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ORIGINAL PAPER

Biodiversity, dynamics, and characteristics of *Propionibacterium freudenreichii* in Swiss Emmentaler PDO cheese

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Abstract Propionibacteria are naturally present in raw milk at low levels, but little is known regarding the influence of these wild-type strains on cheese quality. To evaluate the prevalence of wild strains of propionibacteria in Emmental cheese, three cheeses were manufactured from the same raw milk using three commercial *Propionibacterium freudenreichii* starters (A, B, and C). A total of 165 isolates from the raw milk and 479, 436, and 476 isolates from cheeses A, B, and C, respectively, were typed by insertion sequence-restriction fragment length polymorphism or by multiple-locus variable number of tandem repeat analysis in order to determine the composition of the propionibacterial flora in the raw milk, in the curd, and in the cheeses after 2, 4, 6, and 8 months of ripening. Nine starter strains and 17 wild *P. freudenreichii* strains isolated from the curd and cheese were characterized with regard to their specific activity of aspartase and for their ability to grow at low temperature (11 °C). After 8 months of ripening, more than 80% of wild-type strains of *P. freudenreichii* were isolated from cheese A, whose starter strains had low aspartase activity. Although warm room storage (23 °C) stimulated the growth of various wild-type strains in all cheeses, strains of starters B and C, which had high aspartase activity or fast growth at 11 °C, remained dominant over the whole ripening period. In all cheeses, the growing wild-type strains exhibited high aspartase activity, indicating that this strain-specific property is a key factor in the control of propionic acid fermentation and in the prevention of late fermentation in Swiss-type cheeses.

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PDO 瑞士多孔干酪中 *Propionibacterium freudenreichii* 的生物多样性、动力学及特性

摘要 原料乳中存在着少量的丙酸菌量，而且人们对野生行丙酸菌对干酪质量影响的了解是非常有限。的了解价存在于瑞士多孔干酪中的野生丙酸菌的特性，以同样的原料奶为原料，使用了3种 *Propionibacterium freudenreichii* 商业发酵剂制造了3个干酪样品(A、B、C)。从原料奶中共分离出165个菌株，而从3个干酪样品A、B和C中分别分离出479、436和476个菌株，为了确定在原来奶、凝块以及成熟2、4、6、8月的干酪中丙酸菌菌群的组成，采用插入序列-限制片段长度多态性分析(IS-RFLP)或者多位点可变数目串联重复序列分析(MLVA)方法对上述菌株进行分型。以菌株特殊的天门冬氨酸酶活性以及在低温（11 °C）生长的能力为评价指标，从凝乳和干酪中分离获得了9个发酵剂菌株和17株野生 *P. freudenreichii* 菌株。成熟8个月后，从干酪A中分离出80%多的野生型 *P. freudenreichii*，而干酪A的发酵剂菌株具有较低的天门冬氨酸酶活性。尽管较高的贮藏温度(23 °C)刺激了在所有干酪中不同类型野生菌株的生长，但是干酪B和C中的发酵剂菌株具有较高的天门冬氨酸酶活性和在 11 °C下能够快速增长的特性，并且是整个成熟期内的优势菌群。在所有的干酪中，野生型菌株呈现出较高的天门冬氨酸酶活性，表明这些菌株的特性是这类瑞士多孔干酪中控制丙酸发酵酵和防止后发酵的关键因素。

Keywords Propionic acid bacteria · Aspartase activity · Emmentaler PDO cheese · MLVA · IS-RFLP

关键词 丙酸菌 · 天门冬氨酸活活性 · 瑞士PDO多孔干酪 · MLVA · IS-RFLP

1 Introduction

Starters of *Propionibacterium freudenreichii* are used in the manufacture of various Swiss-type cheeses and impart the characteristic nutty sweet flavor and eye formation (Fröhlich-Wyder and Bachmann 2004). In the traditional manufacture of Emmentaler PDO (Protected Designation of Origin) cheese, raw milk is inoculated with low concentrations (10^3-10^4 cfu.mL^{-1}) of *P. freudenreichii*. Commercial grades of Emmentaler PDO cheese, aged for 4, 8, or 12 months contain *P. freudenreichii* at a level of 10^8-10^9 cfu.g^{-1} (Steffen et al. 1987). Despite the use of starters of *P. freudenreichii,* the storage quality of Emmentaler PDO cheese also seems to be influenced by the composition and the numbers of naturally occurring propionibacteria present in raw milk.

The term “dairy propionibacteria” includes the species *P. freudenreichii*, *Propionibacterium acidipropionici*, *Propionibacterium jensenii*, and *Propionibacterium thoenii*, which are frequent constituents of the natural flora of raw milk. Various strain-specific characteristics, such as heat resistance, salt resistance, growth at cold temperatures, aspartase activity, amino acid catabolism, and interactions with lactic acid bacteria, complicate the prediction of growth and survival of individual strains during cheese ripening. Overall, *P. freudenreichii* shows the most resistance to cold and heat stresses, which may explain its dominance in hard cheeses with high scalding temperature (Chamba and Irlinger 2004). The minimal pH for growth is around 5.0–5.2 (optimum 6.0 to 7.5), while optimal growth temperature ranges from 25 °C to 35 °C. Park et al. (1967) reported that 11 out of 14 tested strains of *P. freudenreichii* grew at 7.2 °C.

Three different pathways are known to utilize lactate as an energy source, but only one of these pathways uses aspartate as an electron acceptor (Boyaval and Deborde 2000; Crow and Turner 1986). In cheeses with propionic acid fermentation,

the content of aspartate is always low (Sieber et al. 1988). The ability to metabolize aspartate and other free amino acids is associated with a stronger growth rate and therefore becomes a key factor in the selection of starters that will impart a good storage quality to Emmentaler PDO cheese (Fröhlich-Wyder and Bachmann 2004; Piveteau et al. 1995), Fröhlich-Wyder et al. (2002) showed that systems that included starters of *P. freudenreichii* with moderate aspartase activity and starters of lactic acid bacteria with limited proteolytic activity, as well as adjunct cultures, allowed a good control of propionic acid fermentation. In practice, the introduction of these systems resulted in a drastic reduction of cases of late fermentation during the long-term ripening of Emmentaler PDO cheese. Propionibacteria are naturally present in raw milk at concentrations of 10^1-10^3 cfu.mL^{-1}, and little is known regarding their influence on Emmentaler PDO cheese quality (Fessler et al. 1997; Fessler et al. 1998).

A better understanding of the influence of wild-type strains on cheese quality and of the behavior of multi-strain starters of *P. freudenreichii* during cheese ripening would allow a more systematic development of suitable starters. The objective of this study was therefore to investigate the biodiversity and composition of the propionibacterial flora in raw milk and in corresponding Emmentaler PDO cheeses and to study the characteristics of individual strains with regard to their growth during cheese ripening. For this purpose, two established typing methods, insertion sequence-restriction fragment length polymorphism (IS-RFLP; Petrovic et al. 2006; Ricci and Fortina 2006) and multiple-locus variable number of tandem repeat analysis (MLVA; Gierczynski et al. 2007; Quénée et al. 2005), were used to allow the typing of propionibacterial strains originating from raw milk and starter cultures.

