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Philosophiae Doctor (PhD) Thesis 2012:21

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A study of potential energy sources used by cheese ripening bacteria

En studie av potensielle energikilder brukt av bakterier involvert i ostemodning

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Ås, March, 2012

Kim Marius Moe

SUMMARY

Non-starter lactic acid bacteria (NSLAB) continue to grow in cheese during ripening even though lactose and its monosaccharide moieties (glucose and galactose (Gal)) are depleted. This indicates that the NSLAB are able to utilize other energy sources present in the cheese during ripening. Components from the starter bacteria, amino acids (AA), citrate and carbohydrates from the milk fat globule membrane have all been suggested as potential sources of energy for NSLAB, however, the substrates used during cheese ripening are currently not clearly defined. The objective of this work was to study potential energy sources used by cheese ripening bacteria during cheese ripening, with the hypothesis that carbohydrates from the glycoconjugates found in the milk fat globule membrane (MFGM) are essential for the development of bacteria during cheese ripening.

The growth and metabolism of selected *Lactobacillus* (*Lb.*) *paracasei, Lb. plantarum* INF15D*, Lactococcus lactis* subsp. *lactis* ML8 and *Enteroccus* (*Ec.*) *hirae* INFE1 when grown in a model system added MFGM or its monosaccharide components as the only potential carbon sources was studied. Analysis of catabolic- as well as anabolic products have been conducted during growth of the bacteria, using high performance liquid chromatography for organic acids, carbohydrates and AA. Gas chromatography was used to monitor volatile compounds. Two dimensional gel electrophoresis was used to chart the protein expressions of *Lb. plantarum* INF15D when grown on the single monosaccharides found in the MFGM. Matrix assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry was ultimately used to identify proteins of interest from the two dimensional gels. The effect of the difference in MFGM monosaccharide utilization between NSLAB was explored in a cheese making trial using MFGM rich buttermilk powder as an addition to the cheese milk.

The lactic acid bacteria (LAB) studied, all relevant to cheese production and ripening, were able to grow and survive for an extended period of time with a bovine MFGM isolate as the only potential carbohydrate source. All isolates were able to effectively utilize most of the monosaccharides found in MFGM glycoconjugates. Growth of *Lb. plantarum* INF15D on the acylated aminosugar *N*-acetylglucosamine (GlcNAc) led to induction of enzymes involved in the degradation of this sugar through the GlcNAc pathway, and this yielded large amounts of acetate as a degradation product. Presumably, the large amounts of acetate released from the degradation of *N*-acetylgalactosamine (GalNAc) and GlcNAc induced a decrease of cultivable number of bacteria at an earlier stage than when incubated on any of the other

monosaccharides. The potential cheese contaminant *Ec. hirae* INFE1 showed superior growth and survival abilities compared with the other LAB when grown on the MFGM media, and on several of the MFGM monosaccharides.

Enzymes involved in two different pathways yielding energy from serine (Ser) were found in *Lb. plantarum* INF15D, whereas none of the studied *Lb. paracasei* strains decreased the levels of Ser when grown on any of the monosaccharides. Growth of *Lb. plantarum* INF15D on Gal resulted in a metabolic shift expressed as different fates of the produced pyruvate compared to growth on the other monosaccharides, as well as initiation of degradation of some AA known to potentially take part in energy production, specifically arginine and tyrosine. Enzymes involved in ribose metabolism as well as phosphoketolase pathway enzymes were upregulated, leading to the production of ethanol and acetate. These findings indicate that *Lb. plantarum* INF15D are able to utilize several different substrates for energy at the same time to secure growth and survival during sub-optimal conditions. The cell shape determining protein MreB, that leads to elongation of the cells was also found to be upregulated during growth of *Lb. plantarum* INF15D on Gal.

Cheeses were made with the addition of butter milk, and low initial numbers (log 2 cfu/mL cheese milk) of two different *Lb. paracasei* adjunct cultures (INF448 and INF456) known to be different in their utilization of the MFGM monosaccharide GalNAc. A low initial number was used to mimic the content of NSLAB normally found in cheese milk. Even if the *Lb. paracasei* adjuncts have the ability to utilize the MFGM components, they did not dominate the NSLAB microflora in the cheese in the later stages of ripening. The starter bacteria were located within the protein networks in clusters which were distributed homogeneously throughout the cheese matrix regardless of additive, while lactobacilli were rare, and when found, they were in huge clusters of clearly elongated cells.

SAMMENDRAG

Bakterier som ikke er en del av den tilsatte syrekulturen (NSLAB) fortsetter å vokse i ost selv om laktose og dets monosakkaridkomponenter (glukose og galaktose (Gal)) er oppbrukt. Dette indikerer at NSLAB er i stand til å utnytte andre energikilder som er tilstede i osten under modning. Komponenter fra syrekulturbakterier, aminosyrer, sitrat og karbohydrater fra melkens fettkulemembran har alle blitt foreslått som mulige energikilder for NSLAB. Hvilke substrater som blir benyttet av NSLAB under ostemodningen er imidlertid ikke klart definert. Formålet med dette arbeidet var å studere mulige energikilder som benyttes av bakteriene i osten under modning. Hypotesen var at karbohydrater fra glukokonjugater som finnes i melkens fettkulemembran er avgjørende for utviklingen av bakterier under ostens modning.

Vekst og metabolisme av utvalgte *Lactobacillus* (*Lb.*) *paracasei*, *Lb. plantarum* INF15D*, Lactococcus lactis* subsp. *lactis* ML8 og *Enteroccus* (*Ec.*) *hirae* INFE1 dyrket i et modellsystem med melkens fettkulemembran eller dens monosakkaridkomponenter ble studert. Analyser av katabolske- samt anabolske produkter ble utført under bakterienes vekst ved bruk av høytrykks væskekromatografi til organiske syrer, karbohydrater og aminosyrer. Gasskromatografi ble benyttet til analyse av flyktige forbindelser. Todimensjonal gel elektroforese ble brukt til å kartlegge proteinuttrykkene til *Lb. plantarum* INF15D dyrket på de enkelte monosakkaridene som finnes i melkens fettkulemembran. Matrix assisted laser desorption/ionization time-of-flight (MALDI-TOF) massespektrometri ble brukt til å identifisere proteiner av interesse fra de todimensjonale gelene. Effekten av forskjellene mellom bakterienes egenskaper til å utnytte monosakkaridene som finnes i melkens fettkulemembran ble utforsket i et ystingsstudie, hvor ystemelken ble tilsatt kjernemelk rik på fettkulemembrankomponenter.

Melkesyrebakteriene som ble studert, som alle er relevante i osteproduksjon og modning, var i stand til å vokse og overleve i en lengre periode med fettkulemembran, isolert fra kumelk, som eneste potensielle karbohydratkilde. Alle isolatene var i stand til å effektivt utnytte de fleste monosakkaridene som finnes i glykokonjugatene i melkens fettkulemembran. Vekst av *Lb. plantarum* INF15D på det acetylerte aminosukret *N*-acetylglukosamin (GlcNAc) førte til en høyere produksjon av enzymer som er involvert i nedbrytningen av dette sukret via GlcNAc nedbrytningsveien, og dette førte til at store mengder eddiksyre ble frigjort som et nedbrytningsprodukt. Det er trolig at de store mengdene eddiksyre som ble frigjort under

nedbrytningen av *N*-acetylgalaktosamin (GalNAc) og GlcNAc førte til en nedgang i antallet dyrkbare bakterier på et tidligere stadium, enn ved vekst på noen av de andre monosakkaridene. Sammenliknet med de andre melkesyrebakteriene som ble studert hadde den potensielle ostekontaminanten *Ec. hirae* INFE1 overlegne vekst- og overlevelsesegenskaper da den vokste på fettkulemembranmedie og på flere av fettkulemembranens monosakkarider.

Enzymer involvert i to ulike nedbrytningsveier, som begge gir energi fra serin (Ser), ble funnet i *Lb. plantarum* INF15D, mens ingen av de studerte *Lb. paracasei* stammene reduserte nivået av Ser da de ble dyrket på noen av monosakkaridene. Vekst av *Lb. plantarum* INF15D på Gal resulterte i et metabolsk skift, hvor pyruvat ble degradert via andre nedbrytningsveier enn ved vekst på noen av de andre monosakkaridene. Nedbrytning av aminosyrer som potensielt kan utnyttes til energi (arginin og tyrosin) ble i tillegg initiert. Enzymer som er involvert i nedbrytningen av ribose samt i fosfoketolase nedbrytningsveien ble oppregulert, noe som førte til produksjon av etanol og eddiksyre. Disse funnene indikerer at *Lb. plantarum* INF15D er i stand til å samtidig utnytte flere forskjellige substrater til produksjon av energi for å sikre vekst og overlevelse under forhold som ikke er optimale. Proteinet MreB som er med på å bestemme bakteriecellens form ble i tillegg oppregulert da *Lb. plantarum* INF15D ble dyrket på Gal.

Oster ble produsert med ystemelk tilsatt kjernemelkpulver og med lavt antall (log 2 kde/mL) av to ulike tilsatte *Lb. paracasei* kulturer som det var kjent at hadde ulik evne til å utnytte monosakkarider fra melkens fettkulemembran. Et lavt antall tilsatte bakterier ble benyttet for å simulere innholdet av NSLAB som normalt finnes i ost. Selv om *Lb. paracasei* stammene kunne benytte seg av komponenter fra melkens fettkulemembran, så førte dette ikke til at de dominerte mikrofloraen i osten på et sent stadium i modningen. Syrekulturbakterier ble lokalisert i osten i kolonier som var homogent distribuert i hele ostematrixen uavhengig av tilsetning til ystemelken, mens lactobasiller var distribuert i få og store ansamlinger med tydelig forlengede celler.

ABBREVIATIONS

AA – Amino acid

ADI – Arginine deiminase

Ala – Alanine

Arg – Arginine

Asn – Aspartate

CD – Cluster of differentiation

CEP – Cell-envelope proteinase

Cit – Citrulline

CN – Casein

Gal – D-galactose

GalNAc – *N*-acetyl-D-galactosamine

GalNAcol – *N*-acetylgalactosaminitol

Glc – D-glucose

GlcNAc – *N*-acetyl-D-glucosamine

His – Histidine

LAB – Lactic acid bacteria

MALDI – Matrix assisted laser desorption/ionisation

Man – D-mannose

MFGM – Milk fat globule membrane

MUC – Mucin

NANA – *N*-acetyl-D-neuraminic acid

NSLAB – Non-starter lactic acid bacteria

Orn – Ornithine

PAS – Periodic acid Schiff

PMF – Proton motive force

PTS – Phosphotransferase system

SDS – Sodium dodecyl sulphate

Ser – Serine

Thr – Threonine

TOF – Time of flight

LIST OF PAPERS

Paper I

Moe, K. M., Faye, T., Abrahamsen, R. K., Østlie, H. M. and Skeie, S. (2012). Growth and survival of cheese ripening bacteria on milk fat globule membrane isolated from bovine milk and its monosaccharides. International Dairy Journal. doi:10.1016/j.idairyj.2011.12.014.

Paper II

Moe, K. M., Porcellato, D. and Skeie, S. (2012). Metabolism of milk fat globule membrane components by nonstarter lactobacilli isolated from cheese. Submitted.

Paper III

Moe, K. M. (2012). Regulation of proteins in the cheese nonstarter lactic acid bacteria *Lb. plantarum* INF15D when grown on monosaccharides found in the milk fat globule membrane. Manuscript.

Paper IV

Martinovic, A., Moe, K. M., Romeih, E. A., Aideh, B., Vogensen, F. K., Østlie, H. and Skeie, S. (2012). Growth of adjunct *Lactobacillus casei* in Cheddar cheese with added buttermilk powder. Submitted.

Paper V

Romeih, E. A., Moe, K. M. and Skeie, S. (2012). The influence of fat globule membrane components on the microstructure of low-fat Cheddar cheese. Accepted for publication in International Dairy Journal.

INTRODUCTION

Cheese ripening is a process of concerted biochemical and physiochemical changes where the bland curd is converted into mature cheese having the flavour, texture and aroma characteristics of the intended variety. Cheese undergoes a series of complex changes during ripening that are caused by indigenous milk proteinases, milk-clotting enzymes, lactic acid bacteria (LAB) starter cultures and other adventitious or added microorganisms. The primary function of cheese starter LAB is to rapidly acidify the cheese milk during the cheese making process. The starter bacteria typically grow to high numbers (~log 8 cfu/g) during the cheese making process (Beresford et al., 2001; Peterson and Marshall, 1990) by efficiently fermenting the milk sugar lactose, generating ATP needed for cell growth and metabolism. After the readily available lactose is depleted in the cheese matrix, the dominating starter culture usually declines, while a secondary culture takes the dominating role in the cheese microflora. This secondary flora is called non-starter lactic acid bacteria (NSLAB), since they traditionally are not added to the cheese milk during the cheese making process. During cheese ripening, the proteinases and peptidases of both the starter and NSLAB play a role in the degradation of casein (CN), and proteolysis plays a vital role in the textural changes in cheese during ripening.

The substrates utilized by NSLAB in ripening cheese are not clearly defined yet, however, several studies conducted have proposed potential substrates. These include amino acids (AA), specifically arginine (Arg; Laht et al., 2002) and serine (Ser; Liu et al., 2003b), components from lysed starter cells (Thomas, 1987b), citrate (Palles et al., 1998) and carbohydrates from the milk fat globule membrane (MFGM) glycoconjugates (Adamberg et al., 2005; Fox et al., 1998).

As a consequence of consumer demands, cheese producers have developed and launched reduced fat counterparts of traditional cheeses. However, consumers often regard cheeses with reduced fat content to be of inferior quality (Guinee et al., 1998). A major objective of the industry is to produce low-fat cheeses that are similar in characteristics to full-fat cheeses. Significant advances in understanding the biochemical and physiochemical characteristics of reduced fat cheeses have to be undertaken to achieve this objective.

Low-fat cheeses are made from milk with reduced fat content, and as a consequence the protein to fat ratio of low fat cheeses are higher than of full-fat cheeses (Drake et al., 2010). Fenelon et al., (2000) demonstrated that the growth rate of NSLAB in Cheddar

decreased as a consequence of reduced fat content in cheese up to six months of ripening. By removing the fat from the cheese milk, the potential energy source of the MFGM glycoconjugates are also removed. This may indicate that the components of the MFGM could be a source of energy for NSLAB in cheese.

OBJECTIVES

The objective of this PhD work was to study potential energy sources used by cheese ripening bacteria during cheese ripening, with the hypothesis that carbohydrates from the glycoconjugates found in the milk fat globule membrane (MFGM) are essential for the development of bacteria during cheese ripening, by conducting the following studies:

- *Differentiation of growth of cheese related bacteria when utilizing MFGM monosaccharides or MFGM material in a model system.*
  The growth and survival of cheese LAB on MFGM isolated from bovine milk and on monosaccharides found in MFGM glycoconjugates were studied. The LAB studied were selected lactobacilli strains isolated from Norwegian semi-hard cheese, a starter *Lactococcus* (*Lc.*) sp. and the potential cheese contaminant *Enterococcus* (*Ec.*) *hirae*.

- *Analysis of metabolites of NSLAB when utilizing MFGM monosaccharides or MFGM material in a model system.*
  The objective of this study was to investigate how components present in the MFGM may be used, and how they influence growth and survival by cheese ripening lactobacilli. This was achieved by analysing metabolites produced during incubation on appropriate media.

- *Analysis of protein regulation in the NSLAB, Lactobacillus plantarum INF15D, when utilizing MFGM monosaccharides.*
  The proteomes of the cheese NSLAB *Lb. plantarum* INF15D was studied when grown on different monosaccharides found in MFGM glycoconjugates. Proteins were separated and identified using 2D gel electrophoresis and MALDI-TOF/TOF.

- *Analysis of reduced fat cheese made from cheese milk enriched with milk fat globule membrane material in the form of buttermilk.*
  The characteristics of reduced fat Cheddar cheese differing in the content of MFGM components achieved by addition of either buttermilk powder or skim-milk powder to the cheese milk were studied.

The work is tied together with an introductory synopsis.

BACKGROUND, RESULTS AND DISCUSSION

Milk fat globule membrane

Nearly all of the lipids in milk are present in separate microscopic globules covered by a MFGM, as an oil in water emulsion. The MFGM consists of a complex mixture of proteins, phospholipids, glycoconjugates, diacylglycerols, sterols, sterol esters, enzymes and other minor components (Keenan and Mather, 2006). The innermost layer of the MFGM derives from the endoplasmatic reticulum where the fat droplets apparently originates (Zaczek and Keenan, 1990) and is a monolayer of proteins and polar lipids that covers the triacylglycerol core and acts as the outermost layer before secretion (Dylewski et al., 1984). During secretion into the lumen, the fat globules are enveloped by a bi-membrane derived from the apical plasma membrane from the mammary secretory cell (Fig. 1; Bargmann and Knoop, 1959). The lumen is a receptacle for the milk from a sphere shaped arrangement of mammary secretory cells called the alveolus. All the lumens are connected by ducts leading the milk to the skin surface at the teat (Patton and Keenan, 1975).

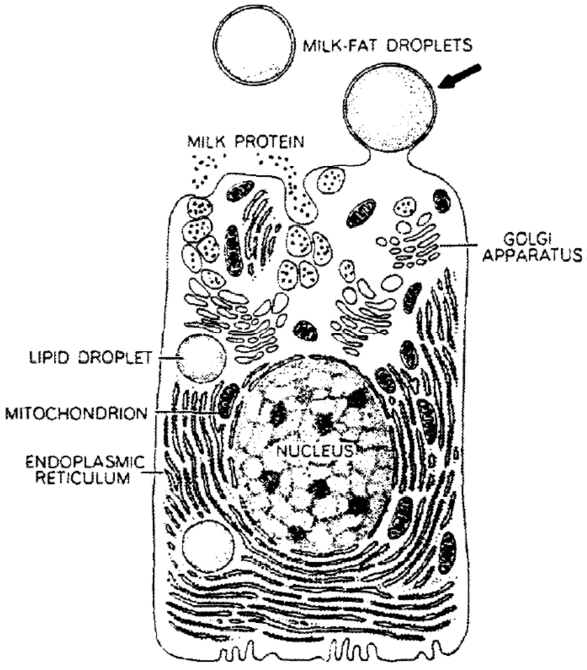

Fig. 1. The formation of milk by the lactating cell (Patton and Keenan, 1975).

The glycoconjugates found in the MFGM contains numerous complex carbohydrates (Liu et al., 2005; Mather, 2000). The structure of the MFGM glycoconjugates as well as their carbohydrate side chains have been studied extensively and are well documented (Hvarregaard et al., 1996; Nakata et al., 1993; Pallesen et al., 2007; Sato et al., 1995; Snow et al., 1977). The monosaccharides of the MFGM glycoconjugate side chains include *N*-acetyl-D-neuraminic acid (Sialic acid; NANA), D-galactose (Gal), L-fucose (Fuc), D-mannose (Man), *N*-acetyl-D-glucosamine (GlcNAc) and *N*-acetyl-D-galactosamine (GalNAc). It has been suggested that the carbohydrates found in MFGM glycoconjugates can serve as a potential energy source for NSLAB during cheese ripening (Fox et al., 1998). The ability for *Lb. rhamnosus* GG to grow efficiently with mucin as the only available carbon source has been demonstrated by Sánchez et al. (2010). Williams and Banks (1997) found that several species of mesophilic lactobacilli possess glycolytic enzymes (α- and β-galactosidases, α- and β-galactosaminidases and *N*-acetyl-α- and *N*-acetyl-β-D-neuraminidase), which may be capable of releasing sugars from the glycoproteins. Experiments by Gagnaire et al. (2004) showed that the glycolytic enzyme β-galactosidase was present in the aqueous phase of a 76 day old Emmental cheese. This enzyme is capable of both hydrolyzing lactose as well as releasing sugars from MFGM glycoconjugates (Liu et al., 2005). The carbohydrate side chains of MFGM glycocoproteins are either O-linked or N-linked. The O-linked oligosaccharides are comprised of a core unit of Galβ1-3GalNAcol (Wilson et al., 2008), a backbone and a terminal region (Fig. 2; Patton et al., 1995). The GalNAcol of the core unit is covalently bound to the AA Ser or threonine (Thr) in the glycoprotein (Zinn et al., 1977; Patton et al., 1995). The N-linked oligosaccharides are attached to the protein backbone through bonds between GlcNAc and asparagine residues. They all contain a common core pentasaccharide consisting of two GlcNAc and three Man (Fig. 2).

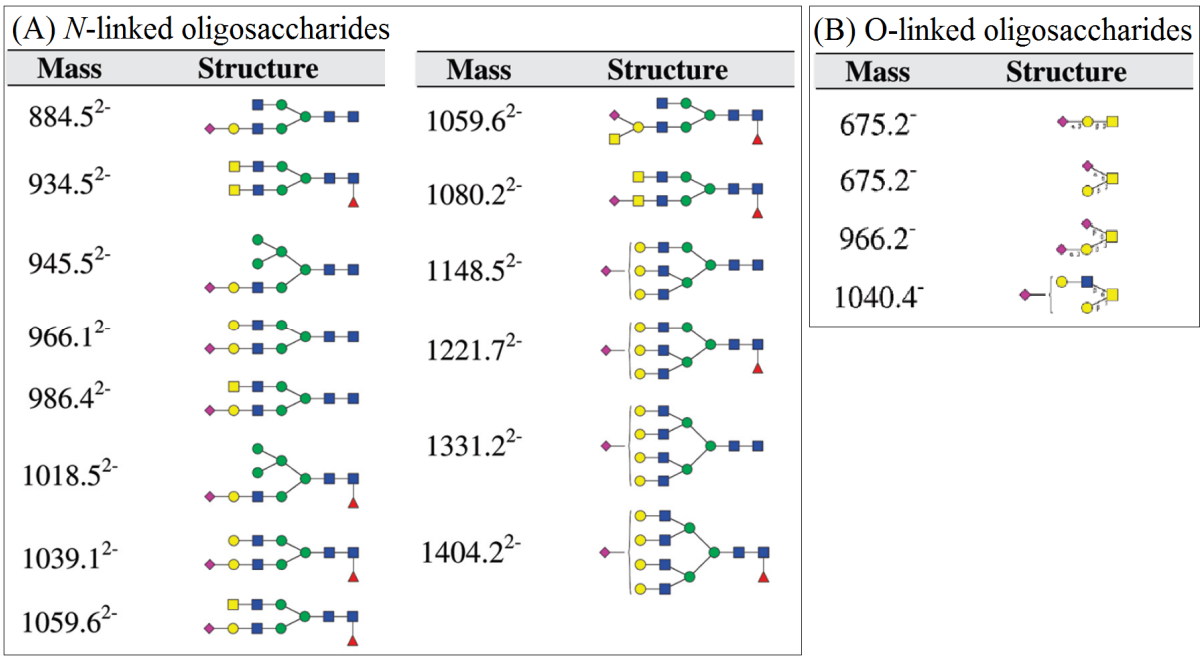

Fig 2. Examples of bovine N-linked (A) and O-linked (B) oligosaccharides. ■, *N*-acetylgalactosamine; ■, *N*-acetylglucosamine; ●, galactose; ●, mannose; ▲, fucose; ◆, sialic acid. Modified from Wilson et al. (2008).

The mucins are heavily glycosylated proteins, and have been estimated to contain 50 % carbohydrates by weight. However, 30 % of the carbohydrates in mucins are NANA (Snow et al., 1977), which none of the cheese associated LAB studied in Paper I were able to utilize for growth. The oligosaccharide side-chains of mucins are mainly O-linked but N-linked oligosaccharides are also observed. Bovine mucins are integral parts of epithelial membranes (Pallesen et al., 2001), however normal handling of milk, such as cooling and agitation have shown to transfer MUC1 into the skim milk fraction (Peterson et al., 1998). The mucins are high in the AA Ser, proline, alanine (Ala) and glycine (Snow et al., 1977; Cawston et al., 1976). The function of mucins is not known, but it has been speculated that they may serve as a competitive binding site for pathogen microorganisms, protecting infants from infections (Peterson et al., 1998). Schroten et al. (1992) found that *Escherichia* (*E.*) *coli* agglutinated human milk fat globule, indicating active binding between the two. Mucins are major constituents of the luminal surfaces of epithelial organs, and by binding to receptor analogues in the MFGM, *E. coli* binding to intestinal mucins were inhibited (Schroten et al., 1992). It has also been speculated that the strong negative charge as conferred by the high NANA content of the mucins, may prevent wall to wall adherence and thereby keep the milk ducts and alveoli open (Patton, 1999).

Mucin 1 (MUC1) is found to be a major consistuent of bovine MFGM (Pallesen et al., 2001). The peptide chain in the extracellular filament of MUC1 consists of a tandemly repeated segment of 20 AA (Patton, 1999), with three absolutely conserved Ser and one absolutely conserved Thr, as well as several well conserved Ser and Thr residues (Fig. 3; Sando et al., 2009). Because of its polymorphism and the fact that the carbohydrate side chains are of a variable nature, MUC1 is not of a specific molecular mass or structure (Patton et al., 1995). Patton et al. (1995) estimated that MUC1 constituted up to 40 mg/mL of bovine milk. Treatment of skim milk whey samples containing mucins and β-galactosidase slightly increased the mobility of MUC1 and MUCX in sodium dodecyl sulphate (SDS) poly acrylamide gels (Liu et al., 2005), indicating that β-galactosidase is able to release carbohydrates from mucin.

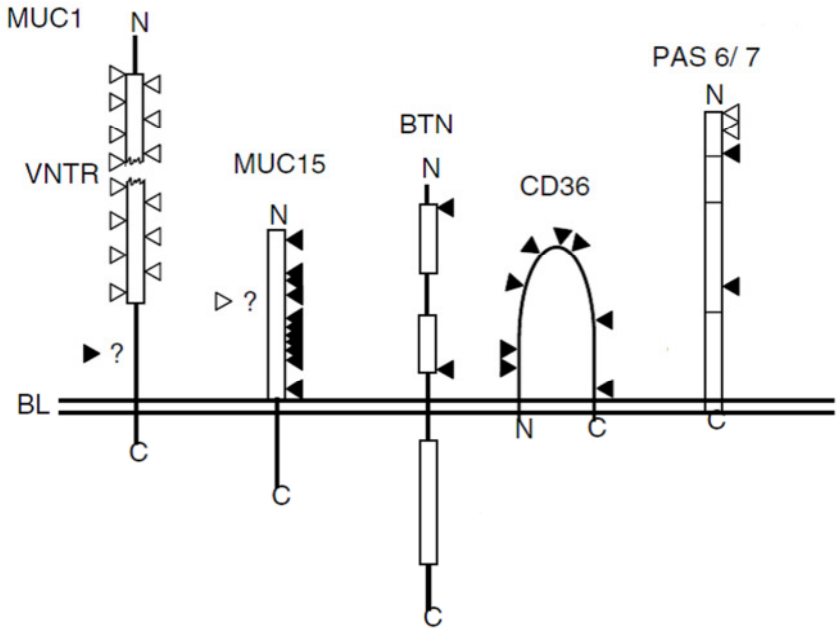

Fig. 3. Topology and proposed structure of glycosylated bovine MFGM proteins. Open triangles show possible O-glycosylation sites, while closed triangles show possible N-glycosylation sites. MUC, Mucin; BTN, Butyrophilin; CD, Cluster of differentiation; PAS, Periodic acid Schiff; VNTR, variable-number tandem repeats; BL, bi-layer. Modified from Keenan and Mather (2006).

A variant of mucin called MUCX have been found in bovine skim milk (Liu et al., 2005). However, an attempt to identify it in the bovine MFGM was unsuccessful (Patton et al., 1989), indicating a more loose attachment to the MFGM than MUC1.

Bovine MFGM has been found to contain a third heavily glycosylated protein classified as a mucin and designated MUC15 (Pallesen et al., 2002). Like MUC1, MUC15 has an extensive exoplasmic domain, a single membrane span and a short cytoplasmic tail, however, unlike MUC1, MUC15 have no tandemly repeated AA sequences. Among the 15 potential N-glycosylation motifs on MUC15 isolated from bovine milk, 11 of them have been shown to be glycosylated (Fig. 3; Pallesen et al., 2002). The heavy glycosylation of mature MUC15 is evident from the large divergence of its calculated mass of 33,317 Da compared to its mass of 130,000 Da approximated from electrophoretic mobility (Pallesen et al., 2007). This indicates that carbohydrates might constitute as much as 67 % of the relative molecular mass of MUC15 (Pallesen et al., 2002).

Butyrophilin (BTN) is the major glycoprotein of bovine MFGM, accounting for over 40 % of the total MFGM protein weight (Jack and Mather, 1990). However, BTN is likely to have only two N-glycosylation sites and no O-glycosylation sites (Jack and Mather, 1990), and contains only ~5 % (w/w) carbohydrate (Fig. 3; Heid et al., 1983). Interactions between plasma membrane BTN and BTN in the milk secretory granule phospholipid monolayer are believed to be controlling the milk fat globule excretion from the lactating cell (Robenek et al., 2006).

Cluster of Differentiation 36 (CD36) constitute 2-5 % of the MFGM proteins (Greenwalt et al., 1992). Eight potential N-glycosylation sites have been identified in CD36, and it was calculated to contain 24 % (w/w) carbohydrates (Berglund et al., 1996). Both involvement of CD36 in milk fat secretion (Reinhardt and Lippolis, 2006) and in import of fatty acids in bovine mammary cells (Bionaz and Loor, 2008) have been suggested as possible functions of this glycoprotein.

Periodic acid Schiff 6 and 7 (PAS-6/7) is the most abundant glycoprotein in bovine MFGM after butyrophilin (Hvarregaard et al., 1996), but is found to be only loosely associated with the MFGM (Kanno and Kim, 1990). Only three and two glycosylation sites have been identified in PAS-6 and PAS-7 respectively (Hvarregaard et al., 1996), and the carbohydrate content have been reported as 7 % and 5.5 % (w/w) respectively. Only N-glycosylation was found in PAS-7, whereas both N-glycosylation and O-glycosylation sites were found in PAS-6 (Fig. 3; Kim et al., 1992).

Cheese related bacteria

The general description of bacteria included in the LAB group is Gram-positive, non-spore-forming, catalase-negative, devoid of cytochromes, of nonaerobic habit but aerotolerant, fastidious, acid-tolerant, strictly fermentative cocci or rods, which produce lactic acid as the major end product during fermentation of carbohydrates (Axelsson, 2004). From a food-technology point of view, the genera presented in Fig. 4 are considered the principal LAB. All LAB are found in the phylum *Firmicutes*, class *Bacilli*, order *Lactobacillales*. Historically *Lb.* sp., *Leuconostoc* (*Ln.*), *Pediococcus* and *Streptococcus* (*Sc.*) form the core of the LAB group.

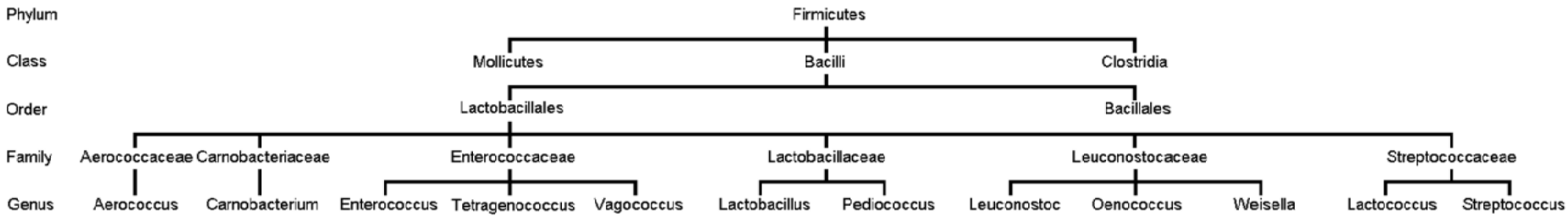

Fig. 4. Classification of lactic acid bacteria associated with food products.

For cheese making purposes LAB from only a few genera are used. It is a prerequisite for successful cheese making that the starter acidifies the cheese milk rapidly using the lactose in the milk as fermentable carbohydrate. The LAB added during cheese making are called starter LAB, and either mesophilic or thermophilic starters are used, depending on the cheese variety. Starter strains commonly used in cheese making are members of the genera *Lc.*, *Lb.* sp., *Sc.* Sp., *Ln.* sp. and *Ec.* sp. (Beresford et al., 2001). The main function of the starter bacteria is to acidify the milk, however they have been found to contribute significantly during ripening as well, where their enzymes are involved in proteolysis and conversion of AA into flavour compounds (Fox and Wallace, 1997).

Adventitious bacteria called NSLAB are thought to originate from the raw milk, surviving pasteurization, or from the production environment through post pasteurization contamination (Beresford et al., 2001; Cogan et al., 2007). They can be present in the fresh cheese in undetectable numbers, but continue to grow during ripening even though lactose and its monosaccharide moieties (Glc and Gal) are depleted. This indicates that the NSLAB are able to utilize other energy sources present in the cheese (Beresford and Williams, 2004). Not all LAB grow well with lactose as the main substrate, and some NSLAB have been shown to grow poorly in milk (Cogan et al., 1997). It is, however, most probable that the residual lactose of the fresh cheese curd is an important energy source during the initial stages in the establishment of the NSLAB flora (Williams et al., 2000). The energy sources utilized by NSLAB have been widely discussed (Fox et al., 1998; Laht et al., 2002; Palles et al., 1998; Thomas, 1987b), but the issue is still unresolved.

The NSLAB found in cheese are mostly mesophilic lactobacilli and sometimes pediococci, and they form a significant portion of the microbial flora of most cheese varieties during ripening (Cogan et al., 1997). Lactobacilli in cheese usually don't reach numbers higher than log 7 cfu/g (Peterson and Marshall, 1990; Fox et al., 1998; Lindberg et al., 1996; Ardö, 1993; Crow et al., 2001), while rich growth media supports the growth of lactobacilli up to log 8-9 cfu/mL. The reason why lactobacilli are not able to reach the same numbers in cheese as in rich growth media is unclear. However, it was observed that lactobacilli in cheese were severely elongated and grouped together in large clusters (Fig. 5; Paper IV).

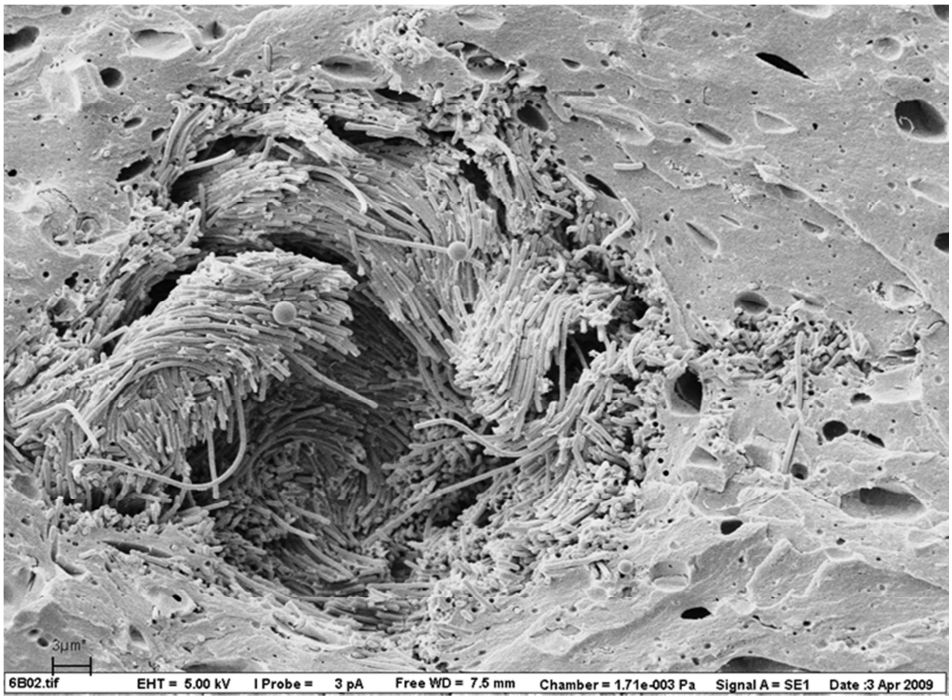

Fig. 5. Electron micrograph (5000 x) of a colony of severely elongated lactobacilli in low-fat Cheddar cheese ripened for 24 weeks (Paper IV).

The cell shape determining protein MreB was found to be significantly ($P < 0.05$) upregulated in *Lb. plantarum* INF15D when it was grown in a model system with Gal as the only added sugar (Paper III). Doi et al. (1988) established that the MreB protein is involved in the formation of the rod shape of the bacterial cell. The shape determining genes are also thought to have a negative influence on cell division, as expression of additional copies of the *mreB* gene in *E. coli* produced elongated, multinucleated cells (Wachi and Matsuhashi, 1989). The MreB protein is a bacterial actin homologue, and it is thought to polymerize by the use of ATP into long filaments (van den Ent et al., 2001). The MreB filaments were first described as helical structures encircling the cell cytoplasm directly under the membrane (Jones et al., 2001). Later work questions this hypothesis (Swulius et al., 2011) and instead suggests that MreB makes up discrete patches moving circumferential in the bacterial cell, inserting radial hoops of new peptidoglycans during their transit (Garner et al., 2011; Domínguez-Escobar et al., 2011). The reason for the elongated lactobacilli cells observed in cheese (Paper IV) could very well be a consequence of the upregulation of the production of the MreB protein as was observed in *Lb. plantarum* INF15D when grown on Gal (Paper III). The fact that it has been observed that the cells elongated by the overproduction of MreB also could be multinucleated (Wachi and Matsuhashi, 1989) makes it reasonable to hypothesize that the elongated state of

lactobacilli obtained in cheese could be an explanation of the relative low maximum cell number obtained. It was shown that *Lb. plantarum* INF15D did obtain slightly lower maximum cell numbers when grown on Gal, in which MreB was up-regulated, than in Glc, GlcNAc and Man (Paper I).

Carbohydrate metabolism of LAB

Lactic acid bacteria are utilizing carbohydrates efficiently, producing ATP by substrate level phosphorylation (Axelsson, 2004). The generated ATP is subsequently used for biosynthesis and transport. Lactic acid bacteria have the ability to adapt to a variety of conditions and are able to utilize a large number of different carbohydrates. Glucose is the preferred hexose sugar of most LAB, however Gal, GlcNAc and Man are also fermented by many LAB (Paper I). Of the strains studied during this work, only *Lb. paracasei* INF448 was able to utilize the amino sugar GalNAc for growth (Paper I).

Glycolysis and the phosphoketolase pathway

The two major pathways for utilization of hexoses in LAB are glycolysis (Embden-Meyerhof-Parnas pathway; Fig. 6A) and the phosphoketolase (PK) pathway (Fig. 6B). The glycolytic pathway yields lactate from Glc and is termed homolactic fermentation, while the PK pathway yields other fermentation products such as CO_2, ethanol and acetate, and is termed heterolactic fermentation. The difference on the enzyme level between these two fermentation pathways is the presence or absence of key enzymes of the glycolysis and the phosphoketolase pathway, fructose bisphosphate aldolase and phosphoketolase respectively.

The glycolytic pathway is used by all LAB except *Ln.* sp. and heterofermentative group III lactobacilli, oenococci and weisellas (Axelsson, 2004). The glycolytic pathway is usually divided into two parts called the energy investment phase and the energy generation phase. In the energy investment phase ATP is used to phosphorylate the hexose in two steps. The hexose is then cleaved into two triose phosphate molecules. In the energy generation phase the formed glyceraldehyde-3-phosphate are further metabolized, producing ATP by substrate-level phosphorylation of 1,3-diphosphoglycerate and phosphoenolpyruvate. A net gain of two moles each of ATP, NADH and pyruvate is formed during glycolysis.

