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High Genetic Diversity among Strains of the Unindustrialized Lactic Acid Bacterium *Carnobacterium maltaromaticum* in Dairy Products as Revealed by Multilocus Sequence Typing

Abdur Rahman,^a Catherine Cailliez-Grimal,^a Cyril Bontemps,^{b,c} Sophie Payot,^{b,c} Stéphane Chaillou,^d Anne-Marie Revol-Junelles,^a Frédéric Borges^a

Université de Lorraine, Laboratoire d'Ingénierie des Biomolécules, ENSAIA, Vandœuvre-lès-Nancy, France^a; INRA, DynAMic, UMR1128, Vandœuvre-lès-Nancy, France^b; Université de Lorraine, DynAMic, UMR1128, Vandœuvre-lès-Nancy, France^c; INRA, UMR1319 Micalis, Jouy-en-Josas, France^d

Dairy products are colonized with three main classes of lactic acid bacteria (LAB): opportunistic bacteria, traditional starters, and industrial starters. Most of the population structure studies were previously performed with LAB species belonging to these three classes and give interesting knowledge about the population structure of LAB at the stage where they are already industrialized. However, these studies give little information about the population structure of LAB prior their use as an industrial starter. *Carnobacterium maltaromaticum* is a LAB colonizing diverse environments, including dairy products. Since this bacterium was discovered relatively recently, it is not yet commercialized as an industrial starter, which makes *C. maltaromaticum* an interesting model for the study of unindustrialized LAB population structure in dairy products. A multilocus sequence typing scheme based on an analysis of fragments of the genes *dapE*, *ddlA*, *glpQ*, *ilvE*, *pyc*, *pyrE*, and *leuS* was applied to a collection of 47 strains, including 28 strains isolated from dairy products. The scheme allowed detecting 36 sequence types with a discriminatory index of 0.98. The whole population was clustered in four deeply branched lineages, in which the dairy strains were spread. Moreover, the dairy strains could exhibit a high diversity within these lineages, leading to an overall dairy population with a diversity level as high as that of the nondairy population. These results are in agreement with the hypothesis according to which the industrialization of LAB leads to a diversity reduction in dairy products.

Food fermentation has been used for at least 7,000 years for the preservation and the manufacture of foods (1). Since the major role played by microorganisms was unraveled in the late 19th century, the commercial production and the use of starter cultures expanded and were widespread in the 20th century (2). Since then, these commercial bacteria massively populated dairy products in addition to traditional starters and opportunistic contaminants, including the natural milk microbiota. The advantages of using commercial starters were obvious; they could be used to optimize and standardize the manufacturing process. Later, the industrial production of food bacteria raised the question of its impact on the diversity of food microflora and thereby on the quality, and more specifically, on the typicality of the food product. In addition, several studies support the idea that industrialization of the dairy manufacturing led to a diversity reduction (3). In line with this, the study of intraspecific diversity by multilocus sequence typing (MLST) in the industrialized lactic acid bacteria (LAB) *Lactococcus lactis*, *Streptococcus thermophilus*, and representatives of the genus *Lactobacillus* revealed a strikingly low diversity. Within the species *Streptococcus thermophilus* a low diversity was recorded compared to the closely related species of the *salivarius* group that share other ecological niches (4). Similarly, *Lactococcus lactis* strains originating from cheeses exhibit a strikingly low diversity compared to the strains isolated either from raw milk or nondairy environments (5, 6). The relative low level of diversity is not restricted to starter LAB since the industrialized species *Lactobacillus casei*, a nonstarter LAB, exhibit a relatively low level of diversity as well (7).

Investigating the impact of LAB industrialization on population structure is not an easy task when bacteria are already on the market for decades. Even when studies are focused on traditional cheeses, it cannot be completely ruled out that commercial start-

ers have been used to solve technological problems such as occasional acidification failure. Apart industrialized LAB, cheeses can be populated with LAB that are still not industrialized at a wide scale. An interesting representative of these species is *Carnobacterium maltaromaticum*, which is a psychrotrophic and an alkaliphilic bacterium that can be isolated from a wide range of environments such as the sea, meat, or dairy products. In dairy products, *C. maltaromaticum* has been detected in pasteurized and raw milk soft cheeses manufactured with ewe, cow, or goat milk and can predominate in some cheeses at the end of the ripening when the pH values increases (8, 9). On the other hand, this weak milk-acidifying bacterium can also sustain low pH values and does not interfere with the acidifying activity of LAB starters (10). The cheeses known to contain *C. maltaromaticum* are Pérail, Brique de Jussac, Picodon, Petit Munster, Epoisse, Brie, Camembert, and Petit Livarot (8, 9). The presence of this bacterium has been associated with the development of malty flavors in milk (11) and cheese (12) and thus contributes to aroma production during the ripening process. Since *C. maltaromaticum* is generally recognized as safe in the United States (13) and is on the list of microorganisms with technological beneficial use (14), it could be used as an adjunct culture in the dairy industry. However, since it was discovered relatively lately in 1974 as *Lactoba-*

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Address correspondence to Frédéric Borges, frederic.borges@univ-lorraine.fr.

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TABLE 1 Characteristics of the *C. maltaromaticum* strain collection

