Elsevier Editorial System(tm) for International Dairy Journal

Manuscript Draft

Manuscript Number: INDA-D-12-00029R3

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

Article Type: Research Article

Corresponding Author: Dr. Siv Borghild Skeie, Ph.D.

Corresponding Author's Institution: Norwegian University of Life Sciences

First Author: Aleksandra Martinovic, PhD

Order of Authors: Aleksandra Martinovic, PhD; Kim Marius Moe; Ehab Romeih, PhD; Finn K Vogensen, PhD; Bashir Aideh; Hilde Østlie, PhD; Siv Borghild Skeie, Ph.D.

Abstract: This study describes the effect of two adjunct Lactobacillus casei strains on the lactobacilli population of low-fat Cheddar cheese. 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 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 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 the fresh cheese. Electron micrographs of ripened cheese showed large clusters of clearly elongated lactobacilli.

*Manuscript
Click here to view linked References

# 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 Life 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*

*Corresponding author.* Tel.:+47 64 96 58 44; fax:+47 46 80 15 05

*E-mail address:* siv.skeie@umb.no (S. Skeie)

ABSTRACT

This study describes the effect of two adjunct *Lactobacillus casei* strains on the lactobacilli population of low-fat Cheddar cheese. 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 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 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 the 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, Beresford, Steele, Broadbent, Shah & Ustunol, 2007). The population of NSLAB isolated from Cheddar cheese made from pasteurized milk is dominated by *Lactobacillus* (*Lb.*) *casei/paracasei* (Fox, McSweeney & Lynch, 1998; Beresford et al., 2001). Non-starter lactic acid bacteria, 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 (Fox et al., 1998; Hynes, Bergamini, Suarez & Zalazar, 2003; Cogan et al., 2007; El Soda, Madkor & Tong, 2008). The growth substrate for the NSLAB microflora in cheese is not fully known, but it has been hypothesized that mesophilic lactobacilli are able to utilize, 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; Laloy, Vuillemard, El Soda & Simard, 1996; Williams & Banks, 1997; Fox et al., 1998; Østlie, Vegarud & Langsrud, 1995; Adamberg et al., 2005; Moe, Faye, Abrahamsen, Østlie & Skeie, 2012).

Numerous studies have been carried out in order to improve the overall quality of low-fat cheese (Collins, McSweeney & Wilkinson, 2003; Kilcawley, O'Connell, Hickey, Sheehan, Beresford, & McSweeney, 2007; Liu, Xu & Guo, 2008) which generally is of inferior quality compared to 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 (Fox et al., 1998; Fox, Wallace, Morgan, Lynch, Niland & Tobin, 1996; Lynch, Muir, Banks, Mc Sweeney & Fox, 1999; El Soda, Madkor & Tong, 2000; Beresford et al., 2001). In low-fat cheese, it has been assumed that the NSLAB microflora develops differently compared to 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 experiments made with adjuncts use inoculations > log 4 cfu $mL^{-1}$ (Lynch, Mc Sweeney, Fox, Cogan & Drinan, 1996; Puchades, Lemieux & Simard, 1989; Skeie, Kieronczyk, Eidet, Reitan, Olsen & Østlie, 2008a; Skeie, Kieronczyk, Næs & Østlie, 2008b; Broadbent, Houck, Johnson & Oberg, 2003) 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 made with low inoculation levels of adjunct *Lb. casei/Lb. 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 (Coeuret, Dubernet, Bernardieau, Gueguen & Vernoux, 2003; Berthier & Ehrlich, 1998). 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 (De Urraza, Gomez-Zavaglia, Lozano, Romanowski & Antoni, 2000; Singh, Pawas, Singh & Heller, 2009; Berthier, Beuvier, Dasen & Grappin, 2001; Ben Amor, Vaughan & De Vos, 2007).

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 utilize 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 (powder), was achieved by adding either buttermilk powder (BMP) or skim milk powder (SMP) to the cheese milk. In cheese milk with SMP addition, cream was added to standardize the fat and protein contents to be comparable with that of the BMP cheese milk. Factor 2: Adjunct, was achieved using two different 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), both added in the amount of log 2 cfu $mL^{-1}$ and differing in their ability to utilise MFGM components (Moe et al., 2012). The adjuncts were previously described as *Lb. paracasei* INF 448 and *Lb. paracasei* INF 456 (Østlie,

Eliassen, Florvaag & Skeie, 2004; Skeie et al., 2008b; Moe et al., 2012) according to species specific PCR-identification. The reclassification is based on the observation that the type strain *Lactobacillus 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 buttermilk 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 & Skeie (2012). The BMP had 96.6 % dry matter (DM) and 9.8 % fat and 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 pasteurized buttermilk than in skimmed milk.

142

143 *2.3. Cheese milk*

144 Raw milk was obtained from a local farm in Cork, Ireland, and was separated
145 (45 °C) and standardised to 0.5 % fat using a table top milk separator (Clair, Milky;
146 Althofen, Austria), before pasteurization (72 °C, 15 sec). The six cheese making vats,
147 containing 20 L of milk each, were mixed and supplemented with different amounts
148 of BMP, SMP and cream according to the procedure described by Romeih et al.,
149 (2012). The vats of cheese milk were stored at 4 °C for 17 h to ensure that the proteins
150 of the powders were fully hydrated before further cheesemaking.

151

152 *2.4. Cheese making procedures*

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

161

162 *2.5. Cheese analyses*

163 Cheese sampling was carried out performed as described in IDF (1995) standard
164 50c after 24 h (time 0) and after 2, 4, 6, 10 and 24 weeks of ripening. Microbial
165 counts (Skeie, Lindberg & Narvhus, 2001), pH (Skeie et al., 2001) and dry matter
166 (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 analyzed 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 x 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 x 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 7,500 x.

*2.7. Bacterial isolates*

2.7.1. Morphological and physiological characterization 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 $30^{o}$ 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 $^{o}$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 $^{o}$C.

Isolates were analyzed by observing the morphology using phase contrast microscopy, Gram reaction and catalase reaction (3 % $H_2O_2$). Carbon dioxide production was determined by an infra red gas analyzer (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 $^{o}$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 characterized on the basis of their carbohydrate fermentation patterns by using the API 50 CHL system (BioMérieux, Marcy l'Etoile, France). Analysis of the obtained fermentation profiles was done by 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 synthesized by Invitrogen (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 of 10 x PCR buffer, 1 µL of 10 mM dNTP, 4 µL of 25 mM $MgCl_2$, 2 µL of DNA template and 0.5 µL of 5 U $\mu L^{-1}$ 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 $^{o}$C for 3 min; 34 cycles consisting of denaturation at 94 $^{o}$C for 15 sec, primer annealing at 54 $^{o}$C for 15 sec, elongation at 72 $^{o}$C for 1 min; a final extension step at 72 $^{o}$C for 10 min.

The PCR products were purified using E.Z.N.A.$^{TM}$ Cycle-Pure Kit (Omega Biotek, Norcross, GA, USA) according to the procedure recommended by the supplier. Sequencing was done using a BigDye 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 $^{o}$C for 10 sec; 25 cycles consisting of denaturation at 96 $^{o}$C for 10 sec, primer annealing at 50 $^{o}$C for 5 sec, polymerization and ddNTPs incorporation at 60 $^{o}$C for 4 min. Sequences were edited using BioEdit software (Abbott, CA, USA) and analyzed 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 analyzed in order 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 normalized 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 to 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 (Table 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 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 to 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).

*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 to cheeses with added adjuncts.

