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Analysis of *rpoB* polymorphism and PCR-based approaches for the identification of *Leuconostoc mesenteroides* at the species and subspecies level

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ABSTRACT

Leuconostoc mesenteroides includes the subsp. *cremoris*, subsp. *dextranicum*, subsp. *mesenteroides* and subsp. *jonggajibkimchii*, but the identification at the subspecies level using current phenotypic and/or genotypic methods is still difficult.In this study, a polyphasic approach based on the analysis of *rpoB* gene polymorphism, Multiplex-PCR and phenotypic tests was optimised and used to identify a collection of *Leuc. mesenteroides* strains at the species and subspecies levels. The annotation of published *Leuc. mesenteroides* genomes was also revised.A polymorphic region of *rpoB* gene was effective in separating *Leuc. mesenteroides* strains at the species (*rpoB*-species-specific-PCR) and subspecies (phylogenetic comparison) levels. Multiplex-PCR discriminated the subsp. *mesenteroides* from subsp. *cremoris*, but strains of uncertain attribution were found among subsp. *dextranicum* and subsp. *jonggajibkimchii*. Most of phenotypic features were not suitable for subspecies discrimination.Our assays may provide a rapid and reliable identification of subsp. *mesenteroides* and subsp. *cremoris* strains in fermented foods. The discrimination of subsp. *dextranicum* and subsp. *jonggajibkimchii* suffered from several limitations (e.g. low number of available strains and genomes, phenotypic profile close to subsp. *mesenteroides*, discrepancy between genotypic and phenotypic traits) and further investigations are needed to clarify their delineation and taxonomical position.

1. Introduction

The genus *Leuconostoc* includes heterofermentative lactic acid bacteria which affect the quality of foods either positively through fermentation, or negatively through spoilage (Hemme and Foucaud-Scheunemann, 2004). The taxonomy of the genus *Leuconostoc* has undergone several re-arrangements (Collins et al., 1993; Dicks et al., 1995; Endo and Okada, 2008) and it currently includes fourteen species (http://www.bacterio.net/leuconostoc.html). The classification at subspecies level has been also revised and different polyphasic approaches have been used to identify new subspecies and/or to resolve uncertain delineation. The species *Leuc. gelidum* (spoilage agents of packaged and refrigerated meat products) and *Leuc. mesenteroides* (starter and adjuncts in several fermented foods) suffered the main phylogenetic revisions. *Leuc. mesenteroides* is now divided into subsp. *cremoris*, subsp. *dextranicum*, subsp. *mesenteroides* and subsp. *jonggajibkimchii*. The first three were defined before the introduction of molecular typing methods (Garvie, 1983), while the subsp. *jonggajibkimchii* was recently described (Jeon et al., 2017; based on the features of a single strain) by integrating the Average Nucleotide Identity (ANI), in silico DNA-DNA Hybridization (*i*DDH) and core-genome data. The subsp. *suionicum* (described by Gu et al., 2012) was recently upgraded to species status (*Leuc. suionicum*, type strain DSM20241^T; Chun et al., 2017a).

Over time, several molecular techniques, including RAPD-PCR, rep-PCR, species-specific PCR, multiplex-PCR, 16S PCR-RFLP, ARDRA, MLST and partial sequencing of housekeeping genes (Frantzen et al., 2017; Gu et al., 2012; Rahkila et al., 2014; Sharma et al., 2018), have been proposed to identify the *Leuc. mesenteroides* strains at species and/or subspecies level, with variable degree of success due to some practical limitations (poor reproducibility, low discriminative power). Recent comparative genomic studies (Chun et al., 2017b; Frantzen et al., 2017; Jeon et al., 2017) have confirmed that 16S rRNA gene sequencing, and/or ANI and *i*DDH may be insufficient to classify *Leuc. mesenteroides* at subspecies level, and more powerful approaches (e.g. pan- and core-genome analyses) may be needed to outline strain diversity and delineate subspecies. However, although genome sequencing may provide a robust support, its use requires specific expertise and

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tools and it is unlikely to become routine in food laboratories in the near future. Therefore, faster and readily applicable methods, with a good discriminative power, are desirable to identify the *Leuc. mesenteroides* strains and to provide correct information about *Leuconostoc* diversity in fermented foods.

In this study, a polyphasic approach based on the analysis of RNA polymerase subunit beta (*rpoB* gene) polymorphism, Multiplex-PCR assay (presence/absence of *rpoB*, L-arabinose isomerase, dextransucrase, PTS-sorbose transporter subunit IIC genes) and phenotypic tests (sugar fermentation, dextran production, colony morphology) was used to discriminate a collection of *Leuc. mesenteroides* strains at species and subspecies levels. The subspecies annotation of published *Leuc. mesenteroides* genomes was also revised (ANI, *i*DDH, gene occurrence analysis) and used for comparison.

2. Material and methods

2.1. Strains and culture conditions

Ninety-one strains (Table 1) of *Lactobacillus* (n. 4), *Leuconostoc* (n. 77) and *Weissella* (n. 10) genera were used in this study. The strains were maintained as freeze-dried stocks (11% *w/v* Skim Milk, 0.1% w/v ascorbic acid) in the culture collection of Laboratorio di Microbiologia Industriale, Università degli Studi della Basilicata, and were routinely propagated in modified APT pH 6.8 (mAPT; Sperber and Swan, 1976), for 16 h at 30 °C.

2.2. ANI, iDDH and gene occurrence in Leuconostoc mesenteroides genomes

Pairwise ANI of available *Leuc. mesenteroides* genomes (retrieved from NCBI and Integrated Microbial Genomes databases on April 2018; bold in Supplementary Table 1) was calculated using the IMG tools (https://img.jgi.doe.gov/). *i*DDH values were estimated with the Genome-to-Genome Distance Calculator (http://ggdc.dsmz.de/distcalc2.php).

The gene occurrence (see Supplementary Fig. 3 for gene name) was verified on IMG database. Subspecies annotation resulting from ANI, *i*DDH and gene occurrence was used for further phylogenetic analyses.

2.3. Study of rpoB gene polymorphism as a tool for species and subspecies discrimination

2.3.1. Phylogenetic analysis of rpoB sequences

The complete nucleotide sequences (3609 bp) of *rpoB* gene, retrieved from the *Leuc. mesenteroides* genomes (bold in Supplementary Table 1), were aligned with ClustalW algorithm to generate a phylogenetic tree with the Maximum Likelihood (ML) method, Tamura-Nei model, and 1000 bootstrap replications. Similarly, the complete *rpoB* aminoacid sequences (1202 aa) were aligned with Muscle algorithm and trees were inferred with ML method, Jones-Taylor-Thornton (JTT) model, and 1000 bootstrap replications (MEGA7 software; Kumar et al., 2016).