| Strain code ^a | CC ^b | ST ^c | Allele no. | | | | | | | Origin | Yr | Location | Reference |
|-----------------------------|-----------------|-----------------|-------------|-------------|-------------|------------|-------------|-------------|-------------|-----------------------------|-------|-------------|------------|
| | | | <i>ilvE</i> | <i>dapE</i> | <i>pyrE</i> | <i>pyc</i> | <i>ddlA</i> | <i>glpQ</i> | <i>leuS</i> | | | | |
| F7 | 1 | 4 | 4 | 8 | 4 | 4 | 7 | 3 | 4 | Halloum | 2010 | Lebanon | This study |
| F44 | 1 | 4 | 4 | 8 | 4 | 4 | 7 | 3 | 4 | Mont D'or | 2010 | France | This study |
| LMA28 | 1 | 4 | 4 | 8 | 4 | 4 | 7 | 3 | 4 | Brie | 1994 | France | 16 |
| F29-1 | 1 | 4 | 4 | 8 | 4 | 4 | 7 | 3 | 4 | Lumpfish roe | | Denmark | 17 |
| DSM20342^T | 1 | 4 | 4 | 8 | 4 | 4 | 7 | 3 | 4 | Raw milk | 1974 | USA | 11 |
| F33 | 1 | 6 | 4 | 8 | 4 | 4 | 7 | 3 | 6 | Ste. Maure de Touraine | 2010 | France | This study |
| F86 | 1 | 11 | 4 | 8 | 4 | 4 | 3 | 3 | 4 | Cabrales | 2010 | Spain | This study |
| L1 | 1 | 13 | 4 | 8 | 4 | 6 | 7 | 3 | 4 | Raw milk | 2010 | France | This study |
| CP5 | 1 | 15 | 4 | 8 | 4 | 4 | 7 | 3 | 20 | Brie | 1994 | France | 16 |
| CP23 | 1 | 16 | 4 | 8 | 4 | 4 | 3 | 9 | 10 | Brie | 1994 | France | 16 |
| CP32 | 1 | 17 | 4 | 8 | 4 | 4 | 4 | 3 | 4 | Brie | 1994 | France | 16 |
| F4 | 2 | 3 | 3 | 3 | 3 | 3 | 3 | 2 | 3 | Le Montagnard | 2010 | France | This study |
| F84 | 2 | 3 | 3 | 3 | 3 | 3 | 3 | 2 | 3 | Kernhemmer | 2010 | Netherlands | This study |
| CP14 | 2 | 3 | 3 | 3 | 3 | 3 | 3 | 2 | 3 | Brie | 1994 | France | 16 |
| F87 | 2 | 3 | 3 | 3 | 3 | 3 | 3 | 2 | 3 | Tynjethaler | 2010 | Netherlands | This study |
| CP7 | 2 | 3 | 3 | 3 | 3 | 3 | 3 | 2 | 3 | Brie | 1994 | France | 16 |
| F42 | 2 | 7 | 3 | 3 | 3 | 3 | 4 | 2 | 3 | Rocamadour | 2010 | France | This study |
| N15 | 2 | 7 | 3 | 3 | 3 | 3 | 4 | 2 | 3 | Halibut-sugar salted | 1990s | Denmark | 17 |
| F43 | 3 | 8 | 2 | 4 | 8 | 6 | 7 | 8 | 7 | St. Marcellin | 2010 | France | This study |
| CIP100481 | 3 | 18 | 2 | 4 | 8 | 6 | 4 | 3 | 7 | Human blood | 1983 | France | |
| ATCC 35586 | 4 | 36 | 5 | 15 | 6 | 11 | 4 | 12 | 5 | Diseased rainbow trout | 1987 | USA | 18 |
| 9.4 | 3 | 30 | 2 | 17 | 8 | 6 | 4 | 3 | 14 | Beef | 2003 | Denmark | 17 |
| 710 | 3 | 35 | 12 | 4 | 8 | 6 | 4 | 3 | 7 | Pork | 2009 | France | 19 |
| CIP102035 | 4 | 21 | 5 | 15 | 6 | 11 | 4 | 3 | 5 | Raw milk | 1985 | France | |
| SF1189 | 4 | 27 | 5 | 15 | 6 | 11 | 4 | 10 | 5 | Smoked salmon | 1996 | France | 20 |
| F2 | 5 | 2 | 2 | 2 | 2 | 2 | 2 | 1 | 2 | Camembert | 2010 | France | This study |
| DSM20344 | 5 | 12 | 2 | 2 | 2 | 2 | 2 | 5 | 2 | Raw milk | 1974 | USA | 11 |
| F88 | 5 | 12 | 2 | 2 | 2 | 2 | 2 | 5 | 2 | Pérail | 2005 | France | 8 |
| F48 | 6 | 9 | 7 | 10 | 9 | 9 | 5 | 4 | 7 | Mascarpone gorgonzola | 2010 | Italy | This study |
| F73 | 6 | 10 | 7 | 10 | 9 | 9 | 5 | 4 | 3 | Selles-sur-Cher | 2010 | France | This study |
| F1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | Camembert | 2010 | France | This study |
| C4 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | Soil | 2005 | Canada | 21 |
| F14 | 5 | 13 | 9 | 5 | 5 | 4 | 3 | 5 | 5 | Morbier | 2010 | Tunisia | This study |
| L11 | 14 | 2 | 5 | 10 | 6 | 4 | 5 | 8 | 8 | Raw milk | 2010 | France | This study |
| CIP101342 | 19 | 4 | 11 | 2 | 4 | 7 | 3 | 2 | 2 | Human blood | 1984 | France | |
| CIP101354 | 20 | 8 | 11 | 6 | 7 | 4 | 9 | 11 | 11 | Deer | 1984 | France | |
| DSM20590 | 22 | 9 | 12 | 9 | 8 | 4 | 7 | 17 | 17 | Vacuum-packaged beef | 1985 | | 22 |
| DSM20624 | 23 | 5 | 6 | 7 | 2 | 4 | 3 | 17 | 17 | Vacuum-packaged minced meat | 1983 | | 23 |
| SF692 | 24 | 10 | 6 | 6 | 6 | 6 | 7 | 21 | 21 | Smoked salmon | 1995 | France | 20 |
| SF800 | 25 | 6 | 13 | 1 | 1 | 4 | 7 | 18 | 18 | Smoked salmon | 1995 | France | 20 |
| SF834 | 26 | 14 | 14 | 3 | 10 | 4 | 3 | 12 | 12 | Smoked salmon | 1995 | France | 20 |
| 5.27 | 28 | 2 | 16 | 9 | 12 | 4 | 6 | 13 | 13 | Beef | 2003 | Denmark | 17 |
| 6.2 | 29 | 11 | 7 | 9 | 6 | 9 | 9 | 9 | 9 | Beef | 2003 | Denmark | 17 |
| 3BO4 | 31 | 4 | 8 | 9 | 1 | 4 | 11 | 3 | 3 | <i>Sphagnum</i> moss | 2005 | Denmark | 17 |
| 1A4II | 32 | 15 | 11 | 11 | 6 | 4 | 7 | 19 | 19 | <i>Sphagnum</i> moss | 2005 | Denmark | 17 |
| G97 | 33 | 9 | 5 | 9 | 4 | 8 | 7 | 15 | 15 | Raw milk | 2004 | Israel | 24 |
| G117 | 34 | 9 | 18 | 9 | 6 | 4 | 7 | 16 | 16 | Raw milk | 2004 | Israel | 24 |

^a Dairy strains are indicated in boldface.^b CC, clonal complex.^c ST, sequence type.

cillus maltaromicus (11) and subsequently reclassified as *C. maltaromaticum* in 2003 (15), this bacterium is still not used as a commercial nonstarter LAB. This makes *C. maltaromaticum* an interesting model to use to study the population structure of LAB in cheeses prior industrial utilization.

