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Usage of *gdp*P for improved subspecies differentiation of *Lactococcus lactis*

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Abstract

Lactic acid bacteria (LABs), in particular *Lactococcus lactis* strains, are important for the production of fermented dairy products. Natural sources are interesting reservoirs of new strains with superior performance. Five LAB isolates from Algerian camel milk were characterized phenotypically, i.e. according to metabolic activities and growth characteristics, and by gene sequence-based methods. Phenotypically all isolates were identified as proteolytic *Lactococcus lactis*, able to utilize citrate and lactose and to grow at temperatures up to 45°C, they could not be differentiated at the subspecies level. Sequencing and analysis of their 16S rRNA identified three strains as belonging to *L. lactis* ssp. *lactis*, and two as *L. lactis*ssp. *cremoris*. These results were confirmed by a novel approach, using the conserved, single-copy *gdp*P gene for complementary phylogenetic analysis. *Gdp*P performed better than the 16S rRNA gene in resolving relationships as well as subtle differences at the subspecies level. The isolate HD20A was different from the other *L. lactis*ssp. *lactis* strains, showing are combination event in its *gdp*P gene which proved to be a chimaera between the sequences typical for ssp. *lactis* and ssp. *cremoris*, respectively. This is another example for the importance of horizontal gene transfer in the evolution of *Lactococcus lactis*.

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Introduction

Lactic acid bacteria (LAB), many of them being Generally Recognized as Safe (GRAS) organisms, are involved in the manufacture of a wide variety of fermented foods. They produce exopolysaccharide (Nyyssola *et al.*, 2005), antibacterial agents (Arques *et al.*, 2015), vitamins (Wegkamp *et al.*, 2007) andhigh-value metabolites involved in flavour, texture, development and food preservation. Various species of LAB are claimed to act as probiotics (Marco *et al.*, 2010) andas potential carriers of compounds with therapeutic or prophylactic effects (Amar *et al.*, 2011).

The beneficial microbiota of camel milk represented by LAB is a potential source of biological materials to be used in dairy technology (Khedid *et al.*, 2009). Camel milk is known for its medicinal properties and is widely exploited in several countries (Mal *et al.*, 2006; Konuspayeva *et al.*, 2011; Zuo *et al.*, 2014; Benmechernene *et al.*, 2014; Kadri *et al.*, 2015a; Kadri *et al.*, 2015b;). *Lactococcus lactis* is the best characterized member of the LAB and is considered the model organism of this group.

The two major *L. lactis* subspecies, ssp. *lactis* and ssp. *cremoris*, only differ by a few phenotypic traits (Kelly and Ward, 2002), they are used as starters for the production of fermented dairy food (Holzapfel, 1997).

The ssp. *lactis* is able to metabolize arginine, is tolerant to 40°C and 4% NaCl, but normally sensitive to 45°C and 6.5% NaCl. In addition, strains of the biovar. *diacetylactis* can ferment the citrate present in milk, which constitutes a secondary energy source. In contrast, ssp. *cremoris* is usually sensitive to 40°C and 4% NaCl, and most strains do not display the ability to catabolize arginine.

Discriminating between ssp. *cremoris* and *lactis* is difficult, but is very important in industrial applications, because the activities of the two subspecies in cheese manufacturing differ.

In addition, when newly isolated bacterial strains are registered in public culture collections, these strains have to be identified and discriminated at the subspecies level. From an evolutionary viewpoint, it is reasonable to classify subspecies also by using the divergence of housekeeping genes that are well conserved at the genus or species level (Case *et al.*, 2007).

This study aims to identify *Lactococcus lactis* strains, exhibiting proteolytic abilities, at the subspecies level. Five *Lactococci* isolated from raw camel milk in an arid region in the south of Algeria (Drici *et al.*, 2010) were characterized by phenotypic, physiological and biochemical properties. Furthermore, a molecular characterization is proposed for these isolates to avoid the limitations of phenotypic characterization and to achieve reliable and consistent identification.

Materials and methods

Bacterial strains, growth conditions and phenotypic characterization

The *L. lactis* strains used here, designated HD20A, HD20D, HD12C, HD11D, and HD9B_GL2, isolated from dromedary's milk in South Algeria, were provisionally characterized in a previous study(Drici *et al.*, 2010). They had randomly been selected for identification based on phenotypical characteristics such behavior in Gram stain and catalase test.

