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Original article

Naturally occurring genetic markers in lactobacilli and their use to verify the authenticity of Swiss Emmental PDO cheese

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Abstract – A general method for the detection and identification of specific strains of bacteria is described. The assay is based on the observation that insertion sequences (IS) in different strains of bacteria occur at diverse loci on the bacterial genome. Exclusive PCR primers can be selected for a particular strain where one of the primers is specific for a particular IS element and the other is specific for the adjacent DNA sequence in the genome. Only bacterial strains containing the IS element at the particular point on the genome will yield an amplicon of the expected size after PCR. We have illustrated this method by selecting primers to detect some lactobacilli strains that are used exclusively in the manufacture of Swiss Emmental cheese. Using this method we were able to differentiate cheeses manufactured in Switzerland from those made in other European countries.

transposase / insertion sequence / strain-specific PCR / lactic acid bacteria

摘要 – 乳杆菌中自然产生的遗传标记及鉴别受原产地名号保护的瑞士 Emmental 干酪。本文介绍了一种检测及鉴定细菌菌株的方法。由于插入序列 (IS) 发生在不同菌株基因组上的不同位点，我们对每一株特定的细菌设计了一对专门的 PCR 引物，其中一条只针对插入序列，另一条则只针对其毗邻的 DNA 序列。结果显示只有在基因组特定位点含有插入序列的菌株通过 PCR 扩增后才能产生预期大小的扩增子。通过选择引物对瑞士多孔干酪生产专用乳酸杆菌进行了检测验证，发现使用这种方法能够区分瑞士与其他欧洲国家产的 Emmental 干酪。

转座酶 / 插入序列 / 种特异性 PCR / 乳酸菌

Résumé – Marqueurs génétiques naturellement présents chez les lactobacilles et leur utilisation pour vérifier l'authenticité du fromage Emmental suisse AOC. Une méthode générale pour la détection et l'identification spécifique de souches bactériennes est décrite. Elle est basée sur l'observation que les séquences d'insertion (IS) sont présentes à divers endroits sur le génome bactérien. Des amorces PCR spécifiques à une souche peuvent être conçues, l'une étant située sur un élément IS et l'autre sur la séquence ADN adjacente. Seules les souches avec l'élément IS à cet endroit particulier du génome donneront un produit d'amplification de la taille attendue après PCR. Cette méthode est illustrée ici par des paires d'amorces permettant de détecter spécifiquement des souches de lactobacilles utilisées exclusivement dans la fabrication de l'Emmental suisse. Ainsi, nous avons pu différencier les fromages fabriqués en Suisse des fromages provenant d'autres pays européens.

transposase / séquence d'insertion / PCR souche-spécifique / bactérie lactique

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1. INTRODUCTION

Molecular phylogeny of prokaryotes was initiated by Woese et al. [22] by making use of the small subunit (SSU) ribosomal RNA sequences. The SSU rRNA trees have been considered as the standard tree of life by many biologists. The evolution of distinct species takes place over a long period of time. For example, the last common ancestor of *Salmonella enterica* and *Escherichia coli* existed approximately 140 million years ago [10]. For more closely related species, such as *Mycobacterium bovis* and *Mycobacterium tuberculosis*, divergence took place 15 000 years ago [6].

The comparison of housekeeping gene sequences from a single species will not distinguish between clones of that species. A different method for measuring evolution over shorter periods of time is necessary to distinguish between clones. Insertion sequences (IS) are short (< 2.5-kb) phenotypically cryptic segments of DNA with a simple genetic organization and are capable of inserting at multiple sites in the genome or into plasmids [7]. IS elements have been shown to promote the evolutionary adaptation of hosts [8, 9, 11, 20]. However, various IS elements have shown different transpositional activities [11, 18]. An IS element was even found to be conserved in the same location in all strains of *Lactobacillus helveticus* tested [1]. The restriction fragment length polymorphism (RFLP) associated with the presence of multiple IS elements proved to be suitable for strain typing of lactic acid bacteria at the infraspecies level [12, 19].

We reasoned that the position of one or several IS sequences on the genome could be used not only to determine relatedness between strains but also for their identification. When an insertion locus is specific for a given strain, strain-specific primer pairs can be designed based on a fragment sequence containing the insertion sequence and the neighboring gene sequence, allowing this fragment to serve as a potential marker for monitoring strains.

