Genomic Characterization of Dairy Associated Leuconostoc Species and Diversity of Leuconostocs in Undefined Mixed Mesophilic Starter Cultures

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Undefined mesophilic mixed (DL-type) starter cultures are composed of predominantly Lactococcus lactis subspecies and 1–10% Leuconostoc spp. The composition of the Leuconostoc population in the starter culture ultimately affects the characteristics and the quality of the final product. The scientific basis for the taxonomy of dairy relevant leuconostocs can be traced back 50 years, and no documentation on the genomic diversity of leuconostocs in starter cultures exists. We present data on the Leuconostoc population in five DL-type starter cultures commonly used by the dairy industry. The analyses were performed using traditional cultivation methods, and further augmented by next-generation DNA sequencing methods. Bacterial counts for starter cultures cultivated on two different media, MRS and MPCA, revealed large differences in the relative abundance of leuconostocs. Most of the leuconostocs in two of the starter cultures were unable to grow on MRS, emphasizing the limitations of culture-based methods and the importance of careful media selection or use of culture independent methods. Pan-genomic analysis of 59 Leuconostoc genomes enabled differentiation into twelve robust lineages. The genomic analyses show that the dairy-associated leuconostocs are highly adapted to their environment, characterized by the acquisition of genotype traits, such as the ability to metabolize citrate. In particular, Leuconostoc mesenteroides subsp. cremoris display telltale signs of a degenerative evolution, likely resulting from a long period of growth in milk in association with lactococci. Great differences in the metabolic potential between Leuconostoc species and subspecies were revealed. Using targeted amplicon sequencing, the composition of the Leuconostoc population in the five commercial starter cultures was shown to be significantly different. Three of the cultures were dominated by Ln. mesenteroides subspecies cremoris. Leuconostoc pseudomesenteroides dominated in two of the
cultures while *Leuconostoc lactis*, reported to be a major constituent in fermented dairy products, was only present in low amounts in one of the cultures. This is the first in-depth study of *Leuconostoc* genomics and diversity in dairy starter cultures. The results and the techniques presented may be of great value for the dairy industry.

**Keywords:** dairy, cheese, *leuconostoc*, comparative, genomics, diversity analysis, starter cultures, differentiation

## INTRODUCTION

Mesophilic mixed (DL-type) starter cultures used in the production of Dutch-type cheeses are composed of undefined mixtures of homofermentative *Lactococcus lactis* subsp. *lactis* (*Lc. lactis*), *Lactococcus lactis* subsp. *cremoris* (*Lc. cremoris*), *Lactococcus lactis* subsp. *lactis* biovar. *diacetylactis* (*Lc. diacetylactis*) and heterofermentative *Leuconostoc* spp. The latter two provide aroma and texture by metabolizing citrate, producing diacetyl, acetoin and CO$_2$, while *Lc. cremoris* and *Lc. lactis* are the major acid producers through fermentation of lactose. In many cheeses, diacetyl is an important aroma compound, and CO$_2$ is important for the eye formation (Hugenholtz, 1993). In fermented dairy products, *Leuconostoc* grows in association with the acid-producing lactococci and have been suggested to play a role in promoting the growth of citrate positive *Lactococcus* strains (Vedamuthu, 1994; Bandell et al., 1998; Hache et al., 1999). The importance of *Leuconostoc* in cheese production is widely recognized. DL-type starter cultures are predominantly *Lactococcus* spp., *Leuconostoc* spp. commonly accounting for 1–10% of the starter culture population (Cogan and Jordan, 1994). However, knowledge on the species diversity of *Leuconostoc* included in these starter cultures, or the composition of *Leuconostoc* through the culture production is sparse. Due to the low initial number and relatively weak ability to ferment lactose, *Leuconostoc* spp. are not believed to have a significant effect in the acidification process in the early stages of cheese making (Ardö and Varming, 2010). However, leuconostocs have been shown to dominate the cheese microbiota in the later stages of ripening with added propionic acid bacteria (Porcellato et al., 2013; Østlie et al., 2016). The genus *Leuconostoc* is comprised of 13 species, with the species *Leuconostoc mesenteroides* divided into subspecies *mesenteroides*, *dextranicum*, *cremoris*, and *suisanicum* (Hemme and Foucaud-Scheunemann, 2004; Gu et al., 2012). The *Leuconostoc* species (or subspecies) relevant for dairy production are *Leuconostoc mesenteroides* subsp. *mesenteroides* (*Ln. mesenteroides*), *Leuconostoc mesenteroides* subsp. *dextranicum* (*Ln. dextranicum*), *Leuconostoc mesenteroides* subsp. *cremoris* (*Ln. cremoris*), *Leuconostoc pseudomesenteroides* (*Ln. pseudomesenteroides*) and *Leuconostoc lactis* (*Ln. lactis*) (Cogan and Jordan, 1994; Thunell, 1995)

The bases for *Leuconostoc* taxonomy are results from cultivation-dependent methods, followed by phenotypic/biochemical characterization or non-specific molecular methods. In addition to being tedious and time-consuming, classical cultivation-dependent methods are known to underestimate the number of *Leuconostoc* spp., especially *Ln. cremoris* (Vogensen et al., 1987; Ward et al., 1990; Auty et al., 2001). In addition, concerns on the lack of stability and reproducibility of phenotypical methods have been raised (Thunell, 1995; Barrangou et al., 2002). Several molecular typing methods, such as RAPD, PFGE, RFLP, Rep-PCR, MLST, MALDI-TOF MS, plasmid profiling and 16S rRNA targeted differentiation have been employed to characterize or identify *Leuconostoc* isolates (Villani et al., 1997; Björkroth et al., 2000; Cibik et al., 2000; Pérez et al., 2002; Sánchez et al., 2005; Vihavainen and Björkroth, 2009; Nieto-Arribas et al., 2010; Alegria et al., 2013; Zeller-Péronnet et al., 2013; Dan et al., 2014; Zhang et al., 2015). However, most of these techniques requiring a preliminary stage of cultivation and comparison of results between the methods and between different laboratories remains challenging. Often, these methods were developed to work with only one or two species of *Leuconostoc*, so they do not provide subspecies differentiation, yield inconclusive results, yield results that are hard to reproduce, or provide arbitrary differentiation of isolates not sufficiently tethered to phenotypic traits. So far, the work by Dr. Ellen Garvie on the growth and metabolism of *Leuconostoc* spp. (Garvie, 1960, 1967, 1969, 1979, 1983; Garvie et al., 1974), and DNA-DNA hybridization studies (Farrow et al., 1989) remains the basis for the taxonomical division of dairy relevant leuconostocs.

