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Effect of mesophilic lactobacilli and enterococci adjunct cultures on the final characteristics of a microfiltered milk Swiss-type cheese

Yvette Bouton [a,*], Solange Buchin [b], Gabriel Duboz [b], Sylvie Pochet [b], Eric Beuvier [b]

[a] Comité Interprofessionnel du Gruyère de Comté, Avenue de la Résistance, 39801 Poligny, France
[b] INRA, UR342, Rue de Versailles, 39801 Poligny cedex 1, France

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ABSTRACT

The effect of four associations of adjunct cultures composed of mesophilic lactobacilli and enterococci, either solely or combined, on the microbiological, biochemical and sensory characteristics of Swiss-type cheese made using microfiltered cows' milk and supplemented with propionibacteria was studied. The global pattern of growth was similar to that generally observed in raw milk cheese and interactions between microflora were highlighted during ripening. Enterococci, which negatively affected the survival of streptococci starters, seemed to play a limited role in the formation of volatile compounds, probably due to their low levels throughout ripening. On the contrary, mesophilic lactobacilli, which affected the evolution of propionibacteria, enterococci and *L. delbrueckii* subsp. *lactis* starter counts, modified free amino acid content, production of volatile compounds and organoleptic properties of mature cheese. This population appeared to be of major importance in the formation of cheese flavor as it was positively related to numerous potential flavor compounds such as alcohols and their corresponding esters, acetaldehyde and 4-methyl-4-heptanone. The original mesophilic lactobacilli present in milk could play an important role in the sensorial diversity of raw milk Swiss-type cheeses such as Comte.

1. Introduction

The indigenous microflora in raw milk play an important role in the formation of the cheese flavor diversity (Beuvier and Buchin, 2004; Callon et al., 2005). In Swiss-type cheeses, it has been observed that the flavor intensity is correlated to counts of facultatively heterofermentative lactobacilli (FHL), propionibacteria (PAB) and enterococci, which occur naturally in raw milk (Beuvier et al., 1997). Among the principal cheese flavor compounds formation pathways, amino acid catabolism by cheese-related microorganisms is a major process (McSweeney and Sousa, 2000). Most of the studies dealing with the conversion of amino acids were carried out on Cheddar and Gouda and focused on *Lactococcus lactis* and mesophilic lactobacilli (Yvon and Rijnen, 2001; Kieronczyk et al., 2004). In Swiss-type cheeses such as Gruyère and Emmental, the role of *Proponibacterium* ssp. and thermophilic lactic acid bacteria in the production of flavor compounds is beginning to be elucidated (Helinck et al., 2004; Thierry et al., 2005). However, the contribution of other microorganisms present in these cheeses, namely mesophilic lactobacilli and enterococci in the formation of aroma compounds appears to have been overlooked. The aim of this work was to study the effects of using different adjunct cultures composed of mesophilic lactobacilli and enterococci on the development of the microbiological, biochemical -in particular volatile compounds- and sensory characteristics of a scaled-down Swiss-type cheese made using microfiltered milk and supplemented with propionibacteria.

2. Materials and methods

2.1. Cheese manufacture

Twelve pilot-scale Swiss -type cheeses were manufactured according to a factorial experimental design where two factors were studied: addition of non-starter FHL and addition of non-starter enterococci, resulting in four associations denoted p, ph, phe and pe (Table 1). All the cheeses were manufactured with an additional inoculum of *Propionibacterium freudenreichii* ssp. strain P52. Cheeses "ph" and "phe" contained an additional inoculum of *L. paracasei* Lc45 and *L. rhamnosus* Lr46 strains (equal ratios). Cheeses "phe" and "pe" contained an additional inoculum of *Enterococcus faecium* strain E45. Three batches of each association were manufactured on 3 different days for three associations (p, ph, phe) and on 2 days, for the fourth one (pe). Each vat contained 13 kg of standardized microfiltered cows' milk of the same origin, that is to say fat:protein = 1 with cream pasteurized at 72 °C for 30 s,

* Corresponding author. INRA-URTAL, Rue de Versailles, BP20089, 39801 Poligny cedex, France. Tel.: +33 3 8473 6324; fax: +33 3 8437 3781.
E-mail address: bouton@poligny.inra.fr (Y. Bouton).