2.3.2. Species-specific-PCR and phylogenetic analysis of the polymorphic rpoB-region

The polymorphic site analysis of the complete *rpoB* gene were performed with DnaSP v.6.12.01 software, to identify useful regions for phylogenetic and PCR-based assays.

A *Leuc. mesenteroides* species-specific PCR was developed to amplify the variable region (952 bp) of *rpoB* gene in our strains (Table 1). The genomic DNA was isolated with the GeneElute Bacterial Genomic DNA Kit (Sigma-Aldrich Srl, Milan, Italy) and quantified with a NanoDrop®1000c spectrophotometer (Thermo Scientific, Wilmington, DE). Forward and reverse *rpoB* gene primers (Supplementary Table 2) were designed with Primer Express v.3.0 (Applied Biosystems, Concord, On-

Table 1
Strains (belonging to different species of the genera *Lactobacillus*, *Leuconostoc* and *Weissella*) used in this study.

Species [a]	Strain label and isolation source [b]
Lactobacillus brevis (Chun et al., 2017b)	**575** (FS); B29, F02 (SD); TO62 (RM)
Leuconostoc carnosum (Campedelli et al., 2015)	**102**, 1249 (FS)
Leuconostoc citreum (Campedelli et al., 2015)	D27, V2 (SD)
Leuconostoc mesenteroides (66)	**ACA-DC 0134**, **ACA-DC 0493**, **ACA-DC 0547**, ACA-DC 0750, **ACA-DC 1136**, **ACA-DC 1140**, ACA-DC 1245, DPC1052, DPC167, DPC224, DPC227, DPC2277 DPC2304, **DPC231**, **DPC232**, DPC242, **DPC2963**, **DPC3611**, **DPC3615**, **DPC3617**, **DPC3618**, **DPC3944**, **LE30**, **RF15-2**, **RF60-1**, **RLM4** (AC); **M206**, **M288**, **M300** (AC); **M148**, **M166**, **M16S**, **M17P**, **M203**, **M5P**, **M68**, **M69**, **M6P** (RM); **G2-3**, **MT2A12S**, **MT2A13SP**, **MT2A26L**, **MT2A29S**, **MT2A4SP**, P3, W8, **Z06**, **Z15** (SD)

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Table 1 (*Continued*)

Species [a]	Strain label and isolation source [b]
	subsp. *mesenteroides* **E29**, **LMG19463**, **DSM20240** (UN); **DSM20343** [T] (FV); **LMG18967** (SI); **LMG23111** (HU); **LMG26308** (plant); **LMG30251** (IN); **M176**, **M201** (RM)
	subsp. *cremoris* **E55**, **LMG6909** [T] (cheese starter powder); **LMG7954** (FV)
	subsp. *dextranicum* **E58**, **LMG6908** [T], **LMG11318**, **LMG11320**, **LMG11321** (UN)
Leuconostoc pseudomesenteroides (Endo and Okada, 2008)	1034 (FS); M65 (RM); W2, W4, W5, Z02, Z11 (SD)
Weissella cibaria (Dicks et al., 1995)	E66, D4, O3, MTE22L, MTE5L (SD); DSM15878 (chili)
Weissella confusa (Björkroth et al., 2014)	DSM20196 (sugar cane)
Weissella hellenica (Björkroth et al., 2014)	DSM7378 (FS)
Weissella minor (Björkroth et al., 2014)	DSM20014 (milking machine slime)
Weissella viridescens (Björkroth et al., 2014)	DSM20410 (cured meat products)

Table 1 (*Continued*)

Isolation sources: AC, artisanal cheeses; FV, fermented vegetables; FS, fermented sausages; HU (human); IN, insects; RM, raw milk; SI, silage; SD, sourdough; UN, unknown. Culture collections: DSMZ, Deutsche Sämmlug von Mikroorganismes und Zellkulturen GmbH (Germany); LMG, Bacteria Collection Laboratorium voor Microbiologie Universiteit Gent (Belgium)); strains LE30, RF152, RF601, RLM4 were from University of Verona, Italy; ACA-DC0134, ACA-DC0493, ACA-DC0547, ACA-DC0750, ACA-DC1136, ACA-DC1140, ACA-DC1245 from Agriculture University of Athens, Greece; DPC1052, DPC167, DPC224, DPC227, DPC2277, DPC2304, DPC231, DPC232, DPC242, DPC2963, DPC3611, DPC3615, DPC3617, DPC3618, DPC3944 from Moorepark Teagasc Food Research Center, Ireland; D4, E66, O3, P3, V2, W2, W4, W5, W8 from ISA-CNR, Avellino, Italy; where not specified, the strains belong to the culture collection of the Laboratory of Industrial Microbiology, Università degli Studi della Basilicata (Potenza, Italy). Bold: strains used for the phylogenetic analysis of *rpoB* gene (see Fig. 1).

[a] As classified and stored in the culture collections (see below) before this study. The number of strains for each species is reported in brackets.

[b] The isolation source of each strain is reported in brackets.

tario, Canada). Reaction mixture (50 μL) contained Taq High Fidelity Buffer 1×, 1.5 mM $MgSO_4$, 0.2 mM dNTPs, 0.5 μM of each forward and reverse *rpoB* primers, 0.75 U AccuStart Taq DNA Polymerase HiFi (Quantabio, Beverly, MA, USA), and 1 μL of 25 ng DNA template. PCR was carried out in a T100™ Thermal Cycler Bio-Rad (Bio-Rad Laboratories Srl, Segrate, Milan, Italy) using an initial denaturation step for 5 min at 94 °C, followed by 30 cycles of 30 s at 94 °C, 45 s at 54 °C and 60 s at 72 °C each, and by a final extension of 7 min at 72 °C. PCR products were separated (90 min at 100 V) on 1.2% *w/v* agarose gel, stained with 0.05 μL/mL of GelRed™ (10,000× in water; Botium Inc., Fremont, California) and visualized using GelDoc XR system (Bio-Rad Laboratories).