The *Leuconostoc* genus has also not been subject to extensive genomic research, and information on the genomic diversity or species population dynamics through the cheese production processes is scarce if available at all. Scientific literature and product information on starter cultures pre-dating the genomic age list *Ln. cremoris* and *Ln. lactis* as the key *Leuconostoc* in undefined mixed mesophilic starter cultures (Lodics and Steenson, 1990; Johansen and Kibenich, 1992; Vedamuthu, 1994). However, in recent years, isolation of *Ln. mesenteroides*, *Ln. dextranicum*, and *Ln. pseudomesenteroides* is more common from starter cultures or from cheese derivatives (Olse et al., 2007; Kleppen et al., 2012; Pedersen et al., 2014a,b; Østlie et al., 2016).

Here we present genomic comparative analysis of *Leuconostoc* spp. and present data on the diversity and composition of *Leuconostoc* populations in five commercially available DL-type starter cultures. Using traditional cultivation methods in combination with high-throughput sequencing techniques, we provide robust species and subspecies differentiation, and direct population composition analysis using targeted amplicon-sequencing techniques. To our knowledge, this is the first in-depth genomic work performed on the *Leuconostoc* genus, and the first data published on *Leuconostoc* diversity in DL-type starter cultures.
MATERIALS AND METHODS

Cultivation of Bacterial Strains and Starter Cultures

All bacterial strains used in this study are listed in Supplementary Table S1. The two different media used for cultivation were de Man Rogosa Sharpe (MRS) (Difco, Detroit, Michigan, USA), and modified PCA (MPCA). PCA (Sigma-Aldrich, Oslo, Norway) was supplemented with 0.5 g/L Tween 80, 5.0 g/L ammonium-citrate, 1 g/L skim milk powder (TINE SA, Oslo, Norway), 0.04 g/L FeSO₄, 0.2 g/L MgSO₄, 0.05 g/L MnSO₄, and 10.0 g/L glucose. Glucose was sterile filtered separately and added after autoclaving. Both media were supplemented with 40 µg/mL vancomycin to select for Leuconostoc. Three separate extractions from one batch of each starter cultures (A, B, C, D, and E) were suspended in MPCA to an optical density at 600 nm (OD₆₀₀) of 1.0, serially diluted in 10% (w/v) skim milk and spread plated on MRS and MPCA agar plates in triplicate. The plates were incubated at 22°C for 5 days before colony enumeration. Isolates were transferred to MRS and MPCA broth media, respectively, and cultivated at 22°C for two passages before aliquots were supplemented with 15% (v/v) glycerol (Sigma-Aldrich) and stored at −70°C.

Genome Sequencing, Assembly, and Annotation

Genomic DNA from Leuconostoc isolates was extracted from 1 mL of overnight culture using Qiagen DNeasy Blood & Tissue Kit (Qiagen, Hilden, Germany). The cells were lysed with 40 mg/mL lysozyme (Qiagen, Hilden, Germany) and bead-beating in a FastPrep®-24 (MP Biomedicals, Santa Ana, California) using 0.5 g acid-washed beads (<106 µm) (Sigma-Aldrich) prior to column purification. DNA libraries were made using the Nextera XT DNA Sample Prep kit (Illumina, San Diego, California, USA) according to manufacturer instructions and sequenced with Illumina MiSeq (Illumina, San Diego, California, USA) using V3 chemistry for 33 isolates sequenced at the Norwegian University of Life Sciences, and V2 chemistry for 13 isolates sequenced at the Aarhus University. Raw sequences were adapter trimmed, quality filtered (Q > 20), de novo assembled using SPAdes V3.7.1 (Nurk et al., 2013) and annotated using the Prokka pipeline (Seemann, 2014). Contigs shorter than 1000 bp or with < 5 times coverage were removed from each assembly prior to gene annotation. Thirteen publicly available genomes of Leuconostoc obtained from the National Center for Biotechnology Information (NCBI) database were also included in the dataset (Jung et al., 2012; Meslier et al., 2012; Erkus et al., 2013; Pedersen et al., 2014a,b; Campedelli et al., 2015; Østlie et al., 2016). This whole genome project has been deposited at DDBJ/ENA/GenBank under the BioProject PRJNA352459.

Genomic Analysis

The protein coding sequences of all Leuconostoc isolates were compared by an all-against-all approach using BLASTP (Camacho et al., 2009) and grouped into orthologous clusters using GET_HOMOLOGUES (Version 2.0.10) (Contreras-Moreira and Vinuesa, 2013). Pan and core genomes were estimated using the pan-genomic analysis tool PanGP v.1.0.1 (Zhao et al., 2014). Orthologous groups (OGs) were identified via the Markov Cluster Algorithm (MCL) with an inflation value of 1.5 (Enright et al., 2002) and intersected using the compare_clusters.pl script provided with GET_HOMOLOGUES. The orthologous clusters were curated to exclude significantly divergent singletons, which is likely the result of erroneous assembly or annotation. A presence/absence matrix for each gene cluster and each genome was constructed for the pan-genome before statistical and clustering analysis of the matrix was performed in R (http://www.r-project.org/). Hierarchical clustering of the pan-genome matrix was performed using complete-linkage UPGMA with Manhattan distances, and a distance cut-off for the number of clusters was determined using the knee of the curve approach (Salvador and Chan, 2004), binning the isolates into genomic lineages. The resulting distance-matrix was used to construct a heatmap with dendograms using the heatmap.2 function included in the Gplots package (Version 2.16; Warnes et al., 2015) supplemented by the Dendextend package (Version 0.18.3; Galili, 2015).

Comparative Genomics Analysis

The genetic potential of individual Leuconostoc lineages that were identified by the pan-/core-genome analysis was investigated by producing intra-lineage pan-genomes using GET_HOMOLOGUES (Version 2.0.10). The pan-genome for each lineage was analyzed using Blast2GO v4 (Conesa et al., 2005) to identify functionality, and Geneious 8.1.8 (Kearse et al., 2012) to identify sequence variation within orthologous clusters. The lineage pan-genomes were then compared using KEGG databases (Kanehisa and Goto, 2000) and the functional comparative comparison tool found in The SEED Viewer (Overbeek et al., 2014). CRISPR sequences and spacers were identified using the CRISPRFinder tool (Grissa et al., 2007).