The objective of this study was to develop specific PCR assays for strains of facultative heterofermentative (FH) lactobacilli which are used in the manufacture of Swiss Emmental PDO cheese. All manufacturers of Emmental cheese in Switzerland add one of three available cultures, supplied by our research station, to cheese milk. These bacteria grow in the cheese body during ripening and are still present after six months of ripening. The described method allows the identification of these strains and thus the establishement of the authenticity of Swiss Emmental cheese.

2. MATERIALS AND METHODS

2.1. Bacterial strains, cheese extracts and culture conditions

The complete list of bacterial strains and cultures used in this study as well as their taxonomic classification and origin are presented in Tables I and II. Bacteria were extracted from cheese by suspending 10 g of grated cheese in water and mixing in a Stomacher apparatus at room temperature for 3 min. FH strains and bacteria extracted from cheese were grown anaerobically in FH medium [5] at 30 °C for 48 h. One liter of FH medium contains 10 g proteose peptone, 10 g meat extract, 1 g yeast extract, 20 g mannitol, 0.1 g $MnSO_4$, 0.1 g $MgSO_4$, 1 mL Tween 80, 200 mL acetate buffer 1 mol·L^{-1} pH 5.4 and 0.05 g vancomycine.

2.2. DNA extraction, amplification and analysis

DNA was extracted from 1 mL of cultures with the High Pure PCR Template Preparation Kit (Roche Applied Science,

Table I. Facultative heterofermentative lactobacilli strains examined in this study that are used in the manufacture of Swiss Emmental.

Strain code	Species	Commercial culture	Origin
WS 07.04	*L. casei*	MK3008	Swiss Emmental Cheese
WS 09.05	*L. casei*		Swiss Emmental Cheese
WS 11.30	*L. casei*		Swiss Emmental Cheese
WS 01.02	*L. casei*	MK3010	Swiss Emmental Cheese
WS 10.16	*L. rhamnosus*	MK3012	Swiss Emmental Cheese
WS 13.25	*L. rhamnosus*		Swiss Emmental Cheese
WS 15.23	*L. rhamnosus*		Swiss Emmental Cheese

Table II. Other facultative heterofermentative lactobacilli strains examined in this study.

Species	*n*	Strain code	Origin
L. casei	44	FAM 18095 - FAM 18138	Gruyere Cheese
L. rhamnosus	22	WS 2.08, 3.09, 4.05, 5.18, 6.09, 7.13, 10.02, 11.06, 12.12, 13.24, 14.13, 15.12, 16.05, 17.21, 20.02, 21.02, 23.07, 24.12, 25.01, 26.04, 28.14, 29.07	Swiss Emmental Cheese
L. rhamnosus	6	JCL4384, 4395, 4398, 4967, 4968, 4977	Milk
L. rhamnosus	15	JCL5802, 5803, 6377, 6381, 6383, 6385, 6387, 6388, 6390, 6396, 6409, 6416, 6418, 6428, 6436	Strain collection Rotholz
L. rhamnosus	10	GR11, 20, 24, 25, 33, 45, 48, 50, 57, 59	Grana Cheese
L. rhamnosus	5	1052-73, 871-69, 886-11, 927-10, 935-31	Gruyere Cheese
L. rhamnosus	1	ZL 34	Tilsit Cheese

Mannheim, Germany) after pretreatment of the sample as described previously [2]. Pretreatment consisted of incubation in 0.05 N NaOH for 15 min at room temperature, then in TES buffer (0.1 mol·L^{-1} Tris-HCl, 10 mmol·L^{-1} EDTA, 25% w/v saccharose, pH 8.0) with 1 mg·mL^{-1} lysozyme for 1 h at 37 °C. Amplification was carried out with a 25-µL reaction mixture containing 2.5 µL of 10 X buffer with 15 mmol·L^{-1} $MgCl_2$ (Applied Biosystems, Foster City, CA), 0.5 µL of 10 mmol·L^{-1} dNTPs (Promega Corp., Madison, WN), 0.2 µL of each primer (100 µmol·L^{-1}) (Operon Technologies, Alameda, CA) and 0.2 µL Taq DNA polymerase (5 U·µL^{-1} AmpliTaq Gold) (Applied Biosystems). Amplification was performed with a GeneAmp PCR System 2400 (Applied Biosystems). Amplification products were separated on a DNA 7500 LabChip in an Agilent 2100 Bioanalyzer according to the manufacturer's instructions (Agilent Technologies, Palo Alto, CA).