2 Materials and methods

2.1 Cheese manufacturing and sampling

Three loafs of Emmentaler PDO cheese, each weighing about 90 kg, were manufactured in separate vessels from the same raw milk, but with different commercial starters (A, B, C) of *P. freudenreichii.* The cheeses were manufactured in the experimental cheese factory of the Agroscope Liebefeld-Posieux research station according to requirements listed by the Emmentaler PDO association. Briefly, the fat content of the cheese milk was adjusted by adding skimmed raw milk (225 L) to the raw milk (775 L). After the addition of water (100 L), the cheese milk was warmed in a copper vessel to 32 °C over a 30-min period and then maintained at this temperature for 30 min. Cheese milk in all three vessels was then inoculated with 2.8‰ (*v*/*v*) of a bulk starter containing strains of *Streptococcus salivarius* subsp. *thermophilus* and *Lactobacillus delbrueckii* subsp. *lactis* and 0.2‰ (*v*/*v*) of an adjunct culture containing strains of *Lactobacillus casei*. After pre-ripening (32 °C, 20 min), each milk batch was inoculated with five drops of a different commercial *P. freudenreichii* starter (starter A, B, or C) at a level of approximately 10^3 cfu.mL^{-1} to produce cheeses A, B, and C, respectively. Starters A, B, and C were composed of two, four, and three strains, respectively, from the ALP collection (Table 1). Milk was coagulated in approximately 35 min with a microbial coagulant derived from the fungus *Cryphonectria parasitica*, and the coagulum was cut into grains of about

Table 1 Partial characterization of *P. freudenreichii* strains present in starters A, B, and C

Starter	Strain code (collection ALP)	Genotype	Specific activity of aspartase[a] ($\mu mol.min^{-1}.mg^{-1}$ protein)	Growth at 11 °C[b] (OD_{578} after 24 days)
Starter A	FAM 14176[c]	A1	1.045±0.013	0.126±0.005
	FAM 14177[c]	A1	0.323±0.009	0.356±0.102
Starter B	FAM 14217[d]	B1	32.532±0.117	0.120±0.001
	FAM 14218[d]	B1	41.450±0.179	0.642±0.514
	FAM 14221[e]	B3	1.427±0.015	0.303±0.198
	FAM 14222[e]	B4	1.524±0.024	0.036±0.005
Starter C	FAM 14184	C1	8.087±0.011	1.465±0.168
	FAM 14197	C2	6.209±0.024	1.914±0.086
	FAM 14193	C3	0.269±0.006	0.376±0.106

[a] Mean value±SD from three culture replicates

[b] Mean value±SD from two culture replicates

[c] Strains with identical IS-RFLP and MLVA profiles

[d] Strains with identical IS-RFLP and MLVA profiles

[e] Strains with identical MLVA profile but different IS-RFLP profiles

0.3–0.5 cm in 15 min. After the addition of water (5%), the mixture of curd grains and whey was warmed up to 53 °C over 30 min, scalded at 51–52 °C for 30 min, poured into moulds, pressed (50–35 °C, 1 day), and brine-salted (15 °C, 2 days). After manufacture, the cheeses were initially stored at cold temperature (12–14 °C) for 7 days, then stored in a warm room (22 °C) for 50 days, and finally further ripened at cold temperature (11 °C) up to the age of 8 months. During manufacturing, samples were collected of *P. freudenreichii* starters, raw milk, and curd grains before and after scalding. Cheese samples were taken at a distance of 12 cm from the border of the loaves at the age of 2, 4, 6, and 8 months.

2.2 Enumeration and isolation of propionibacteria

For enumeration of propionibacteria in raw milk, samples were plated onto sodium lactate agar containing 24 $mL.L^{-1}$ sodium lactate solution (Merck, Dr. Grogg Chemie AG, Stettlen, Switzerland), 30 $g.L^{-1}$ Casein-Peptone (Merck), 30 $g.L^{-1}$ yeast extract (Becton Dickinson AG, Allschwil, Switzerland), 12 $g.L^{-1}$ Agar-agar (Oxoid AG, Pratteln, Switzerland), pH 6.8±0.2. The plates were incubated under anaerobic conditions at 30 °C for 9–10 days.

For the recovery of the maximal number of genotypes from raw milk, six samples of 1 mL and nine samples of 100 μL were directly plated onto sodium lactate agar. In addition, three samples each of 100 μL raw milk were first incubated under anaerobic conditions for 48 h at 30 °C in 10 mL sodium lactate broth, then the suspension was plated onto sodium lactate agar and incubated as described above. A total of 165 propionibacterial isolates originating from raw milk were collected and genotyped.

The bacterial count of *P. freudenreichii* starters A, B, and C were determined after incubation of diluted starters on sodium lactate agar. For the determination of the strain-specific composition of the multi-strain starters of *P. freudenreichii*, two series of 40 isolates, randomly picked from two plates of the same dilution, were genotyped and the percentage of individual starter strains was calculated.

For the isolation of *P. freudenreichii* strains from curd grains and cheeses, 10-g samples were homogenized with 90 mL of peptone water (Merck) for 3 min in a stomacher. Diluted suspensions were plated onto sodium lactate agar and incubated as described above. For each sample, two series of 40 isolates were randomly picked from two plates of the same dilution. Prior to genotyping, the isolates of raw milk, curd grains, and cheeses were cultured a second time on sodium lactate agar and then grown in sodium lactate broth. The exact number of genotyped isolates per sample varied slightly from sample to sample. In some cases, fewer than 80 isolates could be picked from two plates of the same dilution, and in other cases not all of the isolates could be attributed to dairy propionibacteria after identification.

2.3 Identification of propionibacteria isolates and molecular genotyping of strains by IS-RFLP and MLVA

The propionibacteria genus was identified based on the polymerase chain reaction (PCR) amplification of 16S rRNA gene sequence as previously described by Rossi et al. (1999) and species were identified according to Fessler et al. (1999). From each sample, 80 isolates were typed using two different methods. One set of 40 propionibacterial isolates was typed by IS-RFLP while the other set of 40 isolates from a second plate was typed by MLVA.

IS-RFLP assay The cultured propionibacteria isolates were harvested by centrifugation (18200×*g*, 10 min, 20 °C) of 2 mL propionibacterial suspension, and the pellet was resuspended in 0.5 mL Tris-EDTA-sucrose (TES) buffer (0.1 M Tris–HCl, 10 mM EDTA, 25% sucrose, pH 8.0) and incubated (1 h, 37 °C) with 2.5 mg lysozyme (Merck). After centrifugation, DNA was extracted according to the manufacturer's instructions with the BioRobot EZ1 workstation (BioRobot® QIAGEN Instruments AG, Hombrechtikon, Switzerland). A 600-ng sample of DNA was incubated (2 h, 37 °C) with 10 U of the *Pst*I restriction enzyme (Roche Diagnostics AG, Rotkreuz, Switzerland). The DNA fragments were separated on a 0.7% agarose gel (Roche Diagnostics) in 0.5× Tris-borate-EDTA (TBE) buffer (80 V, 3 h, 4 °C) and subsequently fixed on a positively charged nylon membrane (Roche Diagnostics). Further steps were performed as described in the user's guide (The DIG System User's Guide for Filter Hybridization, Boeringer Mannheim 1995, Mannheim, Germany). Probe primers (Table 2) were designed based on two different insertion sequences present in the sequenced genome (sequenced by 454 Life Sciences, Brandford, CT, USA) of strain FAM 14176 from starter A using the DS Gene 1.5 software (Accelrys Ltd., Cambridge, UK). The probes were amplified in one reaction using a PCR DIG Probe Synthesis Kit (Roche Diagnostics) according to manufacturer's instructions (T_{an}=62 °C). Conversion, normalization, and further analysis of the patterns were carried out with the GelCompare II software (Applied Maths, Sint-Martens-Latem, Belgium).