The *rpoB* amplicons detected in 57 *Leuc. mesenteroides* strains (bold in Table 1) were purified (GenElute™ PCR Clean-Up Kit; Sigma-Aldrich Srl), sequenced (Genechron Srl, Roma, Italy) and aligned (852 bp-length, after deletion of poor quality bases) with ClustalW to generate a phylogenetic tree with UPGMA method, Tamura-Nei model, and 1000 bootstrap replications (MEGA 7 software). The partial *rpoB* sequences retrieved from the 22 *Leuc. mesenteroides* genomes were used for comparison.

2.4. Multiplex-PCR assay as a tool for species and subspecies discrimination

RNA polymerase subunit beta (*rpoB*; taxonomic marker for species identification), L-arabinose isomerase (*araA*), dextransucrase (*dsr*) and PTS-sorbose transporter subunit IIC (*sorA*) (subspecies-discriminant genes; see decision tree in Supplementary Fig. 1) were selected to develop a Multiplex-PCR assay. Primers are reported in Supplementary Table 2.

The reaction mixture (25 μL) contained EuroTaq Buffer 1×, 2.5 mM of $MgCl_2$, 0.2 mM of dNTPs, 1 μM of each forward and reverse *rpoB* gene primers, 0.5 μM of each forward and reverse *araA* gene primers, 0.3 μM of each forward and reverse *dsr* gene primers, 0.1 μM of each forward and reverse *sorA* gene primers, 2.5 U of EuroTaq polymerase (EuroClone SpA, Pero, Milan, Italy) and 1 μL of 25 ng DNA template. Amplification steps included 5 min at 95 °C, 30 cycles of 30 s at 95 °C, 60 s at 60 °C and 90 s at 72 °C each, and a final extension of 10 min at 72 °C. PCR products (Supplementary Table 2) were separated (90 min at 100 V) on 1.2% *w/v* GelRed-stained (see Section 2.3.2) agarose gel.

2.5. Phenotypic tests

2.5.1. Colony morphology

The overnight mAPT-cultures of all strains (n. 91, Table 1) were streaked on three-sectors Petri dishes with: 1. modified MRS agar with 0.05% w/v cysteine and 0.002% w/v bromophenol blue (BPB) as pH indicator (mMRS; Ricciardi et al., 2015), 2. MRS agar with 50 g/L sucrose as carbon source (sMRS) and 3. MRS agar with 10 g/L L-arabi-

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nose as carbon source and 0.16 g/L of bromocresol purple (BCP) as pH indicator (aMRS). At the end of incubation (anaerobiosis, 48 h, 30 °C), the colony morphology (size, colour) on mMRS, the acidification (yellow colonies) on aMRS and the production of dextran (translucent slimy colonies) on sMRS were evaluated and codified with binary characters (1 or 0) for statistical analyses.

2.5.2. Acid production from carbohydrates

Acid production from carbohydrates (L-arabinose, cellobiose, fructose, galactose, D-glucose, D-xylose, lactose, maltose, ribose, sucrose, trehalose) was evaluated in all strains (n. 91, Table 1) using a 96-well microplate assay (MRS + BCP + 10 g/L of each sugar). Microplates were inoculated (10% *v*/v) with standardised (OD_{650nm} of 0.2) overnight mAPT-cultures and incubated in anaerobiosis at 30 °C for 48 h. The change of BCP from purple to yellow indicated positive results. Ability to grow at 37 °C was evaluated through BCP colour change, after 48 h of incubation (screw-cap tubes with 10 mL of MRS + BCP, inoculated with 1% v/v mAPT-cultures) in water-bath incubator.

2.6. Statistical analyses

All statistical and graphical analyses were carried out using R v3.5.2 (R Core Team, 2019; see Supplementary Materials for the specification of R packages).

3. Results

3.1. Comparative analysis of annotated Leuconostoc mesenteroides genomes

An in silico comparison approach was used to verify the phylogenetic distance and the subspecies annotation of published *Leuc. mesenteroides* genomes. The results of pairwise ANI were used to create a dissimilarity dendrogram (Supplementary Fig. 2) that generated four main groups, each of which included one of the type strains (*cre*_ATCC19254^T, *dex*_DSM20284^T, *mes*_ATCC8293^T, *jon*_DRC1506^T). In many cases the annotation in public databases was in contrast with the ANI-distance among the *Leuc. mesenteroides* genomes. *i*DDH (data not shown) partially supported the ANI results and the original subspecies annotation (see Table 2). Strain P45 was distant (lowest ANI and *i*DDH values) from all others, although the 16S rRNA comparison confirmed its identification at species level (99.86% identity with *mes*_ATCC8293^T; https://www.ezbiocloud.net/identify).

The gene occurrence analysis (Supplementary Fig. 3) clearly separated the subsp. *cremoris* group (lacking several metabolically relevant genes) from the others *Leuc. mesenteroides*. A second major cluster, that included *dex*_DSM20284^T and most of strains without or with ambiguous subspecies (see Table 2), was characterised by the lack of all or some genes involved in arabinose utilization. A third major cluster, with a complete gene pattern, included almost all subsp. *mesenteroides* strains (with exception of LbE16 and FM06, whose original identification was ambiguous) and was very close to *jon*_DRC1506^T. The revised subspecies annotation was reported in the consensus Table 2 and used for phylogenetic comparison.

3.2. rpoB gene polymorphism as a tool for species and subspecies discrimination

Sequence analysis of *rpoB* gene (Supplementary Fig. 4) provided a clear separation of subsp. *cremoris* (cre), subsp. *jonggajibkimchii* (jon) and subsp. *mesenteroides* (mes) genomes, but did not resolve the classification of subsp. *dextranicum* and several strains (amb or mes-amb from in silico analyses) with genomic features close to both subsp. *mesenteroides* and subsp. *jonggajibkimchii*. Comparison of *rpoB* amino acid sequences (Supplementary Fig. 5) separated the subsp. *cremoris* group, but was not conclusive for the other strains. P45 was excluded as its *rpoB* sequence was quite divergent and it impaired dendrogram visualisation.

To improve the discriminative power of *rpoB* gene, the region (544–1496 bp) containing the highest % of parsimony informative sites (2.1%) was identified and used to obtain a 952-bp *rpoB* amplicon needed for phylogenetic and PCR-based assays. The specificity of primers for the *Leuc. mesenteroides* species was confirmed with in silico PCR amplifications (http://insilico.ehu.es/user_seqs/PCR/) and with mismatch detection, using as templates all available genomes of *Leuconostoc* and *Weissella* species (Supplementary Table 1).

