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Dynamics of starter, adjunct non starter lactic acid bacteria and propionic acid bacteria in low-fat and full-fat Dutch-type cheese

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Abstract

The microbial dynamics of Dutch-type cheeses differing in starter (DL or single strain of *Lactococcus (Lc.) lactis* subsp. *cremoris*), adjunct (*Lactobacillus (Lb.)* or *Propionibacteria*) and fat contents (10 or 28% fat) were investigated by culture-dependent and culture-independent analysis. The cheese microbiota was dominated by the adjunct *Lactobacillus* after 4 weeks of ripening and the fat content did not influence the microbial diversity. The *Leuconostoc* spp., presumably from the DL starter, was detected in cheeses made with added *Lb. plantarum* and *Lb. rhamnosus* and was not detected in cheese made with added *Lb. paracasei* after 4 and 7 weeks by denaturing gradient gel electrophoresis. No *Lactobacillus* spp. were detected in cheese with added *Propionibacteria*, while *Leuconostoc* was the only species detected. In cheeses made with *Lc. lactis* subsp. *cremoris* as starter, the *Lactobacillus* microbiota was similar to the cheese milk microbiota after 24 hours while after 4 weeks different species of *Lactobacillus* and *Leuconostoc* were detected.

1. Introduction

The microbial population play a key role during cheese manufacture and ripening, contributing to flavour and texture of the final product (Banks & Williams, 2004; Beresford, Fitzsimons, Brennan, & Cogan, 2001). Lactic acid bacteria (LAB) are present in cheese as an added starter, but may also originate from contamination of the milk from dairy environment (Beresford, Fitzsimons, Cogan, & Condon, 1999). Within the LAB group, the Non Starter LAB (NSLAB) are defined as secondary microbiota. They are not added to the cheese but are able to grow during the harsh conditions found in cheese (Banks & Williams, 2004; Lynch, McSweeney, Fox, Cogan, & Drinan, 1996). The most common NSLAB species found in Cheddar and Dutch-type cheese varieties made from pasteurized or microfiltered milk are mesophilic lactobacilli as *Lactobacillus (Lb.) casei/paracasei, Lb. plantarum* and *Lb. curvatus* (Fitzsimons, Cogan, Condon, & Beresford, 1999; Jordan & Cogan, 1993; Østlie, Eliassen, Florvaag, & Skeie, 2004).

Isolates from the NSLAB flora may be added as adjuncts to the cheese for their ability to survive and affect the cheese flavour. Other secondary microorganisms as the dairy propionic acid bacteria (PAB) are important for eye formation and the typical flavour formation in Swiss-type cheeses (Thierry & Maillard, 2002; Thierry, Maillard, Herve, Richoux, & Lortal, 2004). A reduction of the fat content in cheese affects both flavour and texture, possibly because of the fat removal itself but also because the fat removal changes the environment for cheese microbiota, especially as the moisture content usually is increased. To improve the flavour and texture of low fat cheese, the use of selected starter culture and adjunct flavour-producing strains have been suggested (Beresford et al., 2001; Mistry, 2001; Randazzo, Pitino, De Luca, Scifo, & Caggia, 2008; Van Hoorde et al., 2010).

Molecular techniques have been widely used for the study of microbial dynamics in dairy products for their accuracy and reproducibility. Culture-dependent methods for identification at species and strain level are known to be time-consuming and laborious, while culture-independent methods have been shown to give a faster and more reliable identification of the bacterial community (Quigley et al., 2011). Anyway, the combination of culture-dependent and culture-independent methods have been shown to give a better understanding of the microbial communities in cheese (Bonetta, Bonetta, Carraro, Rantsiou, & Cocolin, 2008; Dolci et al., 2008; Ndoye, Rasolofo, LaPointe, & Roy, 2011; Randazzo, Pitino, Ribbera, & Caggia, 2010). Denaturing Gradient Gel Electrophoresis (DGGE) has been widely applied for the characterization of the microbial dynamics in cheese during ripening (Jany & Barbier, 2008; Ndoye et al., 2011).

The aim of the present study was to perform a screening of the microbial dynamics in a Dutch-type cheese differing in fat content and primary and secondary starter composition during cheese making and ripening. The microbial dynamics of the cheese milk and cheese were followed by a combination of culture-dependent and culture-independent analysis.

2. Materials and methods

2.1 Cheese making

Washed-curd, brine salted cheeses were made in four days with two levels of fat (10 and 28 % fat in cheese) and eight different culture combinations of starter and adjuncts (Table 1). In total, 16 vats of cheese were manufactured. The cheese milk was obtained from the university herd. The skimmed milk was microfiltered (1.4 µm membranes), pasteurized (72 °C, 15 s) and standardized to 1.0 or 2.7 % fat with pasteurized cream (74 °C, 15 s). Cheese

