Metabolism of milk fat globule membrane components by nonstarter lactic acid bacteria 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 (MFGM) may be used for growth and survival by cheese-ripening lactobacilli. This was achieved by analyzing metabolites produced during incubation on appropriate media. The lactobacilli investigated were able to utilize components from the MFGM throughout a 24-d incubation period. We observed an apparent connection between the higher proteolytic activity of *Lactobacillus paracasei* INF448 and its ability to grow in the MFGM media after depletion of readily available sugars. 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 D-galactose resulted in a metabolic shift, expressed as different fates of the produced pyruvate, compared with growth on the other monosaccharides. For *Lb. plantarum* INF15D, the presence of D-galactose also seemed to initiate degradation of some amino acids known to 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 flavors and aromas in cheese during ripening (Marilley and Casey, 2004). However, when lactose is depleted at a very early state of the cheese ripening, it is not clear which substrates ensure the metabolic active state observed in LAB during cheese ripening (Beresford and Williams, 2004).

The fat globules of bovine milk are excreted from secretory mammary cells by the envelopment of the fat in a membrane derived from the plasma membrane (Patton and Keenan, 1975). This bilayer milk fat glob-

ule membrane (MFGM) contains numerous complex glycoconjugates in the form of glycoproteins and glycolipids (Mather, 2000; Liu et al., 2005). The glycoproteins of the MFGM contain different oligosaccharides identified as being composed of *N*-acetyl-D-neuraminic acid, D-galactose (**Gal**), L-fucose, D-mannose (**Man**), *N*-acetyl-D-glucosamine (**GlcNAc**), and *N*-acetyl-D-galactosamine (**GalNAc**; Snow et al., 1977; Nakata et al., 1993; Sato et al., 1995; Hvarregaard et al., 1996; Pallesen et al., 2007). The LAB in cheese are often found close to the entrapped milk fat globule surface (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 growth of nonstarter LAB (**NSLAB**; Martley and Crow, 1993; Crow et al., 1995; Hynes et al., 2001; Di Cagno et al., 2003). 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 casein-derived free AA that did not accumulate during ripening of Swiss-type cheese, and that the Arg degradation products (Cit and Orn) increased. Only LAB able to produce the enzymes involved in the arginine deiminase (**ADI**) pathway are able to convert Arg to Orn (Liu et al., 2003a); however, it has been shown that both ADI and ornithine transcarbamylase were inhibited by D-glucose (**Glc**), whereas the inhibitory effect of Gal was much lower (Crow and Thomas, 1982).

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

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(Kieronczyk et al., 2001). Liu et al. (2003b) showed that only ammonia and acetate were produced by *Lb. plantarum* B3087 in phosphate buffer with added Ser.

Intracellular decarboxylation of amino acids that consume protons has been described in several works as a reaction used by bacteria to reduce intracellular pH (Christensen et al., 1999; Ardö et al., 2002). The products of AA decarboxylation are amines, some of which are regularly detected in fermented food products (Komprda et al., 2008, 2010; Linares et al., 2011). Several research papers suggest the occurrence of antiporters in the bacterial cell wall of LAB (Driessen et al., 1989; Molenaar et al., 1993; Konings et al., 1995; Higuchi et al., 1997; Lucas et al., 2005; 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_0 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 has been described for the cheese bacteria *Lactobacillus buchneri* (Molenaar et al., 1993), and for several meat (Masson et al., 1996) and wine LAB (Moreno-Arribas et al., 2000; Moreno-Arribas and Lonvaud-Funel, 2001).

A previous study has 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 in a carbohydrate-restricted medium (CRM) with added MFGM isolate (Moe et al., 2012). These observations indicate that the bacteria were able to utilize compounds for growth and survival other than the lactose present in the medium. Lactose and its monosaccharide components are exhausted quite rapidly 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 analyzed when the NSLAB were grown with an MFGM isolate or single MFGM carbohydrates as the only carbohydrate source.

MATERIALS AND METHODS

Bacterial Strains

The 4 strains of NSLAB used in this experiment were *Lactobacillus paracasei* INF10, INF448, and 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 (Ås, Norway). Strains INF10 and INF15D were isolated from 90-d-old and strains INF448 and INF456 from 180-d-old Norwegian cheese (a Norwegian, washed-curd, Dutch-type cheese).

