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Propionibacteria flora in Swiss raw milk from lowlands and Alps

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Abstract — The propionic acid bacterial flora in Swiss raw milk was investigated. Four hundred and fifty-three strains from lowland milk and 21 strains from Alpine raw milk were classified to the species level by protein profile analysis and restriction analysis of the 23S ribosomal ribonucleic acid (rRNA). Plasmid profiles and random amplified polymorphic deoxyribonucleic acid (RAPD) was used to differentiate the strains. The flora was found to be extremely diverse. All four dairy *Propionibacterium* species were found in lowland raw milk: 71 % were *P. freudenreichii*, 19 % *P. jensenii*, 8 % *P. acidipropionici* and 2 % *P. thoenii*. *P. acidipropionici* was not found in alpine milk but *P. freudenreichii* made up 55 %, *P. jensenii* 15 % and *P. thoenii* 30 % of the total. Among 278 *P. freudenreichii* strains, 219 (79 %) different strains were identified by RAPD to the strain level. For the other species strain diversity was even greater. Only 30 % of the strains analyzed carried plasmids. The Swiss cheese industry has a large reservoir of *Propionibacterium* strains in raw milk for future applications and developments. © Inra/Elsevier, Paris

***propionibacterium* / milk / electrophoresis / PCR**

Résumé — La flore propionique dans le lait cru suisse en provenance de la plaine et des alpages. La flore propionique dans le lait cru suisse a été analysée. L'analyse des profils protéiques et l'analyse de restriction de l'ARNr 23S ont été faites avec 453 souches bactériennes du lait en provenance de la plaine et 21 souches du lait d'alpages, en vue de les classer en espèces. Leur contenu en plasmides et la méthode de l'amplification génique au hasard de l'ADN ont été utilisés pour la différenciation des souches. La flore propionique dans le lait cru suisse s'est avérée être extrêmement variée. Les quatre espèces laitières de *Propionibacterium* ont été trouvées dans le lait cru de plaine ; 71 % étaient des *P. freudenreichii*, 19 % des *P. jensenii*, 8 % des *P. acidipropionici* et 2 % des *P. thoenii*. Dans le lait cru d'alpage *P. acidipropionici* n'a pas été trouvé, *P. freudenreichii* formait 55 %, *P. jensenii* 15 % et *P. thoenii* 30 % des bactéries propioniques. Parmi les 278 souches de *P. freudenreichii* isolées 219 (79 %) souches différentes ont été identifiées par RAPD. La diversité chez les autres

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espèces était encore plus grande. Seulement 30 % de toutes les souches analysées possédaient des plasmides. L'industrie suisse du fromage dispose grâce au lait cru d'un reservoir important de souches de *Propionibacterium* en vue d'applications et développements futurs. © Inra/Elsevier, Paris

***propionibacterium* / lait / électrophorèse / PCR**

1. INTRODUCTION

Propionibacteria are used in the manufacture of Emmental (or Swiss-type) cheese to produce the characteristic eyes and the nutty flavour [18, 19]. Orla-Jensen [23] first isolated propionibacteria from milk and described them in detail.

Propionibacteria are gram-positive, nonmotile, asporogenous, anaerobic to aerotolerant and generally catalase-positive pleomorphic rods [11]. The genus *Propionibacterium* is separated into eight species. Four have been isolated from human skin and are generally not found in milk. The other four species, the so-called 'dairy propionibacteria', are *P. freudenreichii*, *P. jensenii*, *P. thoenii* and *P. acidipropionici*. Few investigations exist on the natural habitat of dairy propionibacteria. All species have been isolated from soil and silage [5], some species from fodder and dung [21], and anaerobic fermenters [29]. Plastourgos and Vaughn [27] isolated *P. acidipropionici* and *P. jensenii* strains from spoiled olives. In the rumen, *Propionibacterium* sp. are, together with other species, responsible for the breakdown of urea and the release of ammonia [36].

