70, 71 and 72) was isolates from the vats supplemented with *Lb. casei* INF 448 and two isolates from cheese vats without adjuncts (isolates 58

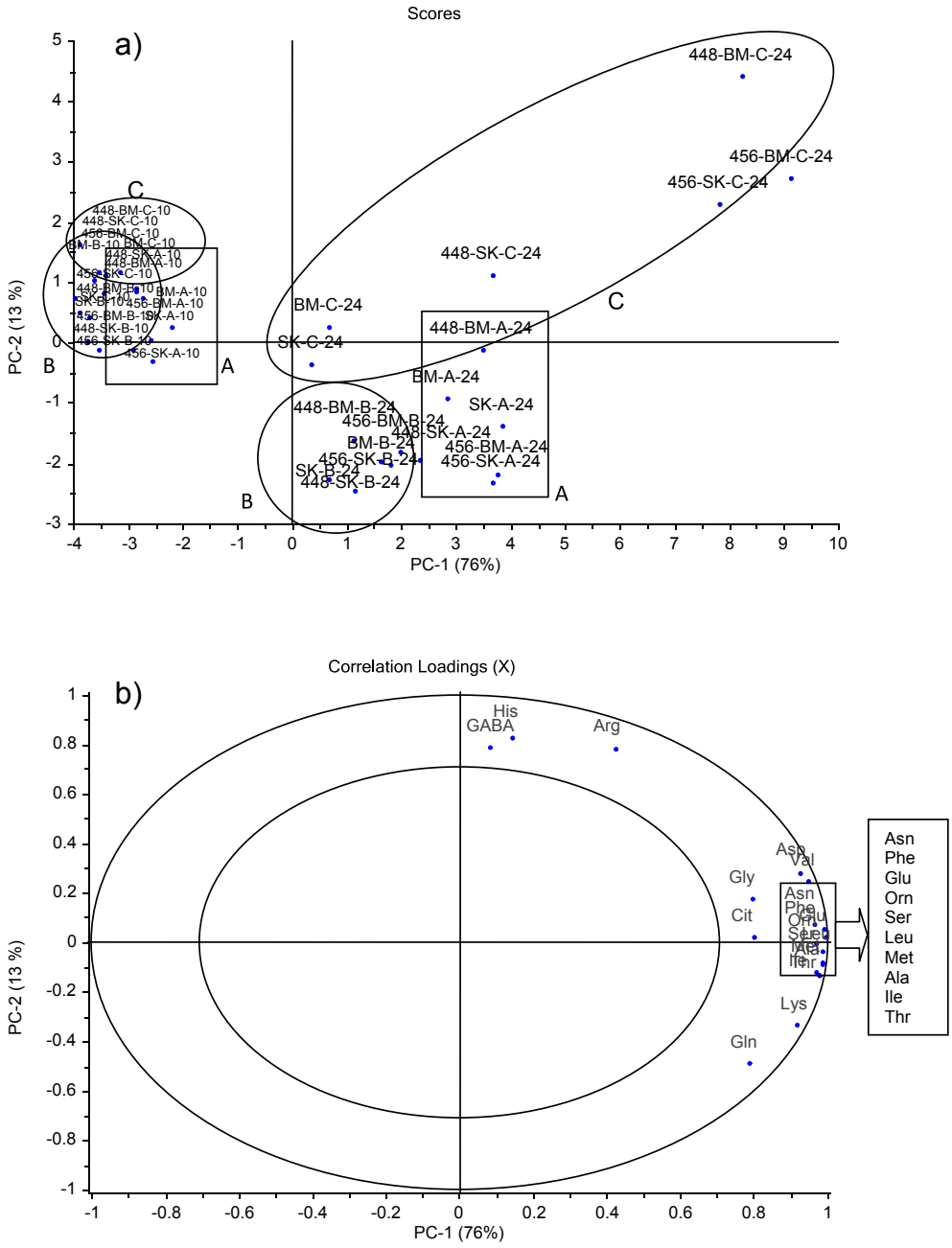

Fig. 5. Principal component analysis (PCA) scores (a) and loadings (b) of the amino acid distribution in the studied cheeses. Samples marking: Adjunct (448 and 456), MFGM components addition (skim milk powder, SK, and butter milk powder, BM), replicate block (A, B, C) and age (10 and 24 weeks). Grouping of the replicate blocks is done by: A; rectangle, B; circle and C; ellipse. GABA: gamma aminobutyric acid.

and 63). Isolates 80 and 81 showed an identical pattern and grouped with 100% similarity. The rest of the isolates from 10 week old cheeses grouped together with less than 80% similarity.

The most uniform grouping was observed among the isolates from the 24 week old cheeses (Fig. 7d), represented by three clusters which grouped with at least 80% similarity; only four of the isolates had less than 80% similarity with the others. The isolates of the first two clusters were isolated from cheese with added BMP. Isolates 89, 90, 91 and 96 clustered together with 100% similarity. At this stage of ripening there seemed to be less clustering according to the experimental factors than earlier in the ripening.

4. Discussion

This work was undertaken to follow the evolution of the microflora of low-fat cheeses with a low initial number of adjunct lactobacilli over a 24 week maturation period. Adjuncts are commonly added at high numbers, around log 5 cfu mL^{-1}, to the cheese milk to dominate the cheese microflora (Fox et al., 1996); however, in this study the adjuncts were added at log 2 cfu mL^{-1} in the cheese milk. Through using a low inoculum, the adjunct colonies were sparsely distributed in the cheese, whereas the lactococci were more densely distributed. During the first 24 h, the number of presumptive lactobacilli increased to

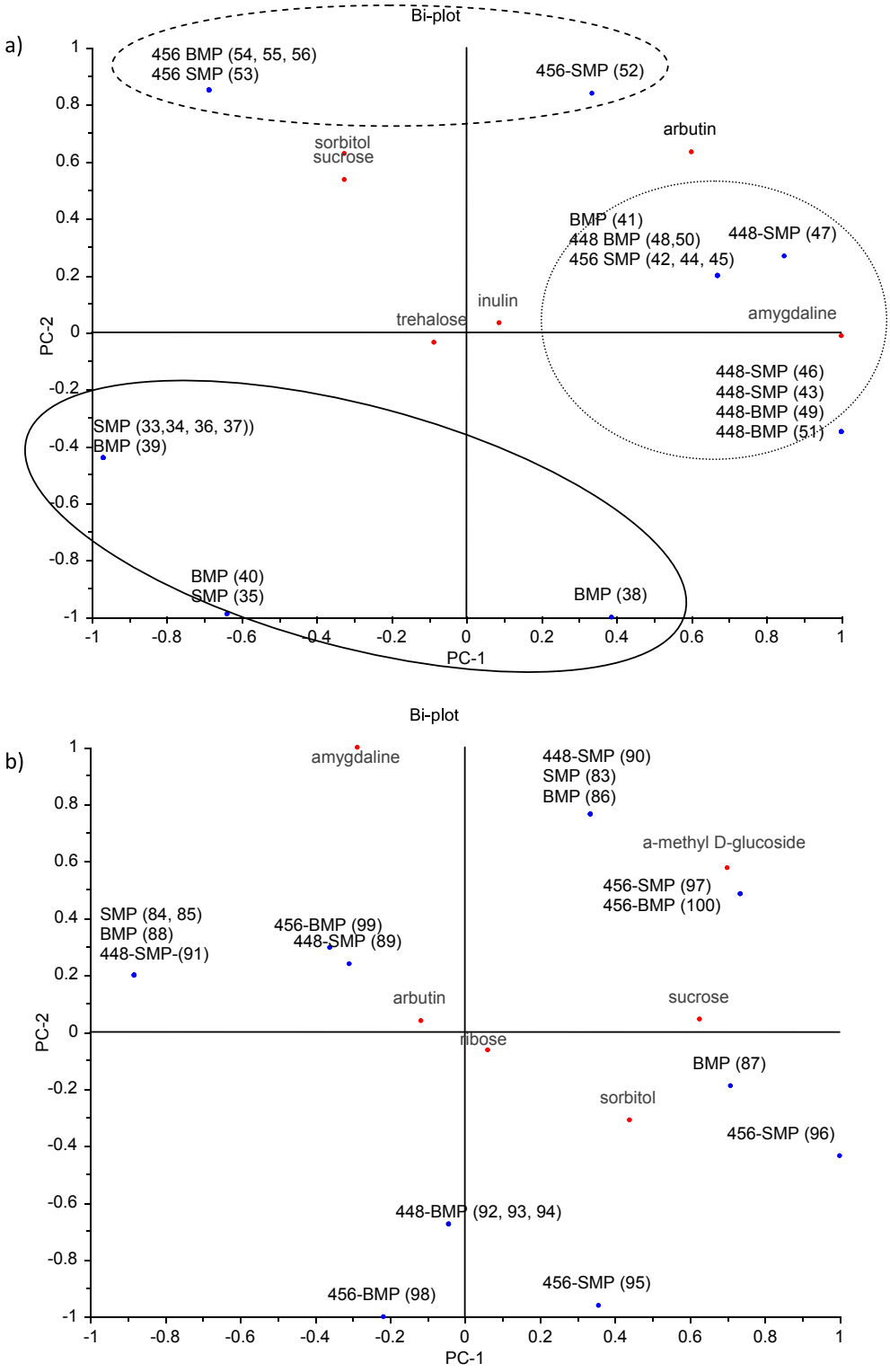

Fig. 6. Principal component analysis (PCA) biplot of the API 50 CHL sugar fermentation of the bacteria isolates. Samples marking: Adjunct (448 and 456), MFGM component addition (skim milk powder, SMP; butter milk powder, BMP) and (isolate number). a) Distribution of the cheese isolates after 4 weeks of ripening (40% and 34% of the variation explained by PC1 and PC2, respectively). Cheese without added adjuncts (solid ellipse), cheese with *Lb. casei* INF 448 (dotted circle) and cheese with *Lb. casei* INF 456 (broken ellipse). b) Distribution of the cheese isolates after 24 weeks ripening (37% and 28% of the variation explained by PC1 and PC2).