Production of 3-methyl butanal was 1.2 times higher in cheeses with added BMP compared to 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 (FAA) were analyzed after 10 and 24 weeks, and the distribution of FAA 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 FAA 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 content of FAA, 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 FAA 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

368 omitted from the dataset, ANOVA revealed that cheeses with BMP had significantly
369 higher levels of Leu after 10 weeks and 24 weeks of ripening than cheeses with added
370 SMP (results not shown). Cheeses with adjuncts had significantly lower contents of
371 Ile than the cheese without adjunct after 10 weeks of ripening. Cheeses added *Lb.*
372 *casei* INF 456 had higher levels of Thr than the cheeses without adjuncts and cheeses
373 with *Lb. casei* 448, both after 10 and 24 weeks. After 24 weeks of ripening the
374 cheeses without adjuncts had lower levels of Asp, Asn and Ser than the cheeses with
375 adjuncts.
376
377 *3.6. Phenotypic characterization*
378 Phenotypic characterization showed that all of the isolates (a total of 993) were
379 Gram positive and catalase negative bacilli showing colony morphology on MRS agar
380 ranging from large round, white colonies to small, opalescent colourless colonies. The
381 $CO_2$ production was at the level of homofermentative organisms (lower than 1,000
382 mg $kg^{-1}$) ranging from 98 mg $kg^{-1}$ to 261 mg $kg^{-1}$.
383 The results obtained by API 50 CHL showed that 97 out of 100 isolates tested in
384 this study were ribose positive. Among the isolates from 24-hour cheeses, the API 50
385 CHL results revealed the highest phenotypic diversity (results not shown), whereas
386 the isolates from the ripened cheeses had a more similar fermentation pattern. From
387 the PCA (Fig. 6) the isolates from 4- (25 isolates) and 24- (18 isolates) week old
388 cheeses were clustered on the basis of their sugar fermentation pattern, whereas the
389 isolates from 24-hour cheese seemed to be randomly distributed (results not shown).
390 A clear clustering of the isolates was observed after four weeks of ripening with
391 respect to the adjuncts as well as to the powder addition (MFGM content; Fig. 6a).
392 Isolates from cheeses with added *Lb. casei* INF 448 formed one cluster, whereas

393 cheeses with added *Lb. casei* INF 456 formed a second cluster. A third cluster was
394 formed with the isolates from the cheeses without added adjunct (only SMP or BMP).
395 The differences between isolates diminished throughout the maturation period and the
396 24-week old cheese isolates (Fig. 6b) made two distinct clusters with no relationship
397 to the experimental design.
398
399 *3.7. Species identification by 16S rRNA sequence analysis*
400 The highest microbial diversity was observed in cheeses at start of maturation
401 (24 hours of ripening), and the population composition became more uniform by the
402 end of maturation (24 weeks of ripening). Most of the isolates were identified as *Lb.*
403 *casei/Lb. paracasei*. At the start of ripening (time 0), 27 out of 32 analyzed isolates
404 from all cheeses were identified as *Lb. casei/Lb. paracasei*, four of the tested isolates
405 were identified as *Lb. brevis* (isolates 5 (SMP+448-0), 13 (BMP+448-0), 23
406 (SMP+456-0) and 24 (SMP+456-0)), whereas one belonged to *Lb. rhamnosus* (17
407 (SMP+456+0)).
408 In the 10 week old cheeses, 24 out of 25 tested isolates belonged to *Lb.*
409 *casei/Lb. paracasei* and only one isolate was characterized as *Lb. curvatus* ( 66 (
410 BMP- 10)). All of the 42 tested isolates from 4 and 24 week old cheeses were
411 identified as *Lb. casei/Lb. paracasei*.
412
413 *3.8. Rep-PCR typing*
414 Rep-PCR gave bands in the range of 0.3 to 8 kbp, which made it possible to
415 cluster the 100 isolates. The REP1R-Dt and REP2R-Dt primer set generated
416 fingerprints containing between 10 and 22 visualized PCR products. The analysis of
417 the generated banding patterns is shown as a dendrogram (Fig. 7 a-d).

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

424 Most of the isolates from the 24-hour old cheese (Fig. 7a) grouped together with
425 at least 40 % similarity, showing little grouping on the basis of the experimental
426 factors. Clustering with at least 80 % similarity was observed in ten small clusters.
427 The clustering of the isolates was rather random and seemed not to correlate with the
428 adjunct addition, however, some clustering was correlated with the MFGM
429 composition of the cheeses. In the 4 week old cheeses (Fig. 7b), clustering based on
430 the different experimental treatments was observed in five clusters with at least 80 %
431 similarity. The first cluster comprised isolates from cheese with added *Lb. casei*
432 INF456 (isolates 52, 53, 54, 55, 56 and 57) with the exception of one isolate from the
433 vat supplemented with *Lb. casei* INF448 (isolate 51). This cluster also had two sub-
434 groups due to the MFGM content of the cheese. The second cluster was grouped
435 according to MFGM content, but seemed to be random regarding the adjuncts. The
436 isolates of the second and third cluster had a random clustering and mostly consisted
437 of isolates from the cheese vats without added adjuncts or the vats supplemented with
438 the adjunct strain *Lb. casei* INF448. The last two clusters were grouped according to
439 the adjunct addition; they all came from vats added *Lb. casei* INF448.

440 Clustering of the isolates from the 10 week old cheeses (Fig. 7c) showed three
441 different clusters with at least 80 % similarity, and also showing also sub-clusters
442 within the clusters. The first group (isolates 60, 61, 62, 64 and 65) represents the

443 isolates from the cheese vats without added adjuncts and one isolate supplemented
444 with *Lb. casei* INF448 (isolate 67). The second group (isolates 68, 69, 70, 71 and 72)
445 was isolates from the vats supplemented with *Lb. casei* INF448 and two isolates from
446 cheese vats without adjuncts (isolates 58 and 63). Isolates 80 and 81 showed an
447 identical pattern and grouped with 100 % similarity. The rest of the isolates from 10
448 week old cheeses grouped together with less than 80 % similarity.

449 The most uniform grouping was observed among the isolates from the 24 week
450 old cheeses (Fig. 7d), represented by three clusters which grouped with at least 80 %
451 similarity; only four of the isolates had less than 80 % similarity with the others. The
452 isolates of the first two clusters were isolated from cheese with added BMP. Isolates
453 89, 90, 91 and 96 clustered together with 100 % similarity. At this stage of ripening
454 there seemed to be less clustering according to the experimental factors than earlier in
455 the ripening.

456

457 **4. Discussion**

458 This work was undertaken to follow the evolution of the microflora of low fat
459 cheeses with a low initial number of adjunct lactobacilli over a 24 week maturation
460 period. Adjuncts are commonly added at high numbers, around log 5 cfu $mL^{-1}$, to the
461 cheese milk to dominate the cheese microflora (Fox et al., 1996), but in this study, the
462 adjuncts were added at log 2 cfu $mL^{-1}$ in the cheese milk. By using a low innoculum,
463 the adjunct colonies were sparsely distributed in the cheese, whereas the lactococci
464 were more densely distributed. During the first 24 h, the number of presumptive
465 lactobacilli increased to log 4.5 cfu $g^{-1}$, indicating growth of adjuncts and/or NSLAB
466 during cheese making. The rep-PCR showed that the adjunct bacteria did not
467 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.