Propionibacteria were enumerated in samples of raw milk from different areas of France over a period of 1year but the species were not identified [34]. Carcano et al. [6] analyzed 306 samples of milk used for Grana cheese production for the presence of propionibacteria. Sixty strains were isolated and the four different species identified. Until now only a limited study on the analysis and classification of the propionibacterial flora in Swiss raw milk has been performed [3]. The wild propionibacterial flora may influence cheese quality and be responsible for splitting [15, 25] and brown spot defects [4]. It may also serve as a reservoir for future applications in the cheese industry.

The differentiation between the four dairy species, especially between *P. jensenii* and *P. thoenii*, using classical biochemical methods is problematic. New methods, which allow a better classification, have been proposed [1–3, 12, 14, 30, 31].

The purpose of this study was to screen a large number of strains, classify them by protein analysis into species and to differentiate them by random amplified polymorphic deoxyribonucleic acid (RAPD) at the strain level in order to obtain information concerning the diversity of the propionibacterial flora in Swiss raw milk. Milks from the lowlands in different areas of Switzerland and a small number of milks from alpine regions were studied, since approximately 20 % of cows sojourn to the Alps in summertime.

2. MATERIALS AND METHODS

2.1. Propionibacterial strains and culture conditions

Four hundred and fifty-three strains from 358 milks, provided by nine laboratories of the Dairy Inspection and Consulting Services, were isolated by plating on YELA (24 mL·L^{-1} sodium lactate (50 % v/v), 30 g·L^{-1} casein peptone, 30 g·L^{-1} yeast extract and 15 g·L^{-1} agar, pH 6.8) and incubated for 10 d at 30 °C. The nine laboratories were situated in different regions of

Switzerland: in the west, Vaud-Geneva/Neuchâtel (VD/NE), Fribourg (FR) and Bern (BE); in the north, Nordwestschweiz (NW), Aargau (AG), Nordostschweiz (NO), Thurgau (TG) and Saint-Gallen/Appenzell Ausserrhoden (SG/AR); in the centre, Zentralschweiz (ZS). Preliminary identification was determined by noting the colour of each colony, which ranged from beige to red to brown, the morphology under the microscope and the presence of catalase tested with 3 % (v/v) H_2O_2. Another 21 strains were isolated from alpine milk from three different areas of Canton Fribourg, where Gruyere cheese is produced. Type strains were obtained from the American Type Culture Collection (ATCC; Rockville, MD, USA) and Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSM).

All strains were purified by re-streaking three times on YELA for 10 d at 30 °C under anaerobic conditions. Strains were subsequently grown on YEL broth for 5 to 7 d at 30 °C.

2.2. Analysis of the soluble cell free protein extracts

Ten mL of a broth culture were centrifuged at 10 000 g for 10 min at 4 °C and the cells suspended in 150 µL TES buffer (10 mmol·L^{-1} Tris-Cl, pH 7.5, 1 mmol·L^{-1} EDTA, 100 mmol·L^{-1} NaCl). Approximately 300 mg glass beads of 100 µm diameter (BioSpec Products) were added and the suspension was vortexed five times at 1-min intervals with a cooling period of 1 min on ice between each vortexing period. Glass beads and intact bacterial cells were removed by centrifugation at 450 g for 10 min. The protein concentration in the supernatant was determined according to the method of Lowry et al. [20].

Polyacrylamide gel electrophoresis was performed according to Laemmli [17] as modified by Baer [1]. The gels were stained according to Morrissey [22], photographed with a Sony CCD camera and the patterns analyzed with GelCompar (Applied Maths, Kortrijk, Belgium). Classification of profiles and their reproducibility were verified using the unweighted pair group method using arithmetic averages [33].

2.3. Preparation of crude DNA extracts for species identification

The preparation of crude DNA was performed as described by Fessler et al. [14].