Relative Quantification of Leuconostoc Species in Starter Cultures

Compositional analysis of Leuconostoc in five commercially available starter cultures was performed in triplicates on total DNA isolated from the starter cultures using 1 mL of starter culture diluted to an OD₆₀₀ of 1. The cultures were treated with 20 mg/mL lysozyme (Sigma-Aldrich) and 3U/L mutanolysin (Sigma-Aldrich), mechanically lysed using FastPrep (MP Biomedicals) with 0.5 g of acid-washed beads (106 µm) (Sigma-Aldrich) and purified using the Qiagen DNeasy Blood & Tissue Kit (Qiagen). A suitable amplicon target was identified by screening the core-genome for nucleotide sequence variation using the sequence alignment metrics functions available in the DECIPHER package v1.16.1 (Wright, 2015). Genes without flanking consensus regions within a 500 bp variable region adequate for differentiation, or did not provide sufficient discrimination from similar sequences in species likely to be present in dairy, were excluded. The locus eno encoding for enolase was amplified by PCR using the KAPA HiFi PCR Kit (KAPA Biosystems, Wilmington, Massachusetts, USA) with primers Eno-F (5’-AACACGAAGCTTGGCGGCGGTTTA-3’), and Eno-R (5’-GCAATATCCATCTCATCAAGA-3’). Forward (5’-TCGTCGGCACCGTCAGATGTGTTAAGAGACAG-3’)} and
reverse (5′GTCTCGTGGGCTCGGAGATGTATAAGAGA CAG-) Illumina adapter overhangs were added to the 5′ end of the primers to allow for Nextera XT DNA indexing of the PCR-products. The resulting libraries were sequenced on an Illumina MiSeq with V3 (2 × 300 bp) reagents. The resulting data were paired-end-joined and quality filtered using PEAR (Zhang et al., 2014) and clustered with a 100% identity level threshold using usearch v7 (Edgar, 2010) with error-minimization from uparse (Edgar, 2013). The resulting sequences were matched against a local BLAST-database produced from the Leuconostoc genomes for identification.

RESULTS

Leuconostoc in Dairy Starters

Enumeration on MRS-agar has been reported to underestimate the number of leuconostocs, especially Ln. cremoris (Vogensen et al., 1987; Ward et al., 1990; Auty et al., 2001). Bacterial counts were compared in five starter cultures (A, B, C, D, and E) commonly used in the production of Dutch-type cheeses using MRS and MPCA agar with 40 µg/mL vancomycin. The results (Figure 1) showed large differences in the counts between starter cultures for the two media. Cultures A and D gave substantially higher counts on MPCA compared to MRS, while cultures B, C, and E had similar counts on both media. Thus, cultures A and D seemed to contain a large number of Leuconostoc strains unable to grow on MRS, while cultures B, C, and E did not.

Genome Sequencing and Pan-Genomic Analysis

Leuconostoc diversity was investigated by whole-genome sequencing of 20 isolates picked from MPCA- and MRS-plates of cultures A and D, and 26 isolates from cheese, including Dutch-type cheese produced using cultures B, C, and E. Lastly, 13 publically available Leuconostoc spp. genomes were included in the dataset. All 59 Leuconostoc genomes were annotated and the coding sequences (CDS) were compared by a blast-all-against-all approach to identify OGs. Pan- and core-genomes were estimated (Figure 2) using the pan-genomic analysis tool PanGP. After curation, the pan-genome was determined to consist of 4415 OGs, and a core-genome was found to compris 638 OGs. Differentiation of isolates using hierarchal clustering on the pan-matrix clearly separated Leuconostoc species and sub-species (Figure 3). Several of the strains previously identified as Ln. mesenteroides subspecies were shown to be Ln. pseudomesenteroides by the genomic analysis. Moreover, the NCBI strain LbT16 previously identified as Ln. cremoris, was an outlier to the Ln. cremoris species branch and was identified in the pan-genomic analysis as Ln. mesenteroides. This was further confirmed by alignment of the full-length 16S rRNA, revealing a 100% identity between Ln. cremoris LbT16 and Ln. mesenteroides type 16S rRNA. Based on sequence similarity and gene content, the pan-genomic clustering divided the 59 leuconostocs into 12 robust Leuconostoc lineages across the genus. These included three lineages of Ln. cremoris (C1-C3), four lineages of Ln. pseudomesenteroides (P1-P4), four lineages of Ln. mesenteroides (M1-M4), and one lineage of Ln. lactis (L1). The Ln. cremoris TIFN8 genome was excluded from further analysis because the genome data contained a high number of fragmented genes and redundant sequences, making it an outlier.

The differences between lineages (Table 1), species and subspecies level (in the case for Ln. mesenteroides subsp.) include significantly smaller genomes for Ln. cremoris and Ln. lactis (1.6–1.8 Mb) compared to Ln. mesenteroides, Ln. dextranicum,
and Ln. pseudomesenteroides (1.8–2.2 Mb). Moreover, the larger genome found in the latter three species contained up to 400 more coding sequences (CDS) than Ln. cremoris and Ln. lactis. Analysis of functional genomics indicated a closer relationship between Ln. lactis and Ln. pseudomesenteroides, than that of Ln. mesenteroides. Comparison of genetic potential within and between the Ln. mesenteroides subspecies showed only minor differences between Ln. mesenteroides and Ln. dextranicum. Rather, as shown in Figure 3, the variation between the isolates was much greater than the difference between Ln. mesenteroides and Ln. dextranicum. On the other hand, substantial difference was found between isolates of dairy origin and non-dairy origin. This environment adaptation was also observed for Ln. lactis, where Ln. lactis 91922, isolated from kimchi was clearly distinguishable from LN19 and LN24 isolated from dairy. Comparison of Ln. cremoris and other Ln. mesenteroides subspecies isolates revealed that a range of genetic elements found in these species that were missing in Ln. cremoris. Apart from some enzymes encoding for rhamnose-containing glucans, Ln. cremoris isolates did not have any genetic functionality absent in Ln. mesenteroides or Ln. dextranicum. Moreover, several truncated genes and deletions were found in Ln. cremoris isolates, likely the result of a degenerative evolutionary process through a long period of growth in the milk environment.

Comparative Genomics of Intra-Species Leuconostoc Lineages

To explore differences in functional genetic potential between the lineages within the species and subspecies, comparative analysis of intra-lineage pan-genomes was performed. The results are included in Supplementary Table S2.

(I) Ln. cremoris Lineages

Comparison of the genetic content for Ln. cremoris lineages showed that Ln. cremoris C1, C2, and C3 were highly similar and differentiated from each other mostly because of sequence variation in shared OGs. Ln. cremoris C1 (MPCA-type), which did not grow on MRS was missing four OGs found in both lineage C2 and C3 (MRS-type). These OGs were annotated rmlA, rmlB, rmlC, and rmlD, encoding for four enzymes identified in the subsystem “rhamnose containing glycans.” These enzymes are associated with polysaccharide biosynthesis and their presence likely does not explain the inability of C1-type strains to grow on MRS.