log 4.5 cfu g^{-1}, indicating growth of adjuncts and/or NSLAB during cheesemaking. The rep-PCR showed that the adjunct bacteria did not dominate the lactobacilli microflora completely in the early stages of ripening. The numbers of lactobacilli (NSLAB) enumerated in the cheeses without added adjuncts was in accordance with previous findings (Beresford et al., 2001) for replicate blocks A and B, whereas replicate block C showed lower numbers of lactobacilli at the start of the ripening period, indicating that the initial NSLAB numbers of replicate block C was lower than that of A and B.

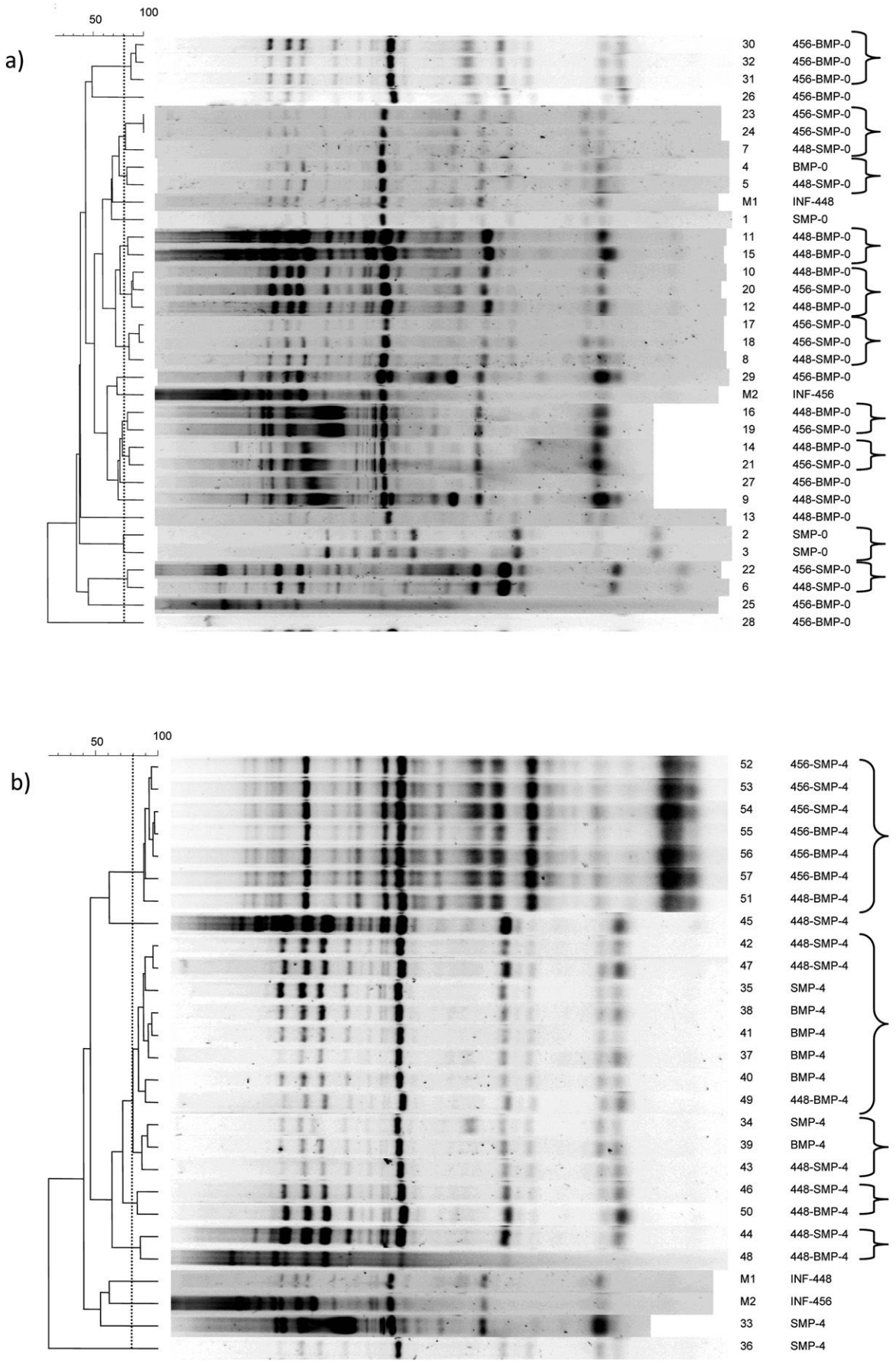

Fig. 7. Dendrogram based on the rep-polymerase chain reaction (PCR) DNA fingerprinting of bacterial isolates from the low-fat Cheddar cheeses. a) 24-h cheese isolates; b) 4 week cheese isolates; c) 10 week cheese isolates; d) 24 week cheese isolates, M1: *Lb. casei* INF 448, M2: *Lb. casei* INF 456. The scale in the upper left corner represents the % of similarity within the clusters. SMP: skim milk powder; BMP: butter milk powder.

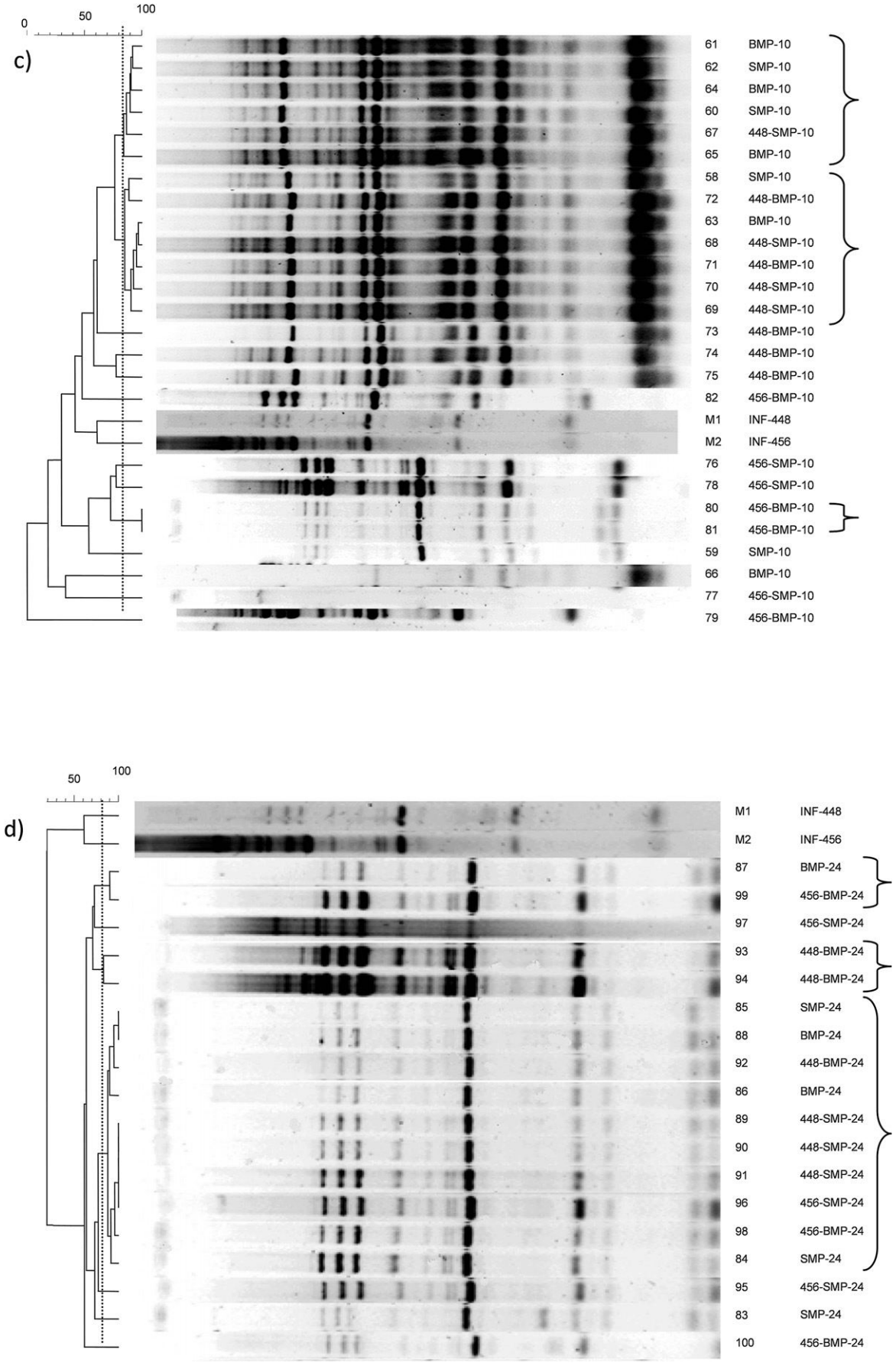

Fig. 7. (*continued*).

