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Characterization of *Lactococcus lactis* subsp. *lactis* Isolated from Surface Waters

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ABSTRACT. A group of nine presumptive enterococci was isolated on enterococcal selective media Slanetz–Bartley agar and/or kanamycin–esculin–azide agar during a screening of *Enterococcus* spp. in surface waters. All strains formed a homogeneous cluster separated from all enterococcal species using rep-PCR fingerprinting with the $(GTG)_5$ primer but they matched fingerprints revealed by *Lactococcus lactis* subsp. *lactis* representatives. Further identification using extensive biotyping and automated ribotyping with *Eco*RI (RiboPrinter® microbial characterization system) confirmed all strains as *L. lactis* subsp. *lactis* in full correspondence with the $(GTG)_5$-PCR. We demonstrated that *L. lactis* subsp. *lactis* strains occur in different surface waters and can be confused with enterococci due to their positive growth on selective enterococcal media as well as positive results in tests commonly used for identification of the genus *Enterococcus* (esculin hydrolysis, acetoin and pyrrolidonyl arylamidase production, growth at 10 °C and in 6.5 % NaCl). The $(GTG)_5$-PCR fingerprinting was revealed as a reliable and fast method for the identification of *L. lactis* subsp. *lactis* while automated ribotyping with *Eco*RI proved to be a good tool for intrasubspecies typing purposes.

Lactococci are Gram-positive, non-motile, catalase-negative cocci belonging among the lactic acid bacteria group. The genus consists of five species inhabiting various environments (Klijn *et al*. 1995; Euzéby 2007). The most important species of the genus is *Lactococcus lactis* that is traditionally considered as a milk-product-associated bacterium. It plays an important role in dairy industry as a common part of many fermented products and as a crucial part of starter cultures. Therefore, this species is mainly associated with dairy environment and these economically important milk-derived strains are studied and characterized (Stiles and Holzapfel 1997). Isolation of *L. lactis* from other sources is reported less commonly. For example, *L. lactis* was retrieved from plant surfaces (Nomura *et al*. 2006), animals (Pot *et al*. 1996), the environment associated with cattle farms and a cheese-production plant (Klijn *et al*. 1995), forest industry wastewaters (Niemi *et al*. 1993) or from human clinical materials (Aguirre and Collins 1993).

This study deals with a group of *L. lactis* subsp. *lactis* strains isolated from different surface waters on enterococcal selective media during a routine microbiological water analysis.

MATERIAL AND METHODS

Bacterial strains were isolated by inoculation of analyzed water samples on Slanetz–Bartley agar (*Oxoid*, UK) and/or on kanamycin–esculin–azide agar (*Merck*, Germany). Individual enterococcus-like colonies were picked up, purified on brain–heart infusion agar (*Oxoid*) and maintained at –70 °C. Analyzed water samples were collected in Czechia from the following sources and localities: strain P1328, peloid bath, spa Karlovy Vary; P1409, storage reservoir in a lime and cement factory, Mokrá (Northern Moravia); P1942, brook Radějovka, Radějov (South-Eastern Moravia); P1944, unnamed pond, Ostrožská Nová Ves (South-Eastern Moravia); P1946, intake of the dihydrogen sulfide water into the reservoir, spa Buchlovice-Leopoldov (South-Eastern Moravia); P2011, swimming beach Rakovec, the Brno dam, Brno; P2013, swimming beach Sokolská, the Brno dam, Brno; P2016 and P2019, two different unnamed little ponds, Čížkrajice (South Bohemia). Reference strains were obtained from the *Czech Collection of Microorganisms, Masaryk University* (Brno, Czechia; **www.sci.muni.cz/ccm**).

Repetitive sequence-based PCR. The rep-PCR fingerprinting using the $(GTG)_5$ primer (5′-GTG GTG GTG GTG GTG-3′) was done according to Gevers *et al*. (2001) with the following modifications: Bacterial DNA was isolated by alkaline extraction procedure according to Švec *et al*. (2001): a 1-μL loopful of bacterial cells was homogenized in 20 μL of lysis buffer (0.25 % sodium dodecyl sulfate + 50 mmol/L

NaOH) and heated for 15 min at 95 °C. The obtained cell lysate was diluted by adding of 180 μL sterile deionized water, centrifuged (220 Hz, 5 min) and maintained at –20 °C. Totally, 1 μL of cell lysate and 24 μL of a PCR mixture were included into PCR reactions performed in Tpersonal ThermoCycler (*Biometra*, Germany). PCR products were separated in 1.5 % agarose gels (200 × 250 mm) for 16 h at 60 V (≈1.7 V cm^{-1}) in 0.5× TBE buffer (*Fluka*, Switzerland). The resulting fingerprints were digitized and processed using BioNumerics v. 4.601 software (*Applied-Maths*, Belgium).