(II) Ln. mesenteroides and Ln. dextranicum Lineages

Comparison of the genetic content showed a large variance between and within the Ln. mesenteroides lineages. Interestingly, no major difference between subspecies Ln. mesenteroides and Ln. dextranicum was found. Ln. dextranicum 20484 is grouped together with Ln. mesenteroides isolates LN32 and LN34, while Ln. dextranicum LbE16 is grouped together with Ln. mesenteroides LbE15 and LN08. This subspecies segregation of Ln. dextranicum and Ln. mesenteroides was based on the phenotypical ability to produce dextran from sucrose. Dextransucrase, the enzyme involved in this process, is a glucosyltransferase that catalyzes the transfer of glucosyl residues from sucrose to a dextran polymer and releases fructose. Several glucosyltransferases were found within all Ln. mesenteroides isolates included in this study, among them several genes encoding for dextransucrases with 40–67% amino acid identity to each other. Genotypically, the potential for dextran production exists within many if not all Ln. mesenteroides isolates, and does not differentiate Ln. mesenteroides from Ln. dextranicum. This finding was manifest by the separation of Ln. mesenteroides and Ln. dextranicum isolates into four lineages. Functional comparative analyses showed that the presence of the cit operon necessary for metabolism of citrate, and the lacLM genes is a characteristic of dairy-associated Ln. mesenteroides, Ln. cremoris and Ln. pseudomesenteroides. In all of the strains in lineages M3 and M4, both the cit operon and the lacLM genes were present, while strains in lineages M1 and M2 were lacking the cit operon, and half of them also lacked the lacLM genes. Furthermore, the strains in lineages M1 and M2 contained the genetic potential for metabolism of arabinose, and the two isolates J18 and ATCC8293 also contained genetic potential for xylose and β-glucoside metabolism. The lineage M4 strains LbT16 and LN05 also contained the deletion in the lacZ gene which is commonly identified in Ln. cremoris type strains. A genetic potential for proteolysis of casein (prtP) was identified in Ln. mesenteroides lineages M1 and M4, but not in M2 or M3.

(III) Ln. lactis Lineages

The pan-genomic differentiation grouped all the Ln. lactis isolates into one lineage. However, differences in genetic potential were found between the kimchi isolate Ln. lactis 91922 and...
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**FIGURE 3** | Differentiation of 59 Leuconostoc genomes using the pan-genome of 4415 OGs. Hierarchal clustering of genomes clearly separated Leuconostoc species and subspecies. Moreover, the high sensitivity of the method produced twelve robust Leuconostoc lineages annotated on the right side of the figure. Four lineages of _Ln. mesenteroides_ (colored orange), three lineages of _Ln. cremoris_ (colored blue), four lineages of _Ln. pseudomesenteroides_ (colored green), and one lineage of _Ln. lactis_ (colored purple) are shown. (*) The _Ln. cremoris_ TIFN8 genome was excluded from further analysis because the genome data contained a high number of fragmented genes and redundant sequences. The heatmap was generated with R using the heatmap.2 function included in the Gplots package supplemented by the Dendextend package.

Dairy isolates LN19 and LN24. _Ln. lactis_ 91922 lacked citrate metabolism genes _citCDEFG_, but carried genetic potential for a maltose and glucose specific PTS system, metabolism of arabinose and a CRISPR-Cas operon, that were not found in the other two _Ln. lactis_ isolates.

**(IV) *Ln. pseudomesenteroides* Lineages**

Despite the significant pan-genomic differences and the sequence variation in shared OGs, the functional differences between lineages of _Ln. pseudomesenteroides_ were surprisingly few. _Ln. pseudomesenteroides_ P4 was different from the other three lineages with regards to genome synteny and genetic potential. Genetic functionality in the category of methionine biosynthesis, β-glucoside metabolism, sucrose metabolism, as well as an additional lactate dehydrogenase was identified in _Ln. pseudomesenteroides_ P4 but not P1, P2, and P3. Moreover, P4 isolates were missing the genes for reduction of diacetyl to acetoin and 2,3-butandiol, and contained genes for a different capsular and extracellular polysaccharide biosynthesis pathway, compared to P1, P2, and P3 isolates.

**Genetic Potential of Leuconostoc**

**(I) Amino Acid Biosynthesis**

The amino acid requirements of leuconostocs have been described as highly variable between strains. Glutamic acid and valine are required by most leuconostocs, methionine usually stimulates growth, while no *Leuconostoc* are reported to require alanine (Garvie, 1967). Comparative analysis of genes involved in amino acid biosynthesis showed that _Ln. cremoris_ and _Ln. mesenteroides_ subspecies carried the genetic potential to produce a wide range of amino acids while _Ln. lactis_ and _Ln. pseudomesenteroides_ did not (Table 2). This included genes encoding biosynthesis of histidine, tryptophan, methionine and lysine. Studies on the amino acid requirement of leuconostocs show that most of the _Ln. mesenteroides_ subspecies do require isoleucine and leucine to grow. The _ilv_ and _leu_ operons involved in biosynthesis of the branched-chain amino acids isoleucine, leucine and valine were present in all _Ln. mesenteroides_ isolates, however both operons were truncated when compared to functional _ilv_ and _leu_ operons from lactococci. The _leuA_ gene in the _leuABCD_ operon is truncated in leuconostocs (391 aa) compared to lactococci (513 aa) likely resulting in an inactive product and a nonfunctional pathway. This has been documented in the dairy strain *Lactococcus lactis* IL1403 where a similar truncation of the _leuA_ gene led to an inactivation of the leucine/valine pathway (Godon et al., 1993). Likewise, the _ilv_ operon of sequenced leuconostocs is missing the _ilvD_ gene, and has truncated _ilvA_ and _ilvH_ genes when compared to the lactococcal _ilv_ operon. The truncation of _ilvA_ has been shown to result in inactivation of the product, and would by itself be sufficient to abort the biosynthesis pathway (Cavin et al., 1999). None of the leuconostocs had genes for biosynthesis of glutamic acid. _Ln. lactis_ isolates also lacked the genetic potential for cysteine biosynthesis.

**(II) Carbohydrate Metabolism**

Differences in the genetic potential within and between the *Leuconostoc* species were analyzed by comparing intra-species
TABLE 1 | Average genome size and coding sequences of Leuconostoc isolates binned into pan-genome lineages.

