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Multiplex-PCR Detection of an Atypical *Leuconostoc mesenteroides* subsp. *jonggajibkimchii* Phenotype Dominating the Terminal Spoilage Microbial Association of a Fresh Greek Whey Cheese Stored at 4 °C in Vacuum

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Abstract: A species-specific multiplex-PCR method and phenotypic tests were combined to evaluate biochemical and genotypic differences between 24 representative *Leuconostoc mesenteroides* diverse isolates previously found to dominate in spoiled, vacuum-packed Anthotyros whey cheeses stored at 4 °C for 40 days and identified by 16S rRNA gene sequencing. Based on their phenotypic (API 50 CHL) profiles, the 24 isolates comprised 6 multi-strain and 7 single-strain biotypes. Only two single-strain biotypes (L4A and L4B) produced slime (dextran) from sucrose, and only four biotypes (L2A–L2C, L3; 7 isolates) fermented L-arabinose; the remaining 15 isolates (biotypes L1A–L1F) were dextran-negative, oligofermenting *Ln. mesenteroides* variants, able to ferment D-xylose and grow at 37 °C. Based on their multiplex-PCR (*rpoB*, *araA*, *dsr*, and *sorA*) gene profiles in comparison with those of the type strains of the four *Ln. mesenteroides* subsp. *cremoris* (*rpoB*), *dextranicum* (*rpoB*/*dsr*), *mesenteroides* (*rpoB*/*araA*/*dsr*/*sorA*), and *jonggajibkimchii* (*rpoB*/*araA*/*dsr*), no isolate was assigned to the first two subspecies and only four isolates (L2A and L2C) to the subsp. *mesenteroides*. Ten isolates shared the subsp. *jonggajibkimchii* profile, while the other ten ones have a fifth atypical profile (*rpoB*/*dsr*/*sorA*), seemingly being closer to the subsp. *dextranicum*. Particularly the atypical biotype L1B representatives of the most prevalent psychrotrophic *Ln. mesenteroides* subsp. *jonggajibkimchii* (*rpoB*/*araA*/*dsr*) genotype at Anthotyros whey cheese spoilage deserve further biochemical and molecular characterization studies.

Keywords: *Leuconostoc mesenteroides* subsp. *jonggajibkimchii*; subspecies identification; *rpoB*; multiplex-PCR; *araA*/*dsr*/*sorA* gene profiles; whey cheese spoilage

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1. Introduction

Traditional cheeses, yogurt, sour cream, and acidified milks are naturally preserved by indigenous starter or non-starter lactic acid bacteria (LAB) strains or consortia [1–3], which are beneficial except in rare cases where they cause bitterness, flavor defects, such as fruity or malty off flavors, or textural defects, such as unwanted gas pockets, slits or excessive blowing by CO_2 formation, and ropiness (slippery mouth-feel) by exopolysaccharides formation in cheese [4–7]. Overall, the term 'dairy spoilage LAB' remains controversial in the milk industry [8] because, in principle, souring and clotting of raw milk at ambient or cold storage temperatures is a milder and often beneficial natural-LAB fermentative spoilage process [9] compared to various offensive types of spoilage manifested when

psychrotrophic Gram-negative bacteria, mainly *Pseudomonas* spp., Gram-positive spore-forming bacteria, or yeast contaminants predominate in raw or processed milk and dairy products [10–13]. In this context, prevention of post-thermal LAB contamination and growth is a prerequisite in relatively few dairy technologies, with fresh, ready-to-eat (RTE) whey cheeses produced by heating the remaining whey after the manufacture of typical rennin-coagulated cheeses at high temperatures (>80–95 °C) and collection of the water-soluble milk protein coagulum, being the most prominent non-fermented dairy product category, preceded by the Italian-style Ricotta-type cheeses globally [14].