2.3. Strategy

Analysis of the *Lactobacillus casei* ATCC 334 (GenBank Acc. No. CP000423) showed that it contained 25 copies of a putative transposase belonging to the IS30 family [8]. Due to the high copy number, this transposase was selected to investigate insertion sequence-associated polymorphism. The selection of an IS with a high copy number increases the chance of finding a strain-specific insertion locus. Among others, IS the genome also had three copies of the transposase IS153, identical to that found in *L. sanfranciscensis* [4]. The DNA segment between the IS was amplified by using upstream and downstream outward facing primers. They were designed for IS30

and IS153 transposases as well as for 16S rDNA and 23S rDNA: IS30 Left – 5' CACAGCGCTTATCAATAAC 3'; IS30 Right – 5' TTGCATCAGTTCTATCTGAG 3'; IS153 Left – 5' CGTAACGAGTTGGCATGT 3'; IS153 Right – 5' CTGTGGAAGGTAAACGC 3'; 16S rDNA Left – 5' CTGAGCCAKGATCAAACT 3'; 23S rDNA Right – 5' GCTGAAAGCATCTAAGTGT 3'. These primers were used alone or in different combinations to amplify the DNA between the different IS or rDNA. The amplification program included a 10-min initial denaturation step at 95 °C; 35 cycles of 95 °C for 30 s, 54 °C for 30 s, and 72 °C for 8 min; and a 10-min final extension step at 72 °C.

2.4. Nucleotide sequencing

Sequencing of PCR products was performed using the BigDye Terminator Cycle sequencing kit and analyzed with a 47-cm capillary in an ABI Prism 310 Genetic Analyzer (Applied Biosystems). The same primers were also used for the sequencing of both strands of amplified DNA.

2.5. Application of the method to commercial cheese samples

Cheese extracts of Swiss-type cheeses manufactured outside Switzerland (n = 18) and of Swiss Emmental PDO cheeses made with one of the three adjunct cultures MK 3008 (n = 17), MK 3010 (n = 19) or MK 3012 (n = 19) were cultured in FH broth for 48 h. DNA was extracted from the resulting cultures and amplified using the strain-specific primers.

3. RESULTS

3.1. Analysis of insertion sequence-associated polymorphism

The outward facing primers for IS30, IS153 and rRNA were used alone or in pairs to amplify genomic DNA sequences from the four different strains of *L. casei* used in the manufacture of Swiss Emmental PDO cheese (Fig. 1). The results from the four bacteria were all different, thus confirming that IS elements are positioned differently on the genome. The greatest differences are to be found in lanes 8–16 which are produced with primers designed for IS30 that was found with a high copy number in *L. casei* (ATCC 334).

The same primers were also used to analyze the genome of three *Lactobacillus rhamnosus* strains used in Swiss Emmental manufacture (Fig. 2). The results from the three strains of *L. rhamnosus* are all different and also differ from those of *L. casei*. The number of amplified segments, nevertheless, is smaller for this species suggesting a smaller copy number of IS30 and IS153.

3.2. Selection of specific sequences of strains present in adjunct cultures

Examination of Figure 1 reveals that, whereas some amplicons were found in more than one *L. casei* strain (e.g. amplicons in lane A4 and lane D4), other amplicons were specific for a particular strain (e.g. amplicon in lane A17 of strain WS 01.02 and amplicon in lane B9 of strain WS 07.04). In order to ascertain that these sequences were specific, the selected amplicons were sequenced using their PCR primers and analyzed.

A BLAST search of the GenBank showed that the first 73 bp of amplicon A17 corresponded to published sequences from several organisms, e.g. *L. casei* insertion sequence ISLC3. No homology for the remaining part of the sequence could be found at the GenBank or in the *L. casei* genome sequence (CP000423). A primer was designed from this sequence in order to obtain a primer pair (IS153 Left/01.02) specific for this strain and producing an