Table 2 List of primers designed for IS-RFLP and MLVA assay

Primers for probes c14-D and c30-D on transposase genes for IS-RFLP assay	
c14-DF	5′-ccc gaa gca cgt acg ggt a
c14-DR	5′-cca cga ggt ccg tcg tca t
c30-DF	5′-ccg aca acg gca tca tct aca
c30-DF	5′-cgt cgt gcc agt gag gtg t
Primers framing tandem repeats for MLVA assay	
MS22-F	5′-cct gcg cta ggc ctc atc a
MS22-R	5′-cga cca gcg atg agt cct ga
MS23-F	5′-ggg ttg ggc caa gct gct
MS23-R	5′-gcg act acg cca tcc tgc t
MS26-F	5′-gcg aac gca tcc gca tcg aa
MS26-R	5′-gga cat ggt gga cgg ttg t
MS28-F	5′-cgt ccc tat ctc gac cag at
MS28-R	5′-cga gga aac gtc acg gac ga
MS29-F	5′-ctc act tgt ggg ttc cac aga
MS29-R	5′-gtc aca ccc cat gag ctt gtt
MS12-F	5′-ggg cgt cgg ggc tag act t
MS12-R	5′-cgt tca gcc atc gtc ccc act t
MS27-F	5′-cga acc cga ggg caa gga tt
MS27-R	5′-cga agg tcc tgc act cga a

MLVA assay Propionibacteria were harvested as described above. The pellet was resuspended in 0.16 mL TES and incubated (1 h, 37 °C) with 1 mg lysozyme, 0.58 mg proteinase K (Macherey-Nagel AG, Oensingen, Switzerland). Then, 0.1 mg RNAse (Macherey-Nagel) was added, followed by further incubation (10 min, 37 °C). Total genomic DNA was extracted using the NucleoSpin 96 Tissue kit (Macherey-Nagel). The primers for tandem repeats (Table 2) were designed using the tandem repeats finder software (online at http://minisatellites.u-psud.fr/) from Quénée et al. (Quénée et al. 2005) and seven selected loci of tandem repeats combined with multiplex PCR, as described by Henegariu et al. (Henegariu et al. 1997). PCR was performed in a total volume of 25 μL including 2 μL target DNA (40–200 ng), 2 U AmpliTaq Gold (Roche Diagnostics), 2.5 μL 10× PCR buffer containing 15 mM $MgCl_2$ (Roche Diagnostics), 0.5 μL PCR nucleotide mix (10 mM; Promega AG, Dübendorf, Switzerland), 0.9 μL 50 mM $MgCl_2$ (Eurogentec, Geneva, Switzerland), 2.5 μL primer mix (4.0 μM of primer MS22, 1.4 μM of primer MS23, 1.4 μM of primer MS26, 2.8 μM of primer MS28, 6.6 μM of primer MS29, 3.7 μM of primer MS12, 3.7 μM of primer MS27), and distilled water. The amplification was carried out at 95 °C for 10 min, followed by 35 cycles at 95 °C for 1 min, 60 °C for 30 s, 72 °C for 30 s, and terminated by one cycle at 72 °C for 7 min. The PCR products were visualized with an Agilent 2100 bioanalyzer using an Agilent DNA 1000 Kit, according to manufacturer's instructions (Agilent Technologies AG, Basel, Switzerland).

2.4 Growth of *P. freudenreichii* strains at cold storage temperature

Individual strains of *P. freudenreichii* isolated from raw milk and cheese were assessed for their ability to grow at 11 °C as follows: Individual strains were first reactivated on a lactate broth (24 mL sodium lactate syrup (Merck 6522), 30 g peptone from casein (Merck 7213), 30 g yeast extract (BBL 11929, Becton Dickinson AG, Allschwil, Switzerland), 976 mL distilled water; pH adjusted to 6.8±0.2; autoclaved for 15 min at 121 °C) at 30 °C for 6 days. Then, the same broth was inoculated at a level of 1% and incubated at 30 °C for 4 days. Finally, tubes containing peptone whey broth (10 g peptone from casein (Merck 7213), 4 g yeast extract (BBL 11929), 166 mL of concentrated acid whey, 834 mL distilled water, pH 5.5; autoclaved for 15 min at 121 °C) were inoculated in duplicate with the pre-culture at a level of 1% and incubated at 11 °C for 24 days. The tubes were vortexed once a day, and growth of the individual strains was monitored by measuring optical density (OD) at 578 nm. The OD_{578} indicated in Table 1 (starter strains) and Table 3 (wild-type strains) represent the final values obtained after 24 days.

2.5 Enzyme analyses

The specific activity of aspartase (L-aspartate ammonia-lyase, EC 4.3.1.1) of the individual strains of *P. freudenreichii* was determined in three culture replicates as follows: A 50-mL volume of sodium lactate broth was inoculated with 1% of a pre-cultured strain. To provide anaerobic conditions, inoculated broth was then placed together with GENbox Anaer generator sachets (BioMérieux, Lyon, France) inside a sealed polycarbonate culture jar (GasPak Anaerobic System, Becton Dickinson AG, Switzerland).

After an incubation at 30 °C for 4 days, bacterial cells were harvested by centrifugation (4000×*g*, 10 min, 20 °C), washed twice with 20 mM HEPES–NaOH (pH 7.4), suspended in 500 μL 20 mM HEPES–NaOH (pH 7.4), and transferred into a 2-mL screw-capped micro-centrifuge tube containing approximately 0.4 g glass-beads (212 to 300 μm, Sigma-Aldrich, Buchs, Switzerland). Tubes were then shaken in the Mini-Beadbeater-8 (Biospec Products, Bartlesville, USA) using six cycles (10 s on at the highest speed followed by 10 s off-time). The extract was cleared by centrifugation (17,900×*g*, 10 min, 4 °C), and metabolites were removed using NAP-5 columns (GE HealthCare, Uppsala, Sweden), which had been equilibrated with 20 mM HEPES–NaOH (pH 7.4) and 5 mM $MgCl_2$ according to the manufacturer's instructions. Protein concentration was determined using a bicinchoninic acid assay with bovine serum albumin as a standard, according to (Smith et al. 1985). Aspartase activity of the cell-free extracts was measured in duplicate in a 2-mL volume containing 50 mM HEPES–NaOH (pH 7.4), 30 mM L-aspartate, 5 mM $MgCl_2$, and 200 μL of the cell-free extract at 24±1 °C. The formation of fumarate was followed by measuring the increase in absorbance at 240 nm. The molar extinction coefficient of 2,530 $M^{-1}.cm^{-1}$ was used to calculate the amount of fumarate produced (Emery 1963). The specific activity of aspartase is defined as the formation of fumarate per minute per milligram protein.