80 was made from 350 L milk (10 % fat) or 300 L milk (28 % fat) as described by Skeie et al.
81 (2001) with some modifications. In short, pre-ripening of milk and starter was 30 min at 32
82 °C for the 28 % fat cheese while for the 10 % fat cheese pre-ripening was 45 min at 30.5 °C.
83 The rennet used was ChyMax Plus (Chr. Hansen, Hørsholm, Denmark) (min. 600
84 International Milk Clotting Units/ml). Whey drainage was 40 % (vol/vol) and water addition
85 was 40 % (vol/vol) for the 28 % fat cheese while for the 10 % fat cheese whey drainage was
86 45 % (vol/vol) and water addition was 20 % (vol/vol). The scalding temperature was 39 °C
87 for 40 min for the 28 % fat cheese while for the 10 % fat cheese the scalding temperature
88 was 36 °C for 45 min. Plastic cheese moulds giving 5 kg cheese (Laude b.v., Ter Apel, The
89 Netherlands) were used. The cheeses were salted in brine for 10 h. The cheese was kept for
90 10 days at 11 °C and plastic coated twice with Ceska-coat (Producan, Kolding, Denmark)
91 during this time, then for 14 days at 19 °C. Then the cheeses were wrapped in plastic bags
92 and stored at 4 °C for the remaining ripening period. On cheesemaking day 1, 2 and 3 (Table
93 1), a commercial freeze-dried DVS mesophilic DL starter, Probat Visbyvac 505 (Danisco,
94 Copenhagen, Denmark) was used as 1% (vol/vol) inoculum. On cheesemaking day 4, two in
95 house lactococci strains were used (Table 1) as starters inoculated as 1% (vol/vol) bulk
96 starter. The bulk starter was made by inoculation of the strain in skimmed milk (heat treated
97 at 90 °C for 30 min) for 18 h at 22 °C. The adjunct lactobacilli were inoculated (1 % vol/vol)
98 in De Man-Rogosa Sharpe broth (MRS, Difco, Sparks , USA) and grown at 30 °C for 20 h
99 and the adjunct propionibacteria were inoculated (1 %) in sodium lactate broth (SLB) as
100 described by Faye et al (2002) and grown at 30 °C for 20 h. The inoculation in the cheese
101 vats was 0.3 % (vol/vol) for both *Lactobacillus* and *Propionibacteria*. Before and after
102 cheesemaking the dairy equipment was washed and disinfected with steam water.

2.2 Gross composition and microbial sampling of milk and cheese

Sampling for gross composition and microbial analysis were made according to IDF-standard 50c (1995). Microbial counts, pH and dry matter were measured immediately after sampling. Dry matter was determined according to IDF standard 4a (1982). The pH was measured as described by Skeie et al. (2001). Lactococci were enumerated on M17 broth (Merck, Darmstadt, Germany) added 15 g L^{-1} Bactoagar (Saveen Wener AB, Malmø. Sweden) after aerobic incubation for 2 days at 30 °C for cheeses made with DL starter while at 22 °C for cheeses made with *Lc. lactis* subsp. *cremoris* strains. Lactobacilli were enumerated on *Lactobacillus* selective agar (LBS agar, Difco) after anaerobic incubation in anaerobic incubator (W.C. Hearaeus GmbH, Hanau, Germany) with 10 % v/v CO_2 for 4 days at 30°C. Propionibacteria were enumerated on sodium lactate broth added 15 g L^{-1} Bactoagar (SLA) (Saveen Wener AB) after anaerobic incubation in anaerobic jars (Oxoid, Hampshire, England) at 30°C for 6 days. The samples of milk were analyzed before rennet addition (CMBR), the fresh cheeses 24 h after starter addition and ripened cheeses after 4 and 7 weeks of ripening. Milk before microfiltration (CMBM), milk after microfiltration (CMAM), the starters (ST), cheese milk before rennet (CMBR) and cheese samples at all sampling times were stored at -80 °C until analysis. Frozen cheese from 7 weeks of ripening were plated, in LBS agar plates at appropriate dilutions, and five colonies were randomly picked and purified by successive subculturing on MRS agar (Difco) before DNA isolation. For cheeses Ar1-28 and Bf2-28 a pre-incubation of the cheese slurries at 30°C for 2 days was performed in MRS broth (Difco) due to difficulties in growing bacteria on LBS agar directly from the cheese slurry.

126 *2.3 DNA isolation of bacteria, 16S rRNA gene sequencing and primer design*

127 DNA from the adjunct strains (Table 1) and cheese isolates was extracted from 1 mL
128 overnight culture grown at 30°C by GelElute Bacterial Genomic DNA kit (Sigma-Aldrich,
129 St. Louis, MO) according to the manufacturer's instructions. Sequencing of the 16S rRNA
130 gene was performed with the universal primers 1F (5'- GAGTTTGATCCTGGCTCAG -3')
131 and 5R (5'-GGTTACCTTGTTACGACTT-3'), used for amplification of a 1460 bp DNA
132 fragment of the 16S rRNA gene. PCR products were purified and sequenced using the
133 BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems). All the 16S rRNA
134 sequences obtained from the pure strains and the adjuncts were aligned with CLC Main
135 Workbench 6 (CLC bio A/S, Aarhus, Denmark), before identifying regions specific for
136 *Propionibacterium* spp. and designing of specific primers. The primer pair specific for the
137 genus *Propionibacterium* was designed around the V3 region of the 16S rRNA with the use
138 of Primer3 Input (version 0.4.0, http://frodo.wi.mit.edu/primer3/).

139 *2.4 Extraction of total DNA from dairy samples and PCR conditions*

140 Extraction of bacterial DNA from milk and cheese was performed as described
141 previously (Porcellato, Grønnevik, Rudi, Narvhus, & Skeie, 2012a). The PCR was
142 performed in a final volume of 20 µL as described by Porcellato et al. (2012a). The PCR
143 programme was performed according to Walter et al. (2000) with some modifications. The
144 PCR amplification was run in a 96 multiwell LightCycler 480 Real-Time instrument
145 (Roche) with initial denaturation at 95 ^{o}C for 5 min. The PCR programme consisted of 30
146 cycles of denaturation at 95 ^{o}C for 30 s, annealing at 61 ^{o}C for 30 s and elongation at 72 ^{o}C
147 for 1 min. Annealing temperature for the PAB specific primer was 60 ^{o}C.

2.5 DGGE and high resolution melt analysis conditions

The DGGE and high resolution melt analysis (HRM) conditions were performed as reported previously (Porcellato et al., 2012a). Selected DGGE bands were excised from the gel with a sterile scalpel blade before transfer to a sterile eppendorf tube containing 50 µL of 0.1x TE buffer and incubation at 4 °C for 4 h. The PCR amplification was performed as described previously, adding 2 µL of the extracted DNA as template. The DGGE band identification by HRM analysis and sequencing was performed as described by Porcellato et al. (2012a).

