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## ***Leuconostoc* Strains Unable to Split a Lactose Analogue Revealed by Characterisation of Mesophilic Dairy Starters\*\***

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### **Summary**

Mesophilic starter cultures used in dairy industry have been traditionally characterised by metabolic and biochemical methods. As closely related species of lactic acid bacteria have often only minor differences in phenotypic traits, which may also be variable within certain species, clear identification is often complicated. Therefore, techniques of molecular biology have been applied for rapid detection and differentiation of lactic acid bacteria. In this work, some bacterial clones isolated from mesophilic starters, which were preliminary identified as lactococci by phenotypic methods, were found to be *Leuconostoc* strains by both PCR and PFGE. According to the results, genotypic differentiation methods used in combination with phenotypic tests provide a fast and convenient way to reliably identify lactic acid bacteria displaying atypical metabolic characteristics.

*Key words:* lactic acid bacteria, differentiation, pulsed-field gel electrophoresis (PFGE), PCR

### **Introduction**

Fermentation of milk by specific microflora has been the primary mean to preserve milk against spoilage. Over the centuries, production methods have evolved from traditional home manufacture to industrial-scale production using specific starters and modern equipment. Today, starter cultures of lactic acid bacteria are widely used in dairy industry for the development of texture and flavour in fermented milk products. The stable composition of starter cultures is important for the dairy industry to manufacture products of uniform quality.

Mesophilic starters used in dairy industry are typically cultures of *Lactococcus lactis* subsp. *lactis*, *L. lactis* subsp. *cremoris*, *L. lactis* subsp. *lactis* biovar *diacetylactis* and *Leuconostoc mesenteroides* subsp. *cremoris*. They can be used as single strains or mixed starter cultures. Traditionally, starter cultures have been characterised using phenotypic methods, like hydrolysis of citrate and the

chromogenic lactose analogue 5-bromo-4-chloro-3-indolyl- $\beta$ -D-galactopyranoside (X-gal), which are also recommended by the International Dairy Federation (I). Yet, closely related species of lactic acid bacteria have often minor differences in phenotypic traits. The phenotypic characteristics may also be variable within certain species, which makes clear identification complicated. Therefore, research has focused on the application of molecular biology techniques for rapid detection and differentiation of lactic acid bacteria. The rRNA genes are generally used to design species-specific oligonucleotide probes and PCR-primers, which are applicable for the differentiation of bacteria mostly at species level. Differentiation at strain level can be achieved by e.g. pulsed-field gel electrophoresis (PFGE) of genomic macrorestriction fragments. The aim of this study was to show if consistent results can be achieved by phenotypic and genotypic characterisation of starter lactic acid bacteria originating from mesophilic dairy starters.

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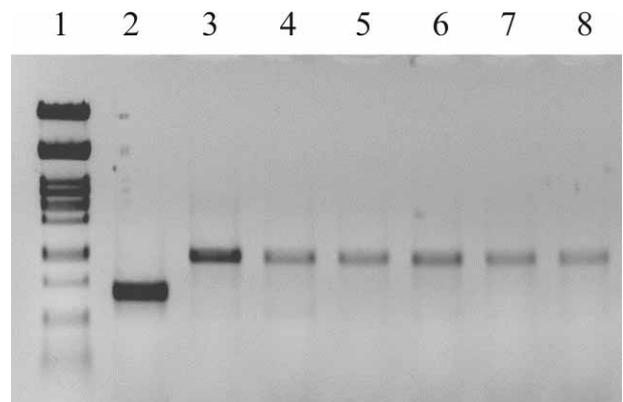
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## Materials and Methods