2.4. Purification of genomic DNA for strain identification

Cultures (10 mL) were harvested at 10 000 g for 10 min and washed in lysis buffer (100 mmol·L^{-1} sodium borate, 10 mmol·L^{-1} EDTA, 25 % saccharose, pH 8.0). One mL of lysis buffer containing 20 mg lysozyme was added, thoroughly mixed and incubated for 1 h at 37 °C. The protoplasts were recovered by centrifugation at 4 000 g for 10 min at 4 °C, suspended in 4.75 mL 20 mmol·L^{-1} EDTA, pH 7.0 and lysed by the addition of 0.25 mL of 20 % sodium dodecyl sulphate (SDS). Protein was removed by the addition of 1.25 mL 5.0 mol·L^{-1} sodium perchlorate and 3.0 mL chloroform followed by incubation for 15 min at room temperature and centrifugation for 10 min at 10 000 g. DNA was precipitated by the addition of 3 mL of propanol to 5 mL of the supernatant, recovered by centrifugation for 10 min at 10 000 *g*, dissolved in TE buffer (10 mmol·L^{-1} Tris-Cl, 1 mmol·L^{-1} EDTA, pH 8.0) and the concentration adjusted to 100 µg·mL^{-1}.

2.5. Polymerase chain reaction (PCR) and restriction analyses

Analysis of the insertion region within the 23S ribosomal ribonucleic acid (rRNA) gene [32] was performed according to Fessler et al. [14].

2.6. Differentiation of *P. freudenreichii* subsp. on minimum growth medium

Minimum growth medium (MGM) was a modification proposed by Baer (personal communication) of that of Crow [10] and included either 14 mmol·L^{-1} lactose or 100 mmol·L^{-1} sodium lactate.

2.7. RAPD with primer SK2

Taq polymerase, PCR buffer and dNTP mix were purchased from Boehringer Mannheim and the primer SK2 (5'-GCC GCC GCC GCC-3') [12] from Microsynth (Balgach, Switzerland). The PCR–reaction mix contained 83.5 µL sterile distilled water, 10 µL PCR buffer, 2 µL dNTP mix, 2 µL of primer SK2 (100 nmol·mL^{-1}), 0.5 µL Taq polymerase (5 U·mL^{-1}) and 2 µL purified DNA.

PCR amplification was performed on a thermocycler (Inotech, Dottikon, Switzerland) using the following conditions: an initial DNA denaturation step at 94 °C for 4 min followed by 45 cycles of denaturation at 94 °C for 1 min, primer annealing at 43 °C for 1 min, and primer extension at 72 °C for 2 min; a final extension was carried out at 72 °C for 10 min.

2.8. RAPD with primer DF4

RAPD analysis beads were purchased from Pharmacia Biotech (Uppsala, Sweden) and the primer DF4 (5'-CGC CGC CGT CGC-3') from MWG Biotech (Ebersberg, Germany). The PCR–reaction mix contained one analysis bead, 24 µL sterile distilled water, 0.6 µL of primer DF4 (50 nmol·mL^{-1}) and 0.4 µL purified DNA. PCR amplification was performed under the same conditions as for primer SK2.

PCR amplification products of both primers were separated on a 4 % agarose gel (NuSieve 3:1 agarose, FMC BioProducts) and visualized with ethidium bromide 1 mg·L^{-1} in 10 mmol·L^{-1} sodium borate, pH 8.0 under UV light. The DNA standard was a 50–2 000 bp ladder (Bio-Rad) with bands of 2 000, 1 500, 1 000, 700, 500, 400, 300, 200, 100 and 50 bp. Photographs were taken with a Sony CCD camera and the patterns analyzed with GelCompar. Classification of profiles and their reproducibility were verified using the unweighted pair group method using arithmetic averages [33].