Table 3 Frequency and partial characterization of wild-type propionibacteria obtained from raw milk, curd grains, and ripened cheeses

Genotype	Raw milk (n=165)	Curd grains and ripened cheeses (n=282)	Specific activity of aspartase[a] ($\mu mol.min^{-1}.mg^{-1}$ protein)	Growth at 11 °C[b] (OD_{578} after 24 days)	Species
WT1	39	15	0.169±0.003	2.043±0.091	*P. freudenreichii*
WT2[c]	1	n.d.	n.a.	n.a.	*P. acidipropionici*
WT3	23	101	35.084±0.031	0.903±0.791	*P. freudenreichii*
WT4	4	n.d.	166.014±0.500	0.010±0.003	*P. freudenreichii*
WT5	3	n.d.	0.331±0.004	0.105±0.033	*P. freudenreichii*
WT6[d]	2	2	0.252±0.005	0.273±0.093	*P. freudenreichii*
WT7[d]	28	82	0.258±0.002	0.545±0.282	*P. freudenreichii*
WT8[c]	2	n.d.	n.a.	n.a.	*P. jensenii*
WT9[c]	2	n.d.	n.a.	n.a.	*P. acidipropionici*
WT10	7	1	8.733±0.038	0.012±0.002	*P. freudenreichii*
WT11	2	n.d.	4.207±0.008	0.957±0.822	*P. freudenreichii*
WT12[c]	2	n.d.	n.a.	n.a.	*P. jensenii*
WT13	17	77	6.021±0.027	0.985±0.704	*P. freudenreichii*
WT14[e]	n.d.	1	2.266±0.012	0.269±0.179	*P. freudenreichii*
WT15[d]	n.d.	1	n.a.	n.a.	*P. freudenreichii*
WT16	n.d.	1	6.630±0.010	0.731±0.630	*P. freudenreichii*
WT17	n.d.	1	1.907±0.012	0.074±0.003	*P. freudenreichii*
Others[f]	33	n.d.	n.a.	n.a.	*P. jensenii*

n.d. not detected, *n.a.* not analyzed

[a] Mean value±SD from three culture replicates

[b] Mean value±SD from two culture replicates

[c] No profile was obtained for these genotypes with the MLVA assay

[d] Genotypes with identical MLVA profiles, but different IS-RFLP profiles

[e] Genotype with an identical MLVA profile as starter strains B3 and B4, but a different IS-RFLP profile

[f] No profile was obtained for these genotypes either by MLVA or IS-RFLP

The identity of the product was confirmed as fumarate by high-performance liquid chromatography. For this purpose, the enzymatic reaction was stopped by the addition of 2 μL of 380 mM H_2SO_4 and the mixture heated at 95 °C for 5 min. Samples were then cleared by centrifugation and filtered through disposable filter holders (0.45 μm). The filtrate was separated on a 300×7.8 mm ion exchange column (HPX-87 H Aminex; Bio-Rad) protected with a cation H+Microguard cartridge. The operating conditions were as follows: flow rate of 0.6 $mL.min^{-1}$, 65 °C, and detection at 210 nm. The mobile phase was 3.8 mM H_2SO_4. Fumarate was identified by comparing its retention time with that of a known standard (Sigma-Aldrich, Switzerland).

D- and L-lactate, succinate, aspartate, and asparagine were analyzed enzymatically according to the instructions of the kit manufacturer (Roche Diagnostics).

2.6 Chemical analyses

Quantitative determination of free amino acids in cheese was performed as described by Bütikofer and Ardö (1999). Determination of volatile carboxylic acids in cheese was performed according to de Jong and Badings (1990).

3 Results and discussion

3.1 Typing of *P. freudenreichii* by IS-RFLP and MLVA

Two different methods, IS-RFLP and MLVA, were used to genotype the isolates, in order to discriminate *P. freudenreichii* strains originating from starters and raw milk and to investigate the biodiversity of the propionibacterial flora during cheese ripening. The combined use of two methods allowed discrimination of 17 wild-type strains of *P. freudenreichii* originating from raw milk and cheese. Nevertheless, the nine strains present in starters A, B, and C, generated only seven different patterns. Our MLVA assay with seven loci of tandem repeats was less discriminatory for strains of *P. freudenreichii* than was typing by IS-RFLP. However, typing by MLVA required less DNA, and the PCR products could be readily analyzed with the Agilent 2100 bioanalyzer, whereas typing by IS-RFLP was very labor-intensive and time-consuming and required a large and standardized quantity of good-quality DNA for PCR (Petrovic et al. 2006).

The use of the sequenced genome of the starter strain FAM 14176 for the development of the probes for IS-RFLP and MLVA resulted in a high discriminatory power for strains of the species *P. freudenreichii* but also caused a partial failure of the two probes for the typing of strains of other species. A total of 33 isolates from raw milk belonging to the species *P. jensenii* could not be typed by IS-RFLP due to the absence of the probe sequences, whereas typing by MLVA yielded specific PCR products only for strains of *P. freudenreichii* (Table 3 and Fig. 2: WT2, WT8, WT9, WT12).

3.2 Stability of IS-RFLP and MLVA patterns

The MLVA and IS-RFLP assay operates with unstable regions of the DNA genome. For this reason, we investigated the stability of the tandem repeats and insertion sequences in four starter strains after 22 passages. The MLVA patterns of 40 clones each of the starter strains FAM14177, FAM14217, FAM14221, and FAM14222 revealed no changes. This finding corresponded with that of Gierczynski et al. (2007), who also observed no changes for seven tandem repeats in *Yersinia enterocolitica* subsp. *palearctica* after 20 passages. In contrast, IS-RFLP patterns showed rearrangements in two clones of FAM14177, in one clone of FAM14217, in two clones of FAM14221, and in three clones of FAM14222. Rearrangements of insertion sequences after growth cycles have been reported in other studies on *Lactobacillus sanfranciscensis* and *Mycobacterium avium* (Bauer and Andersen 1999; Nicoloff and Bringel 2003). Whether these rearrangements occur at the same frequency during the growth of *P. freudenreichii* in cheese as was observed for the

investigated starter strains after 22 passages remains unclear. In the present study, four wild-type strains (WT14–17) were found in cheese, but not in raw milk, and each of these was represented by only a single isolate. Although the possibility cannot be excluded that these genotypes represent clones of starter or wild-type strains with rearrangements of insertion sequences, it seems more likely that these genotypes were present at low concentrations in the raw milk.

3.3 Biodiversity of propionibacteria in raw milk, starters, and Emmentaler PDO cheese

Bacterial communities in fermented foods are usually investigated by culture-dependent methods. Recently, Falentin et al. (2010) developed a culture-independent assay for quantification of *P. freudenreichii* and *Lactobacillus paracasei* in Emmental cheese using real-time reverse transcription PCR. Although the culture-independent assay was shown to yield similar results when compared with plate count assays, the study concluded that plate counts were more accurate than qPCR for cell concentrations below 10^3 cells/g of food. In the present study, sodium lactate agar was used for the initial growth step since this medium was previously shown to enable growth of all species of dairy propionibacteria (Fessler et al. 1998).

Among the 165 isolates from raw milk, a total of 13 wild-type strains (WT1–WT13) could be discriminated that belonged to the species *P. freudenreichii* (WT1, WT3-WT7, WT10-WT11, WT13), *P. acidipropionici* (WT2 and WT9), and *P. jensenii* (WT8, WT12 and others genotypes that could not be typed). The IS-RFLP and MLVA patterns of the individual genotypes are shown in Figs. 1 and 2,

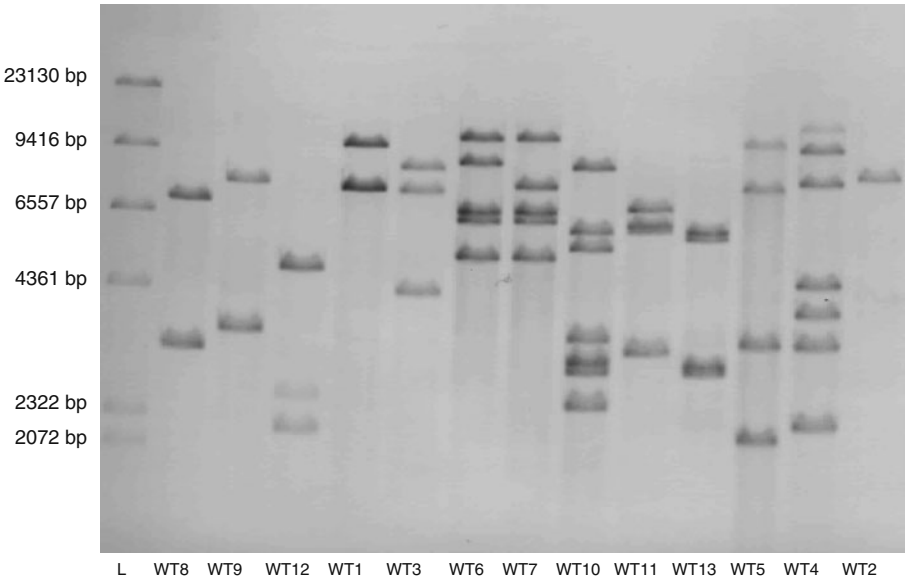

Fig. 1 IS-RFLP profiles of wild-type propionic acid bacteria obtained from raw milk. A total of 13 different genotypes (WT1–WT13) could be discriminated with the IS-RFLP assay (*first lane*: DNA Molecular-Weight Marker II, DIG-labeled, 0.12–23.1 kbp)