They were routinely cultured at 30°C in M17-broth (Terzaghi and Sandine, 1975) supplemented with 5 g/l (w/v) glucose (GM17 brothor agar) under aerobic conditions. Extracellular proteolytic activity was visualized by a transparent halo around bacterial colonies developing on milk agar because of hydrolysis of milk casein. All tests for phenotypic characterization were performed as previously described (Drici *et al.*, 2010).

Genotypic characterization

DNA Isolation, PCR methods and sequence analysis

Bacteria were grown at 30°C on M17 agar plates with 1% of glucose. Total DNA was extracted directly from colonies: a single colony was resuspended in 50 µl 1X PCR buffer containing 1 µl lysozyme (120 mg/ml) (lysozyme from chicken egg, Sigma-Aldrich CHEMIE

GmbH., Steinheim, Germeny) and incubated at 37°C for 15 min. 10 µl proteinase K (Roth, Karlsruhe, Germany) was added (12 mg/ml) and the mixture was incubate at 56°C for 15 min then 5 min at 110°C. 2 µl of treated cells are used for PCR reactions. PCR was performed using Mol Taq DNA polymerase (Taq DNA Polymerase, Mol Zym, Bremen, Germany)in aT3 Thermocycler (Biometra GmbH, Göttingen, Germany). A 251 bp fragment of the 16S rRNA gene was amplified and sequenced using the designed primer pair NG_Lactis_16s_Forward: 5'-GTGCCTAATACATGCAAGTTG-3'and NG_Lactis_16s_Reverse: 5'-CTATGTATCATCGCCTTGGT-3'. PCR conditions were as follows: preheating at 95°C for 4 min; 35 cycles of denaturing at 94°C for 30 sec; annealing at 59°C for 30 sec and extension at 72°C for 1 min, and a final extension at 72°C for 10 min. A 923-bp region of the *gdp*P genes were PCR-amplified and sequenced using the following primer pairs: fwd1 5'-CGTCAAGATTTTTATTCG-3', rev1 5'-AAAATAATGGCGACTGC-3' and fwd2 5'-CGTGCAGTTATTGGTGC-3', rev2 5'-TTGCACGACAACATCTC-3'. PCR conditions were as follows: preheating at 94°C for 5 min; 30 cycles of denaturing at 94°C for 30 sec; annealing at 50°C for 30 sec and extension at 72°C for 30 sec then at 94°C for 30 sec , and a final extension at 72°C for 10 min. The PCR product was electrophoresed on 1% agarose (NEEO Ultra-Quality, Roth, Karlsruhe, Germany) gel in 1X TBE buffer stained with10 µlHD green (INTAS Science Imaging Instruments GmbH, Göttingen, Germany).

The respective bands were purified directly from the gel with a special DNA Purification Kit (DNeasy Blood & Tissue Kit, QIAGEN GmbH, Hilden, Germany) according to the manufacturer's instructions. The sequencing was done by SeqLab (Sequence Laboratories GmbH, Göttingen, Germany) using a Sanger 3730 XL from ABI with the same primers used for PCR reactions.

Sequence alignments of 16S rRNA, gdpP sequences and construction of phylogenetic trees

Multiple sequence alignments of both partial genes were performed with DIALIGN-TX software (Subramanian *et al.*, 2008), trees were generated with PhyML (Criscuolo, 2011) using the maximum likelihood method from the multi-sequence alignment profile we obtained. Visualization resulted from Sea view (Galtier *et al.*, 1996) and Splits tree 4 (Huson and Bryant, 2006).

Database analyses were done at www.ncbi.nlm.nih.gov/genomes/MICROBES/microbial_taxtree.htm

Nucleotide sequence accession numbers

16S ribosomal RNA genes: Sequences of 16S ribosomal RNA genes of strains HD11D, HD12C, HD9B_GL2, HD20A and HD20D have been assigned Genbank accession numbers KR336809, KR336810, EF362617, KR336812, and KR336813. The whole-genome shotgun project of *L. lactis* ssp. *lactisHD9B*_GL2has been deposited in GenBank/EMBL/DDBJ under the accession no. JNCC03000000 (chromosome), consisting of contig sequences JNCC03000001 to JNCC03000048. *gdp*P sequences: In the case of HD9B_GL2,a partial sequence of 743 nt has been deposited in GenBank (see below), however, for phylogenetic treeing the complete sequence (locus tag LGL2_RS03435, 1935 nt), derived from the genome sequence of HD9B_GL2, has been used. The *gdp*P partial sequences accession numbers concerned different *L. lactis* ssp. *lactis* strains HD11D, HD12C, HD9B_GL2, HD20A and HD20D are KR904887, KR904888, KR904891, KR904889, KR904890 respectively.