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

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

502 In Cheddar type cheese which is commonly made with no addition of $Cit^{+}$
503 starter bacteria, the production of the flavour compounds, such as diacetyl and
504 acetoin, was not expected. The amounts of these components were highest in the
505 cheeses without added adjuncts. It has been shown in earlier studies that the
506 concentration of citrate in Cheddar cheese is decreasing rather slowly and its
507 degradation results from the enzymatic activity of the NSLAB microflora at late
508 stages of ripening (Singh, Drake & Cadwallader, 2003; Thomas, 1987a). Diacetyl and
509 acetoin may be synthesized through transamination of Asp and Asn leading to
510 formation of oxaloacetate which can be metabolized to acetoin and diacetyl by some
511 lactobacilli (Kieronczyk, Skeie, Langsrud, Le Bars & Yvon, 2004; Skeie et al.,
512 2008b). In addition, diacetyl can also be produced from oxaloacetate by spontaneous
513 decarboxylation of the intermediate acetolactate. Since the cheeses without added
514 adjuncts had reduced contents of Asp and Asn, it is most probably indigenous
515 NSLAB flora in these cheeses that caused this degradation, resulting in increased
516 levels of diacetyl and acetoin. In the cheeses with added adjunct, the growths of
517 indigenous NSLAB were most probably repressed by the presence of the lactobacilli

518 adjuncts. In addition, it is known that the adjunct *Lb. casei* INF 448 is not able to
519 degrade Asp in cheese (Skeie et al., 2008b). Furthermore, both adjuncts used in this
520 study are not able to metabolize citrate in milk to diacetyl and acetoin (unpublished
521 results).

522 It has been indicated in previous studies of semi-hard cheeses, that mesophilic
523 lactobacilli predominate in the later stages of cheese ripening (Berthier & Ehrlich,
524 1998; Østlie et al., 2004; Crow, Curry & Hayes, 2001). The rep-PCR analysis was
525 applied in order to get a more complete picture of the strain diversity during the
526 period of cheese maturation. Certain strains of *Lb. casei/Lb. paracasei* appeared to be
527 common in all cheeses and they most probably originate from the cheese milk or dairy
528 environment, which is in accordance with findings by other authors (Fitzsimons,
529 Cogan, Condon & Beresford, 1999; Antonsson, Ardö & Molin, 2001; Antonsson,
530 Molin & Ardö, 2003). The most uniform microflora was observed at the end of
531 ripening.

532 As expected, *Lb. casei/Lb. paracasei* represented the most abundant *Lb.* species
533 regardless of experimental factors used, such as powder (MFGM content) or adjunct
534 addition. These results were in accordance with previous findings (Beresford et al.,
535 2001; Banks & Williams, 2004) stating that the NSLAB population of Cheddar
536 cheeses is dominated by *Lb. casei/Lb. paracasei (*approx. 95 % of the isolates belong
537 to *Lb. casei/Lb. paracasei*). The PCA of the API results of the selected isolates were
538 in accordance with the chemical and microbial characterization of the cheeses, with
539 the highest diversity of the lactobacilli in the 24 h cheese, a clear grouping according
540 to the adjuncts in the intermediate phase of ripening (4-10 weeks), whereas after 24
541 weeks the differences between the experimental factors were less apparent.

542 Most of the isolates (97 %) were ribose positive. In model systems, Thomas

543 (1987b) demonstrated that ribose can be used as a carbon source for mesophilic
544 lactobacilli in the later stages of ripening. Lysed lactococcal cells may release ribose
545 from RNA and *N*-acetylgluconsamin from degraded cell walls (Østlie et al., 1995;
546 Adamberg et al., 2005). Another possible source of carbon for mesophilic lactobacilli
547 may be connected to the fact that they possess some glycoside hydrolase activity and
548 can utilize sugars from glycoproteins of the MFGM as an energy source (Williams &
549 Banks, 1997*;* Fox et al., 1998). The electron micrographs showed an even distribution
550 of the lactococcal strains throughout the cheese matrix, most of them connected to fat
551 globules, and the lactobacilli appeared to be surrounding cavities from where milk fat
552 globules were removed.

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

564

565 **5. Conclusion**

566 The diversity of lactobacilli in the studied low-fat Cheddar cheeses were highest
567 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 utilize 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.

**Acknowledgments**

We would like to acknowledge the Norwegian Research Council, the Norwegian Foundation for Research Levy on Agricultural Products, the Norwegian Agricultural Agreement Research Fund and TINE SA for financial support. Prof. Paul McSweeney and Dave Waldron at Dept. of Food and Nutritional Sciences at the University College Cork, Ireland for assistance during cheesemaking and cheese analyses. Kari Olsen at the Norwegian University of Life Sciences for performing the chromatographic analyses.

**References**

Adamberg, K., Antonsson, M., Vogensen, FK., Nielsen, E.W., Kask, S., Moller, P. L., & Ardö, 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.

Antonsson, M., Ardö, Y., & Molin, G. (2001). A comparison between the microflora

593 of Herrgård cheese from three different dairies. *International Dairy Journal, 11*,
594 285–291.
595 Antonsson, M., Molin, G., & Ardö, Y. (2003). *Lactobacillus* strains isolated from
596 Danbo cheese as adjunct cultures in a cheese model system. *International*
597 *Journal of Food Microbiology, 85*, 159-169.
598 Banks, J. M., & Williams, A. G. (2004). The role of nonstarter lactic acid bacteria in
599 Cheddar cheese ripening. *International Journal of Dairy Technology, 57*, 145-
600 152.
601 Ben Amor, K., Vaughan, E. E., & DeVos, W. M. (2007). Advanced molecular tools
602 for the identification of lactic acid bacteria. *Journal of Nutrition, 137*, 741-747.
603 Beresford T. P., Fitzsimons, N. A., Brennan, N. L., & Cogan, T. M. (2001). Recent
604 advances in cheese microbiology. *International Dairy Journal, 11*, 259-274.
605 Berthier, F., & Ehrlich, S.D. (1998). Rapid species identification within two groups of
606 closely related lactobacilli using PCR primers that target 16S/23S rRNA spacer
607 region. *FEMS Microbiology Letters, 161*, 97-106.
608 Berthier, F., Beuvier, E., Dasen, A., & Grappin, R. (2001). Origin and diversity of
609 mesophilic lactobacilli in Comte cheese, as revealed by PCR with repetitive and
610 species-specific primers. *International Dairy Journal, 11*, 293-305.
611 Broadbent, J.R., Houck, K., Johnson, M.E. & Oberg, C.J. (2003). Influence of adjunct
612 use and cheese microenvironment on nonstarter bacteria in reduced-fat
613 Cheddar-type cheese. *Journal of Dairy Science, 86*, 2773-2782.
614 Bütikofer, U., & Ardö, Y. (1999). Quantitative determination of free amino acids in
615 cheese. *Bulletin of the International Dairy Federation, 337*, 24-32.
616 Christiansen, P., Waagner Nielsen, E., Vogensen, F. K., Brogren, C. H. & Ardö, Y.
617 (2006). Heat resistance of *Lactobacillus paracasei* isolated from semi-hard

618 cheese made of pasteurised milk. *International Dairy Journal, 16, 1196*-1204.
619 Cogan, T. M., Beresford, T. P., Steele, J., Broadbent, J., Shah, N. P., & Ustunol, Z.
620 (2007). Invited review: Advances in starter cultures and cultured foods. *Journal*
621 *of Dairy Science, 90,* 4005-4021.
622 Coeuret, V., Dubernet, S., Bernardieau, M., Gueguen, M., & Vernoux, J.P. (2003).
623 Isolation, characterization and identification of lactobacilli focusing mainly on
624 cheeses and other dairy products. *Lait, 83,* 269-306.
625 Collins, Y., McSweeney, P. L. H., & Wilkinson, M. (2003). Lipolysis and free fatty
626 acid catabolism in cheese: a review of current knowledge. *International Dairy*
627 *Journal, 13,* 841-866.
628 Crow, V., Curry, B., & Hayes, M. (2001). The ecology of non-starter lactic acid
629 bacteria (NSLAB) and their use as adjuncts in New Zealand Cheddar.
630 *International Dairy Journal, 11*, 275-283.
631 De Man J.C., Rogosa M., & Sharpe M.E. (1960). A medium for the cultivation of
632 lactobacilli. *Journal of Applied Bacteriology, 23*, 130–135.
633 De Urazza, P.J., Gomez-Zavaglia, A., Lozano, M.E., Romanowski, V., & de Antoni,
634 G. L. (2000). DNA fingerprinting of thermophilic lactic acid bacteria using
635 repetitive sequence-based polymerase chain reaction. *Journal of Dairy*
636 *Research, 67*, 381-392.
637 El Soda, M., S. M. Madkor, & Tong, P. S. (2000). Evaluation of commercial adjuncts
638 for use in cheese ripening: Comparison between attenuated and not attenuated
639 lactobacilli. *Milchwissenschaft, 55,* 260-263.
640 El Soda, M., S. M. Madkor, & Tong, P. S. (2008). Adjunct cultures: recent
641 developments and potential significance to the cheese industry. *Journal of Dairy*
642 *Science, 83,* 609–619.