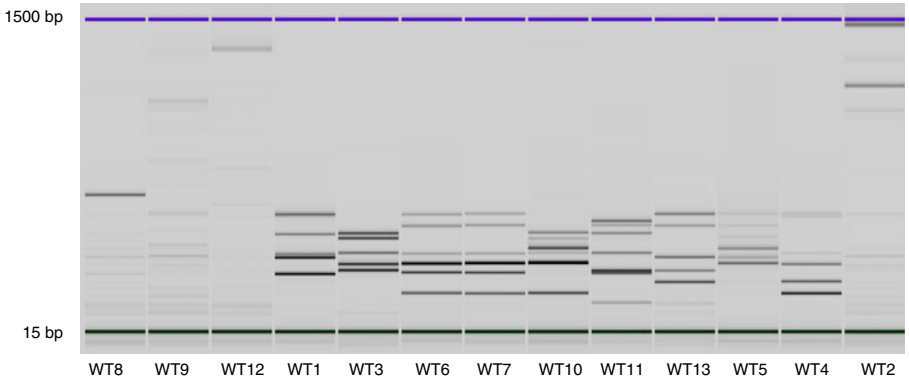

Fig. 2 MLVA profiles of wild-type propionic acid bacteria obtained from raw milk (WT1–WT13). In contrast to the profiles obtained by IS-RFLP (Fig. 1), the MLVA profiles of WT6 and WT7 were identical. MLVA yielded only unspecific PCR products for strains of *P. acidipropionici* (WT2, WT9) and *P. jensenii* (WT8, WT12)

respectively. In contrast to IS-RFLP, the MLVA patterns of the strains WT6 and WT7 were not distinguishable. The frequency of the individual genotypes in raw milk is indicated in Table 3.

A total of seven MLVA and IS-RFLP genotypes were obtained for the nine strains present in starters A, B, and C (Table 1). The IS-RFLP and MLVA patterns of the two strains FAM 14176 and FAM 14177 present in starter A showed identical patterns (both genotype A1). Similarly, the two strains FAM 14217 and FAM 14218 present in starter B could not be discriminated (both genotype B1). According to the entries in the database of the strain collection of the ALP research station, each of these two pairs contained strains that were historically isolated from the same source and thus probably represent duplicates. The two strains of FAM14221 and FAM14222 from starter B showed identical MLVA patterns but could be distinguished by IS-RFLP (results not shown).

Of the 1,391 isolates obtained from samples of curd grains and ripened cheeses, 1,109 isolates (80%) could be attributed to the seven genotypes of the starter strains, whereas 282 isolates (20%) represented wild-type strains (Table 3). The MLVA and IS-RFLP patterns of these 282 isolates allowed the identification of ten different genotypes, of which six genotypes (WT1, WT3, WT6, WT7, WT10, and WT13) could be assigned to the genotypes found in raw milk. With the exception of WT6, the other five genotypes constituted the most frequent strains of *P. freudenreichii* in the propionibacterial flora of the raw milk. In addition, four new genotypes (WT14-17) were found in ripened cheeses. The MLVA pattern of WT14 was identical with the pattern of the starter strains B3 and B4, but its IS-RFLP pattern was different (results not shown). Similarly, genotype WT15 could be discriminated from WT6 and WT7 only by IS-RFLP. The species identification of the ten genotypes revealed that all wild-type strains isolated from curd grains and ripened cheeses belonged to the species *P. freudenreichii*.

These results confirmed that only *P. freudenreichii* strains survived the manufacturing conditions of Emmentaler PDO cheese. This result could be due to the high heat resistance of *P. freudenreichii*, compared with the other dairy propionibacteria (Chamba and Irlinger 2004; Fröhlich-Wyder and Bachmann

2004). The inoculation of cheese milk with low concentrations (10^3-10^4 cfu.mL^{-1}) of *P. freudenreichii* is still the usual practice for the manufacture of Swiss Emmentaler PDO cheese. Due to the use of raw milk and the low inoculation level, the numbers and biodiversity of propionibacteria in raw milk constitute important factors that may influence the storage quality of Emmentaler PDO cheese.

3.4 Characterization of propionic acid fermentation

The results of the microbial and chemical characterization of the propionic acid fermentation in the three investigated cheeses are summarized in Fig. 3 and Tables 4 and 5. During warm room storage, the number of propionibacteria markedly increased up to a typical level of 10^8 cfu.g^{-1} in all three of the investigated cheeses. However, slight differences were observed after a ripening period of 2 months among the three cheeses. The number of propionibacteria slightly decreased in cheese A, but remained rather constant in cheeses B and C during further ripening. The results of the partial chemical characterization of the three cheeses after a ripening period of 6 months confirmed the lower intensity of propionic acid fermentation in cheese A, which showed marked lower levels of propionate and acetate than were found in cheeses B and C (Table 4). Similarly, Fröhlich-Wyder and Bachmann (2004) reported that 12 month aged cheeses made with two different *P. freudenreichii* starters with weak and strong aspartase activity had an average concentration of 63.2 and 83.6 mmol.kg^{-1} propionate, respectively.

The determination of the concentration of the substrates and products of propionic acid fermentation clearly indicated that the higher intensity and ongoing propionic acid fermentation in cheeses B and C were related to aspartate metabolism. Operation of this metabolic pathway results in significantly lower concentrations of aspartate, asparagine, and lactate and increased concentrations of succinate, acetate, and propionate (Table 5). As a result of the ongoing propionic acid fermentation, cheeses B and C showed slight defects in late fermentation after a ripening period

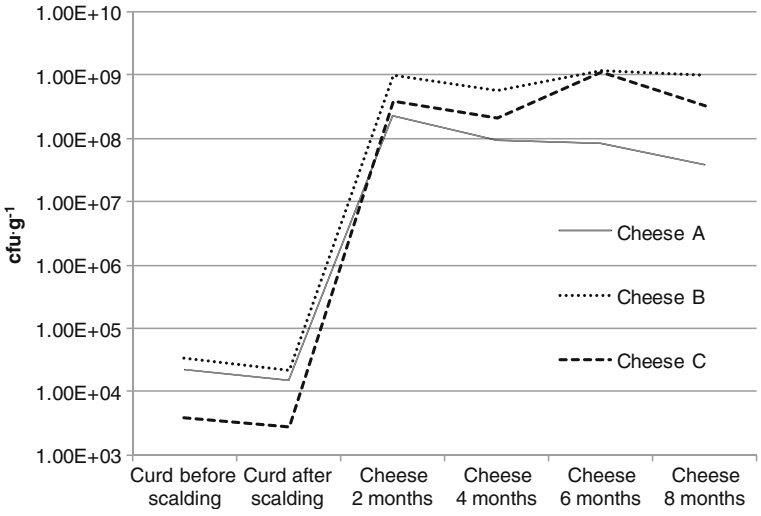

Fig. 3 Enumeration of propionibacteria in curd grains and cheese during ripening

Table 4 Partial chemical characterization of cheeses A, B, and C after 6 months of ripening[a]

	Cheese A	Cheese B	Cheese C
Free amino acids	196.0	170.0	189.0
Total volatile carboxylic acids	102.8	125.7	123.8
Formate	4.7	4.1	4.2
Acetate	42.0	51.9	49.6
Propionate	54.0	68.6	69.0
Butyrate	1.9	0.9	0.9
pH value	5.65	5.62	5.71

[a] Values of free amino acids and volatile carboxylic acids in millimoles per kilogram

8 months. In all three cheeses, the L-(+) isomer of lactate was fermented in preference to the D-(−) isomer (data not shown), which is in good agreement to the findings of Crow (1986).