Grouping due to adjunct addition was shown in the intermediate maturing steps of the cheese by rep-PCR. The microbial composition at the start of ripening influenced the development and composition of the lactobacilli during further ripening. The uniform microorganism distribution observed in the cheeses after 24 weeks of ripening indicated that some strains were able to grow to high numbers and dominate the NSLAB flora.

The enumerated numbers of presumptive lactobacilli were significantly different in cheeses with and without adjuncts throughout the whole period of maturation, while no significant differences between BMP and SMP addition in the lactobacilli counts were found, indicating that the MFGM content had a minor affect on the general lactobacilli growth in cheese. The differences in lactobacilli counts between the replicate blocks of the cheeses without added adjuncts may also explain the broad variety of species and strains found at the start of ripening. Furthermore, this study showed that there was no obvious clustering of the isolates on the basis of experimental factors used in this study. However, a clearer grouping of the isolates could be observed in the mid-stages of ripening in connection to the experimental factors. These findings showed that the adjuncts, when added in low numbers, do not manage to dominate the microflora throughout ripening. However, the different treatments influenced the grouping of the isolates during ripening.

Electron micrographs confirmed the even distribution of lactococci (starter) in the cheese matrix. The lactococci were inoculated at high numbers and were uniformly distributed in the cheese milk. They reached their maximum cell number during cheesemaking, and this explains their uniform distribution in the cheese matrix. In addition, some clustering of cells was seen, indicating further growth in the cheese. The lactobacilli were inoculated at low numbers in the cheese milk, and therefore were more sparsely distributed in the cheese matrix in comparison with the lactococci. The number of lactobacilli increased to log 7–8 cfu g^{-1} during ripening, and since they are not motile in cheese, their growth resulted in large cell clusters with clearly elongated cells as observed by electron microscopy.

In Cheddar type cheese, which is commonly made with no addition of Cit^{+} starter bacteria, the production of the flavour compounds, such as diacetyl and acetoin, was not expected. The amounts of these components were highest in the cheeses without added adjuncts. It has been shown in earlier studies that the concentration of citrate in Cheddar cheese is decreasing rather slowly and its degradation results from the enzymatic activity of the NSLAB microflora at late stages of ripening (Singh, Drake, & Cadwallader, 2003; Thomas, 1987a). Diacetyl and acetoin may be synthesised through transamination of Asp and Asn leading to formation of oxaloacetate which can be metabolised to acetoin and diacetyl by some lactobacilli (Kieronczyk, Skeie, Langsrud, Le Bars, & Yvon, 2004; Skeie et al., 2008b). In addition, diacetyl can also be produced from oxaloacetate by spontaneous decarboxylation of the intermediate acetolactate. Since the cheeses without added adjuncts had reduced contents of Asp and Asn, it is most probably indigenous NSLAB flora in these cheeses that caused this degradation, resulting in increased levels of diacetyl and acetoin. In the cheeses with added adjunct, the growths of indigenous NSLAB were most probably repressed by the presence of the lactobacilli adjuncts. In addition, it is known that the adjunct *Lb. casei* INF 448 is not able to degrade Asp in cheese (Skeie et al., 2008b). Furthermore, both adjuncts used in this study are not able to metabolise citrate in milk to diacetyl and acetoin (unpublished results).

It has been indicated in previous studies of semi-hard cheeses, that mesophilic lactobacilli predominate in the later stages of cheese ripening (Berthier & Ehrlich, 1998; Crow, Curry, & Hayes, 2001; Østlie et al., 2004). The rep-PCR analysis was applied to get a more complete picture of the strain diversity during the period of cheese maturation. Certain strains of *Lb. casei*/*Lb. paracasei* appeared to be common in all cheeses and they most probably originate from the cheese milk or dairy environment, which is in accordance with findings by other authors (Antonsson, Ardö, & Molin, 2001; Antonsson, Molin, & Ardö, 2003; Fitzsimons, Cogan, Condon, & Beresford, 1999). The most uniform microflora was observed at the end of ripening.

As expected, *Lb. casei*/*Lb. paracasei* represented the most abundant *Lb.* species regardless of experimental factors used, such as powder (MFGM content) or adjunct addition. These results were in accordance with previous findings (Banks & Williams, 2004; Beresford et al., 2001) stating that the NSLAB population of Cheddar cheeses is dominated by *Lb. casei*/*Lb. paracasei* (approximately 95% of the isolates belong to *Lb. casei*/*Lb. paracasei*). The PCA of the API results of the selected isolates were in accordance with the chemical and microbial characterisation of the cheeses, with the highest diversity of the lactobacilli in the 24 h cheese, a clear grouping according to the adjuncts in the intermediate phase of ripening (4–10 weeks), whereas after 24 weeks the differences between the experimental factors were less apparent.

Most of the isolates (97%) were ribose positive. In model systems, Thomas (1987b) demonstrated that ribose can be used as a carbon source for mesophilic lactobacilli in the later stages of ripening. Lysed lactococcal cells may release ribose from RNA and *N*-acetylglucosamine from degraded cell walls (Adamberg et al., 2005; Østlie et al., 1995). Another possible source of carbon for mesophilic lactobacilli may be connected to the fact that they possess some glycoside hydrolase activity and can utilise sugars from glycoproteins of the MFGM as an energy source (Fox et al., 1998; Williams & Banks, 1997). The electron micrographs showed an even distribution of the lactococcal strains throughout the cheese matrix, most of them connected to fat globules, and the lactobacilli appeared to be surrounding cavities from where milk fat globules were removed.

Aldehydes in cheese, such as 3-methyl butanal and 2-methyl butanal originate from transamination of branched chain amino acids, as well as from imides that may be decarboxylated to the corresponding aldehydes (Kieronzcyk, Skeie, Olsen, & Langsrud, 2001; McSweeney & Sousa, 2000; Marilley & Casey, 2004). In this study, the highest levels of 3-methyl butanal, most probably derived from Leu, were measured from 6 weeks of ripening in cheeses supplemented with BMP. In addition, the content of Leu was higher in cheeses with BMP than in cheeses with SMP, although the dry matter (and protein) content were lower in the BMP cheeses than in the SMP cheeses. Supplementation of the cheese with buttermilk components seemed to facilitate better growth of microorganisms able to degrade these branched chain amino acids.

5. Conclusion

The diversity of lactobacilli in the studied low-fat Cheddar cheeses were highest at the start of ripening, becoming more uniform by the end of the maturation process with *Lb. casei* being the most dominant lactobacilli species. The *Lb. casei* adjuncts added in low numbers were not able to dominate the cheese microflora at any stage during ripening, even if they have the ability to utilise the MFGM components. However, the experimental factors (adjunct and MFGM components) did influence the development of microorganisms, some flavour compounds and the branched chain amino acid Leu in the cheese during ripening. The lactobacilli were found in the cheese in large clusters of clearly elongated cells.

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References

Adamberg, K., Antonsson, M., Vogensen, F. K., Nielsen, E. W., Kask, S., Moller, P. L., et al. (2005). Fermentation of carbohydrates from cheese sources by non-starter lactic acid bacteria isolated from semi-hard Danish cheese. *International Dairy Journal, 15*, 873–882.

Antonsson, M., Ardö, Y., & Molin, G. (2001). A comparison between the microflora of Herrgård cheese from three different dairies. *International Dairy Journal, 11*, 285–291.

Antonsson, M., Molin, G., & Ardö, Y. (2003). *Lactobacillus* strains isolated from Danbo cheese as adjunct cultures in a cheese model system. *International Journal of Food Microbiology, 85*, 159–169.

Banks, J. M., & Williams, A. G. (2004). The role of nonstarter lactic acid bacteria in Cheddar cheese ripening. *International Journal of Dairy Technology, 57*, 145–152.

Ben Amor, K., Vaughan, E. E., & DeVos, W. M. (2007). Advanced molecular tools for the identification of lactic acid bacteria. *Journal of Nutrition, 137*, 741–747.

Beresford, T. P., Fitzsimons, N. A., Brennan, N. L., & Cogan, T. M. (2001). Recent advances in cheese microbiology. *International Dairy Journal, 11*, 259–274.

Berthier, F., Beuvier, E., Dasen, A., & Grappin, R. (2001). Origin and diversity of mesophilic lactobacilli in Comte cheese, as revealed by PCR with repetitive and species-specific primers. *International Dairy Journal, 11*, 293–305.

Berthier, F., & Ehrlich, S. D. (1998). Rapid species identification within two groups of closely related lactobacilli using PCR primers that target 16S/23S rRNA spacer region. *FEMS Microbiology Letters, 161*, 97–106.

Broadbent, J. R., Houck, K., Johnson, M. E., & Oberg, C. J. (2003). Influence of adjunct use and cheese microenvironment on nonstarter bacteria in reduced-fat Cheddar-type cheese. *Journal of Dairy Science, 86*, 2773–2782.