Fresh whey cheeses (pH > 6.0–6.8; moisture > 60–80%; salt < 1%) have a limited (<7 to max. 33 days) shelf-life when stored aerobically at 6–25 °C [15] because they are prone to post-thermal microbial contamination, with psychrotrophic *Pseudomonas* and *Enterobacteriaceae* spp., and occasionally *Bacillus* spp., being the major spoilers [14,16–18]. Vacuum and MAP are convenient (active) packaging methods to extend the shelf-life of cold-stored RTE Ricotta and other whey cheese types by shifting the microbial spoilage association in favor of autochthonous or intentionally added psychrotrophic LAB strain cultures [14,16,19,20]. Likewise, a progressive numerical dominance of fermentative LAB spoilers over *Pseudomonas*, *Aeromonas*, *Hafnia*, and *Serratia* spp. occurred during refrigerated, vacuum-packed storage of fresh Greek Anthotyros whey cheeses [21]. We reported, for the first time, that after 40 days at 4 °C (i.e., at the commercial sell-by-date of Anthotyros cheese), the terminal spoilage LAB community was dominated by autochthonous *Leuconostoc* spp. (80%), followed by *Carnobacterium maltaromaticum* (10.9%). Specifically, 95.8% of the *Leuconostoc* isolates were identified as typical and mostly atypical *Ln. mesenteroides* strains by 16S rRNA gene sequencing and basic phenotypic tests [21].

Leuconostoc mesenteroides is a taxonomically complex species currently consisting of four subspecies, namely *mesenteroides*, *dextranicum*, *cremoris*, and *jonggajibkimchii*, while *Ln. mesenteroides* subsp. *suionicum* [22] was raised to the species level by Jeon et al. [23]. The first three subspecies are commonly found in milk and dairy products [24], particularly as subdominant members of the diverse autochthonous LAB biota in traditional (raw milk) cheeses [1,3,25–29], including Greek cheeses [30–35]. They exert beneficial catabolic activities during cheese fermentation and ripening and thus are used as flavor formers in mixed dairy starter cultures [3,36]. Especially *Ln. mesenteroides* subsp. *mesenteroides* is ubiquitous and develops in plant material [37], meat [38], fish, and other food products [39,40], either as a natural or commercial starter or as a spoiler [41–43], and the type strain was isolated from fermenting olives [23]. In contrast, *Ln. mesenteroides* subsp. *cremoris* is an exclusive dairy LAB [36,39], with its type strain originating from a Hansen's dried cheese starter powder [23]. Of note, the new subspecies *jonggajibkimchii*, originating from traditional Korean kimchi [23], has started being detected as a subdominant, but important, LAB in dairy foods, such as traditional 'Torta' cheeses from Spain [27] and brine cheeses from Montenegro [29], naturally fermented cow and yakmilk products from India [44], and raw sheep milk from native breeds in Greece [45]. The aforementioned subspecies of *Ln. mesenteroides* are highly intermixed phenotypically and thus cannot be differentiated by biochemical criteria, except for the oligofermenting *Ln. mesenteroides* subsp. *cremoris* industrial starter (type) strain/s [39]. However, neither the atypical *Ln. mesenteroides* isolates from spoiled Anthotyros cheese shared the typical *Ln. mesenteroides* subsp. *cremoris* phenotype nor their 16S rRNA gene profiling by the Sanger sequencing method provided an accurate subspecies identification for any of them [21]. Hence, this research represents an advanced follow-up study aiming to classify our atypical whey cheese spoilage *Ln. mesenteroides* isolates at the subspecies level based on the multiplex-PCR (*rpoB*, *araA*, *dsr*, and *sorA*) method of Ricciardi et al. [46], as it was recently applied for the first detection and genotypic characterization of two *Ln. mesenteroides* subsp. *jonggajibkimchii* isolates from refrigerated raw bulk Epirus sheep milk [46].

2. Materials and Methods

2.1. Whey Cheese Spoilage Isolates and Culture Conditions

Twenty-six *Leuconostoc* spp. isolates from two terminally spoiled Anthotyros batches (C and D) stored at 4 °C without (CN) or with (Ent+) a crude enterocin A-B-P-containing extract added to the fresh whey cheese samples before vacuum packaging [21] were studied (Table 1): 24 isolates were diverse representative strains of the complex species *Ln. mesenteroides*, differentiated into four main biotypes, L1 to L4, on the basis of five key phenotypic traits [21], while two isolates represented a variant biotype L5, identified as *Ln. lactis* (Table 1). Species identifications were based on 16S rRNA gene sequencing of 13 representative isolates [21], indicated in bold in Table 1. Four variable pairs of *Ln. mesenteroides* isolates (WM109A/B; WM110A/B; WM122A/B; and WM125A/B) obtained after re-purification of the original stock cultures were among the selected isolates to elucidate whether their colony size variability following growth on streaked agar plates was due to polymorphism of a pure strain culture or due to the presence of contaminated (mixed) strain cultures.