3.5 Changes in the composition of the *P. freudenreichii* flora during cheese ripening

The *P. freudenreichii* flora present in the three individual Emmentaler PDO cheeses made from starters A, B, and C was investigated during manufacture and ripening up to the age of 8 months (Table 4 and Fig. 4). In all three cheeses, warm room storage (22 °C) favored the growth of wild-type strains. However, during the subsequent cold storage (11 °C), the three cheeses exhibited marked differences with respect to the number of genotypes and the proportion of starter and wild-type strains. In cheese A, the proportion of wild-type strains reached 81% after 8 months of ripening. This strong increase in wild-type strains in the flora of cheese A could have resulted from a decrease in the starter strains and the growth of more adapted wild-type strains during ripening. Surprisingly, starter strain C3 was also found in low concentrations in cheese A, indicating that a cross-contamination had occurred during the simultaneous manufacture of the three cheeses. In cheese B, scalding markedly reduced the share of the starter strain B4. In contrast to cheese A, the share of wild-type strains in cheese B decreased continuously during cold room storage. After a ripening period of 8 months, 100% of the isolates could be attributed to the starter strains B1 (95%) and B3 (5%). The drastic changes in the composition of the *P. freudenreichii* flora in cheese B indicated that genotype B1 was best adapted to the manufacturing and ripening conditions. In cheese C, cold storage resulted in a

Table 5 Changes in asparagine, aspartate, succinate, and lactate during ripening of Emmentaler PDO cheeses made with *P. freudenreichii* starters A, B, and C (in millimoles per kilogram)

	2 months			4 months			6 months			8 months		
	A	B	C	A	B	C	A	B	C	A	B	C
Asparagine	4.5	0.7	4.6	6.9	2.9	5.4	7.6	4.9	5.6	9.4	2.8	4.9
Aspartate	1.3	1.3	1.1	1.2	0.8	1.2	1.0	0.8	1.0	1.6	0.7	1.3
Succinate	1.3	4.1	2.5	2.3	6.3	5.0	3.4	7.1	6.6	4.1	10.9	8.6
Lactate	87	82	93	76	66	56	68	67	44	67	31	15

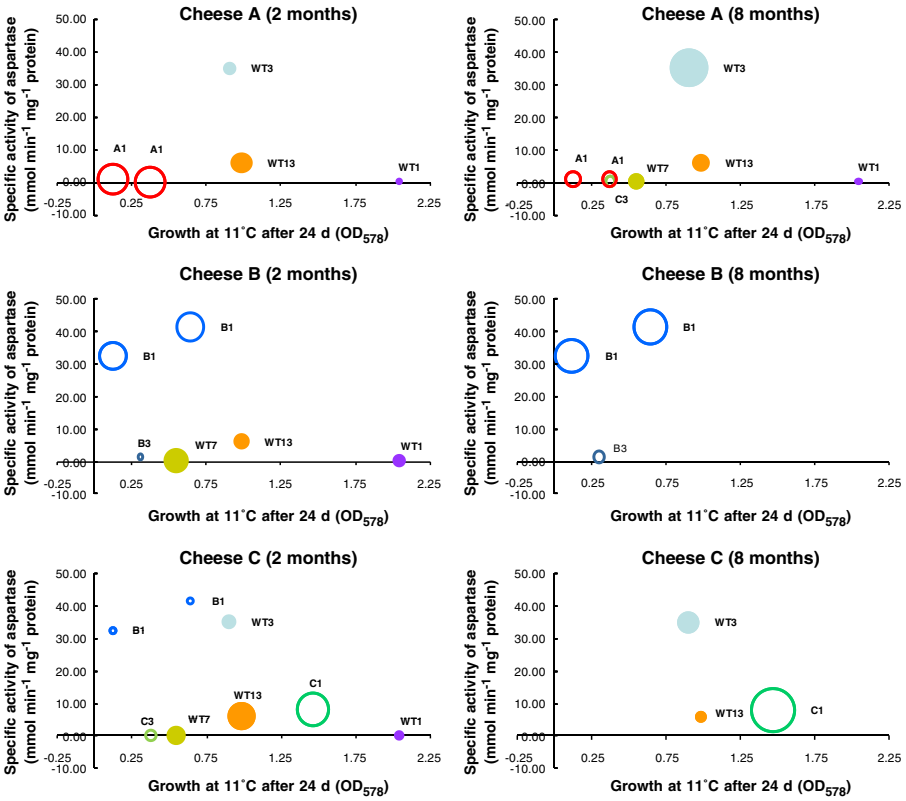

Fig. 4 Composition of the propionibacteria flora in cheese A, B, and C after ripening periods of 2 and 8 months. The *size* of the *circles* corresponds to the percentage of individual starter strains (*A*, *B*, *C*) and wild-type strains (*WT*); the *position* of the *circles* shows the growth potential at 11 °C and the specific activity of aspartase of the strains. (The genotypes A1 and B1 represent each two strains with identical IS-RFLP and MLVA profiles. The *two circles* of the *same size* represent the total percentage of genotypes A1 and B1)

decreased number of genotypes of starter and wild-type strains. After a ripening period of 8 months, the genotypes C1 and WT3 were the dominant strains (81% and 16%, respectively). The observed changes in the composition of the *P. freudenreichii* flora of the three cheeses showed that cheese manufacturing and ripening represents an ongoing selection process and that important differences may result due to the characteristics of starter and wild-type strains.

3.6 Characterization of *P. freudenreichii* strains

In order to explain the observed differences in the survival and growth of individual strains of *P. freudenreichii* during cheese ripening, we investigated the specific activity of aspartase and the growth at cold room storage temperature (11 °C) for the nine starter strains and the 12 wild-type strains of *P. freudenreichii*.

The measurement of the specific activity of aspartase revealed important differences among the investigated strains. The values ranged from 0.27 to 41.45 μmol.min^{-1}.mg^{-1} protein for the nine investigated strains of starters A, B,

and C and from 0.17 to 166.01 μmol.min^{-1}.mg^{-1} protein for the 12 investigated wild-type strains (Tables 1 and 3). Starter A was composed of two strains that showed a low specific activity of aspartase, whereas starter B contained two strains with high and two strains with low specific activity of aspartase. The distinct difference in the specific activity of aspartase of strains FAM 14217 and FAM14218 (both genotype B1) suggests that these strains do not represent duplicates. This presumption is also supported by previous findings (unpublished results) that also revealed distinct differences among the two strains in their abilities for nitrate reduction. However, the small difference between the strains FAM 14176 and FAM 14177 (both genotype A1) did not allow a reliable phenotypic differentiation of the two strains. In cheese A, the proportion of genotype A1 decreased from 100% in the curd grains down to 19% after 8 months of ripening, whereas in cheese B, the proportion of genotype B1 increased in the same period, from 44% to 95%. In addition, strain WT3, which showed a similar high specific activity of aspartase of 35.1 μmol.min^{-1}.mg^{-1} protein, was present in the curd grains of cheeses A and C at a percentage of 1% or lower but became the most frequent wild-type strain at the end of ripening in both cheeses, with a share of 57% and 16%, respectively. These results clearly indicate that specific activity of aspartase plays a key role in the survival or growth of individual strains of *P. freudenreichii* in the Swiss-type cheese ecosystem. However, although strain WT4 exhibited the highest specific activity of aspartase, at 166.01 μmol.min^{-1}.mg^{-1} protein, this strain was not detected in cheese, most likely due to its poor growth at a storage temperature of 11 °C.