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Table 1
Design of the cheese-making experiments.

Cheese trials	*P. freudenreichii* ssp. P52	Non-starter FHL[1]	Non-starter enterococci[2]
p	+	−	−
ph	+	+	−
phe	+	+	+
pe	+	−	+

[1] *L. paracasei* Lc45 and *L. rhamnosus* Lr46 strains (equal ratios).
[2] *Enterococcus faecium* strain E45, (+) addition, (−) no addition.

according to a hard-cooked cheese-making process previously described (Bouton et al., 1993). Milk was heated to 32 °C and inoculated with 0.2% of *Streptococcus thermophilus* starter cultures (equal ratios of strains S2 and S67), 0.15% of *Lactobacillus* starter cultures (equal ratios of *L. helveticus* L122 and *L. delbrueckii* subsp. *lactis* L131) and non-starter cultures were added at a level of 3 log cfu ml^{-1} of cheese milk. All the strains were obtained from the Research Unit for Dairy Technology and Analyses, INRA, Poligny, France. Lactic acid bacteria (LAB) starters were previously characterized and selected to give a suitable acidification and proteolysis (Bouton et al., 1993). Non-starter strains were isolated from a ripened Comté cheese made with raw milk of the same origin as that used in this study and were able to survive cooking during cheese manufacture (Bouton et al., 2000). Absence of growth inhibition between starter and non-starter strains was checked in culture media under temperature conditions similar to those in Comté cheese-making. Using the basal medium described by Joosten and Northolt, 1987, no *in vitro* ornithine, lysine, histidine, phenyalanine, tryptophane and tyrosine decarboxylating strains were detected among FHL while *E. faecium* strain E45 exhibited tyrosine decarboxylase activity. No deoxyribonuclease (Dnase) on methyl green-DNA agar (Batish et al., 1982), no β-hemolysis on 5% sheep blood agar (Giraffa et al., 1995) and no vancomycin resistance genes (Quednau et al., 1998) were observed for *E. faecium* strain E45. The 12 cheeses were ripened at 14 °C for 3 weeks, then placed in a warm room at 17–18 °C for 8 weeks, and finally stored in a cold room at 7 °C for 9 weeks.

2.2. Microbiological enumeration

Milk, starter and cheese samples were taken from each batch. Curds were analysed after 4 h of pressing and on day 1 and cheeses, after 3, 7, 11 and 20 weeks of ripening. Bacterial counts at the beginning of pressing were estimated by adding 1 log to the cell numbers in milk to take into account the concentration due to moulding and pressing. Ten grams of cheese were emulsified in sterile 4% (w/v) trisodium citrate and adjusted to a final concentration of 1/10 by adding sterile distilled water. The detection limit was 1 log cfu ml^{-1} in milk and 2 log cfu g^{-1} in cheese. Aerobic mesophilic bacteria counts were determined on plate count agar (PCA; Difco, Fisher Bioblock Scientific, Illkirch, France) supplemented with 0.5% sterilized milk, after incubation at 30 °C for 3 days. Thermophilic lactobacilli were enumerated on de Man Rogosa Sharpe agar (MRS; Difco) after incubation at 45 °C for 2 days under anaerobic conditions generated by a Merck Anaerocult kit (Dutscher, Brumath, France). *L. delbrueckii* ssp. cultures formed round, smooth, white colonies that could be distinguished from colonies of *L. helveticus* , which appeared rough with indented edges. After 3, 7, 11 and 20 weeks of ripening, an additional MRS plate agar incubated at 48 °C for 2 days under anaerobic conditions was used to prevent FHL from growing. Presumptive thermophilic streptococci were enumerated on M17 agar (Terzaghi and Sandine, 1975) incubated for 2 days at 45 °C. FHL counts were determined on selective agar for "Facultativ Heterofermentativen Laktobazillen" (FH) after incubation at 38 °C for 3 days (Isolini et al., 1990) under anaerobic conditions. To increase the selectivity of the medium, 40 g l^{-1} nalidixic acid was added. Coliforms were counted on Violet Red Bile Agar (Difco) incubated 24 h at 30 °C. Enterococci were enumerated on Slanetz and Bartley agar (m-*Enterococcus*; Difco) after incubation at 37 °C for 2 days. Propionibacteria (PAB) were enumerated on SLAC agar (Drinan and Cogan, 1992) after incubation at 30 °C for 6 days under anaerobic conditions.

