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Short communication

Characterisation of the microflora during ripening of a Norwegian semi-hard cheese with adjunct culture of propionic acid bacteria

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ABSTRACT

The microflora of a semi-hard, washed curd, Norwegian cheese with an added adjunct culture of propionic acid bacteria (PAB) was investigated throughout ripening by phenotypic and physiological tests, API test and 16S rRNA sequencing. Cheeses were made at two commercial Norwegian dairies using different milk treatments (pasteurisation versus microfiltration plus pasteurisation) and the same type of starter cultures. Microflora in the cheese varied according to different plant site, milk treatment, and ripening time. PAB dominated the microflora throughout the ripening process. *Leuconostoc* spp., most probably from the starter, dominated among the isolates from the cheese using microfiltered and pasteurised milk; however, after 40 weeks of ripening non-starter lactic acid bacteria specie *Lactobacillus casei/paracasei* and *Leuconostoc* spp. dominated at the dairy using pasteurised milk. Cheese made at the two plants on two subsequent days showed almost identical microflora throughout ripening.

1. Introduction

In Norway one of the most popular cheeses and also the Norwegian cheese with the greatest international recognition is a semi-hard, washed curd, Dutch-type cheese made with a mesophilic DL-starter with addition of an adjunct culture of propionic acid bacteria (PAB) (Abrahamsen, Byre, Steinsholt, & Strand, 2006). Mesophilic DL-starters are undefined mixed cultures composed of homofermentative *Lactococcus lactis* subsp. *lactis*, *Lc. lactis* subsp. *cremoris*, *Lc. lactis* subsp. *lactis* biovar. *diacetylactis* and the heterofermentative *Leuconostoc mesenteroides* subsp. *cremoris*. The two latter species metabolise citrate, producing diacetyl, acetoin and CO_2. Diacetyl may be important for the aroma of the cheese, and CO_2 is important for the eye formation (Hugenholtz, 1993). Dairy PAB are important for the typical extensive eye formation and for the development of the sweet, nutty flavour in Swiss-type cheeses. Both hard and semi-hard cheese varieties with PAB are available on the market, such as Emmental, Jarlsberg, Grevé and Maasdamer (Fröhlich-Wider & Bachman, 2004).

Since the nineties the role of the non-starter lactic acid bacteria (NSLAB) in cheese ripening has also been a focus in addition to the more traditional factors such as DL-starter bacteria, rennet and pH control. NSLAB may enter the cheese by surviving milk pasteurisation or through post-pasteurisation contamination in the dairy (Martley & Crow, 1993; Naylor & Sharpe, 1958; Reiter & Sharpe, 1971; Turner, Lawrence, & Lelievre, 1986).

In the literature, NSLAB flora of many cheese varieties has been reported (Bautista-Gallego et al., 2014; Beresford & Williams, 2004; Broadbent et al., 2013; Coolbear et al., 2008; Fitzsimons, Cogan, Condon, & Beresford, 1999; Jordan & Cogan, 1993; Lindberg, Christiansson, Rukke, Eklund, & Molin, 1996; Porcellato et al., 2012; Williams & Banks, 1997; Williams, Choi, & Banks, 2002). The microflora of Cheddar cheese has been examined most extensively in recent years and it consists mostly of mesophilic lactobacillus and sometimes pediococci and leuconostoc (Beresford & Williams, 2004; Coolbear et al., 2008; Crow, Curry, & Hayes, 2001; Desfossés-Foucault, LaPointe, & Roy, 2013; Fitzsimons et al., 1999; Martinovic et al., 2013; Peterson & Marshall, 1990; Singh & Singh, 2014; Williams et al., 2002). Much is also known about the development of the microflora in semi-hard, washed curd, Dutch-type cheese made with a mesophilic DL-starter. The NSLAB flora in Norwegian and Swedish semi-hard mature cheese varieties made with mesophilic DL-starter has been reported to consist mainly of the genus *Lactobacillus* and especially *Lactobacillus casei/paracasei* in mature cheese (Antonsson, Ardö, & Molin, 2001; Antonsson, Molin, & Ardö, 2003; Lindberg et al., 1996; Østlie,

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Eliassen, Florvaag, & Skeie, 2004; Østlie, Eliassen, & Skeie, 2005; Porcellato et al., 2012). In semi-hard cheese made with PAB addition, only the evolution of different microorganisms has been studied, and no characterisation or identification of NSLAB throughout ripening has been reported (Laht, Kask, Elias, Adamberg, & Paalme, 2002; Rehn et al., 2011; Sheehan, Wilkinson, & McSweeney, 2008).

The objective of this study was to compare the microflora over time in semi-hard, commercial Norwegian cheese made with the same type of DL-starter and the same PAB adjunct culture at two different Norwegian dairies with different milk treatments. One of the dairies used microfiltered, pasteurised milk and the other used only pasteurised milk. Conventional and PCR-based methods were used to identify the predominant NSLAB species during the overall ripening process in the cheeses.