The species-specific PCR generated the *rpoB*-amplicon only in the *Leuc. mesenteroides* strains (n. 57, bold in Table 1; identification of all strains was verified by partial 16S rRNA gene sequencing), confirming the effectiveness for species classification. The partial *rpoB* sequences (from 57 *Leuc. mesenteroides* strains and 22 published *Leuc. mesenteroides* genomes) were compared to verify the resolution power at subspecies level (Fig. 1). A major cluster of subsp. *mesenteroides* strains (see *mes*_ATCC8293^T) was clearly evident. The smallest subsp. *cremoris* (*cre*_ATCC19254^T) and subsp. *dextranicum* (*dex*_DSM20484^T) groups were close to strains with ambiguous delineation (clusters cl3, cl4, cl5). The subsp. *jonggajibkimchii* (see *jon*_DRC1506^T), on the contrary, was well separated. Cluster cl1 and cl2 included strains with genomic features (see Table 2) related to both *mesenteroides* and *jonggajibkimchii* subspecies. The GenBank accession numbers of representative *rpoB* sequences (* in Fig. 1) are listed in Supplementary Table 3.

3.3. Multiplex-PCR assay as a tool for subspecies discrimination

The specificity of *araA*, *dsr* and *sorA* gene primers for the species *Leuc. mesenteroides* was confirmed with in silico PCR and mismatch analyses. Multiplex assay generated six different profiles among the 57 *Leuc. mesenteroides* strains (Fig. 2), but no amplification bands were observed for the other *Leuconostoc* and *Weissella* strains. With exception of subsp. *cremoris* group (which had only the *rpoB* band, but lacked *araA*, *dsr* and *sorA* amplicons, consistently with the in silico gene profile of Supplementary Fig. 3), the occurrence of *araA*, *dsr* and *sorA* genes (Fig. 3) did not fully reflect the subspecies classification based on *rpoB*-clustering (see confusion matrix, Supplementary Table 4). Some (20%) subsp. *mesenteroides* strains had ambiguous gene pattern, because of *araA* or *araA* and *dsr* band deficiency; while almost all (90%) subsp. *jonggajibkimchii*-related strains grouped with subsp. *mesenteroides* for the presence of *sorA* gene. Some subsp. *dextranicum* strains had the expected pattern of subsp. *jonggajibkimchii* because they lacked *sorA* gene. Multiplex-PCR did not resolve the subspecies classification of strains belonging to cl1, cl4, cl5 (*rpoB*, *dsr*, *sorA*), cl3 (*rpoB*, *araA*, *sorA*) and cl2 (*rpoB*, *araA*, *dsr*, *sorA*, or *rpoB*, *dsr*, *sorA*).

3.4. Phenotypic tests

As for the Multiplex-PCR, the capability to produce acid from arabinose and dextran from sucrose were used to discriminate the *Leuc. mesenteroides* strains with a rapid agar-plate test (Supplementary Fig. 6). For many strains, the presence of *araA* and *dsr* genes did not reflect the production of acid from arabinose and dextran from sucrose (Fig. 4). Growth at 37 °C separated subsp. *cremoris* and cl3 groups (arabinose- and dextran-defective strains) from the other *Leuc. mesenteroides* (Fig. 4). The appearance of colonies on mMRS (Fig. 4; Supplementary Fig. 6) and the sugar fermentation pattern (Supplementary Fig. 7) were not useful for subspecies discrimination. The distribution of phenotypic and genotypic traits among the *rpoB*-based clusters is shown in Fig. 5. The inability to ferment arabinose separated subsp. *cremoris* and subsp. *dextranicum* from subsp. *mesenteroides* and subsp. *jonggajibkimchii*. On

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Table 2
Consensus of the subspecies classification of the published *Leuconostoc mesenteroides* genomes using different comparative genomics criteria.

Genome name [a]	Genome short [b]	Subspecies annotation [c]	Frantzen et al. (2017) [d]	Chun et al. (2017) – ANI [e]	Chun et al. (2017) – core [f]	Chun et al. (2017) – COG [g]	Pairwise ANI [h]	Gene occurrence [i]	*i*DDH70 [l]	*i*DDH79 [m]	Consensus [n]
Leuc. mesenteroides subsp. *jonggajibkimchii* DCR1506 [T]	Lmejo_DCR1506 [T]	jon	jon	jon	jon	jon	jon	jon	jon	jon	jon
Leuc. mesenteroides BD1710	Lmeme_BD1710	mes	–	–	–	–	mes	mes	mes	mes	mes
Leuc. mesenteroides DCR0211	Lmeme_DCR0211	mes	–	amb	jon	jon	jon	amb	mes	mes	amb
Leuc. mesenteroides FM06	Lmeme_FM06	mes	–	–	–	–	cre	dex	dex	dex	dex
Leuc. mesenteroides LK151	Lme_LK151	None	–	–	–	–	dex	mes	mes	mes	mes amb
Leuc. mesenteroides 213 M0	Lme_213M0	None	–	mes	mes	amb	mes	dex	mes	mes	mes
Leuc. mesenteroides 406	Lme_406	None	–	mes	mes	amb	mes	amb	mes	mes	mes
Leuc. mesenteroides AtHG50	Lme_AtHG50	None	–	amb	–	–	mes	amb	–	–	amb
Leuc. mesenteroides subsp. *cremoris* ATCC19254 [T]	Lmecr_ATCC19254 [T]	cre	cre	cre	cre	cre	cre	cre	cre	cre	cre
Leuc. mesenteroides subsp. *cremoris* LbT16	Lmecr_LbT16	cre	mes	dex	cre	amb	cre	dex	dex	dex	dex
Leuc. mesenteroides subsp. *cremoris* T26	Lmecr_T26	cre	cre	cre	cre	cre	cre	cre	cre	cre	cre
Leuc. mesenteroides subsp. *cremoris* TIFN8	Lmecr_TIFN8	cre	cre	cre	–	–	cre	cre	cre	cre	cre
Leuc. mesenteroides subsp. *dextranicum* DSM20484 [T]	Lmedx_DSM20484 [T]	dex	amb	dex	dex	dex	dex	dex	dex	dex	dex
Leuc. mesenteroides subsp. *dextranicum* LbE15	Lmedx_LbE15	dex	mes	amb	mes	amb	dex	dex	amb	amb	amb
Leuc. mesenteroides GL1	Lme_GL1	None	–	amb	mes	dex	dex	mes	mes	mes	mes amb
Leuc. mesenteroides KFRI-MG	Lme_KFRI-MG	None	–	jon	jon	jon	jon	mes	jon	jon	jon