Table 1. Representative *Leuconostoc (Ln.)* spp. isolates from two spoiled batches (C, D) of fresh, untreated (CN) or enterocin (A-B-P)-treated (Ent+), vacuum-packaged, cold-stored (4 °C) Anthotyros whey cheeses used in this study [1].

Group/ Isolate Biotype	Cheese Batch/ Treatment	Isolate Code [2]	Species Identification	Closest Ref. Strain in BLAST	16S rRNA Gene Seq Similarity
L1	C/CN	**WM106**	*Ln. mesenteroides*	MT545072.1	100
	C/CN	WM109A	*Ln. mesenteroides*	NT	-
	C/CN	**WM109B**	*Ln. mesenteroides*	MT545072.1	100
	C/Ent+	WM117	*Ln. mesenteroides*	NT	-
	C/Ent+	WM119	*Ln. mesenteroides*	NT	-
	C/Ent+	**WM123**	*Ln. mesenteroides*	MT545101.1	100
	C/Ent+	WM124	*Ln. mesenteroides*	NT	-
	C/Ent+	WM125A	*Ln. mesenteroides*	NT	-
	C/Ent+	WM125B	*Ln. mesenteroides*	NT	-
	D/CN	**WM136**	*Ln. mesenteroides*	MT545072.1	100
	D/CN	**WM137**	*Ln. mesenteroides*	MT545072.1	100
	D/CN	WM138	*Ln. mesenteroides*	NT	-
	D/Ent+	WM147	*Ln. mesenteroides*	NT	-
	D/Ent+	WM151	*Ln. mesenteroides*	NT	-
	D/Ent+	**WM153**	*Ln. mesenteroides*	MT545072.1	100
L2	C/CN	WM103	*Ln. mesenteroides*	NT	-
	C/CN	**WM105**	*Ln. mesenteroides*	MT545072.1	100
	C/CN	**WM110A**	*Ln. mesenteroides*	MT545113.1	100
	C/CN	WM110B	*Ln. mesenteroides*	NT	-
	C/Ent+	**WM122A**	*Ln. mesenteroides*	MT545072.1	100
	C/Ent+	WM122B	*Ln. mesenteroides*	NT	-
L3	C/Ent+	**WM121**	*Ln. mesenteroides*	MT545113.1	100
L4	C/CN	**WM107**	*Ln. mesenteroides*	MT545113.1	100
	C/CN	**WM108**	*Ln. mesenteroides*	MT545072.1	100
L5	C/Ent+	**WM118**	*Ln. lactis*	MF354765.1	100
	C/Ent+	WM129	*Ln. lactis*	NT	-

[1] All data in Table 1 are adapted from Sameli et al. [21]. Grouping of the *Leuconostoc* spp. isolates in five main biotypes, L1 to L5, based on their reactions to five key phenotypic traits, namely slime formation and fermentation of L-arabinose, raffinose, trehalose, and D-xylose, is presented in Table 3 of the Sameli et al. [21] study. [2] The representative LAB isolates identified by 16S rRNA gene sequencing by Sameli et al. [21] are listed with their code numbers written in bold. NT, not tested.

All isolates were resuscitated from their frozen (−30 °C) stock state in 20% (*w*/*v*) glycerol [21] by subculturing them in 5 mL portions of de Man Rogosa Sharpe (MRS) broth (Neogen Culture Media; formerly Lab M, Heywood, UK) at 30 °C. Following growth, all isolates were streaked on MRS agar (Neogen) plates for 72 h, and one single colony from each isolate was transferred for growth in 10 mL MRS broth, as above, to ensure culture purity. Then, all fresh cultures were activated by two sequent transfers of 100 μLin 10 mL of MRS broth, incubated at 30 °C for 24 h, before use in the experiments.