2.9. Plasmid analysis

Cultures (10 mL) were harvested at 10 000 g for 10 min and washed in lysis buffer. One mL of lysis buffer containing 20 mg lysozyme and 2 000 U mutanolysin was added, thoroughly mixed and incubated 2 h at 37 °C. The protoplasts were recovered by centrifugation at 4 000 g for 10 min at 4 °C and lysed by the addition of 1 mL buffer (50 mmol·L^{-1} sodium borate, 10 mmol·L^{-1} EDTA, 1 % SDS, pH 12.4). 0.5 µL 5 mol·L^{-1} sodium acetate, pH 4.7 was added, incubated at 4 °C for 1 h, and centrifuged at 4 °C for 20 min at 10 000 g. The plasmid in the supernatant was further purified using the Plasmid-Safe kit (Epicentre Technologies, WI, USA) according to the manufacturers instructions. 45 µL of the solution were run overnight on an 0.8 % agarose gel (Bio-Rad, 162–0126) at 50 V in TBE buffer (90 mmol·L^{-1} Tris, 90 mmol·L^{-1} borate, 2 mmol·L^{-1} EDTA, pH 8.4–8.5). The plasmids were visualized under UV light in the presence of ethidium bromide buffer (10 mmol·L^{-1} sodium borate, pH 8.0 containing 1 mg·L^{-1} ethidium bromide). The standard was the supercoiled DNA ladder from Sigma (D5292) with bands of 16, 14, 12, 10, 8, 7, 6, 5, 4, 3 and 2 kb. Photographs were taken with a Polaroid MP4 Land Camera.

3. RESULTS AND DISCUSSION

3.1. *Propionibacterium* species in lowland milk

Of the 453 strains isolated, 379 were identified as being propionibacteria: 267 were identified as *P. freudenreichii*, 32 as *P. acidipropionici*, 72 as *P. jensenii* and 8 as *P. thoenii*. The identification was based on the comparison of the protein profiles with the type strains using the GelCompar software. With SDS-polyacrylamide gel electrophoresis (PAGE) reproducibilities of 92–98 % [7, 8], 95.9 ± 1.7 % [9] and 93–97 % [35] were found for the identification of the species. It was considered that a strain belonged to a particular species when there was more than 70 % identity of the protein profile with the type strain. Species identification was confirmed by PCR and restriction analysis of crude DNA extracts.

Figure 1 shows the protein profiles of the four *Propionibacterium*-type strains, which were used for comparison. The results of restriction analysis of the 23S rRNA gene are to be found in Fessler et al. [14].

With the restriction analysis method a reproducibility of 100 % was achieved. This method has the advantage that with propionibacteria only three or four bands were observed and the species were, therefore, easily recognized. Only 3 % of the strains tested were classified differently by 23S rRNA restriction analysis and protein profile analysis. Restriction enzyme analysis is easier to evaluate and gave better reproducibilities than protein profiling and is, therefore, the preferred method for species identification.

Figure 1. Protein profiles of the type strains of *Propionibacterium.*
1 = *P. acidipropionici* ATCC 25562; 2 = *P. thoenii* ATCC 4874; 3 = *P. freudenreichii* ATCC 6207; 4 = *P. jensenii* ATCC 4868.

Figure 1. Profiles protéiques de souches types de *Propionibacterium.*
1 = *P. acidipropionici* ATCC 25562 ; 2 = *P. thoenii* ATCC 4874 ; 3 = *P. freudenreichii* ATCC 6207 ; 4 = *P. jensenii* ATCC 4868.

Figure 2 shows the distribution of the strains from the areas covered by the Dairy Inspection and Consulting Service laboratories. The distribution of the four *Propionibacterium* species was not the same in each region. However, definite conclusions could not be drawn because the number of strains isolated from some regions was relatively small. Nevertheless, *P. freudenreichii* and *P. jensenii* were encountered in all regions. *P. acidipropionici* was not found in milks from VD/NE, NW, NO or SG/AR, and *P. thoenii* was absent in milks from VD/NE, TG and SG/AR. Only milk from three regions, FR, AG and ZS, harboured all four species of propionibacteria.