2.6 Cheese isolates identification and characterization by HRM

DNA from cheese isolates was isolated according to GelElute Bacterial Genomic DNA kit (Sigma-Aldrich) instructions. Characterization of the isolates was performed as described by Porcellato et al. (2012b) by using HRM analysis of the V1 and V3 regions of the 16S rRNA and sequencing of the 16S rRNA gene. Gene scanning analysis, performed by LightCycler® 480 software Version 1.5 (Roche, Mannheim, Germany), and clustering analysis were used for the characterization of the HRM profiles. Rep-PCR fingerprinting analysis of the isolates and adjuncts were performed using primer $GTG_{(5)}$ according to Porcellato et al. (2012b).

3. Results

3.1 Gross composition and microbial enumeration in agar plates

The pH decreased from 6.7 ± 0.07 in the cheese milk to 5.3 ± 0.11 in the fresh cheese 24 h after starter addition and remained stable or had a slight increase during the 7 weeks of

ripening (Table 2). The dry matter content of the full-fat cheeses increased from 49.35 % ± 2.24 after 24 h to 58.01 ± 1.09 % after 7 weeks of ripening while the dry matter content of the low-fat cheese increased from 48.55 ±1.86 % to 53.67 ± 0.97 after 7 weeks of ripening. Initially, the number of presumptive lactococci, as enumerated on M17, in CMBR was at log number 6-7 cfu ml^{-1} for cheeses made with DL starter and adjunct. The highest numbers were enumerated after 24 h and the numbers subsequently decreased during further ripening (Table 2). In cheeses made with added lactobacilli, the plate counts on LBS agar showed initial numbers of log 6 - 7 cfu mL^{-1} in the cheese milk before rennet addition and an increase to log 8 - 9 cfu mL^{-1} in the cheese after 24 h. During further ripening the numbers on LBS agar in these cheese remained stable (Table 2). In cheese made with added PAB, the counts on LBS agar plates were high after one day but decreased to log 6 – 7 cfu g^{-1} during ripening. In the cheeses made with the two strains of *Lc. lactis* sub. *cremoris*, no growth on the LBS agar plates were observed for cheese milk before rennet addition and in the 24 h old cheese. Growth on the LBS agar plates were detected at low numbers after 4 weeks in the low-fat cheese and after 7 weeks in the full fat cheese (Table 2). A faster decrease of the microbial counts on the M17 and LBS agar plates was observed in cheeses made with added PAB. The enumeration on SLB agar plates showed a fast growth already during the first 24 h of cheese making and a slow growth during the further ripening process.

3.2 DGGE analysis

The two set of primers used (Lac1-Lac2 and Lac3-Lac2) were specific for the *Lactobacillus/Leuconostoc/Pediococcus/weisella* (*Lb./Leu./Ped.*) and the *Lactococcus/Streptococcus/Enterococcus (Lc./St./En.)* genus, respectively, and they

191 amplified the V3 region of the 16S rRNA gene. By using primer Lac3-Lac2, no differences
192 were found between cheese milk, starter culture and cheese samples. The only species
193 detected was *Lc. lactis* subsp. *lactis* and *Lc. lactis* subsp. *cremoris.* Weak DGGE bands were
194 shown in the cheese milk before microfiltration (CMBM) and after microfiltration (CMAM)
195 while intense bands were shown for the starter, CMBR and cheeses (data not shown). In the
196 CMBM at cheesemaking day 3 and 4, two bands were identified by sequencing as
197 *Streptococcus* sp. and *Streptococcus (St.) dysgalactiae* (99% identity, GenBank accession
198 no.: JF789447.1), respectively.

199 More information on the population dynamics of the cheese milk, starters and cheese
200 were found by the Lac1-Lac2 primer pair. The *Lb./Leu./Ped.* population of CMBM and
201 CMAM were constituted of *Lb. kefiri* (band 1 Fig. 1A, Table 3), *Lb. buchneri/parabuchneri*
202 (band 2 Fig. 1A, Table 3) and *Lb. kefiranofaciens* (band 3 Fig. 1A, Table 3) for all 4 days of
203 experimental cheese making. The *Lb. kefiranofaciens* and *Lb. buchneri/parabuchneri* were
204 seen as weak bands while *Lb. kefiri* was the dominant species as indicated by an intense
205 band. The DL-starter samples showed the presence of 2 bands identified as *Leu.*
206 *mesenteroides/pseudomesenteroides* (ex. band 4 and 5 Fig.1A, Table 3). All CMBR samples
207 from cheese made using the DL starter showed also the presence of *Leu. mesenteroides* (ex.
208 band 6 and 7, Fig.1A, Table 3), and the presence of the adjunct *Lactobacillus* sp.. Cheese
209 made with *Lactobacillus* adjuncts showed during the 7 week ripening period an increased
210 intensity of the adjunct bands. The same DGGE gel patterns were shown for cheeses made
211 with added any of the two *Lb. paracasei* strains (Fig. 1A, pattern shown only for *Lb.*
212 *paracasei* INF448). Cheese made with added *Lb. rhamnosus* showed the presence of several

bands, and four of the bands corresponded to *Lb. rhamnosus* as shown by DGGE analysis of the pure strain and sequencing (data not shown).