The same 21 strains were assessed with regard to their ability to grow at a low temperature of 11 °C in a peptone whey broth at pH 5.5 by measuring the optical density (OD_{578}) after an incubation time of 24 days (Tables 1 and 3). The OD_{578} ranged between 0.04 and 1.91 for the starter strains and between 0.01 and 2.04 for the wild-type strains. For a number of strains (WT3, WT7, WT11, WT13, WT16, and FAM 14218), large standard deviations were obtained. This may be related to the circumstance that the temperature of 11 °C is close to the growth limit of most strains. Among the starter strains, the two strains C1 and C2 showed distinctly higher OD_{578} compared with all other strains. The ability of strain C1 to grow at low temperature could explain its increasing dominance during ripening. In contrast to strain C1, strain C2 most likely did not survive the long exposure to heat during scalding, dry stirring, and pressing and therefore disappeared from the flora in the cheese aged for 2 months. Although strain WT1 showed the highest OD_{578} at a growth temperature of 11 °C, this strain was present only at low percentages in the flora of all three cheeses and was even lost in cheeses B and C after prolonged ripening periods. However, this strain also exhibited the lowest specific activity of aspartase of all investigated strains.

In summary, the results obtained for specific activity of aspartase and bacterial growth at 11 °C for the investigated strains of *P. freudenreichii* revealed important differences in both criteria and suggested an explanation for the behavior of individual strains during cheese ripening. Although other criteria, such as pH or salt concentration, will also considerably influence the growth of *P.freudenreichii* in cheese, these criteria are of lower relevance in Emmentaler PDO cheese due to the relatively high pH of this cheese (due to partial removal of lactose during curd washing) and its low salt content (3–5 g.kg^{-1}). The growth and survival of *P.*

freudenreichii in cheese is also influenced by interactions with facultatively heterofermentative lactobacilli such as *L. casei* or *Lactobacillus rhamnosus,* which have been shown to inhibit growth, and by the presence of proteolytic lactobacilli in the starter that stimulate growth (Fröhlich-Wyder and Bachmann 2004; Fröhlich-Wyder et al. 2002). Baer and Ryba (1999) showed that the intensity of late fermentation correlates with the levels of free amino acids in Emmentaler PDO cheese. In addition, *P. freudenreichii* metabolizes other amino acids besides aspartate and asparagine (Thierry and Maillard 2002). However, the presence of proteolytic lactobacilli such as *Lactobacillus helveticus* mainly favors the growth of *P. freudenreichii* strains with a high specific activity of aspartase, whereas the use of adjunct cultures containing facultatively heterofermentative lactobacilli for the control of propionic acid fermentation seems to be more effective in the case of *P. freudenreichii* starters with a low specific activity of aspartase.

3.7 Application properties of the investigated *P. freudenreichii* starters

Starter A has a low aspartase activity and is widely used in practice in order to obtain Emmentaler PDO cheese with excellent storage quality. Starter B contains strains of *P. freudenreichii* with a high specific activity of aspartase and yields cheeses that are prone to late fermentation, with a more intense flavor and a greater number and size of eyes. The combination of the two starters A and B usually yields cheeses with properties that are similar to those of cheeses produced with starter B alone, even when starter B is added at lower levels to the cheese milk. The results of the present study clearly show that strains present in starter A, with a low specific activity of aspartase, are successively replaced by wild-type strains with a higher specific activity of aspartase during cheese ripening. Consequently, the combined use of starters differing in specific activity of aspartase will not result in a stable equilibrium of the propionibacterial flora during cheese ripening. Similarly, the use of a multi-strain starter of *P. freudenreichii* that includes strains with strongly differing characteristics was also not effective for maintaining a high biodiversity, as found with cheeses B and C (Table 6).

Strains of *P. freudenreichii* are naturally present in raw milk at a concentration up to 10^3 cfu.mL^{-1}, but little is known regarding their influence on cheese quality. The contamination of raw milk with propionibacteria is usually not controlled in cheese factories that produce raw milk cheeses with propionic acid fermentation. However, even with the use of starter A, which shows a low specific activity of aspartase and which allows a good control of propionic acid fermentation, sporadic cases of late fermentation still occur in practice. The results of the present study indicate that wild-type strains of *P. freudenreichii* from raw milk may considerably affect the composition of the *P. freudenreichii* flora in cheese and thereby exert an impact on the storage quality of the resulting Emmentaler PDO cheese. Consequently, vigilant control of propionibacteria in raw milk is suggested for Swiss-type cheeses made from raw milk.

4 Conclusions

In the present study, the combination of IS-RFLP and MLVA allowed a good discrimination of *P. freudenreichii* strains originating from raw milk and from

Table 6 Changes in the composition of propionibacterial flora in Emmentaler PDO cheeses made with *P. freudenreichii* starters A, B, and C[a]

	Genotype	Starter A	Curd grains before scalding	Curd grains after scalding	Cheese aged 2 months	Cheese aged 4 months	Cheese aged 6 months	Cheese aged 8 months
Cheese A number of genotypes		(*N*=80)	(*N*=80)	(*N*=80)	(*N*=80)	(*N*=80)	(*N*=80)	(*N*=79)
		1	1	1	4	3	5	6
	A1	100	100	100	78	94	44	19
	WT1				1		3	1
	WT3				5		27	57
	WT6					1		
	WT7					5	21	8
	WT13				16		5	11
	C3							4
	Genotype	Starter B	Curd grains before scalding	Curd grains after scalding	Cheese aged 2 months	Cheese aged 4 months	Cheese aged 6 months	Cheese aged 8 months
Cheese B number of genotypes		(*N*=80)	(*N*=80)	(*N*=80)	(*N*=80)	(*N*=79)	(*N*=40)	(*N*=77)
		3	3	4	6	8	5	2
	B1	41	44	49	66	69	78	95
	B3	36	31	47	1	15	13	5
	B4	23	25	4		4		
	WT1				4	1		
	WT3					1		
	WT7				20	6	5	
	WT13				8	3	2	
	WT14					1		
	WT15				1			
	WT17						2	
	Genotype	Starter C	Curd grains before scalding	Curd grains after scalding	Cheese aged 2 months	Cheese aged 4 months	Cheese aged 6 months	Cheese aged 8 months
Cheese C number of genotypes		(*N*=68)	(*N*=79)	(*N*=80)	(*N*=79)	(*N*=78)	(*N*=80)	(*N*=80)
		3	6	4	7	7	7	3
	C1	33	48	43	46	47	69	81
	C2	41	24	29		3	4	
	C3	26	25	27	5	4		
	WT1				3	6		
	WT3		1	1	6	3	9	16
	WT6						1	
	WT7				11	17	12	
	WT10		1					
	WT13				26	20	4	3
	WT16						1	
	B1				3			
	B3		1					