The only previous study performed on the population of propionibacteria in Swiss raw milk [3] showed that *P. freudenreichii* was almost exclusively present, whereas *P. acidipropionici* and *P. jensenii* were rarely detected and *P. thoenii* not at all.

3.2. Propionibacteria from alpine regions

Twenty-one strains were isolated from 38 milks from three different alpine regions. The techniques used for identification were the same as those used for strains isolated from lowland milk.

One strain was not a PAB and 11 were *P. freudenreichii*, three *P. jensenii* and six *P. thoenii*. *P. acidipropionici* was not found. *Figure 3* shows the distribution of propionibacteria in alpine milk and in lowland raw milk from the FR region. The Alps where the milk originated from are also situated in this region. In both groups, *P. freudenreichii* was the predominant species, with 62 % in lowland raw milk and 55 % in alpine milk. In lowland raw milk, 21 % of the strains were *P. jensenii* and in alpine milk 15 %. Of the alpine milk strains 30 % were classified as *P. thoenii* compared to only 2 % in lowland milk. The large proportion of *P. thoenii* in alpine milk should be confirmed before any definite conclusions may be drawn.

3.3. *P. freudenreichii* subspecies

The two subspecies *P. freudenreichii* subsp. *freudenreichii* and *P. freudenreichii*

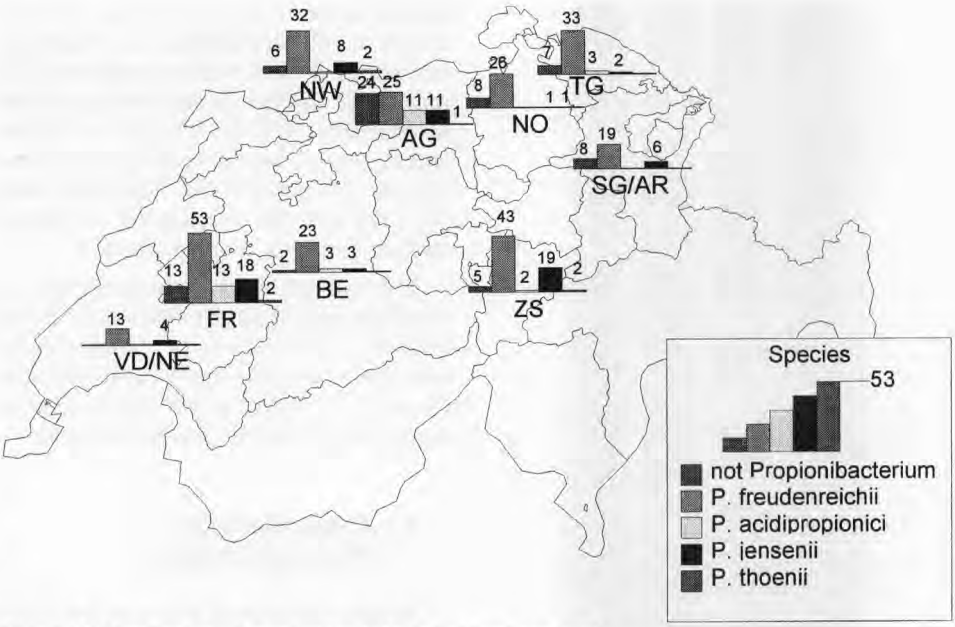

Figure 2. Distribution of propionibacteria in lowland raw milk.
AG = Aargau; BE = Bern; FR = Fribourg; NO = Nordostschweiz; NW = Nordwestschweiz; SG/AR = Saint-Gallen/Appenzell Ausserrhoden; TG = Thurgau; VD/NE = Vaud-Geneva/Neuchâtel; ZS = Zentralschweiz.