643 Fitzsimons, N.A., Cogan, T.M., Condon, S. & Beresford, T. (1999). Phenotypic and
644 molecular characterisation of non-starter lactic acid bacteria in mature
645 commercial Irish Cheddar cheese. *Applied and Environmental Microbiology,*
646 *65*, 3418-3426.
647 Fox, P. F., McSweeney, P. L. H., & Lynch, C.M. (1998). Significance on non-starter
648 lactic acid bacteria in Cheddar cheese. *Australian Journal of Dairy Technology,*
649 *53*, 83-89.
650 Fox, P. F., Wallace, J. M., Morgan, S., Lynch, C.M., Niland, E.J., & Tobin, J. (1996).
651 Acceleration of cheese ripening. *Antonie Leeuwenhoek, 70,* 271-297.
652 Hynes, E., Bergamini, C,V., Suarez, V., & Zalazar, C.A. (2003). Proteolysis on
653 Reggianito cheeses manufactured with natural whey cultures and selected
654 strains of *Lactobacillus helveticus*. *Journal of Dairy Science, 86,* 3831–3840.
655 IDF (1995). *Milk and milk products: Guidance on sampling. IDF standard 50c*.
656 Brussels, Belgium: International Dairy Federation.
657 IDF (1982). *Cheese and processed cheese: Determination of the total solids content.*
658 *IDF Standard 4a*. Brussels, Belgium: International Dairy Federation.
659 Kieronczyk, A., Skeie, S., Olsen, K., & Langsrud, T. (2001). Metabolism of amino
660 acids by resting cells of non-starter lactobacilli in relation to flavor development
661 in cheese. *International Dairy Journal, 11*, 217-224.
662 Kieronczyk, A., Skeie, S., Langsrud, T., Le Bars, D., & Yvon, M. (2004). The nature
663 of aroma compounds produced in a cheese model by glutamate dehydrogenase
664 positive Lactobacillus INF 15D depends on its relative aminotransferase
665 activities towards the different amino acids. *International Dairy Journal, 14,*
666 227-235.

667 Kilcawley, K., O'Connell, P., Hickey, D. K., Sheehan, E. M., Beresford, T. &
668 McSweeney, P. L. H. (2007). Influence of composition on the biochemical and
669 sensory characteristics of commercial Cheddar Cheese of variable quality and
670 fat content. *International Journal of Dairy Technology, 60*, 81-88.
671 Laloy, E., Vuillemard, J.C., El Soda, M, & Simard, R.E. (1996). Influence of the fat
672 content of Cheddar cheese on retention and localization of starters. *International*
673 *Dairy Journal, 6*, 729-740.
674 Larsen, S.K., Aideh, B., Kilstrup, M., Michelsen, O, & Vogensen, F.K. (2008). The
675 nomenclature of *Lactobacillus casei*, an archeology approach. *5th IDF*
676 *Symposium on cheese ripening*, 9-13th March 2008, Bern, Switzerland.
677 Liu, H., Xu, X. M. & Guo, S. D. (2008). Comparison of full-fat and low-fat cheese
678 analogues with or without pectin gel through microstructure, texture, rheology,
679 thermal and sensory analysis. *International Journal of Food Science and*
680 *Technology, 43*, 1581-1592.
681 Lynch, C.M., Mc Sweeney, P. L. H., Fox, P.F, Cogan, T.M., & Drinan, F.D. (1996).
682 Manufacture of Cheddar cheese with and without adjunct lactobacilli under
683 controlled microbiological conditions. *International Dairy Journal, 6*, 851-867.
684 Lynch, C. M., Muir, D.D., Banks, J.M., Mc Sweeney, P. L. H., & Fox, P.F. (1999).
685 Influence of adjunct cultures of *Lactobacillus paracasei* ssp. *paracasei* or
686 *Lactobacillus plantarum* on Cheddar cheese ripening. *Journal of Dairy Science,*
687 *82*, 1618- 1628.
688 Marilley, L. & Casey, M. G. (2004). Flavours of cheese products: Metabolic
689 pathways, analytical tools and identification of producing strains. *International*
690 *Journal of Food Microbiology, 90*, 139–159.
691 McSweeney, P.L.H. & Sousa, M.J. (2000) Biochemical pathways for the production

692 of flavour compounds in cheese during ripening. *Le Lait, 80,* 293–324.
693 Moe, K.M., Faye, T., Abrahamsen, R.K., Østlie, H.M. & Skeie, S. (2012). Growth
694 and survival of cheese ripening bacteria on milk fat globule membrane isolated
695 from bovine milk and its monosaccharides. *International Dairy Journal*, 25, 29-
696 35.
697 Morin, P., Pouliot, Y., & Britten, M. (2008). Effect of buttermilk made from creams
698 with different heat treatment histories on properties of rennet gels and model
699 cheeses. *Journal of Dairy Science, 91*, 871-882.
700 Narvhus, J.A., Hulbækdal, A., Baugerød, H., & Abrahamsen, R.K. (1991).
701 Measurement of $CO_2$ production and $O_2$ metabolism by pure and mixed cultures
702 of lactic acid bacteria growing in milk. *Actes du colloque Lactic, vol. 91,* (p.
703 371), Caen, France: Centre de Publications de l'Universite de Caen.
704 Narvhus, J.A., Østeraas, K., Mutukumira, T. & Abrahamsen, R.K. (1998). Production
705 of fermented milk using a malty compound-producing strain of *Lactococcus*
706 *lactis* subsp. *lactis* biovar. *diacetylactis* isolated from Zimbabwean naturally
707 fermented milk. *International Journal of Food Microbiology, 41,* 73-80.
708 Østlie, H. M., Eliassen, L., Florvaag, A., & Skeie, S. (2004). Phenotypic and PCR-
709 based characterization of the microflora in Norvegia cheese during ripening.
710 *International Journal of Food Microbiology, 94*, 287–299.
711 Østlie, H.M., Helland, M.H. & Narvhus, J.A. (2003). Growth and metabolism of
712 selected strains of probiotic bacteria in milk. *International Journal of Food*
713 *Microbiology, 87,* 17-27.
714 Østlie, H.M., Vegarud, G. & Langsrud, T. (1995). Autolysis of lactococci: detection
715 of lytic enzymes by polyacrylamide gel electrophoresis and characterization in
716 buffer systems. *Applied and Environmental Microbiology, 61,* 3598-3603.