The aim of the present study was therefore to establish an MLST scheme for strain typing in the species *C. maltaromaticum*

and to investigate the population structure of this species with a special focus on the dairy environment.

MATERIALS AND METHODS

Bacterial strains and culture conditions. The *C. maltaromaticum* strains used in the present study are described in Table 1 and were cultured in TSB-YE and CM media (25).

TABLE 2 Genes and primer sequences used for MLST

| Gene | Enzyme function | Primer | Sequence (5'–3') |
|-------------|--|------------------|---|
| <i>ddlA</i> | D-Ala-D-Ala ligase | ddl-f ddl-r | GTTTTCCCAGTCACGACGTTGTTAGAATCGCTGGACCGTGA TTGTGAGCGGATAACAATTTCCGCCATTCTTAAAATAATTTGATCG |
| <i>leuS</i> | Leucyl tRNA synthetase | leus-f leus-r | GTTTTCCCAGTCACGACGTTGTAGCTATTGCTTGGAGCGAAA TTGTGAGCGGATAACAATTTCTTCAACTTCAGATTCAACTAGGAAA |
| <i>ilvE</i> | Branched-chain aminotransferase | ilve-f ilve-r | GTTTTCCCAGTCACGACGTTGATTGCATTATGGGCAGACG TTGTGAGCGGATAACAATTTCACTTTGTAATGCTCGGCAA |
| <i>pyrE</i> | Orotate phosphoribosyltransferase | pyre-f pyre-r | GTTTTCCCAGTCACGACGTTGTACCTTTCACATGGGCTAGTG TTGTGAGCGGATAACAATTTCTCCGCTTAAATGAACCCATTTC |
| <i>dapE</i> | N-Succinyl-L-L-diaminopimelic acid desuccinylase | dape-f dape-r | GTTTTCCCAGTCACGACGTTGTCCGGAGAAGAACTTCTGCAA TTGTGAGCGGATAACAATTTCTCTCAATAGATTGTAATAGGCCTCAA |
| <i>pyc</i> | Pyruvate carboxylase | pyc-f pyc-r | GTTTTCCCAGTCACGACGTTGTCAGATTTTAGCGGATACTCATG TTGTGAGCGGATAACAATTTCTCTGGAGATGTATCAATAAAAGTTGTTT |
| <i>glpQ</i> | Glycerophosphodiester phosphodiesterase | glpq-f glpq-r | GTTTTCCCAGTCACGACGTTGTAATAATTGCCATCGAGGAA TTGTGAGCGGATAACAATTTCTCATTAAATGCTATCTCTGGATAATCTG |

Isolation of *C. maltaromaticum* strains. Totals of 72 cheese and 26 milk samples were screened for the presence of *C. maltaromaticum*. The cheese samples were obtained from diverse origins, and the milk samples were obtained from a dairy cooperative. An enrichment step was performed by inoculating 90 ml of TSB-YE (pH 8.8) with 10 g of each sample and subsequent incubation at 7°C for 7 days. An aliquot was then submitted to DNA extraction (26), and the resulting DNA was used as matrix for *C. maltaromaticum* species-specific PCR as previously described (27) with the following modifications: in a final volume of 50 µl, 3 to 5 ng of bacterial genomic DNA, 10 mM Tris-HCl (pH 9.0), 50 mM KCl, 0.1% Triton X-100, 0.2 mg of bovine serum albumin (BSA)/ml, 200 µM concentrations of each deoxynucleoside triphosphate (dNTP), 2.5 mM MgCl₂, 0.5 µM concentrations of each primer (Cpis and 23S-7), and 1.25 U of *Taq* polymerase (MP Biomedicals) were added. The amplification program started with a denaturation step at 95°C for 5 min, followed by 35 cycles of 95°C for 30 s, 53°C for 30 s, and 72°C for 30 s and a final extension step at 72°C for 7 min. The PCR products were analyzed by agarose gel electrophoresis. The enrichment mixtures for which a PCR product of the expected size was obtained were submitted to an isolation with the CM medium, which is selective for *C. maltaromaticum*, and subsequent incubation at 25°C for 2 to 3 days (25). The colonies obtained were screened by species-specific PCR to identify candidate strains belonging to *C. maltaromaticum* species. The DNA of the candidate isolates were subsequently subjected to PCR amplification of the 16S rRNA gene comprising the V3 region or the 16S-23S rRNA gene spacer region. The V3 region of 16S rRNA gene was amplified with the primers HDA-1-GC and HDA2 as previously described (28) and subsequently modified (29). For some strains, the rD1 and fD1 primers were used for 16S rRNA gene amplification with Emerald Amp GT PCR master mix (TaKaRa). The PCR program was initiated with incubation at 94°C for 5 min, followed by 35 cycles of 94°C for 1 min, 55°C for 1 min, and 72°C for 1 min, followed in turn by a final extension at 72°C for 7 min. The 16S-23S spacer region was amplified with primers 16-sp2 and 23S-7 as previously described (27) with the following modifications. The PCRs were performed in a volume of 50 µl containing 3 to 5 ng of bacterial genomic DNA solution, 10 mM Tris-HCl (pH 9.0), 50 mM KCl, 0.1% Triton X-100, 0.2 mg of BSA/ml, 200 µM concentrations of each dNTP, 2.5 mM MgCl₂, 0.5 µM concentrations of each primer, and 1.25 U of *Taq* polymerase (MP Biomedicals). The obtained PCR products were sequenced according to the Sanger method (GATC Biotech), and the resulting sequence was used to query the non-redundant database with BLASTn for species assignment.