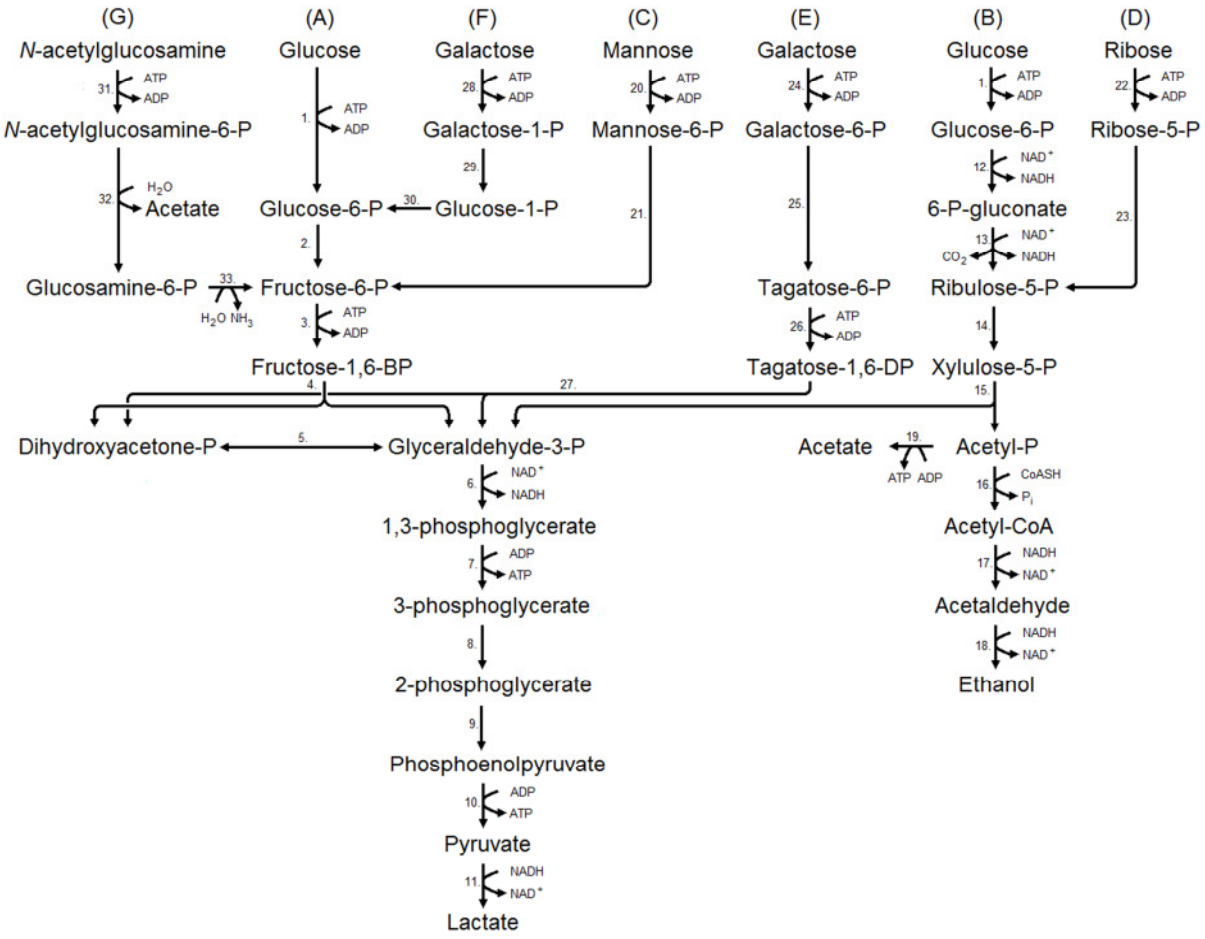

Fig. 6. Fermentation pathways of glucose, mannose, galactose, *N*-acetylglucosamine and Ribose. (A) Homolactic fermentation of glucose (glycolysis, Embden-Meyerhof-Parnas pathway); (B) Heterolactic fermentation of glucose (phosphoketolase pathway); (C) Homolactic fermentation of mannose; (D) Heterolactic fermentation of ribose (phosphoketolase pathway); (E) Tagatose-6-phosphate pathway; (F) Leloir pathway; (G) *N*-acetylglucosamine pathway;. 1. Glucokinase; 2. Phosphoglucoisomerase; 3. Phosphofructokinase; 4. Fructose-1,6-bisphosphate aldolase; 5. Triosephosphate isomerase; 6. Glyceraldehyde-3-phosphate dehydrogenase; 7. Phosphoglycerate kinase; 8. Phosphoglycerate mutase; 9. Enolase; 10. Pyruvate kinase; 11. Lactate dehydrogenase; 12. Glucose-6-phosphate dehydrogenase; 13. 6-phosphogluconate dehydrogenase; 14. Phosphopentose epimerase; 15. Phosphoketolase; 16. Phosphate acetyl transferase; 17. Acetaldehyde dehydrogenase; 18. Alcohol dehydrogenase; 19. Acetate kinase; 20. Hexokinase; 21. Mannose-6-phosphate isomerase; 22. Ribokinase; 23. Ribose-5-phosphate isomerase A; 24. 6-phospho-β-galactosidase; 25. Galactose-6-phosphate isomerase; 26. Tagatose-6-phosphate kinase; 27. Tagatose-1,6-diphosphate aldolase; 28. Galactokinase; 29. Galactose-1-phosphate uridyltransferase; 30. Phosphoglucomutase; 31. *N*-acetylglucosamine kinase; 32. *N*-acetylglucosamine-6-phosphate deacetylase; 33. Glucosamine-6-phosphate deaminase.

The phosphoketolase pathway is characterized by an initial dehydrogenation step with the formation of 6-phosphogluconate followed by decarboxylation. The resulting ribulose-5-phosphate is converted into xylulose 5-phosphate which is split by phoshoketolase into

acetylphosphate and glyceraldehyde-3-phosphate. When no additional electron acceptor is available, acetylphosphate is reduced to ethanol via acetyl-CoA and acetaldehyde. Glyceraldehyde-3-phosphate is metabolised into lactate by the same actions as in the glycolytic pathway. A net gain of one mol each of lactate, ethanol, CO_2 and ATP is obtained by the phosphoketolase pathway.

Mannose enter the major pathways at the level of glucose 6-phosphate or fructose 6-phosphate after isomerisation and/or phosphorylation (Fig. 6C).

Fermentation of ribose

All genera of LAB are able to utilize pentoses, with the exception of the obligate homofermentative group I lactobacilli (Axelsson, 2004). Pentoses are transferred into the cell by specific permeases. Ribose is phosphorylated in the cell to form ribose-5-phosphate, which is further converted by ribose 5-phosphate epimerase into ribulose-5-phosphate (Fig. 6D; Kandler, 1983). Ribulose-5-phosphate enters the phospoketolase pathway, however, in the case of pentose fermentation, no CO_2 is formed. As no dehydrogenation steps are needed to form xylose-5-phosphate, regeneration of NAD^+ are redundant, and acetyl phosphate is used for substrate level phosphorylation yielding acetate and ATP instead of ethanol. *Lb. plantarum* INF15D upregulated the production of ribokinase and ribose-5-phosphate isomerase A when Gal was the only readily available carbohydrate in the media (Paper III). Ribokinase catalyzes the phosphorylation of D-ribose to D-ribose-5-phosphate by the use of ATP (Kandler, 1983). D-ribose-5-phosphate is further converted into D-ribulose-5-phosphate by ribose-5-phosphate isomerase A. Since the only source of ribose in the model system media containing Gal was other dead bacteria cells, these findings indicate that mechanisms for alternative carbohydrate utilization from the dead bacteria cells were induced in *Lb. plantarum* INF15D (Paper III). It has been shown in earlier works that several lactobacilli are able to utilize ribose from dead bacterial cells in model systems (Thomas, 1987b; Rapposch et al., 1999).

Tagatose-6-phosphate pathway and Leloir pathway

Galactose fermentation by LAB that uses the galactose phosphotransferase system (PTS) for uptake of this sugar metabolizes the resulting galactose 6-phosphate through the tagatose 6-phosphate pathway (Bissett and Anderson, 1974). Both *Lc. lactis* and *Lb. casei* uses this pathway for Gal utilization. The tagatose-6-phosphate pathway metabolises galactose 6-phosphate into glyceraldehyde 3-phosphate which is further fermented by the

glycolytic pathway (Fig. 6E). Many LAB transport Gal into the cell by a permease and subsequently convert it into galactose 1-phosphate by the transfer of a phosphate group from ATP. The galactose 1-phosphate is further converted into glucose-6-phosphate that enters the glycolytic pathway (Fig 6F). This metabolic pathway is called the Leloir pathway, and can be used by LAB that is deficient of a galactose PTS system (Kandler, 1983). The Leloir pathway is used for Gal degradation by the NSLAB *Lb. plantarum* INF15D in a model system with Gal as the only readily available carbon source (Paper III).

N-acetylglucosamine pathway

The aminosugar GlcNAc has been reported to be the carbohydrate that gives the highest growth of *E. coli* after Glc (Álvarez-Añorve et al., 2005). It has been shown that several NSLAB are able to utilise GlcNAc for growth (Paper I ; Adamberg et al., 2005; Williams et al., 2000). For several lactobacilli, one *Lc. lactis* subsp. *lactis* strain and one *Ec. hirae* strain it was shown that GlcNAc yielded as high or higher growth rate and/or growth levels compared to Glc (Paper I). The aminosugar GlcNAc can be introduced to the cell via mannose PTS, resulting in GlcNAc-6-P (Postma et al., 1993; Plumbridge and Vimr, 1999). The phosphorylated GlcNAc-6-P is further deacetylated by GlcNAc-6-P deacetylase (Roseman, 1957), releasing one mol acetate per mol sugar (Paper II). The action of GlcN-6-P deaminase releases the ammonia part of the sugar (Comb and Roseman, 1958) yielding fructose-6-phosphate that enters the glycolytic pathway (Fig. 6G). It was observed that LAB grown on GlcNAc as the only readily available carbohydrate source produced large amounts of acetate in a model system (Paper II) and that the enzymes GlcN-6-P deaminase was upregulated in *Lb. plantarum* INF15D grown on GlcNAc (Paper III). This indicates that the same pathway for the degradation of GlcNAc was utilized by the strains studied in Paper II as have been described for several other bacteria in the literature (Foley et al., 2008; Yang et al., 2006; Alice et al., 2003). The large amounts of acetate released from the degradation of unrestricted amounts of GlcNAc appear to be toxic to lactobacilli grown in an unbuffered model system (Paper I and II). The enzyme phosphoglucosamine mutase was found to be upregulated in *Lb. plantarum* INF15D grown on GlcNAc as well (Paper III). This enzyme catalyzes the intermolecular transfer of the phosphate group in glucosamine-6-phosphate to produce glucosamine-1-phosphate. This step is an essential step in the pathway for UDP-GlcNAc biosynthesis used for cell wall catabolism in several bacteria. This indicates that *Lb. plantarum* INF15D was able to use GlcNAc for cell wall biosynthesis directly without metabolizing it for energy first. Topper and Lipton (1953) showed that GlcNAc was

preferentially incorporated into the capsular material in bacterial cells in contrast to Glc when both carbohydrates were present.

Pyruvate metabolism

Lactate dehydrogenase

Lactate dehydrogenase is the last enzyme in the pathway converting sugars to lactate in LAB (Fig. 6 and 7). The conversion of pyruvate to lactate is the main fate of pyruvate in homofermentative LAB, but is also used during the mixed acid fermentation of heterofermentative LAB. This step in the glycolysis is used to regenerate NAD^+ by using pyruvate as a proton acceptor, thereby balancing the redox reaction. Fructose-1,6-bisphosphate functions as an allosteric regulator (activator) of pyruvate kinase and lactate dehydrogenase in *Lc. lactis*, and accumulates during Glc metabolism (Thompson, 1987). The shift to mixed acid fermentation has been interpreted as a result of reduced fructose-1,6-bisphosphate pool, leading to lactate dehydrogenase inactivation, and inhibition relief of pyruvate-formate lyase by triose phosphates (Thomas et al., 1979). Lactate dehydrogenase has been found to have a high negative control on the flux towards production of formate in *Lc. lactis* (Andersen et al., 2001). D-lactate dehydrogenase was found to be down regulated in the NSLAB *Lb. plantarum* INF15D when it was grown in a model system containing Gal as the only fermentable sugar (Paper III). It was also observed that the production of formate was induced in this strain when utilizing Gal (Paper II), suggesting a shift to mixed acid fermentation.

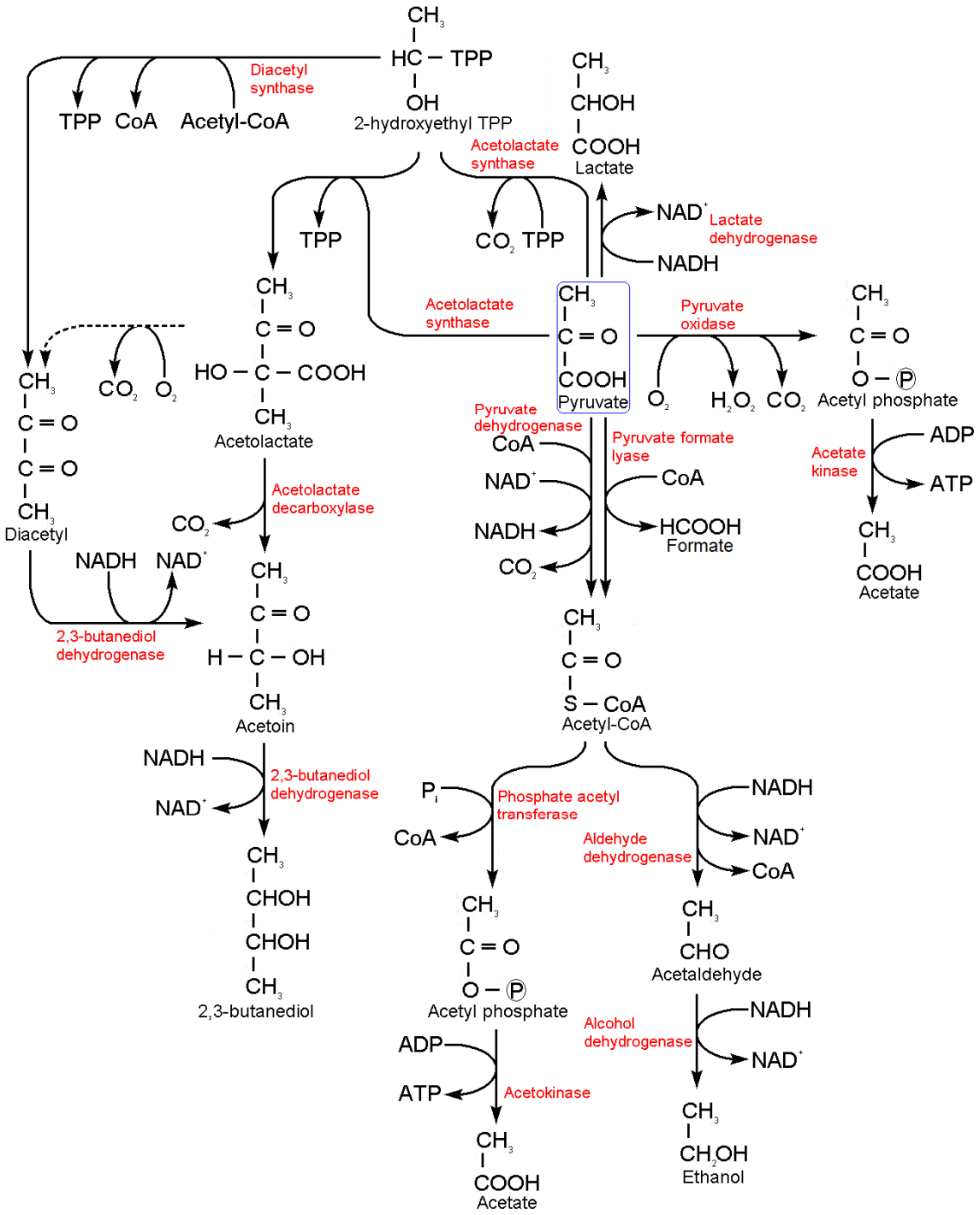

Fig. 7. Pyruvate metabolism in lactic acid bacteria. Broken line denotes a non-enzymatic reaction. TPP, thiamine pyrophosphate; CoA, coenzyme A.

The diacetyl/acetoin pathway

α-acetolactate synthase catalyses the decarboxylation of pyruvate with thiamine pyrophosphate (TPP) acting as a coenzyme, forming 2-hydroxyethyl-TPP, also called active acetaldehyde. Active acetaldehyde either acts as an intermediate that reacts with another

pyruvate molecule and forms α-acetolactate and CO_2 in a TPP dependent condensation, or as a precursor to diacetyl. Diacetyl is formed by the non-enzymatic chemical decomposition of α-acetolactate (Fig. 7). This is a reaction favoured by aeration and low pH. Acetoin can be produced either by decarboxylation of α-acetolactate, or by the dehydrogenation of diacetyl. Acetoin can function as an electron-acceptor, reoxidizing NADH, producing NAD^+ and 2,3-butanediol. The conversion of active acetaldehyde to acetoin by diacetyl synthase was earlier believed to be an important route for the formation of acetoin. However, evidence indicate that this route is of lesser importance (Hugenholtz, 1993).

The diacetyl/acetoin pathway will only proceed to a significant degree if there is a pyruvate surplus in the cell relative to the need for NAD^+ regeneration (Axelsson, 2004). This can happen when other, more effective, pyruvate utilizing enzymes are inhibited or when the rate of puruvate production exceeds its conversion rate (Hugenholtz, 1993).

In fermented milk-products the diacetyl/acetoin pathwas is induced by surplus pyruvate obtained by the additional degradation of citrate to pyruvate (Axelsson, 2004). Bovine milk contain ~8 mM citrate. It is shown that *Ln.* sp., *Lc. lactis* ssp. *lactis* biovar. *diacetylactis*, several *Lb. plantarum* (Thomas, 1987a), *Lb. casei* ATCC 334 (Diaz-Muniz and Steele, 2006) and *Lb. pentosus* (Cselovszky et al., 1992) are all able to metabolize citrate. Torino et al. (2005) have also shown that *Lb. helveticus* ATCC 15807 are able to metabolise citrate in the presence of lactose. In Dutch-type cheeses, the eyes are formed by the release of CO_2 from citrate fermentation by *Ln.* sp. and *Lc. lactis* subsp. *lactis* biovar *diacetylactis*.

In all citrate-utilizing LAB, citrate is transported into the cell by a citrate permease, and is converted to oxaloacetate and acetate by the action of citrate lyase. This enzyme is not found in the LAB unable to utilize citrate. In the citrate utilizing *Lc.* sp. and *Ln.* sp., oxaloacetate is decarboxylated to pyruvate. In *Lb. pentosus* part of the *tricarboxylic acid cycle* (TCA) is used to reduce oxaloacetate to succinate, using the enzymes malate dehydrogenase, fumarase and fumarate reductase (Cselovszky et al., 1992). Both conversion of acetate to succinate and conversion via pyruvate to acetate and formate are known for *Lb. plantarum*.

The pyruvate-formate lyase system

The enzyme pyruvate-formate lyase catalyzes the reaction of pyruvate and CoA to formate and acetyl-CoA. This enzyme is present in most homofermentative LAB, but has not been observed in the heterofermentative *Ln.* sp. Acetyl-CoA can either function as an electron acceptor, or as a precursor for substrate-level phosphorylation via acetyl phosphate. The reduced product of acetyl-CoA is ethanol (Fig. 7). The pyruvate-formate lyase system

contributes to what is called mixed acid fermentation, where formate, lactate, acetate and ethanol are the end products. It is proposed that this pathway is used in LAB as a response to starvation, as energy can be gained by substrate-level phosphorylation instead of the reduction of pyruvate to lactate (Thomas et al., 1979). The production of acetate, formate and ethanol was found to be higher in *Lb. plantarum* INF15D when grown in a model system containing Gal as the fermentable carbohydrate (Paper II), strongly suggesting a shift to mixed acid fermentation, even though a fermentable carbohydrate was present. However, *Lb. plantarum* INF15D utilized Gal at a lower rate (Paper II) and had a lower growth rate on Gal in the model system compared to Glc, Man and GlcNAc (Paper I). A shift from homolactic to mixed acid fermentation occurs in LAB when they grow on slowly fermentable sugars. However, the mechanism regulating this shift is still unclear. Bacteria can benefit from a shift in pyruvate metabolism to regenerate ATP or NAD^+ as needed. A shift to mixed acid fermentation by growth on Gal have been observed in *Lc. lactis* in an earlier experiment (Thomas et al., 1980).

The pyruvate oxidase pathway

Pyruvate oxidase converts pyruvate and O_2 to CO_2, acetyl phosphate and H_2O_2 (Fig. 7). This pathway has been suggested to be involved in aerobic metabolism of *Lb. plantarum*, forming large amounts of acetic acid aerobically (Sedewitz et al., 1984).

The pyruvate dehydrogenase pathway

This enzyme complex produces acetyl-CoA by an alternative pathway to the pyruvate-formate lyase system (Fig. 7). A prerequisite for this metabolism to occur is that the NADH formed during both glycolysis and in the pyruvate dehydrogenase reaction can be reoxidized by NADH oxidases, as NADH has a strong inhibitory effect on the pyruvate dehydrogenase complex (Smart and Thomas, 1987). The pyruvate dehydrogenase complex is also strongly dependent on the presence of the cofactors coenzyme A (CoA) and TPP. It is likely that a pyruvate dehydrogenase enzyme complex is active in lactococci (Smart and Thomas, 1987).

Protein degradation

Bovine milk CN consists of α_{s1}-, α_{s2}-, β- and κ-CN. Lactic acid bacteria degrade CN and large CN-derived peptides produced by milk- and coagulant enzymes by cell-envelope proteinases (CEP). A type of CEP called PrtP is responsible for the proteolytic activity of lactococci associated with milk acidification. All CEPs from LAB described to date, are Ser-

proteinases related to the non-specific protease subtilisin (Parente and Cogan, 2004). The activity of CEPs on α_{s1}-CN, β-CN and κ-CN releases a large variety of oligopeptides of size up to 30 AA residues. However, most oligopeptides released from CEP action are in the 4-10 residues range (Parente and Cogan, 2004). The starter is the principal source of peptidases in cheese, which is responsible for the hydrolysis of short peptides and the liberation of AA (Upadhyay et al., 2004; Fox and Wallace, 1997; Di Cagno et al., 2003). The starter stops growing in cheese soon after the end of manufacture due to the low pH, increasing NaCl concentration, low temperature and lack of a fermentable carbohydrate substrate.

The κ-CN of milk is a glycoprotein and contains ~5 % carbohydrates (Kobata, 1977), however, the glycosylated part of the protein is released by the action of rennet during cheese making, and the glycomacropeptides are mostly drained from the cheese together with the whey.

Amino acid metabolism

It is a general belief that LABs ability to synthesise AA are limited (Liu et al., 2003a). This might be due to the protein rich environment LAB prefers, where the requirements of AA can be fulfilled by proteolysis and peptidolysis of environmental proteins. There are large variations in the AA requirements between both species and strains of LAB. An oligopeptide transport system (Opp) transports the oligopeptides into the cell. Lactic acid bacteria possess several intracellular oligoendopeptidases (PepO and PepF), at least three general aminopeptidases (PepN, PepC and PepG), a glutamyl aminopeptidase (PepA), a pyrolidone carboxyl peptidase (PCP), a leucyl aminopeptidase (PepL), a prolyl-dipeptidyl aminopeptidase (PepX), a proline iminopeptidase (PepI), an aminopeptidase P (PepP), a prolinase (PepR), a prolidase (PepQ), a general dipeptidase (PepV) and a general tripeptidase (PepT; Christensen et al., 1999). These peptidases degrade peptides in the cell into free AA that can be utilized for biosynthesis. Transport of AA is generally mediated by proton motive force (PMF)-dependent systems, antiport systems, or ATP-driven systems (Axelsson, 2004).

The FAA present in milk cannot support growth of LAB to high numbers (10^9-10^{10} cfu/g) in cheese, and the LAB will need to obtain the AA by hydrolysing small peptides in the cheese (McSweeney and Sousa, 2000). It was shown in a model system containing MFGM that *Lb. paracasei* INF448 was more peptidolytic than the other studied strains (Paper II). This strain also had the highest increase in cell numbers during growth after the apparent depletion of lactose compared to the other studied strains in the MFGM medium (Paper I). In

several other experiments it was shown that AA released by proteolytic activity could enhance NSLAB growth (Crow et al., 1995; Di Cagno et al., 2003; Hynes et al., 2001; Martley and Crow, 1993). Our work therefore supports the observations suggesting that a high release of AA from the growth media may support growth of NSLAB (Paper I and paper II).

Arginine

Arginine can be utilized as a source of energy by some LAB in cheese (Laht et al., 2002), but this AA do not support growth in a defined medium deficient of fermentable carbohydrates (Paper I). This implies that Arg cannot be taken up by the cell in absence of a fermentable sugar that generates the energy required to facilitate the initial uptake (Liu and Pilone, 1998). An Arg/ornithine (Orn) antiporter have been reported in *Lc. lactis* (Driessen et al., 1989) able to transport Arg into the cell simultaneously as Orn is transported out. For the antiporter system to be active, a chemiosmotic gradient has to be present by both substrates in opposing directions. If Arg is present in the medium, but no Orn is present in the cell, the antiporter system will be inactive, indicating that initially Arg have to be transported into the cell by another system even if the antiporter system is present. Several studies indicate that the conversion of Arg to Orn and citrulline (Cit) is typically present in heterofermentative lactobacilli (Crow and Thomas, 1982; Liu et al., 1995). In contrast, several heterofermentative lactobacilli and pediococci isolates, do not possess any of the three enzymes involved in this pathway (Liu et al., 1995, Martens et al., 1997). This pathway involves three steps, and Arg is first converted to Cit by arginine deiminase. Ornitihine transcarbamylase further degrade Cit to Orn and Carbamoyl-phosphate that can be used for substrate level phosphorylation by the action of carbamate kinase, producing ATP, ammonia and CO_2 (Fernandez et al., 2004). The ATP formed during Arg catabolism can be used for bacterial growth, and is in theory sufficient to support growth of NSLAB found in ripening cheese (Laht et al., 2002).
It has been shown that both arginine deiminase (ADI) and Ornithine transcarbamylase was inhibited by Glc whereas the inhibitory effect of Gal was much lower (Crow and Thomas, 1982). In *Lb. plantarum* INF15D grown in a model system with Gal as the only readily fermentable carbohydrate, a reduction of Arg and an simultaneous increase in Orn was observed (Paper II). This was not observed in any of the *Lb. paracasei* strains used in this study. This indicates that *Lb. plantarum* INF15D is able to utilize Arg for energy by the use of ADI under certain conditions.

Serine

It was hypothesized that energy can be produced by *Lb. plantarum* from Ser by deamination to form pyruvate and ammonia, and that the pyruvate mainly was catabolized to acetate and formate in a growth media containing lactose as the fermentable carbohydrate (Liu et al., 2003b).

When *Lb. plantarum* INF15D was grown in a model system added the monosaccharides Glc, Gal, GlcNAc or Man, Ser was utilized, whereas none of the studied *Lb. paracasei* strains decreased the levels of Ser when grown on any of the monosaccharides (Paper II). In the study performed by Liu et al. (2003b) it was proposed that the degradation products of pyruvate formed from Ser were formate and acetate. Both of these products as well as small amounts of ethanol were observed when *Lb. plantarum* INF15D was grown on Gal, however, none of these compounds were observed when grown on Glc and Man, even though the levels of Ser decreased (Paper II). When *Lb. plantarum* INF15D was grown on Gal as the only added sugar in the model system, the enzyme L-Serine dehydratase β subunit were significantly ($P < 0.05$) upregulated (Paper III). The enzyme L-Serine dehydratase is involved in the conversion of L-Serine to NH_3 and pyruvate which suggests that this energy yielding pathway was active under the mentioned conditions. The enzyme tryptophan synthase subunit α was found in *Lb. plantarum* INF15D as well, however, this enzyme was found to be unregulated in the different conditions used in this experiment (Paper III). This enzyme is part of an enzyme complex that converts Ser and indole-3-glycerol phosphate into tryptophane, glyceraldehyde-3-phosphate and H_2O. Glyceraldehyde-3-phosphate is a glycolytic pathway intermediate and can be utilized for energy production. These findings indicates that when *Lb. plantarum* INF15D is grown on Gal, Ser may be utilized by more than one potentially energy yielding pathways at the same time.

Decarboxylation

Streptococcus thermophilus, *Lb. brevis, Lb. plantarum, L. lactis* ssp. *lactis* and cheese surface flora is known to regulate the internal pH in an acid environment by producing GABA from Glu by glutamic acid decarboxylase (Ardö et al., 2002; Kieronczyk, 2002). However, several authors have suggested that decarboxylation of AA can be used as a potential indirect source of energy production in bacteria (Higuchi et al., 1997; Konings et al., 1989; Pessione et al., 2010). Several studies report the occurrence of antiporter transport systems in LAB. These include a histidine (His)/histamine antiporter in *Lb. buchneri* (Molenaar et al., 1993) and in *Lb. hilgardii* IOEB 0006 (Lucas et al., 2005), a glutamate/GABA antiporter in *Lb.* sp. strain

E1 (Higuchi et al., 1997), a tyrosine/tyramine antiporter in *Lb. brevis* (Wolken et al., 2006) and *Lb. buchneri* (Konings et al., 1995), a phenylalanine/phenylethylamin exchanger in *Lb. bucheneri* (Konings et al., 1995) and an aspartate (Asn)/Ala exchanger in *Lb.* sp. (Konings et al., 1995). These antiporters are able to transport molecules across cell membranes against their chemiosmotic gradient. The process occurs without energy expenditure as another molecule is transported coincidentally along the chemiosmotic gradient. By exchanging the resulting amine with more substrate via the antiporter, an electrochemical proton gradient is formed that can be used for generation of ATP by pumping protons into the cell via the F_1F_o ATP synthase located in the cell wall. The electrochemical proton gradient is maintained by decarboxylation reactions inside the cell (Higuchi et al., 1997; Konings et al., 1989; Pessione et al., 2010). In cheese, amines can be formed by microbial decarboxylation of AA. Several of these biogenic amines are toxic, and are known to cause headache and dizziness in susceptible individuals. If a decarboxylating strain is present in the cheese, the production of amines is often determined by the production of free AA (Walstra et al., 1999). Propionic acid bacteria are able to decarboxylize His to histamine (Walstra et al., 1999), although this action is dependent of pH and water activity in the cheese. There have been reports of *Lb. plantarum* producing putrescine by decarboxylation of Orn (Arena and de Nadra, 2001). The decarboxylation of Asn to Ala have also been reported in *Lb.* sp. (Abe et al., 1996; Konings et al., 1995). The reduction of Tyr when grown with Gal as the only added carbohydrate in a model system was observed. However, if this AA was decarboxylated to form tyramine or degraded to another product is not known (Paper III).

Proteomics

Proteomics can be used to study the response of a cell to different conditions or treatments, and have with the combination of chromatography and mass spectrometry become a powerful tool for analyzing complex mixtures of proteins. The proteome of a cell is the proteins expressed at a given time under given conditions. By following this definition, the proteome of e.g. a certain cell will vary with time and requirements, or with the stresses the cell undergoes. It is therefore reasonable to study the difference between treatments and/or conditions using proteomics. This is at the same time one of the many challenges with proteomics, as any treatments of the cell will change its proteome. Great care must therefore be taken to avoid additional disturbance of the proteome during sample preparation.

Two-dimensional gel electrophoresis separates proteins in two dimensions where isoelectric point separation is done in one dimension and molecular weight separation in the other. A gel strip with an immobilized pH gradient is most commonly used for the isoelectric focusing (IEF). The proteins enters the gradient gel by absorption from a buffer containing the sample, often by applying an electric current. After sample transfer, an electric field is applied across the pH gradient. The proteins will move in this electric field, initially towards the electrode with the opposite charge. When the protein reaches the point in the pH gradient that is equal to its pI, the net charge of the molecule will become zero, and the migration will end (Eidhammer et al., 2007). The procedure is illustrated in Fig. 7.

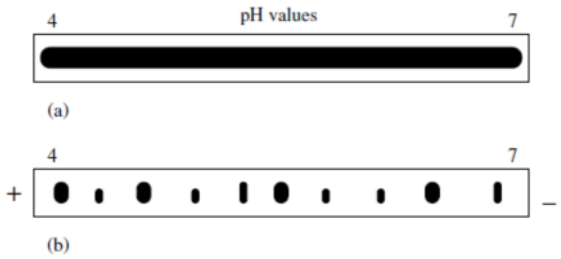

Fig. 7. (a) A protein sample is adsorbed to the pH gradient gel-strip. (b) When an electric field is applied, the proteins move to the point where pH is equal to their pI values. Each line therefore contains a set of proteins with similar pI value (Eidhammer et al., 2007).

After the IEF the gel strip is treated with SDS and placed on top of a polyacrylamide gel. The SDS treatment imparts negative charges to the proteins roughly proportional to the size of the protein. An electric current is applied to the gel and the proteins migrate through the gel with velocities depending on their sizes. Staining of the gels after the electrophoresis makes the protein spots visible.

After enzymatic digestion and removal of the proteins from the gel, the samples are routinely reduced, alkylated, desalted and spotted onto a matrix assisted laser desorption/ionization (MALDI) target plate together with an appropriate matrix material. Identification of the peptides can then be done by ionizing the samples using a pulsating laser beam and introducing it to a time of flight (TOF) mass analyzer. The electrically accelerated ionized sample mass is calculated based on the time the sample uses to travel through a vacuum tube in the mass analyzer. The time the ions use to move through the length of the vacuum tube can be calculated (1).

$$t = \frac{l}{\sqrt{\frac{2ezV}{m}}} \quad (1)$$

t = time, l = the length of the vacuum tube, z = charge of the molecule, V = acceleration charge, m = mass.

Since l and V are constant, t will only be dependent on the mass and charge of the molecules. Molecules with low *m/z* will then use shorter time to travel through the vacuum tube than molecules with high *m/z*. For analyzing proteins excised from gels, trypsination creates peptides that collectively can be identified by the MALDI-TOF, creating of a peptide mass fingerprint that can be used to identify the original protein by using a database.

When samples are ionized in this manner, they become metastable, and the fragmented masses of peptides can also be analysed using a MS/MS mode. By using this mode, complete AA sequences of the peptides can be identified, making confident protein identification easier.

CONCLUSIONS

The LAB studied, all relevant to cheese production and ripening, were able to grow and survive for an extended period of time on a bovine MFGM as the only potential carbohydrate source. All isolates were able to effectively utilize most of the monosaccharides found in MFGM glycoconjugates. However, none of the isolates were able to utilize NANA for growth, and only *Lc. lactis* subsp. lactis ML8 and *Ec. hirae* INFE1 were able to grow on Fuc. Of the studied strains, only *Lb. paracasei* INF448 was able to utilize GalNAc for growth, and large amounts of acetic acid was released during its metabolism. An apparent connection between the higher proteolytic activity of *Lb. paracasei* INF448 and its ability to grow in the MFGM media after the depletion of readily available sugars was observed. Growth of *Lb. plantarum* INF15D on the acylated aminosugar GlcNAc led to induction of enzymes involved in the degradation of this sugar through the GlcNAc pathway, and this gave large amounts of acetate as a degradation product. When the studied lactobacilli were incubated on GlcNAc or GalNAc, a decrease in the number of culturable bacteria was induced at an earlier stage than when incubated on any of the other monosaccharides. This decrease in cultivable bacteria was presumably due to the antimicrobial effect of the acetic acid released during the metabolism of these two sugars in the unbuffered model system. The potential cheese contaminant *Ec. hirae* INFE1 showed superior growth and survival abilities compared with the other LAB when grown on the MFGM media and on several of the MFGM monosaccharides.

When *Lb. plantarum* INF15D was grown in a model system added the monosaccharides Glc, Gal, GlcNAc or Man, Ser was utilized, whereas none of the studied *Lb. paracasei* strains decreased the levels of Ser when grown on any of the monosaccharides. Enzymes involved in two different pathways yielding energy from Ser was found in *Lb. plantarum* INF15D. Growth of *Lb. plantarum* INF15D on Gal resulted in a metabolic shift expressed as different fates of the produced pyruvate compared to growth on the other monosaccharides. For *Lb. plantarum* INF15D the presence of Gal also seemed to initiate degradation of some AA known to potentially take part in energy production, specifically Arg and Tyr. Growth of *Lb. plantarum* INF15D on Gal induced enzymes involved in alternative energy acquisition from carbohydrate sources. Enzymes involved in ribose metabolism as well as phosphoketolase pathway enzymes was upregulated, leading to the production of ethanol and acetate. These findings indicate that *Lb. plantarum* INF15D are able to utilize several different substrates for energy at the same time to secure growth and survival during

sub-optimal conditions. The cell shape determining protein MreB that leads to elongation of the cells was also found to be upregulated during growth of *Lb. plantarum* INF15D on Gal.

Cheeses were made with the addition of butter milk, and low initial numbers (log 2 cfu/mL cheese milk) of two different *Lb. paracasei* adjunct cultures (INF448 and INF456) known to be different in their utilization of the MFGM monosaccharide GalNAc. A low initial numbers was used to mimic the content of NSLAB normally found in cheese milk. The present microorganisms at start influenced the development and composition of the lactobacilli during ripening even if the adjuncts were added at low numbers and did not dominate the microflora at start. Even if the *Lb. paracasei* adjuncts have the ability to utilize the MFGM components, they did not dominate the NSLAB microflora in the cheese in the later stages of ripening. The starter bacteria were located within the protein networks in clusters which were distributed homogeneously throughout the cheese matrix regardless of treatment while lactobacilli was rare and when found they were in huge clusters of clearly elongated cells.

The higher content of MFGM components in the cheese seemed to influence the development of lactobacilli and the composition of free AA during ripening. The amount of leucine and 3-methyl butanal were higher in cheeses with BMP than in cheeses with SMP, indicating that supplementation of the cheese with butter milk components seems to facilitate better growth of flavour producing microorganisms in cheese.

PERSPECTIVES

- It has been shown in this work that *Lb. plantarum* INF15D induced several different energy yielding pathways during growth on Gal. This trait could be further investigated to learn more about alternative pathways used by *Lb. plantarum* that could be relevant for cheese ripening.

- The higher production of the cell shape determining protein MreB in *Lb. plantarum* INF15D as well as the observed elongation of lactobacilli in cheese are interesting traits that warrants further investigations.

- Analysis of oligosaccharides released by deglycosylation from MFGM glycoconjugates is possible by the use of chromatographic equipment. A change in amounts of the detected oligosaccharides after incubation with NSLAB could reveal if and how the bacteria are able to release sugars from the MFGM glycoconjugates.

- Further studies of the occurrence of cell wall antiporters and AA decarboxylation reactions could give insight into the use of F_1F_o ATP synthase as a means to produce energy in NSLAB.

- Metaproteomic studies of the microbial community in cheese could give interesting insight into the different means of energy acquisition of cheese ripening bacteria, by identifying the metabolic pathway enzymes of the community.

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Growth and survival of cheese ripening bacteria on milk fat globule membrane isolated from bovine milk and its monosaccharides

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ABSTRACT

The growth and survival of cheese lactic acid bacteria (LAB) on milk fat globule membrane (MFGM) isolated from bovine milk and on monosaccharides found in MFGM glycoconjugates were studied. The LAB studied were selected lactobacilli strains isolated from Norwegian semi-hard cheese, a starter *Lactococcus* sp. and the potential cheese contaminant *Enterococcus hirae*. The bacteria were able to grow on MFGM isolated from bovine milk and to utilize several of the monosaccharides found in the MFGM. Only one strain of lactobacilli was able to utilize *N*-acetyl-D-galactosamine for growth. Growing lactobacilli on the acylated aminosugars *N*-acetyl-D-glucosamine or *N*-acetyl-D-galactosamine induced a decrease in the number of culturable bacteria at an earlier stage than when grown on any of the other monosaccharides. The potential cheese contaminant *Ec. hirae* showed superior growth and survival abilities compared with the other lactic acid bacteria when grown on the MFGM media and on several of the MFGM monosaccharides.

1. Introduction

The depletion of lactose during the first few days of cheese making and the inhospitable environment (NaCl, 3–6%, w/v; pH, 4.9–5.3; a_w, 0.85–1.0; redox potential, ~−250 mV and free carbohydrates, low or none) are likely reasons for the decline of viable starter lactic acid bacteria (LAB) observed during cheese ripening (Fenelon, O'Connor, & Guinee, 2000; Turcot, St-Gelais, & Turgeon, 2002). Non-starter lactic acid bacteria (NSLAB) do, however, continue to grow in the cheese during ripening even though lactose and its monosaccharide moieties (glucose and galactose) are depleted. This indicates that the NSLAB are able to utilize other energy sources present in the cheese (Beresford & Williams, 2004). The energy sources utilized by NSLAB have been widely discussed (Fox, McSweeney, & Lynch, 1998; Laht, Kask, Elias, Adamberg, & Paalme, 2002; Palles, Beresford, Condon, & Cogan, 1998; Thomas, 1987) but the issue is still unresolved. It is however most probable that the residual lactose of the fresh cheese curd is an important energy source during the initial stages in the establishment of the NSLAB flora (Williams, Withers, & Banks, 2000).

Fenelon et al. (2000) demonstrated that the fat content of Cheddar cheese affects the development of both the starter LAB and NSLAB. Starter LAB were present in a higher initial number in full-fat cheese than in low-fat cheese, which agrees with the results obtained by Laloy, Vuillemard, El Soda, & Simard (1996), who concluded that the amount of milk fat directly influences the retention and the growth, or both, of the starter LAB in the fresh cheese curd. Laloy et al. (1996) suggested that the higher retention rate of starter bacteria in full-fat cheeses could either be explained by fat functioning as a filter retaining the bacteria, or by the existence of an active relationship between fat globules and bacterial cells. Electron microscopic images show that bacteria in cheese most probably are localized in the fat/protein interface (Laloy et al., 1996; Lopez, Maillard, Briard-Bion, Camier, & Hannon, 2006).

The fat globules of bovine milk are enveloped by a membrane derived from the plasma membrane of the secretory mammary cells (Patton & Keenan, 1975). Numerous complex carbohydrates have been identified as glycoconjugates in the form of glycoproteins and glycolipids in the bovine milk fat globule membrane (MFGM) (Liu, Erickson, & Henning, 2005; Mather, 2000).

Fox et al. (1998) suggested that sugars associated with the MFGM can serve as a source of energy for LAB present in cheese during ripening. They found that *Lactobacillus paracasei* subsp. *paracasei* NCDO 1205 was unable to grow in a defined medium containing nucleotides, vitamins and salts, and either the non-fat, water-insoluble fraction or the water-soluble fraction of Cheddar cheese, unless a sugar was added. The same bacteria grew readily in macerated heat treated (65 °C, 30 min) cheese.