717 Puchades, R., Lemieux, L & Simard, R. E. (1989). Evolution of free amino acids
718 during the ripening of Cheddar cheese containing added lactobacilli strains.
719 *Journal of Food Science*, *54*, 885-888.
720 Romeih, E. A., Moe, K.M. & Skeie, S. 2012. The influence of fat globule membrane
721 components on the microstructure of low-fat Cheddar cheese. *International*
722 *Dairy Journal*. 26: 66-72.
723 Singh, T.K., Drake, M.A. & Cadwallader, K.R. (2003). Flavor of cheddar cheese: A
724 chemical and sensory perspective. *Comprehensive Reviews in Food Science and*
725 *Food Safety*, *2*, 166-189.
726 Singh, S., Pawas, G., Singh, R., & Heller, K.J. (2009). Application of molecular
727 identification tools for Lactobacillus, with a focus on discrimination between
728 closely related species: A review. *LWT-Food Science and Technology, 42*, 448-
729 457.
730 Skeie, S., Kieronczyk, A. Eidet, S., Reitan, M., Olsen, K. & Østlie, H. (2008a).
731 Interaction between starter bacteria and adjunct *Lactobacillus plantarum*
732 INF15D on the degradation of citrate, asparagine and aspartate in a washed-curd
733 cheese. *International Dairy Journal, 18*, 169-177.
734 Skeie, S., Kieronczyk, A., Næs, R.M., Østlie. H. (2008b). Lactobacillus adjuncts in
735 cheese: Their influence on the degradation of citrate and serine during ripening
736 of a washed curd cheese. *International Dairy Journal, 18*, 158-168.
737 Skeie, S., Lindberg, C., & Narvhus, J. (2001). Development of amino acids and
738 organic acids in Norvegia, influence of milk treatment and adjunct
739 *Lactobacillus. International Dairy Journal*, *11*, 399-411.

740 Thomas, T.D. (1987a). Acetate production from lactate and citrate by non-starter
741 bacteria in Cheddar cheese. *New Zealand Journal of Dairy Science and*
742 *Technology, 22,* 25-38.
743 Thomas, T. D. (1987b). Cannibalism among bacteria found in cheese. *New Zealand*
744 *Journal of Dairy Science and Technology, 22,* 215-219.
745 Versalovic, J., Schneider, M., de Bruijn, F.J., & Lupski, J.R. (1994). Genomic
746 fingerprinting of bacteria using repetitive sequence based PCR (rep-PCR).
747 *Methods of Cellular Molecular Biology, 5*, 25-40.
748 Williams, A. G., & Banks, J. M. (1997). Proteolytic and other hydrolytic enzyme
749 activities in nonstarter lactic acid bacteria (NSLAB) isolated from Cheddar
750 cheese manufactured in the United Kingdom. *International Dairy Journal, 7*,
751 763–774.
752

**Table 1.** Development of pH and gross composition (DM, FDM, salt, SM) during 24 weeks of ripening and significant effects of the experimental factors[a].

| | pH | | | DM (%) | | | FDM | Salt (%) | SM (%) |
|---|---|---|---|---|---|---|---|---|---|
| Exp. factor | 24 h | 6 weeks | 24 weeks | 24 h | 6 weeks | 24 weeks | 6 weeks | 6 weeks | 6 weeks |
| SMP[b] | 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[c] | 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[d] | 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[e] | 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 ($P$<0.05, ns = non significant) 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[f] | | | 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: 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.

[b] Skim milk powder.

[c] Butter milk powder.

[d] 448 = Adjunct addition of *Lb. casei* INF 448.

[e] 456 = Adjunct addition of *Lb. casei* INF 456.

[f] Tukey's significance test for differences between means within the experimental factor of adjunct type.

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

764 ripening[a].

| Exp. Factor | Thr | Arg | GABA[b] | Met | Ile | Leu | Lys | FAA[c] |
|---|---|---|---|---|---|---|---|---|
| SMP[d] | 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[e] | 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[f] | 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[g] | 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 ($P$<0.05, ns = non significant) 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[h] | 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 |

765 [a] Significance level tested at $P$=0.05; ns: not significant.

766 [b] GABA: Gamma aminobutyric acid,

767 [c] Free amino acids.

768 [d] Skim milk powder.

769 [e] Butter milk powder.

770 [f] 448 = Adjunct addition of *Lb. casei* INF 448.

771 [g] 456 = Adjunct addition of *Lb. casei* INF 456.

772 [h] Tukey's significance test for differences between means within the experimental factor of adjunct type.

773

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

775 of ripening[a].

| Exp. Factor | Asp | Glu | Asn | Ser | Thr | FAA[b] |
|---|---|---|---|---|---|---|
| SMP[c] | 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[d] | 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[e] | 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[f] | 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 ($P$<0.05, ns = non significant) 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[g] | 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 |

776 [a] Significance level tested at $P$=0.05; ns: not significant.

777 [b] Free amino acids.

778 [c] Skim milk powder.

779 [d] Butter milk powder.

780 [e] 448 = Adjunct addition of *Lb. casei* INF 448.

781 [f] 456 = Adjunct addition of *Lb. casei* INF 456.

782 [g] Tukey's significance test for differences between means within the experimental factor of adjunct type.

783

**Legends to Figures**

**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 (5000x) 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.

**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, ---■---SMP 448, ---▲--- 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, ---■---SMP 448, ---▲--- SMP456. Numerals indicate the culture adjunct.

**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.

**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).

**Fig. 7.** Dendrogram based on the rep-polymerase chain reaction (PCR) DNA fingerprinting of bacterial isolates from the low fat Cheddar cheeses. a) 24-hour 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. 1**

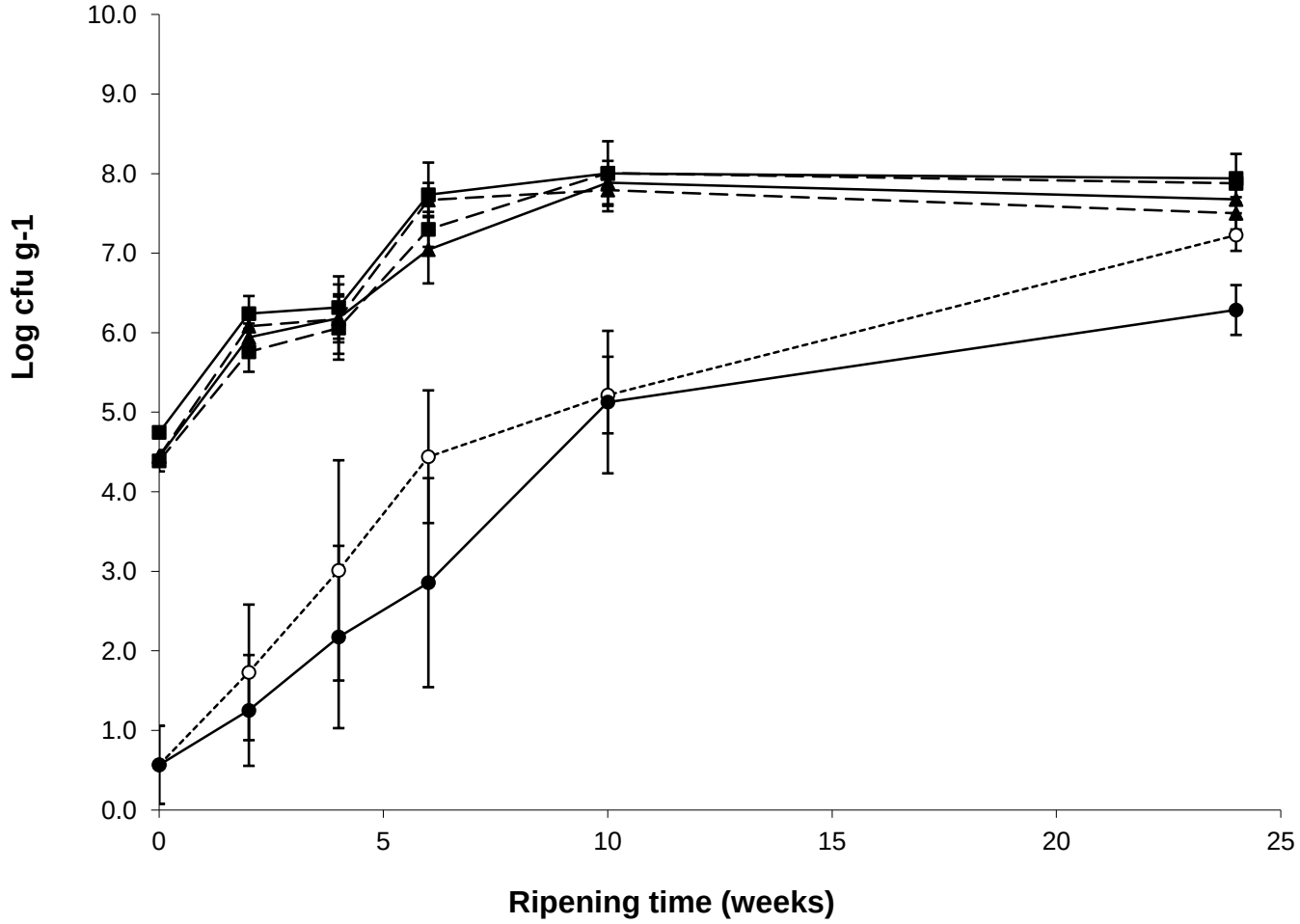
10.0
9.0
8.0
7.0
6.0
5.0
4.0
3.0
2.0
1.0
0.0
Log cfu g-1
0
5
10
15
20
25
Ripening time (weeks)