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Table 2 (*Continued*)

Genome name [a]	Genome short [b]	Subspecies annotation [c]	Frantzen et al. (2017) [d]	Chun et al. (2017) – ANI [e]	Chun et al. (2017) – core [f]	Chun et al. (2017) – COG [g]	Pairwise ANI [h]	Gene occurrence [i]	*i*DDH70 [l]	*i*DDH79 [m]	Consensus [n]
Leuc. mesenteroides subsp. *mesenteroides* ATCC8293 ^{T}	Lmeme_ATCC8293 ^{T}	mes	amb	mes	mes	mes	mes	mes	mes	mes	mes
Leuc. mesenteroides subsp. *mesenteroides* BD3749	Lmeme_BD3749	mes	–	jon	jon	jon	jon	mes	jon	jon	jon
Leuc. mesenteroides subsp. *mesenteroides* J18	Lmeme_J18	mes	mes	amb	jon	jon	jon	mes	mes	mes	amb
Leuc. mesenteroides subsp. *mesenteroides* LbE16	Lmeme_LbE16	mes	mes	amb	mes	amb	dex	dex	jon	jon	amb
Leuc. mesenteroides P45	Lme_P45	None	–	amb	amb	amb	amb	dex	amb	amb	amb
Leuc. mesenteroides Wikim17	Lme_Wikim17	None	–	amb	mes	dex	amb	amb	mes	mes	mes amb

[a] Subspecies annotation and strain label reported on National Center for Biotechnology Information (NCBI) and Integrated Microbial Genomes (IMG) databases.
[b] Short name and label used in Supplementary Figs. 2 and 3. ([c]) Subspecies annotation as reported on NCBI and IMG databases ([c]), or in Frantzen et al. (2017) ([d]), or in Chun et al. (2017b) with pairwise ANI ([e]), core-genome analysis ([f]), GOG analysis ([g]), or in this study with pairwise ANI ([h]), *i*DDH70% ([i]), *i*DDH79%. ([l]), gene occurrence ([m]): none, without subspecies annotation; cre, subsp. *cremoris*; dex, subsp. *dextranicum*; mes, subsp. *mesenteroides*; jon, subsp. *jonggajibkimchii*; mes amb, subsp. *mesenteroides* with ambiguous delineation; amb, ambiguous; –, not available or not investigated. ([n]) Subspecies annotation resulting from the different analyses and used for comparison in Figs. 1 and 3, and in Supplementary Figs. 4 and 5.

the other hand, dextran production (including *dsr* gene) and growth at 37 °C were helpful to distinguish subsp. *cremoris* from subsp. *dextranicum*. None of phenotypic and genotypic traits (including *sorA* gene) separated subsp. *mesenteroides* from subsp. *jonggajibkimchii*. Phenotypic features were not supportive in the discrimination of strains belonging to the ambiguous clusters cl1, cl2, cl3, cl4 and cl5.

4. Discussion

In this study, we optimised PCR-based assays useful for the identification of *Leuc. mesenteroides* strains at the species and subspecies level. Our results were compared with previous comparative genomics studies (Chun et al., 2017b; Frantzen et al., 2017) to verify the effectiveness and adequacy of proposed tests.

The species-specific-PCR we developed successfully discriminated the *Leuc. mesenteroides* strains at species level, confirming that *rpoB* gene is a convenient taxonomic marker and it may offer several advantages compared to the 16S rRNA gene. Multiplex-PCR identified the strains at species level (presence of *rpoB*-band) and discriminated the subsp. *mesenteroides* (presence of *araA*, *dsr*, *sorA* genes) from subsp. *cremoris* (lack of *araA*, *dsr*, *sorA* bands), but did not resolve the ambiguities in the identification of some subsp. *dextranicum* and subsp. *jonggajibkimchii* strains.

Multiplex-PCR assays have been successfully used to separate LAB strains belonging to genetically closely related species (Lee et al., 2000; Torriani et al., 2001; Ventura et al., 2003), but its application for subspecies discrimination is a more difficult task, since the subspecies definition may change in the different taxa and, in most of cases, is based on phenotypic traits.

Compared to other LAB, the identification of *Leuc. mesenteroides* at subspecies level suffers several limitations (e.g. lower number of available genomes and genomic diversity studies; low number of subsp. *cremoris*, subsp. *dextranicum* and subsp. *jonggajibkimchii* isolates compared to the most abundant strains of subsp. *mesenteroides*) that, in some cases, may result in misidentifications. In addition, members of subsp. *cremoris* and subsp. *dextranicum* available in Microbial Culture Collections (e.g. ATCC, DSMZ, LMG) are just a few, and for some of them the isolation source is unknown, limiting information on strain diversity.

The taxonomy of *Leuc. mesenteroides*, even when based on advanced molecular and genome comparison approaches, still refers to type strains (*cre*_ATCC19254^{T}, *dex*_DSM20284^{T}, *mes*_ATCC8293^{T}) described exclusively with metabolic assays and DNA-DNA homology (Garvie, 1983). On the other hand, the description of new subsp. *jonggajibkimchii* is based on phenotypic and genomic features of a single isolate (DR-C1506^{T}, Jeon et al., 2017), suggesting that further investigations and a larger number of strains are needed to reach a confident separation at subspecies level.

In this study, besides to the PCR-based approaches, we analysed and used the *rpoB* gene polymorphism to support the subspecies delineation and to resolve uncertain classification. Contrarily to Multiplex-PCR profiles, *rpoB*-clustering clearly separated subsp. *mesenteroides* from subsp. *jonggajibkimchii*. The delineation of subsp. *dextranicum* group, even integrating Multiplex-PCR and *rpoB* polymorphism, remained ambiguous.

The phenotypic features tested in this study were not supportive for subspecies discrimination since, in most cases, the metabolic traits were affected by strain-to-strain variability and were not consistent with genetic information (as already proven by Chun et al., 2017b).