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The different monosaccharide components found in MFGM glycoproteins are well documented (Hvarregaard, Andersen, Berglund, Rasmussen, & Petersen, 1996; Nakata, Furukawa, Greenwalt, Sato, & Kobata, 1993; Pallesen, Pedersen, Petersen, & Rasmussen, 2007; Sato, Takio, Kobata, Greenwalt, & Furukawa, 1995; Snow, Colton, & Carraway, 1977). The carbohydrates of the MFGM glycoconjugate side chains include *N*-acetyl-D-neuraminic acid (NANA), D-galactose (Gal), L-fucose (Fuc), D-mannose (Man), *N*-acetyl-D-glucosamine (GlcNAc) and *N*-acetyl-D-galactosamine (GalNAc).

Williams and Banks (1997) found that several species of mesophilic lactobacilli possess glycolytic enzymes (α- and β-galactosidases, α- and β-galactosaminidases and *N*-acetyl-α- and *N*-acetyl-β-D-neuraminidase) which may be capable of releasing sugars from glycoproteins.

Adamberg et al. (2005) and Williams et al. (2000) have shown that NSLAB can utilize sugars originating from glycoproteins and glycolipids found in the MFGM, using a chemical reduction indicator or optical density measurements, respectively. It has also been shown that NSLAB are able to utilize bacterial starter cell suspensions for growth (Thomas, 1987). Thomas (1987) concluded that products released during autolysis of the starter cells were utilized for growth. It is well established that the acylated aminosugar GlcNAc is a major bacterial cell wall constituent, and may be released by autolytic enzymes (Doyle, Chaloupka, & Vinter, 1988). However, later studies indicate that high autolysis rate of the starter strain is less important for the growth of the NSLAB microflora (Lane, Fox, Walsh, Folkertsma, & McSweeney, 1997) than indicated by Thomas (1987), and that amino acids released by proteolytic activity could enhance NSLAB growth (Crow et al., 1995; Di Cagno et al., 2003; Hynes, Ogier, & Delacroix-Buchet, 2001; Martley & Crow, 1993).

The objective of this study was to assess and compare the growth and survival of cheese microorganisms *in vitro* in a growth media containing MFGM isolated from bovine milk or monosaccharides found in MFGM glycoconjugates, using three defined growth descriptors. The cheese microorganisms were selected lactobacilli strains isolated from Norwegian semi-hard cheese, a starter *Lactococcus* sp. and the potential cheese contaminant *Enterococcus hirae*.

2. Materials and methods

2.1. Bacterial strains

The nine strains studied, *Lb. paracasei*; INF10 (INF10), INF448 (INF448), INF456 (INF456), INF1052 (INF1052), *Lb. plantarum*; INF15D (INF15D), INF2756 (INF2756), *Lactococcus lactis* subsp. *lactis*; ML8 (ML8), *Lc. lactis* subsp. *cremoris*; P2 (P2) and *Ec. hirae* INFE1 were obtained from the culture collection of the Department of Chemistry, Biotechnology and Food Science, Norwegian University of Life Sciences, Aas, Norway. Strains INF10 and INF15D were isolated from 90 d old, INF448 and INF456 from 180 d old and INF1052 from 270 d old Norvegia cheese (a washed curd Dutch type cheese). Strain ML8 was a gift from the Department of Food Chemistry, University College Cork, Cork, Ireland.

2.2. Carbohydrate-restricted medium

A carbohydrate-restricted medium (CRM) was made according to Adamberg et al. (2005), with minor adjustments. The CRM contained 12.0 g L^{-1} peptone from casein (Merck, Darmstadt, Germany), 1.0 g L^{-1} Tween 80 (Koch-Light Laboratories Ltd., Colnbrook, UK), 0.6 g L^{-1} $MgSO_4 \cdot 7H_2O$ (Merck), 0.3 g L^{-1} $MnSO_4 \cdot 5H_2O$ (Merck), 0.06 g L^{-1} $FeSO_4 \cdot 7H_2O$ (Merck), 0.05 mg L^{-1} biotin (Sigma, Steinheim, Germany), 0.1 mg L^{-1} folic acid (Sigma), 0.1 mg L^{-1} niacinamide (Sigma), 0.1 mg L^{-1} pyridoxal-HCl (Sigma), 0.1 mg L^{-1} riboflavin (Sigma), 0.1 mg L^{-1} thiamine-HCl (Sigma) and 0.2 mg L^{-1} panthothenate (Sigma). The CRM was autoclaved (121 °C, 15 min) prior to use.

2.3. Preparation of carbohydrate stock solutions

The carbohydrates used to supplement the CRM were D-glucose (Glc; Merck), Gal, Fuc, Man, GlcNAc, GalNAc and NANA (all from Sigma). The carbohydrates were prepared as stock solutions (100 mg mL^{-1}), and were filter sterilized (pore size 0.22 μm; Millex, Carrigtwahill, Ireland) prior to addition to CRM (final concentration 5 g L^{-1}). The calculated final levels of the carbohydrates in the supplemented CRM growth media were: GlcNAc, 22.60 mmol L^{-1}, GalNAc, 22.60 mmol L^{-1}, Glc, 27.75 mmol L^{-1}, Gal, 27.75 mmol L^{-1}, Fuc, 30.46 mmol L^{-1}, Man, 27.75 mmol L^{-1}.

2.4. Preparation of purified milk fat globule membrane material

The MFGM material was prepared by a procedure described by Vanderghem et al. (2008) with minor modifications. Fresh unpasteurized milk, cooled to 4 °C was obtained from the herd at the Norwegian University of Life Sciences. The milk was heated to 50 °C, and separated using a tabletop separator (Type R5; HÄKA Buttermaschinen GmbH, Stutensee, Germany). The cream fraction was cooled to 4 °C and gently washed twice with 5 vol phosphate buffered saline (PBS; 0.01 M; pH 7.2; 0.9% NaCl; 4 °C) and once with 5 vol distilled water (4 °C). The cream was subjected to centrifugation between washes (4500× *g*; 10 min; 4 °C). The washed fat globules were added distilled water, and allowed to crystallize for 20 h at 4 °C before heavy agitation induced phase separation. Both the serum and fat fractions were heated to 45 °C for 30 min to melt the fat. The fat fraction was washed with distilled water and centrifuged (4500× *g*; 10 min; 4 °C) to recover the residual serum. The serum from the fat fraction was combined with the serum from the serum fraction and centrifuged twice (5000× *g*; 15 min; 4 °C) to remove the fat. The serum was freeze dried and stored at −22 °C until used.

The freeze dried MFGM powder was rehydrated in distilled water (100 mg mL^{-1}), and added to CRM (final concentration 5 g L^{-1}) before autoclaving (121 °C, 15 min, 2.8 Bar and cooled to 80 °C).

Free mono- and disaccharides in the MFGM media were analysed using high performance liquid chromatography (HPLC), by a modification of the method of Marsili, Ostapenko, Simmons, & Green (1981). Three replicate samples of 1.00 g of the MFGM media was added 2.5 mL deionized water, 0.2 mL 0.5 M H_2SO_4 and 8 mL acetonitrile (Merck) before mixing for 30 min, centrifugation (1470× *g*, 10 min) and filtering (Acrodisc® CR 13 mm Syringe filter with 0.2 μm PTFE membrane; Pall Corporation, Port Washington, NY, USA). The samples were analysed using an Aminex HPX-87H column (Bio Rad, Hercules, CA, USA) held at 32 °C, connected to a Perkin-Elmer HPLC (Perkin-Elmer, Waltham, MA, USA). As mobile phase, 5 mM H_2SO_4 at a flow of 0.4 mL min^{-1} was used. Standard solutions for calibration were prepared the same way as the samples, and carbohydrates were identified according to their retention times compared with the standard solutions using a Perkin-Elmer Series 200 refractive index detector (Perkin-Elmer). The carbohydrates used for standard solutions were: lactose, Gal, GalNAc, Glc, GlcNAc, Fuc and Man. The only free carbohydrate detected in the MFGM media was lactose at 0.34 mM.

2.5. Preparation of bacteria

The lactobacilli and lactococci were sub-cultured at least three times at 30 °C for 24 h in MRS (Merck) and M17 broth (Merck),

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respectively. The *Ec. hirae* INFE1 was also cultured in M17 broth, as experiences showed that this strain recovering in the proposed media was appropriate. Bacterial cultures (1 mL) were harvested by centrifugation at 13,386× *g* for 20 min (Eppendorf Centrifuge 5415D, Eppendorf, Hamburg, Germany). The supernatant was removed with a sterile pipette, and the bacteria pellet was resuspended in 1 mL of CRM. The final concentration of bacteria in the suspension was selected to permit monitoring both a potential increase and decline in cell numbers over time in CRM with carbohydrate added.

2.6. Screening for growth on different carbohydrates

CRM was supplemented with the selected carbohydrates: Glc, Gal, GlcNAc, GalNAc or NANA. The supplemented CRM (190 μL) was transferred to a microtiter plate and the wells were inoculated with 10 μL bacterial suspension (prepared as described above) diluted five-fold in CRM. All combinations of carbohydrates and bacteria were tested, and each combination was repeated four times. Unsupplemented CRM was also inoculated with each strain as a control.

The growth of the bacteria was measured at 620 nm (OD_{620}) for 72 h, using an Ascent Multiskan Photometric microtiterplate reader (Labsystems; Helsinki, Finland) coupled to a computer with Ascent software (Labsystems). The bacterial strain was considered able to utilize the introduced carbohydrate for growth at 30 °C if an increase in OD_{620} above 0.05 was obtained.

2.7. Growth development on different energy sources

Suspensions of five selected bacterial strains were prepared as described earlier. Selected carbohydrates identified as constituents of MFGM glycoconjugates (GlcNAc, GalNAc, Gal, Man, Fuc) were added to CRM. The strains INF456 and INF15D were not analysed for growth on GalNAc, and none of the strains was analysed for growth on NANA, as these strains did not utilize these carbohydrates for growth in the screening experiment. The experiment was done in triplicate.

Glucose was used as a control (5.0 g L^{-1}). Ten millilitres CRM supplied with one carbohydrate was inoculated with 100 μL undiluted bacterial suspension (1.0%, v/v) in a 10 mL tube. Samples for plate count enumeration were taken at selected intervals (0, 2, 4, 8, 12, 24, 48 and 72 h). Lactococci and enterococci were enumerated on M17 agar (30 °C, 2 days, aerobic; Merck), and lactobacilli on MRS agar (30 °C, 3 days, anaerobic; Merck) using pour plate technique.

A lower initial inoculum level was used to monitor the growth on the MFGM medium compared to the initial inoculum level used for the pure monosaccharides. The bacteria suspension was diluted 100 fold in quarter strength Ringers solution before 100 μL was added to 10 mL CRM containing MFGM material. The experiment was done in triplicate and growth was measured for 61 d. To control that the samples were not contaminated during the relative long duration of the growth experiment, all samples grown on MFGM media were controlled by PCR-denaturing gradient gel electrophoresis (PCR-DGGE). When the experiment was done the remains of the samples were centrifuged (15,682× *g*, 15 min; 5415D, Eppendorf) to obtain a cell pellet. The pellet was resuspended in 200 μL spheroplast buffer (10% sucrose (w/v; Merck), 2 mg mL^{-1} lysozyme (Sigma), 0.4 mg mL^{-1} RNase A (Sigma), 25 mM Tris pH 8.4, 25 mM EDTA pH 8.0) followed by lysing at 37 °C for 30 min. Before incubation at 65 °C for 30 min, 75 μL 5% SDS (Merck) and 75 μL 5 M NaCl was added. 150 μL of a protein precipitating solution (60% 5 M potassium acetate, 11.5% glacial acetic acid, 28.5% H_2O) was added before mixing and incubation on ice for 5 min, followed by centrifugation in a tabletop centrifuge (5415D, Eppendorf) at 15,682× *g* at 4 °C for 15 min. Precipitation of the DNA was obtained by adding an identical volume of 2-propanol to the supernatant, followed by incubation for 5 min and centrifugation (5415D, Eppendorf) at 15,682× *g* for 15 min, at room temperature. The pellet was washed with 70% (v/v) ethanol, then dried and resuspended in 50 μL 1 × TE buffer before storage at −20 °C. The V3 region of the 16S rRNA gene was amplified (used as PCR target), using the universal (forward) PRBA338fGC and (reverse) PRUN518r primers (Øvreås, Forney, Daae, & Torsvik, 1997). A denaturant gradient of urea-formamide from 30% to 65% (100% is equivalent to 7 M urea and 40% (v/v) formamide) was used during the PCR-DGGE (INGENYphorU, Ingeny International B.V. Goes, The Netherlands), and PCR products from pure cultures of the experimental bacteria were used as standards for comparison. Samples showing more than one band, or with a band in a different location than the pure PCR product of that strain, were omitted from the data set and further data treatment. The PCR-DGGE method is shown to have a detection limit of at least 10^4 cfu mL^{-1} in a multi strain culture (Ercolini, Moschetti, Blaiotta, & Coppola, 2001; Temmerman, Scheirlinck, Huys, & Swings, 2003) probably as low as 10^3 cfu mL^{-1} (Cocolin, Bisson, & Mills, 2000), depending on the total amount of DNA in the sample.

2.8. Statistical analysis

The results from the analysis of growth were grouped as follows: a) The slope of the growth curve during the logarithmic growth phase was considered the maximum growth rate of the bacteria, per hour (h^{-1}), b) the highest value obtained during stationary phase was considered the maximum growth level of the bacteria (log cfu mL^{-1}), and c) the slope of the steepest decline in the growth curve during the death phase was considered the death rate of the bacteria, per hour (h^{-1}). These descriptors were analysed using one way analysis of variance with the sugars as the classification factor within each strain or the strain as the classification factor within each sugar, and maximum growth rate, maximum growth level and death rate were used as responses ($n = 3$). The differences in growth descriptors between means of the sugars or bacteria were analysed using Tukey's differentiation test ($P < 0.05$). For the MFGM media, the descriptors were analysed using the strains as the experimental factor. All statistical calculations were performed using MiniTab 16 (Minitab Inc., Coventry, UK) statistical software.

3. Results

3.1. Screening

None of the species used in the screening experiment could grow on CRM without any other additions. All of the screened strains were able to utilize Gal, Glc and GlcNAc for growth, but only a few were able to utilize GalNAc. None of the species studied could grow on NANA during screening, and its use was omitted in further studies. Five of the strains studied in the screening experiment (INF10, INF448, INF456, INF15D and ML8) were selected for further studies based on their strain differences and differences in their ability to utilize the MFGM carbohydrates tested. A strain of the potential cheese contaminant *Ec. hirae* was selected for the subsequent studies to assess how this strain performed compared with the starter- and non-starter LAB.

3.2. Growth characteristics on the milk fat globule membrane media

The growth rate of *Lc. lactis* subsp. *lactis* ML8 and *Ec. hirae* INFE1 were significantly ($P < 0.05$) higher than the lactobacilli on the

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Table 1
Maximum growth rate, maximum growth level and death rate of lactic acid bacteria strains grown on MFGM isolate (5 g L^{-1}).

Strain	Isolate	Maximum growth rate (h^{-1})	Maximum growth level (log cfu $mL^{-1}{}_{max}$)	Death rate (h^{-1})
Lb. paracasei	INF10	0.493 ± 0.032^{a}	7.354 ± 0.019^{b}	-0.021 ± 0.003^{ab}
	INF448	0.480 ± 0.072^{a}	6.878 ± 0.055^{a}	-0.040 ± 0.021^{bc}
	INF456	0.461 ± 0.029^{a}	7.578 ± 0.103^{b}	-0.031 ± 0.008^{abc}
Lb. plantarum	INF15D	0.510 ± 0.050^{a}	7.372 ± 0.166^{b}	-0.039 ± 0.021^{bc}
Lc. lactis subsp. *lactis*	ML8	1.145 ± 0.160^{b}	7.548 ± 0.039^{b}	-0.053 ± 0.003^{bc}
Ec. hirae	INFE1	1.129 ± 0.048^{b}	8.423 ± 0.026^{c}	-0.008 ± 0.003^{a}

Values are means of three replicates; values in a column without a common superscript letter differ significantly ($P < 0.05$).

MFGM media (Table 1). The three *Lb. paracasei* all obtained their maximum growth level after 48 h (Fig. 1), however, *Lb. paracasei* INF448 had a significantly ($P < 0.05$) lower growth level than any of the other studied strains when grown on the MFGM media (Table 1). The maximal growth level of *Lb. plantarum* INF15D on the MFGM media was obtained during the first 12 h of incubation (Fig. 1). *Ec. hirae* INFE1 obtained a significantly ($P > 0.05$) higher growth level than the remaining lactobacilli and *Lc. lactis* subsp. *lactis* ML8.

During further incubation the number of cultivable cells decreased slowly. After 19 d of incubation a second growth phase was observed for both INF448 and ML8 increasing the cell numbers with over 1 log cfu mL^{-1} until 29 d of incubation. The numbers of cultivable cells of the INF456 strain decreased until 24 d when a small increase again could be observed. The *Ec. hirae* INFE1 had one of the lowest death rates compared with the other studied strains, and even though all of the studied strains were cultivable after 61 days of incubation on the MFGM media, the cultivable numbers of *Ec. hirae* INFE1 were >log 6 cfu mL^{-1}.

Only in one case contamination was detected in the MFGM media by PCR-DGGE analysis, and this run was discarded.

3.3. Growth characteristics on the milk fat globule membrane monosaccharides

All of the strains could utilize Glc, GlcNAc, Gal and Man for growth (Fig. 2). The growth curves of each different strain was very similar when grown on Glc and Man. At the three parts of the growth curve examined in this study (maximum growth rate, maximum growth level and death rate) the differences between these two sugars were not significant ($P < 0.05$) for any of the strains (Table 2).

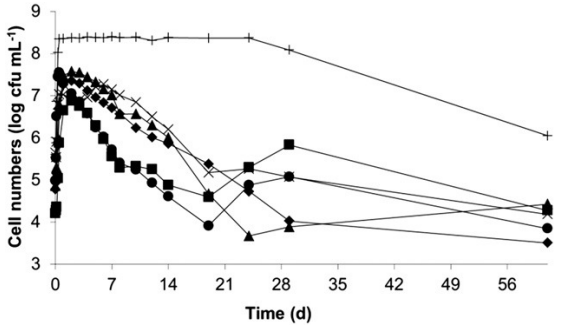

Fig. 1. Growth curves of lactic acid bacteria strains grown on MFGM isolate (5 g L^{-1}). ◆, *Lb. paracasei* INF10 ($n = 3$); ■, *Lb. paracasei* INF448 ($n = 3$); ▲, *Lb. paracasei* INF456 ($n = 2$); ×, *Lb. plantarum* INF15D ($n = 3$); +, *Ec. hirae* INFE1 ($n = 3$); ●, *Lc. lactis* subsp. *lactis* ML8 ($n = 3$).

The maximum growth rate of *Lb. paracasei* INF10 on Gal was significantly ($P < 0.05$) higher than on Fuc, Glc and Man (Table 2), however, a longer lag phase was observed on Gal than on Glc and Man (Fig. 2a).

Even though the growth pattern of *Lb. paracasei* INF448 was comparable with that of INF456 and INF10 on the different monosaccharides (Fig. 2b and c), INF448 obtained a significantly ($P < 0.05$) lower number of cultivable bacteria on all the monosaccharides introduced to the three strains. Significantly ($P < 0.05$) higher numbers of cultivable bacteria were found, for both strains, when grown on GlcNAc compared with growth on Glc, Man, Gal and Fuc. In the screening experiment, only *Lb. paracasei* INF448 was able to grow on GalNAc, and this carbohydrate was therefore only included in the growth study of this strain. *Lb. paracasei INF448* obtained significantly higher ($P < 0.05$) maximum growth level on GalNAc compared with any of the other monosaccharides (Table 2).

The growth rate of *Lb. plantarum* INF15D was significantly ($P < 0.05$) lower when grown on Gal compared with the growth on Glc, GlcNAc and Man (Table 2). No significant ($P < 0.05$) differences between the maximum growth level were obtained between Gal, Glc, GlcNAc and Man (Table 2).

Lc. lactis subsp. *lactis* ML8 obtained a significantly ($P < 0.05$) higher growth level when grown on GlcNAc and Man than when grown on both Fuc and Gal. The maximum growth level was significantly ($P < 0.05$) higher when grown on GlcNAc than both Glc and Fuc. Even though ML8 did not initially seem to be able to utilize Fuc for growth, an increase in cfu mL^{-1} was observed in all three replicates from 24 to 48 h of growth.

When *Ec. hirae* INFE1 was grown on Gal a significantly ($P < 0.05$) higher logarithmic growth rate and maximum growth level were obtained compared with growth on Fuc or GalNAc (Table 2). The *Ec. hirae* INFE1 strain was the only strain studied able to utilize Fuc for growth from $t = 0$ h. However, it did not grow to comparable levels as when grown on Glc, GlcNAc, Gal or Man (Fig. 2e). The maximum growth rate of *Ec. hirae* INFE1 was significantly ($P < 0.05$) higher than all of the lactobacilli on all of the monosaccharides, except for *Lb. plantarum* 15D grown on GlcNAc. This strain obtained significantly ($P < 0.05$) higher maximum growth level than all of the *Lb. paracasei* strains as well as *Lc. lactis* subsp. *lactis* ML8 on all the monosaccharides except GlcNAc, where the numbers were only significantly ($P < 0.05$) higher than INF448 and ML8 (Table 2).

When either of the *Lb. paracasei* was grown on GlcNAc, after the maximum growth level had been reached at 24 h, a decrease was observed in the number of cultivable bacteria at an earlier stage than for any of the other utilized monosaccharides (Fig. 2a–c). The number of cultivable bacteria remained stable at a high level throughout the 72 h incubation on Man and Gal after the maximum growth level was reached. The cultivable number of *Lb. paracasei* INF10 and INF456 decreased slightly after 48 h of growth on Glc. No growth was observed for any of the lactobacilli when Fuc was used as carbohydrate source; however, the number of cultivable *Lb. plantarum* INF15D decreased slower than the *Lb. paracasei* strains studied (Fig. 2a–d).

The number of cultivable *Lb. plantarum* INF15D decreased at an earlier stage when grown on Glc, GlcNAc and Man compared with Gal (Fig. 2d). All the strains grew and remained viable at a high level throughout the 72 h incubation period when Gal was used as carbohydrate source (Fig. 2) with the exception of the starter bacteria ML8 that started to decline after only 12 h on all of the monosaccharides (Fig. 2e). *Ec. hirae* INFE1 was the only strain that showed a stable maximum growth level on all the carbohydrates during the 72 h incubation period (Fig. 2f).

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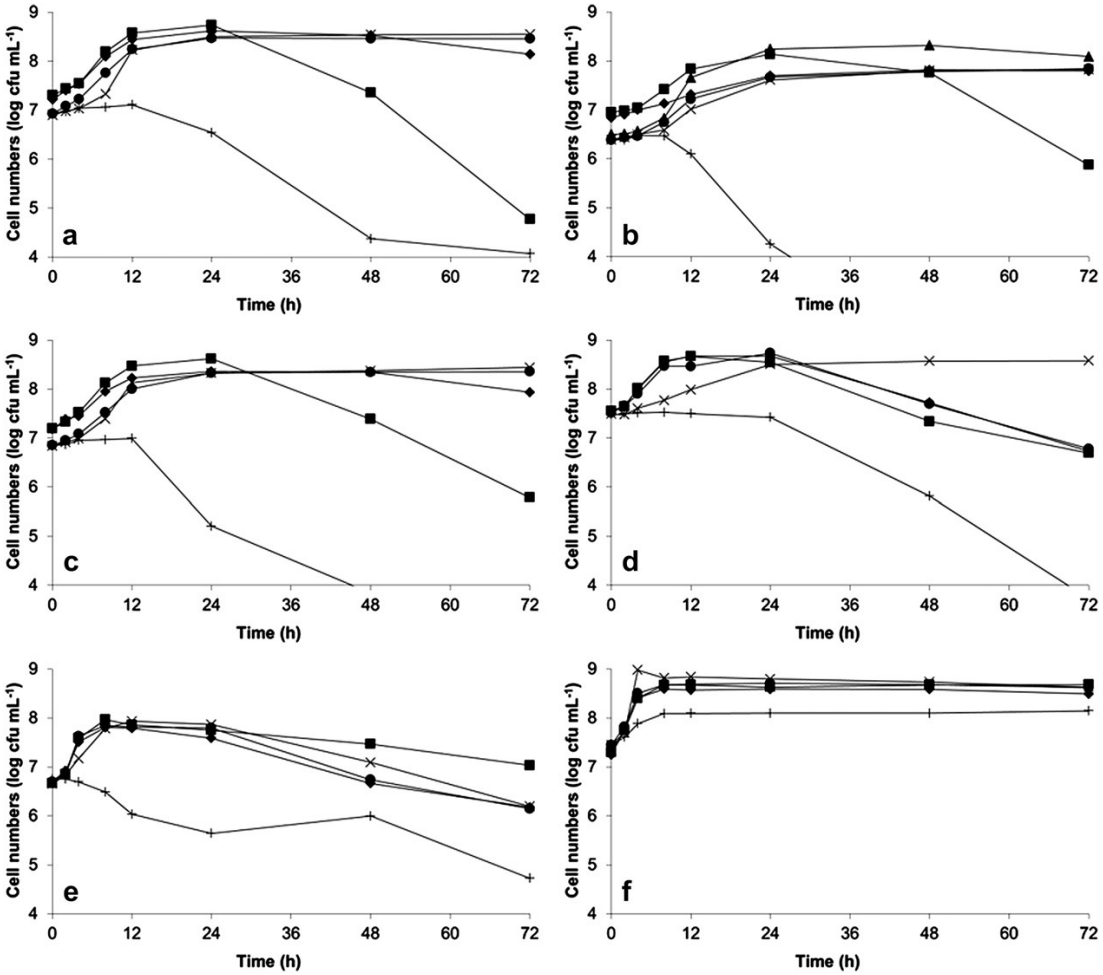

Fig. 2. Growth curves ($n = 3$) of lactic acid bacteria strains (a) *Lb. paracasei* INF10, (b) *Lb. paracasei* INF448, (c) *Lb. paracasei* INF456, (d) *Lb. plantarum* INF15D, (e) *Lc. lactis* subsp. *lactis* ML8 and (f) *Ec. hirae* INFE1, grown on different MFGM carbohydrates (5 g L^{-1}): ◆, D-glucose; ■, *N*-acetyl-D-glucosamine; ▲, *N*-acetyl-D-galactosamine; ×, D-galactose; +, L-fucose; ●, D-mannose.

4. Discussion

It has been shown in this paper that selected LAB strains isolated from cheese were able to grow and survive on CRM added MFGM isolated from bovine milk over 61 days of incubation. No available literature has so far reported such growth and survival. Further it was shown that the selected strains isolated from cheese were able to utilize several of the carbohydrates found in the MFGM for growth when these were present in free form in an otherwise carbohydrate-free growth medium. None of the *Lactobacillus* strains studied was able to utilize either NANA or Fuc for growth. Pronounced differences were found between the ability of the studied *Lactobacillus* strains to utilize the acetylated aminosugar GalNAc. The inability to grow on CRM without added carbohydrate indicated that the method used to prepare the bacterial suspension removed the sugar in the inoculum. The residual lactose present in the MFGM media could, however, as indicated by Williams et al. (2000), be important for the initial growth of the bacteria. This is supported by the fact that ML8 has a significantly ($P < 0.05$) higher growth rate when grown on the MFGM than the lactobacilli (Table 1), and that lactococci are known to grow faster on lactose than lactobacilli. However, when the second observed growth period starts for *Lb. paracasei* INF448, INF456 and *Lc. lactis* subsp. *lactis* ML8, lactose is most likely depleted, so it is reasonable to assume that this growth is supported by other sources of energy.

The simultaneous use of chemically similar compounds as energy sources when present in high concentrations is rare among microorganisms, but it has been shown that GlcNAc is preferentially incorporated into capsular material in bacterial cells compared with Glc when both carbohydrates are present (Topper & Lipton, 1953).

All the carbohydrates were added at a level of 5 g L^{-1}, and the difference in molecular mass of the carbohydrates makes the total mol of added acylated aminosugars lower than for the other carbohydrates added. It would therefore be expected that the acylated aminosugars would be depleted at an earlier stage than the other carbohydrates if the utilization rate was the same. This could be one of several explanations for the observed decrease in cultivable *Lb. paracasei* bacteria cells at an earlier stage when grown on the acylated aminosugars compared with the other carbohydrates. However, if the carbohydrates were depleted and therefore induced a decline in cultivable cells, this would have been expected with all the carbohydrates that supported growth.

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Table 2
Maximum growth rate, maximum growth level and death rate of lactic acid bacteria strains grown on different MFGM carbohydrates (5 g L^{-1}).[a]

Parameter	Carbohydrate	*Lb. paracasei*			*Lb. plantarum*	*Lc. lactis* subsp. *lactis*	*Ec. hirae*
		INF10	INF448	INF456	INF15D	ML8	INFE1
Maximum growth rate (h^{-1})	Fuc	$0.056 \pm 0.011^{a,A}$	$0.106 \pm 0.131^{a,A}$	$0.043 \pm 0.043^{a,A}$	$0.035 \pm 0.032^{a,A}$	$0.043 \pm 0.008^{a,A}$	$0.319 \pm 0.045^{a,B}$
	Gal	$0.520 \pm 0.076^{c,A}$	$0.244 \pm 0.048^{a,A}$	$0.427 \pm 0.043^{b,A}$	$0.145 \pm 0.013^{b,A}$	$0.417 \pm 0.060^{ab,A}$	$1.477 \pm 0.678^{b,B}$
	GalNAc	–	0.477 ± 0.045^{b}	–	–	–	–
	Glc	$0.319 \pm 0.088^{b,A}$	$0.142 \pm 0.085^{a,A}$	$0.287 \pm 0.113^{b,A}$	$0.397 \pm 0.051^{c,AB}$	$0.676 \pm 0.181^{bc,BC}$	$0.748 \pm 0.036^{ab,C}$
	GlcNAc	$0.373 \pm 0.082^{bc,A}$	$0.241 \pm 0.086^{a,A}$	$0.347 \pm 0.119^{b,A}$	$0.424 \pm 0.015^{c,AB}$	$0.845 \pm 0.253^{c,C}$	$0.746 \pm 0.120^{ab,BC}$
	Man	$0.339 \pm 0.022^{b,A}$	$0.270 \pm 0.031^{ab,A}$	$0.296 \pm 0.038^{b,A}$	$0.372 \pm 0.006^{c,A}$	$0.877 \pm 0.019^{c,B}$	$0.785 \pm 0.126^{ab,B}$
Maximum growth level (log cfu $mL^{-1}{}_{max}$)	Fuc	$7.113 \pm 0.103^{a,C}$	$6.515 \pm 0.019^{a,A}$	$6.991 \pm 0.089^{a,BC}$	$7.528 \pm 0.149^{a,D}$	$6.785 \pm 0.060^{a,B}$	$8.145 \pm 0.057^{a,E}$
	Gal	$8.596 \pm 0.037^{bc,B}$	$7.838 \pm 0.039^{b,A}$	$8.440 \pm 0.073^{b,B}$	$8.621 \pm 0.057^{b,B}$	$7.935 \pm 0.044^{bc,A}$	$9.189 \pm 0.318^{b,C}$
	GalNAc	–	8.316 ± 0.079^{d}	–	–	–	–
	Glc	$8.627 \pm 0.037^{bc,C}$	$7.831 \pm 0.065^{b,A}$	$8.370 \pm 0.045^{b,B}$	$8.691 \pm 0.029^{b,C}$	$7.819 \pm 0.034^{b,A}$	$8.623 \pm 0.188^{ab,C}$
	GlcNAc	$8.738 \pm 0.052^{c,B}$	$8.135 \pm 0.101^{c,A}$	$8.618 \pm 0.065^{c,B}$	$8.669 \pm 0.041^{b,B}$	$8.025 \pm 0.135^{c,A}$	$8.719 \pm 0.140^{bc,B}$
	Man	$8.490 \pm 0.021^{b,B}$	$7.852 \pm 0.037^{b,A}$	$8.368 \pm 0.042^{b,B}$	$8.746 \pm 0.050^{b,C}$	$7.871 \pm 0.024^{bc,A}$	$8.728 \pm 0.089^{bc,C}$
Death rate (h^{-1})	Fuc	$-0.221 \pm 0.054^{b,B}$	$-0.354 \pm 0.084^{c,C}$	$-0.342 \pm 0.037^{c,C}$	$-0.206 \pm 0.008^{c,B}$	$-0.122 \pm 0.013^{c,AB}$	$-0.009 \pm 0.008^{a,A}$
	Gal	$-0.002 \pm 0.008^{a,A}$	$0.001 \pm 0.005^{a,A}$	$0.006 \pm 0.005^{a,A}$	$-0.004 \pm 0.007^{a,A}$	$-0.089 \pm 0.017^{b,B}$	$-0.017 \pm 0.005^{a,A}$
	GalNAc	–	-0.022 ± 0.005^{a}	–	–	–	–
	Glc	$-0.037 \pm 0.035^{a,AB}$	$-0.002 \pm 0.008^{a,A}$	$-0.041 \pm 0.064^{a,AB}$	$-0.107 \pm 0.024^{b,B}$	$-0.088 \pm 0.005^{b,AB}$	$-0.009 \pm 0.006^{a,A}$
	GlcNAc	$-0.226 \pm 0.085^{b,C}$	$-0.168 \pm 0.011^{b,BC}$	$-0.194 \pm 0.035^{b,C}$	$-0.116 \pm 0.017^{b,BC}$	$-0.058 \pm 0.002^{a,AB}$	$-0.004 \pm 0.005^{a,A}$
	Man	$-0.003 \pm 0.003^{a,A}$	$0.003 \pm 0.007^{a,A}$	$-0.000 \pm 0.003^{a,A}$	$-0.102 \pm 0.004^{b,B}$	$-0.102 \pm 0.004^{bc,B}$	$-0.007 \pm 0.002^{a,A}$

[a] Carbohydrates abbreviations are: Fuc, L-fucose; Gal, D-galactose; GalNAc, *N*-acetyl-D-galactosamine; Glc, D-glucose; GlcNAc, *N*-acetyl-D-glucosamine; Man, D-mannose. Values are means of three replicates; values in a column without a common superscript lower case letter and in a row without a common superscript upper case letter differ significantly ($P < 0.05$).

Production of growth inhibiting substances when grown on the two different acylated aminosugars could be another explanation for the obvious decrease in cultivable bacteria observed during the utilization of these carbohydrates. Several studies have reported that bacteria able to utilize GlcNAc for growth release acetic acid from the acylated aminosugar (Rogers, 1949; Roseman, 1957; White & Pasternak, 1967). The production of acetic acid in addition to lactic acid during growth on the acylated aminosugars could create a more inhospitable environment detrimental to the viability of LAB during the long incubation periods used in this study.

All the strains remained cultivable at the same high level during the incubation period when grown on Gal as carbohydrate source, except *Lc. lactis* subsp. *lactis* ML8. A longer lag phase combined with no release of acetic acid from the carbohydrate would most probably not present a substantial acid-stress on the bacteria to induce a death phase during the incubation period used in this study. *Lc. lactis* is an important species in cheese starter cultures (Parente & Cogan, 2004), and they are known to be more sensitive to acidic conditions than lactobacilli. β-*N*-acetylglucosaminidases are known to be involved in autolysis of *Lc. lactis* by releasing free reducing groups of GlcNAc from the bacterial cell wall (Huard et al., 2003, 2004; Steen et al., 2005). The autolysis of starter bacteria liberates enzymes that may release carbohydrates from the MFGM glycoconjugates as well as from the cell wall of dead bacterial cells, which may be utilized by the viable LAB. After an initial decrease in cfu in the MFGM media, several of the LAB increased in numbers, however the reason for this increase requires further investigations.

It is interesting to note that the *Ec. hirae* strain studied in this experiment was able to utilize all the carbohydrates studied for growth, except NANA and GalNAc, and that it grew to higher numbers and survived for a longer time than any of the other LAB used in this study when grown on either the pure monosaccharides or on the MFGM media.

All the strains studied were able to utilize Glc, GlcNAc, Gal and Man for growth, but it is not known if any of the bacterial strains involved in cheese ripening are able to take advantage of their ability to utilize MFGM carbohydrates during ripening of cheese. The utilization patterns of Glc, Gal, Man and GlcNAc were quite similar for all the *Lb. paracasei* strains tested, while the patterns of *Lb. plantarum* INF15D, *Lc. lactis* subsp. *lactis* ML8 and *Ec. hirae* were different.

The combination of the results of this study and earlier studies (Williams & Banks, 1997) that showed that several lactobacilli are able to produce enzymes that may release carbohydrates from MFGM glycoproteins, indicates that the MFGM could be postulated as an energy source for LAB during cheese ripening. According to calculations made by Adamberg et al. (2005), full-fat (30% fat, w/w) cheese may contain ~400 mg kg^{-1} bound carbohydrates within MFGM proteins. The results presented in this paper show that not all of the bacteria involved in cheese ripening are able to utilize all these carbohydrates for growth. The amount of bound carbohydrates that could be utilized by LAB in cheese is probably lower than the total amount of these carbohydrates in the cheese.

5. Conclusions

The LAB studied, all relevant to cheese production and ripening, were able to grow and survive for an extended period of time with a bovine MFGM isolate as the only potential carbohydrate source. All isolates were able to effectively utilize most of the monosaccharides found in MFGM glycoconjugates. However, none of the isolates was able to utilize NANA for growth, and only *Lc. lactis* subsp. *lactis* ML8 and *Ec. hirae* INFE1 were able to grow on Fuc. Only *Lb. paracasei* INF448 was able to utilize GalNAc for growth. When lactobacilli were incubated on GlcNAc or GalNAc, a decrease in the number of culturable bacteria was induced at an earlier stage than when incubated on any of the other monosaccharides. The potential cheese contaminant *Ec. hirae* INFE1 showed superior growth and survival abilities compared with the other LAB when grown on the MFGM media and on several of the MFGM monosaccharides. This experiment showed that LAB isolated from cheese may utilize and survive on MFGM components and the mechanisms of energy utilization of the MFGM components by LAB should be further investigated.

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Paper II

INTERPRETIVE SUMMARY

The importance of fat associated components for the well-being of desirable bacteria in ripening cheese.

Moe et al.

This study was conducted to gain insight into the importance of the milk fat associated components in cheese for the development of bacteria involved in the cheese ripening process. Cheese ripening bacteria were grown in different media containing components associated with the milk fat in cheese, and the degradation of these components were monitored. The results from the study showed that cheese ripening bacteria were able to utilize sugars associated with the milk fat in cheese for growth and survival, and that the metabolites produced differed due to which components the bacteria utilized.

Metabolism of milk fat globule membrane components by nonstarter lactobacilli isolated from cheese.

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ABSTRACT

The objective of this study was to investigate how components present in the milk fat globule membrane may be used for growth and survival by cheese ripening lactobacilli. This was achieved by analysing metabolites produced during incubation on appropriate media.

The lactobacilli investigated were able to utilize components from the MFGM throughout an incubation period of 24 days. An apparent connection between the higher proteolytic activity of *Lb. paracasei* INF448 and its ability to grow in the MFGM media after the depletion of readily available sugars was observed.

All the studied strains produced large amounts of acetate when grown on an acylated aminosugar, presumably from deacetylation of the monosaccharides.

Growth of *Lb. plantarum* INF15D on Gal resulted in a metabolic shift expressed as different fates of the produced pyruvate compared to growth on the other monosaccharides. For *Lb. plantarum* INF15D the presence of Gal also seemed to initiate degradation of some AA known to potentially take part in energy production, specifically Arg and Tyr.

Key words: nonstarter lactic acid bacteria, milk fat globule membrane, energy sources in cheese.

INTRODUCTION

The metabolites produced by lactic acid bacteria (LAB) are essential for the development of desirable flavours and aromas in cheese during ripening (Marilley and Casey, 2004). However, when the lactose apparently is depleted at a very early state of the cheese

ripening, it is still not fully understood what substrates that ensures the metabolic active state observed in LAB during cheese ripening (Beresford and Williams, 2004).

The fat globules of bovine milk are excreted from the secretory mammary cells by the envelopment of the fat in a membrane derived from the plasma membrane (Patton and Keenan, 1975). This bi-layer milk fat globule membrane (MFGM) contains numerous complex glycoconjugates in the form of glycoproteins and glycolipids (Liu et al., 2005, Mather, 2000). The glycoproteins of the MFGM contain different oligosaccharides identified as being composed of *N*-acetyl-D-neuraminic acid (NANA), D-galactose (Gal), L-fucose (Fuc), D-mannose (Man), *N*-acetyl-D-glucosamine (GlcNAc) and *N*-acetyl-D-galactosamine (GalNAc) (Hvarregaard et al., 1996, Nakata et al., 1993, Pallesen et al., 2007, Sato et al., 1995, Snow et al., 1977). It has been observed that LAB in cheese are often found in close proximity to the entrapped milk fat globule surface in cheese (Laloy et al., 1996, Lopez et al., 2006). The MFGM contains many different components of which the glycoconjugates could be of special interest as a potential energy source for the cheese ripening microflora. Monosaccharides released from the carbohydrate side-chains of the MFGM glycoconjugates (Fox et al., 1998), as well as from lysed bacterial cells (Thomas, 1987) have been suggested as potential sources of energy for LAB during cheese ripening.