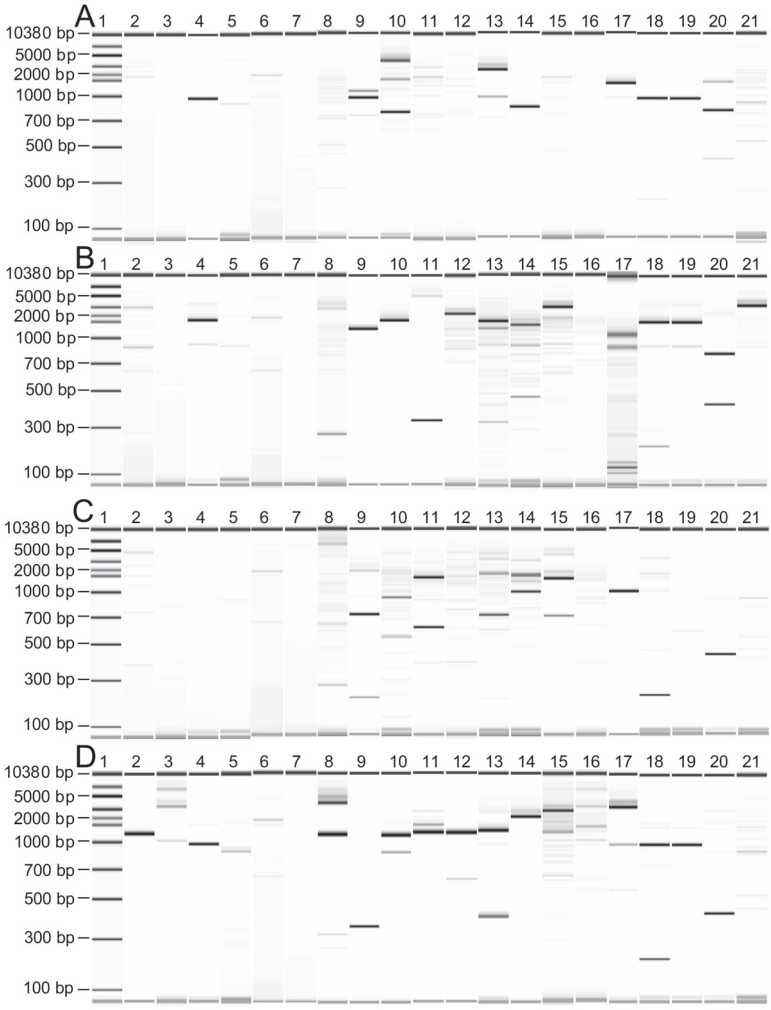

Figure 1. Electrophoresis of PCR products obtained with various primers from four different *L. casei* strains of cultures MK 3008 and MK 3010.

A: *L. casei* WS 01.02 (culture MK 3010), B: *L.casei* WS 07.04 (culture MK 3008), C: *L. casei* WS 09.05 (culture MK 3008), D: *L. casei* WS 11.30 (culture MK 3008).

1: Molecular size markers, 2: IS30 Left, 3: IS30 Right, 4: IS153 Left, 5: IS153 Right, 6: 16S rDNA Left, 7: 23S rDNA Right, 8: IS30 Left and IS30 Right, 9: IS30 Left and IS153 Left, 10: IS30 Left and IS153 Right, 11: IS30 Left and 16S rDNA Left, 12: IS30 Left and 23S rDNA Right, 13: IS30 Right and IS153 Left, 14: IS30 Right and IS153 Right, 15: IS30 Right and 16S rDNA Left, 16: IS30 Right and 23S rDNA Right, 17: IS153 Left and IS153 Right, 18: IS153 Left and 16S rDNA Left, 19: IS153 Left and 23S rDNA Right, 20: IS153 Right and 16S rDNA Left, 21: IS153 Right and 23S rDNA Right.