2.2. Biochemical Differentiation of the Leuconostoc spp. Isolates

All representative isolates (Table 1) were retested for their Gram-positive and catalase-negative reactions, as well as for colony appearance, cell morphology, growth at 4, 10, 37, and 45 °C in MRS broth, gas (CO_2) production from glucose, ammonia (NH_3) production from arginine, slime formation from sucrose, and the fermentation of 13 basic (key) sugars, purchased from Merck (Darmstadt, Germany) or Fluka (Sigma Aldrich Chemie GmbH, Steinheim, Germany) in pre-sterilized 96-well miniplates, as described by Sameli et al. [21]. Retesting was necessary to confirm the genus- and species-specific phenotypic traits of the selected psychrotrophic *Ln. mesenteroides* and *Ln. lactis* isolates. All phenotypic tests were performed twice. Lastly, to improve the biochemical discrimination of the *Leuconostoc* spp. isolates, the entire sugar fermentation profile of each different strain biotype was determined by the API 50 CHL identification kit (BioMerieux, Marcy l' Etoile, Lyon, France), according to the manufacturer's instructions.

2.3. Differentiation and Identification of the Leuconostoc spp. Isolates by Multiplex-PCR

Actively growing cultures of the selected isolates were inoculated into MRS broth and incubated at 30 °C for 24 h. The cell biomass contained in two 1.5 mL portions from each 24h culture was collected by centrifugation (12,000 rpm, 10 min, 4 °C) and washed twice with sterile saline. DNA extraction was performed by modifying the method of Querol et al. [47] according to the analytical procedure detailed by Tsafrakidou et al. [48]. The final clean pellet containing the DNA of each isolate was suspended in 100 μL TE buffer (pH 8.0, 50 mM Tris-HCl, 20 mM EDTA) and kept at −20 °C until the analysis.

The multiplex-PCR method of Ricciardi et al. [46] was used, as described by Sioziou et al. [45]. Three reference *Ln. mesenteroides* strains isolated from traditional Greek cheeses, *Ln. mesenteroides* subsp. *dextranicum* ACA-DC 0231, *Ln. mesenteroides* subsp. *dextranicum* ACA-DC 0493, and *Ln. mesenteroides* subsp. *mesenteroides* ACA-DC 0750, kindly provided by Professor E. Tsakalidou, Laboratory of Dairy Research, Agricultural University of Athens, Greece, were used as positive controls in the multiplex-PCR assays, according to Sioziou et al. [45]. Moreover, to demonstrate the species specificity of this method within the genus *Leuconostoc*, both *Ln. lactis* isolates, WM118 and WM129 (Table 1) were used as negative (i.e., outer species within the same LAB genus) controls.

Briefly, fresh MRS broth cultures (30 °C, 24 h) of the three reference *Ln. mesenteroides* strains were used for DNA extraction, as described above; moreover, the DNA extracts of the selected *Leuconostoc* isolates (Table 1) previously used for the 16S rRNA gene sequencing analysis [21] were subsequently used for the multiplex-PCR assay (presence/absence of the genes: beta subunit of RNA polymerase, *rpoB*; L-arabinose isomerase, *araA*; dextransucrase, *dsr*; PTS-sorbose transporter subunit IIC, *sorA*) [45,46]. The primers for detecting each of the four genes are listed in Table 2. Specifically, PCRamplifications were performed using 25 ng of total bacterial DNA, 1 μM of *rpoB* primers, 0.5 μM of *araA* primers, 0.3 μM of *dsr* primers, and 0.1 μM of *sorA* primers (Table 2) in 25 μL reaction mixtures using the Kapa Taq PCR kit (Kapa Biosystems), according to the manufacturer's instructions. PCR was performed in the DNA Engine Peltier Thermal Cycler (BioRad) using the following steps: 5 min at 95 °C, 30 cycles of denaturation at 95 °C for 30 s, annealing at 60 °C for 60 s, extension at 72 °C for 90 s, and a final extension of 10 min at 72 °C. PCR products were separated in 1.2% agarose gel stained with ethidium bromide [45].