It has also been shown that AA released by proteolytic activity could enhance NSLAB growth (Crow et al., 1995, Di Cagno et al., 2003, Hynes et al., 2001, Martley and Crow, 1993). Both Arg and Ser are known to be involved in substrate-level phosphorylation reactions by LAB. Laht et al. (2002) found that Arg was the only CN derived free AA that did not accumulate during ripening of Swiss-type cheese, and that the Arg degradation products, Cit and Orn, increased. Only LABs able to produce the enzymes involved in the Arg deiminase (ADI) pathway are able to convert Arg to Orn (Liu et al., 2003a), however, it has

been shown that both ADI and Orn transcarbamylase was inhibited by D-glucose (Glc) whereas the inhibitory effect of Gal was much lower (Crow and Thomas, 1982).

It was hypothesized that energy can be produced by *Lactobacillus* (*Lb.*) *plantarum* from Ser by deamination to form pyruvate and ammonia, and that the pyruvate mainly was catabolized to acetate and formate in growth media containing lactose as the fermentable carbohydrate (Liu et al., 2003b). Previously it has been shown that *Lb. plantarum* INF15D reduces the amount of Asn, Ser and Gln in phosphate buffer supplemented with AA (Kieronczyk et al., 2001). Liu et al. (2003b) showed that only ammonia and acetate were produced by *Lb. plantarum* B3087 in phosphate buffer added Ser.

Intracellular decarboxylation of AA that consumes protons have been described in several works as a reaction used by bacteria to reduce intracellular pH (Ardö et al., 2002, Christensen et al., 1999). The products of AA decarboxylation are amines, some of which are regularly detected in fermented food products (Komprda et al., 2008, Komprda et al., 2010, Linares et al., 2011). Several research papers also suggest the occurrence of antiporters in the bacterial cell wall of LAB (Driessen et al., 1989, Higuchi et al., 1997, Konings et al., 1995, Lucas et al., 2005, Molenaar et al., 1993, Wolken et al., 2006). These antiporters are able to transport molecules across cell membranes against their chemiosmotic gradient. The process occurs without energy expenditure as another molecule is transported coincidentally along the chemiosmotic gradient. By exchanging the resulting amine with more substrate via an antiporter, an electrochemical proton gradient is formed that can be used for generation of ATP by pumping protons into the cell via the F_1F_o ATP synthase located in the cell wall. The electrochemical proton gradient is maintained by decarboxylation reactions inside the cell (Konings et al., 1995, Pessione et al., 2010). This potentially energy producing reaction have been described for the cheese bacteria *Lb. buchneri* (Molenaar et al., 1993), and for several

meat (Masson et al., 1996) and wine LAB (Moreno-Arribas and Lonvaud-Funel, 2001, Moreno-Arribas et al., 2000).

A previous study have shown that all of the LAB used in this experiment were able to utilize monosaccharides present in the MFGM glycoconjugates or in the bacterial peptidoglycan for growth. In addition they were able to survive for an extended period of time in a carbohydrate restricted medium (CRM) added MFGM isolate (Moe et al., 2012). These observations indicate that the bacteria were able to utilize other compounds for growth and survival than the lactose present in the media. Lactose and its monosaccharide components are known to be exhausted quite fast during cheese ripening (Turner and Thomas, 1980).

To further explore the metabolic activities of NSLAB during cheese ripening, the objective of the current study was to investigate how components present in the MFGM may be used for growth and survival by NSLAB and to explore which metabolites they may produce during long term incubation. The production and utilization of organic acids, volatile compounds and amino acids were analysed when the NSLAB were grown with a MFGM isolate or single MFGM carbohydrates as the only carbohydrate source.

MATERIALS AND METHODS

Bacteria Strains

The four strains of NSLAB used in this experiment were: *Lb. paracasei* INF10, INF448, INF456 and *Lb. plantarum* INF15D. These strains were obtained from the culture collection of the Department of Chemistry, Biotechnology and Food Science, Norwegian University of Life Sciences, Aas, Norway. Strains INF10 and INF15D were isolated from 90 d old, and INF448 and INF456 from 180 d old Norvegia cheese (a Norwegian washed curd Dutch type cheese).

98

99 ***Carbohydrate-restricted Medium (CRM)***

100 A carbohydrate-restricted medium (CRM) was made according to Adamberg et al.
101 (2005), with minor adjustments as described by Moe et al., (2012). In short, a CRM was made
102 with: 12.0 g/L peptone from CN (Merck, Darmstadt, Germany), 1.0 g/L Tween 80 (Koch-
103 Light Laboratories Ltd., Bucks, UK), 0.6 g/L $MgSO_4 \cdot 7H_2O$ (Merck), 0.3 g/L $MnSO_4 \cdot 5H_2O$
104 (Merck), 0.06 g/L $FeSO_4 \cdot 7H_2O$ (Merck), 0.05 mg/L biotin (Sigma, Steinheim, Germany),
105 0.1 mg/L folic acid (Sigma), 0.1 mg/L niacinamide (Sigma), 0.1 mg/L pyridoxal-HCl
106 (Sigma), 0.1 mg/L riboflavin (Sigma), 0.1 mg/L thiamine-HCl (Sigma) and 0.2 mg/L
107 panthothenate (Sigma). The CRM was autoclaved (121 °C, 15 min) prior to use.

108

109 ***Preparation of Carbohydrate Stock Solutions***

110 The monosaccharides used to supplement the CRM were prepared as stock solutions
111 (100 mg/mL), and were filter sterilised (pore size 0.22 μm; Millex, Carrigtwahill, Ireland).
112 The MFGM carbohydrates used in this experiment were Gal, Man, GlcNAc and GalNAc (all
113 from Sigma). The monosaccharide Glc (Merck) is not found in the MFGM glycoconjugate
114 sidechains and was used as standard. All monosaccharides were added to a final concentration
115 of 5 g/L.

116

117 ***Preparation of Milk Fat Globule Membrane Media***

118 The MFGM material was prepared by a procedure described by Vanderghem et al.
119 (2008) with minor modifications as described by Moe et al. (2012). In short, fresh
120 unpasteurized milk, cooled to 4 °C was obtained from the herd at the Norwegian University of
121 Life Sciences. The cream was washed twice with 5 volumes of phosphate buffered saline
122 (PBS; 0.01 *M*; pH 7.2; 0.9 % NaCl; 4 °C) and once with 5 volumes of distilled water (4 °C).

123 The cream was subjected to centrifugation between washes (4,500 × *g*; 10 min; 4 °C). The
124 washed fat globules were allowed to crystallize for 20 h at 4 °C before heavy agitation
125 inducing phase separation. The collected serum was centrifuged twice (5,000 × *g*; 15 min; 4
126 °C) to remove the fat. The serum was freeze dried and stored at – 22 °C until used.
127 The freeze dried MFGM powder was rehydrated in distilled water (100 mg/mL), and
128 added to CRM (final concentration 5 g/L) before autoclaving (121 °C, 15 min).
129
130 ***Preparation of Bacteria suspension***
131 The bacteria were sub-cultured at least three times at 30 °C for 24 h in MRS (Merck).
132 Bacterial cultures (1 mL) were harvested by centrifugation at 13,400 × *g* for 20 min
133 (Eppendorf Centrifuge 5415D; Eppendorf, Hamburg, Germany). The supernatant was
134 removed with a sterile pipette, and the bacteria pellet was resuspended in 1 mL of CRM. The
135 final concentration of bacteria in the suspension was selected to be the same as earlier
136 experiments done with the same bacteria and growth media (Moe et al., 2012).
137
138 ***Development of Metabolites***
139 Ten mL CRM supplied with the MFGM monosaccharides (5 g/L) was inoculated with
140 100 µL undiluted bacteria suspension (1.0 % v/v) in a 10 mL tube. Only *Lb. paracasei* INF
141 448 of the strains used in this experiment was incubated in CRM with GalNAc, as it was
142 shown earlier that this was the only strain investigated able to grow on GalNAc (Moe et al.,
143 2012). Samples for metabolite analysis were taken at selected time intervals (0, 4, 12 and 48
144 h) corresponding to early lag-, early log-, early stationary and late stationary phase, compiled
145 from earlier experiments (Moe, et al., 2012). The CRM added Glc (5 g L^{-1}) was used as a
146 control.

147 The MFGM media were added a diluted bacteria suspension (100 fold in 1/4 strength
148 Ringer's solution) and the development of metabolites in the MFGM media were followed for
149 a longer time with analysis after 0, 4, 12, 48, 168, 336 and 576 h of incubation. The analysis
150 of carbohydrates, organic acids and AA were done from three replicate incubations. Because
151 of scarcity of MFGM material, volatiles were only measured at 576 h for all the strains, as
152 well as in the 0 h sample without added bacteria. The analysis of volatiles were done from
153 two replicate incubations, and all of the biological samples were controlled for contamination
154 after the end of the experiment with the use of denaturing gradient gel electrophoresis
155 (DGGE), as described by Moe et al. (2012). Samples showing more than one band, or with a
156 band in a different location than the pure PCR product of the strain were omitted from the
157 data set and further data treatment. The growth of the bacteria was confirmed by enumeration
158 by pour-plate technique.
159
160 ***Analysis of Organic Acids and Carbohydrates by HPLC***
161 Organic acids and carbohydrates were analysed using HPLC, by a modification of the
162 method of Marsili et al. (1981). The procedure used was as described by Narvhus et al.
163 (1998), with the following modifications: The samples were analysed using an Aminex HPX-
164 87H column (Bio Rad, Hercules, CA, USA) held at 32 °C, connected to a Perkin-Elmer
165 HPLC (Perkin-Elmer, Waltham, MA, USA). As mobile phase, 5 m*M* H_2SO_4 at a flow of 0.4
166 mL/min was used. Standard solutions for external calibration were prepared the same way as
167 the samples, and the compounds were identified according to their retention times compared
168 with the standard solutions using a Perkin-Elmer Series 200 refractive index detector (Perkin-
169 Elmer) for carbohydrates, and a Perkin Elmer Series 200 UV/VIS detector (Perkin-Elmer) for
170 the organic acids. The carbohydrates used for standard solutions were lactose (Merck), Glc
171 (Merck), Gal (Merck), GlcNAc (Sigma), GalNAc (Sigma) and Man (Merck), and the organic

acids were citric, orotic, pyruvic, succinic, lactic, formic, acetic, uric and propionic acids (Sigma).

Analysis of Volatile Compounds

Volatile compounds were analysed using a headspace gas chromatography (HSGC) system according to the method of Narvhus et al. (1993). The test sample was 10 g of growth media with homogenously dispersed bacteria cells weighed directly into a headspace vial (N20-20 PE, Machery Nagel, Düren, Germany) sealed with a teflon-coated septum and aluminum shrink cap (20-CT3 and 20-ACB, Chromacol Ltd, Welwyn Garden City, UK). The vial was flushed with nitrogen gas (AGA AS; Oslo, Norway) before capping and immediately frozen (-22 °C) until moment of analysis.

Peaks were externally identified using standard solutions of the following compounds: acetaldehyde, 2-pentanone, 2-butanone, ethyl acetate (all from Fluka); 2-methyl-1-propanol, 2-methyl-butanal, 3-methyl-butanal, 3-methyl-1-butanol, 2-methyl-1-butanol, 2-methyl-1-propanal, diacetyl (all from Sigma); 2-propanol, 1-butanol, 2-butanol, acetoin, iso-butylacetat, dimethylsulphide, acetone, 2.3-pentadion (all from Merck); ethanol (Arcus, Oslo, Norway).

Analysis of Free AA

Free AA were analysed by immediately adding 2.0 g of internal standard solution (0.1 *M* HCl; 0.4 µmol/mL L-norvalin; Sigma, St. Louis MO, USA) to 2.0 g of sample followed by sonication for 30 min. After the sample was centrifuged (40 min; 4 °C; 3000 × *g*), 0.5 mL of the supernatant was added 0.5 mL 4 % TCA before mixing in a mini shaker (Gene2, New York, NY, USA) and placed on ice for 30 min. After centrifugation (5 min; 5 °C; 15,700 × *g*)

196 the samples were filtered (0.2 μm, cellulose acetate filter, Advantec, Dublin, CA, USA) and
197 stored in a freezer (-24 °C) until analysis (Bütikofer and Ardö, 1999).
198 Separation of AA was performed using a Perkin Elmer series 410 pump (Perkin
199 Elmer), an Agilent Technologies 1200 series autosampler (Agilent Technologies, Waldbronn,
200 Germany), a Perkin Elmer 200 column oven and an Agilent Technologies 1200 series
201 thermostat. The system was driven by EZChrom Elite (Agilent Technologies) software. A
202 XTerra RP 18 column with 150 x 4.6 mm (Waters, MA, USA) was used for separation of AA
203 at 42 °C.
204
205 ***Statistical Treatment of Data***
206 The relationship between the substrate components and their metabolic products were
207 analysed by principal component analysis (PCA) using Unscrambler X10.1 (Camo,
208 Trondheim, Norway). All results were weighted (1/SD) prior to the PCA. The results from the
209 bacteria grown on the separate sugars and on the MFGM media were analysed separately due
210 to differences in incubation time and inoculation. As the replicates were similar, with low SD,
211 the results of their average values are shown in the PCA scores and loadings plot (Figure 4).
212
213
214 **RESULTS**
215 ***LAB Growth and Metabolism in MFGM Media***
216 All the strains used in this experiment grew on the MFGM media. After preparation of
217 the MFGM media, 34 mmol/L remaining lactose was found (Figure 1). The lactose was
218 depleted by all *Lb. paracasei* strains after 48 h of growth, however samples with *Lb.*
219 *plantarum* 15D still had remaining lactose in the media after 168 hours of growth (Figure 1).
220 The level of lactate continued to increase after the apparent depletion of lactose for *Lb.*

221 *paracasei* INF10 and INF456, however towards the end of the growth period the amount of
222 lactate in general decreased or remained at a constant level in the MFGM media.
223 Acetate was produced by *Lb. plantarum* INF15D and *Lb. paracasei* INF456 after 168
224 h with *Lb. plantarum* 15D producing the highest level, whereas *Lb. paracasei* INF10 and *Lb.*
225 *paracasei* INF448 only produced acetate at low levels after 576 h of incubation.
226 The general release of AA in the MFGM media from 336 to 576 h was higher by *Lb.*
227 *paracasei* INF448 compared to any of the other investigated strains. For samples with *Lb.*
228 *paracasei* INF456, *Lb. paracasei* INF10 and *Lb. plantarum* INF15D the level of Ser was
229 lower after 576 h of incubation than after 336 h of incubation of the MFGM-media. The
230 decrease in the amount of Ser was however more pronounced in samples with *Lb. paracasei*
231 INF456 than in samples with the two other strains.
232 The Gln content was increased by all strains throughout the growth period. However,
233 samples with *Lb. paracasei* INF448 had a lower content of Gln and a higher content of Glu
234 than the other strains studied, and the Gln was almost exhausted at 576 h incubation of *Lb.*
235 *paracasei* INF448. The DL-pyroglutamic acid content decreased drastically after 336 h of
236 growth in samples with *Lb. paracasei* INF448 (Figure 1b).
237 The level of Asn was relatively stable throughout the incubation period, but a decrease
238 was observed in the later stages of growth by all strains.
239 The levels of Arg remained at the same level throughout the incubation period, but the
240 amount was decreased at 556 h for all strains. A slight increase of Orn was observed at 556 h
241 of incubation for all strains except for *Lb. paracasei* INF448.
242
243 ***LAB Growth and Metabolism on MFGM Monosaccarides***
244 All the strains used in this experiment grew on Gal, Glc, GlcNAc and Man. The
245 metabolic patterns when incubated on Glc and the MFGM monosaccharides GlcNAc, Gal and

246 Man were fairly similar for all the *Lb. paracasei* strains. The development of metabolites
247 produced by *Lb. paracasei* INF448, as an example, is shown in Figure 2. However, of the
248 studied strains of *Lb. paracasei* only *Lb. paracasei* INF448 could utilize GalNAc. The main
249 product from the degradation of the monosaccarides was lactate but when GalNAc or GlcNAc
250 was utilized, acetate was also produced in the ratio 2:1 lactate:acetate. In all samples, the
251 levels of Asp and Glu increased in the later stages of incubation, while the level of Asn
252 decreased. None of the *Lb. paracasei* strains changed the levels of Ser, Arg or Tyr during
253 growth on any of the carbohydrates.

254 The development of metabolites of *Lb. plantarum* INF15D differed from the *Lb.*
255 *paracasei* strains (Figure 2 and Figure 3). For *Lb. plantarum* INF15D the PCA revealed a
256 clear difference in the metabolic patterns between the different MFGM monosaccharides
257 (Figure 4). No development of metabolites were observed after 4 h of growth, but after 12 h
258 of growth the metabolites produced when grown on Gal separated from those produced when
259 grown on the other sugars (Figure 4). The incubation time explained the variation in principal
260 component 1 (PC1), whereas PC2 separated *Lb. plantarum* INF15D grown on Gal from its
261 growth on the other monosaccharides investigated.

262 *Lb. plantarum* 15D utilized Glc, Man and GlcNAc at a higher rate compared to Gal
263 and consequently produced lactate at a higher rate on the three first sugars (Figure 3), whereas
264 *Lb. plantarum* 15D grew on Gal; formate and ethanol was produced at a higher rate.

265 Apart from when the strains were grown on an acetylated aminosugar, *Lb. plantarum*
266 INF15D was the only strain that produced acetate on any of the other monosaccharides during
267 the 48 h incubation period. Acetate was produced by *Lb. plantarum* INF15D when grown on
268 both Gal and GlcNAc (Figure 3), however the amounts of acetate produced were very low on
269 Gal compared to GlcNAc.

Even though none of the lactobacilli decreased the levels of Tyr when grown on the MFGM media (Figure 1), *Lb. plantarum* INF15D utilized Tyr when grown on Gal, whereas the three *Lb. paracasei* strains did not utilize Tyr under any of the conditions used in this experiment.

The AA Ser was used by *Lb. plantarum* INF15D when grown on all sugars. Furthermore the major difference when *Lb. plantarum* INF15D was grown on Gal compared to the other monosaccharides was, in addition to the utilization of Tyr, its use of Arg and the following production of Cit and Orn, but also an increase of Gly, Glu and Ala in the media. However the production of Gln was lower when *Lb. plantarum* INF15D was grown on Gal compared to Man, Glc and GlcNAc.

DISCUSSION

Previous work with the same strains as those investigated in this study showed that the cultivable number of *Lb. paracasei* INF448, *Lb. paracasei* INF 456 and *Lb. plantarum* INF15D started to increase after an initial period of decline when incubated for a relatively long time in CRM containing MFGM isolate (Moe et al., 2012). The results presented in this paper show that the surplus lactose was exhausted rapidly by all the *Lb. paracasei* strains but not by *Lb. plantarum* INF15D.

The surplus lactate produced after the apparent depletion of lactose and its monosaccharide moieties in the MFGM media indicate that this additional lactate is produced from alternative sources.

The results presented in this paper confirm that all of the studied strains were able to utilize GlcNAc for growth as proposed by Moe et al. (2012). When any of the strains studied in this experiment were incubated with GlcNAc as the only added carbohydrate source, large

295 amounts of acetate was produced. Rogers (1949) showed that *Streptococcus pyogenes*
296 produced acetate, lactate and ammonia when utilizing GlcNAc. It was later established by
297 Roseman (1959) that the enzyme GlcNAc deacetylase catalyzes the release of acetate from
298 GlcNAc during its metabolism. It is therefore very likely that the large amounts of acetate
299 produced in the media with added GlcNAc is mainly released from this acylated aminosugar
300 during its metabolism.

301 For the NSLAB to be able to utilize GlcNAc as a source of energy in cheese during
302 ripening they have to be able to release the monosaccharides from either the peptidoglycan of
303 dead bacteria or from MFGM glycoconjugates. It has been shown that several species of
304 mesophilic lactobacilli possess glycolytic enzymes which may be able to release these sugars
305 (Williams and Banks, 1997), and that the production of enzymes involved in the breakdown
306 of GlcNAc was upregulated in the stationary growth phase in *Lb. plantarum* WCFS1 when
307 grown in MRS medium (Cohen et al., 2006) and in lactose starved *Lb. casei* (Hussain et al.,
308 2009).

309 If utilization of GlcNAc occurs in cheese, acetate is expected to be produced. In a
310 study where *Lactobacillus paracasei* ATCC 334 was grown on ripening Cheddar cheese
311 extract it was concluded that the metabolic products formed (i.e. acetate and lactate) could not
312 be explained by residual levels of lactose, Gal or citrate present in the medium (Budinich et
313 al., 2011). The authors further suggested that milk derived complex carbohydrates or starter-
314 derived components could be the precursors of the metabolites found in the media after
315 growth of the NSLAB strain. The results presented in this paper shows that the metabolism of
316 GlcNAc could be the source of the excess lactate and acetate, and therefore supports the
317 hypothesis presented by Budinich et al., (2011).

318 Roseman (1957) found that a crude extract of *Escherichia coli* only deacetylated
319 GalNAc at 10 % of the rate of GlcNAc and suggested that this effect might be due to the

effect of one unspecific enzyme involved in the deacetylation of both GlcNAc and GalNAc. However, Reizer et al. (1996) found that the deacetylation of GalNAc-6-P was due to a specific deacetylase from the gene *agaA* in *E. coli*. According to the KEGG database this gene is not found in any of the sequenced LAB genomes to this date, and has to the best of our knowledge not been described in any LAB in literature previously. *Lb. paracasei* INF448 was the only studied strain able to utilize GalNAc for growth, and the growth rate was found to be significantly ($P < 0.05$) lower than when grown on GlcNAc (Moe et al., 2012). However large amounts of acetate was also produced when GalNAc was utilized, strongly indicating the action of a deacetylating enzyme in *Lb. paracasei* INF448 acting on GalNAc during its utilization.

The general release of AA from the MFGM media late in the incubation period was higher by *Lb. paracasei* INF448 compared to any of the other investigated strains. This strain also had the highest increase in cell numbers during growth after the apparent depletion of lactose of any of the studied strains in an earlier experiment (Moe et al., 2012). It is known that LAB has an extensive battery of proteolytic and peptidolytic enzymes able to release AA from the environment (Christensen et al., 1999). It has been stated that the peptide and AA fractions present in cheese only serves as a source of nitrogen, and cannot be used by LAB as a source of energy (Fox et al., 1998). However other studies and the results presented in this paper indicate that AA both directly and indirectly could serve as a source of energy for LAB, and that AA released by proteolytic activity could enhance NSLAB growth (Crow et al., 1995, Di Cagno et al., 2003, Hynes et al., 2001, Martley and Crow, 1993). Our work therefore supports the observations suggesting that a high release of AA from the growth media may support growth of NSLAB.

The *Lb. plantarum* INF15D strain utilized Ser when grown on all of the monosaccharides in this study, whereas none of the *Lb. paracasei* strains decreased the levels

345 of Ser when grown on any of the monosaccharides. A study performed by Liu et al. (2003b)
346 proposed that the breakdown products of Ser were formate and acetate. Both of these products
347 as well as small amounts of ethanol were observed when *Lb. plantarum* INF15D was grown
348 on Gal, however none of these compounds were observed when grown on Glc and Man, even
349 though the levels of Ser decreased. Degradation of Ser produces pyruvate (Liu et al., 2003b)
350 which potentially could be further metabolized to lactate or acetate, formate and ethanol.
351 Adamberg et al., (2006) suggested that *Lb. plantarum* improved ATP and biomass yield by
352 shifting the pyruvate breakdown from lactate to acetate. It is reasonable to assume that the
353 acetate, ethanol and formate produced by *Lb. plantarum* INF15D when grown on Gal were
354 derived from Ser, as have been observed by Liu et al. (2003b). However, the acetate was most
355 likely produced from pyruvate via the pyruvate-formate lyase pathway. These results indicate
356 that *Lb. plantarum* INF15D had a metabolic shift when grown on Gal, resulting in different
357 fates of the produced pyruvate when grown on the different monosaccharides. Skeie et al.
358 (2008b), incubated *Lb. plantarum* INF15D in phosphate buffer added only Ser as the
359 potential energy source, and acetate and formate were produced during incubation. The results
360 presented in this paper indicates that *Lb. plantarum* INF15D metabolises Ser by the same
361 pathway when grown on Gal as in a medium completely devoid of fermentable carbohydrates,
362 in contrast to media containing either Glc, GlcNAc or Man.

363 When incubated on the MFGM media acetate was found late in the incubation period,
364 however the decrease of Ser was only apparent by some of the strains and it was difficult to
365 relate acetate and Ser to each other.

366 When *Lb. plantarum* INF15D was grown on Gal, the Arg level decreased whereas the
367 level of Orn increased. Cunin et al. (1986) suggested that energy depletion is a key triggering
368 factor for the induction of the ADI pathway in most bacteria. However the regulation differs
369 among LAB, and the differences seems to correlate with the adaption to different habitats

370 (Fernández and Zúñiga, 2006). In the experiment described in this paper, *Lb. plantarum*
371 INF15D utilized Arg when grown on Gal, whereas Arg did not decrease when grown on any
372 of the other monosaccharides. Manca de Nadra et al. (1986) showed that the specific activity
373 of the three ADI pathway enzymes were higher in *Lb. buchneri* when grown on Gal than on
374 other sugars, and that observation may explain the induction of Arg metabolism in *Lb.*
375 *plantarum* INF15D when grown on Gal observed in the work presented in this paper as well.
376 The decrease of Arg when the lactobacilli grew on the MFGM media was larger than
377 the increase of Cit and Orn combined, indicating either that Arg have been converted by some
378 other pathway than the ADI pathway, or that Cit or Orn or both have been further converted.
379 Arena and de Nadra, (2001) reports that *Lb. plantarum* produced putrescine by
380 decarboxylation of Orn.
381 Transamination reactions usually involve α-ketoglutarate as an amino group acceptor,
382 resulting in the production of Glu (Ardö, 2006). The level of Glu was higher in *Lb. plantarum*
383 INF15D grown on Gal than on any of the other monosaccharides studied. This was also the
384 only condition where Tyr was degraded in this experiment, making transamination a likely
385 reaction for the Tyr catabolism observed.. Gummalla and Broadbent (2001) described the
386 catabolism of Tyr in the Cheese LAB *Lb. casei* LC202 and LC301, and *Lb. helveticus* LH212
387 and CNRZ32. They found that the strains studied, degraded Tyr by a transamination reaction,
388 and that they did not decarboxylate Tyr in Cheddar cheese (Gummalla and Broadbent, 1999).
389 Amino acid decarboxylases are induced in LAB by low pH media during late
390 exponential growth phase (Molenaar et al., 1993). They are thought to favor the adaption to
391 growth in acidic environments, as it fixate protons that can be expelled from the cell, and
392 thereby increasing the pH (Lonvaud-Funel, 2001). Lonvaud-Funel (2001) suggested that the
393 AA decarboxylating LAB strains may survive longer in food products as a consequence of
394 their presumed ability to produce energy by the decarboxylating action by creating a

electrochemical proton potential that could fuel the cell wall associated F_1F_o ATP synthase system.

The AA decarboxylation activity is strain specific, and the ability to produce amines varies within the different species (Lonvaud-Funel, 2001). The results obtained for *Lb. plantarum* INF15D also indicate that Tyr breakdown is only active during some specific conditions. However, what specific conditions that induced the Tyr breakdown in the CRM added Gal is not known. Tyr decarboxylating enzymes have been found in *Lb. brevis* (Moreno-Arribas and Lonvaud-Funel, 2001) and in several other lactobacilli, including *Lb. paracasei* (Arena et al., 2007).

When the strains used in this study were incubated in MFGM media, no decrease in the Tyr levels was observed. The low levels of readily fermentable sugars in the MFGM media led to low levels of acid production which in turn led to a relative high pH in the media. As indicated by earlier literature, a low pH is a prerequisite for induction of AA decarboxylating enzymes (Molenaar et al., 1993). However the expected low pH value of the media added Glc, Man or GlcNAc did not alone seem to be inducing Tyr breakdown in *Lb. plantarum* INF15D.

Ganesan et al. (2007) observed that the induction of genes associated with AA metabolism led to the depletion of Gln in *Lactococcus lactis*. They further suggest that the depletion of Gln over time indicates the use of alternative energy sources because the transport of Gln is energy driven, presumably by the use of ATP or any other energy-rich phosphate intermediate. The depletion of Gln was observed in sample with *Lb. paracasei* INF448 during its incubation on the MFGM media, and it is therefore reasonable to presume that alternative energy producing pathways were active at the later stages of incubation of this strain in the MFGM media.

419 It is known that Asn and water can be transformed into Asp and ammonia by L-
420 asparaginase (Ardö, 2006) and the conversion of Asn to Asp have been observed in *Lb.*
421 *plantarum* INF15D earlier (Skeie et al., 2008a). All strains investigated decreased the levels
422 of Asn whereas the levels of Asp increased when grown on all monosaccharides used in this
423 study. It seems likely that this happens as a consequence of the action of L-asparaginase as
424 described by Ardö (2006). The levels of Asn decreased at the end of incubation in the MFGM
425 media. However, because of the apparent differences in peptidolytic activity among the
426 studied strains, no conclusions could be made concerning the end product of the Asn
427 metabolism in the MFGM media. This reaction does not seem to be involved in energy
428 production, but could be a way to regulate internal pH in the bacterial cell by the release of
429 ammonia.
430
431
432 **CONCLUSIONS**
433 In this study it was shown by the production of metabolites throughout an incubation
434 period of 24 days that the lactobacilli investigated were able to utilize components from the
435 MFGM. An apparent connection between the higher proteolytic activity of *Lb. paracasei*
436 INF448 and its ability to grow in the MFGM media after the depletion of readily available
437 sugars was also observed.
438 All the studied strains produced large amounts of acetate when grown on an acylated
439 aminosugar, presumably from the deacetylation of these monosaccharides during the
440 metabolism.
441 The results presented indicate that *Lb. plantarum* INF15D had a metabolic shift when
442 grown on Gal, resulting in different fates of the produced pyruvate compared to growth on the
443 other monosaccharides. For *Lb. plantarum* INF15D the presence of Gal also seemed to initiate

444 degradation of some AA known to potentially partake in energy production, specifically Arg
445 and Tyr.
446 Based on the results presented in this paper, further studies of *Lb. plantarum* INF15
447 grown on Gal could increase the knowledge of the potential energy sources in ripening
448 cheese.
449
450
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Figure legends

Figure 1. Development of selected metabolites during incubation in carbohydrate restricted media with added milk fat globule membrane. *Lb. paracasei* INF10 (n=3), a; *Lb. paracasei* INF448 (n=2), b; *Lb. paracasei* INF456 (n=2), c; *Lb. plantarum* INF15D (n=3), d. GABA, γ-aminobutyric acid.

Figure 2. Development of the added carbohydrates, organic acids and AA during incubation of *Lb. paracasei* INF448 with D-glucose, a and b; D-galactose, c and d; *N*-acetyl-D-galactosamine, e and f; *N*-acetyl-D-glucosamine, g and h; D-mannose, i and j. GABA, γ-aminobutyric acid.

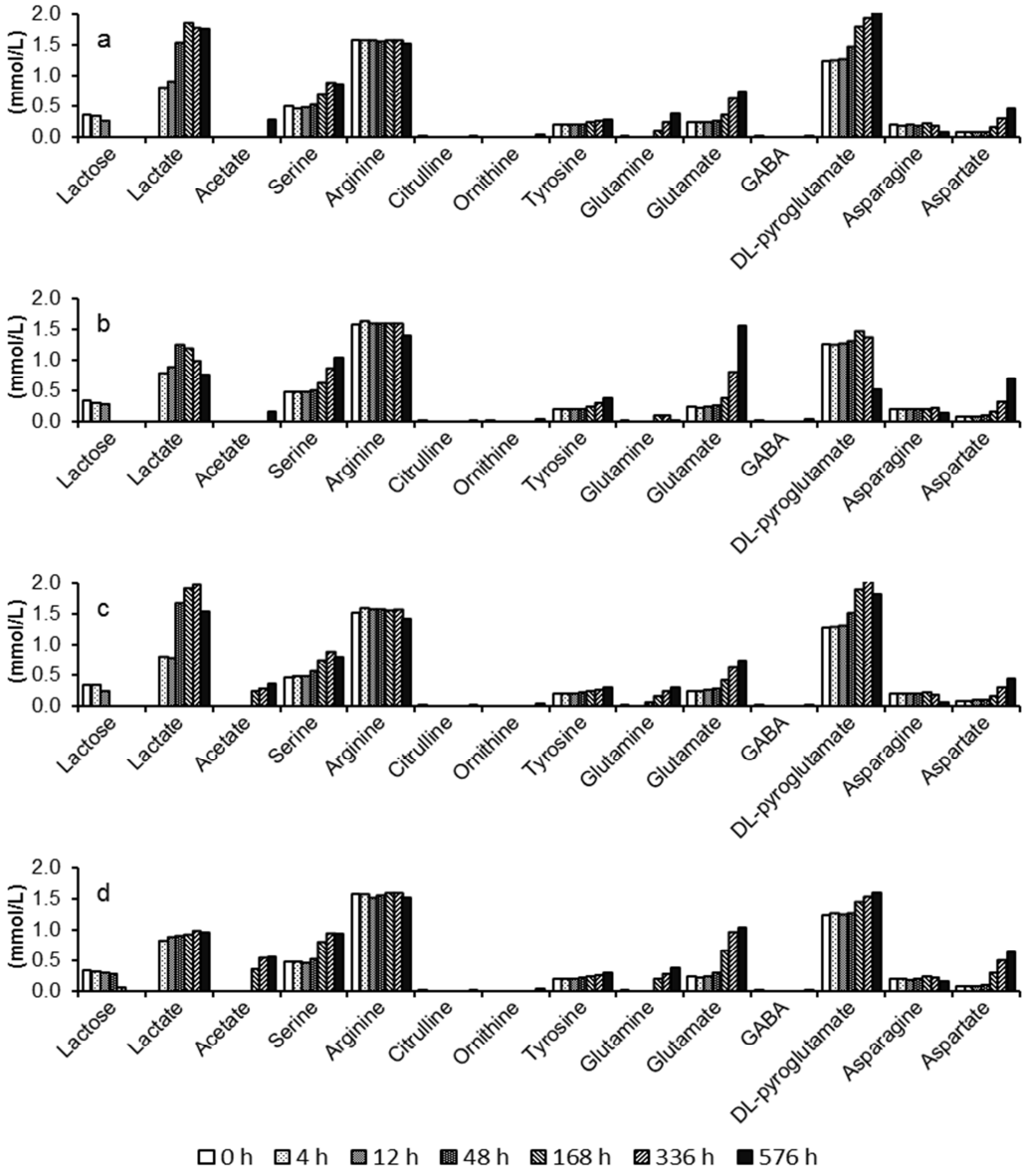
Figure 3. Development of the added carbohydrates, organic acids and AA during incubation of *Lb. plantarum* INF15D with D-glucose, a and b; D-galactose, c and d; *N*-acetyl-D-glucosamine, e and f; D-mannose, g and h. GABA, γ-aminobutyric acid.

Figure 4. Principal component analysis biplot of *Lb. plantarum* INF15D strains incubated in carbohydrate restricted media with MFGM monosaccarides at 0, 4, 12, 48, 168, 336 and 576 h. Principal component 1 (PC1) explains 46 % of the variation and PC2 explains 23 % of the variation. Each point represents the average of three replicate samples. GABA, γ-

aminobutyric acid; Glc, D-Glucose; Gal, D-Galactose; GlcNAc, *N*-acetyl- D-glucosamine; Man, D-Mannose.

Moe

Figure 1.


a
b
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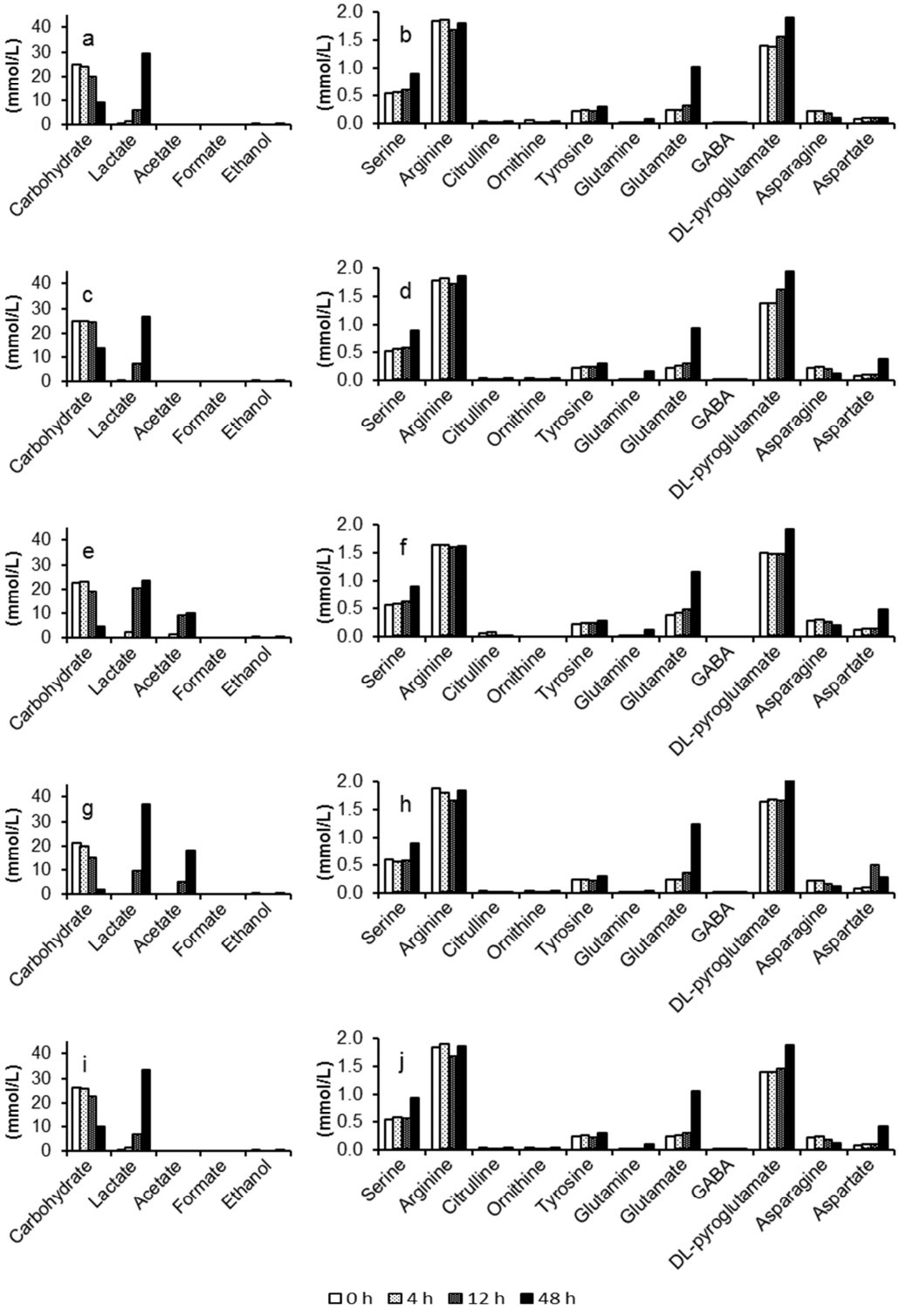
(mmol/L)
2.0
1.5
1.0
0.5
0.0
Lactose
Lactate
Acetate
Serine
Arginine
Citrulline
Ornithine
Tyrosine
Glutamine
Glutamate
GABA
DL-pyroglutamate
Asparagine
Aspartate
0 h
4 h
12 h
48 h
168 h
336 h
576 h

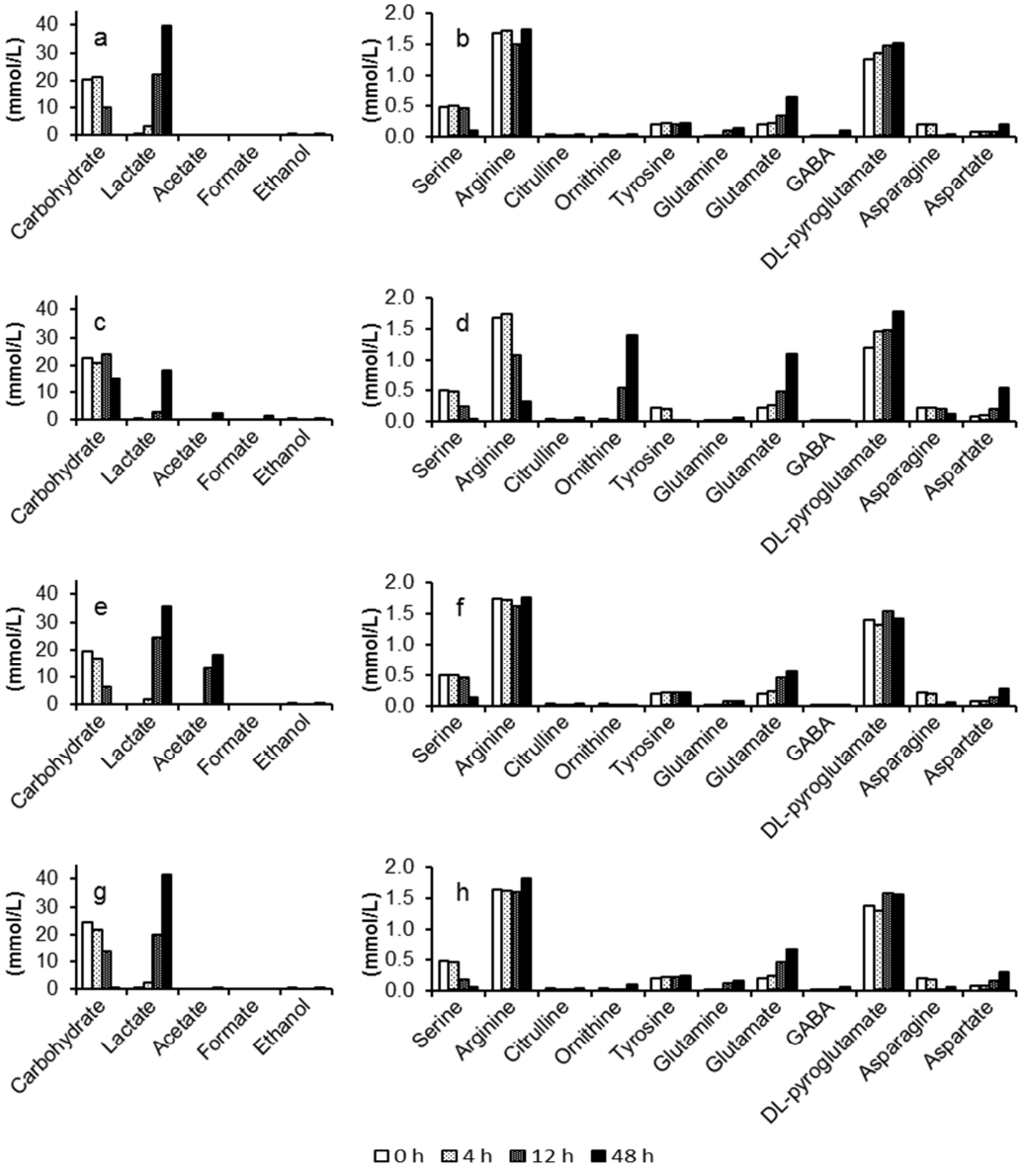
Moe

Figure 2.


a
b
c
d
e
f
g
h
i
j
(mmol/L)
Carbohydrate
Lactate
Acetate
Formate
Ethanol
Serine
Arginine
Citrulline
Ornithine
Tyrosine
Glutamine
Glutamate
GABA
DL-pyroglutamate
Asparagine
Aspartate
0 h
4 h
12 h
48 h

Moe

Figure 3.


a
b
c
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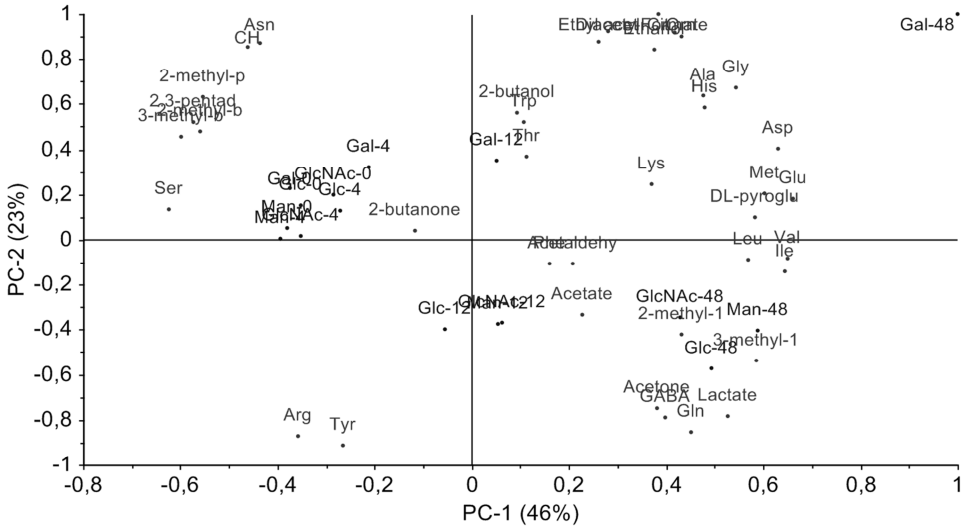
e
f
g
h
(mmol/L)
Carbohydrate
Lactate
Acetate
Formate
Ethanol
Serine
Arginine
Citrulline
Ornithine
Tyrosine
Glutamine
Glutamate
GABA
DL-pyroglutamate
Asparagine
Aspartate
0 h
4 h
12 h
48 h

Moe

Figure 4.