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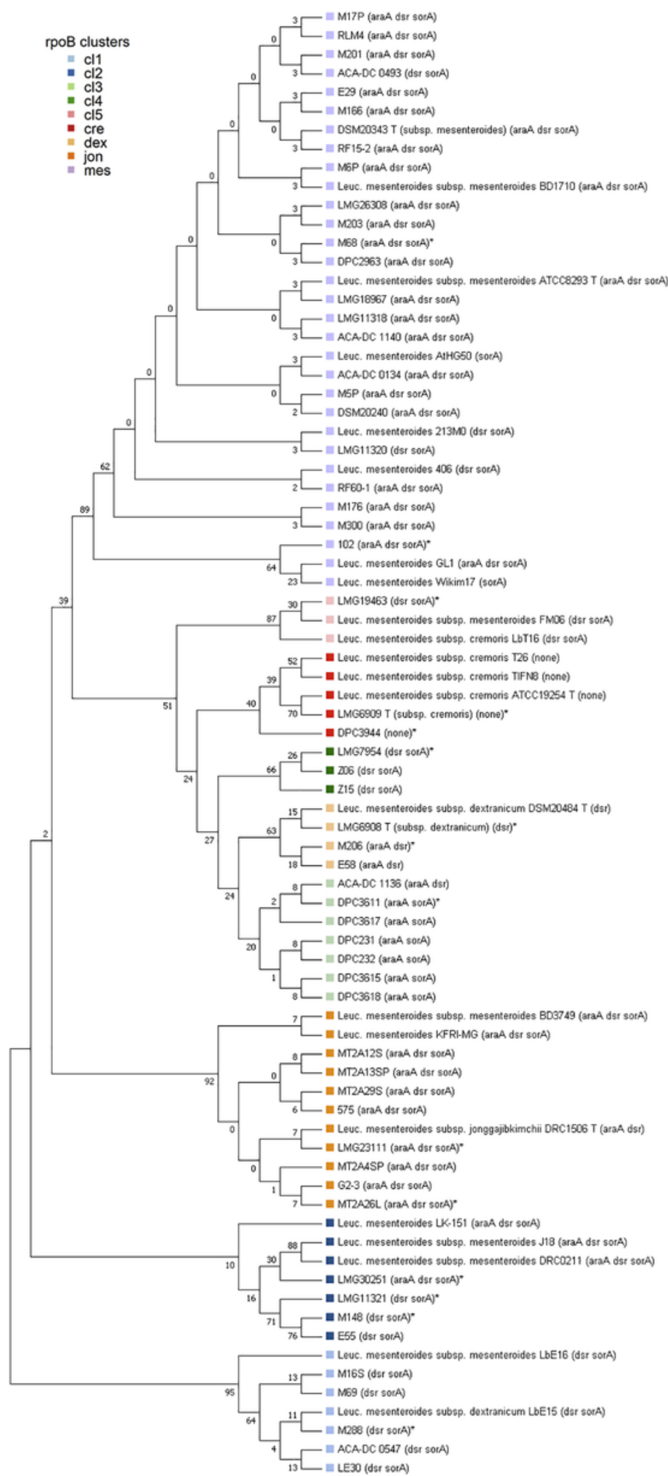

Fig. 1. Phylogenetic tree of partial *rpoB* gene sequences retrieved from the *Leuc. mesenteroides* strains (bold in Table 1) and genomes (database annotation was reported near the strain label). The presence of *araA*, *dsr* and/or *sorA* genes, verified in silico (Supplementary Fig. 2) or with Multiplex-PCR (Fig. 3) was reported in brackets. Cluster colour: cl1, light blue; cl2, blue; cl3, light green; cl4, green; cl5, pink; cre, red; dex, light orange; jon, orange; mes, purple. The colour scale indicating the membership to *rpoB*-cluster was used in Figs. 3, 4, 5. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Our results are consistent with those of Chun et al. (2017b) and Frantzen et al. (2017). The pan-genome analysis of 46 dairy-associated *Leuconostoc* spp. and 13 available *Leuconostoc* genomes (Frantzen

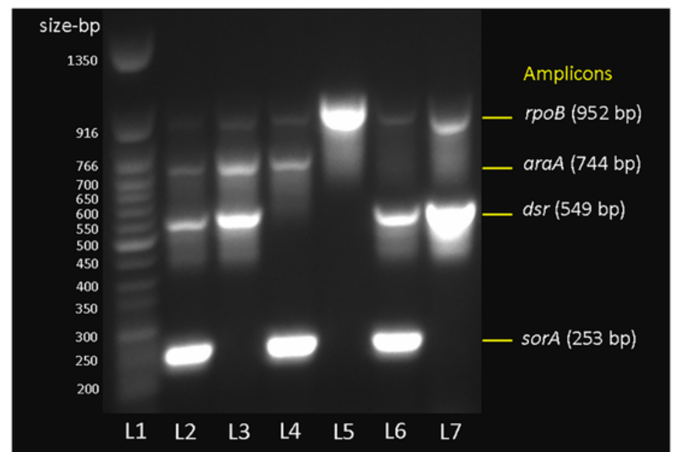

Fig. 2. Amplicon profiling obtained with Multiplex-PCR. Lane 1: 50-bp DNA Ladder (New England BioLabs Inc., Ipswich, MA, US); lane 2: subsp. *mesenteroides*; lane 3: subsp. *jonggajibkimchii*; lane 4: ambiguous profile; lane 5: subsp. *cremoris*; lanes 6: subsp. *dextranicum*; lane 7: ambiguous profile. Interpretation of Multiplex-PCR profiles referred to the gene occurrence in the four type strains (*cre*_ATCC19254^{T}, *dex*_DSM20284^{T}, *mes*_ATCC8293^{T}, *jon*_DRC1506^{T}; Supplementary Fig. 2).

et al., 2017) was unable to separate the subsp. *dextranicum* from subsp. *mesenteroides*, because of high within-subspecies variability; the subsp. *cremoris* strains were clearly discriminated because of smaller genome size and gene loss evolution due to adaptation to the dairy environment. Successively, Chun et al. (2017b), using a robust comparative approach (pan-, core-, accessory- and unique-genome, COG, ANI and *i*DDH analyses on 17 available *Leuc. mesenteroides* genomes), discriminated the subsp. *mesenteroides* from subsp. *jonggajibkimchii*, and subsp. *cremoris* from subsp. *dextranicum*. Genomic criteria (Chun et al., 2017b; Frantzen et al., 2017) confirmed the diversity of P45 (highest number of unique genes), suggesting that the strain had undergone several HGT events. Also LbT16, LbE15 and LbE16, respectively annotated as subsp. *cremoris*, subsp. *dextranicum* and subsp. *mesenteroides* (Campedelli et al., 2015), were characterised by a significant number of unique genes that could explain the ambiguous classification found in this study and before (Chun et al., 2017b; Frantzen et al., 2017).