Carbohydrate-Restricted Medium

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

Preparation of Carbohydrate Stock Solutions

The monosaccharides used to supplement the CRM were prepared as stock solutions (100 mg/mL), and were filter sterilized (pore size 0.22 μm ; Millex, Carriagtwahill, Ireland). The MFGM carbohydrates used in this experiment were Gal, Man, GlcNAc, and GalNAc (all from Sigma). The monosaccharide Glc (Merck) is not found in MFGM glycoconjugate side-chains and thus was used as a standard. All monosaccharides were added at a final concentration of 5 g/L.

Preparation of MFGM Medium

The MFGM material was prepared by a procedure described by Vanderghem et al. (2008), with minor modifications as described by Moe et al. (2012). In short, fresh unpasteurized milk, cooled to 4°C, was obtained from the herd at the Norwegian University of Life Sciences. The cream was washed twice with 5 volumes of PBS (0.01 M, pH 7.2, 0.9% NaCl; 4°C) and once with 5 volumes of distilled water (4°C). The cream was subjected to centrifugation between washes (4,500 $\times g$ for 10 min at 4°C). The washed fat globules were

allowed to crystallize for 20 h at 4°C before heavy agitation, inducing phase separation. The collected serum was centrifuged twice ($5,000 \times g$ for 15 min at 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) and added to CRM (final concentration 5 g/L) before autoclaving (121°C, 15 min).

Preparation of Bacterial Suspensions

The bacteria were subcultured at least 3 times at 30°C for 24 h in de Man, Rogosa, and Sharpe broth (Merck). Bacterial cultures (1 mL) were harvested by centrifugation at $13,400 \times g$ for 20 min (Eppendorf Centrifuge 5415D, Eppendorf, Hamburg, Germany). The supernatant was removed with a sterile pipette, and the bacterial pellet was resuspended in 1 mL of CRM. The final concentration of bacteria ($\log 6.38$ – 7.55 cfu/mL for the monosaccharides and $\log 4.20$ – 5.65 cfu/mL for the MFGM material) in the suspension was selected to be the same as in earlier experiments with the same bacteria and growth media (Moe et al., 2012).

Development of Metabolites

Ten milliliters of CRM supplied with the MFGM monosaccharides (at 5 g/L) was inoculated with 100 μ L of undiluted bacteria suspension (1.0% vol/vol) in a 10-mL tube. Of the strains used in this experiment, only *Lb. paracasei* INF 448 was incubated in CRM with GalNAc, because we showed earlier that this was the only strain able to grow on GalNAc (Moe et al., 2012). Samples for metabolite analysis were taken at selected time intervals (0, 4, 12, and 48 h), corresponding to early lag, early log, early stationary, and late stationary phases, compiled from earlier experiments (Moe et al., 2012). The CRM with added Glc (5 g/L) was used as a control.

The diluted bacterial suspension (100-fold in quarter-strength Ringer's solution) was added to the MFGM medium, and the development of metabolites in the MFGM medium was followed for 0, 4, 12, 48, 168, 336, and 576 h of incubation. The analysis of carbohydrates, organic acids, and AA was done from 3 replicate incubations. All of the biological samples were controlled for contamination after the end of the experiment with the use of denaturing gradient gel electrophoresis, as described by Moe et al. (2012). Samples showing more than one band or a band in a different location than the pure PCR product of the strain were omitted from the data set and further data treatment. Bacterial growth

was confirmed by enumeration using the pour-plate technique.

Analysis of Organic Acids and Carbohydrates by HPLC

Organic acids and carbohydrates were analyzed using HPLC, following a modification of the method of Marsili et al. (1981). The procedure used was as described by Narvhus et al. (1998), with the following modifications. The samples were analyzed using an Aminex HPX-87H column, (Bio-Rad Laboratories, Hercules, CA) held at 32°C, connected to a Perkin-Elmer HPLC (Perkin-Elmer, Waltham, MA). As mobile phase, 5 mM H₂SO₄ at a flow of 0.4 mL/min was used. Standard solutions for external calibration were prepared in the same way as the samples, and the compounds were identified according to their retention times compared with the standard solutions using a Perkin-Elmer Series 200 refractive index detector for carbohydrates, and a Perkin Elmer Series 200 UV/VIS detector for the organic acids. The carbohydrates used for standard solutions were lactose (Merck), Glc (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 (all from Sigma).

Analysis of Volatile Compounds

Volatile compounds were analyzed using a headspace gas chromatography system according to the method of Narvhus et al. (1993). The test sample was 10 g of growth medium with homogeneously dispersed bacterial 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 the moment of analysis.