Multilocus sequencing. The primers used to amplify the MLST loci are presented in Table 2. They consist of a 3' region targeting the targeted locus and a 5' tail region—either 5'-GTTTTCCCAGTCACGACGTTG T-3' for the forward MLST primers or 5'-TTGTGAGCGGATAACAATT TC-3' for the reverse MLST primers, which are the sequences of the se-

quencing primers. This allowed sequencing the PCR products of all MLST loci with only two sequencing primers. The DNA was extracted as previously described (26). The PCRs were performed in a volume of 50 µl containing 10 mM Tris-HCl (pH 9.0), 50 mM KCl, 0.1% Triton X-100, 0.2 mg of BSA/ml, 3 to 5 ng of bacterial genomic DNA solution, 200 µM concentrations of each dNTP, 2.5 mM MgCl₂, 0.5 µM concentrations of each primer, and 1.25 U of *Taq* polymerase (MP Biomedicals). The PCR conditions for the *dapE*, *ddlA*, *pyrE*, *leuS*, and *ilvE* gene fragments were initiated with a denaturation step at 94°C for 5 min, followed by 35 cycles of 94°C for 1 min, 52°C for 1 min, and 72°C for 1 min, and a final extension step at 72°C for 5 min. The *pyc* and *glpQ* gene fragments were amplified by using initial denaturation at 94°C for 4 min, followed by 35 cycles of 95°C for 30 s, 56°C for 30 s, and 72°C for 1 min, and ended with a final extension step at 72°C for 7 min. Both strands of the PCR products were sequenced according to the Sanger method (Eurofins).

Data analysis. Sequence handling, including sequencing electropherograms, sequence alignments by using the integrated CLUSTAL W program, was performed with Bioedit (30). Split decompositions were performed with SplitsTree4 v4.12.8 by selecting the network parameter SplitDecomposition option (31). Allele number assignments were performed by sequentially building a local nucleotide database with BLAST query in Bioedit (30). Sequence concatenation was performed with START2 (32). STs were manually defined and the absence of redundancy was checked with eBURST (33). The *dN/dS* ratio, the π and the Tajima's *D* values were calculated with DnaSp v5.1 (34). The discriminatory index was calculated using the Simpson's index of diversity (35). Clonal complex analysis was performed with eBURST (33). Neighbor-joining-based phylogeny reconstruction was performed using MEGA v5.1 (36) by using the Kimura two-parameter model, including transitions and transversions. The candidate tree was statistically tested with 1,000 bootstrap replications. The dissociation index was calculated with Start2 (32). Recombination analysis and phylogenetic tree inference under the neutral coalescent model were performed with ClonalFrame as previously described (37).

Nucleotide sequence accession numbers. The MLST sequence data are hosted at GenBank under the following accession numbers: KF637011 to KF637017 for *pyrE*, KF637018 to KF637064 for *pyc*, KF637065 to KF637111 for *leuS*, KF637112 to KF637158 for *ilvE*, KF637159 to KF637205 for *glpQ*, KF637206 to KF637250 for *ddlA*, and KF637251 to KF637297 for *dapE*. The MLST data for the strain *C. maltaromaticum* ATCC 35586 were obtained from the available complete genome sequence (38).

RESULTS

Strain collection. The collection of *C. maltaromaticum* strains used in the present study contained 47 strains (Table 1). Sixteen of

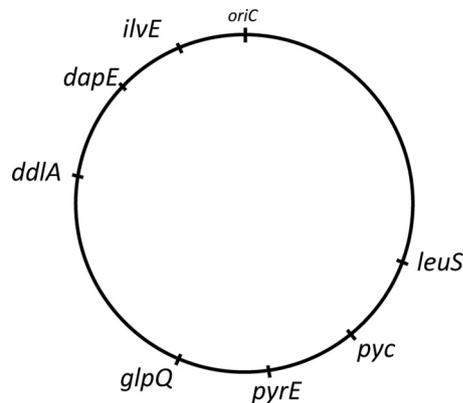


FIG 1 Localization of the MLST loci on the chromosome of *C. maltaromaticum* LMA 28. In addition to the seven MLST gene fragments, the localization of the putative replication origin *oriC* of the chromosome is represented.

these strains were isolated from dairy products during this study. The screening was performed on 72 cheese and 26 raw milk samples, among which 15 and 10, respectively, were found to contain *C. maltaromaticum* according to species-specific PCR results (data not shown). From the positive samples, 16 strains (14 from cheese and 2 from raw milk) were isolated during 2010 with only one strain per sample. The identity of the newly isolated strains was confirmed by partial sequencing of either the 16S rRNA gene or the 16S-23S spacer region. Six other dairy strains (strains LMA 28, CP5, CP23, CP32, CP14, and CP7) were added to our samples. They were all isolated in 1994 from independent Brie cheeses, with the exception of CP5 and CP7, which came from the same cheese sample. Although these strains were isolated from the same manufacture, pulsed-field gel electrophoresis (PFGE) analyses previously revealed that they belonged to different pulsotypes and therefore were different strains (12). By including 6 strains from other collections, the study was performed using a total of 28 strains from a dairy origin.

The remaining 19 strains of the collection were provided from public and laboratory collections and were isolated from other food products such as sea and meat products or from the environment, more precisely from *Sphagnum* moss and soil. Among these strains, the SF692, SF800, and SF834 were isolated from the same industrial smoked salmon lot and exhibited different PFGE pulsotypes (Françoise Leroi, unpublished data). In addition, three strains associated with animal pathologies were included: one fish pathogen (ATCC 35586) and two human blood isolates (CIP100481 and CIP101342). The 47 strains were isolated from 1974 to 2010 and originated from nine different countries.

Descriptive analysis of MLST loci and allelic diversity. Seven genes were selected from the complete chromosome sequence of *C. maltaromaticum* LMA 28 (39) (Fig. 1). The selected genes encode functions associated with cell wall biosynthesis (*ddlA*), amino acid metabolism (*dapE* and *ilvE*), nucleotide metabolism (*pyrE*), sugar metabolism (*pyc* and *glpQ*), and translation (*leuS*) (Table 2). The length of the sequences for the MLST scheme goes from 309 bp (*ddlA*) to 522 bp (*glpQ*) (Table 3). The split decomposition analysis (31) revealed that the individual genes *dapE*, *ddlA*, *glpQ*, *ilvE*, and *pyc* exhibited a limited network structure and that, once concatenated, no network structure was detected, showing that this gene set is suitable for MLST analysis (data not shown).

The ratio of mean nonsynonymous substitutions and mean synonymous substitutions (dN/dS) was calculated and remained <0.1 for all loci, but *ddlA* and *glpQ*, for which values of 0.24 and 0.30, were, respectively, found (Table 3). However, these latter values remain low, indicating higher amount of synonymous substitutions over nonsynonymous substitutions, which is typical of housekeeping genes. Consistently, the Tajima's D values ranged from -1.10 to 0.64 , and since the values are between -2 and 2 the evolution of these genes was likely driven by neutral selection (40). The number of alleles ranged from 9 to 21, and the number of polymorphic sites S ranged from 13 to 42 (Table 3). Considering the 2,985 nucleotides obtained after the concatenation of the seven gene fragments, an overall 155 polymorphic sites were identified. The nucleotide diversity (π), which is defined as the average number of nucleotide differences per site between two randomly selected sequences, ranged from 0.012 for *glpQ* to 0.023 for *ilvE* (Table 3).