Bütikofer, U., & Ardö, Y. (1999). Quantitative determination of free amino acids in cheese. *Bulletin of the International Dairy Federation, 337*, 24–32.

Christiansen, P., Waagner Nielsen, E., Vogensen, F. K., Brogren, C. H., & Ardö, Y. (2006). Heat resistance of *Lactobacillus paracasei* isolated from semi-hard cheese made of pasteurised milk. *International Dairy Journal, 16*, 1196–1204.

Coeuret, V., Dubernet, S., Bernardieau, M., Gueguen, M., & Vernoux, J. P. (2003). Isolation, characterization and identification of lactobacilli focusing mainly on cheeses and other dairy products. *Lait, 83*, 269–306.

Cogan, T. M., Beresford, T. P., Steele, J., Broadbent, J., Shah, N. P., & Ustunol, Z. (2007). Invited review: advances in starter cultures and cultured foods. *Journal of Dairy Science, 90*, 4005–4021.

Collins, Y., McSweeney, P. L. H., & Wilkinson, M. (2003). Lipolysis and free fatty acid catabolism in cheese: a review of current knowledge. *International Dairy Journal, 13*, 841–866.

Crow, V., Curry, B., & Hayes, M. (2001). The ecology of non-starter lactic acid bacteria (NSLAB) and their use as adjuncts in New Zealand Cheddar. *International Dairy Journal, 11*, 275–283.

De Man, J. C., Rogosa, M., & Sharpe, M. E. (1960). A medium for the cultivation of lactobacilli. *Journal of Applied Bacteriology, 23*, 130–135.

De Urazza, P. J., Gomez-Zavaglia, A., Lozano, M. E., Romanowski, V., & de Antoni, G. L. (2000). DNA fingerprinting of thermophilic lactic acid bacteria using repetitive sequence-based polymerase chain reaction. *Journal of Dairy Research, 67*, 381–392.

El Soda, M., Madkor, S. M., & Tong, P. S. (2000). Evaluation of commercial adjuncts for use in cheese ripening: comparison between attenuated and not attenuated lactobacilli. *Milchwissenschaft, 55*, 260–263.

El Soda, M., Madkor, S. M., & Tong, P. S. (2008). Adjunct cultures: recent developments and potential significance to the cheese industry. *Journal of Dairy Science, 83*, 609–619.

Fitzsimons, N. A., Cogan, T. M., Condon, S., & Beresford, T. (1999). Phenotypic and molecular characterisation of non-starter lactic acid bacteria in mature commercial Irish Cheddar cheese. *Applied and Environmental Microbiology, 65*, 3418–3426.

Fox, P. F., McSweeney, P. L. H., & Lynch, C. M. (1998). Significance on non-starter lactic acid bacteria in Cheddar cheese. *Australian Journal of Dairy Technology, 53*, 83–89.

Fox, P. F., Wallace, J. M., Morgan, S., Lynch, C. M., Niland, E. J., & Tobin, J. (1996). Acceleration of cheese ripening. *Antonie Leeuwenhoek, 70*, 271–297.

Hynes, E., Bergamini, C. V., Suarez, V., & Zalazar, C. A. (2003). Proteolysis on Reggianito cheeses manufactured with natural whey cultures and selected strains of *Lactobacillus helveticus*. *Journal of Dairy Science, 86*, 3831–3840.

IDF. (1982). *Cheese and processed cheese: Determination of the total solids content. IDF Standard 4a*. Brussels, Belgium: International Dairy Federation.

IDF. (1995). *Milk and milk products: Guidance on sampling. IDF standard 50c*. Brussels, Belgium: International Dairy Federation.

Kieronczyk, A., Skeie, S., Langsrud, T., Le Bars, D., & Yvon, M. (2004). The nature of aroma compounds produced in a cheese model by glutamate dehydrogenase positive *Lactobacillus* INF 15D depends on its relative aminotransferase activities towards the different amino acids. *International Dairy Journal, 14*, 227–235.

Kieronczyk, A., Skeie, S., Olsen, K., & Langsrud, T. (2001). Metabolism of amino acids by resting cells of non-starter lactobacilli in relation to flavor development in cheese. *International Dairy Journal, 11*, 217–224.

Kilcawley, K., O'Connell, P., Hickey, D. K., Sheehan, E. M., Beresford, T., & McSweeney, P. L. H. (2007). Influence of composition on the biochemical and sensory characteristics of commercial Cheddar cheese of variable quality and fat content. *International Journal of Dairy Technology, 60*, 81–88.

Laloy, E., Vuillemard, J. C., El Soda, M., & Simard, R. E. (1996). Influence of the fat content of Cheddar cheese on retention and localization of starters. *International Dairy Journal, 6*, 729–740.

Larsen, S. K., Aideh, B., Kilstrup, M., Michelsen, O., & Vogensen, F. K. (2008). The nomenclature of *Lactobacillus casei*, an archeology approach. In *5th IDF Symposium on cheese ripening, 9–13th March 2008, Bern, Switzerland*.

Liu, H., Xu, X. M., & Guo, S. D. (2008). Comparison of full-fat and low-fat cheese analogues with or without pectin gel through microstructure, texture, rheology, thermal and sensory analysis. *International Journal of Food Science and Technology, 43*, 1581–1592.

Lynch, C. M., Mc Sweeney, P. L. H., Fox, P. F., Cogan, T. M., & Drinan, F. D. (1996). Manufacture of Cheddar cheese with and without adjunct lactobacilli under controlled microbiological conditions. *International Dairy Journal, 6*, 851–867.

Lynch, C. M., Muir, D. D., Banks, J. M., McSweeney, P. L. H., & Fox, P. F. (1999). Influence of adjunct cultures of *Lactobacillus paracasei* ssp. *paracasei* or *Lactobacillus plantarum* on Cheddar cheese ripening. *Journal of Dairy Science, 82*, 1618–1628.

McSweeney, P. L. H., & Sousa, M. J. (2000). Biochemical pathways for the production of flavour compounds in cheese during ripening. *Lait, 80*, 293–324.

Marilley, L., & Casey, M. G. (2004). Flavours of cheese products: metabolic pathways, analytical tools and identification of producing strains. *International Journal of Food Microbiology, 90*, 139–159.

Moe, K. M., Faye, T., Abrahamsen, R. K., Østlie, H. M., & Skeie, S. (2012). Growth and survival of cheese ripening bacteria on milk fat globule membrane isolated from bovine milk and its monosaccharides. *International Dairy Journal, 25*, 29–35.

Morin, P., Pouliot, Y., & Britten, M. (2008). Effect of buttermilk made from creams with different heat treatment histories on properties of rennet gels and model cheeses. *Journal of Dairy Science, 91*, 871–882.

Narvhus, J. A., Hulbækdal, A., Baugerød, H., & Abrahamsen, R. K. (1991). Measurement of CO_2 production and O_2 metabolism by pure and mixed cultures of lactic acid bacteria growing in milk. In*Actes du colloque lactic*, Vol. 91, (pp. 371). Caen, France: Centre de Publications de l'Universite de Caen.

Narvhus, J. A., Østeraas, K., Mutukumira, T., & Abrahamsen, R. K. (1998). Production of fermented milk using a malty compound-producing strain of *Lactococcus lactis* subsp. *lactis* biovar. *diacetylactis* isolated from Zimbabwean naturally fermented milk. *International Journal of Food Microbiology, 41*, 73–80.

Østlie, H. M., Eliassen, L., Florvaag, A., & Skeie, S. (2004). Phenotypic and PCR-based characterization of the microflora in Norvegia cheese during ripening. *International Journal of Food Microbiology, 94*, 287–299.

Østlie, H. M., Helland, M. H., & Narvhus, J. A. (2003). Growth and metabolism of selected strains of probiotic bacteria in milk. *International Journal of Food Microbiology, 87*, 17–27.

Østlie, H. M., Vegarud, G., & Langsrud, T. (1995). Autolysis of lactococci: detection of lytic enzymes by polyacrylamide gel electrophoresis and characterization in buffer systems. *Applied and Environmental Microbiology, 61*, 3598–3603.

Puchades, R., Lemieux, L., & Simard, R. E. (1989). Evolution of free amino acids during the ripening of Cheddar cheese containing added lactobacilli strains. *Journal of Food Science, 54*, 885–888.

Romeih, E. A., Moe, K. M., & Skeie, S. (2012). The influence of fat globule membrane components on the microstructure of low-fat Cheddar cheese. *International Dairy Journal, 26*, 66–72.

Singh, T. K., Drake, M. A., & Cadwallader, K. R. (2003). Flavor of cheddar cheese: a chemical and sensory perspective. *Comprehensive Reviews in Food Science and Food Safety, 2*, 166–189.

Singh, S., Pawas, G., Singh, R., & Heller, K. J. (2009). Application of molecular identification tools for Lactobacillus, with a focus on discrimination between closely related species: a review. *LWT – Food Science and Technology, 42*, 448–457.

Skeie, S., Kieronczyk, A., Eidet, S., Reitan, M., Olsen, K., & Østlie, H. (2008a). Interaction between starter bacteria and adjunct *Lactobacillus plantarum* INF15D on the degradation of citrate, asparagine and aspartate in a washed-curd cheese. *International Dairy Journal, 18*, 169–177.