Leuconostoc spp. from the DL-starter was detected in the cheeses made with added *Lb. paracasei* 448 (Fig. 1A) and *Lb. paracasei* INF456 from day 1, shown with a clear and strong band, but *Leuconostoc* was not detected in the cheese after 4 and 7 weeks of ripening. On the contrary after 4 and 7 weeks in the cheeses made with added *Lb. plantarum* 15D and *Lb. rhamnosus* GG, bands corresponding to *Leuconostoc* spp. were still detected but with reduced and weak intensity (band 10 and 11 Fig 1A, Table 3). In cheeses made with added propionibacteria, only strong bands corresponding to *Leuconostoc* spp. (bands 1 and 2 Fig. 1B, Table 3) were detected. No lactobacilli were detected in cheese made with added PAB (Fig. 1B). The *Lb./Leu./Ped.* population in cheese made with a single strain starter of *Lc. lactis* subsp. *cremoris* showed after 24 h, a DGGE pattern of *Lb./Leu./Ped.* identical to the cheese milk (Fig. 1C). After 4 weeks of ripening the *Lb./Leu./Ped.* microbiota was totally changed. None of the lactobacilli species identified in the cheese milk were detected after 4 and 7 weeks while species of *Lb. paracasei* and *Lb. plantarum* were found (band 1 and 2 Fig. 1C, Table 3) together with *Lb. rhamnosus, Leu. mesenteroides* and other *Lb.* sp. The fat content of the cheese did not seem to influence the microbial composition. The same band intensity and the presence of representative species bands were seen between cheeses made with different fat contents (Fig. 1A, 1B, 1C).

3.3 Isolate identification and characterization

A total of 80 strains were purified from LBS plates after plating of the cheese samples at 7 weeks of ripening. Comparison of the V1 and V3 16S rRNA region HRM profiles with the

235 reference strain profiles and sequencing allowed identification at the species level. Isolates
236 from cheese made with added *Lb. paracasei* were identified as the same species as the
237 adjunct or as *Leu. mesenteroides/pseudomesenteroides* (1-2 isolates out of 5). All the
238 isolates from cheese made with added *Lb. rhamnosus* and *Lb. plantarum* as adjuncts, were
239 identified as the same species as the adjunct. Isolates from cheese with added PAB were
240 identified as *Leu. mesenteroides/pseudomesenteroides* as well as for cheeses made with the
241 single strain culture of lactococci. One isolate from the cheese made with *Lc. lactis* subsp.
242 *cremoris* Ar1 was identified as *Lb. sakei* by sequencing of the 16S rRNA gene. From cheese
243 with *Lc. lactis* subsp. *cremoris* Ar1 and 28 % fat all isolates from the pre-incubated cheese
244 slurry were identified as *Enterococcus (En.)* spp.. Rep-PCR fingerprinting analysis by HRM
245 profiles of the isolates and the adjuncts with a $(GTG)_5$ primer showed how the added strains
246 of *Lb. paracasei* 448, *Lb. paracasei* 456, *Lb. plantarum* INF15D and *Lb. rhamnosus* GG
247 were isolated in the various cheese after 7 weeks (Figure 3).

248 *3.4 Identification of propionibacteria*

249 The designed PAB specific primers, PABV3F (5'-ACGGCCTTCGGGTTGTAA-3') and
250 PABV3R (5'- CACGTAGTTAGCCGGTGCTT- 3'), were tested for PAB specificity by
251 qPCR and HRM on the strains listed in Table 1. Amplification on qPCR and DGGE bands
252 were shown only for the PAB strains. The DGGE migration distance and the HRM profiles
253 were specific for each of the PAB strains analysed allowing the specific characterization.
254 The primer pair was used further to detect PAB in the milk and cheese samples. The DGGE
255 pattern and HRM profiles identified the presence of the added PAB species in the CMBR
256 and cheese samples, (Fig 2, band 1 and 2, Table 3).

4. Discussion

The microbial dynamics during manufacture and ripening of Dutch-type cheese with two different fat contents and with different adjuncts and starter combination were screened by culture-dependent and culture-independent methods. Combination of plate-counting, culture-dependent HRM and 16S rRNA sequencing and culture-independent DGGE gave a detailed description of the development of lactic acid and propionic acid bacteria during cheese manufacture and ripening. The plate counts showed the development of the LAB and PAB communities in the cheese without qualitative information of the species present, but when associated with the culture-independent DGGE the description of the species was achieved.

Lactobacillus and *Propionibacteria* adjuncts were added at 6 log cfu mL^{-1} in the cheese milk and they grew to level found previously in during ripening (Beresford et al., 2001; Rehn et al., 2011). The microbial counts of *Lactobacillus* and *Lactococcus* in cheese made with added propionibacteria showed a large decrease during ripening compared to cheese made with different *Lactobacillus* species as adjunct, however, a high amount of propionibacteria was detected in these cheeses. Cheeses made with added *Propionibacterium* spp. usually have a high content of propionic acid, and the amount of propionic acid may be correlated with the inhibition of other microorganism (Baer & Ryba, 1999; Beresford et al., 2001; Rehn et al., 2011).

The HRM analysis has previously been described as a method for characterization of NSLAB in cheese (Porcellato et al., 2012b). After isolation from the LBS agar plates the isolates from the 7 weeks old cheese were identified by HRM and 16S rRNA gene sequencing. Agreement between the species identification of the isolates and the DGGE

results were found for the cheeses made with added PAB, where *Leu.* spp. were identified as the dominating organism among the *Lb./Leu./Ped.* population during ripening. However, opposite results were found between cheeses made with added *Lb. paracasei*, *Lb. plantarum* and *Lb. rhamnosus.* DGGE analysis showed the presence of weak bands corresponding to *Leu.* in cheeses made with added *Lb. plantarum* INF15D and *Lb. rhamnosus* GG, while in cheeses made with added *Lb. paracasei* only bands corresponding to *Lb. casei/paracasei* where seen. These results may indicate that in cheese, the growth of *Leuconostoc* is dependent on the dynamics of the microbiota during ripening. The selection of the colonies from the agar plates was made according to morphological differences and might have influenced the results of the species characterization. In addition, the incubation temperature of the agar plates used may have affected the results. However, the *Lb.* adjuncts may also repress growth of *Leuconostoc* from the DL-starter and dominate the microbiota during the ripening process. The presence and predominance of *Lb. paracasei* in the NSLAB flora of mature semi-hard Dutch-type and Cheddar cheese has been reported by many authors (Antonsson, Molin, & Ardo, 2003; Crow, Curry, & Hayes, 2001; Østlie et al., 2004; Østlie, Eliassen, Florvaag, & Skeie, 2005). Few studies reports how the NSLAB microbiota is influences by PAB in semi-hard Dutch-type cheese made with added PAB, Rehn et al. (2011) showed higher count of PAB compared to starter and NSLAB.