[a] Results expressed as percentage of individual genotypes, number of genotyped isolates indicated in brackets

starters. The typing of isolates obtained from cheeses during ripening revealed that a considerable biodiversity exists in Emmentaler PDO cheese made from raw milk and that wild-type strains can constitute a substantial proportion of the *P. freudenreichii* flora present in these cheeses. Drastic changes can occur in the composition of the flora during cheese manufacture and ripening. The characterization of nine starter strains and 12 wild-type strains of *P. freudenreichii* indicated that cheese ripening represents a continuous selection process that favors the growth of strains with a high specific activity of aspartase and good growth at cold room storage temperatures. Not surprisingly, then, most of the commercially available *P. freudenreichii* starters exhibit these characteristics, since most of the strains were initially isolated from ripened cheeses produced by propionic acid fermentation. However, the selection of these strains has made it difficult to obtain suitable starters that allow a good control of propionic acid fermentation. Consequently, most of the commercially available starters still yield cheeses that are prone to late fermentation and thus show poor storage quality. The results of the present study indicate that a more specific selection of wild-type strains would facilitate the development of new *P. freudenreichii* starters to yield cheeses with good sensorial properties, tailor-made openings, and optimal storage qualities. However, despite the use of adapted starters, good control of propionibacteria in raw milk also appears to be essential in order to limit the impact of the indigenous flora in milk used for cheese.

References

Baer A, Ryba I (1999) Interactions between propionic acid bacteria and thermophilic lactic acid bacteria. Lait 79:79–92

Bauer J, Andersen SB (1999) Stability of insertion sequence IS1245, a marker for differentiation of *Mycobacterium avium* strains. J Clin Microbiol 37:442–444

Boyaval P, Deborde C (2000) Metabolic behaviour of lactic and propionic acid bacteria. In: Barbotin JN, Portais JC (eds) NMR in Microbiology: Theory and Applications. Horizon Scientific Press, Wymondham, UK, pp 283–307

Bütikofer U, Ardö Y (1999) Quantitative determination of free amino acids in cheese. In: Ardö Y (ed) Bulletin IDF 337, Chemical Methods for Evaluating Proteolysis in Cheese Maturation. International Dairy Federation, Brussels, Belgium, pp 24–32

Chamba JF, Irlinger F (2004) Secondary and adjunct cultures. In: Fox PF, McSweeney PLH, Cogan TM, Guinee TP (eds) Cheese Chemistry, Physics and Microbiology, Vol. 2, Major Cheese Groups. Elsevier Academic Press, Amsterdam, The Netherlands, pp 191–206

Crow VL (1986) Utilization of lactate isomers by *Propionibacterium freudenreichii* subsp. *shermanii*: regulatory role for intracellular pyruvate. Appl Environ Microbiol 52:352–358

Crow VL, Turner KW (1986) The effect of succinate production on other fermentation products in Swiss-type cheese. New Zeal J Dairy Sci 21:217–227

de Jong C, Badings HT (1990) Determination of free fatty acids in milk and cheese: procedures for extraction, clean up, and capillary gas chromatography. J High Res Chromatog 13:94–98

Emery TF (1963) Aspartase-catalyzed synthesis of *N*-hydroxyaspartic acid. Biochem Moscow 2:1041–1045

Falentin H, Postollec F, Parayre S, Henaff N, Le Bivic P, Richoux R, Thierry A, Sohier D (2010) Specific metabolic activity of ripening bacteria quantified by real-time reverse transcription PCR throughout Emmental cheese manufacture. Int J Food Microbiol 144:10–19

Fessler D., Characterisation of propionibacteria in Swiss raw milk by biochemical and molecular-biological methods, PhD thesis, ETH Zürich, Switzerland (1997).

Fessler D, Casey MG, Puhan Z (1998) Rapid identification of dairy *Propionibacterium* species by restriction analysis of the insertion within the 23S rRNA gene. Lait 78:203–216

Fessler D, Casey MG, Puhan Z (1999) Propionibacteria flora in Swiss raw milk from lowlands and alps. Lait 79:201–209

Fröhlich-Wyder MT, Bachmann HP (2004) Cheeses with propionic acid fermentation. In: Fox PF, McSweeney PLH, Cogan TM, Guinee TP (eds) Cheese Chemistry, Physics and Microbiology, Vol. 2, Major Cheese Groups, 3rd edn. Elsevier, Academic Press, Amsterdam, The Netherlands, pp 141–156

Fröhlich-Wyder MT, Bachmann HP, Casey MG (2002) Interaction between propionibacteria and starter/non-starter lactic acid bacteria in Swiss-type cheeses. Lait 82:1–15

Gierczynski R, Golubov A, Neubauer H, Pham JN, Rakin A (2007) Development of multiple-locus variable-number tandem-repeat analysis for *Yersinia enterocolitica* subsp. *palearctica* and its application to bioserogroup 4/O3 subtyping. J Clin Microbiol 45:2508–2515

Henegariu O, Heerema NA, Dlouhy SR, Vance GH, Vogt PH (1997) Multiplex PCR: critical parameters and step-by-step protocol. Biotechniques 23:504–511

Nicoloff H, Bringel F (2003) ISLpl1 is a functional IS30-related insertion element in *Lactobacillus plantarum* that is also found in other lactic acid bacteria. Appl Environ Microbiol 69:6032–6040

Park HS, Reinbold GW, Hammond WS, Clark WS Jr (1967) Growth of propionibacteria at low temperatures. J Dairy Sci 50:589–591

Petrovic T, Niksic M, Bringel F (2006) Strain typing with ISLpl1 in lactobacilli. FEMS Microbiol Lett 255:1–10

Piveteau PG, Condon S, Cogan TM (1995) Interactions between lactic and propionic acid bacteria. Lait 75:331–343

Quénée P, Lepage E, Kim WS, Vergnaud G, Gruss A (2005) Minisatellite polymorphism as a tool to distinguish closely related *Lactococcus lactis* strains. FEMS Microbiol Lett 248:101–109

Ricci G, Fortina MG (2006) Characterization of *Lactobacillus helveticus* strains isolated from cheeses by distribution studies of insertion sequences. Int J Food Microbiol 112:112–119

Rossi F, Torriani S, Dellaglio F (1999) Genus- and species-specific PCR-based detection of dairy propionibacteria in environmental samples by using primers targeted to the genes encoding 16S rRNA. Appl Environ Microbiol 65:4241–4244

Sieber R., Collomb M., Lavanchy P., Steiger G., Beitrag zur Kenntnis der Zusammensetzung schweizerischer konsumreifer Emmentaler, Greyerzer, Sbrinz, Appenzeller und Tilsiter. (Composition of some marketable Swiss Emmental, Gruyere, Sbrinz, Appenzell and Tilsit cheeses), Schweiz. Milchwirt. Forsch. 17 (1988) 9–16.

Smith PK, Krohn RI, Hermanson GT, Mallia AK, Gartner FH, Provenzano MD, Fujimoto EK, Goeke NM, Olson BJ, Klenk DC (1985) Measurement of protein using bicinchoninic acid. Anal Biochem 150:76–85

Steffen C, Flückiger E, Bosset JO, Rüegg M (1987) Swiss-type varieties. In: Fox PF (ed) Cheese: Chemistry, Physics and Microbiology, Vol. 2, Major Cheese Groups. Elsevier Applied Science Publ, London, UK, pp 93–120

Thierry A, Maillard MB (2002) Production of cheese flavour compounds derived from amino acid catabolism by *Propionibacterium freudenreichii*. Lait 82:17–32