The headspace sampler used was an HP 7694 with a 6890 GC system (Agilent Technologies, Santa Clara, CA), Series 900 interface connector clips (Perkin Elmer, Shelton, CT), a hydrogen generator (model 75-32, Whatman, Haverhill, MA) with 160 kPa of pressure, and TotalChrom LC software (Perkin Elmer). Volatile compounds were separated on a CP-SIL 5CB GC column (25 m $\times$ 0.53 mm i.d., 5- μ m film thickness (Varian, Middelburg, the Netherlands), and N₂ (5 mL/min; AGA AS) was used as carrier gas. The GC was fitted with a flame-ionization detector at 200°C. Peaks were externally identified and quantified using standard

solutions of the following compounds: acetaldehyde, 2-pentanone, 2-butanone, ethyl acetate (all from Fluka, Seelze, Germany); 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, isobutylacetate, dimethylsulfide, acetone, 2,3-pentadion (all from Merck); and ethanol (Arcus, Oslo, Norway).

Analysis of Free AA

Free AA were analyzed by immediately adding 2.0 g of internal standard solution (0.1 M HCl; 0.4 μ mol/mL L-norvalin; Sigma, St. Louis, MO) to 2.0 g of sample, followed by sonication for 30 min. After the sample was centrifuged ($3,000 \times g$ for 40 min at 4°C), 0.5 mL of 4% TCA was added to 0.5 mL of the supernatant, mixed in a mini shaker (Gene2, New York, NY), and placed on ice for 30 min. After centrifugation ($15,700 \times g$ for 5 min at 5°C), the samples were filtered (0.2- μ m cellulose acetate filter, Advantec, Dublin, CA) and stored in a freezer (−24°C) until analysis.

Before separation, 350 μ L of borate buffer (0.4 M, pH 10.2; Agilent Technologies) was added to the samples. Separation of AA was performed using a Perkin Elmer series 410 pump (Perkin Elmer), an Agilent Technologies 1200 series autosampler (Agilent Technologies), a Perkin Elmer 200 column oven, and an Agilent Technologies 1200 series thermostat. The system was driven by EZChrom Elite (Agilent Technologies) software. An XTerra RP 18 column (150 $\times$ 4.6 mm; Waters, Milford, MA) was used for separation of AA at 42°C. Derivatization with *o*-phthaldialdehyde (OPA) was done according to Bütikofer and Ardö (1999).

Statistical Treatment of Data

The relationship between the substrate components and their metabolic products were analyzed by principal component analysis (PCA) using Unscrambler X10.1 (Camo, Trondheim, Norway). All results were weighted (1/SD) before the PCA. The results from the bacteria grown on the separate sugars and on the MFGM medium were analyzed separately because of differences in incubation time and inoculation. Because the replicates were similar, with low SD, the results of their average values are shown in the PCA scores and loadings plot (Figure 4).

RESULTS

LAB Growth and Metabolism in MFGM Medium

All strains used in this experiment grew on the MFGM medium. After preparation of the MFGM me-

dium, 34 mmol/L lactose remained (Figure 1). Lactose was depleted by all *Lb. paracasei* strains after 48 h of growth; however, samples with *Lb. plantarum* INF15D had lactose remaining in the medium after 168 h of growth (Figure 1). The level of lactate continued to increase after apparent depletion of lactose for *Lb. paracasei* INF10 and INF456; however, toward the end of the growth period, the amount of lactate in general decreased or remained at a constant level in the MFGM medium.

Acetate was produced by *Lb. plantarum* INF15D and *Lb. paracasei* INF456 after 168 h, with *Lb. plantarum* INF15D producing the highest level, whereas *Lb. paracasei* INF10 and *Lb. paracasei* INF448 produced acetate at low levels only after 576 h of incubation.

The general release of AA in the MFGM medium from 336 to 576 h was greater by *Lb. paracasei* INF448 than with any of the other investigated strains. For samples with *Lb. paracasei* INF456, *Lb. paracasei* INF10, and *Lb. plantarum* INF15D, the level of Ser was lower after 576 h of incubation than after 336 h of incubation of the MFGM medium. The decrease in Ser was more pronounced, however, in samples with *Lb. paracasei* INF456 than in samples with the other strains.

The content of Gln was increased by all strains throughout the growth period. However, samples with *Lb. paracasei* INF448 had a lower content of Gln and a higher content of Glu than the other strains studied, and Gln was almost exhausted after 576 h incubation of *Lb. paracasei* INF448. The content of DL-pyroglutamic acid decreased drastically after 336 h of growth in samples with *Lb. paracasei* INF448 (Figure 1b).