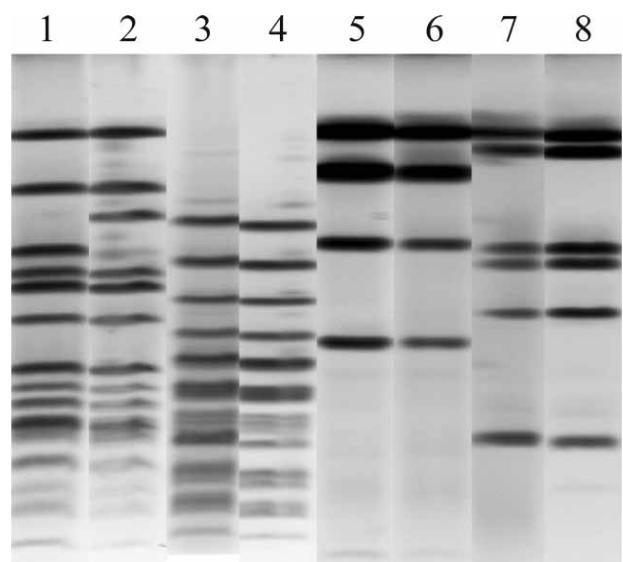
*L. lactis* and *Leuconostoc* strains studied were isolated from two mesophilic dairy starters. *Lactococcus* strains were grown at 25 °C in M17 broth (Difco Laboratories), skimmed milk or on M17 plates solidified with 1.5 % agar. *Leuconostoc* strains were grown at 25 °C in MRS broth (Difco Laboratories) or on MRS plates. The isolated strains were phenotypically characterized by studying their ability to hydrolyze citrate and X-gal on modified Nickels and Leesment's agar plates (2). In order to get clones originating from a single cell, colonies were picked from Nickels and Leesment's agar plates and purified twice on MRS (*Leuconostoc* strains) or M17 (*Lactococcus* strains) plates. For genotypic identification, selected strains were subjected to PCR and pulsed field gel electrophoresis (PFGE). The differentiation between *L. lactis* subsp. *lactis* and *L. lactis* subsp. *cremoris* was based on PCR using species-specific primers, targeting the glutamate carboxylase gene (*gadB*), followed by *AseI* digestion of the PCR products (3). The PCR reaction mixture consisted of the DNA template (0.5 µL of the bacterial lysate obtained by disintegration of the cells with glass beads), 1 × F-511 PCR buffer (Finnzymes, Espoo, Finland), 200 µM of each deoxyribonucleoside triphosphate, 1 µM of each primer and 0.02 U/µL Dynazyme II DNA polymerase (Finnzymes). The following PCR protocol was used: a primary denaturation cycle at 95 °C for 30 s, annealing at 50 °C for 60 s and elongation at 72 °C for 60 s. The final extension step (72 °C for 7 min) was followed by incubation at 4 °C. The PCR products were visualized on a 0.8 % agarose gel. *Leuconostoc* strains were identified by PCR utilizing species-specific primers, targeting the variable regions of 16S rDNA sequences (4). PCR amplification was performed as described above for lactococci. The preparation of the genomic DNA in agarose blocks for PFGE was performed as described by Tynkkynen *et al.* (5). *Lactococcus* and *Leuconostoc* strains were grown in M17 and MRS broth, respectively, to an absorbance of 0.6–1.0 at 600 nm ( $A_{600nm}$ ). Cells were harvested by centrifugation, washed with the solution of 10 mM Tris, 20 mM NaCl and 50 mM EDTA (pH=7.2), and resuspended in 100 µL of the same buffer. The suspension was mixed with 100 µL of 2 % agarose (Certified Megabase Agarose, Bio-Rad) before solidifying in the molds. The agarose blocks were treated with lysis buffer (10 mM Tris, 20 mM NaCl, 100 mM EDTA, 1 % sarcosyl and 0.2 % deoxycholate, containing 2.5 mg/mL lysozyme (Roche)) at 37 °C overnight. The blocks were then incubated for 18 h at 50 °C in the buffer containing 100 mM EDTA, 1 % sarcosyl and 0.2 % deoxycholate (pH=8), supplemented with proteinase K (1 mg/mL). The blocks were soaked four times in the solution of 20 mM Tris and 50 mM EDTA (pH=8), the first two washes containing 1 mM phenylmethylsulfonyl fluoride (Sigma). The blocks were then washed twice with TE buffer and equilibrated with the restriction buffer recommended for the enzyme by the manufacturer. After restriction enzyme digestion, the electrophoresis of the digested DNAs was performed on 1 % Certified Megabase Agarose (Bio-Rad) in 0.5 × TBE buffer using CHEF DRIII apparatus (Bio-Rad Laboratories). The conditions applied to electrophoresis were as recommended by the manufacturer (Bio-Rad Laboratories).