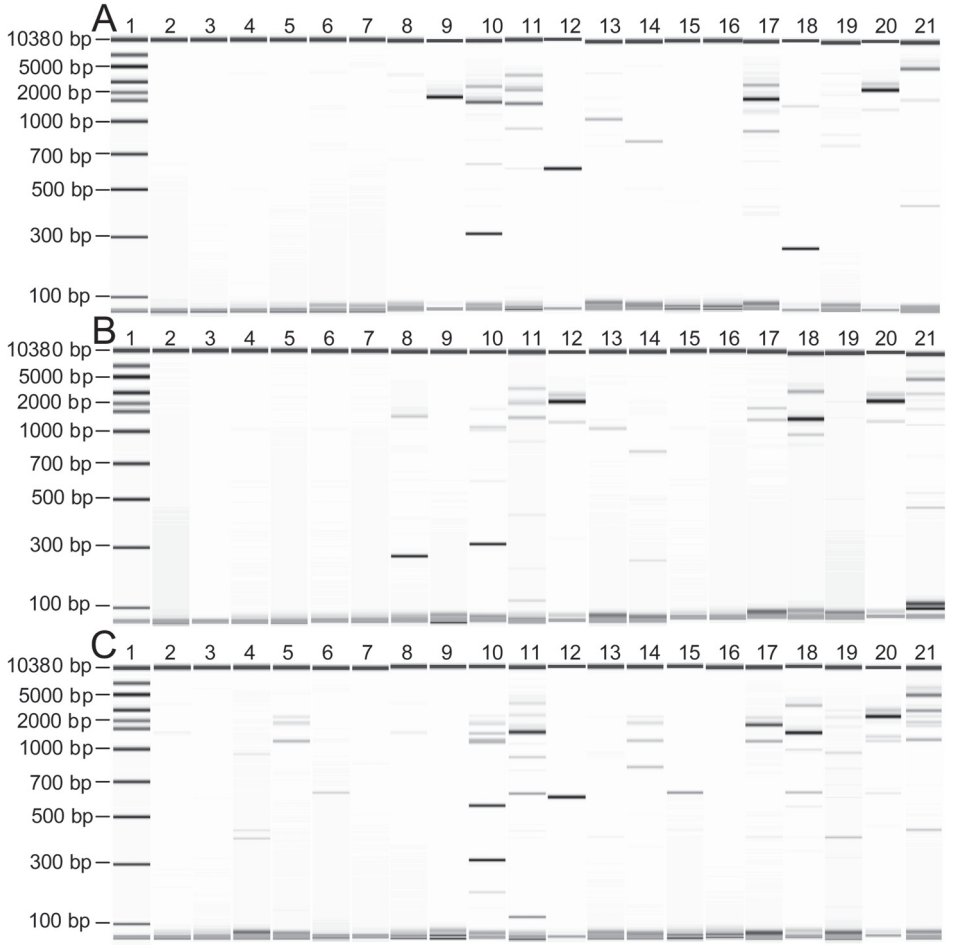

Figure 2. Electrophoresis of PCR products obtained with various primers from three different *L. rhamnosus* strains of culture MK 3012.
A: *L. rhamnosus* WS 10.16, B: *L. rhamnosus* WS 15.23, C: *L. rhamnosus* WS 13.25.
1: Molecular size markers, 2: IS30 Left, 3: IS30 Right, 4: IS153 Left, 5: IS153 Right, 6: 16S rDNA Left, 7: 23S rDNA Right, 8: IS30 Left and IS30 Right, 9: IS30 Left and IS153 Left, 10: IS30 Left and IS153 Right, 11: IS30 Left and 16S rDNA Left, 12: IS30 Left and 23S rDNA Right, 13: IS30 Right and IS153 Left, 14: IS30 Right and IS153 Right, 15: IS30 Right and 16S rDNA Left, 16: IS30 Right and 23S rDNA Right, 17: IS153 Left and IS153 Right, 18: IS153 Left and 16S rDNA Left, 19: IS153 Left and 23S rDNA Right, 20: IS153 Right and 16S rDNA Left, 21: IS153 Right and 23S rDNA Right.

amplicon of 365 bp. Similarly, a strain-specific primer pair could also be obtained for strain WS 11.30 of culture MK 3008, yielding an amplicon of 262 bp. The specificity of the primer pairs was tested with genomic DNA isolated from 44 other *L. casei* strains isolated from cheese as listed in Table II. None of these 44 strains yielded an amplicon of 365 bp or 262 bp, respectively.

Examination of Figure 2 revealed some amplicons which were specific for a particular *L. rhamnosus* strain (e.g. amplicon in lane A9 of strain WS 10.16). A BLAST search of the GenBank showed that the first 47 bp of amplicon A9 corresponded to an IS from *L. casei* (Genbank Accession No. AF322594). There was no homology for the remaining part of the sequence. A primer was designed from this sequence in order to obtain a primer pair (IS130 Left/10.16) specific for this strain and producing an amplicon of 210 bp. The specificity of the primer pair was tested with genomic DNA isolated from 59 other *L. rhamnosus* strains isolated from cheese as listed in Table II. None of the other strains yielded an amplicon of 210 bp.