<table>
<thead>
<tr>
<th>Profile name</th>
<th>Average genome size (Mb)</th>
<th>Average CDS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ln. cremoris C1 (MPCA-type)</td>
<td>1.680 (±5)</td>
<td>1760 (±20)</td>
</tr>
<tr>
<td>Ln. cremoris C2 (MRS-type)</td>
<td>1.741 (±40)</td>
<td>1822 (±30)</td>
</tr>
<tr>
<td>Ln. cremoris C3</td>
<td>1.765 (±124)</td>
<td>1956 (±198)</td>
</tr>
<tr>
<td>Ln. mesenteroides M1</td>
<td>1.869 (±19)</td>
<td>1851 (±7)</td>
</tr>
<tr>
<td>Ln. mesenteroides M2</td>
<td>2.150 (±123)</td>
<td>2212 (±162)</td>
</tr>
<tr>
<td>Ln. mesenteroides M3</td>
<td>2.014 (±19)</td>
<td>2074 (±18)</td>
</tr>
<tr>
<td>Ln. mesenteroides M4</td>
<td>2.061 (±219)</td>
<td>2101 (±173)</td>
</tr>
<tr>
<td>Ln. pseudomesenteroides P1</td>
<td>2.028 (±47)</td>
<td>2081 (±61)</td>
</tr>
<tr>
<td>Ln. pseudomesenteroides P2</td>
<td>1.921 (±25)</td>
<td>1952 (±46)</td>
</tr>
<tr>
<td>Ln. pseudomesenteroides P3</td>
<td>2.063 (±44)</td>
<td>2133 (±60)</td>
</tr>
<tr>
<td>Ln. pseudomesenteroides P4</td>
<td>2.032 (±61)</td>
<td>2046 (±60)</td>
</tr>
<tr>
<td>Ln. lactis L1</td>
<td>1.718 (±28)</td>
<td>1700 (±43)</td>
</tr>
</tbody>
</table>

Information on each individual isolate is included in Supplementary Table S1.

pan-genomes using Blast2GO and the Seed Viewer. The Leuconostoc genus is composed of heterofermentative bacteria that use the phosphoketolase pathway to ferment hexoses. Therefore, it was not surprising to find that none of the isolates contained the gene for phosphofructokinase, a key enzyme in the Embden-Meyerhof pathway. However, a gene encoding fructose-bisphosphate aldolase class II was present in Ln. lactis and Ln. pseudomesenteroides. This could indicate a potential for synthesis of fructose-1,6-bisphosphate and glyceraldehyde-3-phosphate through fructose-1-phosphate, and hence homofermentative breakdown of fructose in Ln. lactis and Ln. pseudomesenteroides.

Comparative analysis of genes related to carbohydrate metabolism revealed big differences between the species (Table 3). All leuconostocs in this study encode beta-galactosidase, enabling utilization of lactose. Interestingly, the dairy Ln. mesenteroides have two different beta-galactosidases, lacZ and the plasmid-encoded lacLM (Obst et al., 1995), while the non-dairy isolates only contain lacZ. In Ln. cremoris, lacZ contains a large central deletion of 1200 bp between positions 740–1940. The Ln. lactis isolates only encode beta-galactosidase through lacZ, while the Ln. pseudomesenteroides isolates only encode beta-galactosidase through lacLM. In Leuconostoc, lactose is taken up by the lactose-specific transporter LacS, which couples lactose uptake to the secretion of galactose. LacS contains a C-terminal EIIAGlc-like domain and in S. thermophilus it has been shown that this domain can be phosphorylated, causing an increased lactose uptake rate (Gunnepijk and Poolman, 2000). All Leuconostoc isolates have this gene, but in Ln. cremoris LacS is truncated and lacks the C-terminal domain, possibly affecting lactose uptake and hence, growth rate on lactose. Alignment of all lacS sequences from this study revealed a close relationship between Ln. pseudomesenteroides, Ln. lactis, and Ln. mesenteroides isolates of non-dairy origin. In fact, lacS of non-dairy associated Ln. mesenteroides is more similar to the lacS from Ln. lactis and Ln. pseudomesenteroides (>75% identity) than that of dairy-associated Ln. mesenteroides or Ln. cremoris (<36% identity). Genes coding for maltose-phosphorylase (malP) and sucrose-6-phosphate hydrolase (srb) were found in Ln. lactis, Ln. pseudomesenteroides P4, and Ln. mesenteroides, but not Ln. cremoris. These enzymes are central to the metabolism of maltose and sucrose. Isolates containing malP also contained genes malR and malL, as well as a maltose epimerase. Ln. lactis and Ln. pseudomesenteroides also contained the malEFG gene cluster encoding for an ABC transporter, however the malEFG genes were truncated in Ln. pseudomesenteroides. Genes encoding for β-glucosidase (bglA) enabling utilization of salicin and arbutin was found in all Ln. pseudomesenteroides and Ln. lactis isolates, as well as in Ln. mesenteroides M2 isolates. The bglA gene, was found to be present in all Ln. cremoris isolates, as well as Ln. mesenteroides M1, M3, and M4 isolates, however the gene was truncated and was identified as inactive by the Seed Viewer. A genetic potential for metabolism of trehalose was found, annotated as treA in Ln. mesenteroides and the Ln. lactis of dairy origin, and as TrePP in Ln. pseudomesenteroides and Ln. lactis 91922. Genes encoding for trehalose transport were not found in Ln. mesenteroides M3 and M4, indicating that these lineages are not able to metabolize trehalose from the environment. Xylose isomerase (xyIA) and xylose kinase (xyIB) genes were found in all Leuconostoc isolates, but the genes were heavily truncated in Ln. cremoris isolates and Ln. mesenteroides M3 and M4 isolates. Isolates with full length xyIA and xyIB genes also contained the gene xylG, encoding for a xylose transport protein.

TABLE 2 | Presence of genes encoding enzymes for amino acid biosynthesis.