The level of Asn was relatively stable throughout the incubation period, but a decrease was observed in the later stages of growth by all strains. The levels of Arg remained constant throughout the incubation period, but the amount was slightly decreased at 576 h for all strains. A slight increase of Orn was observed at 576 h of incubation for all strains except for *Lb. paracasei* INF448.

LAB Growth and Metabolism on MFGM Monosaccharides

All strains used in this experiment grew on Gal, Glc, GlcNAc, and Man. The metabolic patterns when incubated on Glc and the MFGM monosaccharides GlcNAc, Gal, and Man were similar for all *Lb. paracasei* strains. The development of metabolites produced by *Lb. paracasei* INF448, as an example, is shown in Figure 2. However, of the studied strains of *Lb. paracasei*, only *Lb. paracasei* INF448 could utilize GalNAc. The main product from the degradation of the monosaccharides was lactate but when GalNAc or GlcNAc

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

was utilized, acetate was also produced, in the ratio 2:1 lactate:acetate. In all samples, the levels of Asp and Glu increased in the later stages of incubation, whereas the level of Asn decreased. None of the *Lb. paracasei* strains changed the levels of Ser, Arg, or Tyr during growth on any of the carbohydrates.

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

Lactobacillus plantarum INF15D utilized Glc, Man, and GlcNAc at a higher rate than it did Gal and consequently produced lactate at a higher rate on the 3 first sugars (Figure 3), whereas formate and ethanol were produced at a higher rate when *Lb. plantarum* INF15D was grown on Gal compared with Glc, Man, or GlcNAc.

Except when the strains were grown on an acetylated aminosugar, *Lb. plantarum* INF15D was the only strain that produced acetate on any of the other monosaccharides during the 48-h incubation period. Acetate was produced by *Lb. plantarum* INF15D when grown on both Gal and GlcNAc (Figure 3); however, the amounts of acetate produced were very low on Gal compared with GlcNAc.

Even though none of the lactobacilli decreased the levels of Tyr when grown on MFGM medium (Figure 1), *Lb. plantarum* INF15D utilized Tyr when grown on Gal, whereas the 3 *Lb. paracasei* strains did not utilize Tyr under any of the conditions used in this experiment.

Serine was used by *Lb. plantarum* INF15D when grown on all sugars. Furthermore, the major difference when *Lb. plantarum* INF15D was grown on Gal compared with the other monosaccharides was, in addition to the utilization of Tyr, its use of Arg and the resultant production of Cit and Orn, as well as increases of Gly, Glu, and Ala in the medium. However, production of Gln was lower when *Lb. plantarum* INF15D was grown on Gal compared with Man, Glc, and GlcNAc.

DISCUSSION

Previous work with the strains investigated in this study showed that the cultivable number of *Lb. para-*

casei INF448, *Lb. paracasei* INF456, and *Lb. plantarum* INF15D started to increase after an initial period of decline when incubated for 61 d in CRM containing MFGM isolate (Moe et al., 2012). The results presented here show that surplus lactose was exhausted rapidly by all *Lb. paracasei* strains but not by *Lb. plantarum* INF15D. The surplus lactate produced after apparent depletion of lactose and its monosaccharide moieties in the MFGM medium indicated that this additional lactate was 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 was incubated with GlcNAc as the only added carbohydrate source, large amounts of acetate was produced. Rogers (1949) showed that *Streptococcus pyogenes* produced acetate, lactate, and ammonia when utilizing GlcNAc. It was later established by Roseman (1959) that the enzyme GlcNAc deacetylase catalyzes the release of acetate from GlcNAc during its metabolism. It is therefore very likely that the large amounts of acetate produced in the medium with added GlcNAc are mainly released from this acylated aminosugar during its metabolism.

For the NSLAB to be able to utilize GlcNAc as a source of energy in cheese during ripening, they have to be able to release the monosaccharides either from the peptidoglycan of dead bacteria or from MFGM glycoconjugates. Several species of mesophilic lactobacilli possess glycolytic enzymes that may be able to release these sugars (Williams and Banks, 1997), and production of enzymes involved in the breakdown of GlcNAc is upregulated in the stationary growth phase in *Lb. plantarum* WCFS1 when grown in de Man, Rogosa, and Sharpe medium (Cohen et al., 2006) and in lactose-starved *Lactobacillus casei* (Hussain et al., 2009).