Strain discrimination through ST definition. The allele combination for the seven MLST gene fragments was determined for each strain, and for each original combination a unique sequence type (ST) number was assigned (41) (Table 1). The results show that 36 different STs could be distinguished among the 47 *C. maltaromaticum* strains (Table 1). The discriminatory index obtained was of 0.98, revealing the high-resolution displayed by the MLST scheme. Approximately 80% of the STs were represented by only one strain, while 5 STs embedded at least two strains. For instance, the two strains associated with animal pathologies CIP100481 and ATCC 35586 were distinguished as ST18 and ST36, respectively. The most represented STs were ST3 and ST4, for which five strains were identified as exhibiting these genotypes.

MLST scheme optimization for strain typing purpose. In order to optimize the MLST scheme for future use, the discriminatory redundancy of the genes was estimated by omitting each gene from the scheme and determining the resulting allelic profiles. When *ilvE* was deleted, ST18 was no more distinguished from ST35 and therefore the ST number was reduced from 36 to 35. Similarly, when *ddlA*, *glpQ*, *leuS*, or *pyc* was deleted, the ST number was also reduced. However, when *dapE* and *pyrE* were together deleted, no reduction of the ST number was observed. This shows that the MLST scheme can be reduced to the five gene fragments *ilvE*, *ddlA*, *glpQ*, *leuS*, and *pyc* without resolution loss in this sample set.

Impact of recombination on the population structure. The influence of recombination on the population structure was investigated by analyzing the standardized association index, which

TABLE 3 Allelic diversity of *C. maltaromaticum* MLST loci^a

| Gene | Size (bp) | D | dN/dS | π | % G+C | S | No. of alleles |
|-------------|-----------|---------|---------|--------|--------|----|----------------|
| <i>dapE</i> | 399 | -0.80 | 0.0796 | 0.0151 | 0.3982 | 26 | 18 |
| <i>ddlA</i> | 309 | 0.08049 | 0.2387 | 0.0182 | 0.3403 | 14 | 9 |
| <i>glpQ</i> | 522 | -0.3327 | 0.2983 | 0.0117 | 0.3894 | 18 | 12 |
| <i>ilvE</i> | 426 | -1.0963 | 0.0129 | 0.0231 | 0.4114 | 42 | 15 |
| <i>leuS</i> | 408 | -0.5676 | 0.0692 | 0.0116 | 0.3836 | 19 | 21 |
| <i>pyc</i> | 516 | -0.0324 | 0.0058 | 0.0147 | 0.3580 | 23 | 12 |
| <i>pyrE</i> | 405 | 0.6434 | 0.0356 | 0.0126 | 0.4008 | 13 | 11 |

^a D , Tajima's D value; dN/dS , ratio of mean nonsynonymous to synonymous substitutions; π , nucleotide diversity; G+C, percentage of G plus C content; S, number of variable sites.

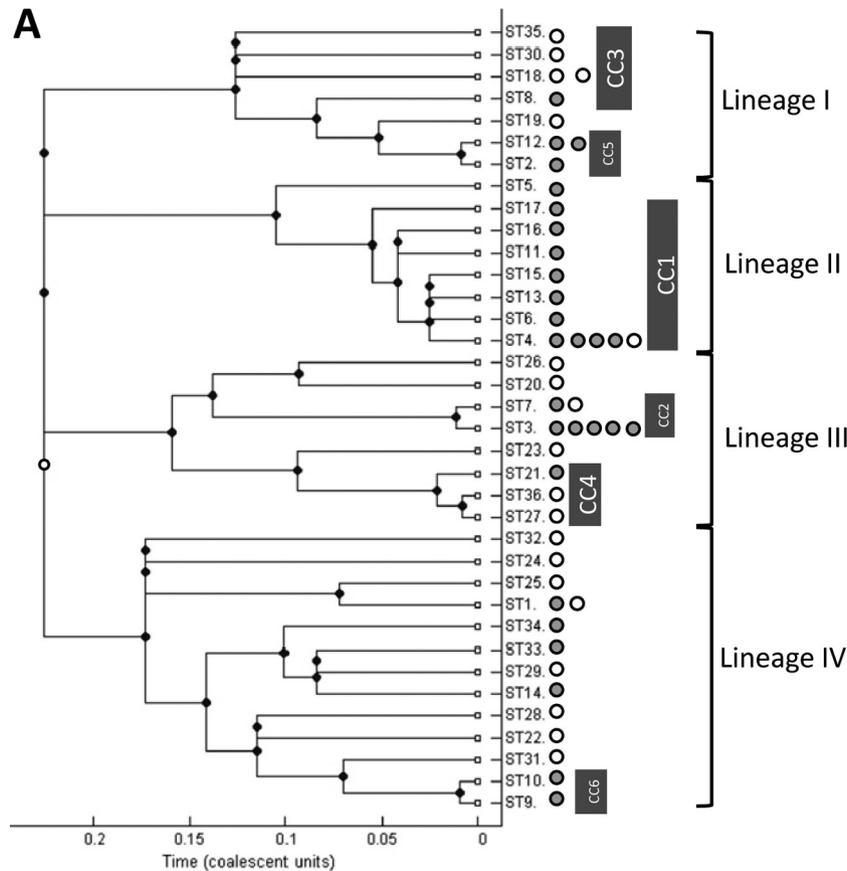


FIG 2 Phylogeny. The trees were constructed based on the alignment of the concatenated sequence of the 36 STs. The gray and white circles symbolize dairy and nondairy strains, respectively. STs belonging to clonal complexes (CCs) are enclosed by a gray rectangle in which the name of the clonal complex is indicated. (A) Phylogenetic reconstruction based on the neutral coalescent model with ClonalFrame according to the 50% majority rule. The posterior probability of the main four lineages is higher than 0.95. Gray dots represent coalescent nodes and the white dot represents the most recent common ancestor. (B) Phylogenetic tree constructed with the neighbor-joining method and statistically tested with 1,000 bootstrap replications. The bootstrap values are given as percentages; values below 70 are not shown.