3.3. Application of the method to determine the authenticity of Swiss Emmental cheese

DNA extracted from the cultures of cheese extracts of 18 Swiss-type cheeses manufactured outside Switzerland and of Swiss Emmental PDO cheeses made with the adjunct cultures MK 3008 (n = 17), MK 3010 (n = 19) and MK 3012 (n = 19) was amplified using the strain-specific primers. The presence of specific PCR products of the size of 262 bp for WS 11.30 (culture MK 3008), 365 bp for WS 01.02 (culture MK 3010), or 210 bp for WS 10.16 (culture MK 3012) allowed the differentiation of cheeses manufactured in Switzerland from those made in other European countries since the three cultures are used exclusively in Switzerland. Effectively, none of the 18 cultured cheese extracts of the foreign cheeses yielded an amplicon that corresponded to the strains of the three cultures MK 3008, MK 3010 and MK 3012. In contrast to this, all of the investigated samples of Swiss Emmental PDO cheese yielded a PCR product that corresponded to the strains of one of the three adjunct cultures. Figure 3 illustrates the application of the method to determine the authenticity of Swiss Emmental PDO cheese. For example, only Swiss Emmental PDO cheeses manufactured with culture 3010 (strain WS 01.02) yielded a PCR product of the size of 365 bp, whereas such an amplicon was not obtained in the cultured extracts of the 18 foreign cheeses.

4. DISCUSSION

In this paper, a PCR assay for the identification or detection of specific strains of facultative heterofermentative lactobacilli is described. The assay is based on the observation that IS elements in different strains of bacteria occur at diverse loci on the bacterial genome. Primers were selected, one of which was complementary to the IS element and the other to an adjacent sequence on the genome, both of which were particular for each strain. The size of the products of the PCR assay were specific only for strains which contained the IS element at that precise position on the genome. The IS and adjacent DNA sequence was selected by screening large numbers of strains for unique sequences. This was performed by amplifying the DNA regions between the IS and selecting unique products for each strain.

Strains of *L. casei* or *L. rhamnosus* used in the manufacture of Swiss Emmental were chosen as an illustration. The method developed allowed us to detect these