Our data (ANI, *i*DDH, gene occurrence, *rpoB* polymorphism) confirmed that possible inaccuracies in the annotation of some *Leuc. mesenteroides* genomes may be present (as already pointed out by Chun et al., 2017b and Frantzen et al., 2017), making difficult other taxonomic comparisons. This discrepancy could be due to technical biases (e.g. choice of molecular identification technique; quality of genome sequencing and comparison processes) or to the inherent diversity of strains.

Our approach could be useful to reveal interesting aspects on the biodiversity of *Leuc. mesenteroides* strains and on the taxonomic procedures applied for species/subspecies description and naming thereof. The species-specific-PCR and Multiplex-PCR may provide a rapid identification of *Leuc. mesenteroides* strains and a reliable separation among subsp. *mesenteroides* and subsp. *cremoris* in fermented foods, and are easier than other culture-dependent methods used to date for the genetic characterisation of *Leuc. mesenteroides*. *rpoB* gene polymorphism was helpful to distinguish a significant group of strains that, despite having phenotypic traits similar to those of subsp. *mesenteroides*, had a different *rpoB*-based phylogeny, supporting the new subspecies *jonggajibkimchii*. The polymorphic region of *rpoB*, then, could be used as gene-target for a targeted-amplicon sequencing approach to detect evolution of *Leuconostoc* population in fermented foods.

Despite these goals, the identification of some subsp. *dextranicum* and subsp. *jonggajibkimchii* strains remained uncertain, and several limi-

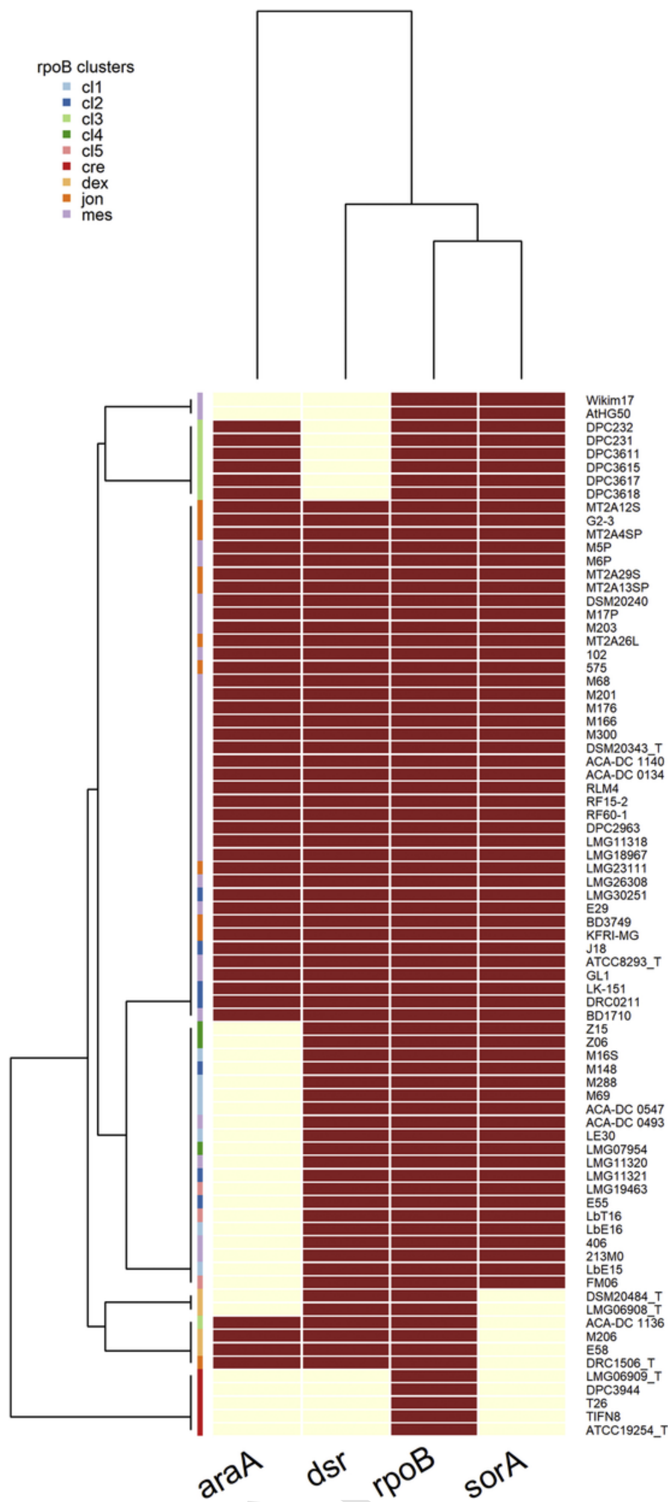

Fig. 3. Occurrence of *rpoB*, *araA*, *dsr* and *sorA* genes in *Leuc. mesenteroides* strains (bold in Table 1; Multiplex-PCR bands) and genomes (as from Supplementary Fig. 2). Heat map was created using a Jaccard similarity matrix of binary data (0, absence, yellow box; 1, presence, red box) and clustered using the UPGMA method. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