Figure 2. Distribution des bactéries propioniques dans le lait cru de la plaine.
AG = Aargau ; BE = Bern ; FR = Fribourg ; NO = Nordost-Schweiz ; NW = Nordwest-Schweiz ; SG/AR = Saint-Gallen/Appenzell Ausserrhoden ; TG = Thurgau ; VD/NE = Vaud/Neuchâtel ; ZS = Zentralschweiz.

subsp. *shermanii* could not be distinguished by the molecular methods used in this work. Protein profiles and restriction profiles with *Msp* I of the 23S rRNA gene did not show any difference. However, for the dairy industry the difference is important: *P. freudenreichii* subsp. *freudenreichii* does not grow on lactose, whereas *P. freudenreichii* subsp. *shermanii* does. In order to distinguish between the two subspecies, the utilization of lactose by *P. freudenreichii* strains was determined. Of the total 278 *P. freudenreichii* strains, 136 did not grow on lactose and were classified as *P. freudenreichii* subsp. *freudenreichii.* The remaining 142 strains grew on lactose and were classified as *P. freudenreichii* subsp. *shermanii.*

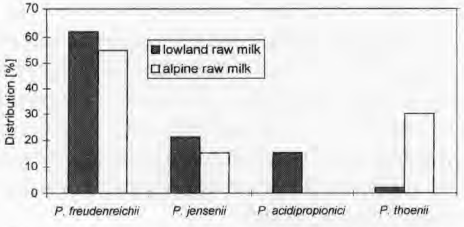

Figure 3. Distribution of propionibacteria in alpine and lowland raw milk from the Fribourg (FR) region.

Figure 3. Distribution des bactéries propioniques dans le lait cru de la plaine et d'alpages de la region FR.

The existence of the two *P. freudenreichii* subspecies is disputed among workers [13, 31]. The differentiation of *P. freudenreichii* subsp. *freudenreichii* and *P. freudenreichii* subsp. *shermanii* on the basis of

nitrate reduction and lactose fermentation may be significant for the dairy industry but questionable at the genetic level, since Johnson and Cummins [16] reported a high DNA homology between the two subspecies. The 23S rRNA results in the present study confirm that there is no difference at the genetic level.

3.4. Plasmid profiles

Plasmid profile analysis was carried out on the majority of propionibacteria from lowland milk. Of 373 strains tested, only 30 % contained plasmids. Of the plasmid-containing strains, 83 % carried one, 13 % two and 4 % more than two plasmids. Three strains possessed five plasmids.

Of the *P. freudenreichii* strains, 29 % carried plasmids, of *P. jensenii* 37 % and of *P. acidipropionici* 30 %. All eight strains of *P. thoenii* lacked plasmids. The size of the plasmids ranged from 1.2 kb to more than 16 kb. Eighty-nine percent of all the plasmid-carrying strains contained plasmids > 16 kb. Plasmids with a size between 6 and 8 kb were relatively frequent (62 %) in *P. jensenii* strains.

Perez Chaia et al. [26] found plasmids in 25 % of the propionibacteria strains analyzed by them. However, their study included only 27 *P. freudenreichii* and 3 *P. acidipropionici* and no *P. jensenii* strains compared to the 373 strains in this study. In the present study, *P. jensenii* had the highest percentage of plasmid-carrying strains. The size of plasmids varied between 3.2 and 47 kb; smaller plasmids were not observed. Panon [24] detected plasmids in 38 % of a total of 53 strains, which included all the propionibacterial species.

The functional properties of *Propionibacterium* plasmids are not known. It was suggested [28] that lactose utilization in *P. freudenreichii* might be plasmid linked. This was not supported by the results in the present study since only 38 of the 78 plasmid-carrying *P. freudenreichii* strains were able to ferment lactose and were, consequently, classified as *P. freudenreichii* subsp. *shermanii*, and 40 were identified as *P. freudenreichii* subsp. *freudenreichii*. In addition, 137 *P. freudenreichii* subs *P. shermanii* strains and 141 *P. freudenreichii* subsp. *freudenreichii* carried no plasmids. Since not all the strains carried plasmids, it was not possible to differentiate all the strains by plasmid analysis.