PC-2 (23%)
PC-1 (46%)
Asn
2-methyl-p
Ser
Gal-4
2-butanone
Gal-12
2-butanol
Trp
Thr
Ala
His
Gly
Asp
Lys
Met
Glu
DL-pyroglu
Leu
Val
Ile
Acetate
GlcNAc-48
2-methyl-1
Man-48
3-methyl-1
Glc-48
Acetone
GABA
Lactate
Gln
Arg
Tyr
Gal-48

Paper III

Regulation of proteins in the cheese nonstarter lactic acid bacteria *Lb. plantarum* 15D when grown on monosaccharides found in the milk fat globule membrane.

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ABSTRACT

The proteomes of the cheese nonstarter lactic acid bacteria *Lactobacillus plantarum* INF15D were studied when grown on different monosaccharides found in milk fat globule membrane glycoconjugates.

Growth on D-galactose induced enzymes involved in alternative energy acquisition from carbohydrate and amino acid sources. This was expressed as a metabolic shift leading to a different fate of pyruvate, where ethanol and acetate were produced. Degradation of some amino acids known to potentially take part in energy production, specifically arginine and tyrosine, was also induced. Enzymes involved in ribose metabolism as well as in the phosphoketolase pathway was upregulated during growth on D-galactose. The cell shape determining protein MreB, that leads to elongation of the cells was also found to be upregulated during growth of *Lb. plantarum* INF15D on D-galactose.

Growth of *Lb. plantarum* INF15D on the acylated aminosugar *N*-acetyl-D-glucosamine led to induction of enzymes involved in the degradation of this sugar through a pathway that yields large amounts of acetate as a degradation product.

These findings indicate that *Lb. plantarum* INF15D was able to utilize several different substrates for energy at the same time to secure growth and survival during sub-optimal conditions.

INTRODUCTION

Lactobacillus (Lb.) plantarum is a facultative heterofermentative lactic acid bacteria involved in fermentation of food products. It is often found to be part of the nonstarter lactic acid bacteria (NSLAB) flora dominating in cheeses during ripening (Beresford and Williams, 2004;

Østlie et al., 2004). The *Lb. plantarum* INF15D strain has been found to totally dominate the NSLAB flora of Norvegia (a Norwegian semi-hard Dutch-type cheese) after extended ripening (3 years and more; Skeie, unpublished data). This suggests that *Lb. plantarum* INF15D is particularly able to utilize components in the cheese environment for survival. The substrates that NSLAB uses for energy during cheese ripening are currently not clearly defined. However, several previous papers have focused on growth of NSLAB on potential substrates from the cheese environment (Beresford et al., 2001; Fox et al., 1998). Several studies suggest that *Lb. plantarum* is able to utilize the AA Arg and Ser from the cheese environment for energy by substrate-level phosphorylation during ripening (Adamberg et al., 2006; Liu et al., 2003; Skeie et al., 2008; Skeie et al., 2001; Moe et al., submitted).

The MFGM contains several different types of glycoconjugates including mucin (Keenan and Mather, 2006), and earlier research have indicated that bacteria in cheese are found in association with the MFGM (Laloy et al., 1996; Lopez et al., 2006). Sánchez et al. (2010) showed that *Lb. rhamnosus* GG was able to grow with the porcine gastric mucin (a glycoconjugate) as the only carbohydrate source, indicating that carbohydrates were released from mucin by the bacterium to be utilized for growth. Proteomic experiments by Gagnaire et al. (2004) found that the glycolytic enzyme β-galactosidase was present in the aqueous phase of a 76 day old Emmental cheese. This enzyme is capable of both hydrolyzing lactose as well as releasing sugars from MFGM glycoconjugates (Liu et al., 2005). Several earlier studies have shown that NSLAB are able to utilize several of the monosaccharides that potentially could be released from MFGM glycoconjugates (Adamberg et al., 2005; Williams et al., 2000; Moe et al., 2012).

47 In a model system, Thomas, (1987) demonstrated that several strains of NSLAB were
48 able to grow on products released from autolysed starter cells. Ribose is considered to be the
49 most readily released sugar present in the starter cells (Thomas and Batt, 1969), and Rapposch et
50 al. (1999) later showed that several different facultative heterofermentative lactobacilli decreased
51 the amount of ribose from lysed *Lb. helveticus* cells in the media.
52 By comparing the proteome of cells growing under different conditions, it is possible to
53 study the regulation of the different proteins by the defined conditions. The proteins extracted
54 from cells can be separated using chromatographic techniques, and by the use of mass
55 spectrometry (MS) it is possible to sequence and identify the proteins with a high degree of
56 certainty. Studies of the proteome from different growth phases of several *Lb. plantarum* strains
57 have been published (Cohen et al., 2006; Koistinen et al., 2007) and are useful as reference maps
58 for subsequent studies.
59 Two previous experiments showed that *Lb. plantarum* 15D was able to utilize the
60 monosaccharides found in the MFGM glycoconjugates for growth (Moe et al., 2012; Moe et al.,
61 submitted). The growth of *Lb. plantarum* INF15D also showed a growth pattern on galactose
62 (Gal) as the only readily available carbohydrate that could be interpreted as a state where
63 alternative energy yielding pathways were induced (Moe et al., 2012; Moe et al. submitted).
64 Therefore, the objective of the present study was to investigate *Lb. plantarum* INF15D in more
65 depth with more powerful proteomic tools. The proteomes of the bacteria when grown on
66 MFGM monosaccharides were analyzed and compared to the proteome from growth on a
67 glucose standard by the up-/down regulation of enzymes involved in the metabolism of the
68 MFGM monosaccharides or other substrates in the growth media.
69

70

71 **MATERIALS AND METHODS**

72 *Bacterial strains and their preparation*

73 The studies were performed on the NSLAB *Lb. plantarum* INF15D, isolated from a 90 days old
74 Norvegia, a Norwegian semi-hard Dutch-type cheese. The isolate was kept dormant at -80 °C
75 suspended in MRS broth added 15 % (v/v) glycerol. The bacterium was sub cultured at least
76 three times at 30 °C for 24 h in MRS for each of the three replicates. Cultures (1 mL) were
77 harvested by centrifugation at 13,400 × *g* for 20 min (Eppendorf Centrifuge 5415D; Eppendorf,
78 Hamburg, Germany). The supernatant was removed with a sterile pipette, and the bacterium
79 pellet was resuspended in 1 mL of carbohydrate-restricted medium (CRM) without any
80 carbohydrates added.

81

82 *Preparation of growth media*

83 A CRM was made according to Adamberg et al. (2005), with minor adjustments (Moe et al.,
84 2012). The carbohydrates used to supplement the CRM were Gal, Man and GlcNAc (All from
85 Sigma), and D-glucose (Glc; Merck) was used as standard. The carbohydrates were prepared as
86 stock solutions (100 mg mL^{-1}), and were filter sterilized (pore size 0.22 μm; Millex,
87 Carrigtwahill, Ireland) prior to the addition to CRM (final concentration 5 g L^{-1}).

88

89 *Growth conditions and extraction of proteins*

90 The resuspended bacteria (1 %) were pre-cultured in CRM supplemented with 5.0 g L^{-1} of one
91 of the four monosaccharides: Glc, Man, Gal, or GlcNAc at 30 °C for 24 h. The bacteria were
92 then inoculated at an initial OD_{600} of 0.07 in 100 mL fresh CRM containing the same

93 monosaccharide at the same level (5.0 g/L) as in the CRM used for the pre-culturing. The OD_{600}
94 was monitored using a Hach DR/2500 spectrophotometer (Hach Company; Loveland, CO, USA)
95 and the bacterial cells were harvested at mid-log (OD_{600} 0.5-0.6) by centrifugation (2800 × *g*; 4
96 °C; 15 min.). The cells were washed twice with 50 m*M* phosphate buffered saline (PBS; 0.9 %
97 NaCl; pH 7.5) and subjected to centrifugation (2800 × *g*; 4 °C; 15 min.) between washes. The
98 harvested cells were added ~500 mg glass beads (Sigma; acid-washed, <106 microns), 500µL
99 UTCD solution (8 *M* urea, 2 *M* thiourea, 2 % (w/v) 3-[(3-cholamidopropyl)dimethylammonio]-
100 1-propanesulfonate (CHAPS; Sigma) and 50 m*M* DTT) and were mechanically disrupted using a
101 FP120 FastPrep cell disruptor (4 × 30 sec.; speed 6.5; 4 °C; Thermo Savant; Waltham, MA,
102 USA). The cell lysates were kept on ice for one minute between runs to counteract a rise in
103 temperature during cell disruption. The samples were centrifuged (20800 × *g*; 4 °C; 30 min) to
104 remove glass beads and insoluble cell wall components. The protein concentration of the
105 supernatant was measured using a colorimetric RC DC Protein assay (Bio-Rad; Hercules, CA,
106 USA), using bovine serum albumin (BSA) for preparation of a standard curve according to the
107 manufacturer's instructions. The supernatants were stored in aliquots at -80 °C. The experiment
108 was done in 3 biological replicates.
109
110 *Two dimensional gel electrophoresis*
111 Cell extracts corresponding to 75 µg (analytical gel) and 250 µg (preparative gel) of protein were
112 diluted by adding UTCD and 0.5 % immobilized pH gradient (IPG) buffer pH 4-7 (GE
113 Healthcare BioSciences; Little Chalfont, UK) to a final volume of 450 µL. A tracking dye
114 consisting of a 0.5 % bromophenol blue solution (BTB; 5 µL) was also added to the sample. The
115 sample solution was used to actively rehydrate a 24 cm pH 4-7 IPG strips (GE Healthcare) for 16

hours at 50 mV at 20 °C in a Protean IEF system (Bio-Rad) under mineral oil (Bio-Rad). The isoelectric focusing was then performed as follows: linear ramping to 250 V during 30 min, rapid ramping to 500 V for 1 h, rapid ramping to 1000 V for 1 h, rapid ramping to 10000 V for 70000 Vh. The IPG strips were equilibrated for 15 min in an equilibration buffer (6 *M* urea, 50 m*M* tris-HCl pH 8.8, 30 % glycerol, 2 % sodium dodecyl sulfate (SDS)) supplemented with 1 % (w/v) dithiothreitol (DTT), followed by 15 min in equilibration buffer added 2.5 % (w/v) iodoacetamide (IAA).

The IPG strips were placed on top of a 12.5 % continuous SDS-polyacrylamide gel for electrophoresis (SDS-PAGE) using an Ettan DALT II system (GE Healthcare Bio-sciences; Uppsala, Sweden). The IPG strips were sealed with an overlay of a 0.3 % agarose solution supplied with BTB as a tracking dye. Proteins were separated at 10 °C at 5 mA/gel for 3 h and then 15 mA/gel until the BTB tracking dye reached the bottom of the gel. Silver staining of the analytical gels were done according to Blum et al., (Blum et al., 1987) modified by Görg et al. (Görg et al., 2002), and preparative gels according to Shevchenko et al. (1996). One analytical gel was made from each of the three biological replicates of each monosaccharide.

Image and statistical analysis

The gels were scanned (Epson Perfection 4990 Photo, Epson; 16-bit greyscale; 400 dpi) and imported, unaltered into Delta 2D (DECODON; Greifswald, Germany) as the uncompressed *.tif file format. For each strain and carbohydrate, one image from each of the three trials were aligned automatically in the software. The automatic warping was checked manually and edited if the images were unaligned. Spots were edited manually, and presumably artifact spots removed. The visualized proteins of *Lb. plantarum* INF15D grown on each of the MFGM

monosaccharides were compared against the Glc standard. Spots which were significant ($P < 0.05$) differently expressed between growth on the two monosaccharides were identified by the software for later identification.

Protein identification

The proteins that were significantly ($P < 0.05$) up- or down-regulated compared to the Glc standard, according to spot intensity were excised from the preparative gels for identification. The proteins were prepared using an adaption of a method by Shevchenko et al. (2006). The excised proteins were cut into ~1 mm pieces and transferred to an Eppendorf tube and added 100 µL 50 % acetonitrile (ACN), 50 m*M* ammonium bicarbonate (AmBic) before a 15 min incubation with shaking at ~20 °C. The solution was discarded, and 200 µL 100 % ACN was added for 15 min. The solution was again discarded, and the gel pieces were completely dehydrated by leaving the tubes open in a fume hood for ~15 min. To reduce the disulphide bridges, 50 µL of a 0.1 *M* AmBic and 10 m*M* DTT solution was added to the gel pieces, and the tubes were incubated for 30 min at 56 °C. After discarding the reduction solution, 50 µL of an alkylation solution (0.1 *M* AmBic and 55 m*M* IAA) was added and incubation commenced in the dark for 30 min at ~20 °C. All of the alkylation solution was discarded, and the gel pieces were incubated with 200 µL ACN for 15 min. After discarding the ACN, the gel pieces were again dehydrated in a fume hood for ~15 min before the tubes were transferred to ice, and the gel pieces were completely covered in a trypsin solution (25 m*M* AmBic, 10 % ACN and 10 ng mL^{-1} modified porcine trypsin (ProMega; Fitchburg, WI, USA) for 1 hour. The trypsin solution was replaced with 25 mM AmBic, 10 % ACN, and the proteins were digested at 37 °C for ~16 h. After incubation, 50 µL of an extraction solution containing 1.67 % formic acid and 66.7 %

ACN was added before incubation commenced at 37 °C for 15 min. The samples were sonicated for 10 min and centrifuged (5 min, 16,000 × *g*) before the supernatant was transferred to a clean Eppendorf tube. The gel pieces were added 50 µL 100 % ACN, and incubated for 15 min at ~20 °C with shaking before the supernatant was pooled with the supernatant from the previous step. The protein extract was dried in a centrifugal evaporator.

Desalting of the digested proteins were done by the method described by Rappsilber et al. (2003) using a plunge of C18 extraction material (3M Empore C18 extraction disc, Varian) into a gel loader tip (20 µl, Eppendorf). The samples were eluted directly on a MALDI target plate (Bruker Daltonics; Billerica, MA, USA) using 0.5 µL of a 0.1 % TFA, 50 % ACN solution saturated with α-cyano-4-hydroxycinamic acid mixed 1:1 with 100 % ACN.

The proteins were identified using peptide mass fingerprints (PMF) or MS/MS fragmentation spectra or both obtained by an Ultraflex MALDI-TOF/TOF (Bruker Daltonics) and Mascot software (Matrix Science Inc.; Boston MA, USA). Sequence coverage, search scores, and agreement between theoretical and experimental isoelectric point (pI) and molecular weight (MW) were considered when positively identifying the proteins. Some proteins were considered positively identified after comparison with results found in literature or if they were identified on more than one gel with the same result or both, even if the sequence coverage was not satisfactory.

RESULTS AND DISCUSSION

In this study the proteins expressed by *Lb. plantarum* INF15D when grown on several different monosaccharides found in MFGM were separated and visualized using 2-DE. The *Lb.*

185 *plantarum* INF15D strain has been isolated from a cheese considered to be of good quality by a
186 panel of certified sensory judges (Narvhus et al., 1993).
187 Statistical comparison of the spot intensity of the different gels revealed that *Lb.*
188 *plantarum* INF15D grown on Gal had a higher number of significantly ($P < 0.05$) differently
189 regulated proteins in the pI 4-7 than Gal or GlcNAc. The proteins that were significantly ($P <$
190 0.05) up- or down regulated compared to the Glc standard gels were prepared for identification
191 by MALDI-TOF/TOF. Proteins yielding a significant ($P < 0.05$) identification are presented in
192 table 1. A total of 31 of the identified proteins were found to be significantly ($P < 0.05$)
193 upregulated and 15 downregulated when *Lb. plantarum* INF15D was grown on Gal. The 20
194 identified proteins that were differently regulated when *Lb. plantarum* INF15D was grown on
195 GlcNAc were mostly involved in carbohydrate metabolism.
196 The glycolytic enzymes fructose bisphosphate aldolase, glyceraldehyde 3-phosphate
197 dehydrogenase and enolase were significantly ($P < 0.05$) downregulated in *Lb. plantarum*
198 INF15D grown on Gal, GlcNAc and Man as compared to Glc. The ATP utilizing and the ATP
199 yielding reactions catalyzed by 6-phosphofructokinase and phosphoglycerate kinase remained
200 unregulated. However the enzyme pyruvate kinase, yielding pyruvate and ATP from
201 phoshoenoylpyruvate and ADP, was found to be significantly upregulated in *Lb. plantarum*
202 INF15D grown on Gal compared to Glc.
203 However, the enzyme phospho acyltransferase was significantly upregulated when *Lb.*
204 *plantarum* INF15D was grown on Gal. This enzyme is involved in the conversion of acetyl-CoA
205 and pyrophosphate to CoA and acetylphosphate (Kandler, 1983). Acetylphosphate can further be
206 converted to acetate, yielding ATP. At the same time, D-lactate dehydrogenase was significantly
207 ($P < 0.05$) downregulated when *Lb. plantarum* INF15D was grown on Gal, strongly indicating a

shift from lactate to acetate production when grown on Gal compared to Glc. This finding is in agreement with several other studies, showing a partial shift from lactate to acetate production when LAB were grown on Gal (Adamberg et al., 2006; Dirar and Collins, 1973; Thomas et al., 1980; Moe et al. submitted). Fructose 1,6-bisphosphate functions as an allosteric regulator (activator) of pyruvate kinase and lactate dehydrogenase in *Lc. lactis*, and accumulates during glucose metabolism (Thompson, 1987). The shift to a mixed acid fermentation has been interpreted as a result of a reduced fructose 1,6-bisphosphate pool, leading to lactate dehydrogenase inactivation, and an inhibition relief of pyruvate-formate lyase by triose phosphates (Thomas et al., 1979; Thomas et al., 1980). A study by Melchiorsen et al. (2002) have shown that a shift to mixed acid fermentation occurred in *Lc. Lactis* when it was grown on Gal, due to a 3.4 fold increase in the production of pyruvate-formate lyase. Pyruvate-formate lyase was not positively identified in this experiment, however several of the effects of it upregulation as described by Melchiorsen et al. (2002) was observed.

The production of NADH oxidase was also upregulated in *Lb. plantarum* INF15D grown on Gal. Lopez de Felipe et al. (1998) and Hugenholtz et al. (2000) showed that when NADH oxidase was overproduced, pyruvate was converted by acetolactate synthase and pyruvate dehydrogenase instead of lactate dehydrogenase. The enzyme pyruvate dehydrogenase complex, E1 component, alpha subunit was observed, however, it was not found to be significantly ($P < 0.05$) differently regulated in *Lb. plantarum* INF15D when grown on any of the MFGM monosaccharides.

It has been shown that several lactobacilli are able to utilize ribose from dead bacterial cells in a model system (Rapposch et al., 1999; Thomas, 1987). It was found in this experiment that ribokinase was significantly ($P < 0.05$) upregulated in *Lb. plantarum* INF15D when grown

231 on Gal. Ribokinase catalyzes the phosphorylation of D-ribose to D-ribose-5-phosphate by the use
232 of ATP (Kandler, 1983). The enzyme ribose-5-phosphate isomerase A, which may further
233 convert the D-ribose-5-phosphate into D-ribulose-5-phosphate was also found to be significantly
234 ($P < 0.05$) upregulated in *Lb. plantarum* INF15D grown on Gal. It is interesting to note that the
235 enzyme 6-phosphogluconate dehydrogenase which converts 6-phosphocluconate into D-ribulose-
236 5-phosphate was found to be upregulated by growth on Gal as well. The precursor to 6-
237 phosphogluconate could be either gluconate or glucose 6-phosphate. This finding could indicate
238 that some Gal fist was converted into glucose 6-phosphate, which was further converted into 6-
239 phosphocluconate. This suggests that Gal was metabolized by the phosphoketolase pathway
240 instead or in addition to the Leloir pathway (Kandler, 1983; Posthuma et al., 2002). This agrees
241 with the observation that several of the enzymes involved in the earlier steps of the glycolytic
242 pathway were down-regulated as described earlier, as well as the observation that ethanol was
243 produced by *Lb. plantarum* INF15D when grown on Gal.
244 D-Ribose is only available as a potential substrate for *Lb. plantarum* INF15D from other
245 dead bacteria cells in the CRM medium, and therefore the presented results suggests that when
246 *Lb. plantarum* INF15D was grown on Gal, mechanisms for alternative carbohydrate utilization
247 from the dead bacteria cells was induced. The finding of purine nucleosidase in the proteome of
248 *Lb. plantarum* INF15D indicates that ribose can be released from bacterial RNA.
249 The facultative heterofermentative lactobacilli, such as *Lb. plantarum*, ferment hexoses
250 via the glycolytic pathway, and pentoses via the phosphoketolase pathway (PKP), and reports of
251 repression of PKP enzymes by Glc and mannose PTS has been reported (Posthuma et al., 2002).
252 The bifunctional protein guanine monophosphate (GMP) synthetase/ Glutamine
253 amidotransferase catalyses the reaction:

ATP + xanthosine 5-phosphate + L-glutamine + H_2O ↔ AMP + diphosphate + GMP + L-glutamate.

When *Lb. plantarum* 15D was grown on Gal, this protein was significantly downregulated, and this is in agreement with earlier research done on *Lb. plantarum* INF15D, where it was shown that the Glu levels were higher when it was grown on Gal compared to Glc, Man and GlcNAc (Moe et al., submitted). Guanosine monophosphate is a nucleotide monomer found in RNA that consists of a phosphate group, ribose and guanine.

When *Lb. plantarum* INF15D was grown on GlcNAc, glucosamine 6-phosphate isomerase was significantly ($P < 0.05$) upregulated. This enzyme converts glucosamine 6-phosphate into the glycolytic pathway intermediate fructose-6-phosphate and ammonia (Comb and Roseman, 1956). Bates et al. (1965) found that both glucosamine 6-phosphate isomerase and GlcNAc 6-phosphate deacetylase were induced in *Bacillus subtilis* by GlcNAc. This was seemingly the case with *Lb. plantarum* INF15D as well, however, glucosamine-6-phosphate isomerase has also been found to be upregulated by lactose starvation in *Lb. casei* GCRL163 as well (Hussain et al., 2009). Cohen et al. (2006) found that glucosamine-6-phosphate isomerase was upregulated in the stationary phase of *Lb. plantarum* WCFS1, even if the growth media did not contain any added free GlcNAc. The aminosugar GlcNAc is known to be abundant in the peptidoglycan of bacterial cell walls. Cohen et al. (2006) therefore suggested that a decrease of the glucose content in the MRS media would induce use of alternative energy sources and pathways.

An earlier experiment showed that *Lb. plantarum* INF15D and some *Lb. paracasei* produced large amounts of acetic acid when grown on GlcNAc (Moe et al., submitted). The identification of both GlcN-6-P isomerase and phosphoglucosamine mutase, where GlcN-6-P is the substrate for both enzymes, strongly suggest the presence of GlcNAc-6-P deacetylase, even though this enzyme was not identified in this experiment.

A higher maximum cell number have been observed when *Lb. plantarum* INF15D was grown on GlcNAc compared to other MFGM monosaccharides (Moe et al., in press). Phosphoglucosamine mutase was found to be significantly ($P < 0.05$) upregulated in *Lb. plantarum* INF15D grown on GlcNAc as compared to Glc. This enzyme catalyzes the intermolecular transfer of the phosphate group in glucosamine-6-phosphate to produce glucosamine-1-phosphate. This step is an essential step in the pathway for UDP-GlcNAc biosynthesis used for cell wall construction in several bacteria. This indicate that *Lb. plantarum* INF15D was able to use GlcNAc for cell wall biosynthesis directly without metabolizing it for energy first. This agrees with the findings of Topper and Lipton (1953) which showed that GlcNAc was preferentially incorporated into the capsular material in bacterial cells in contrast to Glc when both carbohydrates were present.

Phospho acyltransferase was also significantly ($P < 0.05$) upregulated when *Lb. plantarum* INF15D was grown on GlcNAc, however in contrast to growth on Gal, D-lactate dehydrogenase was also significantly ($P < 0.05$) upregulated .

In earlier studies of the metabolism of *Lb. plantarum* INF15D, it was shown that this bacteria used Ser, Asn, Arg and Tyr when grown on Gal (Moe et al., submitted). When *Lb. plantarum* INF15D was grown on Gal, the enzymes L-Serine dehydratase beta subunit and tryptophan synthase subunit alpha were significantly ($P < 0.05$) upregulated. The enzyme L-

299 Serine dehydratase is involved in the conversion of Ser to NH_3 and pyruvate. Earlier studies
300 done on *Lb. plantarum* INF15D have shown that this bacterium utilizes serine during growth
301 (Skeie et al., 2008). However, the analysis of the AA utilization during growth of *Lb. plantarum*
302 INF15D showed no apparent differences in the Ser utilization between the growth on the
303 different monosaccharides used in this study (Moe et al., submitted). This indicate that Ser was
304 used for energy in *Lb. plantarum* INF15D regardless of which of the four carbohydrates it
305 metabolized, and its utilization appear unregulated by the conditions used in this experiment.
306 Tryptophan synthase subunit alpha converts indole-3-glycerol phosphate into indole and
307 glyceraldehyde-3-phosphate. Glyceraldehyde-3-phosphate is a glycolytic pathway intermediate
308 and can be utilized for energy production. The overall reaction of both tryptophan synthase
309 subunits is:
310
311 L-serine + 1-C-(indol-3-yl)glycerol 3-phosphate = L-tryptophan + glyceraldehyde 3-phosphate +
312 H_2O.
313
314 This indicates that when *Lb. plantarum* INF15D is grown on Gal, Ser may utilized by
315 more than one pathway at the same time. Both of the reactions are potentially energy yielding.
316 The AA Lys, Met, Thr and Ile are synthesized via a branched pathway, where Asp is
317 converted into aspartate-4-semialdehyde by the action of the two enzymes aspartokinase and
318 aspartate semialdehyde dehydrogenase. Aspartate semialdehyde dehydrogenase was found in *Lb.*
319 *plantarum* INF15D. However, no significant difference in regulation of this enzyme was found
320 on growth of the bacterium on any of the monosaccharides. Both dihydropicolinate synthetase
321 and homoserine dehydrogenase was found in *Lb. plantarum* INF15D when grown on Gal. These

two enzymes are the first step in the further conversion of aspartate-4-semialdehyde into Lys and Thr respectively. All of the three enzymes found, uses one proton during their action. In a proteomic study of *Streptococcus thermophilus* LMG 18311 during the late stage of milk fermentation, Herve-Jimenez et al. (2008) also identified dihydropicolinate synthetase and homoserine dehydrogenase, however no regulation was observed as a response to the conditions used.

Cohen et al., (2006) found atpA to be present at increasing proportions of the total protein complement in *Lb. plantarum* WCFS1 from log to late stationary phase. Hamon et al. (2011) found F_1F_o ATP synthase subunit delta to be upregulated when *Lb. plantarum* was exposed to bile. The authors suggested that the F_1F_o ATP synthase complex was used for proton expulsion from the cell as a protection against the bile in the environment. However, Higuchi et al. (1997) suggested that F_1F_o ATP synthase is used for energy production by pumping protons into the cell. The protons are then removed from the cytoplasm by decarboxylation reactions and cell wall antiporters instead of by the energy consuming F_1F_o ATP synthase.

Tyr was used by *Lb. plantarum* INF15D when grown on Gal, but not on Glc, GlcNAc or Man (Moe et al., submitted). Some report (Arena et al., 2007; Masson et al., 1996) that *Lb. plantarum* are able to decarboxylize tyrosine to form tyramine. Further, the F_1F_o ATP synthase subunit alpha, beta and delta was found in *Lb. plantarum* INF15D, however only the delta subunit was found to be significantly upregulated when grown on Gal. The F_1F_o ATP synthetase complex make the bacteria able to produce ATP from ADP + Pi and 3 protons. This indicates that *Lb. plantarum* INF15D was able to generate energy by the use of decarboxylation reactions to form a proton gradient across the cell wall to fuel the F_1F_o ATP synthetase.

344 The cell shape determining protein MreB was significantly ($P < 0.05$) upregulated in *Lb.*
345 *plantarum* INF15D when it was grown on Gal. Doi et al. (1988) established that the MreB
346 protein was involved in the formation of the rod shape of the bacterial cell. The shape
347 determining genes are also thought to have a negative influence on cell division, as expression of
348 additional copies of the *mreB* gene in *Escherichia coli* produced elongated, multinucleated cells
349 (Wachi and Matsuhashi, 1989). The MreB protein is a bacterial actin homologue, and it is
350 thought to polymerize by the use of ATP into long filaments (van den Ent et al., 2001). The
351 MreB filaments were first described as helical structures encircling the cell cytoplasm directly
352 under the membrane (Jones et al., 2001). Later work questions this hypothesis (Swulius et al.,
353 2011) and instead suggests that MreB makes up discrete patches moving circumferential in the
354 bacterial cell, inserting radial hoops of new peptidoglycans during their transit (Domínguez-
355 Escobar et al., 2011; Garner et al., 2011).
356 It has been shown that lactobacilli visualized in cheese can be severely elongated
357 (Martinovic et al., submitted). The reason for the elongated cells in cheese could very well be a
358 consequence of the upregulation of the production of the MreB protein as observed in *Lb.*
359 *plantarum* INF15D when grown on Gal.
360 Rich growth media supports the growth of lactobacilli up to log 8-9 cfu/mL, while
361 lactobacilli in cheese usually don't reach numbers higher than log 7 cfu/mL (Ardö, 1993; Crow
362 et al., 2001; Fox et al., 1998; Lindberg et al., 1996; Peterson and Marshall, 1990). The fact that it
363 has been observed that the cells elongated by the overproduction of MreB also could be
364 multinucleated (Wachi and Matsuhashi, 1989) makes it reasonable to hypothesize that the
365 elongated state that lactobacilli obtain in cheese could be an explanation of the relative low
366 maximum cell number obtained. It was shown that *Lb. plantarum* INF15D did obtain slightly

lower maximum cell numbers when grown on Gal compared to Glc, GlcNAc and Man (Moe et al., In press), however no apparent elongation could be observed of the cells by observation using light contrast microscopy (results not shown) of cells grown for 24 h on Gal compared to Glc.

The cell surface protein elongation factor Tu is suggested to be involved in the attachment of lactobacilli to human intestinal cells and mucin (Granato et al., 2004). Although this protein was identified in *Lb. plantarum* INF15D, it was not found to be regulated by any of the conditions used in this study. However this finding is worth mentioning, as it has been observed in earlier studies that bacteria in cheese are preferentially located at the surface of the MFGM (Laloy et al., 1996; Lopez et al., 2006). Laloy at al. (1996) further suggested that it may exist an active binding by the bacteria to the MFGM that retains the bacteria in cheese during cheesemaking.

CONCLUSION

When *Lb. plantarum* INF15D was grown on Gal a number of enzymes involved in proton consuming reactions were upregulated. This finding strengthens the hypothesis that *Lb.* are able to fuel the F_1F_o ATP synthase complex by introducing protons to the cytosol where they are consumed by used in enzymatic reactions. The results presented also indicates that several different alternative pathways can be induced in *Lb. plantarum* INF15D at the same time, utilizing more than one substrate to ensure energy requirements in harsh environments.

388 The results presented also introduces the hypothesis that the upregulation of the cell
389 shape determining protein MreB was involved in the elongation of lactobacilli cells that have
390 been observed in cheese.
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Table 1. Identified proteins in *Lb. plantarum* INF15D grown on D-galactose (Gal), *N*-acetyl-D-glucosamine (GlcNAc) and D-mannose (Man) compared with D-glucose. Proteins significantly ($P < 0.05$) up- (↑) or downregulated (↓) from the D-glucose standard by growth on the assigned monosaccharide are shown. ORF, Open reading frame; MW, Molecular weight; PMF, peptide mass fingerprint.