aim at measuring the extent of the bias of allele association between the genes of the MLST scheme. In the investigated population of *C. maltaromaticum*, the association index (I_A^S) was 0.182 ($P < 0.01$) and was thus significantly positive, showing that recombination played a role in shaping the population. The extent of recombination was then estimated with ClonalFrame (42). The occurrence of recombination was low ($\rho/\theta = 1.15$), and the analysis of the relative impact of recombination versus mutation in generating genetic diversity confirmed the involvement of recombination; however, it indicated that recombination played a minor role in shaping the population of *C. maltaromaticum* ($r/m = 3.13$). This was consistent with the phylogenetic tree obtained with ClonalFrame according to the 50% majority rule (Fig. 2A). The obtained tree was indeed mainly congruent with the NJ tree (Fig. 2A and B): only the branching of ST20 and ST26 was incongruent between the two trees, indicating that when recombination was included in the analysis, minor changes were obtained. Overall, these results strongly support that, even if recombination played a role in shaping the population, this role was minor.

Distribution of the dairy strains in multiple clonal complexes. A clonal complex enable to measure the relatedness of isolates and is defined as a group of STs in which two connected STs differ by one locus (single locus variant) or by two loci (double

locus variant) (33). Among our 36 identified STs, 16 ST singletons were obtained, most of them being isolated from nondairy origin (Fig. 3 and Table 1). The remaining 20 STs split up into six clonal complexes and encompassed a total of 30, 23 of which were isolated from dairy products. The clonal complex CC1 was the most extended, with 7 STs representing 11 strains, mostly of dairy origin. Clonal complexes CC5 and CC6 were exclusively populated by dairy strains, and clonal complexes CC2, CC3, and CC4 contain both dairy and nondairy strains. Consequently, the dairy population of *C. maltaromaticum* is structured into several different clusters, each formed by closely related strains.

High diversity of the dairy population of *C. maltaromaticum*. The genetic diversity of the population of *C. maltaromaticum* was investigated by calculating the mean nucleotide diversity π . The mean nucleotide diversity (π) for the overall population of *C. maltaromaticum* based on concatenated ST sequences was ~ 0.012 (Fig. 4). As expected, a dramatic decrease in nucleotide diversity was obtained within each clonal complex since the strains belonging to clonal complexes were closely related ($0.00034 < \pi < 0.0025$; Fig. 4). When we considered the subpopulation of dairy strains, the nucleotide diversity was only slightly lower ($\pi = 0.011$; Fig. 4). This result reveals the high genetic diversity of the dairy population. In addition, when the genotypes

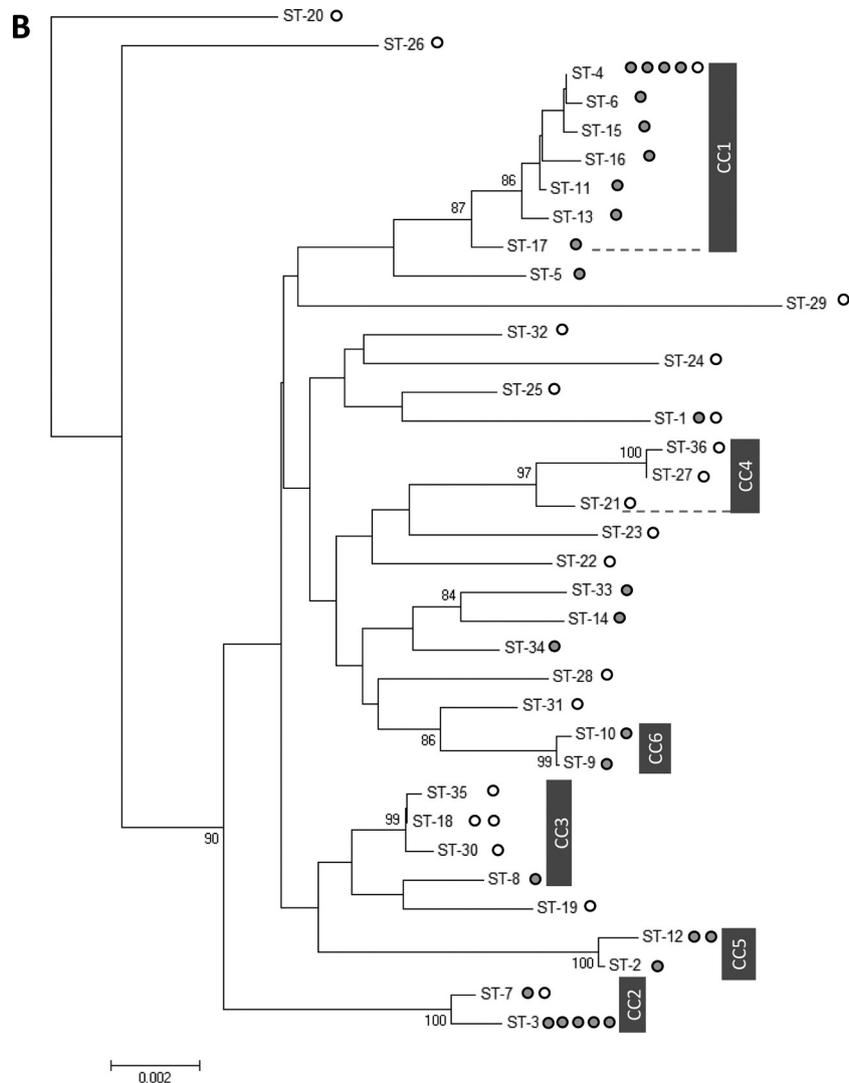


FIG 2 continued

of raw milk strains were removed from the dairy population, the nucleotide diversity only slightly decreased (Fig. 4). This result shows that the high diversity of the dairy population was not strongly biased by the strains isolated from raw milk, which is considered a less selective environment than cheese.