Enterococcus spp. were identified in cheese Ar1-28 after pre-incubation of the cheese slurry in MRS broth. Species of enteroccoci may be found in cheeses made from raw and pasteurized milk. They may be used as a part of the cheese starter culture or they may come from environmental contamination (Giraffa, 2003). The *Enterococcus* spp. were not detected

by DGGE analysis probably due to the low amount present and thus below the DGGE detection limit as showed in previous work (Dolci et al., 2008).

Rep-PCR fingerprinting analysis of the isolates and the adjuncts with the GTG_5 primer by HRM profiles was performed to compare the isolates from the 7 weeks old cheese made with added adjuncts. The same melting profiles were seen for the *Lb*. adjunct strains and the isolates from the 7 week old cheese, indicating that the isolates were the same as the ones added, and that the adjuncts were among the predominant microbiota.

The identification of the DGGE bands was performed by HRM profile comparison with reference strains and by sequencing. The combination of both primer sets used, achieved a higher description of the species present in the samples compared to the use of universal primers (Endo & Okada, 2005). The cheesemakings of the experiment was performed at four different days, therefore different batches of milk were used at each cheese production. The microbial community detected in the raw milk before microfiltration and pasteurisation showed the presence of *St. dysagalactiae,* which is a mastitis pathogen that may be found in milk (Calvinho, Almeida, & Oliver, 1998; Dolci, Alessandria, Rantsiou, Bertolino, & Cocolin, 2010) and the presence of a *Streptococcus* sp. which could not be identified by sequencing. The two bands were not identified in the cheese milk after microfiltration and pasteurisation. The detection of *Lb. kefiri, Lb. kefiranofaciens* and *Lb. buchneri* in all cheese milk used in the experiments may be related to environmental contamination from the dairy plant throughout the whole cheese making process (Kagkli, Vancanneyt, Hill, Vandamme, & Cogan, 2007; Somers, Johnson, & Wong, 2001). *Lb. kefiri* and *Lb. kefiranofaciens* have previously been detected in raw milk cheeses and Ricotta cheese (Baruzzi, Morea, Matarante, & Cocconcelli, 2000; Dolci et al., 2008; Henri-Dubernet, Desmasures, &

Gueguen, 2008). *Lb. kefiri* was also detected by DGGE analysis in samples of cream from the same dairy pilot plant and as the skimmed milk was microfiltered the *Lb. kefiri* most probably were transferred to the cheese milk with the cream (result not shown).

Addition of selected *Lactobacillus* sp. during cheese making influences the cheese flavour and texture (Di Cagno et al., 2011; Hynes et al., 2003; Milesi, Wolf, Bergamini, & Hynes, 2010; Settanni & Moschetti, 2010). The DGGE analysis throughout the cheese ripening up to seven weeks showed the influence of the adjunct NSLAB on the microbial dynamics. The three different species of lactobacilli added in the first two days of cheese making clearly dominated the microbiota after 4 and 7 weeks. Due to the adaptation of the surviving bacteria in the cheese microenvironment, facultative heterofermentative *Lactobacillus* have been shown to dominate the cheese microbiota during ripening in Cheddar and Dutch-type cheeses (Antonsson, Ardo, & Molin, 2001; Beresford et al., 2001; Østlie et al., 2004, 2005). The DGGE of the cheese made with only *Lactococcus* spp. showed the presence of the same adjunct species used in the previous days of the cheesemaking experiment. Mesophilic *Lactobacillus* may survive the cleaning and disinfection with hot water process in the dairy plant and might be a source of NSLAB contamination of the cheese (Kagkli et al., 2007; Somers et al., 2001).

Similar DGGE patterns were seen in low-fat and full-fat cheeses made with the same added adjunct. The microbial diversity detected by both primer sets was not influenced by the fat content, although low-fat and full-fat cheeses differs in their moisture content and salt in moisture. However, differences between low-fat and full-fat cheeses have previously been shown in their microbiota, volatile compounds and flavours suggesting an influence of the fat contents on the NSLAB microbiota and their metabolism (Drake, Miracle, & McMahon,

2010; Oberg, Moyes, Domek, Brothersen, & McMahon, 2011; Urbach, 1995). Molecular approaches based on RNA analysis may, however, contribute to the study of the metabolic active communities during cheese ripening as showed previously (Masoud et al., 2011).

In conclusion, the present study shows how the starter, adjuncts of *Lactobacillus* and PAB might influence the microbial dynamics in a Dutch-type cheese differing in fat content. This study was performed as a screening experiment of the microbial dynamics of the cheese and although each adjunct was not replicated over several days, the results highlight how the fat content did not seem to influence the microbial diversity nor the amount of LAB and PAB in the cheese. After 4 weeks of ripening, the microbiota was dominated by the adjunct *Lb. paracasei* while in cheese with added *Lb. plantarum* and *Lb. rhamnosus* the presence of *Leuconostoc* was also found. In cheese with added PAB, *Leuconostoc* was the only species identified among the *Lb./Leu./Ped.* group. These results showed that the *Leuconostoc* development in cheese was influenced by the microbial dynamics of the cheese.