Phenotype characterization. Phenotypical testing for catalase production, growth at 10, 42 and 45 °C and in 6.5 % NaCl was done according to Švec *et al*. (2001). Pyrrolidonyl arylamidase production and Voges–Proskauer reaction were tested using PYRA test and VP test, respectively (both *PLIVA–Lachema*, Czechia). Group D antigen was tested using a Streptococcal grouping kit (*Oxoid*, UK). Biochemical tests were performed using the API 50CH kit according to manufacturer's instructions (*bioMérieux*, France). Identification was done using the Internet identification tool Apiweb (*bioMérieux*, **https://apiweb.biomerieux.com**).

Ribotyping. Bacterial cells used for ribotyping were cultivated overnight in a 5 % CO_2 atmosphere at 37 °C on brain heart infusion agar (*Oxoid*). Automated ribotyping with *Eco*RI restriction enzyme was carried out using a RiboPrinter® microbial characterization system (*DuPont Qualicon*, USA). Standard procedure intended for lactic acid bacteria was performed in accordance with the protocol provided by the manufacturer. The ribopatterns were normalized and compared to a DuPont Qualicon database DUP 2004 containing 6448 different ribotype profiles by using the RiboExplorer v. 2.1.4216.0 operating software (*DuPont Qualicon*). Numerical analysis of the ribopatterns and dendrogram construction was performed with Bionumerics v. 4.601 software (*Applied-Maths*). Import of the RiboPrinter data into the BioNumerics software was achieved by using the Load samples import script obtained from *Applied-Maths*.

RESULTS AND DISCUSSION

All strains grew well on Slanetz–Bartley agar in typical enterococcus-like small dark-red colonies and were esculin-positive on kanamycin–esculin–azide agar. Both these traits presumtively assigned the analysed strains as members of the genus *Enterococcus*. Further characterization using rep-PCR with the $(GTG)_5$ primer generated DNA fragments ranging from 200 to 2400 bp and clearly separated analyzed strains from all enterococci included in the in-house CCM database (*data not shown*). All strains revealed visually nearly identical fingerprints very close to the *L. lactis* subsp. *lactis* representatives and clustered them into a homogeneous group at the similarity level of >62 % (Fig. 1). The closest, but visually clearly different fingerprint, was shown by *Lactococcus garvieae* CCM 7413^T revealing 33 % similarity to *L. lactis* subsp. *lactis* cluster.

Rep-PCR using the $(GTG)_5$ primer was successfully used for identification of different lactic acid bacteria (*e.g.*, Gevers *et al*. 2001; Švec *et al*. 2005) including lactococci (Ouadghiri *et al*. 2005; Prodělalová *et al*. 2005; Huys *et al*. 2006; Zamfir *et al*. 2006). Our results confirmed this simple and fast method as a good taxonomic tool for identification of *L. lactis* subsp. *lactis* and imply $(GTG)_5$-PCR as a suitable method for identification of other lactococcal species as well as for their differentiation from enterococci.

Further characterization using physiological and biochemical tests confirmed the obtained results and clearly assigned the analyzed strains to *L. lactis* subsp. *lactis.* All the nine strains were catalase-negative, ovoid cocci arranged in groups, pairs and short chains and grew at 10 °C and weakly at 42 °C. Growth at 45 °C was negative (except strain P2013). Variable reaction was revealed for growth in 6.5 % NaCl (weakly positive P1944, P1946, P2011) and pyrrolidonyl arylamidase production (weakly positive P1944, P2016, P2019). Acetoin production (Voges–Proskauer reaction) was positive; group D antigen was negative. The API 50CH numerical profiles calculated for individual strains are as follows: P1328, P1944 and P1946: 0070 1702 0377 0611 0004; P1409, P2011 and P2019: 0030 1702 0377 0611 0004; P2013: 0030 1702 0377 0611 0000; P2016: 0030 1702 0377 0611 0004; and P1942: 0020 1700 0377 0401 0000. Evaluation of these results using the Apiweb identification tool assigned all strains to *L. lactis* subsp. *lactis*.