## Results and Discussion

The modified Nickels and Leesment's agar with the added X-gal was a convenient method to differentiate the aroma producers *L. mesenteroides* subsp. *cremoris* (blue colonies with or without a clear zone) and *L. lactis* subsp. *lactis* biovar *diacetylactis* (white colonies with a clear zone) from *L. lactis* subsp. *lactis* and *L. lactis* subsp. *cremoris* (white colonies without a zone). During cultivation for pure cultures originating from a single cell, some lactococcal strains showed retarded growth on M17 agar plates, but grew normally on MRS agar. A PCR assay performed with primers designed to detect lactococci (3) failed to produce any specific bands when the products were analysed on 0.8 % agarose. Instead, a multiplex PCR experiment carried out with primers specific for



**Fig. 1.** Multiplex PCR identification with *Leuconostoc mesenteroides* and *Leuconostoc lactis* specific primers. Lanes: 1, molecular weight marker; 2, *L. lactis* DSM 20202; 3, *L. mesenteroides* DSM 20346; 4–7, isolated starter strains A3, A4, A5 and A6, respectively, identified as lactococci by phenotypic methods; 8, isolated starter strain A2 identified as *Leuconostoc* by phenotypic methods



**Fig. 2.** PFGE patterns of total DNA from the selected strains digested with *SmaI* (lanes 1 to 4) and *NotI* (lanes 5 to 8). Lanes: 1 and 5, isolate A1 (*Lactococcus lactis* subsp. *cremoris*); 2 and 6, isolate A7 (*L. lactis* subsp. *cremoris*); 3 and 7, isolate A2 (*Leuconostoc mesenteroides*); 4 and 8, isolate A3 identified as *Lactococcus* by phenotypic methods

leuconostocs (4) resulted in a PCR-product typical of *L. mesenteroides* subsp. *cremoris* (Fig. 1). A further analysis of the genotypes by PFGE was in agreement with the results obtained by PCR and verified the misclassification by phenotypic methods. The fingerprints of these strains were indistinguishable from the fingerprints of strains identified as *L. mesenteroides* (Fig. 2).

As the accuracy achieved in classification of lactic acid bacteria by phenotypic methods has proven limited, there is great interest in developing molecular typing methods. For closely related species with very similar phenotypic characteristics, correct classification can be ensured only by the use of genotypic techniques (5–7). In this study, *Leuconostoc* strains which do not split X-gal could be identified with the use of genotypic methods in reference with a phenotypic assay. The inability of *L. mesenteroides* subsp. *cremoris* strain to split X-gal has been reported earlier for a type strain obtained from a culture collection (2). In the present study, it was shown that this type of change in phenotypic pattern may occur also in starter cultures used in dairy industry, and must be taken into account when typing strains originating from dairy starters. The  $\beta$ -galactosidase enzyme, responsible for the activity to split lactose and X-gal, is plasmid-encoded in dairy leuconostocs (8,9), which may be the reason for the detected instability of this trait.

The evolution from traditional home-made fermented milks to industrial large-scale production has raised a growing demand for the development of starter cultures with desirable application-specific properties. Classification of lactic acid bacteria based on phenotypic characteristics is of significance, because phenotypes directly reveal the abilities required in dairy industry. However, in order to avoid misclassification, it is of primary importance to use more than just a single trait for species-specific identification of starter strains. With strains displaying unusual metabolic characteristics, like the atypical leuconostocs detected in this study, genotypic differentiation methods used in combination with phenotypic tests provide a fast and convenient way for reliable typing of starter lactic acid bacteria.

### Acknowledgements

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## Sojevi *Leuconostoc* koji ne cijepaju analogon laktoze otkriveni karakterizacijom mezofilnih starter kultura u mljekarstvu

### Sažetak

Mezofilne starter kulture u proizvodnji mlijeka najčešće se određuju biokemijskim metodama. Budući da su različite vrste bakterija mliječne kiseline vrlo srodne, gotovo da se ne razlikuju po fenotipskim značajkama koje se također mogu mijenjati unutar određenih vrsta, njihova je točna identifikacija vrlo komplicirana. Stoga su se počeli primjenjivati postupci molekularne biologije za brzo otkrivanje i razlikovanje bakterija mliječne kiseline. U radu su neki bakterijski sojevi, izolirani iz mezofilnih startera, prethodno identificirani fenotipskim postupcima kao laktokoki, a primjenom PCR i PFGE (engl. pulsed field gel electrophoresis) utvrđeni kao sojevi *Leuconostoc*. Stoga metode genotipske diferencijacije u kombinaciji s fenotipskim testovima omogućavaju pouzdani način identifikacije bakterija mliječne kiseline s atipičnim metaboličnim značajkama.