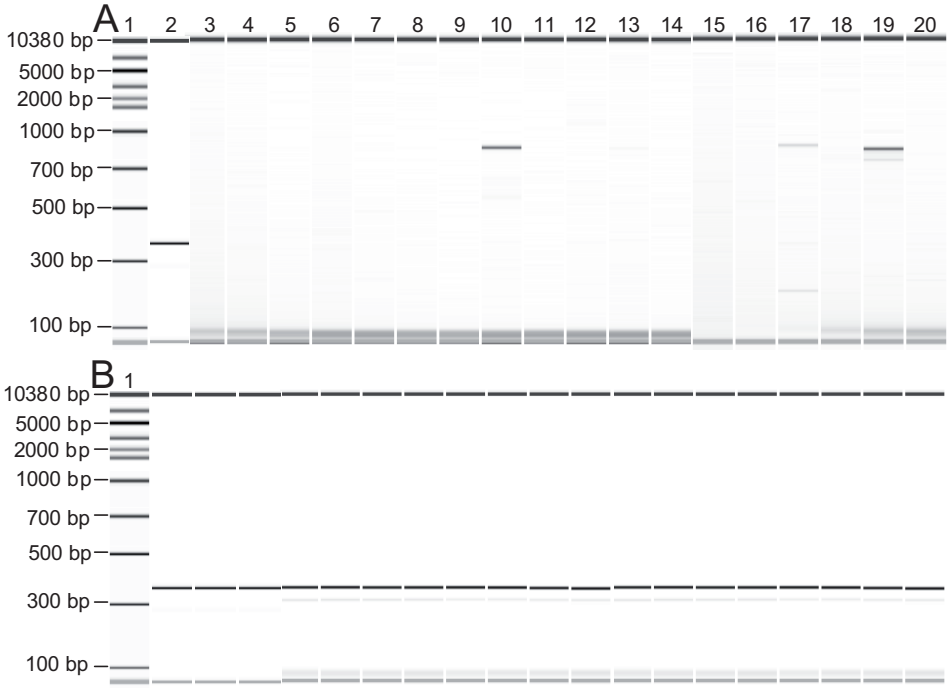

Figure 3. Electrophoresis of PCR products obtained with the primer pair (IS153 Left/01.02) from bacteria of Emmental cheese manufactured in different countries.
A: Emmental cheeses from different cheese factories manufactured outside Switzerland.
1: Molecular size markers, 2: Strain WS 01.02 (culture MK3010), 3: Finland – 9 months, 4: Finland – 17% fat, 5: Finland – 6 months, 6: Finland – organic, 7: Finland – 6 months, 8: Finland – 14 months, 9: Finland – 9 months, 10: France, 11: France, 12: Czech Republic, 13: Finland, 14: Germany – Allgauer, 15: Germany – Oberallgauer, 16: Finland, 17: Germany – Bavaria, 18: Germany – Allgauer, 19: Austria, 20: Slovenia.
B: Emmental cheeses manufactured in different cheese factories in Switzerland with culture MK3010 showing the strain-specific amplicon of WS 01.02 with a size of 365 bp.
1: Molecular size markers, 2–20: Cheeses from 19 different Emmental manufacturers located in Switzerland.

strains in six-month-old cheeses and it was possible to distinguish Emmental PDO cheese manufactured in Switzerland from that produced in other countries. Changes in the arrangement of IS elements may occur in individual cells during culture production or cheese ripening, but not in the whole population. However, to minimize a drift of the commercial cultures, fresh supplies are prepared and delivered weekly from stocks of cultures preserved in milk and stored at –40 °C. Stocks are prepared once a year from freeze-dried cultures which are stored at –40 °C for decades. Under these conditions the position of the IS elements remains stable, allowing the strain-specific detection in cheese. With few exceptions, most of the strain-specific

methods usable to identify strains in cheese are typing methods that require pure cultures of isolates. The advantages of a strain-specific PCR are the rapidity and sensitivity of detection. Tilsala-Timisjarvi and Alatossava [21] used the randomly amplified polymorphic DNA (RAPD) technique to produce potential strain-specific markers for a probiotic *L. rhamnosus* strain and obtained a specific sequence that showed a significant similarity to transposases of other lactic acid bacteria. Similarly, Coudeyras et al. [3] developed a strain-specific identification for a probiotic *L. rhamnosus* strain using subtractive hybridization and obtained with this method among four other specific sequences a sequence that corresponded to an IS. Our approach to developing strain-specific PCR is particularly efficient when a complete genome sequence of the species or of related organisms is available and it should also be applicable to other types of bacteria which need to be traced over relatively short time periods. A direct extraction of the DNA from the samples is a possible variation of the method that allows a further reduction of the time for analysis and reduces the contamination risks in the laboratory.

Pillonel et al. [13–17] recently developed a sophisticated chemometric approach for the determination of the geographic origin of Emmental cheese. This approach is based on the investigation of primary indicators that are dependent on the geographic origin (e.g. distribution of isotopes) and secondary indicators that are influenced by processing (e.g. copper content, presence of *L. helveticus*), and allowed a correct classification of more than 95% of the samples originating from seven production regions of Europe using multivariate statistical analysis and discriminant analysis. Based on a total of 15 factors even a 100% correct discrimination between Swiss and foreign Emmental cheeses was achieved. However, there are some limitations for the application of this powerful approach in practice. The use of secondary indicators may be rather susceptible to unforeseeable modifications in cheese-making by the individual manufacturers (e.g. omission of copper sulfate addition due to legal changes or omission of *L. helveticus* cultures). Further, the chemometric approach demands several different types of assays involving a lot of practical laboratory work and so is rather costly (estimated costs: 700 €/sample). These are hindering factors for its application in high-speed routine control. For this reason, the possibilities for the use of monitoring strains in the manufacturing of Swiss Emmental PDO cheese was evaluated. The use of naturally occurring genetic markers in lactic acid bacteria offers an alternative approach to determine the authenticity of Swiss Emmental PDO cheese. The major advantages are the specificity of this approach and the speed and costs at which the analysis can be carried out. However, for the successful application of this approach it is essential that all manufacturers of a PDO association agree to use cultures containing strains that can be detected with the described method and that such cultures are exclusively used by registered manufacturers. The uniqueness of developed specific primers is questionable as only a limited number of other strains and cheese samples could be examined in order to verify the reliability of the method. The occurrence of wild-type strains in raw milk cheeses may lead in rare cases to a wrong classification. In order to enhance the reliability and robustness of the approach it will be favorable to apply concepts in practice that include the simultaneous detection of two or even more strains. Further, it should be mentioned, that only qualitative results are obtained with this approach. For example, the verification of the correct composition of cheese mixtures (e.g. grated cheese) remains a challenging task.

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