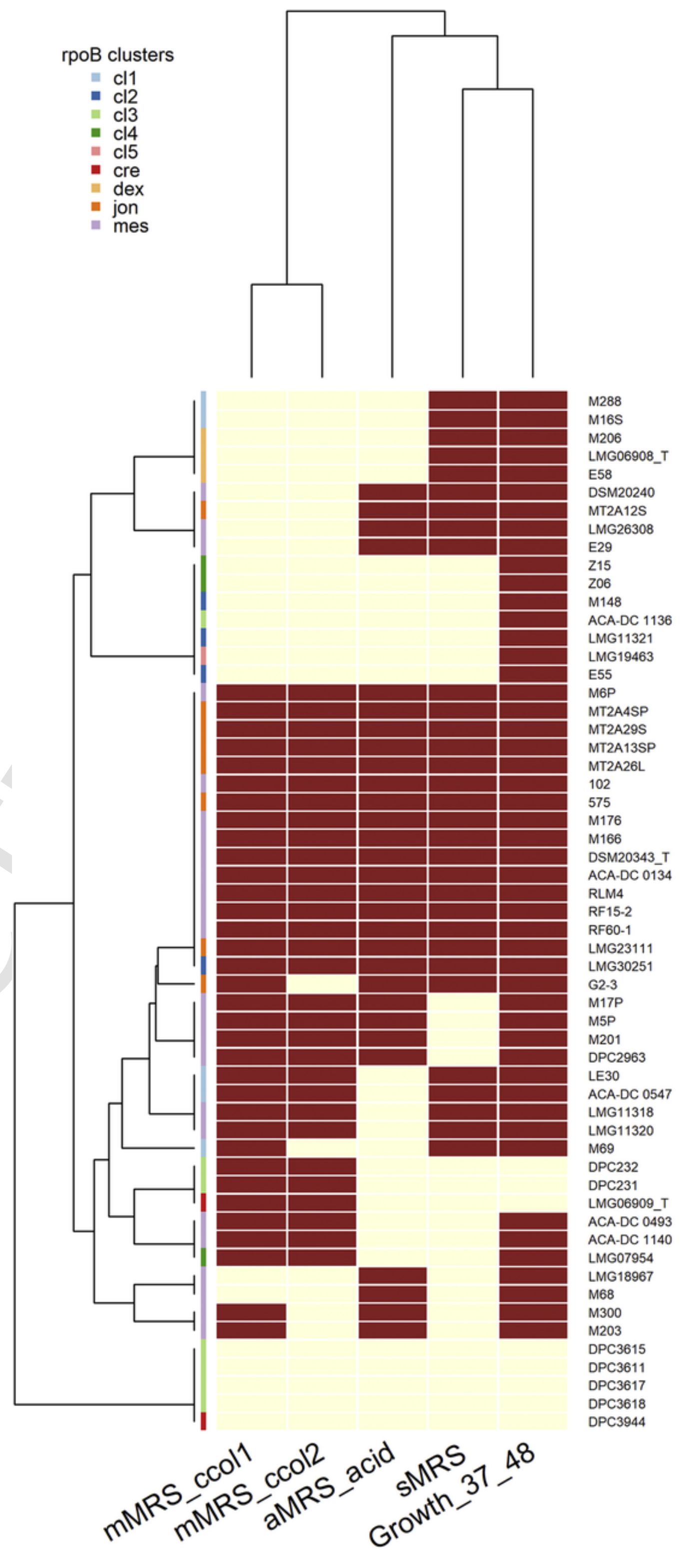

Fig. 4. Heat map of colony morphology on mMRS (col1: 0, white colony, 1, coloured colony; col2: 0, light blue; 1, blue), aMRS (acid production) and sMRS (dextran production). Dendrogram was created using Jaccard similarity matrix of binary data (0, negative, yellow box; 1, positive, red box) and UPGMA clustering method. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

tations (e.g. low number of available strains and genomes, phenotypic profile close to subsp. *mesenteroides*) made their delineation difficult. We believe that many subsp. *dextranicum* isolates could be classified as defective-subsp. *mesenteroides* strains, which lost some metabolic capa-

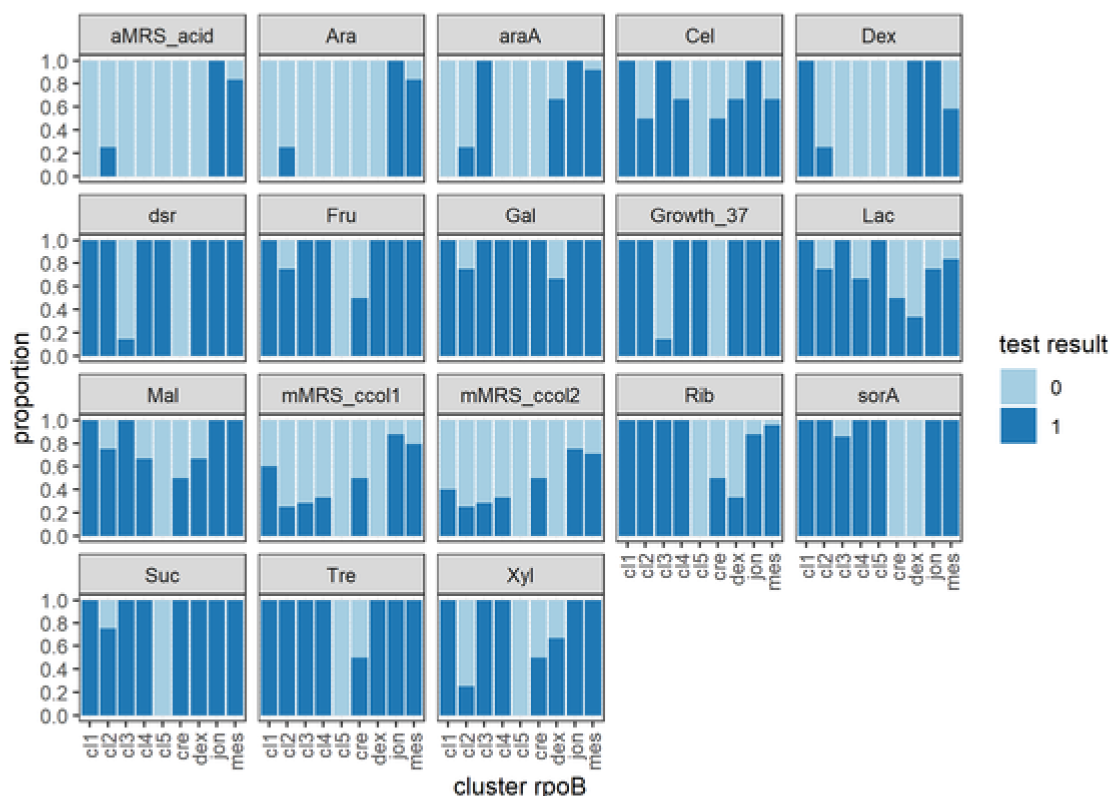

Fig. 5. Distribution of phenotypic (acid from carbohydrates, dextran production, colour colony, growth at 37 °C) and genotypic (*ara*, *dsr*, *sorA* genes) traits among the *rpoB*-based clusters (cl1, cl2, cl3, cl4, cl5, cre, dex, jon, mes).

bilities after ecological niche adaptation events. As for subsp. *jonggajibkimchii* it should be underlined that the description of a subspecies based on the genomic and phenotypic features of a single strain (Jeon et al., 2017) has limited biological meaning and further investigations (i.e. comparison of *jon*_DRC1506^T with new isolates/newly deposited genomes) are needed before asserting the presence of this new subspecies.

Therefore, we suggest that the genome-based classification of *Leuc. mesenteroides* should be supported by comparative metabolic diversity studies in order to identify molecular markers (e.g. taxonomically and functionally relevant genes) convenient for the rapid detection and discrimination of strains.

Declaration of competing interest

None.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijfoodmicro.2019.108474.

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