Skeie, S., Kieronczyk, A., Næs, R. M., & Østlie, H. (2008b). Lactobacillus adjuncts in cheese: their influence on the degradation of citrate and serine during ripening of a washed curd cheese. *International Dairy Journal, 18*, 158–168.

Skeie, S., Lindberg, C., & Narvhus, J. (2001). Development of amino acids and organic acids in Norvegia, influence of milk treatment and adjunct *Lactobacillus*. *International Dairy Journal, 11*, 399–411.

Thomas, T. D. (1987a). Acetate production from lactate and citrate by non-starter bacteria in Cheddar cheese. *New Zealand Journal of Dairy Science and Technology, 22*, 25–38.

Thomas, T. D. (1987b). Cannibalism among bacteria found in cheese. *New Zealand Journal of Dairy Science and Technology, 22*, 215–219.

Versalovic, J., Schneider, M., de Bruijn, F. J., & Lupski, J. R. (1994). Genomic fingerprinting of bacteria using repetitive sequence based PCR (rep-PCR). *Methods of Cellular Molecular Biology, 5*, 25–40.

Williams, A. G., & Banks, J. M. (1997). Proteolytic and other hydrolytic enzyme activities in nonstarter lactic acid bacteria (NSLAB) isolated from Cheddar cheese manufactured in the United Kingdom. *International Dairy Journal, 7*, 763–774.

Paper III

Letters in Applied Microbiology

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ORIGINAL ARTICLE

Survival of lactic acid and propionibacteria in low- and full-fat Dutch-type cheese during human digestion *ex vivo*

A. Martinovic[1], M.E. Brede[2], G.E. Vegarud[3], H.M. Østlie[3], J. Narvhus[3] and S.B. Skeie[3]

1 Faculty of Food Technology, Food Safety and Ecology, University of Donja Gorica, Podgorica, Montenegro
2 TINE Meieriet Verdal, Verdal, Norway
3 Institute of Chemistry, Biotechnology and Food Science, Norwegian University of Life Sciences, Ås, Norway

Significance and Impact of the Study: Cheese can function as a suitable vehicle for the delivery of a variety of food-related micro-organisms to the intestine. Young cheese as well as low-fat cheeses are better carrier matrixes than full-fat and/or more well-ripened cheeses. Most of the lactobacilli and all the propionibacteria survived well during digestion of the low-fat cheeses. This study also showed the ability of cheese lactobacilli and PAB to survive the severe conditions of GIT.

Keywords
cheese, *ex vivo* digestion, lactic acid bacteria, propionic acid bacteria, survival rate.

Correspondence
Siv Borghild Skeie, Institute of Chemistry, Biotechnology and Food Science, Norwegian University of Life Sciences, Postboks 5003 NMBU, 1432 Ås, Norway.
E-mail: siv.skeie@nmbu.no

Research performed at: Department of Chemistry, Biotechnology and Food Science, Norwegian University of Life Sciences, Postbox 5003 NMBU, 1432 Ås, Norway.

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Abstract

The survival of selected bacteria in semi-hard experimental cheeses was studied after exposure to human gastric and duodenal juices in an *ex vivo* model. Experimental cheeses (10 and 28% fat) were supplemented with different strains of *Lactobacillus* sp. and *Propionibacterium* sp. and ripened for 7 and 70 weeks. After digestion, greater numbers of the adjunct bacteria we rerecorded in the 7-week-old cheeses compared to the well-matured cheeses (70 weeks). The bacterial survival was strain dependent, and influenced by the fat content of the cheese. Lactobacilli showed better survival, especially when in low-fat cheeses. The strains of propionibacteria also survived well during the digestion of the low-fat cheeses. The results confirmed that cheese can potentially be a good carrier matrix for bacteria to the intestine. In addition, it has been shown that different strains present in cheese have different abilities to survive the conditions of the gastrointestinal tract. Younger cheese was indicated to be a better carrier, possibly because the bacteria present in those cheeses have had shorter exposure to the stress conditions occurring in cheese during prolonged maturation.

Introduction

The microbiome of the human gut represents a specific ecosystem, having complex metabolic activities that, to great extent, influence physiology and susceptibility to inflammation and diseases of every individual (Fujimura *et al.* 2010). Changes in the normal balance of the gut microflora may result in not only changes in energy harvest from foods but also numerous physiological disorders and diseases (Lozupone *et al.* 2012; Schloissnig *et al.* 2013).

Recent research is focusing on improvement of the gut health by maintaining the microbial balance in the gastro intestinal tract (GIT) via addition of a suitable microflora (Cotillard *et al.* 2013; Smid and Lacroix 2013). Complex relations in the microbial ecosystem of the human gut emphasize the significance of the interactions between the host and the existing microbiome, as well as the interactions between the existing microbiome of the human gut and bacteria introduced via food (Eckburg *et al.* 2005; Schloissnig *et al.* 2013). The main prerequisite for the efficient incorporation of exogenous micro-organisms into the existing microflora is survival and adaptation to diverse and severe GIT conditions. Different fermented foods, especially fermented dairy products, may act as carrier foods that preserve the viability of the introduced

bacteria, thus enabling them to survive the severe conditions in the gastrointestinal tract (GIT) and potentially attain a synergism with the existing gut microflora (Sharp *et al.* 2008; Karimi *et al.* 2011; Pitino *et al.* 2012).

Cheese might be a good carrier of beneficial/probiotic bacteria, as it has a firmer texture, higher pH, and a greater buffering capacity compared to fermented milks and yoghurts (da Cruz *et al.* 2009). Research conducted by Sumeri *et al.* (2012) on open texture cheeses ripened for 22 weeks, showed that cheese was a better choice for the administration of viable beneficial bacteria to the gut when compared to fermented milk products.

In testing of potentially probiotic bacteria, they have primarily been exposed to *in vitro* tests which assess their ability to survive the severe conditions in the digestive tract (Borchers *et al.* 2009). However, when bacteria are subjected to standard *in vitro* testing, the results of such tests usually only indicate their tolerance to digestive conditions without taking into account other stress factors that also affect their survival in the GIT (Faye *et al.* 2012). Therefore, recent experiments have shown that a more valid suitable option is to use digestion model systems *ex vitro*, since in such models, bacteria are given a more realistic exposure to the complex conditions in the upper part of the human digestive tract (Faye *et al.* 2012; Sumeri *et al.* 2012; Adouard *et al.* 2016).

The aim of this work was to evaluate the *ex vivo* survival of selected micro-organisms in cheeses of different fat content and after 7 and 70 weeks maturation. Cheese samples with different adjunct bacteria of lactic acid bacteria and propionic acid bacteria (PAB) were exposed to the conditions of the human upper GIT in a simulated digestion using human gastric and duodenal juices (*ex vivo* digestion).

Results and discussion

In this study, young and aged cheeses differing in fat content and supplemented with different culture combinations of starter and adjunct bacteria, were tested for the survival of cheese microflora after exposure to an *ex vivo* model digestion. Human gastrointestinal enzymes were used in order to simulate realistic conditions in the GIT, since commercial nonhuman enzymes differ in various characteristics such as the content and types of amylases, lipases, bile salts, cell mucus, etc. (Ulleberg *et al.* 2011).

pH development during the first 7 weeks of cheese ripening was uniform in all of the cheeses regardless of fat content and adjuncts (Porcellato *et al.* 2013). At 7 weeks, the pH in the 10% fat cheese was significantly lower than in the 28% fat cheese, an effect not seen after 70 weeks of maturation (Table 1). After 7 weeks of ripening, the cheeses with the added PAB showed the highest pH ($5{\cdot}55 \pm 0{\cdot}03$) ($P < 0{\cdot}01$), while the cheeses with added lactobacilli obtained the lowest pH ($5{\cdot}47 \pm 0{\cdot}02$) (results not shown). The dry matter was significantly higher in cheeses with 28% fat than in those with 10% fat (Table 1).

Table 1 pH and dry matter (mean (SD)) of the experimental cheeses at 7 and 70 weeks of ripening

		Age in weeks	
	Fat	7	70
pH	10	5·48 (0·04)*	5·66 (0·07)
	28	5·52 (0·04)	5·62 (0·05)
DM (%)	10	53·98 (0·72)***	54·82 (1·35)**
	28	58·16 (1·24)	57·83 (1·24)

Asterisks represent significant differences between the two levels of fat (*$P < 0{\cdot}05$, **$P < 0{\cdot}01$, ***$P < 0{\cdot}001$).

Porcellato *et al.* (2013) have described the development of the microbial population in the cheeses during the first 7 weeks of ripening in detail. Through further ripening from 7 to 70 weeks, the number of presumptive lactobacilli, as enumerated on LBS agar, was similar in cheeses with added adjuncts of lactobacilli regardless of their fat content (Table 2).

The numbers of presumptive lactobacilli were lower in cheeses supplemented with propionibacteria compared with cheeses supplemented with lactobacilli. In cheeses with added *Lactococcus lactis* ssp. *cremoris* starter, the number of bacteria growing on LBS agar increased by 1·3–1·8 log from 7 to 70 weeks of ripening in the 28% fat cheeses, but did not increase in the 10% fat cheeses (Table 2).