<table>
<thead>
<tr>
<th>Amino acid pathway</th>
<th>Ln. cremoris</th>
<th>Ln. mesenteroides</th>
<th>Ln. lactis</th>
<th>Ln. pseudomesenteroides</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alanine</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Arginine</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Aspartate</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Cysteine</td>
<td>+</td>
<td>+</td>
<td>−</td>
<td>+</td>
</tr>
<tr>
<td>Glutamine</td>
<td>−</td>
<td>−</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Glutamic acid</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>+</td>
</tr>
<tr>
<td>Glycine</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Histidine</td>
<td>+</td>
<td>+</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>Isoleucine</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>Leucine</td>
<td>−</td>
<td>−</td>
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<td>−</td>
</tr>
<tr>
<td>Lysine</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>−</td>
</tr>
<tr>
<td>Methionine</td>
<td>+</td>
<td>+</td>
<td>−</td>
<td>−</td>
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<tr>
<td>Phenylalanine</td>
<td>+</td>
<td>+</td>
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<td>+</td>
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<tr>
<td>Proline</td>
<td>+</td>
<td>+</td>
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</tr>
<tr>
<td>Serine</td>
<td>+</td>
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<tr>
<td>Threonine</td>
<td>+</td>
<td>+</td>
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<td>+</td>
</tr>
<tr>
<td>Tryptophan</td>
<td>+</td>
<td>+</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Valine</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
</tr>
</tbody>
</table>

+, presence of predicted pathway functionality; −, absence of predicted pathway functionality.
### (III) Citrate Metabolism

All the dairy strains in this study contained the genes necessary for uptake and metabolism of citrate. These genes are found in an operon comprised of *citC* (citrate lyase ligase), *citDEF* (citrate lyase), *citG* (holo-ACP synthase), *citO* (transcriptional regulator) and *citS* (Na+ dependent citrate transporter). A citrate/malate transporter annotated *cimH* was present in *Ln. mesenteroides* subspecies isolates, but was not present in any of the *Ln. lactis* or *Ln. pseudomesenteroides* isolates. In the *Ln. cremoris* and *Ln. pseudomesenteroides* genomes, the *cit* operon is flanked by two IS116/IS110/IS902 family transposases, suggesting it may have been acquired by horizontal gene transfer. In these bacteria, the operon appears to be located on the chromosome, a finding supported by the genome assembly, which organizes the *cit* operon on a contig containing a number of essential genes, and by read coverage analysis that shows a continuous gapless coverage through the contig, with no elevation in read coverage across the *cit* operon. The *citCDEFGOS* operons of *Ln. mesenteroides* and *Ln. lactis*, however, appear to be located on a plasmid, since in all cases they assembled on a contig, which includes a site of replication and not essential genes. The *cit* operon is highly conserved in the *Ln. cremoris* and *Ln. pseudomesenteroides* genomes with >97% DNA sequence identity between all the isolates. The likely to be plasmid-encoded cit operon found in *Ln. mesenteroides* and *Ln. lactis* genomes is also highly conserved between the isolates (>99% identity), however it is significantly different from the chromosomally encoded *cit* operon present in *Ln. cremoris* and *Ln. pseudomesenteroides* (50–65% DNA sequence identity for each gene). None of the strains of non-dairy origin included in this study contained the citrate genes, indicating that the ability to metabolize citrate plays an important role in the successful adaption to the milk environment.

### (IV) Proteolytic Activity

Leuconostocs grow in association with the lactococci in dairy fermentations, and commonly grow poorly in milk without the presence of lactococci. The general explanation for this poor growth is their lack of proteinase activity, making them dependent on small peptides from lactococcal proteinase activity. Screening all the isolates for genes involved in peptide and proteinolytic activity revealed a number of differences between the lineages (Table 4). The genes encoding for the OppABCDF...
system were found in all *Leuconostoc* genomes. However, in *Ln. cremoris* genomes, the oppA gene was missing, and the oppB gene was severely truncated. A gene encoding for a PII-type serine proteinase (PrtP) best known for its action on caseins was found in all *Ln. pseudomesenteroides* genomes, dairy *Ln. lactis* genomes, *Ln. mesenteroides* M4 and 33% of *Ln. mesenteroides* M1 genomes. All the sequenced *Leuconostoc* strains coded for a range of peptidases and aminotransferases. The *Ln. cremoris* isolates did not contain the pepN gene, but had the other general aminopeptidase gene, pepC, which was found to be missing from *Ln. lactis* genomes. The pepX gene, encoding for the enzyme x-prolyl dipetidyl aminopeptidase was truncated in *Ln. cremoris* (534 amino acids) compared to the pepX of other *Leuconostoc* strains (778–779 amino acids). The pepA, pepF, pepO, pepQ, pepS, and pepT genes were present in all *Leuconostoc* isolates. Finally, all *Ln. pseudomesenteroides* have the pepV gene, encoding β-aladipeptidase. This dipeptidase has been shown to cleave dipetidases with an N-terminal β-Ala or D-Ala residue, such as carnosine and to a lesser extent, was shown to catalyze removal of N-terminal amino acids from a few distinct tripeptides in *Lactobacillus delbrueckii* subsp. *lactis* (Vongerichten et al., 1994).

**CRISPR-Cas in *Ln. lactis* and *Ln. pseudomesenteroides***

*Ln. lactis* 91922 and all the *Ln. pseudomesenteroides* isolates included in this study contained CRISPR-Cas genes with repeat regions.

**Composition of Leuconostocs in Starter Cultures**

The *Leuconostoc* core gene library was used to devise a scheme for species and subspecies quantification in starter cultures by amplicon sequencing. Core genes were screened for sequence variation and for targeted-amplicon suitability. After curation, the top three candidates were 16S rRNA, *rpoB*, and *eno*. While the full-length 16S rRNA sequence enables differentiation of species and subspecies, any region shorter than 500 bp is only able to differentiate between species, and then only when using the nucleotides between position 150–550, encompassing the V2 and V3 regions of 16S rRNA. However, the sequences of 16S rRNA and the *rpoB* loci were too similar to the same genes in lactococci to allow for primer design specific for leuconostocs, and thus were unsuitable for quantification of leuconostocs. The gene encoding enolase (*eno*) did allow for *Leuconostoc* specific primer design, and was used in targeted-amplicon sequencing to analyze the diversity of leuconostocs in the five starter cultures. The analysis revealed great differences between the starter cultures (Figure 4). *Ln. cremoris* dominated the *Leuconostoc* populations in cultures A, D and E, *Ln. pseudomesenteroides* was most abundant in cultures B and C. Most of the *Ln. cremoris* in cultures A and D were of the MPCA type (*Ln. cremoris* C1) unable to grow on MRS, while MRS type *Ln. cremoris* dominated in culture E (data not shown). Relatively low levels of *Ln. mesenteroides* and *Ln. dextranicum* were found in all cultures, the highest being 14% in culture B. *Ln. lactis* was only found in one of the starter cultures, culture E, where it constituted 17% of the leuconostocs.