2.3. Typing of isolates

Growth of starter and adjunct cultures were studied by identifying six to 10 isolates taken from MRS (incubated at 45 and 48 °C), M17, FH, m-*Enterococcus* and SLAC agar at each sampling point. A total of 692 isolates were taken from milk and cheese samples after 3 and 20 weeks of ripening from two batches of each association (p, ph, phe, pe). Total DNA of lactobacilli, streptococci and enterococci was extracted using the phenol-chloroform technique. The Instagen Matrix (Bio-Rad Laboratories, Hercules, CA, USA) was also used for lactobacilli. Rep-PCR was performed with REP primer pairs according to Berthier et al. (2001) and species-specific PCR was applied to representative isolates of each rep-PCR profile, as previously described (Bouton et al., 2002; Depouilly et al., 2004). Enterococci were identified from their fermentation profiles determined using the API 20 STREP (API Bio-Mérieux, Marcy l'Etoile, France) and from acid production in basal M17 broth (lacking lactose) supplemented with 1% (w/v) arabinose and 1.7% of bromocresol purple. *Propionibacterium* DNA samples were prepared according to Gautier et al., 1996. Genomic DNA digestion was carried out using 20 U restriction endonuclease XbaI for 8 h at 37 °C with the buffer indicated by the suppliers. Samples were electrophoresed through 1% agarose (Gibco BRL) with TBE buffer (Bio-Rad) at 200 V for 20 h with a 3–30 s pulse time at 14 °C in a Bio-Rad CHEF DRII electrophoresis cell. The band patterns of the gels were analysed with the Pearson correlation coefficient (r) and UPGMA by using Bionumerics v3.0 software (Applied Maths, Kortrijk, Belgium).

2.4. Chemical analyses

Chemical analyses were carried out after removing of 10 mm of rind using the methods described by Pochet, 1999. The pH was measured with a pH meter equipped with a KCl electrode. Dry matter content (DM, g.100 g^{-1} cheese) was determined by a gravimetric method. Fat content (g kg^{-1} cheese) was measured using an acido-butyrometric procedure. Sodium chloride content (g.100 g^{-1} moisture) was determined by a potentiometric method using a chloride analyser (Model 926, Corning limited, Halstead, Essex, Great Britain) equipped with soluble silver electrodes. Calcium content (g.100 g^{-1} dry matter) was determined using a complexometric method. d-/l-Lactic acid and citrate concentrations were determined from a water extract of cheese using Boehringer kits (ref 1112821 and ref 139076 respectively, Boehringer Mannheim, Meylan, France). Proteolysis in cheese was determined by trinitrobenzensuphonic acid method (TNBS, mM -NH_2 100 g^{-1} DM) as previously described (Bouton et al., 1993).

2.5. Analysis of free amino acids (FAA)

FAA were determined at 20 weeks of ripening as follows: 5 g grated cheese were added to 40 ml of 0.2 N sodium citrate buffer pH 2.2 containing 0.01% (w/v) pentachlorophenol, 0.02% (w/v) EDTA and 1.25 mM l-norleucine as an internal standard. The mixture was magnetically stirred for 15 min then homogenized with an Ultra-Turrax for 5 min at 13,500 rpm and centrifuged 20 min at 2 000 × g. After filtration on Whatman 2 V filter paper 10 ml of filtrate were diluted with the same volume of 7,5% (w/v) sulphosalicylic acid