Cheeses supplemented with PAB contained approx. log 9 CFU g^{-1} cheese of PAB after 7 weeks of ripening (Porcellato *et al.* 2013), but a decrease to 5 log CFU g^{-1}occurred during further ripening to 70 weeks (Fig. 2).

The numbers of lactobacilli enumerated in the digestive fluids (DF) was higher following digestion of the younger cheeses (7 weeks) than following digestion of the well-matured cheeses (70 weeks) (Fig. 1).

After digestion of both the 7- and 70-week-old cheeses, a greater decrease in numbers of lactobacilli and PAB ($\boldsymbol{P} < 0{\cdot}01$) was found in the DF of the digested 28% fat cheeses compared to the 10% fat cheeses (Figs 1 and 2). After digestion of the 7-week-old cheese, a decrease of only 1–2 log CFU g^{-1} cheese were observed during digestion of the 10% fat cheeses, compared to a decrease of 2–3 log CFU g^{-1} cheese during digestion of the 28% fat cheeses. The numbers of lactobacilli and PAB were only 4–5 log CFU g^{-1} before digestion of cheeses ripened for 70 weeks. However, during digestion, the growth rate of the bacteria was similar to that found by digestion of the

7-week-old cheeses in the 10% fat cheeses, while a decrease of 3–4 log CFU g^{-1} cheese was found for the 28% fat cheeses.

No significant differences between the adjunct bacteria were found with respect to the survival of the different lactobacilli or PAB adjuncts during digestion.

A marked difference in the survival of the cheese bacteria during the digestion of 7- and 70-week-old cheeses was observed, and can most probably be explained by the initial numbers of lactobacilli cells in the 70-week-old cheeses at the start of digestion and the physical condition of the bacterial cells in those cheeses. Survival of lactobacilli during digestion was also higher in the low-fat cheeses (10% fat) when compared to full-fat cheeses (28% fat). The low-fat cheeses had higher moisture content, and thereby a higher a_w value, most probably creating better conditions for bacterial growth. Similar findings on better survival rate in low-fat cheeses were observed by Ganesan *et al.* (2014).

Current trends towards healthy diets cause an increased interest in reduced-fat cheeses. However, these have different technological characteristics when compared to full-fat cheeses (Gerdes 2005; Drake *et al.* 2010). During the manufacture of low-fat cheese, the fat is replaced by moisture and protein resulting, among other factors, also in higher moisture content in these cheeses and thereby higher a_w value (Esteban and Marcos 1989; Beresford *et al.* 2001; Drake *et al.* 2010).

Nonstarter Lactic Acid Bacteria (NSLAB), especially lactobacilli, are only slightly inhibited by the conditions in cheese and they can increase during cheese maturation (Beresford *et al.* 2001, Banks and Williams 2004; Beresford and Williams 2004). Lactobacilli are able to survive for over 3 years in cheese at a storage temperature of

Table 2 Numbers of lactobacilli and lactococci in the experimental cheeses after 7 and 70 weeks of ripening

	LBS 30°C (log CFU g^{-1})		M 17 30°C (log CFU g^{-1})	
Cheese	7 weeks	70 weeks	7 weeks	70 weeks
448-10	8·3	6·2	8·5	5·3
448-28	8·2	6·1	8·1	5·4
456-10	7·7	5·0	7·5	5·1
456-28	7·9	5·5	7·4	5·9
15D-10	8·2	4·7	8·5	6·0
15D-28	8·6	4·9	8·0	4·4
GG-10	8·3	5·2	8·5	4·8
GG-28	7·3	5·1	8·5	5·1
P203-10	7·3	5·3	7·3	5·2
P203-28	7·3	4·3	7·0	4·5
P303-10	7·4	6·1	7·8	4·8
P303-28	6·7	5·1	6·2	3·8

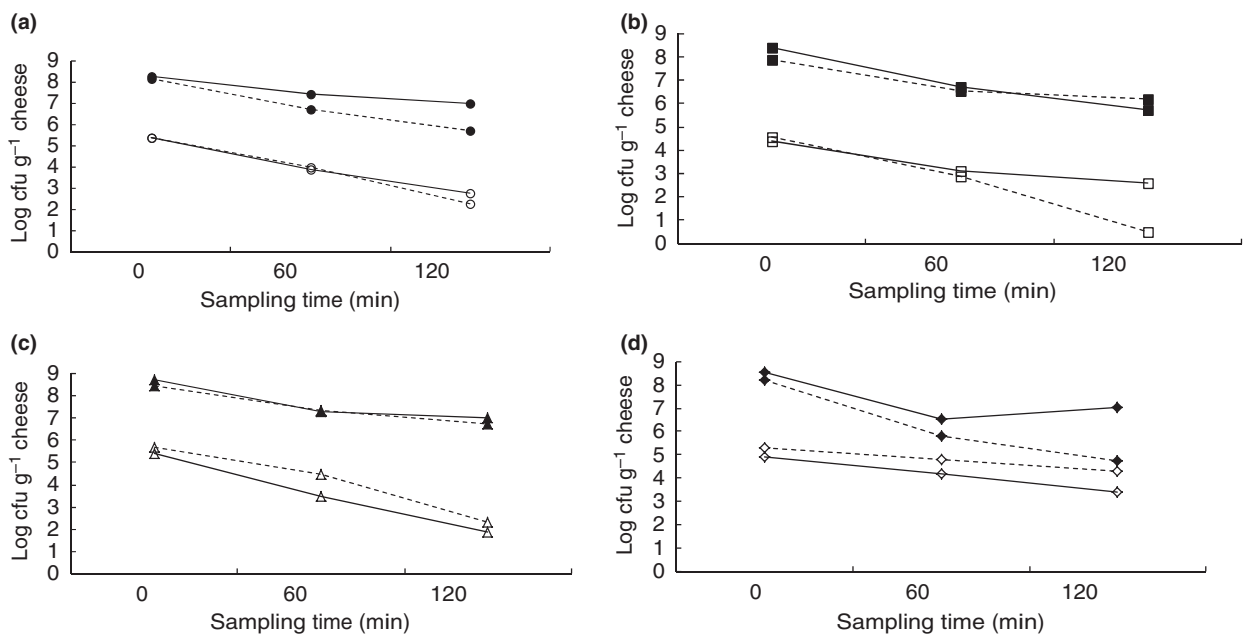

Figure 1 Numbers of *Lactobacilli* during digestion in human gastric (HGJ) and duodenum (HDJ) juice of the studied cheeses of 10 (——) and 28 (----) % fat after 7 (closed symbols) and 70 (open symbols) weeks of ripening. Enumeration made at start of digestion (0 min) and after digestion in HGJ (5–60 min) and HDJ (60–120 min). (a) *Lactobacillus paracasei* INF448, 7 weeks (●) and 70 weeks (○), (b) *Lact. paracasei* INF456, 7 weeks (■) and 70 weeks (□), (c) *Lactobacillus plantarum* INF15D, 7 weeks (▲) and 70 weeks (△) and (d) *Lactobacillus rhamnosus* GG, 7 weeks (♦) and 70 weeks (◊).

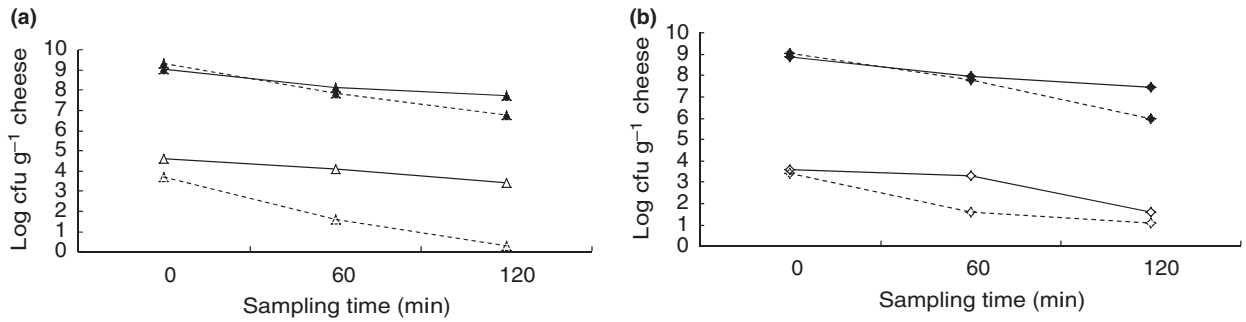

Figure 2 Numbers of PAB during digestion in human gastric (HGJ) and duodenum (HDJ) juice of the studied cheeses with 10 (——) and 28 (----) % fat after 7 (closed symbols) and 70 (open symbols) weeks of ripening. Enumeration at start of digestion (0 min) and after the digestion in HGJ (5–60 min) and HDJ (60–120 min). (a) *Propionibacterium freudenreichii* subsp. *shermanii* INF P203, 7 weeks (▲) and 70 weeks (△) and (b) *Propionibacterium jensenii* INF P303, 7 weeks (♦) and 70 weeks (◊).