Identification of lactic acid bacteria using biochemical tests is not often straightforward and reliable. Lactococci can be most often confused with enterococci due to possible combination of positive so-called "classical" tests used for differentiation of the genera of Gram-positive, catalase-negative cocci. Lactococcal strains can be positive for the Voges–Proskauer reaction, esculin hydrolysis, pyrrolidonyl arylamidase production and growth at 10 and 45 °C and in 6.5 % NaCl (Devriese *et al*. 1993; Facklam and Elliott 1995). Especially in the case of analysis of bacteria isolated from nondairy sources it is possible to misidentify lactococci as enterococci (Elliott and Facklam 1996). However, genus identification should necessarily follow species identification (Devriese *et al*. 1993) so that extensive biotyping using a wide variety of biochemical

tests could clarify the genus identification and/or lead to the correct species identification as shown in our study.

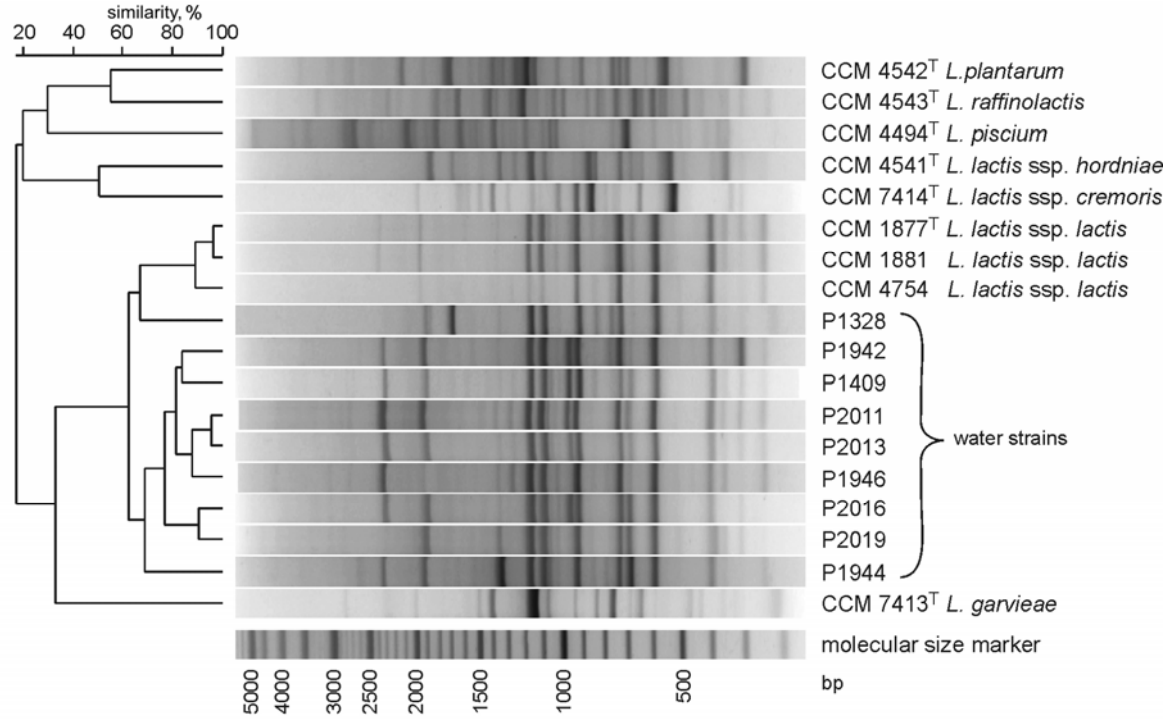

Fig. 1. Dendrogram based on cluster analysis of $(GTG)_5$-PCR fingerprints obtained from analyzed and reference strains, representing all recognized lactococcal species; the dendrogram was calculated with Pearson's correlation coefficients using UPGMA clustering method (% – *r*, expressed for convenience as percentage similarity values); the fingerprint images are inverted due to the software requirements.