If utilization of GlcNAc occurs in cheese, acetate should be produced. In a study where *Lactobacillus paracasei* ATCC 334 was grown on ripened Cheddar cheese extract, it was concluded that the metabolic products formed (i.e., acetate and lactate) could not be explained by residual levels of lactose, Gal, or citrate present in the medium (Budinich et al., 2011). Those authors further suggested that milk-derived complex carbohydrates or starter-derived components could be the precursors for the metabolites found in the medium after growth of the NSLAB strain. The results presented in the current paper show that the metabolism of GlcNAc could be the source of the excess lactate and acetate, and therefore support the hypothesis presented by Budinich et al. (2011).

Roseman (1957) found that a crude extract of *Escherichia coli* deacetylated GalNAc at only 10% of the rate at which *E. coli* deacetylated GlcNAc and suggested

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

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

Figure 4. Principal component analysis biplot of *Lactobacillus plantarum* INF15D strains incubated in carbohydrate-restricted medium with D-glucose (Glc), D-mannose (Man), N-acetyl-D-glucosamine (GlcNAc; dashed ellipse), or D-galactose (Gal; solid ellipse), at 0, 4, 12, and 48 h. Principal component 1 (PC 1) explains 46% of the variation and PC 2 explains 23% of the variation. Each point represents the average of 3 replicate samples. GABA = γ -aminobutyric acid.

that this difference might be caused by 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 *agaA* gene in *E. coli*. According to the KEGG (Kyoto Encyclopedia of Genes and Genomes) database, this gene is not found in any of the sequenced LAB genomes to date and has not, to our knowledge, been described in any LAB in literature previously. *Lactobacillus paracasei* INF448 was the only studied strain able to utilize GalNAc for growth, and the growth rate was found to be significantly lower than when this strain was grown on GlcNAc (Moe et al., 2012). However, large amounts of acetate were produced when GalNAc was utilized, 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 medium late in the incubation period was higher by *Lb. paracasei* INF448 compared with 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 have 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 serve only as a source of nitrogen and cannot be used by LAB as a source of energy (Fox et al., 1998). However, our current results and those of other studies indicate that AA could serve, both directly and indirectly, as a source of energy for LAB, and that AA released by proteolytic activity could enhance NSLAB growth (Martley and Crow, 1993; Crow et al., 1995; Hynes et al., 2001; Di Cagno et al., 2003). Our work therefore supports the observations that a high release of AA from the growth medium 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 level of Ser when grown on any of the monosaccharides. Liu et al. (2003b) proposed that the breakdown products of 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 was observed when the strain was grown on Glc and Man, even though the levels of Ser decreased. Degradation of Ser produces pyruvate (Liu et al., 2003b), which could be further metabolized to lactate or acetate plus formate and ethanol. Adamberg et al. (2006) suggested that *Lb. plantarum* improved ATP and biomass yield by shifting the pyruvate breakdown from lactate to acetate. It is reasonable to assume that the acetate, ethanol, and formate produced by *Lb. plantarum* INF15D when grown on Gal were derived from Ser, as was observed by Liu et al. (2003b). However, the acetate was most likely produced from pyruvate via the pyruvate-formate lyase pathway. These results indicate that *Lb. plantarum* INF15D had a metabolic shift when grown on Gal, resulting in different fates of the produced pyruvate when grown on the different monosaccharides. Skeie et al. (2008b) incubated *Lb. plantarum* INF15D in phosphate buffer containing only Ser as the potential energy source, and acetate and formate were produced during incubation. The results presented in this paper indicate that *Lb. plantarum* INF15D metabolizes Ser by the same pathway when grown on Gal as in a medium completely devoid of fermentable carbohydrates, in contrast to media containing Glc, GlcNAc, or Man. When incubated on the MFGM medium, acetate was found late in the incubation period; however, the decrease in Ser was only apparent by some of the strains and it was difficult to relate acetate and Ser to each other.

When *Lb. plantarum* INF15D was grown on Gal, the level of Arg decreased, whereas that of Orn increased. Cunin et al. (1986) suggested that energy depletion is a key triggering factor for induction of the ADI pathway in most bacteria. However, the regulation differs among LAB and the differences seem to correlate with the adaptation to different habitats (Fernández and Zúñiga, 2006). In the current study, *Lb. plantarum* INF15D utilized Arg when grown on Gal, whereas Arg did not decrease when the strain was grown on any of the other monosaccharides. Manca de Nadra et al. (1986) showed that the specific activity of the 3 ADI pathway enzymes was higher in *Lactobacillus buchneri* when grown on Gal than on other sugars, an observation that may explain the induction of Arg metabolism in *Lb. plantarum* INF15D grown on Gal observed in the current study.