**DISCUSSION**

Decades have passed since Dr. Ellen Garvie laid the foundation for the taxonomy of dairy relevant leuconostocs, and Dr. John Farrow expanded this list to include *Ln. pseudomesenteroides*. Their work has been the basis for classification of leuconostocs since then.

The *Ln. pseudomesenteroides* species was described for the first time in 1898 (Farrow et al., 1989), however its presence in a dairy starter culture was not described before 2014 (Pedersen et al., 2014b). Identification of leuconostocs by phenotypical traits or by partial 16S rRNA sequencing does not reliably distinguish between all species and misidentification has been
common. After genomic analysis, several isolates previously identified as *Ln. mesenteroides* subspecies proved to be *Ln. pseudomesenteroides* and isolates may have been misidentified in other studies as well. Surprisingly, the strain LbT16 (Accession. No: LAYV00000000) reported to be *Ln. cremoris* by Campedelli et al. (2015) was identified as *Ln. mesenteroides* when characterized by its genomic content and its full length 16S rRNA sequence. Misidentification of *Ln. cremoris* is also uncommon. Compared to other dairy leuconostocs, *Ln. cremoris* grow slower, to a lower density and not at temperatures of 30°C or higher. In addition, a large proportion of *Ln. cremoris* type strains are not able to grow on MRS. These characteristics provide the means for reliable phenotypical identification of *Ln. cremoris*. However, phenotypical differentiation between other *Ln. mesenteroides* subspecies, *Ln. lactis* and *Ln. pseudomesenteroides* remains unreliable. In this study, dairy relevant leuconostocs are characterized using a genomics approach and the diversity of leuconostocs in five commercial DL-type starter cultures is analyzed.

The genomic analysis clearly separated leuconostocs by species, subspecies, and enabled intra-species differentiation. Interestingly, the genomic analysis did not distinguish *Ln. dextranicum* from *Ln. mesenteroides*. The strain-to-strain variation was higher than the differences between subspecies. The *dextranicum* subspecies has been previously defined by phenotypical traits only and separate subspecies distinction is not justified by the genomic data of this study. On the other hand, the pan-genomic analysis separated *Ln. mesenteroides* isolates by habitat. The dairy strains clearly differ from those isolated from plant material, the former have smaller genomes and utilize a more restricted range of carbohydrates. The two subspecies *Ln. mesenteroides* and *Ln. cremoris* share a large amount of genetic content with high identity scores, reflecting a close phylogenetic relationship. However, many genes present in *Ln. mesenteroides* are found to be truncated, contain deletions or are completely missing in *Ln. cremoris*. Adaptation of dairy strains to the milk environment involved acquisition of the plasmid-encoded lacLM by horizontal gene transfer (Obst et al., 1995), which in turn permitted loss of a functional lacZ. Some of the dairy *Ln. mesenteroides*, and all of the *Ln. cremoris* isolates carry a deletion in the lacZ gene. The dairy *Ln. mesenteroides* and in particular *Ln. cremoris* display telltale signs of a prolonged degenerative evolution, likely the result of a long period of growth in milk. In this environment, the leuconostocs have evolved alongside lactococci. All the dairy strains included in this study contain the cit operon comprised of citC (citrate lyase ligase), citDEF (citrate lyase), citG (holo-ACP synthase), citO (transcriptional regulator) and citS (Na+ dependent citrate transporter). The citCDEFGS operon organization is different from the operon in *Lactococcus lactis*, which lacks citO and the citS transporter (Drider et al., 2004). In citrate positive *Lactococcus lactis*, homologs of citO (citR) and the citS (citP) are located on a plasmid (Magni et al., 1994). The presence of the citCDEFGS genes enable so-called citrolactic fermentation, co-metabolism of sugar and citrate providing the cells with higher energy yield and proton motive force (Marty-Teysset et al., 1996). In *Ln. lactis* and *Ln. mesenteroides*, this operon has been linked to a ~22-kb plasmid, inferred by phenotypical studies in combination with monitoring the presence of mobile genetic elements (Lin et al., 1991; Vaughan et al., 1995). In the study by Vaughan et al. (1995), *Ln. mesenteroides* was shown to retain its ability to metabolize citrate after losing three of its four plasmids. Moreover, after curing, a derivative isolate without the ability to degrade citrate still contained the fourth plasmid. Our data indicates that for *Ln. cremoris* and *Ln. pseudomesenteroides*, this is not the case. In all the *Ln. cremoris* and *Ln. pseudomesenteroides* genomes included in this study, the cit operon is located on the chromosome in a region with mobile element characteristics. A low level of genetic drift is indicated by the high sequence similarity between the cit operons of *Ln. cremoris* and *Ln. pseudomesenteroides* suggesting that the acquisition of these genes is quite recent.
possibly from a common donor. The chromosomally encoded \textit{cit} operon of \textit{Ln. cremoris} and \textit{Ln. pseudomesenteroides} was significantly different from the highly conserved and likely to be plasmid-encoded \textit{cit} operon found in \textit{Ln. lactis} and \textit{Ln. mesenteroides}. These results indicate that the plasmid encoded \textit{cit} operon originates from a different source and time. None of the strains of non-dairy origin included in this study contained the citrate metabolism genes, indicating that the ability to metabolize citrate also plays an important role in the successful adaption to the milk environment. The manufacture of Dutch-type cheeses has been going on for centuries and the starter cultures have been maintained by so-called “back slopping” for the last one and a half century, where new milk is inoculated with whey from the previous batch. This technique for propagating starter cultures is still being used and recent studies have shown that the complex starter cultures maintain a highly stable composition with regards to lactococci (Erikus et al., 2013). Culture composition may change over a short period of time depending on growth conditions and bacteriophage predation, but the microbial community is sustained in the long run. In this study, we show a large variation in the amount and composition of the \textit{Leuconostoc} populations in cheeses starter cultures. Three of the starter cultures (A, D, and E) were dominated by \textit{Ln. cremoris}, and for culture A and D, the majority of these were unable to grow on MRS. The other two starter cultures (B and C) were dominated by \textit{Ln. pseudomesenteroides}. Interestingly, the cultures dominated by \textit{Ln. cremoris} also contain \textit{Ln. pseudomesenteroides} strains. \textit{Ln. pseudomesenteroides} growth rates in pure culture are significantly higher than that of \textit{Ln. cremoris} at temperatures above 20°C, so the microbial community is preserved, either by the starter culture developers, or by the microbial community itself. Little knowledge exists on how the diversity of leuconostocs is affected by manufacturing procedures. According to Thunell (1995) and Vedamuthu (1994) the only leuconostocs prevalent in dairy are \textit{Ln. cremoris} and \textit{Ln. lactis}, but in this study, \textit{Ln. lactis} was detected only in culture E, which was dominated by \textit{Ln. cremoris}. In two of the starter cultures studies in this work, \textit{Ln. pseudomesenteroides} was the dominating \textit{Leuconostoc}, which shows that they are highly relevant in the production of cheese. This is also reflected by recent studies, where the presence of \textit{Ln. pseudomesenteroides} is more frequently reported (Callon et al., 2004; Porcellato and Skeie, 2016; Østlie et al., 2016). It is tempting to speculate that starter culture manufacturers have altered the conditions for culture propagation or manipulated the strain collections, thereby altering the culture dynamics between strains in favor of \textit{Ln. pseudomesenteroides}.