The GenBank/EMBL/DDB accession nos. of the genome sequences of the reference strains are IL1403: NC_002662.1/AE005176.1; KLDS4.0325:NC_022593.1/CP006766.1; NCDO2118: NZ_CP009054.1/CP009054.1; MG1363: NC_009004.1/AM406671.1; SK11: NC_008527.1/CP000425.1

Results and discussion

Phenotypic characterization

We were interested in proteolytic LAB strains isolated from the indigenous flora of raw milk of *Camelus dromedarius* in the south of Algeria. Five isolates were identified as *Lactococci* since they displayed the following properties, as summarized in Table 1: Gram-positive, oxidase and catalase negative, able to utilize lactose and citrate and to grow rapidly on agar milk medium. All these proteolytic strains exhibited a tolerance to 45°C and alkaline pH. Thus the phenotypic characterization did not allow for subspecies discrimination. Rapid growth in milk and at a higher temperature, in addition to casein, lactose and citrate-utilizing abilities, reflects a diversity of these strains unknown for usual dairy Lactococcal starter strains and drove us to further investigate them in order to characterize their taxonomic position. For this, molecular methods were used, suited to identify strains at the subspecies level.

Table 1. Main properties of the five camel milk isolates.

Strain	Cat	Oxy	HHD	Citrate	Lac	M17-Glu	45°C	Milk-Glu	pH 9,6
HD20A	-	-	Homo	+	+	+	+	+T	+
HD20D	-	-	Homo	+	+	+	+	+T	+
HD12C	-	-	Homo	+	+	+	+	+T	+
HD11D	-	-	Homo	+	+	+	+	+T	+
HD9B_GL2	-	-	Homo	+	+	+	+	+T	+

Cat: catalase. Oxy: oxydase. HHD: homofermentative-heterofermentative differential medium. Citrate: citrate-fermenting capacity (+) or incapacity (-) after growth on KMK agar medium. Lac: lactose utilization. M17-Glu: growth on M17 supplemented with 5 g/l (w/v) glucose. Milk-Glu: growth on milk supplemented with 5 g/l (w/v) glucose.45°C: slow but noticeable growth at 45°C. T: presence of a transparent halo around bacterial colonies developing on milk agar because of hydrolysis of milk casein. Bacterial growth (+) or absence of growth (-).All strains were gram-positive.

Genotypic characterization

16S rDNA sequencing and analysis

16S rRNA gene sequencing is the most common technique currently used to identify species(Balcazar *et al.*, 2007; Tanigawa *et al.*, 2010). Sequences of a 251-bp fragment of the 16S rRNA genes, providing discrimination between *lactis* and *cremoris* subspecies, were compared.

It is known that the sequences of *lactis* and *cremoris* subspecies differ by 10-bp within the first 200-bp of the sequence(Ward *et al.*, 1998).

The respective gene regions of the strains under study were PCR-amplified and sequenced. Based on the obtained sequences, phylogenetic trees have been calculated.

The HD9B_GL2, HD11D, HD12C strains were identified as ssp. *Lactis* (Fig. 1A.). The HD20A and HD20D strains were identified as ssp. *cremoris* (Fig. 1B).

GdpP gene sequencing and analysis

Lactococcus lactis strains often contain multiple alleles of the 16S rRNA gene which may complicate the phylogenetic analysis.

It has therefore been suggested to complement the 16S RNA-based approach by the use of a conserved single-copy gene (Case *et al.*, 2007). *Gdp*P of *L. lactis* (for GGDEF domain protein containing phosphodiesterase) encodes a membrane-bound stress signaling protein of a protein family which exhibits cyclic dimeric AMP (c-di-AMP)-specific phosphodiesterase activity (Smith *et al.*, 2012).

Database analysis showed that a *gdp*P gene with the particular sequence found in *L. lactis*is present in this species only, the functional homologs in other firmicutes have largely different gene sequences.