ORF	Gene	Function	MW	p*I*	Mascot Score	Sequence coverage	Matched peptides	Identification type	Regulation
Carbohydrate metabolism									
		Glycolysis							
lp_1898	*pfk*	6-Phosphofructokinase	34244	5.13	133	47	17	PMF	
lp_0330	*fba*	Fructose-bisphosphate aldolase	31016	5.07	242	88	24	PMF	Gal↓, GlcNAc↓, Man↓
lp_0330	*fba*	Fructose-bisphosphate aldolase	31016	5.07	76	28	6	PMF	Gal↓, GlcNAc↓, Man↓
lp_0789	*gapB*	Glyceraldehyde-3-phosphate dehydrogenase	36644	5.30	172	55	17	PMF	Gal↓, GlcNAc↓, Man↓
lp_0789	*gapB*	Glyceraldehyde-3-phosphate dehydrogenase	36644	5.30	112	72	13	PMF	Gal↓, GlcNAc↓, Man↓
lp_0789	*gapB*	Glyceraldehyde-3-phosphate dehydrogenase	36644	5.30	126	47	11	PMF	Gal↓, GlcNAc↓, Man↓
lp_0789	*gapB*	Glyceraldehyde-3-phosphate dehydrogenase	36644	5.30	86	33	8	PMF	
lp_0790	*pgk*	Phosphoglycerate kinase	42770	5.04	124	40	14	PMF	
lp_3170	*pmg9*	Phosphoglyceromutase/ Phosphoglycerate mutase	26071	4.94	112	61	11	PMF	GlcNAc↑
lp_3170	*pmg9*	Phosphoglyceromutase/ Phosphoglycerate mutase	26071	4.94	172	63	15	PMF	GlcNAc↑
lp_0792	*enoA1*	Phosphopyruvate hydratase	48057	4.61	349	59	27	PMF	Gal↓, GlcNAc↓, Man↓
lp_0792	*enoA1*	Phosphopyruvate hydratase (Enolase)	48057	4.61	295	56	24	PMF	Gal↓, GlcNAc↓, Man↓
		Phosphopyruvate hydratase (Enolase)	48000	4.61	271	51	23	PMF	Gal↓, GlcNAc↓, Man↓
lp_1897	*pyk*	Pyruvate kinase	66572	5.05	118	31	17	PMF	Gal↑
		Pentose phosphate pathway							
lp_0602	*rpiA1*	Ribose-5-phosphate isomerase A	24627	4.79	77	40	7	PMF	Gal↑
		Ribokinase	31749	5.18	172	50	15	PMF	Gal↑
lp_1541	*gnd2*	6-Phosphogluconate dehydrogenase (decarboxylating)	53063	5.04	145	45	19	PMF	Gal↑
		Pentose and glucuronate interconversations							
lp_0757	*galU*	UTP--glucose-1-phosphate uridylyltransferase	34227	6.04	89	5	1	MS/MS	GlcNAc↑
		Fructose and mannose metabolism							
lp_2384	*pmi*	Mannose-6-phosphate isomerase	36041	5.36	202	64	19	PMF	Gal↑, GlcNAc ↑, Man↑
lp_3545	*gutB*	L-iditol-2-dehydrogenase	38523	4.93	86	35	10	PMF	Gal↑, Man↑
		Galactose metabolism							
lp_3482	*galK*	Galactokinase	42847	4.83	168	51	17	PMF	Gal↑
lp_3482	*galK*	Galactokinase	42847	4.83	72	29	10	PMF	Gal↑
lp_3481	*galE4*	UDP-glucose 4-epimerase	36378	5.43	151	50	15	PMF	Gal↑
		UDP-glucose 4-epimerase	36127	5.06	96	39	9	PMF	
		Aminosugar and nucleotide sugar metabolism							

lp_0226	*gnp*	Glucosamine-6-phosphate isomerase	25922	4.92	101	46	9	PMF	GlcNAc↑
lp_0226	*gnp*	Glucosamine-6-phosphate isomerase	25922	4.92	93	43	9	PMF	GlcNAc↑
lp_0226	*gnp*	Glucosamine-6-phosphate isomerase	25922	4.92	98	14	2	MS/MS	GlcNAc↑
lp_0820	*glmM*	Phosphoglucosamine mutase	48640	4.79	157	47	20	PMF	GlcNAc↑
		Pyruvate metabolism							
lp_2057	*ldhD*	D-lactate dehydrogenase	37158	4.89	140	52	21	PMF	Gal↓, GlcNAc↑
lp_2057	*ldhD*	D-lactate dehydrogenase	37158	4.89	96	35	17	PMF	Gal↓, GlcNAc↑
lp_2057	*ldhD*	D-lactate dehydrogenase	37158	4.89	99	23	13	PMF	Gal↓, GlcNAc↑
lp_0537	*ldhL1*	L-Lactate dehydrogenase	34242	4.80	152	64	18	PMF	
lp_2154	*pdhA*	Pyruvate dehydrogenase complex E1 component Alpha sub unit	41443	5.15	85	25	11	PMF	
lp_2154	*pdhA*	Pyruvate dehydrogenase complex E1 component Alpha sub unit	41443	5.15	56	19	6	PMF	
		L-2-hydroxyisocaproate dehydrogenase	32680	5.16	145	63	17	PMF	Gal↑
lp_0807	*eutD*	Phospho acetyltransferasease (Phosphotransacetylase)	34515	5.04	98	45	10	PMF	Gal↑, GlcNAc ↑
Energy metabolism									
		Oxidative phosphorylation							
		Fumarate reductase, flavoprotein subunit	48740	5.33	135	48	15	PMF	Gal↓
lp_2366	*atpA*	F0F1 ATP synthase subunit alpha	54545	4.95	116	27	12	PMF	
lp_2366	*atpA*	F0F1 ATP synthase subunit alpha	54545	4.95	134	27	17	PMF	
lp_2364	*atpD*	F0F1 ATP synthase subunit beta	50800	4.58	177	58	17	PMF	
lp_2367	*atpH*	H(+)-transporting 2 sector ATPase, delta subunit	20009	5.45	218	91	19	PMF	Gal↑
Lipid metabolism									
		Fatty acid biosynthesis							
		Short chain dehydrogenase/ oxidoreductase	32128	5.15	72	26	8	PMF	Gal↑
		Short chain dehydrogenase/ oxidoreductase	32071	5.15	110	50	12	PMF	Gal↑
		Short chain dehydrogenase/ oxidoreductase	31609	4.85	78	36	7	PMF	
		Glycerolipid metabolism							
lp_0370	*glpK*	Glycerol kinase	55783	5.26	96	39	18	PMF	
		Glycerophospholipid metabolism							
lp_0371	*glpD*	Glycerol-3-phosphate dehydrogenase	66572	5.05	149	40	19	PMF	Gal↑
lp_0371	*glpD*	Glycerol-3-phosphate dehydrogenase	66572	5.05	145	40	19	PMF	Gal↑
Nucleotide metabolism									
		Purine metabolism							
lp_0363		Purine nucleosidase	34206	4.41	98	7	2	MS/MS	Gal↑, GlcNAc ↑, Man↑
		Purine nucleosidase	35712	4.39	68	39	10	PMF	Gal↑, GlcNAc ↑, Man↑
		Deoxyguanosine kinase	24918	4.81	96	53	10	PMF	Gal↑, GlcNAc ↑
lp_2720	*purH*	Bifunctional phosphoribosylaminoimidazole carboxamide formyltransferase-IMP cyclohydrolase	55386	5.89	233	67	29	PMF	Gal↓, Man↓
lp_2720	*purH*	Bifunctional phosphoribosylaminoimidazole carboxamide formyltransferase-IMP cyclohydrolase	55386	5.89	83	21	9	PMF	Gal↓, Man↓

lp_2720	*purH*	Bifunctional phosphoribosylaminoimidazole carboxamide formyltransferase-IMP cyclohydrolase	55386	8.89	103	28	11	PMF	Gal↓, Man↓
		Phosphoribosylaminoimidazole carboxylase, ATPase subunit	40478	5.99	87	31	7	PMF	
lp_2725	*purQ*	Phosphoribosylformylglycinamide synthase I	24107	4.86	48	12	2	PMF	GlcNAc↑
		Pyrimidine metabolism							
lp_2702	*pyrC*	Dihydroorotase	45638	5.72	126	40	10	PMF	Gal↓
	pyrD	Dihydroorotate oxidase/ PyrD protein	31318/31343	6.14	67	22	7	PMF	Man↓
Amino acid metabolism									
		Alanine, aspartate and glutamate metabolism							
lp_2738	*asnA*	L-asparaginase AnsA	36906	5.81	72	20	7	PMF	
lp_2703	*pyrB*	Aspartate carbamoyltransferase	34689	6.06	128	44	12	PMF	GlcNAc↑
		Glycine, serine and threonine metabolism							
lp_2570	*asd2*	Aspartate-semialdehyde dehydrogenase	38903	5.50	111	42	10	PMF	
lp_0571	*hom2*	Homoserine dehydrogenase	46700	5.10	133	32	12	PMF	
lp_2790	*serA3*	Phosphoglycerate dehydrogenase	34238	6.13	82	8	2	MS/MS	GlcNAc↑, Man↑
lp_0505	*sdhB*	L-serine dehydratase Beta subunit	23888	5.56	103	72	9	PMF	Gal↑
lp_1658	*trpA*	Tryptophan synthase subunit alpha	28349	5.44	15	4	1	MS/MS	Gal↑
		Cysteine and methionione metabolism							
lp_0774	*luxS*	S-ribosyl-homocysteinase	17588	6.06	134	67	8	PMF	GlcNAc↑
		Lysine biosynthesis							
lp_2264	*dapD*	2,3,4,5-tetrahydropyridine-2-carboxylate-N-succinyltransferase	24537	5.02	112	38	11	PMF	
lp_1874	*dapB*	Dihydrodipicolinate synthetase	30631	5.13	48	24	7	PMF	
Metabolism of other amino acids									
		Gluthatione metabolism							
lp_0369	*gshR1*	Glutathione reductase	47365	5.29	121	48	11	PMF	Gal↓, Man↓
lp_0369	*gshR1*	Glutathione reductase	48343	5.19	71	24	7	PMF	
Translation									
		Ribosome							
lp_1882	*rpsA*	30S ribosomal protein S1	47131	4.79	149	52	14	PMF	Gal↑, GlcNAc ↑
lp_1040	*rpsC*	30S ribosomal protein S3	24193	9.95	57	30	7	PMF	
lp_0621	*rplJ*	50S Ribosomal protein L10	17916	5.08	110	58	9	PMF	Gal↑
		Aminoacyl-tRNA biosynthesis							
lp_1391	*argS*	arginyl tRNA synthetase	62870	5.29	96	31	13	PMF	Gal↓, GlcNAc↓
		Seryl tRNA synthetase	48220	5.16	135	38	12	PMF	
lp_1149	*gatB*	Aspartyl/glutamyl tRNA amidotransferase subunit A	52056	4.96	84	26	14	PMF	
Membrane transport									
		Phosphotransferase system (PTS)							

lp_0575	*pts9AB*	Mannose PTS EIIAB	35261	5.75	46	17	5	MS/MS	Gal↑, GlcNAc ↑
Cell growth and death									
lp_2319	*mreB1*	Cell shape determining protein MreB	35294	4.79	89	37	10	PMF	Gal↑
lp_2319	*mreB1*	Cell shape determining protein MreB	35294	4.79	78	18	6	PMF	Gal↑
		Translation							
lp_2054	*tsf*	Elongation factor Ts	31616	4.91	65	41	9	PMF	GlcNAc↑
lp_2119	*tuf*	Elongation factor Tu	43350	4.95	189	58	24	PMF	
lp_2119	*tuf*	Elongation factor Tu	43350	4.95	133	43	16	PMF	
		Replication and repair							
		Stress induced DNA binding protein	18010	4.70	92	38	6	PMF	Gal↑
		Folding							
lp_0728	*groEL*	Chaperonin GroEL	57402	4.69	98	30	12	PMF	Gal↓, Man↓
lp_0728	*groEL*	Chaperonin GroEL	57402	4.69	302	60	29	PMF	Gal↓, Man↓
		Stress proteins							
lp_0930	*asp2*	Alkaline shock protein	17556	5.11	72	50	7	PMF	Gal↑
		Cold shock protein CspC	7303	4.57	82	78	4	PMF	Gal↓
		Cold shock protein CspI	7252	4.50	98	89	6	PMF	Gal↓
		Hypotetical proteins							
lp_1974		Hypotetical protein lp_1974	30646	5.50	129	52	11	PMF	Gal↑
lp_2507		Hypotetical protein lp_2507	46845	4.99	94	37	12	PMF	
lp_2340		Hypothetical protein lp_2340	17597	5.91	87	61	8	PMF	Gal↑, Man↑
		Miscellaneous							
lp_2544	*npr2*	NADH peroxidase	48283	5.36	168	72	20	PMF	Gal↑
lp_2544	*npr2*	NADH peroxidase	48283	5.36	143	43	14	PMF	Gal↑
lp_3403		Oxidoreductase	36130	5.94	69	32	10	PMF	Gal↓, Man↓
lp_3403		Oxidoreductase	33804	5.57	69	48	12	PMF	Gal↑, Man↑
		Oxidoreductase	31667	5.16	111	37	9	PMF	Gal↑, Man↑
		Oxidoreductase (Putrative)	22778	5.12	97	37	9	PMF	Gal↑
		Oxidoreductase (Putrative)	37086	5.03	98	28	7	PMF	Gal↑
lp_2631		Lipase/ esterase (Putrative)	27324	6.40	79	38	5	PMF	
lp_1321	*pepV*	Dipeptidase PepV	50767	4.47	114	30	15	PMF	
lp_0061	*adc*	Acetoacetate decarboxylase (putrative)	30785	4.92	83	39	12	PMF	Gal↑
		Nitroreductase	24368	5.52	102	43	9	PMF	Gal↑
		NADH oxidase	49526	5.06	80	26	8	PMF	Gal↑
		NADH oxidase	49526	5.06	74	26	9	PMF	Gal↑

Paper IV

Growth of adjunct *Lactobacillus casei* in Cheddar cheese with added buttermilk powder

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Abstract

The development of the lactobacilli population during ripening of low fat Cheddar cheese differing in the content of milk fat globule membrane (MFGM) components and with a low initial number of adjunct *Lb. casei* strains differing in their ability to utilize MFGM components was studied. Two different adjuncts were added to the cheese milk: *Lactobacillus* (*Lb.) casei* INF 448 or *Lb. casei* INF 456, in the amount of log 2 cfu mL^{-1}. The composition of MFGM components was varied by the addition of butter milk powder (BMP) or skim milk powder (SMP) to the cheese milk. Regardless of adjunct addition the most diverse microbial composition was revealed at the beginning of the cheese ripening, while it became more uniform in the later stages of the ripening process (24 weeks). The present microorganisms at start did influence the development and composition of the lactobacilli during ripening even if the adjuncts were added at low numbers and did not dominate the flora at start. Even if the *Lb. casei* adjuncts have the ability to utilize the MFGM components, they still did not dominate the microflora in the cheese in later stages of ripening. A higher content of MFGM components in the cheese, however seemed to influence the development of lactobacilli and the composition of free amino acids during ripening.

The amount of Leu was higher in cheeses with BMP than in cheeses with SMP. This indicates that the presence of MFGM components was significantly influencing the flavour development in the studied cheeses.

Keywords: Cheddar cheese, buttermilk, adjunct lactobacilli, Rep-PCR

1. Introduction

Non starter lactic acid bacteria (NSLAB) in cheese originate from the raw milk or the production plant environment (Beresford, Fitzsimons, Brennan & Cogan, 2001; Cogan, Beresford, Steele, Broadbent & Ustunol, 2007). The population of NSLAB isolated from Cheddar 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 (FHL), used as adjuncts may contribute to the development of desirable cheese flavour and replace the indigenous NSLAB microflora resulting in a more controlled cheese production (Fox, McSweeney & Lynch, 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 and citrate, 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, In press).

Numerous studies have been performed in order to improve the overall quality of low fat cheese (Collins, McSweeney & Wilkinson, 2003; Kilcawley, O'Connell, Hickey, Sheehan, Beresford, & McSweeney, 2007; Hickey, Kilcawley, Beresford & Wilkinson, 2007; Banks & Williams, 2004) 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 a desirable flavour of reduced fat Cheddar cheese due to their metabolic activities, resulting in the absence of off flavours (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 develop differently from full fat cheese (Laloy et al., 1996). Low fat cheese has a lower content of MFGM which is a possible energy source for NSLAB. Addition of buttermilk 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 utilize MFGM for growth (Moe et al., in press).

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) to repress growth of the indigenous NSLAB flora and dominate the cheese microflora. However, if the adjunct is added at low numbers mimicking the normal number of NSLAB in cheese milk (~log 1 cfu mL^{-1}), they will presumably develop in dynamics with the indigenous NSLAB flora. To our knowledge experiments made with low inoculation level 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 species level (Coeuret, Dubernet, Bernardieau, Gueguen & Vernoux, 2003; Berthier & Ehrlich, 1998). In addition, PCR amplification of repetitive bacterial DNA elements (rep-PCR) has been proven to be a reliable technique for typing of different bacteria at strain level and has been applied for

93 studying LAB communities of different food products including cheese (De Urraza,
94 Gomez-Zavaglia, Lozano, Romanowski & Antoni, 2000; Singh, Pawas, Singh & Heller,
95 2009; Berthier, Beuvier, Dasen & Grappin, 2001; Ben Amor, Vaughan & De Vos, 2007).
96 The objective of this study was to investigate the development of the lactobacilli
97 population during ripening of low fat Cheddar cheese differing in the content of MFGM
98 components and with a low initial number of adjunct *Lb. casei* strains differing in their
99 ability to utilize MFGM components.
100
101 **2. Materials and methods**
102
103 *2.1. Experimental design*
104 Low fat Cheddar cheese was made with two experimental factors in three replicate
105 blocks (cheesemaking days). The first experimental factor, MFGM content, was addition
106 of different contents of MFGM components by adding either buttermilk powder (BMP)
107 or skim milk powder (SMP) and cream to the cheese milks. In cheese vats with SMP,
108 cream was added in addition to adjust the fat and protein content to be comparable with
109 that of the BMP cheese vats. The second experimental factor, adjunct, was two different
110 adjunct cultures, *Lb. casei* INF 448 and *Lb. casei* INF 456, both added in the amount of 1
111 x 10^2 cfu mL^{-1} and differing in their ability to utilise fat globule membrane components
112 (Moe et al., In press). The adjuncts were previously described as *Lb. paracasei* INF 448
113 and *Lb. paracasei* INF 456 (Østlie, Eliassen, Florvaag & Skeie, 2004; 2008b; Moe et al.,
114 In press) according to species specific PCR-identification. The reclassification is based
115 on the observation by one of us that the type strain *Lactobacillus casei* ATCC 393^T is

most probably a contamination and not the original Orla-Jensen strain #7 as claimed by ATCC (Vogensen et al. manuscript in preparation). The different vats were denoted: SMP (Control, cheese milk with SMP, cream and without adjunct), BMP (Control, 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 performed with SAS (SAS Institute Inc., Cary, NC USA) using proc GLM with replicate block, powder and adjuncts as classification variables. Tukey's studentized range test was used to test for differences between the adjuncts.

2.2. Milk powders

For production of the powders, 900 L whole milk was separated (45°C) and the cream was standardized to 36 % fat by addition of skimmed milk. The cream (~95 L) was pasteurized (73°C, 15 sec), directly cooled to 7°C and stored at 4°C overnight. It was churned to butter in 30 min, with a rise in temperature from 8.5°C to 13.8°C. The buttermilk was sieved through a sterilized cloth bag. The churning yielded ~50 L buttermilk (1.6 % fat) that was stored at 4°C overnight. Then it was heated to 50 °C, and separated to yield 45.8 kg buttermilk with a fat content of 0.6 %. The buttermilk (0.6 % fat) was spray dried directly after separation to yield 3.3 kg BMP. The 50 L skimmed milk was standardized to 0.5% fat before pasteurization and spray drying to yield 3.8 kg powder (SMP). The spray drying was performed with a temperature of 185-190°C on the

139 inlet air, and a constant temperature of 85 °C on the outlet air.

140

141 *2.3. Cheese milks*

142 Raw milk was separated (45 °C) and standardised to 0.5 % fat using a table top milk
143 separator (Claire, Milky; Althofen, Austria), before pasteurization (72°C, 15 sec). Three
144 20 L cheese making vats were added 20 L of milk and 263 g BMP, and the remaining
145 three vats were added 19.95 L of milk, 240 g SMP and 48 g cream (42 % fat; pasteurized
146 at 80°C). The vats added SMP were added cream to adjust for the fat content of the BMP.
147 The milks were stirred every 30 min until the powders were fully dissolved. The cheese
148 milk was then stored at 4°C for 17 h to ensure that the proteins of the powders were fully
149 hydrated.

150

151 *2.4. Cheese making procedures*

152 The cheese milk was heated to 30°C, and a single strain starter culture (*Lactococcus*
153 (*Lc.*) *lactis* ssp. *lactis* ML-8) cultured for 24 h in 200 mL reconstituted skim milk (10 %
154 w/v) was added to each of the vats. The adjunct cultures were added 15 min after the
155 addition of the starter culture. The adjuncts were grown in MRS broth overnight (ON)
156 and were diluted in Ringer's solution. To each vat, 10 mL of the solution was added to
157 achieve ~10^2 cfu mL^{-1} of adjuncts in the cheese milk. After 45 min, rennet (7 mL diluted
158 to 40 mL with pasteurized distilled water; Chy-Max™ Plus; 190 mcu/mL; Chr. Hansen,
159 Hørsholm, Denmark) and calcium chloride (18 mL, 0.1 M) were added to each of the
160 vats. The cheese milk was coagulated for 40-50 min, and after cutting, the curd was left
161 undisturbed for 5 min before gentle stirring was started. The temperature was increased to

39°C 10 min after cutting. The whey was drained at pH 6.20, and the cheese was cut into blocks. During cheddaring, the cheese blocks were inverted every 15 min, until pH 5.40 was reached. The cheese blocks were milled (~2 x 2 x 15 cm), 2.5 % salt (w/w) was added and gently mixed into the cheese curd. The curd was transferred to cylindrical cheese moulds lined with cheese cloth before pressing at 1.0 bar for 30 min, then at 2.5 bars for 18 h. The cheeses were vacuum packed, and ripened at 8°C for 24 weeks. The curd from one vat was used to make one single cheese.

2.5. Cheese analyses

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

Volatile compounds were determined in headspace vials containing 10 g of grated cheese sealed with 20-CBT-3 teflon coated septa and aluminum crimp caps and measured using 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.

184 Amino acids were analyzed using HPLC with O-phthaldialdehyde (OPA) and
185 fluorenylmethyl chloroformate (FMOC) derivatisation according to a modified method of
186 that previously described by Bütikofer & Ardö (1999). To 1.5 gram of cheese 15.0 mL
187 0.1 M HCl was added. The 0.1 M HCL contained 0.4 μmol mL^{-1} L-norvalin (Sigma, St.
188 Louis, MO USA) and 0.4 μmol mL^{-1} Piperidine-4-carboxylic acid (PICA) (Fluka, St.
189 Louis, MO USA) as internal standards. The sample was homogenised by an Ultra-Turrax
190 (Pro Scientific Inc, Monroe, CT USA) for 5 min at 20 000 rpm, sonicated for 30 min
191 (Branson, Soest, The Netherlands), centrifuged (40 min; 4 °C; (4700 rpm) ~3000 x g)
192 (Beckman J2-MC, GMI Inc., MN USA) and 1.0 mL of the supernatant was added 1.0
193 mL 4 % trichloracetic acid (Merck, Darmstadt, Germany) before mixing on a Vortex-
194 Genie 2 (Aldrich, St. Louis, MO USA) and placed on ice for 30 min before further
195 analysis. After centrifugation (5 min; 5 °C; (13 000 rpm) ~11148 x g) (Eppendorf 5415
196 D, Hamburg, Germany) the samples were filtered with a 0.2 μm MFS-13 mm CA filter
197 (Advantec, CA, USA) and analysed directly or stored in the freezer (-20 °C) until
198 analysis. The separation of the amino acids was performed using a Perkin Elmer series
199 410 pump (Perkin Elmer, Waltham, MA USA), an Agilent Technologies 1200 series
200 autosampler (Agilent Technologies, Waldbronn, Germany), a Perkin Elmer 200 column
201 oven and an Agilent Technologies 1200 series thermostat. The system was driven by an
202 EZChrom Elite (Agilent Technologies). An XTerra RP 18 column with 150 x 4.6 mm
203 (Waters, MA USA) was used and separations were carried out at 42 °C.
204
205 *2.6. Cheese electron micrographs*
206 From the center of the cheese blocks ripened for 24 weeks, small cubic samples

(approximately 3 x 3 mm) were prepared as described by Liu, Xu and Guo (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 center into two pieces using a fine scalpel. Samples were then mounted on aluminum 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 CB1 3JS, UK) was used to examine the samples at 5 kV and a magnification of 7500x.

2.7. Bacterial isolates

2.7.1. Morphological and physiological characterization of the isolates

From Rogosa agar approximately 16 colonies were randomly picked from each of the six cheeses at each time point of cheese ripening, from each replicate block, transferred to 1.0 mL De Man Rogosa and Sharpe (MRS, Merck, Darmstadt, Germany) broth (de Man, Rogosa & Sharpe, 1960) and grown at 30^{o} C for 24 h before addition of 0.3 mL glycerol (60%) and further storage at -80^{o}C. All the isolates were subsequently purified three times on MRS-agar and in total 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 ON 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

230 Narvhus, Hulbækdal, Baugerød and Abrahamsen (1991) with modifications as described
231 by Østlie, Helland and Narvhus (2003) after 20 h incubation at 30°C in MRS broth.
232 In total 100 isolates were chosen for further investigation on the basis of their
233 cultivation and morphological differences. These isolates are further in this paper
234 described by their isolation number and the cheese from which they were isolated; xx
235 (powder+adjunct-weeks of ripening) e.g. 23 (SMP+456-0). Presumptive lactobacilli were
236 characterized on the basis of their carbohydrate fermentation patterns by using the API 50
237 CHL system (BioMérieux, Marcy l'Etoile, France). Analysis of the obtained fermentation
238 profiles was done by using the APILAB Plus version 4.0 program (BioMérieux).
239 Principal component analysis of API 50 CHL results was made by using The
240 Unscrambler Client 9.5 (CAMO Process AS, Oslo, Norway).
241
242 *2.7.2. 16S rRNA sequence analysis*
243 The universal primers 5'-GAGTTTGATCCTGGCTCAG-3' and 5'-
244 AGAAAGGAGGTGATCCAGCC-3' (*Escherichia coli* positions 9-27 and 1544-1525
245 respectively) were used for amplification of an approximately 1540 bp DNA fragment of
246 the 16S rRNA gene. The primers were synthesized by Invitrogen (Invitrogen Ltd,
247 Scotland). PCR reactions were performed in a 50 µL reaction mixture containing 2 µL of
248 each 20 pmol primer, 5 µL of 10 x PCR buffer, 1 µL of 10mM dNTP, 4 µL of 25 mM
249 $MgCl_2$, 2 µL of DNA template and 0.5 µL of 5U/µL Taq Polymerase (Applied
250 Biosystems, Carlsbad, CA USA). The PCR reaction was performed in a DNA-Thermal
251 Cycler (Perkin Elmer Cetus, Waltham, MA USA) using the following program: 1 cycle
252 of denaturation at 97°C for 3 min; 34 cycles consisting of denaturation at 94°C for 15 sec,
253 primer annealing at 54°C for 15 sec, elongation at 72°C for 1 min; a final extension step

254 at 72°C for 10 min.

255 The PCR products were purified using E.Z.N.A.™ Cycle-Pure Kit (Omega
256 Biotek, Norcross, GA USA) according to the procedure recommended by the supplier.
257 Sequencing was done using a BigDye v3.1 terminator cycle sequencing kit, the primers
258 5'-CAGCMGCCGCGGTAATWC-3', 5'-TAACACATGCAAGTCGAACG-3' and 5'-
259 ACGGGCGGTGTGTRC-3' (*E. coli* positions 519-536, 50-70 and 1406-1392,
260 respectively) and the sequencing device ABI Prism 377 DNA (Applied Biosystems).
261 PCR reactions were performed using the following program: 1 cycle of denaturation at
262 96°C for 10 sec; 25 cycles consisting of denaturation at 96°C for 10 sec, primer annealing
263 at 50°C for 5 sec, polymerization and ddNTPs incorporation at 60°C for 4 min. Sequences
264 were edited using BioEdit software and analyzed using BLAST (basic local alignment
265 search tool).

266 *2.7.3. Repetitive sequence based PCR analysis of FHL isolates*

267 The Rep-PCR method was adapted from the method described by Versalovic,
268 Schneider, de Brujin and Lupski (1994) and it was used for the confirmation of NSLAB
269 strain identity. Bacterial isolates from MRS agar plates were incubated in MRS broth for
270 24 h at 30°C. In addition, the adjuncts used in this study and the strain *Lb. casei* 7R1,
271 previously described as *Lb. paracasei* 7R1 in Christiansen, Waagner Nielsen, Vogensen,
272 Brogren and Ardö (2006) were also analyzed in order to compare Rep-PCR profiles. The
273 Rep-PCR protocol was as described by Christiansen et al. (2006) using the rep PCR
274 primers REP1R-Dt: (5'-III NCG NCG NCA TCN GGC-3'), and REP2R-Dt: (5'-NCG
275 NCT TAT CNG GGC CTA C-3').

276 Banding patterns of Rep-PCR products were normalized using the *Lb. casei* 7R1

277 profile and clustered by Bionumerics 4.5 (Applied Maths, Saint-Martens-Latem,
278 Belgium) and the dendrogram was constructed on the basis of Dice's Coefficient of
279 similarity with the unweighted pair group method with arithmetic averages clustering
280 algorithm (UPGMA).
281
282 ## 3. Results
283 *3. 1. Cheese composition*
284 The development of the gross composition during ripening is shown in Table
285 1. The BMP significantly reduced the content of dry matter in the cheese compared to the
286 cheese added SMP by 0. 08 % and increased the pH by 0.05 after 24 weeks of ripening
287 ($P<0.05$). The adjuncts significantly lowered the pH in the cheese ripened for 24 weeks.
288 Significant differences ($P<0.05$) between the replicate blocks were found explaining the
289 large standard deviation found for some of the analyzed parameters. Of special
290 importance is that replicate block C had a 1 % higher moisture content than replicate
291 block A and B, resulting in higher microbial activity and higher levels of most volatile
292 compounds, free amino acids and organic acids.
293 Salt and fat were measured at 6 weeks of ripening and the cheeses had a similar
294 salt content regardless of treatment.
295
296 *3.2. Microbial development*
297 At the start of ripening, the cheeses showed up to log 9.6 cfu g^{-1} of presumptive
298 lactococci as measured on M17 agar, from 4 weeks on these numbers were reduced and
299 after 24 weeks the numbers were around log 6.9 cfu g^{-1} (results not shown). In the early

300 phases of ripening (6 weeks) the numbers of lactococci were slightly but significantly
301 ($P<0.05$) higher in cheeses with adjunct *Lb. casei* INF 456.

302 The growth of lactobacilli as enumerated on Rogosa agar (Fig. 1) showed
303 significant differences in the growth between cheeses with or without added adjuncts
304 ($P<0.001$). Generally, the number of lactobacilli increased during 0 to 10 weeks of
305 ripening in all the experimental cheeses. Presumptive lactobacilli in cheeses with added
306 adjuncts reached the maximum cell numbers, log 8 cfu g^{-1}, after 10 weeks of ripening. In
307 cheeses without adjuncts the number of lactobacilli were 3-4 log cfu g^{-1} lower from 0 to
308 10 weeks of ripening and was still increasing up to 24 weeks of ripening.

309 When comparing the control cheeses without adjuncts, significant ($P<0.001$)
310 differences were found between the replicate blocks at the start of ripening until 6 weeks
311 of age (results not shown). Cheese from replicate block A showed no growth on Rogosa
312 agar after 24 h, but showed log 1.4 and 3.7 cfu g^{-1} after 2 weeks and 6 weeks of ripening,
313 respectively, and cheese from replicate block B showed log 1.7 cfu g^{-1} already after 24 h
314 and log 3.1 and 5.7 cfu g^{-1} after 2 weeks and 6 weeks, respectively. However, in replicate
315 block C no growth were seen on Rogosa agar before 6 weeks of ripening and even at that
316 stage the numbers were low, log 1.48 cfu g^{-1}.

317

318 *3.3. Electron micrographs of matured cheese*

319 As shown in the electron micrographs in Fig. 2 the starter lactococci (black
320 arrows) were displayed in the form of clusters immersed and dispersed uniformly
321 throughout the protein matrix after 24 weeks of ripening.

322 The removed fat globules can be seen as smooth surface concave areas, spherical

323 in shape (white arrows). The lactococcal cells seem to be connected to these areas. The
324 adjunct lactobacilli were very difficult to find and when found they appeared in the shape
325 of a huge cluster of long bacilli cells embedded in the protein matrix (Fig. 2b).
326
327 *3.4. Development of flavour compounds*
328 The concentration of diacetyl (results not shown) and acetoine (Fig. 3) decreased
329 until six weeks of ripening in all cheeses, but increased from 6 to 10 weeks of ripening
330 and then again decreased until 24 weeks of ripening. The measured levels of acetoine in
331 the fresh cheeses were two times higher ($P<0.05$) in the control cheeses without adjuncts
332 compared to cheeses with added adjuncts.
333 Production of 3-methyl butanal was 1.2 times higher in cheeses added BMP
334 compared to cheeses added SMP throughout ripening (Fig. 4). The differences were
335 significant after 6 and 10 weeks of ripening ($P<0.05$). After 10 weeks of ripening the
336 contents of 3-methyl butanal was significantly higher ($P<0.05$) in cheeses with BMP and
337 adjunct *Lb. casei* INF 456 than in cheese with SMP and the adjunct *Lb. casei* INF 448.
338
339 *3.5 Amino acid composition*
340 Free amino acids (FFA) were analyzed after 10 and 24 weeks and the distribution
341 of FAA were evaluated by principal component analysis (PCA). The PCA showed that
342 Tyr and Trp were not significant for the distribution of the samples, they were therefore
343 omitted from the further statistical analysis. The PCA plot (Fig 5) showed that PC1
344 distributed the cheese samples due to age and replicate block, while PC2 distributed the
345 cheeses due to the experimental treatments. The cheese made in replicate block C

346 (elipses) had significantly ($p<0.01$) higher levels of FAA after 24 weeks of ripening and
347 this is also reflected in the PCA. However, the relation between the experimental factors
348 within each replicate block was fairly similar both after 10 and 24 weeks (A; rectangle,
349 B; circle and C; ellipse). The cheeses with adjuncts in replicate block C had a higher
350 content of FAA, while no such clear difference could be seen in replicate block A and B.
351 The content of GABA, His, Arg, Glu and Lys was responsible for the separation along
352 PC2 and some of them were also significantly influenced by the treatment factors.

353 The FAA significantly influenced by the experimental factors in cheeses ripened
354 for 10 weeks are shown in Table 2 and for 24 weeks in Table 3. Cheeses with BMP had a
355 significantly higher content of GABA and significantly lower levels of Lys than cheese
356 with SMP after 10 weeks of ripening. Cheeses added *Lb. casei* 456 had significantly
357 lower levels of Arg after 10 weeks of ripening and higher levels of Glu after 24 weeks of
358 ripening than the control cheese and cheese added *Lb. casei* 448. This was also reflected
359 in the PCA with cheeses added *Lb. casei* 456 being placed more opposite Arg along PC2
360 than cheeses added *Lb. casei* 448. Most other FAA were clustered along PC1 and were
361 not separated by the experimental treatments. However, ANOVA revealed that cheese
362 with BMP had significantly higher levels of Leu after 10 weeks of ripening and also after
363 24 weeks of ripening when replicate block C was omitted than cheeses added SMP
364 (results not shown). Cheeses with adjuncts had lower contents of Ile than the control
365 cheese after 10 weeks of ripening. Cheeses added *Lb. casei* INF 456 had higher levels of
366 Thr than the control and cheese with *Lb. casei* 448, both after 10 and 24 weeks. After 24
367 weeks of ripening the control cheeses had lower levels of Asp, Asn and Ser than the
368 cheeses with adjuncts.

369

370 *3.6. Phenotypic characterization*

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

376 The results obtained by API 50 CHL showed that 97 out of 100 isolates tested in
377 this study was ribose positive. Among the isolates from 0-week cheeses, the API 50 CHL
378 results revealed the highest phenotypic diversity (results not shown), while the isolates
379 from the ripened cheeses had a more similar fermentation pattern. By PCA (Fig. 6) the
380 isolates from 4 (25 isolates) and 24 (18 isolates) weeks old cheese were clustered on the
381 basis of their sugar fermentation pattern, while the isolates from week 0 seemed to be
382 randomly distributed (results not shown). A clear clustering of the isolates was observed
383 after 4 weeks of ripening with respect to the adjuncts as well as to the powder addition
384 (MFGM content) (Fig. 6a). Isolates from cheeses added *Lb. casei* INF 448 formed one
385 cluster, while cheeses added *Lb. casei* INF 456 formed a second group. A third cluster
386 was formed with the isolates from the control vats (only SMP or BMP). The differences
387 between isolates were diminishing through the maturation time and the 24 week old
388 cheese isolates (Fig. 6b) made two distinct clusters with no relation to the experimental
389 design.

390

391 *3.7. Species identification by 16S rDNA sequence analysis*

392

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

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

404

405 *3.8. Rep- PCR typing*

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

410 The Rep-PCR analysis was in accordance with the sequencing data revealing that
411 most of the isolates formed a joined cluster with at least 45% similarity, with the
412 exception of three isolates (66 (BMP-10), 13 (BMP+448-0) and 77 (SMP+456-10).

413 It can be observed that some isolates from the same maturation time clustered
414 together in separate sub-clusters. Formed sub-clusters showed at least 80% similarity.
415 This indicates the change in microbial composition of the studied cheeses over the

416 maturation period. However, a grouping at the basis of adjunct addition was observed,
417 especially in the later stages of cheese maturation (10 and 24 week cheeses).

418 Isolates 23 (SMP+456-0) and 24 (SMP+456-0) grouped together at 100%
419 similarity and isolate 13 (BMP+448-0) showed less similarity with the other isolates.
420 They were characterized as *Lb. brevis* by 16S rRNA analysis. This sub-cluster included
421 also isolate 17 (SMP+456-0) identified by 16S rRNA sequencing as *Lb. rhamnosus*.
422 Isolate 66 (BMP-10), showed only 10 % similarity with the other isolates studied by
423 Rep-PCR which is also in accordance with the sequencing data indicating that this isolate
424 belongs to the species *Lb. curvatus*.

425 Isolates from the 0 week old cheese grouped together with at least 50 % similarity
426 showing no significant grouping on the basis of the experimental factors. However,
427 grouping according to experimental factors was influenced by the maturation stage of the
428 cheese. In 4 week old cheeses, clustering in three sub-clusters with at least 45 % of
429 similarity was observed based on different experimental treatments. The first sub-cluster
430 (box 4-1) comprised isolates from the 4 week old cheese added adjunct *Lb. casei* 456 (but
431 including one isolate from BMP+448-4). The second sub-cluster (box 4-2) included
432 isolates from the control cheeses (BMP-4 and SMP-4) and cheese SMP+448-4, showing
433 that isolates from the SMP and BMP vats without added adjuncts clustered together. The
434 third sub-cluster (box 4-3) was represented by isolates from 4 week old cheeses added *Lb.*
435 *casei* INF 448

436 Most of the isolates from the 10 week old cheeses made two different sub-clusters
437 with at least 85% similarity, showing grouping also within separate sub-clusters mainly
438 according to experimental factors. The first group (isolates 68, 69, 70, 71, 75 and 72)

represents the isolates from vats supplemented with *Lb. casei* INF 448. The second group (isolates 58, 60, 61, 62, 63, 64, 65, 67 and 74) was mainly isolates from vats without adjuncts (SMP and BMP vats), with the exception of isolate 67 (SMP+448-10) and 74 (BMP+448-10). The rest of the 10 week old cheese isolates were from vats added *Lb. casei* INF 456 (76, 78, 79, 80, 81), with the isolates 80 and 81 (BMP+456-10) representing the same strain.

The most uniform grouping was observed among the isolates from the 24 week old cheeses, represented by 2 sub-clusters which grouped with at least 85 % similarity. Isolates 89, 90, 91 (SMP+448-24), 96 (SMP+456-24), 85 (SMP-24) and 88 (BMP-24) represent only two different strains and belonged to the same sub-cluster.

4. Discussion

This work was undertaken to follow the evolution of the microflora of low fat Cheddar cheeses over a 24 week maturation period. Normally adjuncts are added at high numbers, around log 5 cfu mL^{-1}, to the cheese milk, and they will then dominate the cheese microflora (Fox et al., 1996). In this present work they were added at log 2 cfu mL^{-1} in the cheese milk, but the number of presumptive lactobacilli was log 4.5 cfu g^{-1} already after 24 h, indicating growth of adjuncts and/or NSLAB. The Rep PCR showed that the adjuncts did not dominate the microflora completely in the early stages of ripening. The lactobacilli (NSLAB) growth in the control cheeses without adjuncts was in accordance with previous findings (Beresford et al., 2001) for replicate blocks A and B, while replicate block C showed lower numbers of lactobacilli at the start of the ripening period.

462 A clear grouping due to adjunct addition was shown in the mature cheese by Rep-
463 PCR. The present microorganisms at start influenced the development and composition
464 of the lactobacilli during ripening. The uniform composition observed after 24 weeks of
465 ripening in the cheeses added adjuncts indicate that some strains were able to grow to
466 high numbers and dominate the NSLAB flora. Available nutrients are important for
467 growth and Moe et al. (in press) have shown that *Lb. casei* INF 456 is able to utilize
468 monosaccharides found in the MFGM.
469 The enumerated numbers of presumptive lactobacilli was different in cheeses with
470 and without adjuncts throughout the whole period of maturation. There were no
471 significant differences between BMP and SMP cheeses in the lactobacilli counts
472 indicating that the MFGM content had minor influence on the general lactobacilli growth
473 in cheese. The revealed differences in lactobacilli counts between the replicate blocks of
474 the control cheeses may also explain the broad variety of species and strains found at the
475 start of ripening.
476 Electron micrographs confirmed the even distribution of lactococci (starter) in the
477 cheese matrix. The lactococci were inoculated at high numbers and were uniformly
478 distributed in the cheese milk. They reached their maximum cell number during
479 cheesemaking (cheddaring), and this explains their uniform distribution in the cheese
480 matrix. In addition, some clustering around the original cell was seen, indicating further
481 growth in the cheese. The lactobacilli were inoculated at low numbers in the cheese milk,
482 and therefore they were distributed more rarely in the cheese matrix. The lactobacilli are
483 not motile, but their numbers increased to log 7-8 cfu g^{-1} during ripening and a large
484 cluster of lactobacilli was observed by electron microscopy with clearly elongated cells.

In Cheddar type cheese which is normally made with no addition of aromatic starter bacteria, the production of the flavour compounds diacetyl and acetoin was not expected. The amounts of these components were highest in the control cheeses. Diacetyl and acetoin may be synthesized through transamination of Asp and Asn leading to formation of oxaloacetate which can be metabolized to acetoin and diacetyl by some lactobacilli (Kieronczyk, Skeie, Langsrud, Le Bars & Yvon, 2004; Skeie et al., 2008b). Since the control cheeses had reduced contents of Asp and Asn, it is most probably the indigenous NSLAB flora of these cheeses that caused this degradation, while these strains have been repressed in cheeses added adjuncts. In addition, it is known that the adjunct *Lb. casei* INF 448 is not able to degrade Asp in cheese (Skeie et al., 2008b). Furthermore, both adjuncts used in this study are not able to metabolize citrate in milk to diacetyl and acetoin (unpublished results).

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

It has been indicated in previous studies of semi-hard cheeses, that mesophilic lactobacilli predominate in the later stages of cheese ripening (Berthier & Ehrlich, 1998;

508 Østlie et al., 2004; Crow, Curry & Hayes, 2001). The Rep-PCR analysis was applied in
509 order to get a more complete picture of the strain diversity during the period of cheese
510 maturation. Certain strains of *Lb. casei/paracasei* appeared to be common in all cheeses
511 and they most probably originate from the cheese milk or dairy environment, also shown
512 by others (Fitzsimons, Cogan, Condon & Beresford, 1999; Antonsson, Ardö & Molin,
513 2001; Antonsson, Molin & Ardö, 2003). The most uniform microflora was observed at
514 the end of ripening.

515 As expected *Lb. casei/paracasei* represented the most abundant *Lactobacillus*
516 species in all the examined cheeses regardless of experimental factors used such as
517 powder (MFGM content) or adjunct addition. The obtained results were in accordance
518 with previous findings (Beresford et al., 2001; Banks & Williams, 2004) stating that the
519 NSLAB population of Cheddar cheeses is dominated by *Lb. casei/paracasei (*approx.
520 95% of the isolates belong to *Lb. casei/paracasei*). The results of the principal
521 component analysis of the API results of the selected isolates were in accordance with the
522 chemical and microbial characterization of the cheeses with the highest diversity of the
523 lactobacilli in the fresh cheese, a clear grouping according to the adjuncts in the early
524 phase of ripening (4-10 weeks) while after 24 weeks the differences between the
525 experimental factors were more vague.

526 In model systems, Beresford et al. (2001) demonstrated that ribose can be used as
527 a carbon source for mesophilic lactobacilli in the later stages of ripening. The results here
528 showed that most of our isolates (97%) were ribose positive. Lysed lactococcal cells may
529 release ribose from RNA and N-acetylgluconsamin from degraded cell walls (Østlie et
530 al., 1995; Adamberg et al., 2005). Another possible source of carbon for mesophilic

lactobacilli may be connected to the fact that they posses some glycoside-hydrolase activity and can utilize sugars from glycoproteins of the milk fat globule membrane as an energy source (Williams & Banks, 1997*;* Fox et al., 1998). The electron micrographs showed an even distribution of the lactococcal strains through the cheese matrix, all connected to fat globules and the lactobacilli were clearly surrounding a removed milk fat globule.