3.5. RAPD

The method of choice for investigating strain variety in Swiss raw milk proved to be RAPD. Several primers were studied and the two (SK2 and DF4) that gave the most consistent number and clearest bands were chosen. Both were 12-mers with 100 % G + C. Primer SK2 was used for all propionibacteria strains, whereas primer DF4 was used for *P. freudenreichii* strains only. The average intra-gel reproducibility with 15 strains was 94 ± 2 % and inter-gel reproducibility with 12 strains was 90 ± 2 %. The total of 278 *P. freudenreichii* strains yielded, at the 88 % similarity level, 154 different profiles with primer DF4 and 112 profiles with primer SK2.

The identification of the strains was primer dependent, because with RAPD only a small part of the total DNA is amplified. The RAPD profiles for two strains may, therefore, be identical with one primer, but different with another primer.

Only 59 strains gave the same profile with both primers, so that finally 219 different *P. freudenreichii* strains were obtained. Identical strains usually came from the same region. The *P. freudenreichii* strain diversity in Swiss raw milk is important to note, since it offers a large reservoir of strains for use in the dairy industry. *Figure 4* shows an example of the RAPD profiles from *P. freudenreichii* strains from lowland milk.

Primer SK2 permitted classification of the 32 *P. acidipropionici*, 14 *P. thoenii* and

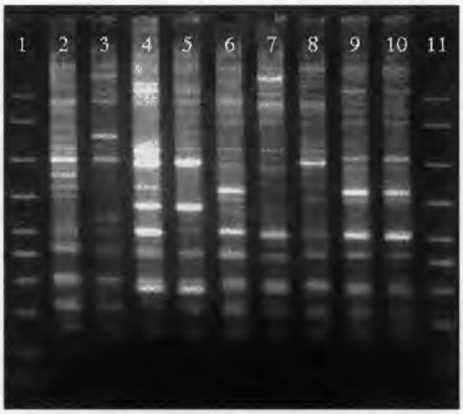

Figure 4. RAPD profiles with primer SK2 of *P. freudenreichii* strains from lowland raw milk. 2, 3, 4, 5, 6, 7, 8, 9, 10 = *P. freudenreichii* strains; 1, 11 = 50–2 000 bp ladder.

Figure 4. Profiles de RAPD avec amorce SK2 de souches de *P. freudenreichii* du lait cru de la plaine (2, 3, 4, 5, 6, 7, 8, 9, 10 = *P. freudenreichii* strains ; 1, 11 = 50–2000 bp ladder).

75 *P. jensenii* strains into 30, 12 and 50 different groups, respectively. Because of the lesser significance of these species compared to *P. freudenreichii* in Emmental cheese, only one primer was used to differentiate the strains.

It is also interesting, that of the four pairs of strains having identical RAPD patterns and originating from the same milk, two did not have the same plasmid profile. This may indicate horizontal transfer of plasmids between bacteria and/or spontaneous loss of plasmids.

4. CONCLUSION

The propionibacterial flora in Swiss raw milk is extremely diverse. In raw milk from the lowlands, all four dairy species were found. In raw milk from the Alps, *P. acidipropionici* was absent and *P. thoenii* made up 30 % of the strains compared to only 2 % in lowland raw milk. The reason for the diversity of *Propionibacterium* species in Swiss milks can only be guessed at, since different factors such as grass feeding, sojourn of cows on the Alps during the summer, propionibacterial flora on the farm and in the cheese factory could play a role. Most of the strains were only detected once by RAPD. As only 30 % of all analyzed propionibacteria carried plasmids, plasmid content was not useful for further strain identification. The plasmids might, however, be interesting for future studies, as the characteristics coded on them remain unknown and there is a possibility of horizontal transfer and/or spontaneous loss. The cheese industry has in Swiss raw milk a large reservoir of *Propionibacterium* strains for future applications and developments.

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