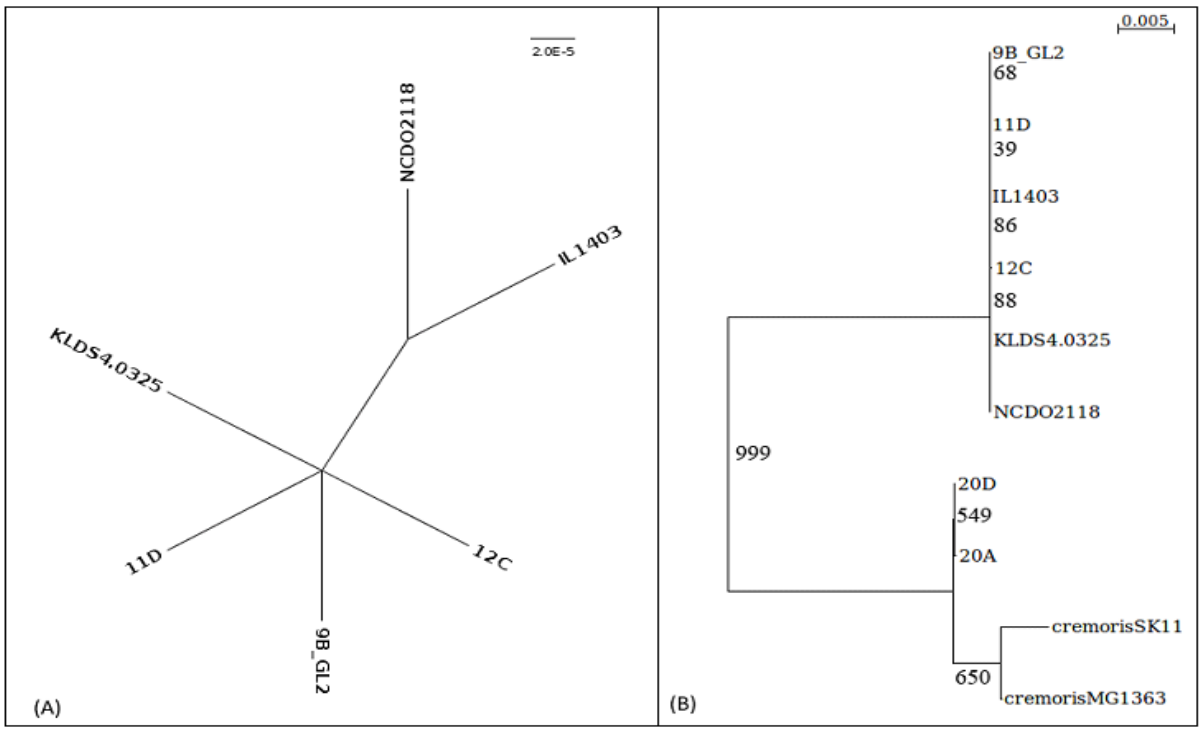

Fig. 1. Phylogenetic analysis based on 16S rRNA sequences. (A) Unrooted tree illustrates phylogenetic relationship among *Lactococcuslactis* ssp. *lactis* strains investigated in this study. (B) Rooted maximum likelihood tree with bootstrap values indicates the taxonomic position of the LAB species investigated here and of reference strains.