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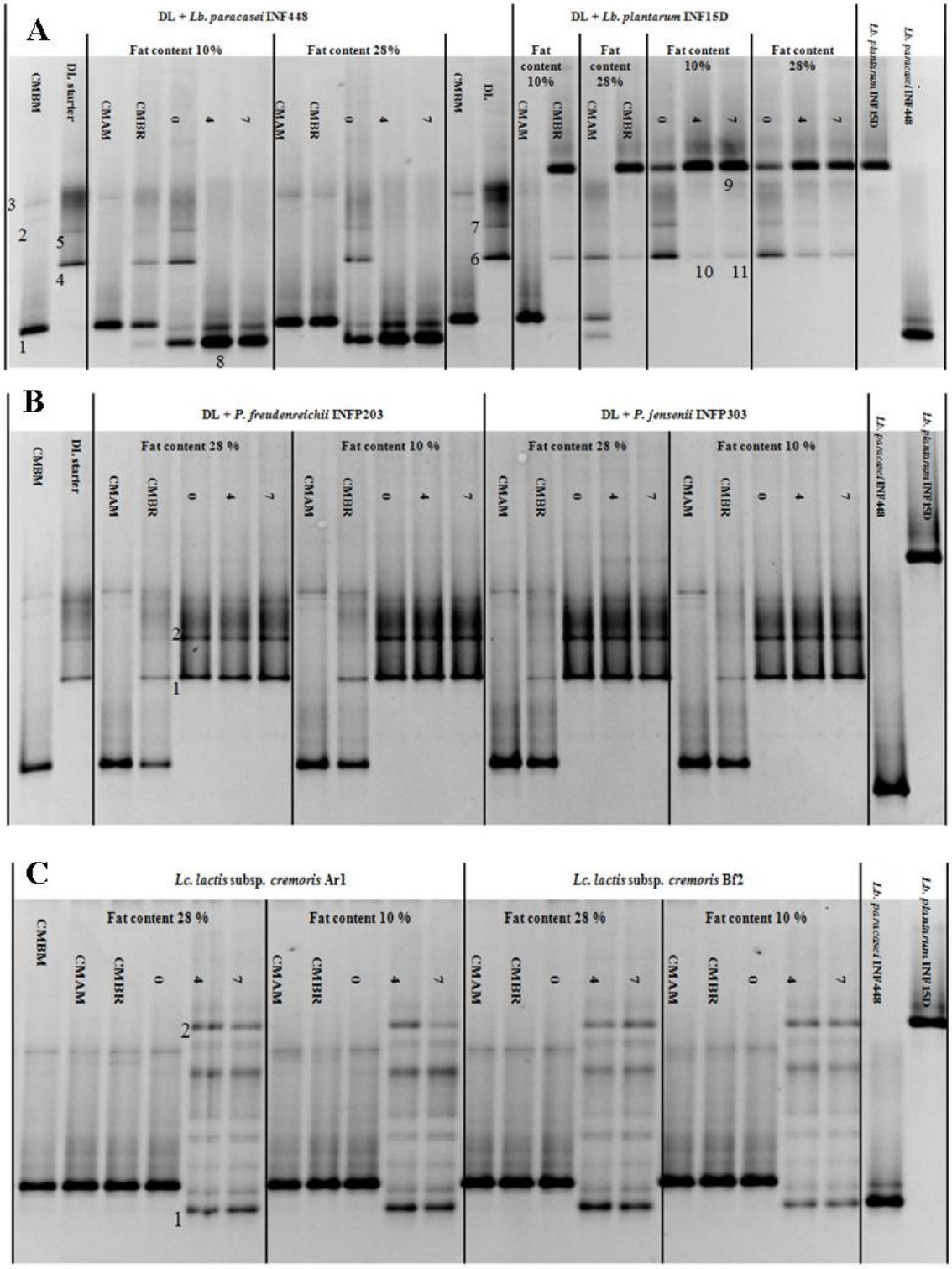
Figure caption

Fig. 1. Denaturing gradient gel electrophoresis profile obtained with primer LAC1F and LAC2R of milk, starter and cheeses made with (A) adjunct *Lb. paracasei* INF448 and *Lb. plantarum* INF15D, (B) adjunct *P. freudenreichii* INFP203 and *P. jensenii* INFP303, (C) starter *Lc. lactis* subsp. *cremoris* Ar1 and *Lc. lactis* subsp. *cremoris* Bf2. CMBM: cheese milk before microfiltration; CMAM: cheese milk after microfiltration; CMBR: cheese milk before rennet addition; 0: cheese after 24 hours; 4: cheese after four weeks; 7: cheese after seven weeks.

Fig. 2. Denaturing gradient gel electrophoresis (DGGE) profile and high resolution melting (HRM) profiles obtained with primer specific for propionic acid bacteria for milk and cheese with added *P. Freudenreichii* INFP203 and *P. jenseii* INFP303. (a) DGGE analysis with primer pair PABV3F and PABV3R. CMBM: cheese milk before microfiltration. CMAM: cheese milk after microfiltration; CMBR: cheese milk before rennet addition; 0: cheese after 24 hours; 4: cheese after four weeks; 7: cheese after seven weeks. (b) HRM melting profiles of *P. freudenreichii* INFP203 and cheeses made at day 3, vat 1 and 2 (profile P1) and of *P. jenseii* INFP303 and cheeses made at day 3, vat 3 and 4 (profile P2).

Fig. 3. Melting peak genotypes of reference strains (black) used in cheese making and isolates from cheese after 7 weeks obtained by high resolution melting-rep-PCR fingerprinting with primer GTG_5. (a) *Lb. paracasei* INF448 and *Lb. paracasei* isolates from cheese DL-448-10/28. (b) *Lb. paracasei* INF456 and *Lb. paracasei* isolates from cheese DL-456-10/28. (c) *Lb. plantarum* INF15D and *Lb. plantarum* isolates from cheese DL-15D-10/28. (d) *Lb. rhamnosus* GG and *Lb. rhamnosus* isolates from cheese DL-GG-10/28.