5. Conclusion

The microbial diversity in the studied low fat cheeses was decreasing during the maturation period, becoming more uniform by the end of the maturation process and *Lb. casei* being the most dominating lactobacilli species. The present microorganisms at start did influence the development and composition of the lactobacilli during ripening even if the adjuncts were added at low numbers and did not dominate the flora at start. However, when *Lb. casei* adjuncts were added in low numbers to the low fat cheeses, even if they have the ability to utilize the MFGM components (Moe et al., in press), they still did not dominate the cheese microflora in the later stages of ripening.

The concentration of some flavour compounds was also influenced by the experimental factors used in this study. The amount of these compounds and the branched chain amino acid Leu was higher in cheeses with BMP than in cheeses with SMP. This indicates that the presence of MFGM components was significantly influencing the flavour development in the studied cheeses.

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753

Table 1. pH development and gross composition (dry matter (DM); fat in dry matter (FDM); salt; salt in moisture (SM) kg^{-1} cheese) during 24 weeks of ripening and significant effects of the experimental factors ($P<0.05$).

	pH			DM (%)			FDM^{-1}	Salt (%)	SM^{-1} (%)
	24 h	6 weeks	24 weeks	0 weeks	6 weeks	24 weeks	6 weeks	6 weeks	6 weeks
SMP	5.16 ±0.02	5.24±0.16	5.39 ±0.11	53.52±0.82	53.00±1.51	52.58 ±1.04	13.82 ±0.69	1.66 ±0.33	3.53
BMP	5.13±0.03	5.19±0.16	5.31 ±0.14	52.56±0.65	52.09±0.53	51.54 ±0.61	13.44 ±0.22	1.78 ±0.22	3.69
SMP+448*	5.19±0.03	5.24±0.17	5.32 ±0.11	53.47±0.33	52.51±0.92	52.35 ±0.56	13.96 ±1.03	1.76 ±0.03	3.71
BMP+448	5.14±0.01	5.16±0.15	5.26 ±0.14	52.35±1.29	51.25±0.91	50.99 ±0.97	13.66 ±0.24	1.59 ±0.26	3.26
SMP+456**	5.15±0.03	5.24±0.12	5.30 ±0.12	53.37±0.99	52.68±0.84	52.29 ±0.76	13.91 ±0.9	1.51 ±0.08	3.19
BMP+456	5.18±0.01	5.23±0.13	5.28 ±0.13	52.84±0.21	51. 94±0.60	51.52 ±1.1	13.48±0.15	1.60 ±0.37	3.33
Significance, P<0.05 (ns= not significant)									
Powder	ns	0.05	0.001	0.01	0.01	0.001	ns	ns	ns
Adjunct	ns	ns	0.001	ns	ns	ns	ns	ns	ns
Diff. betw. adj			0>448,456						
Rep block	ns	0.001	0.001	0.05	0.001	0.001	ns	0.01	0.01

* 448= Adjunct addition of *Lb. casei* INF 448

**456= Adjunct addition of *Lb. casei* INF 456

Table 2. The content of free amino acids (µmol g^{-1}) significantly (P<0.05) influenced by experimental factors in the cheeses after 10 weeks of ripening.

	Thr	Arg	GABA	Met	Ile	Leu	Lys	FAA
SMP	0.76±0.05	0.74±0.06	0.4±0.05	0.64±0.06	0.4±0.06	4.2±0.1	2.5±0.4	31.9±1.4
BMP	0.75±0.01	0.78±0.04	0.5±0.1	0.63±0.03	0.4±0.04	4.4±0.2	2.2±0.2	31.7±1.1
SMP+448*	0.71±0.1	0.7±0.04	0.4±0.1	0.62±0.05	0.3±0.02	4.2±0.3	2.2±0.1	31.4±1.2
BMP+448	0.68±0.03	0.68±0.03	0.6±0.1	0.56±0.02	0.3±0.02	4.3±0.2	2.1±0.1	30.5±0.4
SMP+456**	0.76±0.1	0.6±0.1	0.5±0.1	0.62±0.04	0.3±0.05	4.2±0.3	2.3±0.02	31.2±1.8
BMP+456	0.81±0.1	0.62±0.1	0.5±0.1	0.61±0.03	0.3±0.03	4.4±0.4	2.2±0.2	32.1±1.9
Significance, P<0.05 (ns= not significant)								
Powder	ns	ns	0.01	0.05	ns	0.04	0.03	ns
Adjunct	0.009	0.0017	ns	0.02	0.02	ns	ns	ns
Diff. betw. adj	456>448	0>456		0>448				
Rep.block	0.01	ns	ns	0.0008	0.0003	0.0002	0.03	0.0013

* 448= Adjunct addition of *Lb. casei* INF 448

**456= Adjunct addition of *Lb. casei* INF 456

763 **Table 3.** The content of free amino acids (µmol g^{-1}cheese) significantly (P<0.05) influenced by experimental factors in the cheeses

764 after 24 weeks of ripening.

Exp. factor	Asp	Glu	Asn	Ser	Thr	FAA
SMP	1.2±0.1	8.4±1.0	3.3±0.5	1.4±0.3	1.3±0.2	46.4±5.8
BMP	1.3±0.1	8.7±0.4	3.4±0.3	1.4±0.2	1.3±0.1	47.4±2.9
SMP+448*	1.8±0.6	8.8±1.0	3.5±0.4	1.5±0.1	1.2±0.1	48.0±4.5
BMP+448	1.8±0.6	9.8±2.0	3.9±0.8	1.7±0.3	1.4±0.3	54.4±11.4
SMP+456**	1.6±0.6	10.0±1.8	4.1±0.7	1.7±0.2	1.5±0.2	54.2±9.7
BMP+456	1.7±0.6	10.5±2.3	4.3±0.8	1.8±0.2	1.6±0.2	56.3±11.7
Significance, P<0.05 (ns= not significant)						
Powder	ns	ns	ns	ns	ns	ns
Adjunct	0.05	0.05	0.01	0.05	0.02	ns
Diff. betw. adj.	0<448,456	ns	0<456	ns	456>0,448	
Rep.block	0.0003	0.01	0.007	ns	ns	0.01

765 * 448= Adjunct addition of *Lb. casei* INF 448

766 **456= Adjunct addition of *Lb. casei* INF 456

767 **Legends to Figures**

768 **Fig. 1.** Development of lactobacilli as enumerated on Rogosa agar (log cfu g^{-1}) during
769 cheese ripening. Each data point represents mean ± standard deviation.

770

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

775

776 **Fig. 3**. Development of acetoin (area g^{-1} cheese) during cheese ripening. Each data point
777 represents mean ± standard deviation.

778

779 **Fig. 4.** Development of 3-methyl butanal (area g^{-1} cheese) during cheese ripening. Each
780 data point represents mean ± standard deviation.

781

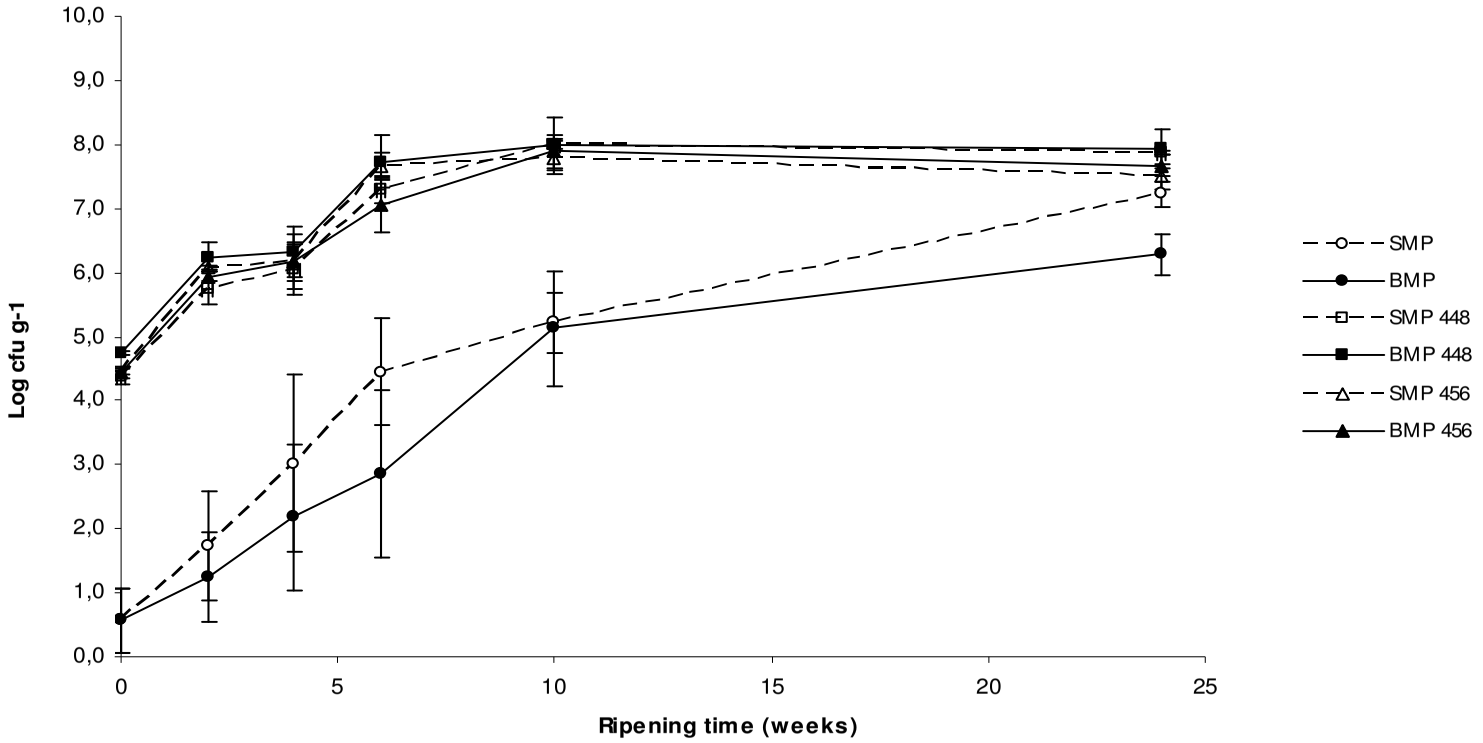
782 **Fig. 5.** PCA scores (a) and loadings (b) of the amino acid distribution in the studied
783 cheeses. Samples marking: Adjunct (448 and 456), powder addition (SMP and BMP),
784 replicate block letter (a, b, c) and age (10 and 24 weeks). Rectangles; replicate block A,
785 circle; replicate block B and ellipse; replicate block C.

786

787 **Fig. 6.** PCA biplot of the API 50 CHL sugar fermentation of the bacteria isolates.
788 Samples marking: Adjunct (448 and 456), powder addition (SMP and BMP) and (isolate
789 number). a) Distribution of the cheese isolates after 4 weeks of ripening (40 % and 34 %

790 of the variation explained by PC1 and PC2, respectively). Control cheese (solid ellipse),
791 cheese with *Lb. casei* INF 448 (dotted circle) and cheese with *Lb. casei* INF 456 (broken
792 ellipse). b) Distribution of the cheese isolates after 24 weeks ripening (37 % and 28 % of
793 the variation explained by PC1 and PC2).
794
795 **Fig. 7.** Dendrogram based on the Rep-PCR DNA fingerprinting of bacterial isolates from
796 the low fat Cheddar cheeses.
797 Boxes: 0: 0 week cheese isolates, 4: 4 week cheese isolates (groups 4-1, 4-2 and 4-3), 10:
798 10 week cheese isolates (groups 10-1 and 10-2), 24: 24 week cheese isolates, M1: *Lb.*
799 *casei* INF 448, M2: *Lb. casei* INF 456, 7R1: *Lb. casei.*
800

801 **Fig. 1.**



802

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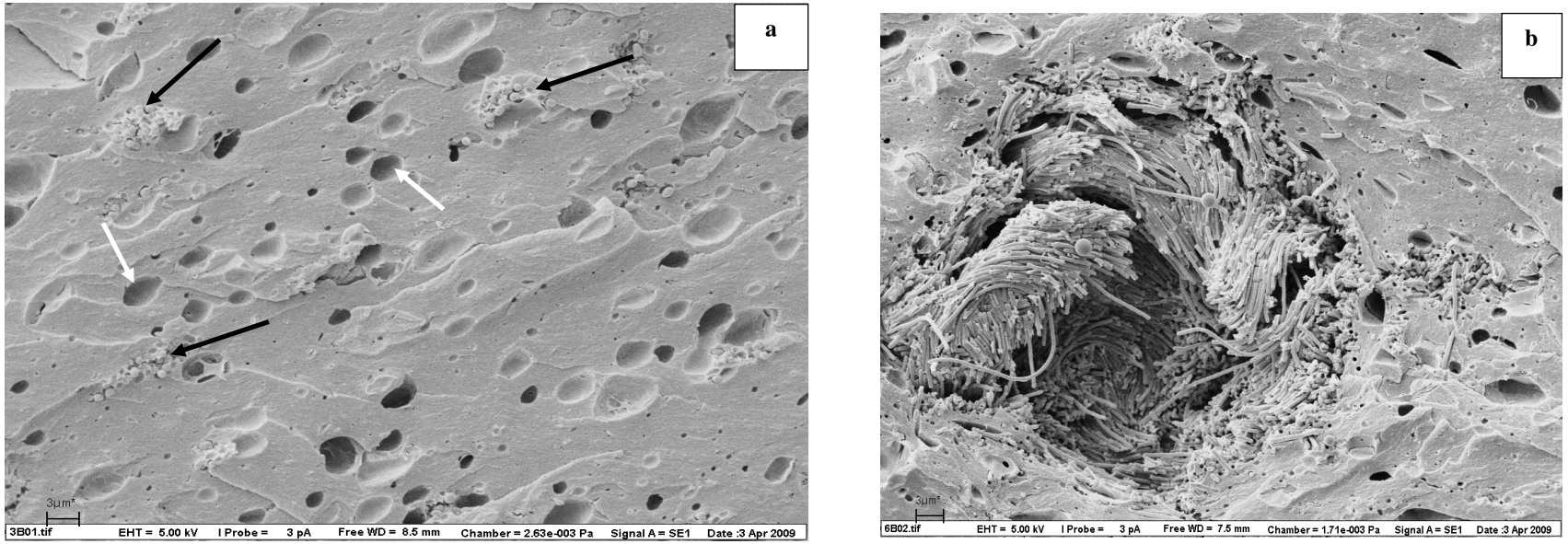
805

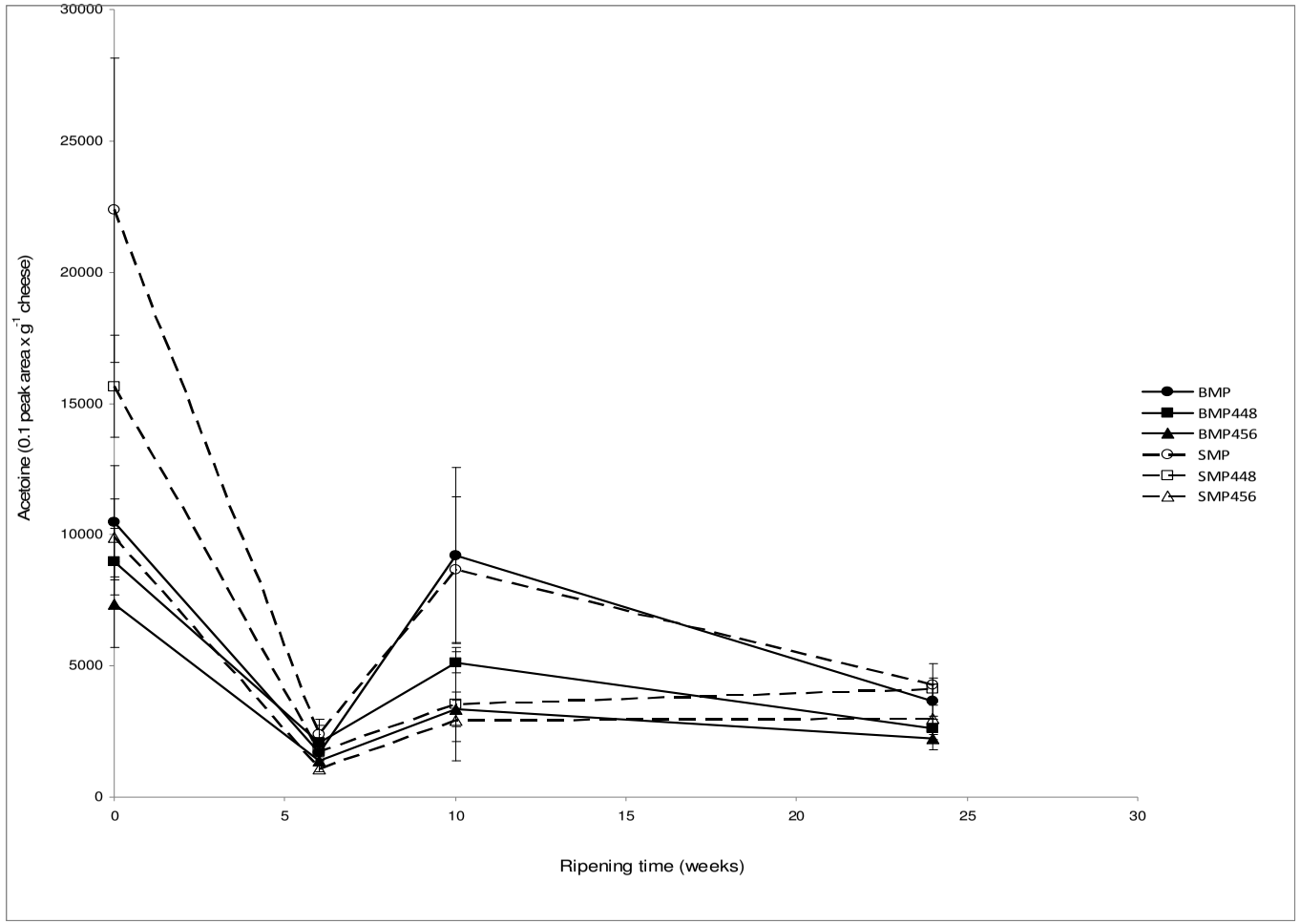
806 **Fig. 2.**

807

808



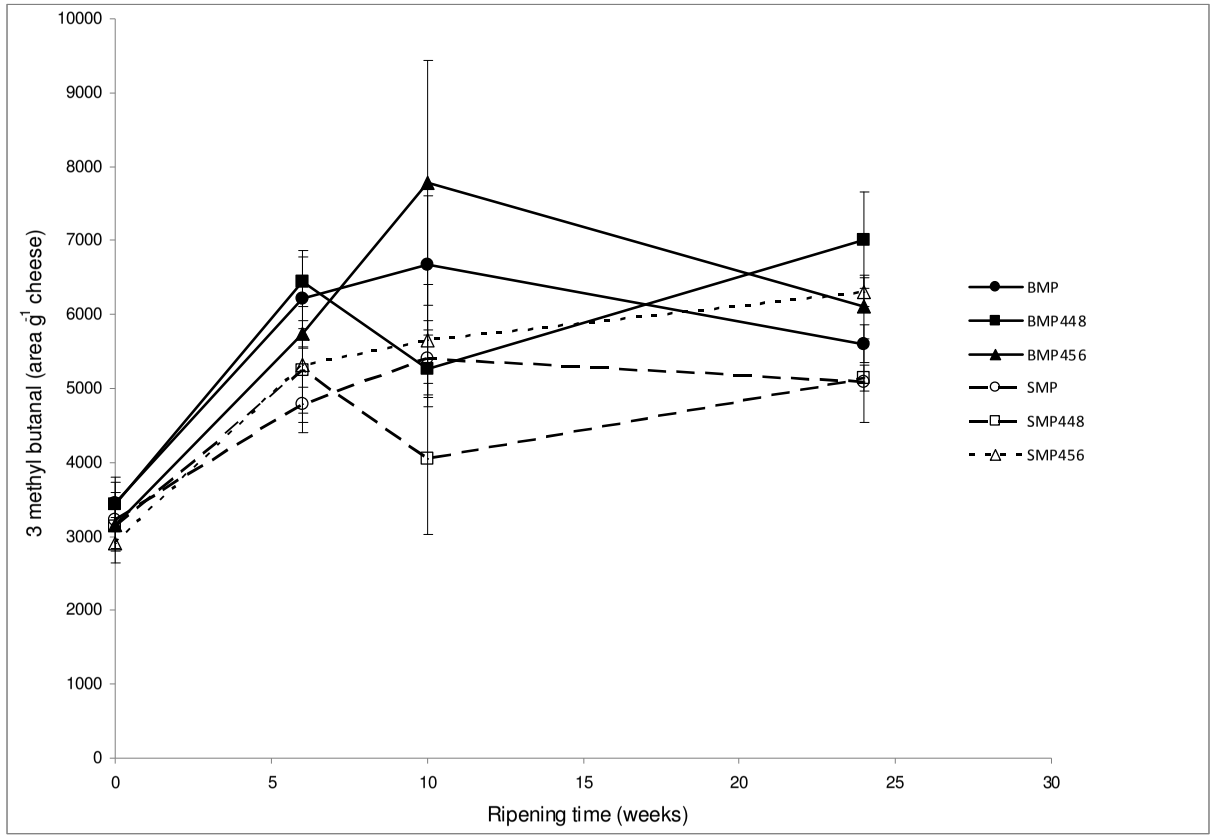
809 **Fig. 3.**



810

811

812 **Fig. 4.**

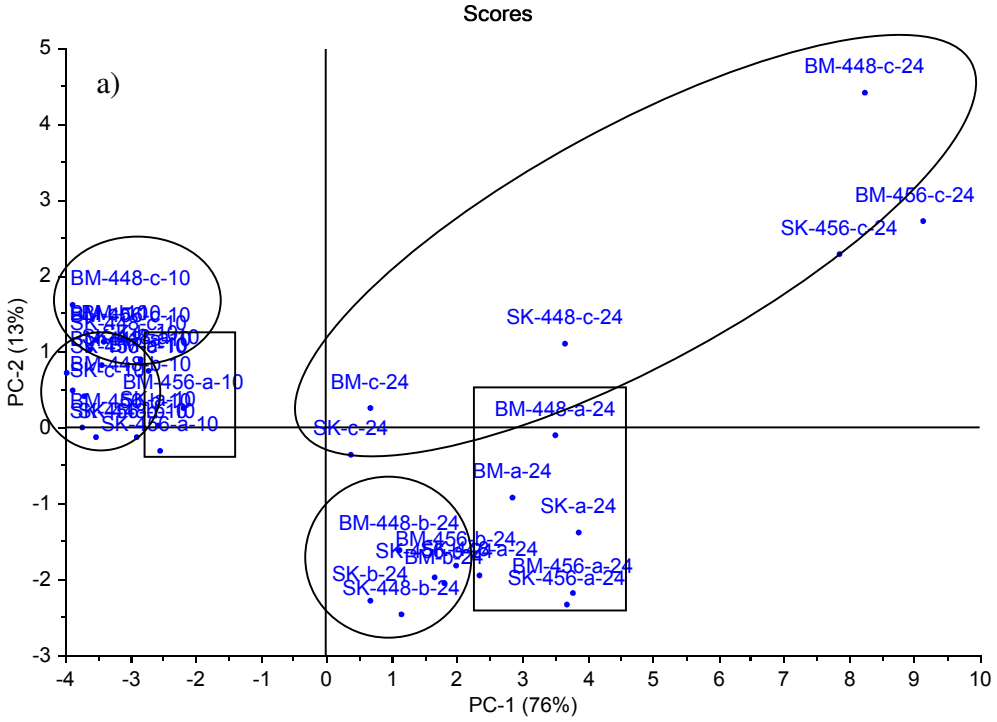

10000
9000
8000
7000
6000
5000
4000
3000
2000
1000
0
3 methyl butanal (area g-1 cheese)
0
5
10
15
20
25
30
Ripening time (weeks)
BMP
BMP448
BMP456
SMP
SMP448
SMP456

813

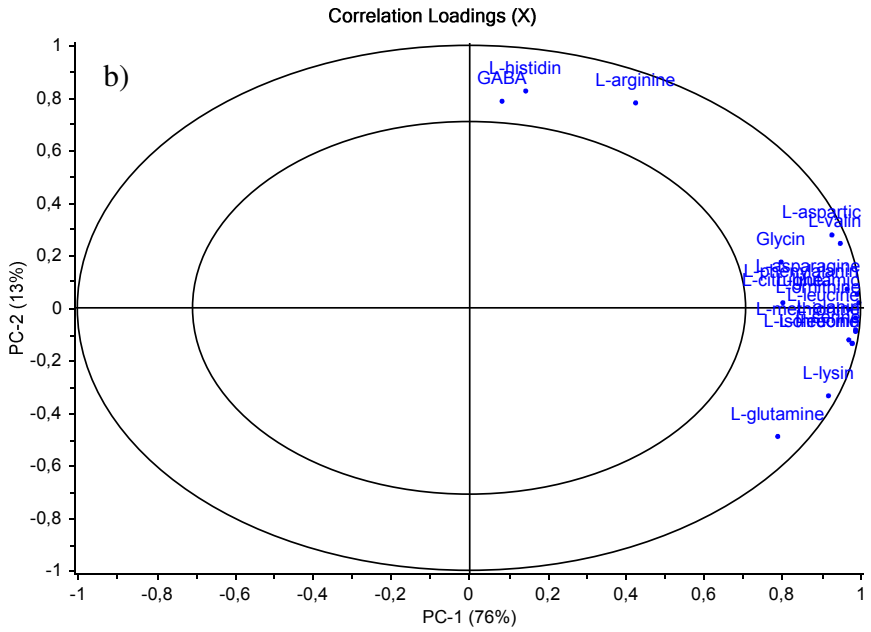
814

815 **Fig. 5.**

816



817



818

Fig. 6.

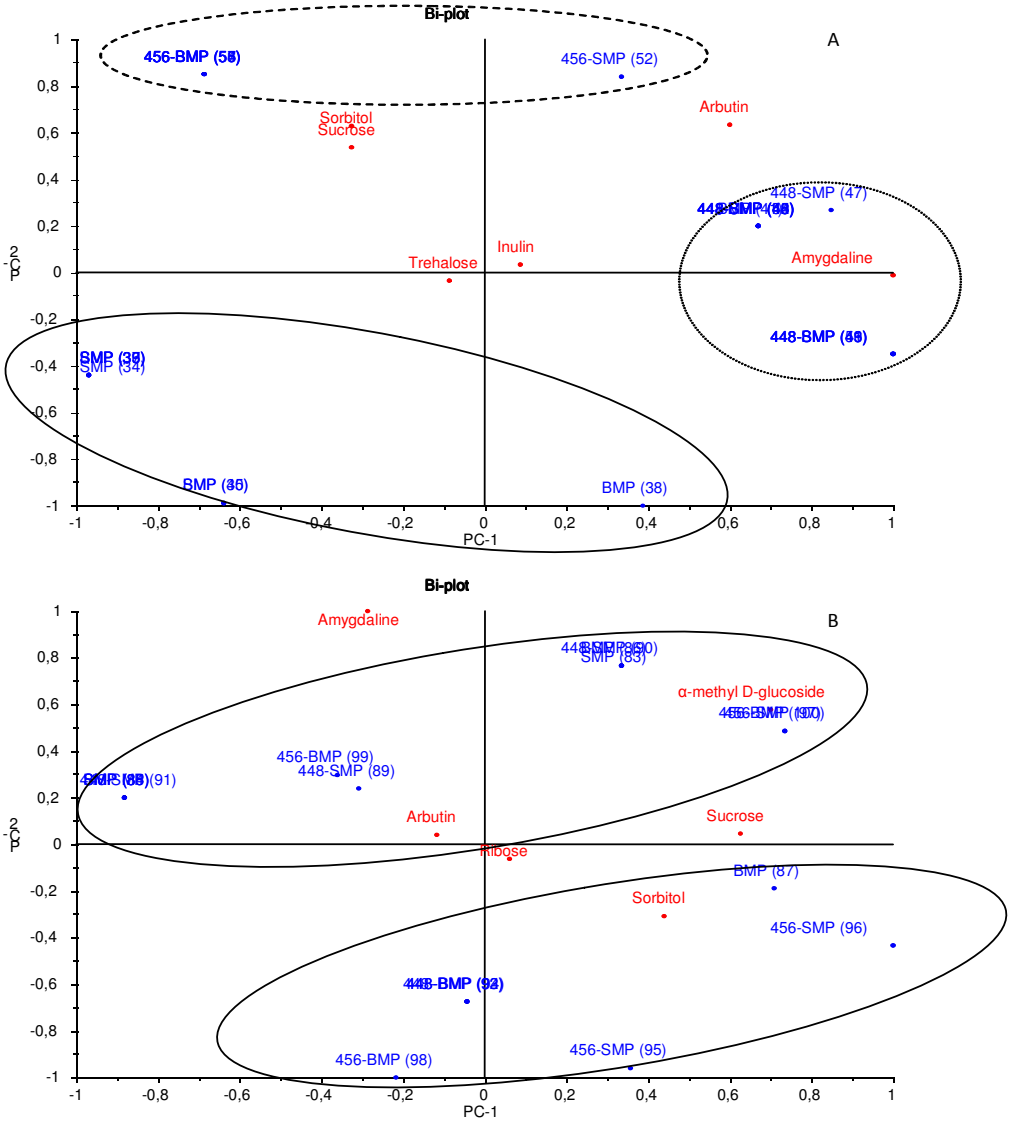
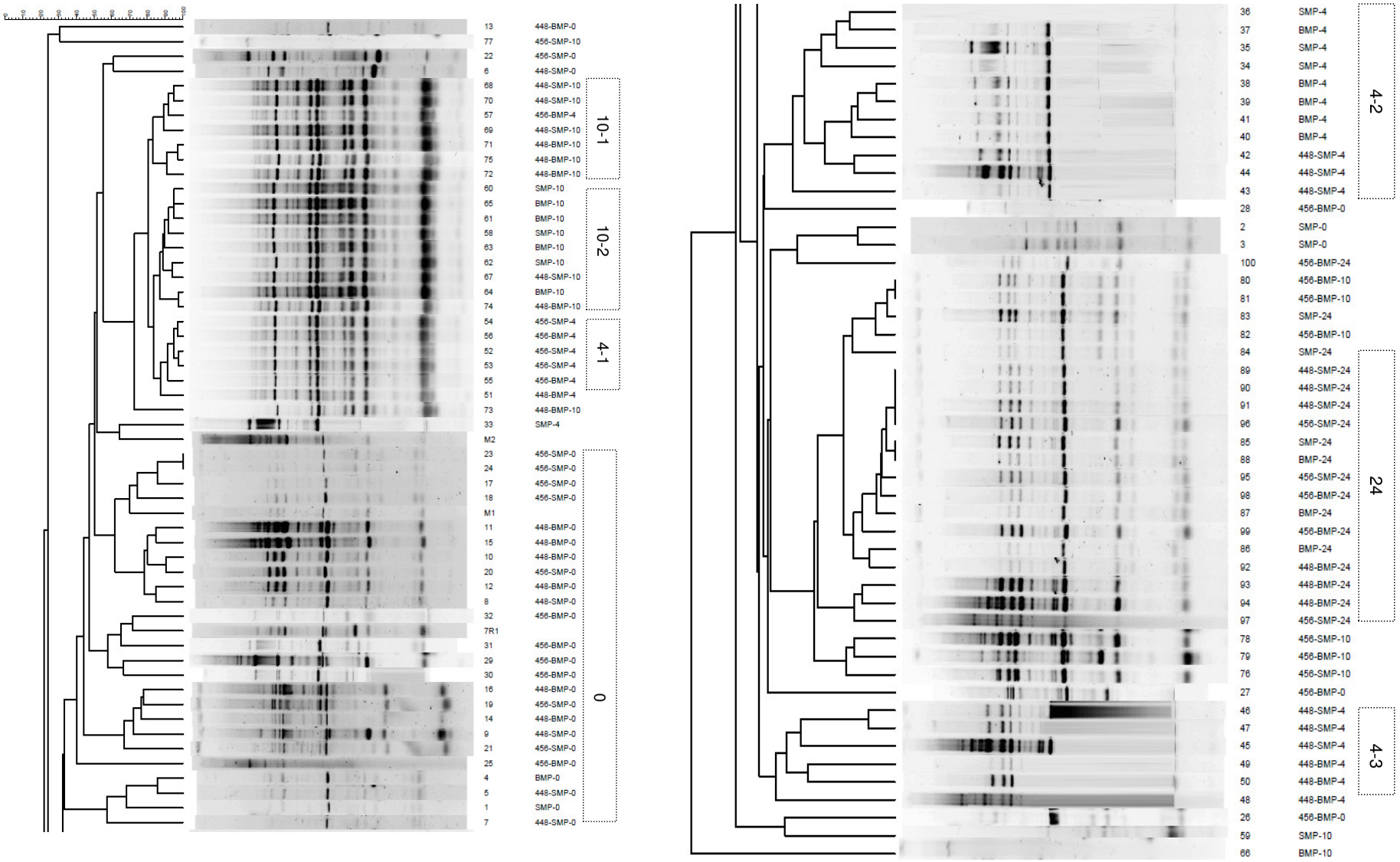

Bi-plot
A
456-BMP (56)
456-SMP (52)
Arbutin
Sorbitol
Sucrose
448-SMP (47)
Inulin
Trehalose
Amygdaline
448-BMP (48)
SMP (34)
BMP (38)
PC-1
Bi-plot
B
Amygdaline
SMP (83)
α-methyl D-glucoside
456-BMP (99)
448-SMP (89)
Arbutin
Sucrose
Ribose
BMP (87)
Sorbitol
456-SMP (96)
456-BMP (98)
456-SMP (95)
PC-1

Fig. 7.


13 448-BMP-0
77 456-SMP-10
22 456-SMP-0
6 448-SMP-0
68 448-SMP-10
70 448-SMP-10
57 456-BMP-4
69 448-SMP-10
71 448-BMP-10
75 448-BMP-10
72 448-BMP-10
10-1
60 SMP-10
65 BMP-10
61 BMP-10
58 SMP-10
63 BMP-10
62 SMP-10
67 448-SMP-10
64 BMP-10
74 448-BMP-10
10-2
54 456-SMP-4
56 456-BMP-4
52 456-SMP-4
53 456-SMP-4
55 456-BMP-4
51 448-BMP-4
73 448-BMP-10
4-1
33 SMP-4
M2
23 456-SMP-0
24 456-SMP-0
17 456-SMP-0
18 456-SMP-0
M1
11 448-BMP-0
15 448-BMP-0
10 448-BMP-0
20 456-SMP-0
12 448-BMP-0
8 448-SMP-0
32 456-BMP-0
7R1
31 456-BMP-0
29 456-BMP-0
30 456-BMP-0
16 448-BMP-0
19 456-SMP-0
14 448-BMP-0
9 448-SMP-0
21 456-SMP-0
25 456-BMP-0
4 BMP-0
5 448-SMP-0
1 SMP-0
7 448-SMP-0
0
36 SMP-4
37 BMP-4
35 SMP-4
34 SMP-4
38 BMP-4
39 BMP-4
41 BMP-4
40 BMP-4
42 448-SMP-4
44 448-SMP-4
43 448-SMP-4
28 456-BMP-0
4-2
2 SMP-0
3 SMP-0
100 456-BMP-24
80 456-BMP-10
81 456-BMP-10
83 SMP-24
82 456-BMP-10
84 SMP-24
89 448-SMP-24
90 448-SMP-24
91 448-SMP-24
96 456-SMP-24
85 SMP-24
88 BMP-24
95 456-SMP-24
98 456-BMP-24
87 BMP-24
99 456-BMP-24
86 BMP-24
92 448-BMP-24
93 448-BMP-24
94 448-BMP-24
97 456-SMP-24
24
78 456-SMP-10
79 456-BMP-10
76 456-SMP-10
27 456-BMP-0
46 448-SMP-4
47 448-SMP-4
45 448-SMP-4
49 448-BMP-4
50 448-BMP-4
48 448-BMP-4
26 456-BMP-0
59 SMP-10
66 BMP-10
4-3

Paper V

The influence of fat globule membrane material on the microstructure of low-fat Cheddar cheese

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Abstract

The microstructural characteristics of low-fat Cheddar cheese differing in the content of milk fat globule membrane (MFGM) material achieved by addition of either buttermilk powder (BMP) or skim-milk powder (SMP) to the cheese milk were investigated. Scanning electron microscopy (SEM) and confocal scanning laser microscopy (CSLM) were used to study the cheese structure and the distribution of the starter culture and fat globules.

Variations in the microstructure were observed relating to the MFGM content. The structure of the control cheese (SMP) was more irregular with inhomogeneous large voids. Whereas, cheese with BMP had a homogeneous protein network with small voids, showing a smoother, more compact and less coarse structure accompanied by more pronounced fat globules that were uniformly scattered throughout the protein matrix. The starter bacteria were located within the protein networks in clusters which were distributed homogeneously throughout the cheese matrix regardless of treatment.

1. Introduction

Consumers often regard cheese with reduced fat content to be of inferior quality (Banks, 2004). Nevertheless, novel technology in cheese manufacture and considerable advances in understanding the biochemical and physicochemical characteristics of low-fat cheese variants have led to potential improvements in terms of flavour, texture and functionality, with major achievements in the area of fresh and soft cheeses. However, there is still work to be done in the area of semi-hard and hard ripened cheeses. Besides, seeking new dairy products that possess health effects beyond the nutritional components has become a way of life for consumers during the last decade.

Buttermilk, a byproduct of butter making released during churning of cream, is very rich in milk fat globule membrane (MFGM) (Morin, Pouliot, & Britten, 2008). The MFGM is mainly composed of phospholipids, sphingolipids, glycoproteins and other minor compounds and Morin et al. (2008) found that the phospholipid content was 8.5 times higher in sweet pasteurized buttermilk than in skimmed milk (0.119 % and 0.014 %, respectively). The MFGM fragments have previously been suggested to carry many beneficial health effects (Dewettinck et al., 2008; Spitsberg, 2005). It has been reported that MFGM fractions may inhibit colon cancer, suppress gastrointestinal pathogens and may be involved in stress responses (McDaniel, Maier, & Einstein, 2003; Parodi, 2001).

Buttermilk has been used as a functional ingredient in many food products, such as salad dressings, chocolate, cheese and yoghurt (Govindasamy-Lucey, Lin, Jaeggi, Johnson, & Lucey, 2006; Mistry, Metzger, & Maubois, 1996; Morin et al., 2008; Trachoo & Mistry, 1998). Many studies have used ultrafiltered or concentrated buttermilk in cheese manufacture (Govindasamy-Lucey et al., 2006; Poduval & Mistry, 1999; Raval & Mistry, 1999). Commercial buttermilk is often subjected to process conditions that are severe and variable (pasteurization temperatures of cream, fermentation, holding time through the evaporation and spray-drying process), which are likely to have major impact on buttermilk functionality (Morin et al., 2008). Accordingly, most studies have not been able to fully explain the effects and changes in physical and structure characteristics resulting from adding

64 commercial buttermilk to cheese milk. Accordingly, in this present experiment, we produced the
65 buttermilk powder having control of the full processing history from the raw milk.

66 Cheese is stated to have a microstructure consisting mostly of the casein matrix in which the fat
67 globules are entrapped; water or serum is both bound to casein and fills interstices of the matrix that
68 forms a network (Hort & Grys, 2001). Thus knowledge and understanding of the way in which milk
69 components and functional ingredients affect cheese microstructure make it possible to direct the
70 industrial processes towards the production of high-quality low-fat cheeses. Electron microscopy is one
71 of the disciplines which may contribute to this objective.

72 Scanning electron microscopy (SEM) has been used extensively as a high resolution analysis to
73 elucidate the state of the casein micelles, type of aggregates and the formation of network during
74 cheese making and of the final cheese products, (Dabour, Kheadr, Benhamou, Fliss, & LaPointe, 2006;
75 Guinee, Auty, & Fenelon, 2000; Kalab, 1985; Kalab, Allan-Wojtas, & Miller, 1995; Kaláb, Yang, &
76 Chabot, 2008; Lopez, Camier, & Gassi, 2007; Poduval & Mistry, 1999; Morin et al., 2008). Findings
77 obtained by SEM have made useful contributions to a better understanding of the complex structure-
78 function relationships of cheese components. However, it is worthwhile to note that SEM operates
79 under high vacuum where the cheese sample is exposed to a high electron beam and, owing to the high
80 moisture and fat content of the cheese, an extensive sample preparation is required prior to analysis
81 with such steps as sectioning, chemical fixation and dehydration. Additionally, it has been reported that
82 the well-defined structures, e.g. fat globules, can be reliably seen by high resolution topographical SEM
83 images, and thereby ensure their identification. However, less well-defined particles, e.g. starch or
84 other food additives, were better observed using specific staining microscopy and/or advanced
85 examination techniques, e.g. confocal scanning laser microscopy “CSLM” (Montesinos-Herrero,
86 Cottell, O’Riordan, & O’Sullivan, 2006).