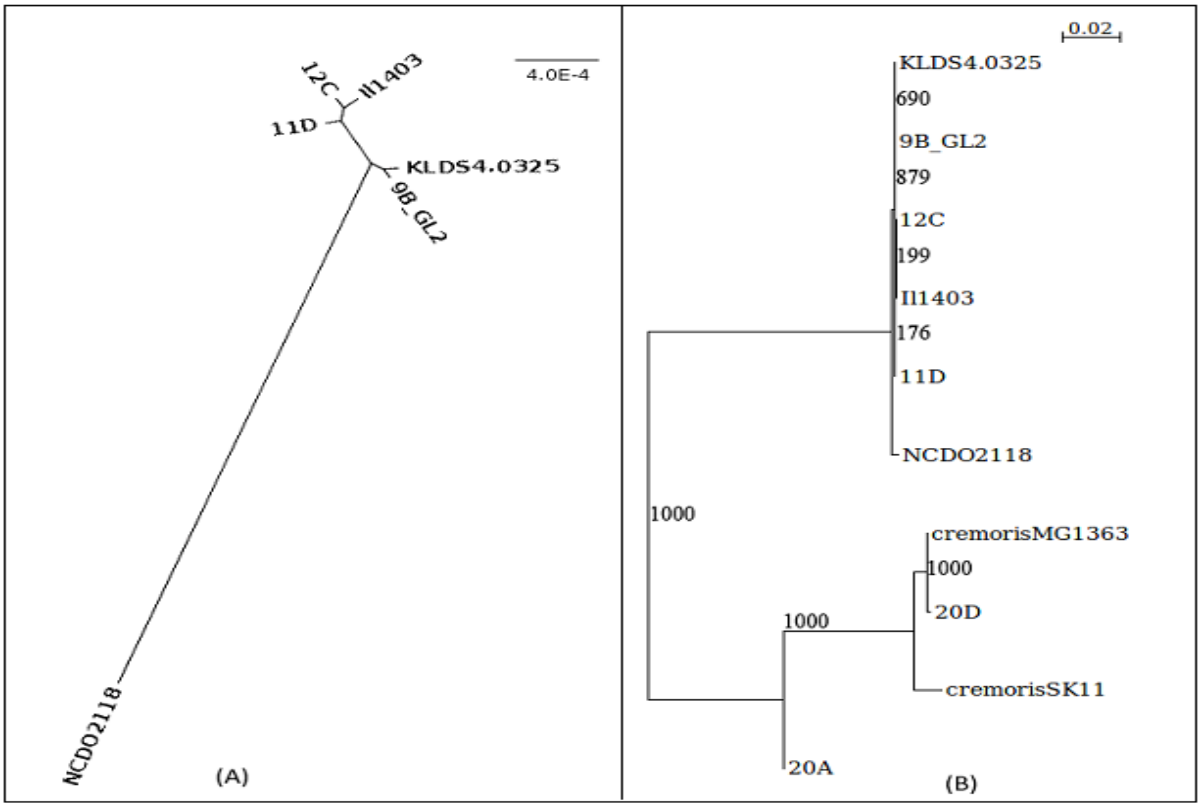

Fig. 2. Phylogenetic analysis based on *gdp*P sequences.(A) Unrooted tree illustrates the distance among the *Lactococcus lactis* spp. *lactis* strains. (B) Rooted tree with bootstrap values, constructed using the maximum likelihood method, of all strains and of reference strains.

It is absent in *L. garvieae*, *L. fujiensis*, *L. raffinolactis* and *L. piscium*. No statement can be made, due to the lack of complete genome sequences, for *L. lactis* ssp. *tructae* and ssp. *hordniae* as well as for other newly described *Lactococcus* species. The gene is well conserved, but shows enough variation over its entire length to allow for a fine resolution of strain differences. We PCR-amplified and sequenced a central part of the *gdp*P gene from the strains under study. Fig. 2A describes the phylogenetic relationships among the *Lactococcus lactis* ssp. *lactis* strains. HD9B_GL2 appears closest to KLDS4.3025 and relatively close to HD11D, HD12C and Il1403. All remain rather distinct from NCDO2118, based on *gdp*P sequences, a fact which could not be identified by the 16S rRNA approach.