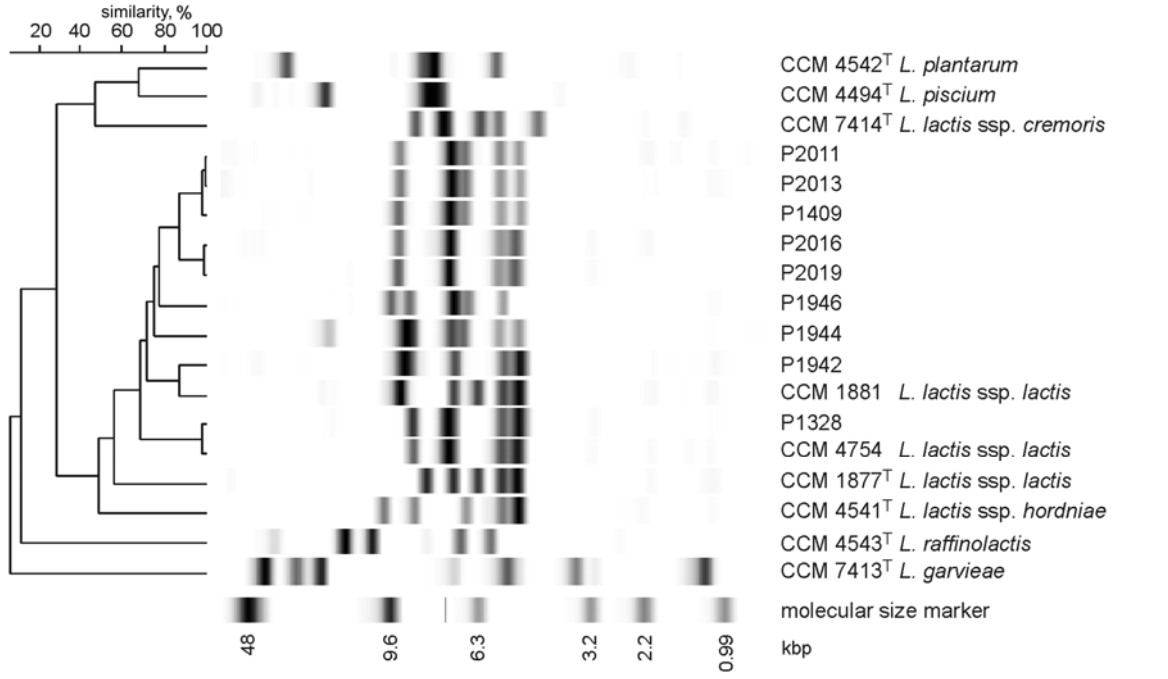

Fig. 2. Dendrogram based on cluster analysis of *Eco*RI ribotype patterns obtained by automated ribotyping with the RiboPrinter® microbial characterization system; the dendrogram was calculated with Pearson's correlation coefficients using UPGMA clustering method (% – *r*, expressed for convenience as percentage similarity values).

Automated ribotyping with the *Eco*RI clustered reference and water *L. lactis* subsp. *lactis* strains into a single group separated from all other lactococcal taxa at the similarity level of >56 % (Fig. 2). In total, six ribopatterns were found among the water isolates. All strains (except P1946) shared two typical bands (5.3 and 5.7 kbp) separating clearly all *L. lactis* subsp. *lactis* from other lactococci except *L. lactis* subsp. *hordniae* CCM 4541^T which revealed the closest ribopatterns to *L. lactis* subsp. *lactis* strains (48 % similarity). Identification obtained with the RiboExplorer software assigned strains P1942, P1944, P1946, P2016 and P2019 as *L. lactis* subsp. *lactis*; the remaining four strains were not identified. No correlation between clustering obtained with rep-PCR, ribotyping and biotyping was revealed but two couples of strains (P2011 and P2013, P2016 and P2019) isolated from different sampling places on the same locality revealed identical ribopatterns. Automated ribotyping separated the analyzed strains into visually clearly different ribopatterns in contrast to rep-PCR which revealed visually very close fingerprints. Considering these results, the $(GTG)_5$-PCR fingerprinting is a reliable and fast method for identification of *L. lactis* subsp. *lactis* while automated ribotyping with *Eco*RI was shown to be a better tool for intrasubspecies typing purposes.

Our short study demonstrates that *L. lactis* subsp. *lactis* is more abundant in different surface waters than generally considered, and that this bacterium can be commonly recovered during a routine microbiological water analysis. Although lactococci are fastidious microorganisms they can survive in waters providing sufficient nutrition. Water environment probably does not favor multiplication of lactococci and it is likely that the strains analyzed in this study represent transient microflora originating from plants or from dairy environment. *L. lactis* subsp. *lactis* strains can be confused with enterococci due to their growth on selective enterococcal media as well as possible positive "classical" tests commonly used for identification of the genus *Enterococcus*. Molecular methods, *e.g.*, rep-PCR used in this work or extensive biotyping, should be used for reliable differentiation of these two genera.

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