In order to explain this high diversity, the structure of the dairy population was assessed in more detail. The ClonalFrame phylogenetic tree revealed that dairy strains are distributed, even if unequally, in the four main lineages inferred for the species *C. maltaromaticum* (Fig. 2A). Indeed, even if 8 STs over the 20 dairy STs are found within lineage II, the remaining 12 STs are spread into the other three lineages, leading to a high genetic diversity in the dairy population (Fig. 2A). The genetic diversity of the dairy strains within each lineage is heterogeneous, the highest diversity being recorded for the dairy strains of lineage IV and the lowest for lineage II, which contains clonal complex CC1 (Fig. 4). Moreover, no decrease in nucleotide diversity was recorded when the dairy genotypes from lineage II were omitted from the population, indicating that it was the dairy genotypes from lineages I, III, and IV (Fig. 4) that contributed the most to the observed diversity. Fur-

thermore, when only strains isolated from cheeses sampled in 2010 were used for π calculation, no diversity reduction was observed compared to the overall dairy population, indicating that this high diversity is observable in today's cheeses (Fig. 4). Overall, these results show that the population of *C. maltaromaticum* is characterized by a high genetic diversity in the dairy environment.

DISCUSSION

In this study, a *C. maltaromaticum* MLST scheme based on an analysis of seven gene fragments is described. The discriminatory power of the scheme is high since 36 different genotypes were distinguished among the 47 strains. A typing tool for *C. maltaromaticum* based on 114 phenotypic unit characters is already available and was tested on another collection (17). It allowed distinguishing 46 *C. maltaromaticum* strains into 10 clusters with one major cluster containing 37 strains. Among these strains, the two strains—6.2 and F29-1—were indistinguishable on the basis of these phenotypic traits (17) but could be distinguished here by MLST as exhibiting two unrelated STs: ST4 and ST29. This suggests that the MLST scheme might be better adapted for strain

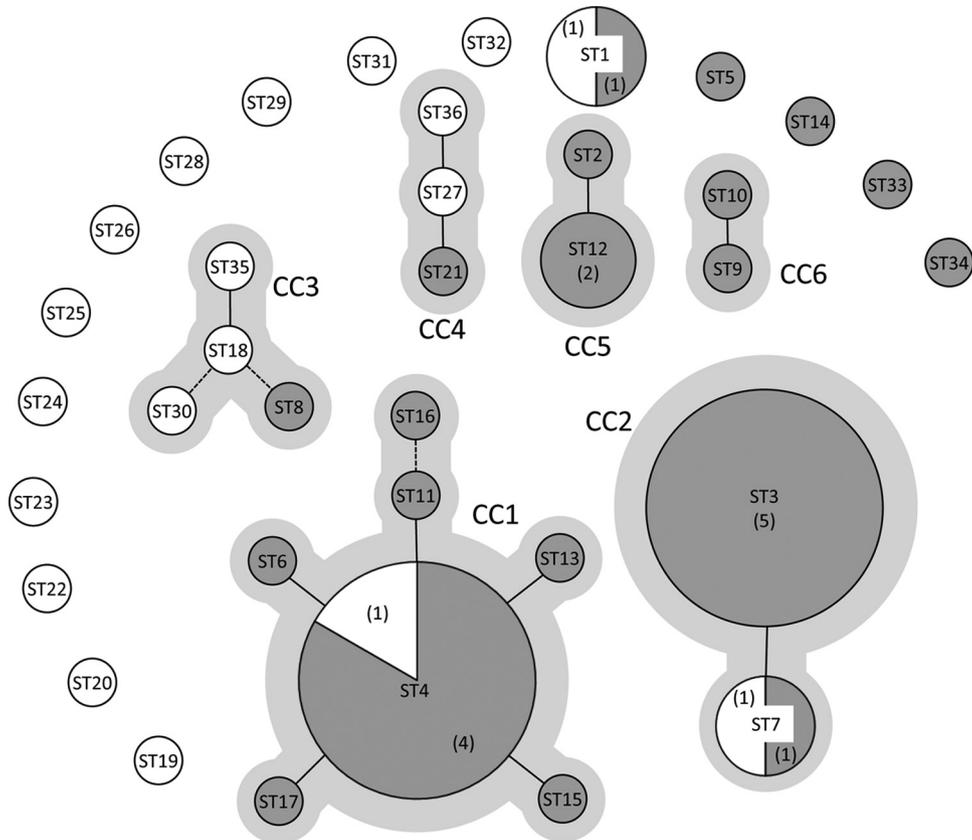


FIG 3 eBURST analysis of 47 *C. maltaromaticum* strains. Each sequence type ST is represented by a circle, the size of the circle is proportional to the number of strains belonging to a given ST. The number of strains is also indicated in brackets when the ST is represented by more than one strain. Dairy and nondairy strains are represented by dark gray and white circles, respectively. Solid lines connect single locus variants, and broken lines connect double locus variants. Clonal complexes (CC) are indicated in light gray.

typing and that phenotyping is better adapted to technological surveys of *C. maltaromaticum* strains.

The MLST scheme could be reduced to five gene fragments: *ilvE*, *ddlA*, *glpQ*, *leuS*, and *pyc*, since the deletion of *dapE* and *pyrE* did not lead to a decrease in ST number. Similarly, one gene was removed from the MLST scheme in *Lactobacillus plantarum* without discriminatory loss (7). However, since the majority of the mutations are synonymous in these fragments, i.e., do not lead to amino acid change, it cannot be ruled out that still unknown subpopulations of *C. maltaromaticum* might require *dapE*, *pyrE*, or both gene fragments for typing. It could be consequently relevant for future strain typing to use the five genes *ilvE*, *ddlA*, *glpQ*, *leuS*, and *pyc* in a first attempt and then *dapE* and *pyrE* in cases where isolates would be indistinguishable with the five genes.

This MLST analysis revealed a high diversity within the dairy population of *C. maltaromaticum*. Indeed, approximately the same nucleotide diversity (π) was found between the dairy strains and the strains originating from all other environments, including other food product categories, soil, and live fish. It was previously described for *Lactococcus lactis* that diversity of strains originating from raw milk was dramatically higher than in cheese opening the possibility for a strong bias in our study (5). However, when we removed the strains isolated from raw milk, no drastic diversity reduction was recorded, indicating that the high diversity was not biased by these strains. Moreover, when only the strains isolated

from cheeses sampled in 2010 were considered, again no decrease in genetic diversity was observed, showing that a high diversity characterizes the current dairy population of *C. maltaromaticum*. All of these results suggest that soft cheeses are weakly selective for this species. They also suggest that instead of having a specialized subpopulation able to colonize cheese, a wide range of *C. maltaromaticum* strains might be able to colonize soft cheeses.