**Fig. 2.**

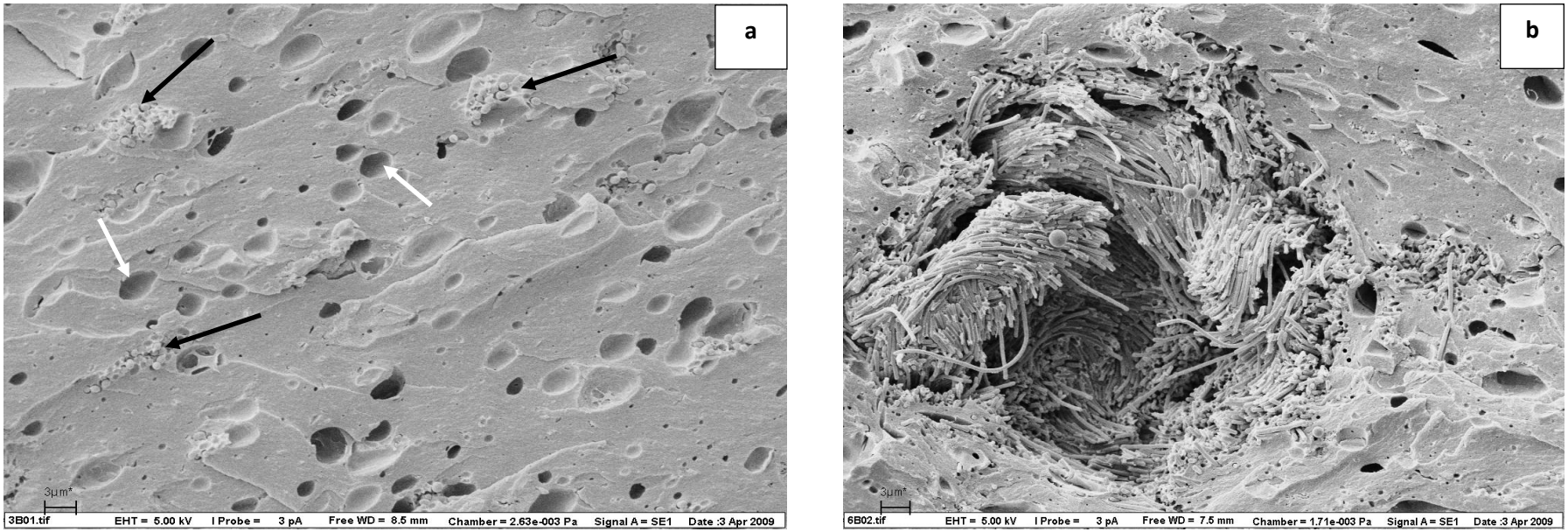
a
3µm
3B01.tif EHT = 5.00 kV I Probe = 3 pA Free WD = 8.5 mm Chamber = 2.63e-003 Pa Signal A = SE1 Date :3 Apr 2009
b
3µm
6B02.tif EHT = 5.00 kV I Probe = 3 pA Free WD = 7.5 mm Chamber = 1.71e-003 Pa Signal A = SE1 Date :3 Apr 2009

**Fig. 3.**

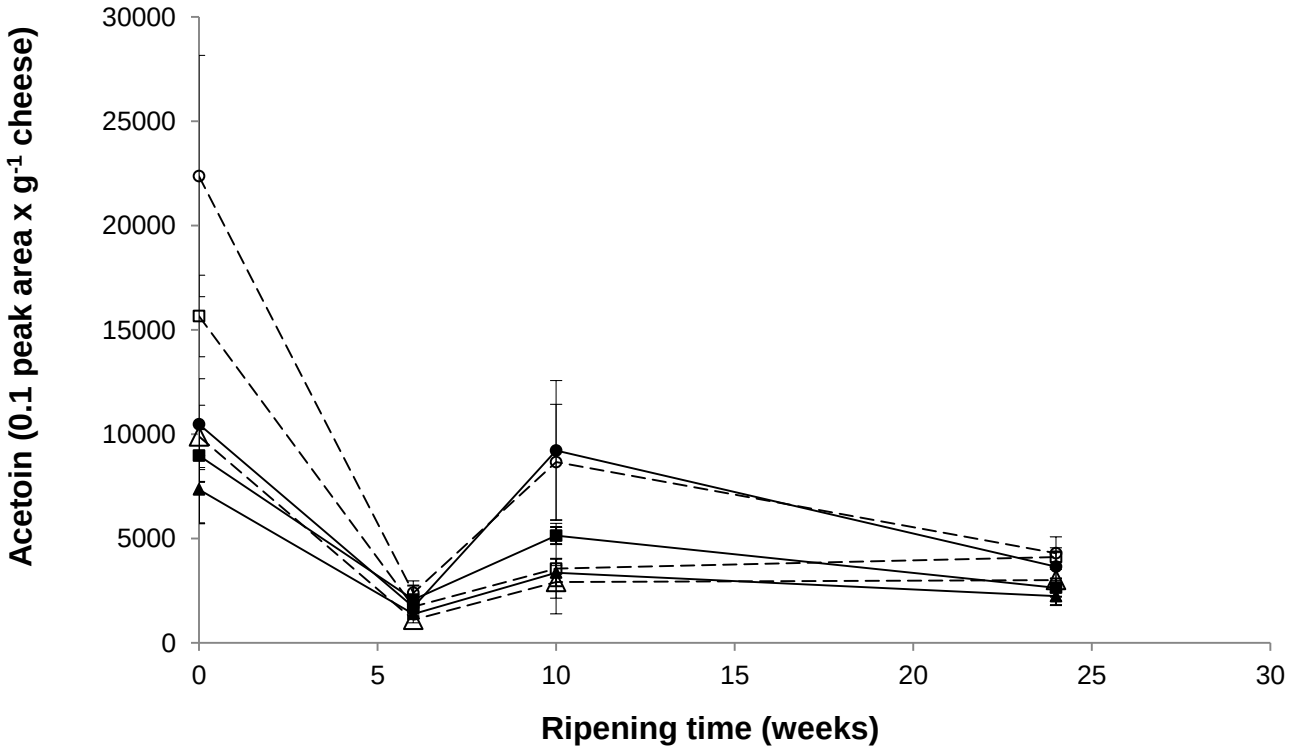
Acetoin (0.1 peak area x g-1 cheese)
30000
25000
20000
15000
10000
5000
0
0
5
10
15
20
25
30
Ripening time (weeks)