10°C (Beresford and Williams 2004) and they have even been found at high numbers in a cheese stored for 6 years at 4°C (S. Skeie, personal communication). Lactobacilli are able to use other energy sources beside lactose and therefore may increase in numbers during the later stages of maturation (Martinovic *et al.* 2013). This may also explain their good survival during digestion in cheese ripened for 7 weeks, since they may have been metabolically active and in good physical condition. The lower numbers of lactobacilli in 70-week-old cheese is most probably a result of increasing stress conditions in older cheeses due to the lack of easily accessible nutrients and a lowered a_w. Our results of survival during digestion of *Lactobacillus paracasei* INF 448 and *Lact. paracasei* INF 456 in 7-week-old cheese are in agreement with findings obtained earlier for these same strains when tested under similar conditions during *ex vivo* digestion of fermented milk (Faye *et al.* 2012).

In cheese, lactobacilli develop as large clusters of cells entrapped in the cheese matrix (Martinovic *et al.* 2013). The microenvironment around such clusters regulates the nutrient supply and thereby the metabolic activity of the bacteria. Our results showed that the fat content of the cheese influenced the survival of lactobacilli during the digestion of cheeses supplemented with different lactobacillus adjuncts. The lactobacilli decreased only slightly in numbers following digestion of the 10% fat cheeses, whereas in the 28% fat cheeses, they decreased further by 1–2 log following exposure to the acidic digestive conditions. Digestion with duodenal juice, following 60 min of digestion with gastric juice, caused a further decrease in lactobacilli. However, the numbers of lactobacilli remained more stable during digestion of the 10% fat cheeses, indicating that these bacteria could enter the duodenum in substantial numbers.

PAB are used as adjunct cultures in Swiss-type cheese. They tolerate high scalding temperatures of the curd and can be found in high numbers ranging from 10^8 to 10^9 CFU g^{-1} in the cheese 2 weeks after cheesemaking (Rossi *et al.* 2000; Beresford *et al.* 2001; Fröhlich-Wyder *et al.* 2002). When the pH of the cheese is higher than 5 and the salt concentration in the moisture phase is lower than 5%, good growth of PAB can be expected (Walstra *et al.* 2006; Poonam *et al.* 2012). This was also observed in the cheeses added PAB in this study, where the numbers of these bacteria increased to around log 9 CFU g^{-1} cheese at 7 weeks of maturation (Porcellato *et al.* 2013). During digestion, the propionibacteria survived well, especially in the 7-week-old cheese. However, the numbers of PAB in the cheese decreased from 7 to 70 weeks indicating that nutrient availability probably decreased in the microenvironment around the bacteria in the cheese. During digestion, the survival rate of PAB was not influenced by the age of the cheese. However, as the initial numbers were lower, such cheese ripened for 70 weeks would contribute with fewer PAB to the intestine than similar cheese ripened for only 7 weeks. Furthermore, the fat content in the 7-week-old cheeses did not influence the numbers of PAB in cheese after digestion, while in 70-week-old cheese the numbers were somewhat higher in the low-fat cheeses, probably due to higher moisture content. The obtained results suggest that the propionic acid bacteria are less exposed to stress conditions in younger cheeses than in more aged cheeses. This can most probably be explained by a high availability of lactate, which PAB utilizes as an energy source in the younger cheeses. Subsequently, its local exhaustion around the cell during further maturation most probably caused the reduced numbers of PAB in the well-matured cheeses. Porcellato *et al.* (2013) showed that the PAB used also in this experiment maintained their numbers throughout the first 7 weeks of ripening regardless of the fat content of the cheeses. Cheese with PAB consumed at an early stage of ripening may, therefore, contribute to a higher transfer of PAB to the intestine than consumption of a well-matured cheese.

Similar studies on PAB in *in vivo* and *ex vivo* simulations with gastric and small intestinal juices have shown that they can survive the severe conditions in the GIT (Jan *et al.* 2001; Huang and Adams 2004).

Fermented foods are rich in micro-organisms that may be found in the human GIT (van Hylckama Vlieg *et al.* 2011; Tremaroli and Bäckhed 2012). These foods may, therefore, contribute to the microbiota in the human GIT provided that the viability and functionality of the microflora is preserved through the severe conditions in the human GIT (Huang and Adams 2004; da Cruz *et al.* 2009; Coeuret *et al.* 2003). Dairy products have a high buffer capacity their bacteria may be enclosed in a protein matrix.

This study demonstrated that cheese can function as a vehicle for delivery of a variety of food-related micro-organisms to the intestine and that the younger cheese was a better carrier matrix of the cheese bacteria. Besides the age of the cheese, the survival rate was influenced by the fat content of the cheese. Most of the lactobacilli and all the PAB survived well during digestion of the low-fat cheeses. This study also showed the ability of cheese lactobacilli and PAB to survive the severe conditions of GIT.

The initial numbers of lactobacilli and PAB in the cheeses at the two maturation stages before digestion were different, and the numbers were lower in the 70-week-old cheeses. This is most probably a consequence of the bacteria being more stressed due to exposure to the different stress factors affecting the bacterial growth and survival in well-matured cheeses for a longer time period. These bacteria, already stressed in the cheese matrix, were unable to fully recover after the exposure to gastric juices and to resist additional stress conditions of the duodenum.

Materials and methods

Cheesemaking procedure and experimental design of the cheese makings

Cheese was made over 3 days using two levels of fat (10 and 28% fat in cheese) and six different combinations of the starter culture with the different adjuncts as described by Porcellato *et al.* (2013) (Table 3). Using this experimental design, the effect of the adjunct had two replicates, and the fat content six replicates. The same starter (Probat Visbyvac 505; Danisco, Copenhagen, Denmark) was used in all vats.

Analysis of the cheese

In the cheese, lactococci were enumerated on M17 broth (Merck, Darmstadt, Germany) added 15 g l^{-1} Bactoagar (Saveen Werner AB, Malmø, Sweden) with aerobic incubation for 2 days at 30°C. Lactobacilli were enumerated on *Lactobacillus*-selective agar (LBS) (Difco, Sparks, MD) with anaerobic incubation for 4 days at 30°C. The number of presumptive PAB in the cheese was enumerated on sodium lactate agar (SLA) as described by Østlie *et al.* (1995), with anaerobic incubation at 30°C for 6 days.

Table 3 Experimental design with combination of adjunct and fat content of the cheeses

Adjunct	Fat content (%)	Cheese code
Lactobacillus paracasei INF448	10	448-10
Lact. paracasei INF448	28	448-28
Lact. paracasei INF456	10	456-10
Lact. paracasei INF456	28	456-28
Lactobacillus plantarum INF15D	10	15D-10
Lact. plantarum INF15D	28	15D-28
Lactobacillus rhamnosus GG*	10	GG-10
Lact. rhamnosus GG*	28	GG-28
Propionibacterium freudenreichii subsp. *shermanii* INF P203	10	P203-10
P. freudenreichii subsp. *shermanii* INF P203	28	P203-28
Propionibacterium jensenii INF P303	10	P303-10
P. jensenii INF P303	28	P303-28

*Valio Ltd, Helsinki, Finland.

The cheese was sampled and analysed after 7 and 70 weeks of ripening. Microbial counts, dry matter (IDF standard 4a 1982) and pH (Skeie *et al.* 2001) were measured at all sampling times. Sampling was done according to IDF-standard 50c (1995).

Model digestion of the cheese samples

Human gastric juice (HGJ; 36·9 U ml^{-1}) and duodenal juices (HDJ; 12·4 Uml^{-1}) were collected from 20 individual healthy volunteers, following the procedure described by Ulleberg *et al.* (2011). Enzyme activities were calculated by the pepsin activity assay for HGJ (Sanchez-Chiang *et al.* 1987) and by the total proteolytic activity assay for HDJ according to Krogdahl and Holm (1979).

Cheese samples, after 7 and 70 weeks of ripening, were digested with HGJ and HDJ. Eleven grams of cheese was placed into a sterile omni mixer (Omni Int., Waterbury, CT), supplemented with sterile citrate water (99 ml, 48°C) and mixed at 4500 rev min^{-1} for two min. The cheese suspension (0·5 ml) was transferred to two sterile plastic tubes containing sterile magnetic stirrers before incubation in a water bath circulator (Julabo MB-7a; Julabo Labortechnik GmbH, Seelbach, Germany) preheated at 37°C, simulating the conditions of the human digestive system. The digestion process was followed according to Faye *et al.* (2012) with the following modifications to adapt to the cheese matrix. The pH in the first

step was adjusted to 3·0 using 1 mol l^{-1}HCl. After 5 min incubation, 178 μl of HGJ was added. After 60 min digestion, the sample pH was adjusted to 7·0 using 1 mol l^{-1} NaOH and then 1569 μl of the HDJ was added and incubated further for 60 min. Samples for determination of the viable cell counts were taken in duplicates, after 0, 60 and 120 min of incubation.

Statistical analysis

To investigate the effects of the experimental factors adjunct culture and fat % at each ripening stage (7 and 70 weeks of ripening) and digestion stage (0, 60 and 120 min of digestion), Analysis of Variance (ANOVA) was carried out using MINITAB 16.2.2 (Minitab Inc., State College, PA), using the general linear model (GLM) procedure. In addition to investigating differences in the decrease of bacteria during digestion, the 0–120 min decrease in bacteria during digestion was used in calculations. Differences between the means of the adjunct culture were found by Tukey's honest significant difference (HSD) test for pairwise comparison of the means.