It could be considered that other target genes exhibiting different evolutionary rate might have led to different results. For instance, genes exhibiting a slower evolutionary rate would have likely ended with a poor level of strain discrimination. On the other hand, with genes showing higher evolutionary rate, it can be predicted that the relative diversity between dairy and nondairy strains would still have been approximately the same despite a higher level of absolute diversity. In conclusion, our targeted genes showed enough diversity and resolution to discriminate a dairy population from a nondairy population.

The high diversity of the dairy population of *C. maltaromaticum* strikingly contrasts with the low diversity of the dairy LABs, for which a detailed analysis of the diversity has been performed in dairy products, i.e., *Lactococcus lactis* and *Streptococcus thermophilus* (4–6, 43, 44). Several hypotheses could explain the low diversity observed in cheese for these latter species. One hypothesis could be that only limited number of genotypes within a given species are well adapted to cheese and would have emerged thanks

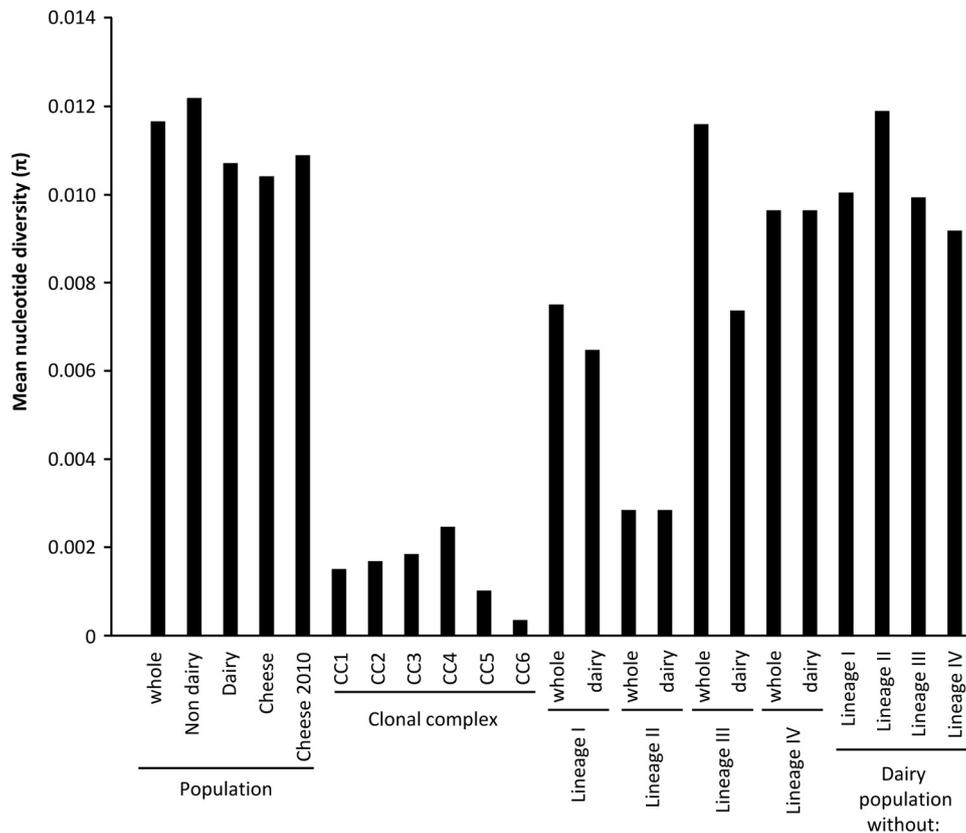


FIG 4 Mean nucleotide diversity. The mean nucleotide diversity π was calculated for the overall population and for subpopulations.

to this better fitness (5). Another hypothesis would be that the wide use of commercial starters representing a limited number of genotypes would have outcompeted other genotypes. According to this hypothesis, the competitive advantage of the commercial LABs likely results from several factors, including (i) the wide geographic commercialization of these bacteria, (ii) the high concentration at which commercial LABs are added to milk during cheese manufacture, and (iii) the processes that lead to the reduction of the bacterial load in milk, i.e., drastic hygienic practices, milk thermal treatment, or microfiltration. The strikingly high diversity of the dairy population of the noncommercialized species *C. maltaromaticum* is in agreement with the idea that the massive use of commercial starters contributes significantly to the reduction of diversity in cheeses.

Our analysis indicated that the dairy *C. maltaromaticum* population is diverse and structured in four main phylogenetic lineages. Lineages I, III, and IV contribute the most to the whole population diversity, whereas lineage II is characterized by a lower diversity. This lineage is almost exclusively composed of clonal complex I. In this clonal complex, the major genotype is ST4, which is represented by strains isolated from diverse environments: raw milk, cheeses, and lumpfish roes. In addition, the cheese isolates exhibiting the genotype ST4 were obtained from sealed cheeses that were manufactured in different factories, and all originated from diverse countries (Lebanon, France, Denmark, and the United States). This environmental diversity suggests that the phylogenetic relatedness of the strains from ST4 is not due to a sample bias. In addition, ST4 could be the founder genotype of

CC1, which connects 7 different STs and represents 11 strains of the 47 studied. Although we demonstrated that a large diversity of *C. maltaromaticum* strains can colonize cheese, one hypothesis to explain the over-representation of CC1 would be that strains populating this clonal complex are particularly well adapted to the dairy environment and would therefore highly proliferate in this environment. Another hypothesis would be that *C. maltaromaticum* is subjected to domestication and would thus be a “species bred in captivity and thereby modified from its wild ancestor in ways making it more useful to humans who control its reproduction and its food supply” (45). This definition fits well with food LAB, which were selected via ancient backslipping practices and now via the use of isolated starter and adjunct cultures. In *Lactococcus lactis*, the domestication process led to the drastic reduction of genetic diversity (5). Indeed, an MLST analysis of this LAB revealed that the species can be subdivided into “environmental” and “domesticated” strains. Domesticated strains were exclusively isolated from cheese products (excluding strains from raw milk), were closely related, and formed a major clonal complex (5). Further investigations are required to determine whether, and to what extent, domestication is taking part in population structuring within the species *C. maltaromaticum*.

Since *C. maltaromaticum* is a LAB with high technological potential for dairy products (12), it can be expected that representatives of the species will become commercialized and therefore widely distributed in the dairy industry in the future. This could provide the unprecedented opportunity to investigate on the impact of the industrialization process on the evolution of the pop-

ulation structure of a given LAB species in cheese. Such study could be useful to define future strategies that would reconcile standardization requirement inherent to the industrial production of cheese and product diversity.

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