87 Since the early 1990s, CSLM has complemented SEM in cheese microstructure studies
88 (Everett, 2007). CSLM is a technique that has great potential as a tool to improve our understanding of

89 milk and cheese microstructure, and offers a number of advantages over conventional techniques. One
90 of the advantages of this technique is that it can both visualize and chemically differentiate cheese
91 components through the use of specific stains. The basis of staining specimens relies on a number of
92 different mechanisms, e.g. acidic dyes will bind to basic groups and vice versa. In other cases,
93 differential solubility will cause dyes to accumulate according to polarity (Hassan, Frank, Farmer,
94 Schmidt, & Shalabi, 1995; Ong, Dagastine, Kentish, & Gras, 2011). Also, structural information can be
95 obtained in a nondestructive manner and with minimal sample preparation through this technique. In
96 particular, CSLM has proven to be very useful for examination of highly-hydrated and high-fat foods
97 which are difficult in sample preparation using the conventional microscopic techniques without the
98 loss or migration of their components.

99 Defining the structural properties and their relative magnitude with respect to other similar
100 products will increasingly become a critical criterion for cheese manufacturers seeking to design new
101 products, to maintain the quality of current ones or understand the strengths and weaknesses of the new
102 relative to their competitors.

103 The objective of this study was to investigate the microstructural characteristics of low-fat
104 Cheddar cheese differing in the content of MFGM material. SEM was used to characterize cheese
105 structure as well as the features of the pure starter culture in the cheese. A further aim was to use
106 CSLM to see and to differentiate the distribution of fat globules and bacterial colonies within the
107 protein matrices, providing complementary insights into the evaluation of cheese microstructure.

108

109 ***2. Material and methods***

110 ***2.1 Experimental design***

111 Cheddar cheeses were made in a replicate block design with two experimental factors; Factor 1.
112 Replicate block, which was milk obtained at three different cheesemaking days; Factor 2. MFGM
113 composition, achieved by adding either buttermilk powder (BMP) or skim milk powder (SMP) and

114 cream to the cheese milk. SMP and cream were added to standardize the fat and protein contents in the
115 SMP cheese vats to that in BMP cheese vats. Six cheese vats were made in each of the three replicate
116 blocks; three vats with SMP in the milk and three vats with BMP in the milk.
117
118 ***2.2. Production of skim milk powder (SMP) and buttermilk powder (BMP)***
119 A quantity of 900 L whole milk from the University farm at the Norwegian University of Life
120 Sciences was separated, and the cream was standardized to 36 % fat by addition of skimmed milk. The
121 cream (~95 L) was pasteurized (73°C, 15 s), directly cooled to 7°C and stored at 4°C overnight. The
122 cream was churned to butter in 30 min, with a rise in temperature from 8.5°C to 13.8°C. The buttermilk
123 was sieved through a sterilized cloth bag. The churning yielded ~50 L buttermilk with 1.6 % fat. The
124 buttermilk was stored cold overnight, and then heated to 50°C, and separated to yield 45.8 kg
125 buttermilk with a fat content of 0.6 %. The buttermilk was spray dried directly after separation to yield
126 3.3 kg BMP (96.6 % dry matter (DM) and 9.8 % fat). From the original milk, 50 L skimmed milk
127 (0.5% fat) was pasteurised (73°C, 15 s) and spray-dried to yield 3.8 kg SMP (97.1 % DM and 2.26 %
128 fat). The SMP and BMP were produced by spray drying (Niro Atomizer, GEA NIRO, Søborg,
129 Denmark) with an inlet air temperature of 185-190°C, and a constant outlet air temperature of 85 °C.
130 The spray drier rotary atomizer had a drying rate of 15 L liquid h^{-1}.
131
132 ***2.3. Cheese milk***
133 The raw milk was obtained from a local farm in Cork, Ireland. The raw milk was separated (45
134 °C) and standardized to 0.5 % fat using a table top milk separator (Claire, Milky; Althofen, Austria),
135 before pasteurization (72°C, 15 s). Before each cheese making session, three cheese making vats were
136 mixed containing 20 L of milk and 263 g BMP each. Further three vats contained 19.95 L of milk and
137 240 g SMP and 48 g cream (42 % fat; pasteurized at 80 °C) each, to standardise these to the increase in
138 dry matter and fat in vats with BMP. The vats of cheese milk were stored at 4 °C for 17 h and were

139 stirred every 30 min until the powders were fully dissolved to ensure complete hydration of the milk
140 proteins of the powders.
141
142 ***2.4. Cheese making and ripening***
143 The cheese milk was heated to 30°C, and 1 % of a single strain starter culture (*Lactococcus*
144 *lactis* ssp. *lactis* ML8), cultured for 24 h in 200 mL reconstituted skim milk (10 % w/v); was added to
145 each of the vats. The adjunct starters, cultured for 24 h in MRS broth, were added 15 min after starter
146 addition.
147 After 45 minutes, rennet (Chy-Max™ Plus; 190 IMCU mL^{-1}; Chr. Hansen, Hørsholm,
148 Denmark), 35 mL $100L^{-1}$ milk (7 mL diluted to 40 mL with pasteurized distilled water), and $CaCl_2$, 0.1
149 g $100L^{-1}$ (18 mL 0.1 M), was added to each of the vats.
150 The cheese milk was coagulated for 40-50 min, and the firmness of the gel was checked before
151 cutting. After cutting the curd was left undisturbed for 5 min before gentle stirring commenced, and
152 then stirred for 5 min followed by increasing the temperature to 39°C over the course of 10 min. The
153 whey was drained at pH 6.2, and the cheese was cut into blocks that were stacked at each side of the
154 cheese vat. The cheese blocks were inverted every 15 min during cheddaring, until pH 5.4 was reached.
155 The cheese blocks were milled (~2 x 2 x 15 cm) and 2.5 % salt (w/w) was added to the cheese curd,
156 and gently mixed in. The curd was transferred to cylindrical cheese moulds lined with cheesecloth, and
157 pressed at 1.0 bar for 30 min. The pressure was then increased to 2.5 bar, and the pressing continued
158 for another 18 h. The cheeses were vacuum packed, and ripened at 8°C over a period of 24 weeks.
159
160 ***2.5. Compositional and statistical analysis***
161 After 24 weeks of ripening the gross composition of the cheese was analysed. Sampling was
162 undertaken according to IDF Standard 50C (IDF/FIL, 1995). Microbial counts, pH and dry matter

were measured immediately after sampling. The cheese for analysis of fat was wrapped in aluminum foil and packed in plastic bags sealed under vacuum and then frozen (-20 °C) until analysis.

Dry matter was determined according to IDF Standard 4A (IDF/FIL, 1982). pH was measured as described by Skeie, Lindberg, and Narvhus (2001). Fat was analysed by the van Gulik method according to IDF Standard 222 (ISO 3433) (IDF/FIL, 2008). Total Nitrogen (TN) of cheese was determined by the Kjeldahl method according to IDF standard 20 (IDF/FIL, 1993). Total protein content was calculated by multiplying the TN by 6.38. Salt content was measured according to IDF Standard 88 (IDF/FIL, 2004).

The microorganisms were enumerated on specific media giving the presumptive genera of lactococci on M17 agar (Oxoid, Basingstoke, UK) after aerobic incubation at 30 °C for 4 days.

Cheese hardness was measured using the texture profile analysis (TPA) on between 3 - 9 samples for each cheese (24 weeks of aging). The TPA was performed according to Romeih, Michaelidou, Biliaderis, and Zerfiridis (2002) with a TA-XT2i Texture Analyser equipped with a flat aluminum plunger 75mm in diameter, produced by Stable Micro Systems (Godalming, UK). Cylindrical samples, prepared using a cylindrical sharp hand cutter, were taken from at least 20 mm deep in the cheese blocks, and their dimensions were 35 mm in diameter and 20 mm in height. Samples were compressed axially in two consecutive cycles without yield, with 35% deformation from the initial sample's height at 120 $mm.min^{-1}$ rate of force application. The force required to attain a given deformation or the maximum force during the first compression in TPA technique, is the TPA hardness measured in Newton.

Analysis of variance (ANOVA) was performed using the SAS Enterprise guide 4.0 (SAS Institute Inc., Cary, NC, USA). The treatment factors replicate block and MFGM content formed the statistical model. When analysing the TPA hardness, 7 outliers were removed from the data having been defined as outliers by analysis of the normal distribution.

2.6. Scanning electron microscopy

2.6.1. Starter cultures

The starter culture was activated in MRS broth media. After incubation for 24 h at 30 ºC, 1 mL of the broth was spun down and the supernatant was decanted while the sediment was fixed by addition of 1 mL of the fixation mixture consisting of 1.25 % (v/v) glutaraldehyde and 2 % (w/v) para-formaldehyde in 0.1 M cacodylate buffer for 2 h. A 8 mm glass slide of poly-l-lysine was submerged in the fixed bacterial solution and held for 2 h to carry the bacterial cells on both sides. The glassy film of bacteria was dehydrated in series of aqueous ethanol solutions (70%, 90%, 96% and 100%, 5 min in each), and then dried to critical point using CO_2 in a BAL-TEC CPD 030 Critical Point Dryer (BAL-TEC AG, Balzers, Liechtenstein), and mounted on aluminum SEM stubs, followed by gold coating in a sputter coater Polaron SC 7640 (Quorum Technologies Ltd, Ringmer, UK). A high vacuum Zeiss scanning electron microscope EVO-50-EP (Carl Zeiss SMT Ltd., Cambridge, UK) was used to view the strains at 10 kV and magnification of 5000x.

2.6.2. Cheese

Small cubic samples from the center of the Cheddar blocks (approximately 3 x 3 mm) were prepared using a surgical blade. The protein network of the cheese cubes was fixed overnight in 4% (v/v) glutaraldehyde in 0.1 M sodium cacodylate buffer at pH 6.8. The samples were washed several times in 0.1 M sodium cacodylate buffer (pH 6.8) at 15 min intervals, and then the fat was fixed in 2% (w/v) osmium tetraoxide (OsO_4) in 0.1 M sodium cacodylate for 1-2 h. The cheese samples were re-washed several times in 0.1 M sodium cacodylate buffer at 15 min intervals, followed by dehydration in increasing concentrations of aqueous ethanol solutions (25%, 50%, 75%, 90% and 100%, 15 min in each). Samples were then dried to critical point using CO_2 in a BAL-TEC CPD 030 Critical Point Dryer (BAL-TEC AG, Balzers, Liechtenstein). Following the drying step, some of the dried cubic

213 samples of cheese from each treatment were gently cut from the center into two pieces using a fine
214 scalpel. This was done to explore the internal structure of the cheese cubes, which is free from the fat
215 globules as a result of using series concentrations of ethanol in the dehydration step. This modified step
216 may help in investigating the localization and incorporation of the bacterial cells into the cheese matrix
217 without any interruption from overlapping fat globules. Finally, both samples of complete cubes and
218 divided cubes were mounted individually on aluminum SEM stubs, followed by gold coating as
219 described previously and examined at 5 kV and magnification of 5000x.
220
221 ***2.7. Confocal laser scanning microscopy***
222 Cheese cubes (~ 4 x 4 x 10 mm) were prepared and fixed overnight in 4% (v/v) glutaraldehyde
223 as described previously for the SEM method. Cryo-sections, 50 μm in thickness, were taken from the
224 cheese cubes using a cryotome (Microm HM 560 MV, Microm International GmbH, Walldorf,
225 Germany). The sections were then incubated for 10 min in the dark at ambient temperature with a mix
226 of three fluorescent dyes (50μl of each) consisting of 0.2% (w/v) Fast Green FCF (Sigma-Aldrich,
227 UK), 0.01% (w/v) Nile Red (Sigma-Aldrich, Madison WI, USA) and 0.2% (w/v) Hoechst 33342
228 (AnaSpec Inc., San Jose, CA, USA) for labeling protein, fat and bacteria respectively. Each cheese
229 section was placed between a microscope slide and a cover slip. Samples were then examined at 25 ºC
230 with a 63x oil objective lens and sequential scan using a Leica TCS SP5 confocal laser scanning
231 microscope (Leica Microsystems CMS GmbH, 68165 Mannheim, Germany), using a helium neon
232 (HeNe) laser with excitation wavelength of 633 nm for proteins (an excitation peak of 622-626 nm and
233 the maximum emission was at 640 nm) and an argon (Ar) laser with an excitation wavelength of 488
234 nm for fat (an excitation peak of 515-530 nm and an emission of 525-605 nm), while employing a UV
235 laser with excitation wavelength of 405 nm for the bacterial cells (an excitation peak of ~ 350 nm and
236 maximum emission at 461 nm). In the CLSM micrographs, the protein network, fat and bacterial cells
237 were labeled in gray, green and blue, respectively, while the aqueous phase appears as black areas.

238

239 **3. Results and discussion**

240 Only the effects of the first experimental factor the milk fat globule membrane compounds are
241 covered by this paper. The effects of the adjuncts are described in another manuscript where the
242 microbial development of the cheeses is characterised.

243

244 ***3.1. Cheese composition and texture***

245 Significant differences in the gross composition and the TPA hardness of the cheeses after 24
246 weeks of ripening are shown in Table 1. The BMP considerably reduced the content of dry matter by 1
247 % and the pH by 0.05 in the ripened cheese.. Additionally, no influence from the experimental factors
248 was found on the protein content, fat in dry matter or the salt content which were on average 32.11±
249 0.64 %, 13.7 ± 0.6 % and 1.6 ± 0.2 %, respectively. The content of presumptive lactococci was on
250 average log 6.9 ± 0.5 cfu g^{-1}.

251 The TPA hardness measurements (Table 1) clearly revealed a considerable reduction in the
252 textural hardness as a function of added BMP. The SMP cheese was the hardest, reflecting the potential
253 effect of BMP in softening the cheese texture. The TPA hardness is affected by cheese composition,
254 such as protein content, protein degradation and the interaction between casein and fat and/or other
255 cheese components (Heertje, 1993; Tunick, 2000). No significant difference between SMP and BMP
256 was found on the proteolysis as measured by the content of free amino acids (results not shown). The
257 effect of buttermilk in reducing cheese hardness appears to be linked to its MFGM fragments, which
258 were incorporated with the casein matrices, and played a lubricant role that provided a smoother and a
259 soft texture. This physical function of buttermilk has also been demonstrated by other studies; i.e.
260 Poduval and Mistry (1999) for reduced-fat Mozzarella cheese and Trachoo and Mistry (1998) for low-
261 fat yoghurt. Also, Mistry et al. (1996) reported that reduced-fat Cheddar cheese made with 5% UF-

sweet buttermilk had lower hardness values after 4 weeks of ripening than its control counterpart cheese.

3.2. Scanning electron microscopy

3.2.1 Conventional technique micrographs (surface scanning of cheese cube samples)

The SEM micrographs of low-fat Cheddar cheese ripened for 6 months are shown in Fig. 1. The protein matrix (gray area) formed a continuous phase permeated by amorphous voids (black areas), and spherical fat globules of different sizes. As these micrographs show, an obvious variation in the cheese microstructure was obtained between low-fat Cheddar cheeses made with addition of either SMP (Fig. 1A) or BMP (Fig. 1B). An extremely porous, open and coarse structure was obtained in low-fat Cheddar cheese with SMP addition, while cheese with added BMP was more dense and homogeneous.

Despite the fact that a uniform protein content was achieved in both cheese treatments, cheese with addition of SMP had irregularly aggregated protein folds and large matrix clusters interrupted by large pores filled with serum which appeared as cavities embedded in the cheese matrix. The trend to formation of apparent cavities was expected according to the age of the cheese. Earlier studies (El-Zeini, El-Aasser, Anis, & Romeih, 2006; Poduval & Mistry, 1999; Tunick et al., 1993) have indicated that an increase in the size of the cavities would occur during aging in different cheese varieties due to the weakening of the paracasein matrix caused by proteolysis or CO_2 production by starter or non-starter bacteria.

In contrast, the microstructure of the low-fat Cheddar cheese with BMP added, as shown in Fig. 1B, reveals a homogeneous systematic protein aggregate network. The protein matrix appeared as a smooth continuous phase of aggregated micelles, characterized by a compact fusion and a dense structure. The BMP cheese structure was more regular and had fewer voids compared to that of cheese with SMP, and the effect of the BMP addition was most probably attributed to the high levels of

MFGM material in the BMP. The BMP used in this study was prepared from cream pasteurized at 73°C. This promotes a higher retention of MFGM material in the buttermilk than is obtained from raw-cream as stated by Morin, Jimenez-Flores, and Pouliot (2007). Furthermore, it has been reported that MFGM fragments may physically be entrapped within the paracasein network (Morin et al., 2008). It could induce direct interactions with casein by folding casein micelles inside reconstituted aggregates reflecting the functional properties of buttermilk in dairy product structures (Morin et al., 2008; Ong et al., 2011). In this context, Lopez et al. (2007) have stated that cross-links can be formed between MFGM material and the casein matrix, which in turn strongly affects the rheological and microstructure properties of milk gels. In addition, BMP cheese tended to have a higher moisture content compared to that of SMP cheese (Table 1), which is mainly attributed to the increased hydration capacity of the buttermilk components, particularly its phospholipids. This result is in agreement with those of Morin et al. (2008), Raval and Mistry (1999) and Turcot, Turgeon, and St-Gelais (2001) who reported that addition of buttermilk increased the moisture content of low-fat cheese.

Although a uniform fat-in-dry-matter basis was achieved in all cheese treatments, the spherical fat globules were more pronounced and more uniformly scattered throughout the protein matrices in the BMP cheese compared to the SMP cheese structures (Fig. 1). Addition of BMP tended to cause inclusion of a higher number of discrete fat globules differing in size within the protein matrix, whereas fat globules were more often trapped and embedded within the protein matrix of the SMP cheeses. The more hydrated the texture, the more systematically distributed were the fat globules and the presence of MFGM material and these, taken all together, may contribute to a softer and less firm texture of the BMP cheese compared to its SMP counterparts. This finding goes in parallel with the hardness values (Table 1) for these treatments and is in agreement with the results of Mistry et al. (1996) and Turcot, St-Gelais and Turgeon (2002) who concluded that addition of UF-buttermilk to cheese milk led to softer texture properties of reduced and low fat Cheddar cheeses.

312 Surprisingly, the observation of starter within these cheese micrographs was infrequent and
313 difficult to clearly define (Fig 1). The starter culture (*Lc. lactis* subsp. *lactis* ML8) appeared attached
314 and embedded within the protein network and distributed all over the cheese matrix. This finding leads
315 us to investigate and develop other techniques to explore the distribution and localization of these
316 bacterial cells within the cheese matrix.
317
318 ***3.2.2 Modified technique micrographs (entire matrix of cheese cube samples)***
319 The uneven clarity of the starter culture (*Lc. lactis* subsp. *lactis* ML8) in the cheese micrographs
320 (Fig. 1) stimulated a development and improvement in the microstructure examination in order to
321 explore the manner of distribution of the bacterial cells within the cheese matrix. By cutting the dried
322 cubic cheese samples from the center; the internal structure was exposed, and the fat globules were
323 removed by the ethanol series extraction during sample preparation (see section 2.6.1). The
324 microstructure obtained with this technique as shown in Fig. 2 clearly revealed that the protein matrices
325 (gray area) appeared as a continuous phase of a smooth, flat consolidated surface permeated by
326 heterogeneous voids (black area), without the appearance of the network obtained in the previous
327 examination technique (Fig. 1). The appearance shown in Fig. 2 resulted from the fine scalpel cut step
328 of the fixed and dried cubic cheese samples (see section 2.6.1).
329 In this context, the distribution of milk fat globules can be clearly seen through the numerous
330 smooth surfaced concave areas, which are mostly spherical in shape and were originally occupied by
331 fat globules (white arrows) as described above.
332 These micrographs show that the cocci, most probably the starter culture (*Lc. lactis* subsp. *lactis*
333 ML8), appeared in clusters immersed and uniformly dispersed throughout the protein matrix (black
334 arrows), which is in parallel to the appearance of the pure strain (Fig 3). The starter cells appeared as
335 discrete globular cocci gathered in clusters randomly distributed. Besides in the cheese micrographs, a

336 collection of hollows appeared, which most probably are areas of cells removed during the sample
337 preparation process in particular with regard to the cutting of the dried cheese cube samples.
338 Overall, it can be observed from the microstructure revealed in Fig. 2 that the protein matrix
339 formed a continuous uniform phase in both SMP cheese (Fig. 2A) and BMP cheese (Fig. 2B).
340 However, the pronounced uneven size and shapes of voids were noticeably less marked and fewer in
341 number in the BMP cheese than in the SMP cheese, reflecting the higher fusion of casein aggregates
342 and the increased network formation obtained by addition of BMP. This effect of buttermilk was also
343 identified by Lopez et al. (2007) and Morin et al. (2007) as a vital factor influencing the microstructure
344 characteristics of the cheese. These structure are similar to those obtained by the previous formal
345 examination technique presented in this work.
346 By the conventional SEM it was difficult to see the distribution and localization of bacterial
347 colonies, most probably owing to the sample preparation. However, by the modified SEM technique
348 applied in the current study we were able to see and describe the bacteria with a degree of high
349 resolution.
350
351 ***3.3. Confocal laser scanning microscopy***
352 CLSM was used to differentiate between the structural components of the cheese and to make
353 the preferential localization of bacterial colonies visible throughout this cheese matrix in comparison
354 with the findings of SEM.
355 The CLSM micrographs presented in Fig. 4 reveal a cheese structure with features that
356 resemble those observed by the SEM techniques (Fig. 1 and Fig. 2). Fig. 4 shows the protein matrix
357 (gray area) as a continuous uniform phase permeated by heterogeneous voids (black area) representing
358 the aqueous serum pores. The milk fat globules appear as discrete green spots differing in shape and
359 size. Previously, CLSM micrographs of Emmental cheese showed that fat was dispersed in the cheese
360 matrix in three phases; as individual fat globules, as coalesced fat globules resulting from the fusion of

individual fat globules, and finally as nonglobular fat (free fat) of larger size than the other phases (Lopez et al., 2007). This may explain the different sizes and shapes of fat in Fig. 4. An obvious finding was that the localization of fat noticed in CLSM images (Fig. 4) suggest that the globules are not only entrapped in the protein network, but also protrude into the serum pores. According to Ong, Dagastine, Kentish, and Gras (2010) the native MFGM acts as a natural emulsifying agent that enables the fat to remain in the aqueous phase.

Furthermore, Fig. 4 shows that the clusters of starter cells were of uneven size and had different shapes (blue spots) distributed randomly in the cheese matrix. This finding is compatible with Fig. 3 of the pure starter strain image and Figs. 2A and 2B of the Cheddar cheese structure by the modified SEM technique.

Consistent with the microstructural characteristics obtained by SEM, under CLSM the cheeses with added BMP (Fig. 4B) appeared homogenous, with rather small and evenly distributed pores and a network consisting of a relatively fused protein phase. Cheeses with added SMP (Fig. 4A), however, had noticeably rather large pores which appeared as cavities embedded in the cheese matrix, reflecting the porous and coarse structure of the SMP cheese.

Lopez (2005) has stated that milk caseins are able to associate with the fat globule membrane, forming a protein layer which in turn enables the newly formed phase to behave as pseudo-protein particles, becoming an integral part of the protein matrix during coagulation. Also, Ong et al. (2011) have suggested that chemical bonds may exist between the fat globule membrane components and the protein matrix. This might allow for more rearrangement to take place in the cheese matrix, again favouring the formation of a more homogeneous and compact structure in the BMP cheeses.

In summary, CLSM provided structural information compatible with that from SEM. Furthermore, CLSM allowed a visualization of the cheese chemical composition as well as the distribution and localization of bacteria within the cheese matrix. Structure imaging was achieved by

385 the combination of specific stains, which effectively avoided any artifacts due to possible cross-
386 reactions of the multi-stains used.
387
388 **4. Conclusion**
389 Addition of BMP softens the texture of low fat Cheddar cheese as shown by decreased hardness
390 values, and the microstructure analysis supported these findings. The structural network of the BMP-
391 added cheeses was characterized by a smooth and dense protein matrix, in which spherical fat globules
392 exhibited a more uniform dispersion and were more pronounced compared to those of SMP cheeses.
393 The modified SEM technique used, provided a peerless tool over the conventional technique for
394 monitoring the genuine localization and distribution of bacterial colonies in the cheese matrix without
395 disturbance of other cheese structure compounds.
396 Qualitatively, the microstructure attributes revealed by CLSM were similar to the structure
397 observed using SEM, but CLSM also had the capacity to specifically distinguish the different
398 components of the cheese. The staining procedure we used has shown protein, fat and starter clusters
399 and their manner of incorporation in the cheese matrix.
400 Together these techniques provide a complementary and more thorough assessment of the
401 microstructure of cheese and of other more hydrated dairy products. The results of this study offer a
402 better understanding of the functional impact of BMP on cheese structure, which may lead to a better
403 comprehension of the functional properties and quality attributes of low-fat Cheddar cheese. Addition
404 of BMP proved to be a promising option to direct the industrial processes to the production of high-
405 quality low-fat cheeses with additional nutritional properties.
406
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415

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507 **Figure headings**

508

509 **Fig. 1.** SEM micrographs (5000x) of low-fat Cheddar cheese (surface of cubic samples): **(A)** Cheese of
510 skim milk (SMP), **(B)** Cheese of butter milk (BMP). Scale bar is 2 µm.

511

512 **Fig. 2.** SEM micrographs (5000x) of low-fat Cheddar cheese (entire matrix of cubic samples): **(A)**
513 SMP and **(B)** BMP. Black arrows indicate the starter cluster cells and white arrows indicate
514 voids of removed fat globules. Scale bar is 2 µm.

515

516 **Fig. 3.** SEM micrographs (5000x) of *Lc. lactis* ssp. *lactis* ML8. Scale bar is 3 µm.

517

518 **Fig. 4.** CLSM micrographs (63x) of low-fat Cheddar cheese treatments: **(A)** Cheese of skim milk
519 (SMP), **(B)** Cheese of butter milk (BMP). Protein is labeled in gray, fat is in green and bacterial
520 cells are in blue. The aqueous phase appears in black. Scale bar is 10 µm.

Table 1. Dry matter (DM %), pH and the texture properties as measured hardness on a Texture analyzer (TPA hardness) of the cheeses after 24 weeks of ripening (Values are means ± SD, n = 9). The *p*-statistics of each experimental factor is shown in the last two rows of the table.

	DM (%)	pH	Hardness (N)
SMP	52.41 (±0.71)	5.33 (±0.11)	148.2 (±12.7)
BMP	51.34 (±0.84)	5.28 (±0.12)	132.6 (±15.3)
Powder	0.0002	0.01	0.0024
Rep block	0.0002	0.0001	ns

* ns: not significant

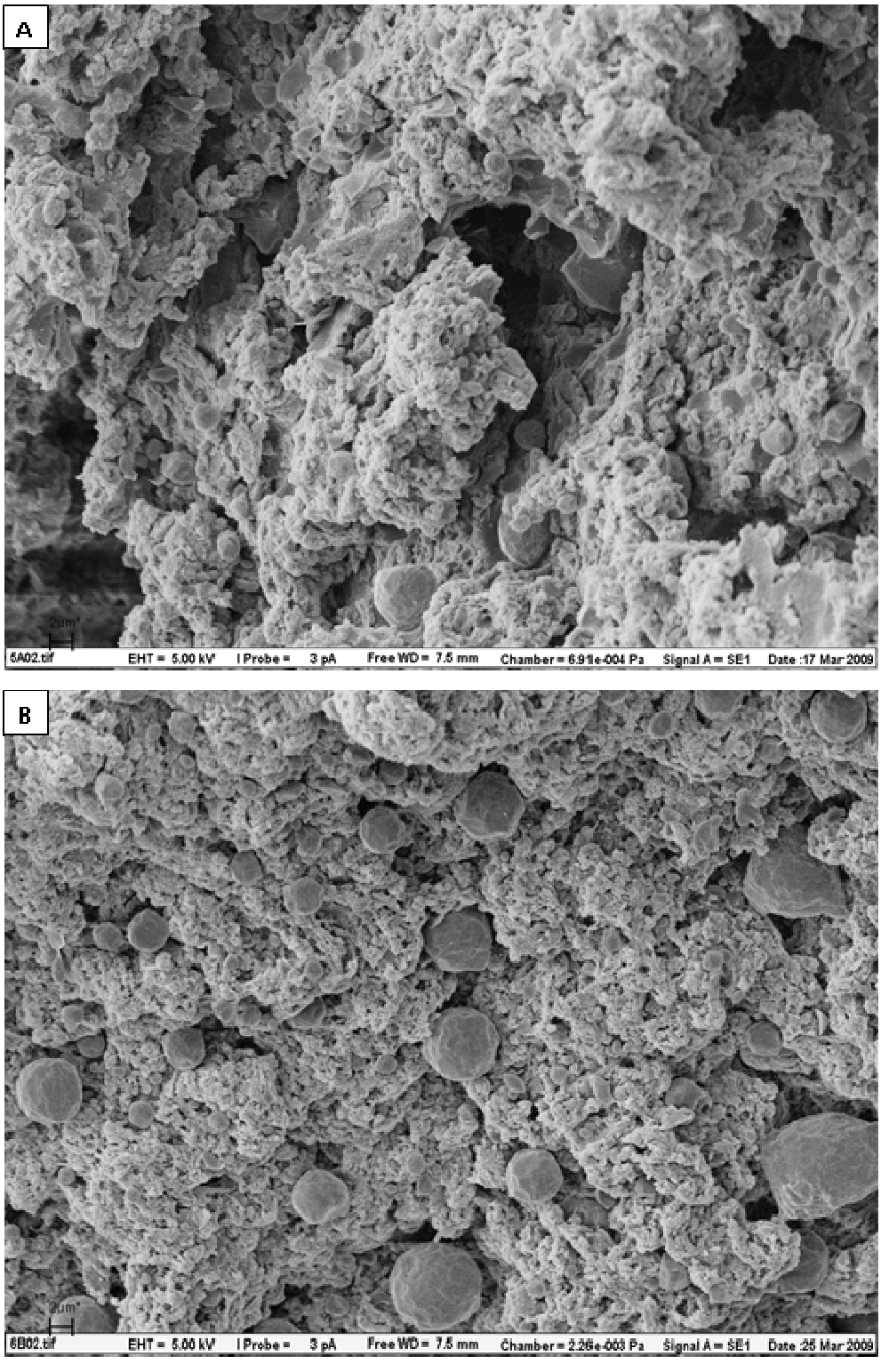
A
5A02.tif EHT = 5.00 kV I Probe = 3 pA Free WD = 7.5 mm Chamber = 6.91e-004 Pa Signal A = SE1 Date :17 Mar 2009
B
6B02.tif EHT = 5.00 kV I Probe = 3 pA Free WD = 7.5 mm Chamber = 2.26e-003 Pa Signal A = SE1 Date :25 Mar 2009

Fig. 1.

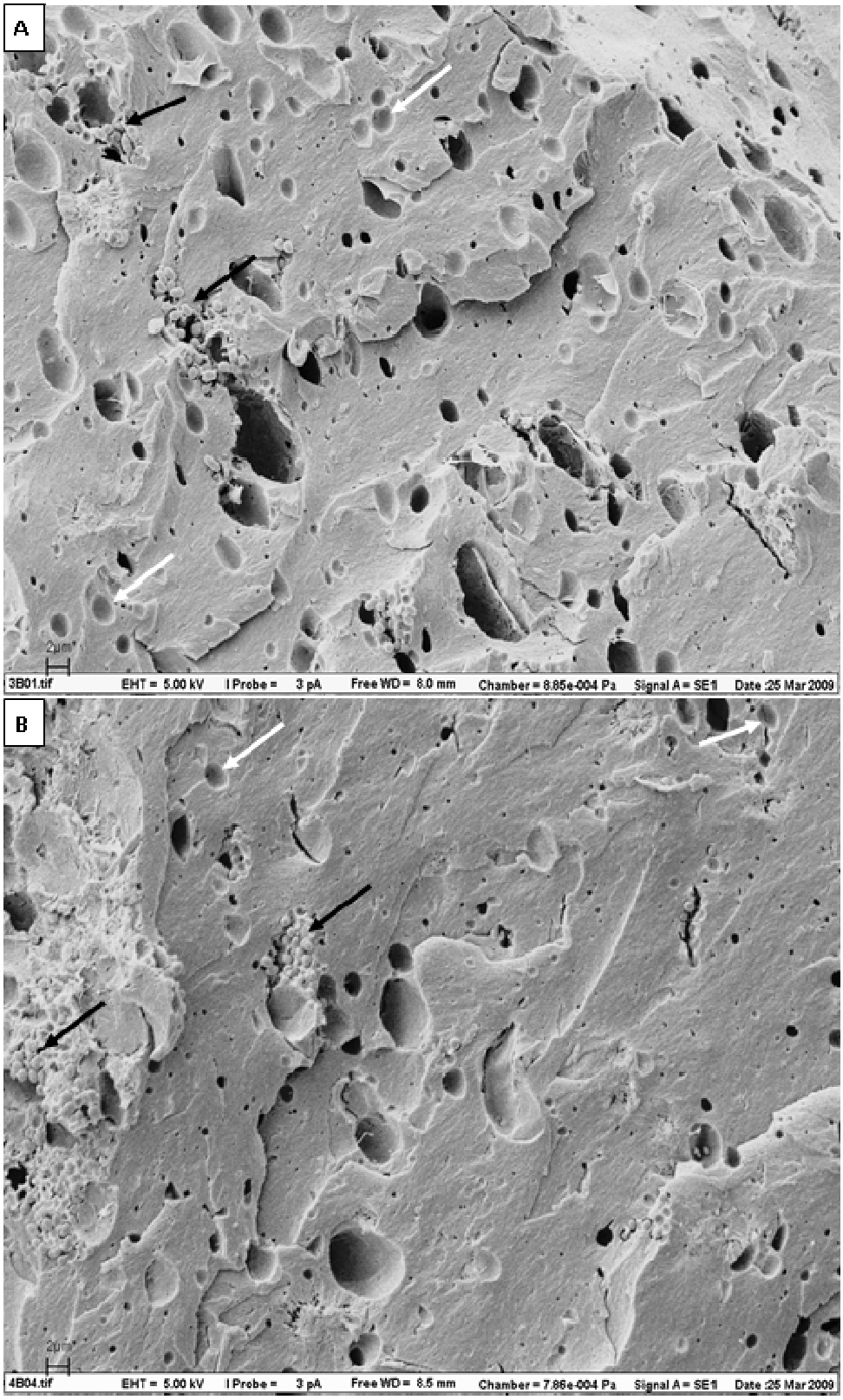
A
2µm
3B01.tif EHT = 5.00 kV I Probe = 3 pA Free WD = 8.0 mm Chamber = 8.85e-004 Pa Signal A = SE1 Date :25 Mar 2009
B
2µm
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Fig. 2.

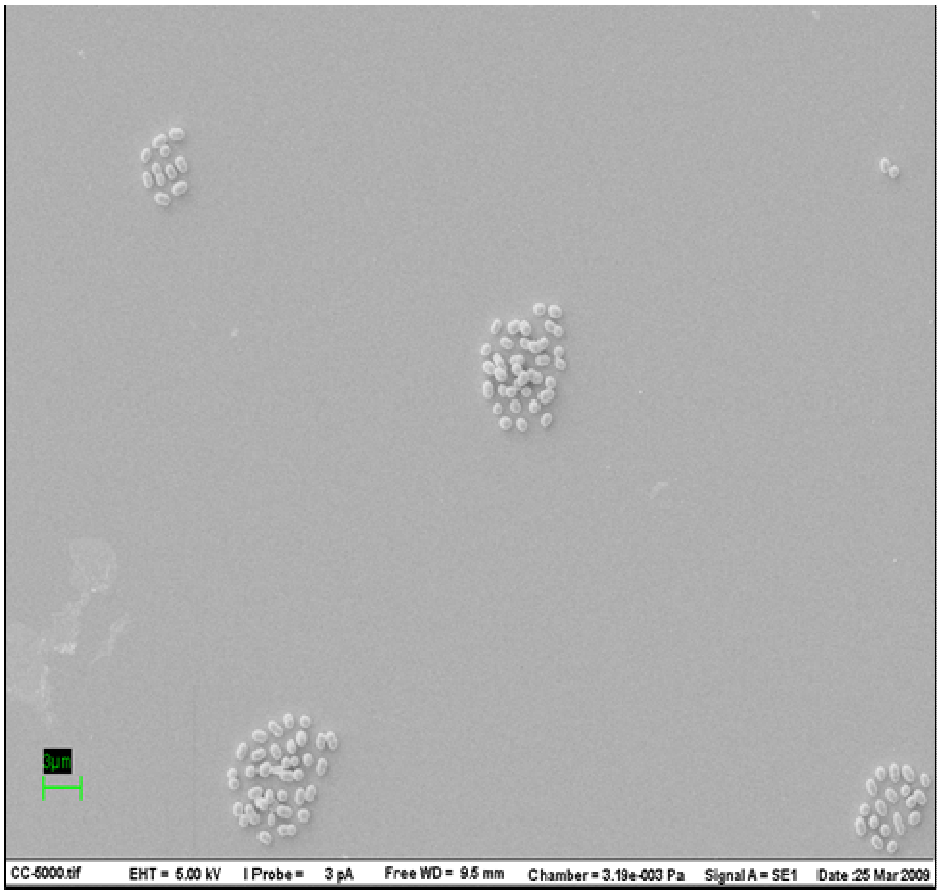
3µm
CC-5000.tif EHT = 5.00 kV I Probe = 3 pA Free WD = 9.5 mm Chamber = 3.19e-003 Pa Signal A = SE1 Date :25 Mar 2009

Fig. 3.

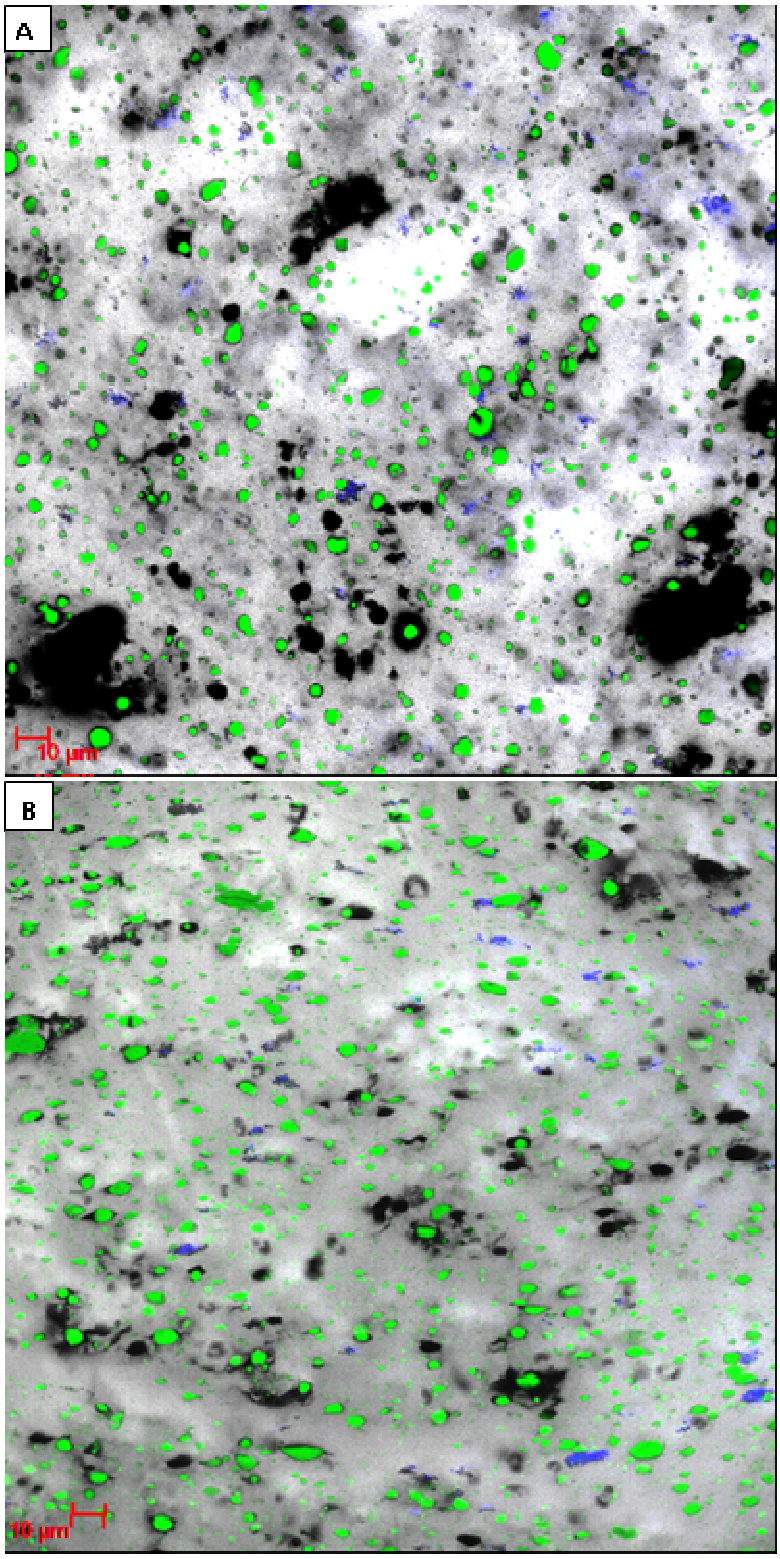

Fig. 4.