2. Materials and methods

2.1. Cheese samples

Cheese samples from two commercial Norwegian dairy plants (A and B) located in the southern and middle part of Norway were analysed. Washed curd, brine-salted cheese was made with similar fat (27%) and dry matter (59%) content. The cheeses were made in the summer over two subsequent production days (dairy A: day 229 and 230; dairy B: day 228 and 229) to test short term stability of the NSLAB using a mesophilic DL-starter and an adjunct culture of PAB. Cheese coding according to dairy, production day and ripening time is shown in Table 1. Dairy A used microfiltered pasteurised milk and dairy B used pasteurised milk. The cheeses were made with the same type of DL-starter. Dairy A used the starter Probat Visbyvac 606 (Danisco, Copenhagen, Denmark; starter A) and dairy B used CHN-19 (Christian Hansen, Hørsholm, Denmark; starter B). The cheeses from both dairies were ripened for 1 week at 12 °C, 3 weeks at 21 °C, before storage at 4 °C for further maturation. All cheeses were taken out of the warm room 1 month after production. A 5-kg cheese wrapped in plastic foil was analysed after 1–3 days, 8, 24 and 40 weeks of ripening.

2.2. Microbiological sampling of cheese

Cheese sampling was done according to IDF standard 50C (IDF, 1995) using a cork borer (1–1.5 cm in diameter) before homogenising, diluting and plating according to Østlie et al. (2004). Aerobic mesophilic bacteria were enumerated on plate count agar (PCA, Oxoid Ltd, Basingstoke, Hampshire, England) after incubation for 4 days at 30 °C and lactococci were enumerated on M17 agar (Oxoid), after incubation for 2 days at 30 °C. Presumptive lactobacilli were enumerated on *Lactobacillus* selective agar (LBS agar, Becton Dickinson Microbiology Systems, Sparks, MD, USA) incubated anaerobically in anaerobic jars for 4 days at 30 °C (BBL GasPakPlus System, Becton Dickinson Microbiology Systems). PAB were enumerated on sodium lactate broth (Østlie, Vegarud, & Langsrud, 1995) with 1.5% (w/v) agar, sodium lactate agar (SLA), after anaerobic incubation in anaerobic jars for 5 days at 30 °C (BBL GasPakPlus System). The number of colonies developed in each medium was expressed as cfu g^{-1}.

2.3. NSLAB isolation

From LBS agar, 10 single colonies were randomly picked from each cheese after 8, 24 and 40 weeks of ripening. In total, 120 isolates were recovered from 12 cheeses made at both dairies, i.e., 2 cheeses and 20 isolates per dairy at each ripening time. All colonies were sub-cultured to purity on De Man Rogosa Sharpe agar (MRS; Difco Laboratories, Detroit MI, USA) before freezing and storing the cultures according to Østlie et al. (2004). The bacteria were inoculated (1%, v/v) in MRS broth before incubation overnight at 30 °C at least twice before use.

2.3.1. Phenotypic characterisation

Gram reaction, catalase reaction (3% H_2O_2) and the morphology of the isolates by phase contrast microscopy were investigated. Growth at 15 °C was studied in MRS broth up to 7 days. Ethanol and acetoin production were analysed after 18 h incubation in MRS broth at 30 °C by headspace gas chromatography (HSGC, HP Agilent 7694, Santa Clara, CA, USA) by the method of Narvhus, Osteraas, Mutukumira, and Abrahamsen (1998) as described by Østlie, Helland, and Narvhus (2003). Carbon dioxide production was determined after 18 h incubation at 30 °C in MRS by an infra-red (IR) gas analyser (ADC 225 Mk3, Analytical development Co. Ltd., Hoddesdon, Hertfordshire, UK) according to the method of Narvhus, Hulbækdal, Baugerød, and Abrahamsen (1991) as described by Østlie et al. (2003). Sugar fermentation of the isolates was investigated by using the API 50 CH system (BioMérieux, Marcy l'Etoile, France) and the results were analysed by the APILAB Plus version 4.0 program (BioMérieux). Acetic acid, lactic acid and propionic acid used for PAB identification were analysed by high performance liquid chromatography (HPLC, Perkin Elmer, Norwalk, CT, USA) using a modification of the method of Marsili, Ostapenko, Simmons, and Green (1981) as described by Østlie et al. (2003).

2.3.2. 16S rRNA sequence analysis

Aliquots of 5 mL of MRS broth, grown overnight at 30 °C, were centrifuged in a microfuge at 9300 × *g* for 10 min. The cells were resuspended in 100 μL of spheroplast buffer containing 10%

Table 1
Physiological and biochemical characteristics of the non-starter lactic acid bacteria flora isolated from cheese made at dairy A and B during ripening (% positive strains) and cheese coding.[a]

Dairy	Production day	Age of cheese (weeks)	Cheese coding	Number of isolates	Acetoin production (%)	Homofermentation (%)
A	229	8	A-229-8	8	12.5	25
		24	A-229-24	10	0	0
		40	A-229-40	10	10	12
	230	8	A-230-8	10	10	40
		24	A-230-24	10	10	10
		40	A-230-40	10	0	0
B	228	8	B-228-8	10	0	0
		24	B-228-24	10	40	40
		40	B-228-40	10	60	60
	229	8	B-229-8	12	0	16.7
		24	B-229-24	10	30	30
		40	B-229-40	10	40	40

[a] All isolates (100%) grew at 15 °C in MRS; a positive for acetoin production is defined as levels above 3 mg kg^{-1}, a positive for homofermentation is defined as below 1000 mg kg^{-1} CO_2 and 1000 mg kg^{-1} ethanol.