538 **Fig.1**



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541 **Fig. 2**

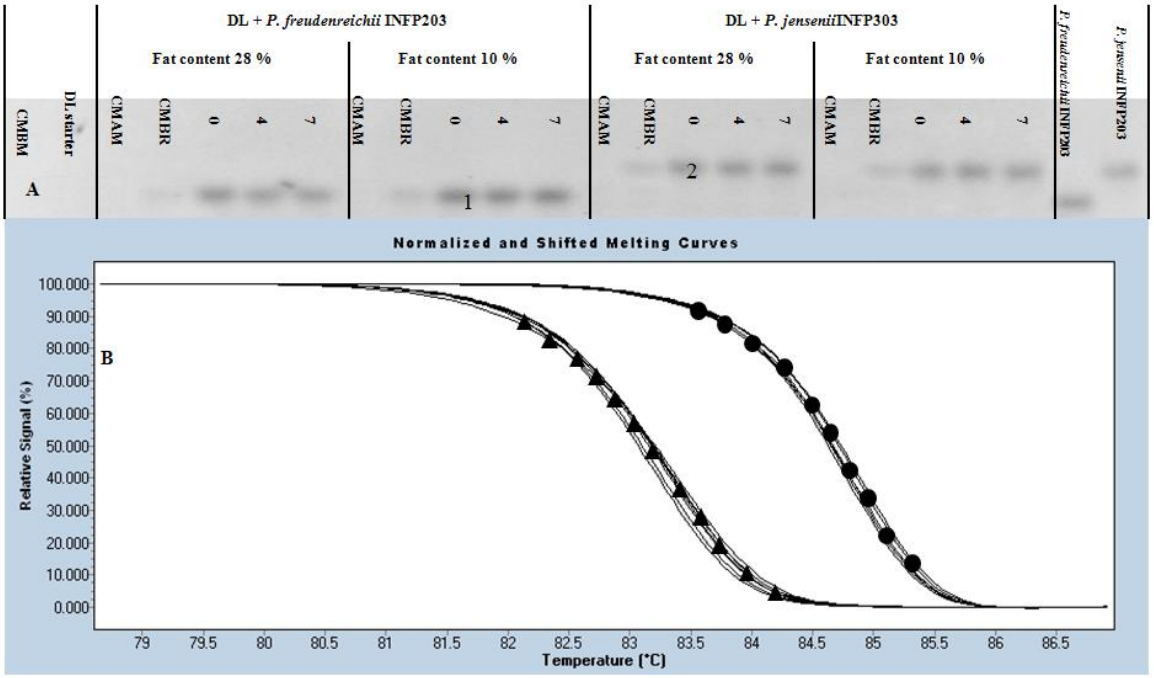

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Fig. 3.

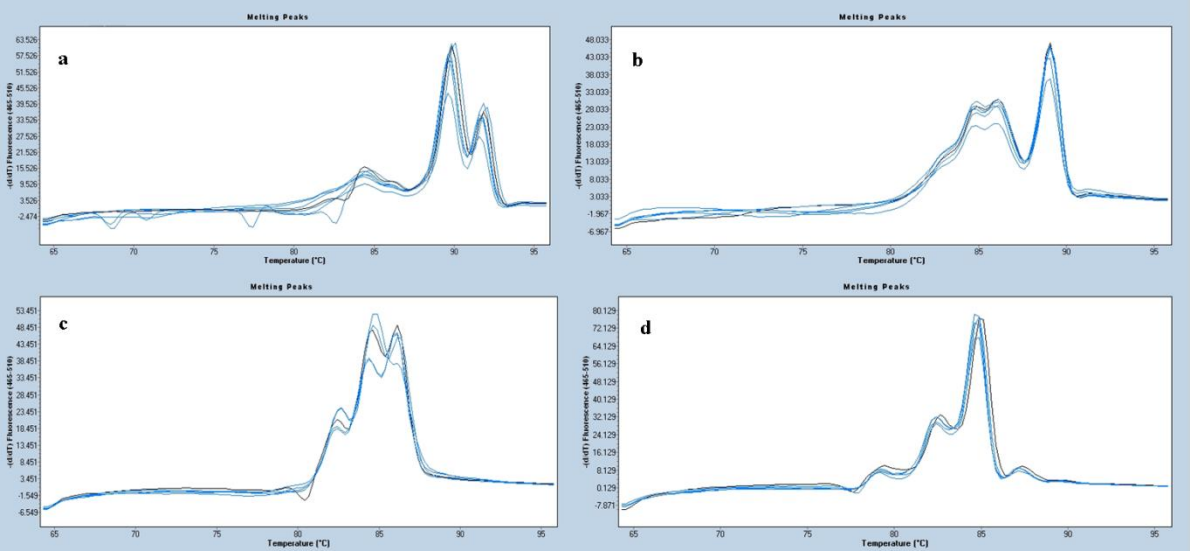

Table 1. Experimental design with combination of starter, adjunct and their source, fat content and cheese coding.

Day	Vat	Starter[A]	Adjunct	Source of the starter / adjunct	Fat content	Coding
1	1	DL	*Lb. paracasei* INF448[C]	Cheese	10	DL-448-10
1	2	DL	*Lb. paracasei* INF448 [C]	Cheese	28	DL-448-28
1	3	DL	*Lb. paracasei* INF456 [C]	Cheese	10	DL-456-10
1	4	DL	*Lb. paracasei* INF456 [C]	Cheese	28	DL-456-28
2	1	DL	*Lb. plantarum* INF15D [C]	Cheese	28	DL-15D-28
2	2	DL	*Lb. plantarum* INF15D [C]	Cheese	10	DL-15D-10
2	3	DL	*Lb. rhamnosus* GG	[B]	28	DL-GG-28
2	4	DL	*Lb. rhamnosus* GG	[B]	10	DL-GG-10
3	1	DL	*P. freudenreichii* INFP203 [C]	Cheese	28	DL-P203-28
3	2	DL	*P. freudenreichii* INFP203 [C]	Cheese	10	DL-P203-10
3	3	DL	*P. jensenii* INF P303 [C]	Unknown	28	DL-P303-28
3	4	DL	*P. jensenii* INF P303 [C]	Unknown	10	DL-P303-10
4	1	*L.lactis* subsp. *cremoris* INFAr-1 [C]	-	Fermented milk	28	Ar1-28
4	2	*L.lactis* subsp. *cremoris* INFAr-1 [C]	-	Fermented milk	10	Ar1-10
4	3	*L. lactis* subsp. *cremoris* INFBf-2 [C]	-	Fermented milk	28	Bf2-28
4	4	*L. lactis* subsp. *cremoris* INFBf-2 [C]	-	Fermented milk	10	Bf2-10