Ethical standards

The manuscript does not contain clinical studies or patient data.

Conflict of Interest

The authors report no conflict of interest and are alone responsible for the content and writing of this manuscript.

References

Adouard, N., Magne, L., Cattenoz, T., Guillemin, H., Foligné, B., Picque, D. and Bonnarme, P. (2016) Survival of cheese-ripening microorganisms in a dynamic simulator of the gastrointestinal tract. *Food Microbiol* **53**, 30–40.

Banks, J.M. and Williams, A.G. (2004) The role of nonstarter lactic acid bacteria in Cheddar cheese ripening. *Int J Dairy Technol* **57**, 145–152.

Beresford, T. and Williams, A. (2004) The microbiology of cheese ripening. In *Cheese: Chemistry, Physics and Microbiology* ed. Fox, P.F., McSweeney, P.L.H., Cogan, T.M. and Guinee, T.P. pp. 287–318. London: Elsevier Academic Press.

Beresford, T.P., Fitzsimons, N.A., Brennan, N.L. and Cogan, T.M. (2001) Recent advances in cheese microbiology. *Int Dairy J* **11**, 259–274.

Borchers, A.T., Selmi, C., Meyers, F.J., Keen, C.L. and Gershwin, M.E. (2009) Probiotics and immunity. *J Gastroenterol* **44**, 26–46.

Coeuret, V., Dubernet, S., Bernardieau, M., Gueguen, M. and Vernoux, J.P. (2003) Isolation, characterization and identification of lactobacilli focusing mainly on cheeses and other dairy products. *Lait* **83**, 269–306.

Cotillard, A., Kennedy, S.P., Kong, L.C., Prifti, E., Pons, N., Le Chatelier, E., Almeida, M., Quinquis, B. *et al.* (2013) Dietaryintervention impact on gut microbial gene richness. *Nature* **500**, 585–588.

da Cruz, A.G., Buriti, F.C.A., de Souza, C.H.B., Faria, J.A.F. and Saad, S.M.I. (2009) Probiotic cheese: health benefits, technological and stability aspects. *Trends Food Sci Technol* **20**, 344–354.

Drake, M.A., Miracle, R.E. and McMahon, D.J. (2010) Impact of fat reduction on flavor and chemistry of Cheddar cheeses. *J Dairy Sci* **93**, 5069–5081.

Eckburg, P.B., Bik, E.M., Bernstein, C.N., Purdom, E., Dethlefsen, L., Sargent, M., Gill, S.R., Nelson, K.E. *et al.* (2005) Diversity of the human intestinal microbial flora. *Science* **30**, 1635–1638.

Esteban, M.A. and Marcos, A. (1989) Chemical prediction of water activity in processed cheese. *J Dairy Res* **56**, 665–668.

Faye, T., Tamburello, A., Vegarud, G.E. and Skeie, S. (2012) Survival of lactic acid bacteria from fermented milks in an in vitrodigestion model exploiting sequential incubation in human gastric and duodenum juice. *J Dairy Sci* **95**, 558–566.

Fröhlich-Wyder, M.T., Bachmann, H.P. and Gerard Casey, M. (2002) Interaction between propionibacteria and starter/non-starter lactic acid bacteria in Swiss-type cheeses. *Lait* **82**, 1–15.

Fujimura, K.E., Slusher, N.A., Cabana, M.D. and Lynch, S. (2010) Role of the gut microbiota in defining human health. *Expert Rev Anti Infect Ther* **8**, 435–454.

Ganesan, B., Weimer, B.C., Pinzon, J.Dao., Kong, N., Rompato, G., Brothersen, C. and McMahon, D.J. (2014) Probiotic bacteria survive in Cheddar cheese and modify populations of other lactic acid bacteria. *J Appl Microbiol* **116**, 1642–1656.

Gerdes, S. (2005) Dairy detective: new life for lower-fat cheese. *Dairy Foods* **48**, (November) http://www.dairyfoods.com/articles/83169-dairy-detective-new-life-for-lower-fat-cheese.

Huang, Y. and Adams, M.C. (2004) In vitro assesment of the upper gastrointestinal tolerance of potential probiotic dairy propionibacteria. *Int J Food Microbiol* **91**, 253–260.

van Hylckama Vlieg, J.E.T., Veiga, P., Zhang, C., Derrien, M. and Zhao, L. (2011) Impact of microbialtransformation of food on health- from fermented foods to fermentation in the gastro-intestinaltract. *Curr Opin Biotechnol* **22**, 1–9.

IDF (1982) *Cheese and Processed Cheese: Determination of the Total Solids Content. IDF Standard 4a*. Brussels, Belgium: International Dairy Federation.

IDF (1995) *Milk and Milk Products: Guidance on Sampling. IDF standard 50c*. Brussels, Belgium: International Dairy Federation.

Jan, G., Leverrier, P. and Roland, N. (2001) Survival and beneficial effects of *propionibacteria* in the human gut: in vivo and in vitro investigations. *Lait* **82**, 131–144.

Karimi, R., Mortazavian, A., Gomes, M. and Da Cruz, A. (2011) Viability of probiotic microorganisms in cheese during production and storage: a review. *Dairy Sci Technol* **91**, 283–308.

Krogdahl, A. and Holm, H. (1979) Inhibition of human and rat pancreatic proteinases by crude and purified soybean proteinase inhibitors. *J Nutr* **109**, 551–558.

Lozupone, C.A., Stombaugh, J.I., Gordon, J.I., Jansson, J.K. and Knight, R. (2012) Diversity, stability and resilience of the human gut microbiota. *Nature* **489**, 220–230.

Martinovic, A., Moe, K.M., Romeih, E., Aideh, B., Vogensen, F.K., Østlie, H. and Skeie, S. (2013) Growth of adjunct *Lactobacillus casei* in Cheddar cheese differing in milk fat globule membrane components. *Int Dairy J* **31**, 70–82.

Østlie, H.M., Vegarud, G.E. and Langsrud, T. (1995) Autolysis of lactococci: detection of lytic enzymes by polyacrylamide gel electrophoresis and characterization in buffer systems. *Appl Environ Microbiol* **61**, 3598–3603.

Pitino, I., Randazzo, C.L., Cross, K.L., Parker, M.L., Bisignano, C., Wickham, M.S.J., Mandalari, G. and Caggia, C. (2012) Survival of *Lactobacillus rhamnosus* strains inoculated in cheese matrix during simulated human digestion. *Food Microbiol* **31**, 57–63.

Poonam, Pophaly, S.D., Tomar, S.K. and Singh, R. (2012) Multifaceted attributes of dairy propionibacteria: a review. *World J Microbiol Biotechnol* **28**, 3081–3095.

Porcellato, D., Østlie, H.M., Brede, M.E., Martinovic, A. and Skeie, S. (2013) Dynamic of adjunct non starter lactic acid bacteria and propionic acid bacteria in low-fat and full-fat cheese. *Int Dairy J* **33**, 104–111.

Rossi, F., Dellaglio, F., Peluso, M. and Torriani, S. (2000) Dairy propionibacteria: occurrence-resistance to technological stresses and antagonistic properties (cheesemaking). *Ind Aliment* **39**, 553–557.

Sanchez-Chiang, L., Cisternas, E. and Ponce, O. (1987) Partial purification of pepsins from adult and juvenile salmon fish *Oncorhynchus keta*. Effect of NaCl on proteolytic activities. *Comp Biochem Physiol* **87**, 793–797.

Schloissnig, S., Arumugam, M., Sunagawa, S., Mitreva, M., Tap, J., Zhu, A., Waller, A., Mende, D.R. *et al.* (2013) Genomic variation landscape of the human gut microbiome. *Nature* **493**, 45–50.

Sharp, M., McMahon, D.J. and Broadbent, J. (2008) Comparative Evaluation of Yogurt andLowFat CheddarCheeseas Delivery Media for Probiotic *Lactobacillus casei*. *J Food Sci* **73**, 375–377.

Skeie, S., Lindberg, C. and Narvhus, J. (2001) Development of amino acids and organic acids in Norvegia, influence from milk treatment and adjunct *Lactobacillus*. *Int Dairy J* **11**, 399–411.

Smid, E.J. and Lacroix, C. (2013) Microbe- microbe interactions in mixed culture food fermentations. *CurrOpin Biotechnol* **24**, 148–154.

Sumeri, I., Adamberg, K., Uusna, R., Sarand, I. and Paalme, T. (2012) Survival of cheese bacteria in gastrointestinal tract simulator. *Int Dairy J* **25**, 36–41.

Tremaroli, V. and Bäckhed, F. (2012) Functional interactions between the gut microbiota and hostmetabolism. *Nature* **489**, 242–249.

Ulleberg, E.K., Comi, I., Holm, H., Herud, E.B., Jacobsen, M. and Vegarud, G.E. (2011) Human gastrointestinal juices intended for use in *in vitro* digestion models. *Food Dig* **2**, 52–61.

Walstra, P., Wouters, J.T.M. and Geurts, T.J. (2006) *Dairy Science and Technology*, 2nd edn. Boca Raton: CRC Press, Taylor & Francis Group, LLC.