The differences between the starter cultures could have an impact on the characteristics of the cheese product. \textit{Ln. cremoris} lacks a wide range of genes involved in carbohydrate metabolism and proteolytic activity, and studies have shown that \textit{Ln. cremoris} and \textit{Ln. pseudomesenteroides} differ significantly in their ability to produce a wide range of volatile compounds (Pedersen et al., 2016). Most notably, the amount of acetoin and diacetyl in model-cheeses produced with only \textit{Ln. pseudomesenteroides} was negligible. This was supported by our data, which showed that the \textit{Ln. pseudomesenteroides} P4 isolates lack the genes necessary for reduction of diacetyl to acetoin and 2,3-butandiol. In addition, these isolates lacked the genes \textit{ilvB} and \textit{ilvH} encoding acetalactate synthetase large and small subunits, which is found in all \textit{Ln. mesenteroides} subspecies isolates. However, a different gene \textit{alsS}, encoding the same function, was found in all leuconostocs, including \textit{Ln. pseudomesenteroides}. Studies on \textit{α}-acetalactate synthase (ALS) and \textit{α}-acetalactate decarboxylase (ALDC) activity in \textit{Ln. mesenteroides} subspecies and \textit{Ln. lactis} showed that the activity of both ALS and ALDC was higher for \textit{Ln. lactis} (which does not have the \textit{ilv} or \textit{leu} operon) than that of \textit{Ln. cremoris} (which does have part of these two operons) (Monnet et al., 1994). For comparison, the ALS activity of \textit{Lc. lactis} biovar \textit{diacetylactis} was comparable or in some cases even higher than that of \textit{Ln. lactis}. \textit{Ln. pseudomesenteroides} was not included in the study, but data from semi-hard cheeses comparing the acetoin and diacetyl concentrations revealed lower concentrations in mock starters containing \textit{Ln. pseudomesenteroides} compared to mock starters containing \textit{Ln. cremoris} (Pedersen et al., 2016). This observation could be attributed to the rapid growth rate of \textit{Ln. pseudomesenteroides} when compared to that of \textit{Ln. cremoris}. The presence of the degenerated \textit{ilv} and \textit{leu} operons could somehow be negative to \textit{Ln. cremoris} growth rate. Indeed, when cloning of the \textit{ilv} operon into \textit{Escherichia coli}, the presence of \textit{Leuconostoc ilvB} was strongly detrimental to growth, while recombinant strains with an insertion in the \textit{Leuconostoc ilvB} genes displayed normal growth. Their hypothesis was that expression of \textit{ilvB} without a functional branched chain amino acid biosynthesis mechanism could interfere with energy metabolism via pyruvate (Cavin et al., 1999).

In dairy fermentations, the leuconostocs grow in association with the lactococci. Whether the associative growth is of mutual benefit to the leuconostocs and lactococci has not been determined. Literature often attributes the poor growth of leuconostocs to the lack of protease activity (Vedamuthu, 1994; Thunell, 1995). However, the ability to acidify milk in pure culture has been described for \textit{Ln. pseudomesenteroides} (Cardamone et al., 2011), and we identified genetic potential for caseinolytic activity in \textit{Ln. pseudomesenteroides} in our data. This would enable \textit{Ln. pseudomesenteroides} to grow better in milk than \textit{Ln. cremoris}, which lacks the capacity for protease, as well as a functional peptide uptake system due to the lack of OppA, which is responsible for the uptake of extracellular peptides. An argument for mutually beneficial growth has been made by superimposing metabolic pathways from lactococci and leuconostocs, indicating a potential for metabolic complementation between the two genera (Erikus et al., 2013). One can be forgiven for thinking \textit{Ln. pseudomesenteroides} the better bacteria of the two based on these tidbits of information alone. However, both \textit{Ln. cremoris} and \textit{Ln. pseudomesenteroides} have shown to be significant to the production of cheeses. It is difficult to conclude which \textit{Leuconostoc} species produces the highly subjective matter of the better cheese product. The concentration of volatile compounds, fatty acid derivatives, acetoin, diacetyl, and amino acid derivatives in products have been shown to diverge significantly, depending on which \textit{Leuconostoc} species is added to the mixture of lactococci (Pedersen et al., 2016).
In conclusion, the dairy-associated leuconostocs are highly adapted to grow in milk. Comparative genomic analysis reveals great differences between the *Leuconostoc* species and subspecies accustomed to the dairy environment, where they grow in association with the lactococci. The composition of the *Leuconostoc* population is significantly different between commercial starter cultures, which ultimately affects the characteristics and quality of the product. A better understanding of *Leuconostoc* microbial dynamics and the functional role of different dairy leuconostocs could be of great importance and be an applicable tool in ensuring consistent manufacture of high quality product. Currently, no detailed information on the relative amount or diversity of the *Leuconostoc* population in starter cultures is available to the industry. We provide a culture independent method for robust identification and quantification of *Leuconostoc* species in mixed microbial communities, enabling quantification of leuconostocs in starter cultures, as well as monitoring the diversity of leuconostocs through the cheese production process.

**AUTHOR CONTRIBUTIONS**

CF isolated and sequenced bacterial strains, performed the sequencing work in Norway (of all CF and H-isolates in addition to all amplicon sequencing), analyzed the data, wrote the R-scripts, devised the methods and wrote the manuscript. FB, HØ, TP, HK, and HN provided bacterial isolates for a larger diversity. WK and LH performed the sequencing of isolates in Denmark. Supervision of danish activities was provided by FV. Supervision of Norwegian activities was provided by HK, HØ, and HH. All co-authors were involved in reviewing and commenting on the manuscript prior to its submission. A large contribution to final editing was made by HN and JB.

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**SUPPLEMENTARY MATERIAL**

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fmicb.2017.00132/full#supplementary-material

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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