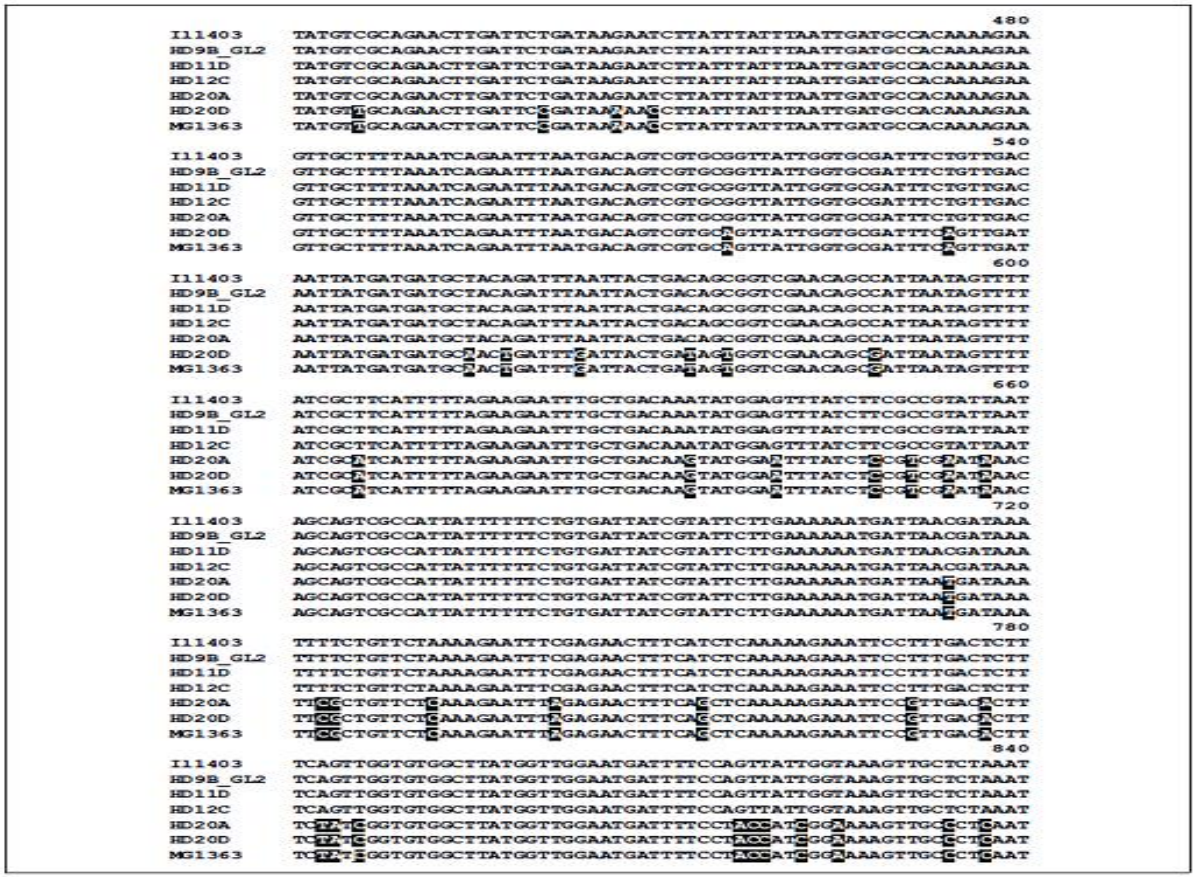

Fig. 3. *gdp*P gene variations. The local sequence alignment in the variable region highlights the recombination event. All positions where the HD strains and/or *L. lactis* ssp. *cremoris* MG1363 deviate from the *L. lactis* ssp. *Lactis* reference strain IL1403 are marked with white characters on black background. The figure shows nucleotides 420-840 only, but the observation that the sequence for HD20A in its 5´part is identical to *L. lactis* ssp. *lactis* and from nt 606 on identical to *L. lactis* ssp. *cremoris* holds true for the entire sequenced part of the gene.

Our phylogenetic analysis confirmed the close relationship, already seen by 16S rRNA analysis, between the novel strains HD20A and HD20D and *L. lactis* subsp. *cremoris* strains SK11 and MG1363 (Fig. 2B). However, HD20A behaved somewhat different from the other strains. A detailed sequence comparison (Fig. 3) revealed that the *gdp*P sequence of HD20A obviously resulted from a recombination event, its 5´part is identical to *L. lactis* ssp. *lactis* and from nt 606 on identical to *L. lactis* ssp. *cremoris,* this holds true for the entire sequenced part of the gene (Fig. 3). Our results resemble those found in another study where the analysis of the *ycd*B gene revealed that two strains of *the L. lactis* ssp. *Cremoris* MG1363 cluster, strains QA5 and QA30 contain alleles of the ssp. *lactis*IL1403 type.

This indicated that the *ycd*B gene was horizontally transferred among *L. lactis* strains (Bolotin *et al.*, 2004)and one can assume that such a gene transfer also gave rise to the chimaeric *gdp*P gene in HD20A. *Gdp*P provided a phylogenetic resolution comparable to that of the 16S rRNA gene at the species level, however, it showed better resolution at the subspecies level. This is particularly relevant in the context of a growing number of studies focusing on subspecies diversity, in which single-copy protein-encoding genes such as *gdp*P could complement the information provided by the 16S rRNA gene, as it enables deciphering of fine-scale phylogenetic relationships that go undetected using the 16S rRNA gene.

Up to now, nomad farmers in North Africa are preparing traditional fermented dairy products as "raib" or "lben" by using ancestral techniques, which are an important part of the tradition. Consequently, studying the microflora of traditional fermented dairy products is useful for organizing industrial production of traditional fermented products with local strains (Bensalah *et al.*, 2009). Moreover, the link between microflora population and variation factors as species or regions could be investigated to understand the gene transfer in *lactococci*.

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