**Fig.4.**

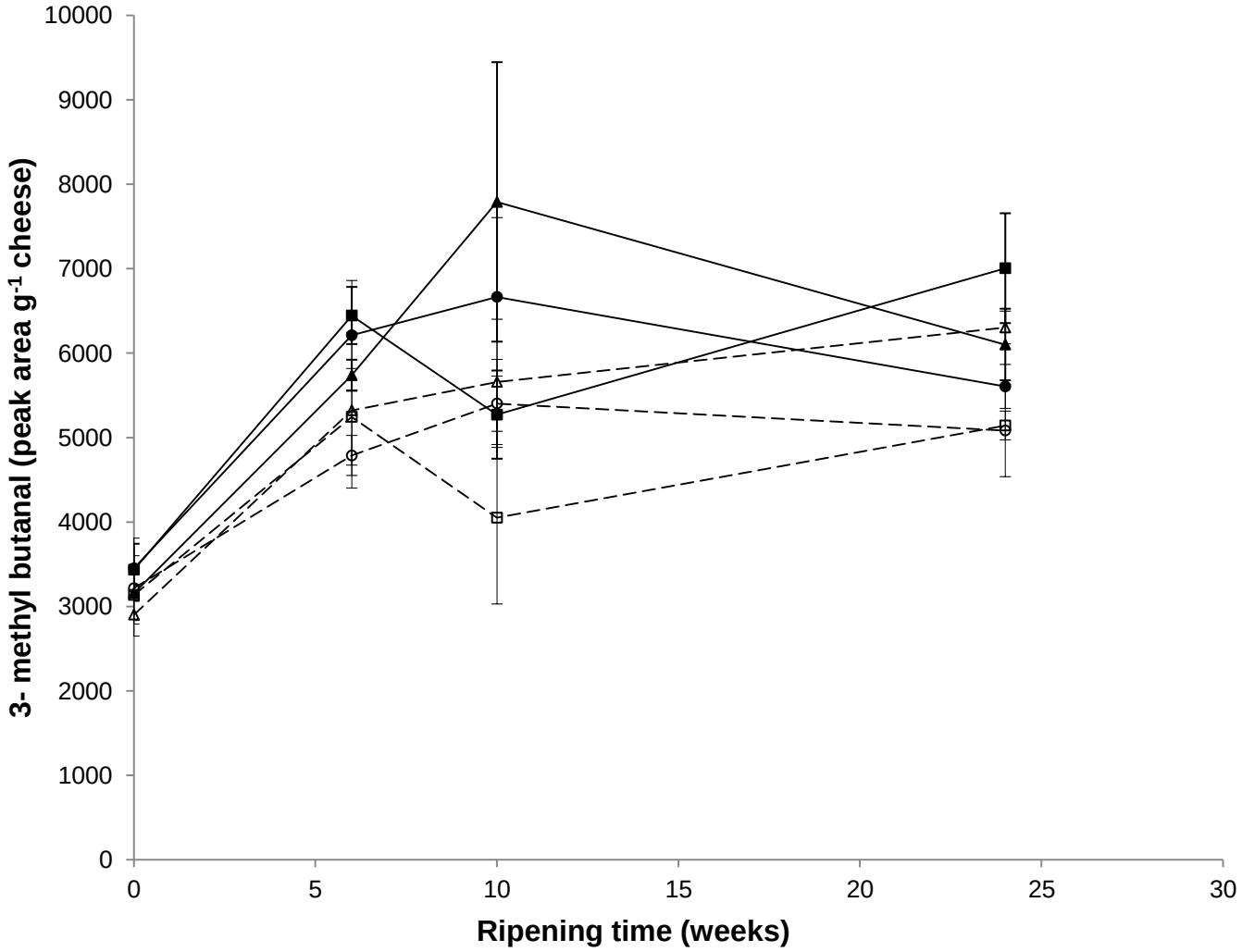
10000
9000
8000
7000
6000
5000
4000
3000
2000
1000
0
3- methyl butanal (peak area g-1 cheese)
0
5
10
15
20
25
30
Ripening time (weeks)