[A] DL starter: Probat Visbyvac 505, Danisco, Copenhagen, Denmark
[B]:Valio Ltd, Helsinki, Finland
[C] :Department of Chemistry, Biotechnology and Food Science, Norwegian University of Life Science, Norway

Table 2. pH development and microbial plate count of the cheese milk and cheese during ripening. ,

	pH					LBS 30 °C (log CFU g^{-1})				M17 30 °C (log CFU g^{-1})				SLB 30 °C (log CFU g^{-1})			
Cheese	CMBR[b]	AP[b]	0w[b]	4w[b]	7w[b]	CMBR[b]	0w[b]	4w[b]	7w[b]	CMBR[b]	0w[b]	4w[b]	7w[b]	CMBR[b]	0w[b]	4w[b]	7w[b]
DL-448-28	6.7	6.1	5.4	5.5	5.5	6.6	9.0	8.2	8.2	6.7	9.4	8.3	8.1	-	-	-	-
DL-448-10	6.7	5.7	5.5	5.5	5.5	6.5	8.6	8.3	8.3	6.9	8.9	8.4	8.5	-	-	-	-
DL-456-28	6.7	6.0	5.4	5.5	5.5	6.8	9.0	8.0	7.9	6.8	9.5	7.8	7.4	-	-	-	-
DL-456-10	6.7	5.6	5.4	5.5	5.5	6.8	8.6	8.2	7.7	6.9	8.7	8.7	7.5	-	-	-	-
DL-15D-28	6.6	6.1	5.2	5.4	5.5	7.0	8.5	8.3	8.6	7.2	9.4	8.2	8.0	-	-	-	-
DL-15D-10	6.6	5.7	5.1	5.4	5.5	6.9	7.9	8.2	8.2	7.2	8.3	8.7	8.5	-	-	-	-
DL-GG-28	6.6	6.0	5.2	5.4	5.5	6.7	8.8	8.2	7.3	6.6	9.2	8.9	8.5	-	-	-	-
DL-GG-10	6.7	5.6	5.2	5.5	5.5	6.6	8.9	8.0	8.3	6.8	8.8	8.6	8.5	-	-	-	-
DL-P203-28	6.7	5.9	5.4	5.5	5.6	6.0	8.3	7.0	7.3	6.7	9.2	7.1	7.0	7.2	9.2	9.2	9.4
DL-P203-10	6.7	5.7	5.3	5.4	5.5	6.0	8.2	7.5	7.3	6.6	8.8	8.2	7.3	7.2	9.0	9.1	9.1
DL-P303-28	6.7	6.0	5.4	5.4	5.6	5.7	8.3	6.9	6.7	6.6	9.2	6.4	6.2	7.0	9.1	9.1	9.2
DL-P303-10	6.7	5.8	5.4	5.4	5.6	5.9	8.2	7.4	7.4	6.7	8.8	8.0	7.8	7.1	8.9	8.9	8.9
Ar1-28[a]	6.5	5.8	5.3	5.3	5.5	<2	<2	<4	4.1	6.6	8.8	7.1	6.6	-	-	-	-
Ar1-10[a]	6.5	5.7	5.2	5.3	5.5	<2	<2	5.4	5.3	6.7	8.9	6.5	6.1	-	-	-	-
Bf2-28[a]	6.5	6.1	5.3	5.3	5.5	<2	<2	<4	3.0	7.2	9.2	7.2	7.2	-	-	-	-
Bf2-10[a]	6.5	5.8	5.3	5.4	5.5	<2	<2	4.8	5.4	7.1	9.3	5.2	5.1	-	-	-	-

[a] Microbial count in M17 agar plates was performed at 22 °C

[b] CMBR: cheese milk before rennet; AP: cheese after pressing; 0w: cheese after 24 hours; 4w: cheese after four weeks; 7w: cheese after 7 weeks.

565 **Table 3. Band sequencing information and comparison with GenBank reported**
566 **sequences**

Band number	Closest identification	% identity	Accession number
Fig 1A band 1	*Lb. kefiri*	100	HM218551
Fig 1A band 2	*Lb. buchneri*	99	HM058035
Fig 1A band 3	*Lb. kefiranofaciens*	99	AB690261
Fig 1A band 4	*Leu. mesenteroides*	98	AB669420
Fig 1A band 5	*Leu. mesenteroides*	97	JF727530
Fig 1A band 6	*Leu. mesenteroides*	98	AB669418
Fig 1A band 7	*Leu. mesenteroides*	99	HM218757
Fig 1A band 8	*Lb. casei*	99	HF562841.1
Fig 1A band 9	*Lb. plantarum*	97	HF562839
Fig 1A band 10	*Leu. mesenteroides*	100	JQ286945
Fig 1A band 11	*Leu. mesenteroides*	100	AB671574
Fig 1B band 1	*Leu. mesenteroides*	98	AB671574
Fig 1B band 2	*Leu. mesenteroides*	99	HM218757
Fig 1C band 1	*Lb. casei*	98	JX561105
Fig 1C band 2	*Lb. plantarum*	97	JX861200
Fig 2 band 1	*Prop. freudenreichii*	99	NR044816
Fig 2 band 2	*Prop. jensenii*	98	NR042269