The decrease of Arg when the lactobacilli were grown on MFGM medium was greater than the increase of Cit and Orn combined, indicating either that Arg was converted by some pathway other than the ADI pathway, or that Cit, Orn, or both, were further converted. Arena and de Nadra (2001) reported that *Lb. plantarum* produced putrescine by decarboxylation of Orn.

Transamination reactions usually involve α -keto-glutarate as an amino group acceptor, resulting in the

production of Glu (Ardö, 2006). The level of Glu was higher in *Lb. plantarum* INF15D grown on Gal than in the strain grown on any of the other monosaccharides studied. This was also the only condition where Tyr was degraded in this experiment, making transamination a likely reaction for the Tyr catabolism observed. Gummalla and Broadbent (2001) described the catabolism of Tyr in the cheese LAB *Lb. casei* LC202 and LC301, and *Lactobacillus helveticus* LH212 and CNRZ32. They found that these strains degraded Tyr by a transamination reaction and that they did not decarboxylate Tyr in Cheddar cheese (Gummalla and Broadbent, 1999).

Amino acid decarboxylases are induced in LAB by low-pH media during the late exponential growth phase (Molenaar et al., 1993). They are thought to favor adaptation to growth in acidic environments, as they fixate protons that can be expelled from the cell, thereby increasing the pH (Lonvaud-Funel, 2001). Lonvaud-Funel (2001) suggested that AA-decarboxylating LAB strains may survive longer in food products because of their presumed ability to produce energy by the decarboxylating action by creating an electrochemical proton potential that could fuel the cell wall-associated F_1F_0 ATP synthase system.

The AA decarboxylation activity is strain specific, and the ability to produce amines varies with different species (Lonvaud-Funel, 2001). The results obtained for *Lb. plantarum* INF15D indicate that Tyr breakdown is only active under specific conditions. However, the specific conditions that induced the Tyr breakdown in the CRM with added Gal are not known. Tyrosine-decarboxylating enzymes have been found in *Lactobacillus 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 medium, we observed no decrease in the Tyr levels. The low levels of readily fermentable sugars in the MFGM medium led to low levels of acid production, which in turn led to a relatively high pH in the medium. As indicated by earlier literature, a low pH is a prerequisite for induction of AA-decarboxylating enzymes (Molenaar et al., 1993). However, acid production in the medium with added Glc, Man, or GlcNAc did not alone seem to induce Tyr breakdown by *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. In the current study, the depletion of Gln was observed in the sample with *Lb. paracasei* INF448 during its incubation

on MFGM medium, 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 medium.

It is known that Asn and water can be transformed into Asp and ammonia by L-asparaginase (Ardö, 2006) and the conversion of Asn to Asp has been observed in *Lb. plantarum* INF15D (Skeie et al., 2008a). All strains investigated here showed decreased levels of Asn, whereas levels of Asp increased when strains were grown on all monosaccharides used. It is likely that this occurred because of the action of L-asparaginase, as described by Ardö (2006). Levels of Asn decreased at the end of incubation in the MFGM medium. However, because of the apparent differences in peptidolytic activity among the studied strains, no conclusions could be reached concerning the end-product of Asn metabolism in the MFGM medium. This reaction does not seem to be involved in energy production, but it could be a way to regulate internal pH in the bacterial cell by release of ammonia.

CONCLUSIONS

In this study, we showed, by the production of metabolites throughout a 24-d incubation period, that the lactobacilli investigated were able to utilize components from the MFGM. We also observed an apparent connection between the higher proteolytic activity of *Lb. paracasei* INF448 and its ability to grow in the MFGM medium after depletion of readily available sugars. All studied strains produced large amounts of acetate when grown on an acylated aminosugar, presumably from the deacetylation of these monosaccharides during metabolism. Our results indicate that *Lb. plantarum* INF15D underwent a metabolic shift when grown on Gal, resulting in different fates of the pyruvate produced compared with growth on the other monosaccharides. For *Lb. plantarum* INF15D, the presence of Gal seemed to initiate degradation of some AA known to partake in energy production, specifically Arg and Tyr. Based on these results, further studies of *Lb. plantarum* INF15 grown on Gal could increase our knowledge of the potential energy sources in ripening cheese.

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