Figure 5

**Fig. 5.**

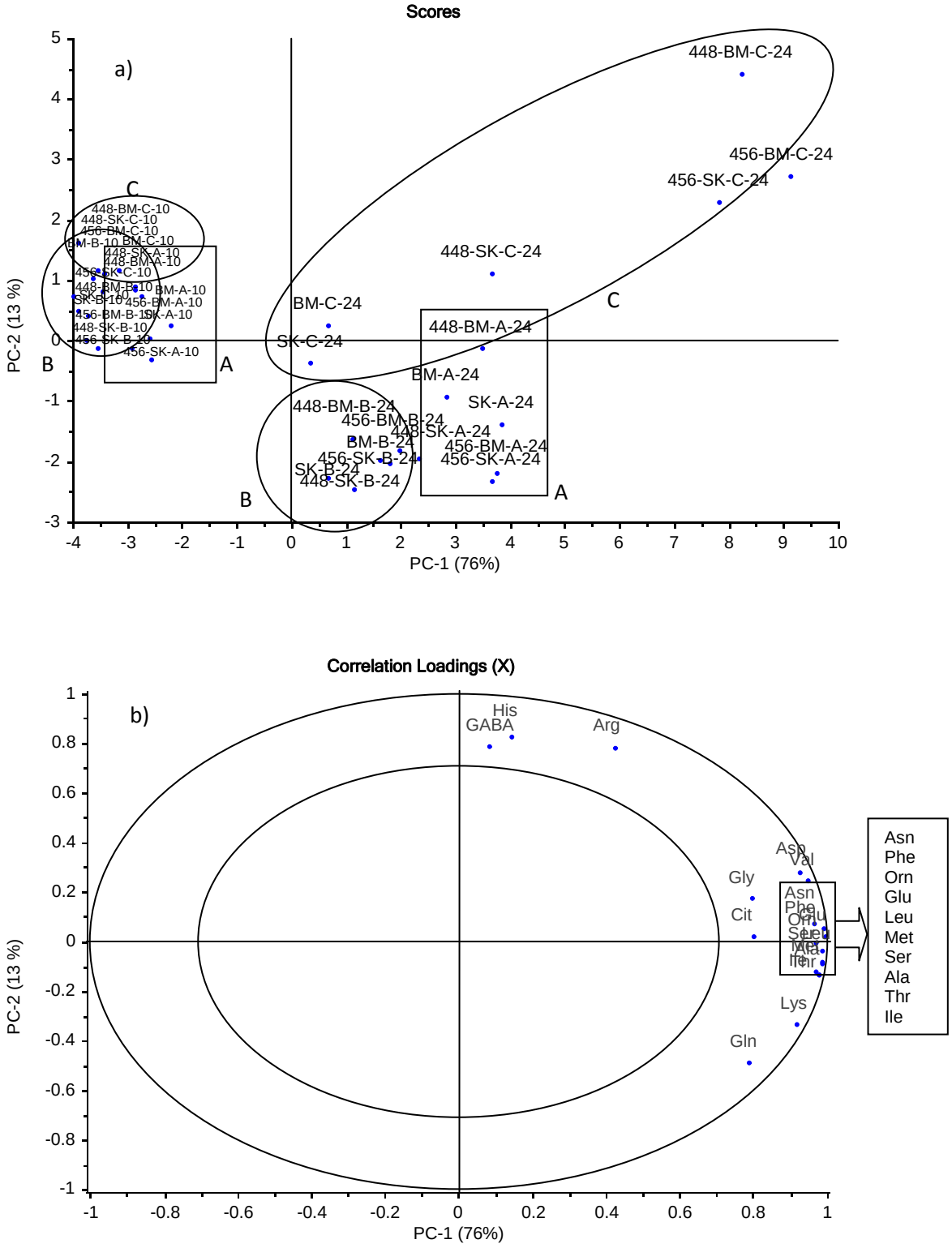

Figure 6

**Fig. 6.**

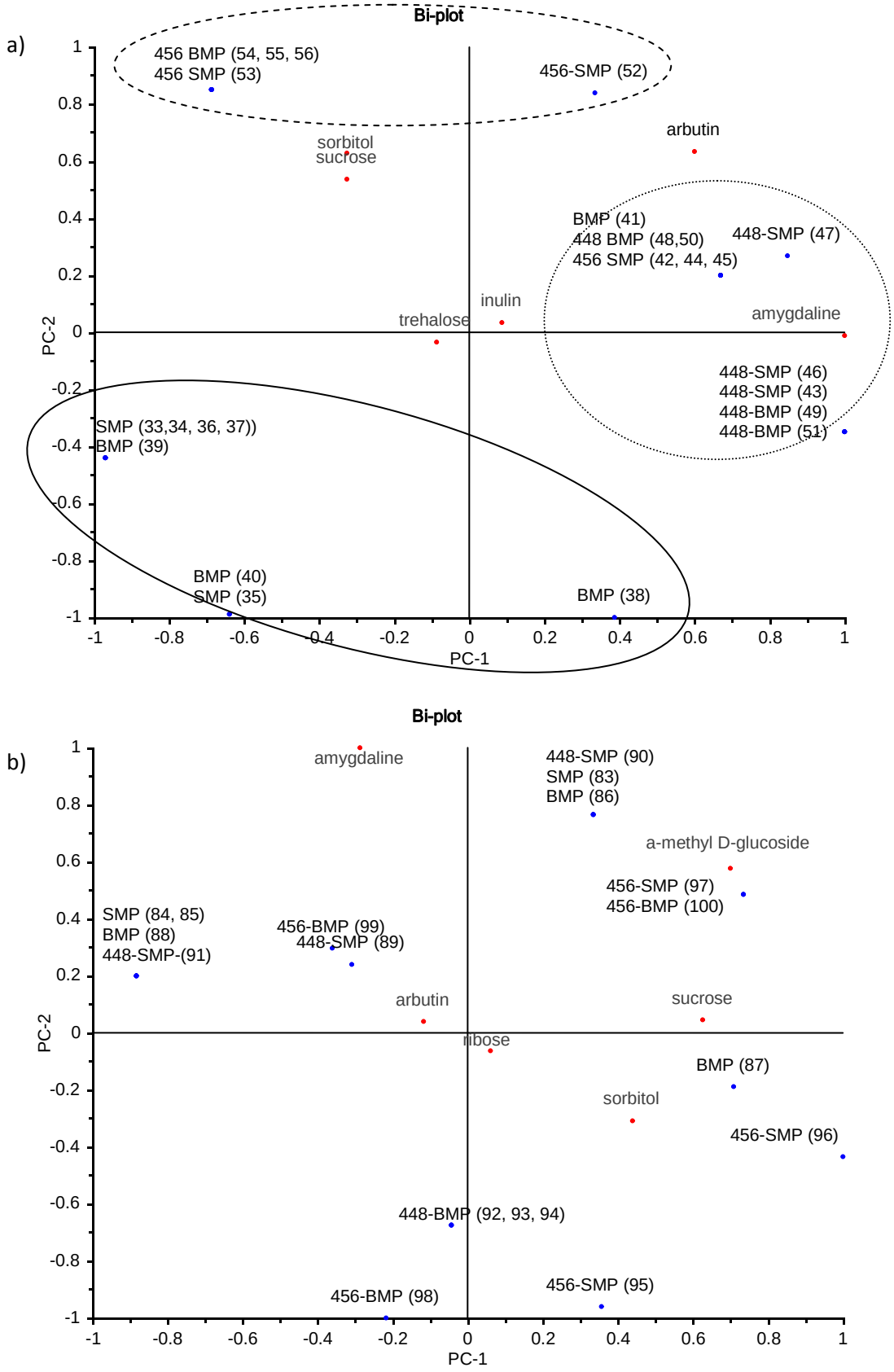

**Figure 7 ab.docx**

Figure 7ab

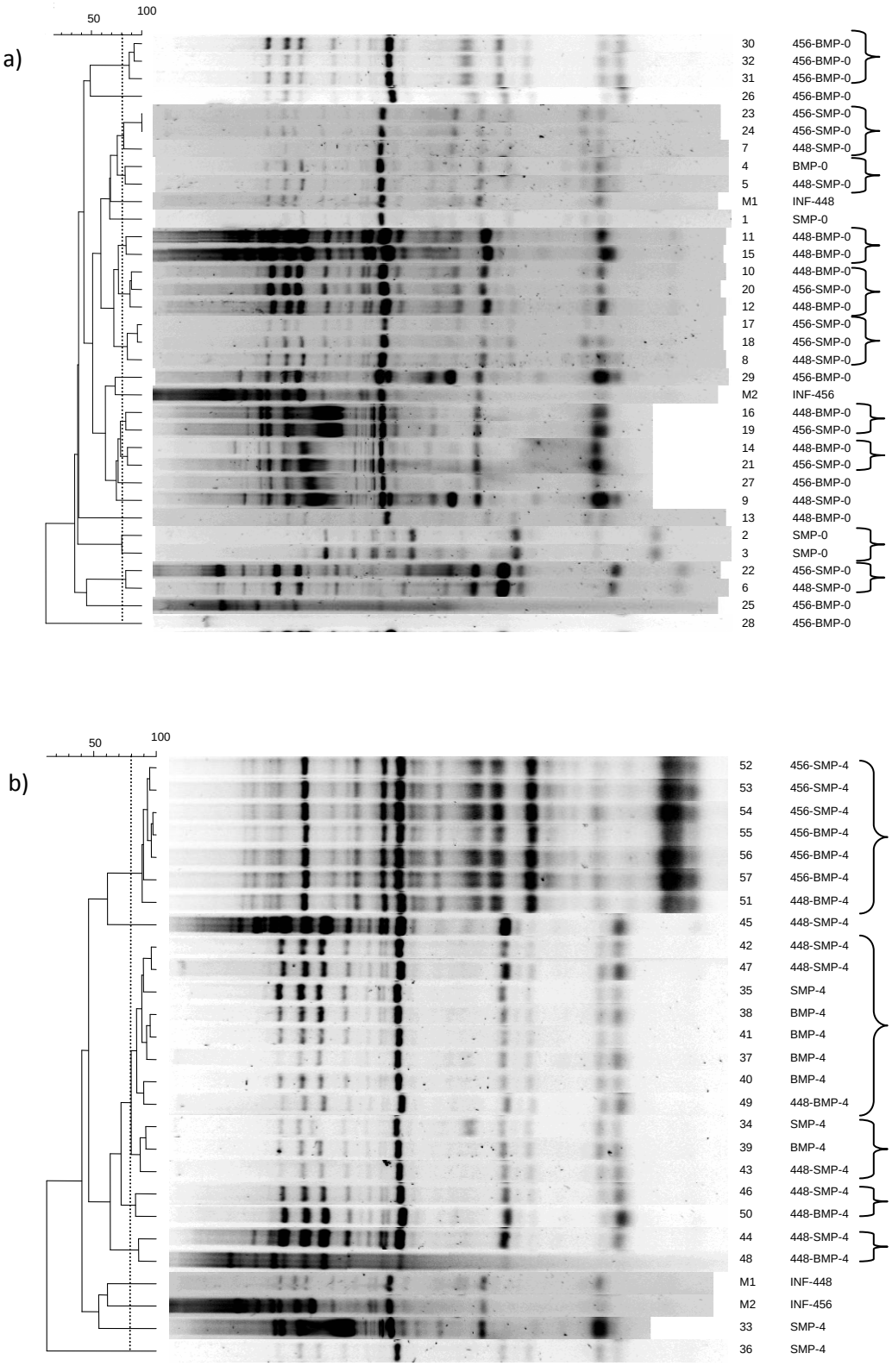

**Figure 7 cd.docx**

Figure 7 cd