Table 2. List of primers used for the multiplex-PCR in this study.

Gene	Primer	Sequence (5′-3′)	Amplicon Size (bp)	Annealing Temperature (°C)	Reference
rpoB	rpob-F rpob-R	GTCCGCATTGATCGCACGC CACCCGGTCCAAGAGCTGAC	952	60	Ricciardi et al. [46]
araA	L-ara-F L-ara-R	TTTGGCTGGACGGTTGACT TGTTGTGTGATGTCCGCCAC	744		
dsr	dextran-F dextran-R	TGGCACCATTACCATAACGAACT TGCCAGCAGTCGATCAATATGG	549		
sorA	PTS-sorb-F PTS-sorb-R	GTGCCTTACTCCCCTGTGTAG TCCTCGTCTTCCTCATCATCGT	253		

3. Results

3.1. Biotyping of the Whey Cheese Spoilage Leuconostoc spp. Isolates

All 26 isolates (Table 1) were confirmed to be obligatory heterofermentative, arginine-negative LAB cocci or coccobacilli, able to promote weak visible growth in MRS broth after 10 days at 4 °C, and good to excellent growth after 2 to 7 days at 10 °C or after 16 to 48 h at 37 °C, respectively, while none grew at 45 °C. Additionally, as shown in Figure 1, all *Leuconostoc* spp. isolates shared the following important biotechnological traits: (i) in addition to the glucose fermentation in MRS broth, they fermented lactose and galactose rapidly (i.e., at ca. 6 h of incubation at 30 °C in miniplates), reflecting their high affinity for growth in milk and (fresh whey) cheese; (ii) also, while all isolates fermented sucrose, only two of them, WM107 and WM108, produced slime from sucrose, i.e., the primary (key) phenotypic trait for their assignment to biotype L4 (Figure 1) originally defined to include all *Ln. mesenteroides* isolates from the spoiled Anthotyros cheese products that were slime (dextran)producers [21].

In addition to that, the two major slime (dextran)-negative *Ln. mesenteroides* biotypes, L1 (L-arabinose- and raffinose-negative) and L2 (L-arabinose- and raffinose-positive), were split into six (L1A to L1F) and three (L2A to L2C) strain biotypes (Figure 1). Of note, both isolates of two *Ln. mesenteroides* pairs, WM110A/B and WM125A/B, were assigned to the same biotype, L2B or L1E, respectively, whereas each of the remaining two pairs, WM109A/B and WM122A/B, included diverse isolates assigned to the separate biotypes L1B or L1D and L2A or L2C, respectively. Additional constant traits were the ability of all *Ln. mesenteroides* biotype L1, L2, and L3 isolates to ferment trehalose and D-xylose, but not cellobiose and sorbitol; maltose, mannitol, melibiose, and ribose were fermented variably (Figure 1).

Solely based on the key phenotypic taxonomic criteria for the differentiation of the oligofermenting *Ln. mesenteroides* subsp. *cremoris* from the subsp. *dextranicum* and *mesenteroides* [39,49], it was noteworthy that the most prevalent, slime (dextran)-negative, L-arabinose-negative isolates in biotypes L1A to L1F, as well as the two most atypical, slime (dextran)-positive but L-arabinose-negative isolates in biotypes L4A and L4B, fermented fewer basic sugars compared to the also atypical, L-arabinose-positive but slime (dextran)-negative, biotypes L2A to L2C and L3 (Figure 1). Clearly, for taxonomic and biotechnological reasons discussed in later paragraphs, the most interesting *Ln. mesenteroides* biotype was L1B due to its high prevalence in the Anthotyros batches C and D at spoilage [21] and because all L1B isolates failed to ferment D-maltose despite being able to ferment D-xylose strongly (Figure 1). Lastly, the isolates WM118 and WM129 comprised a constant biotype L5, typical of *Ln. lactis*, which differed from the *Ln. mesenteroides* biotypes L1A to L4B in failing to ferment trehalose (Figure 1).

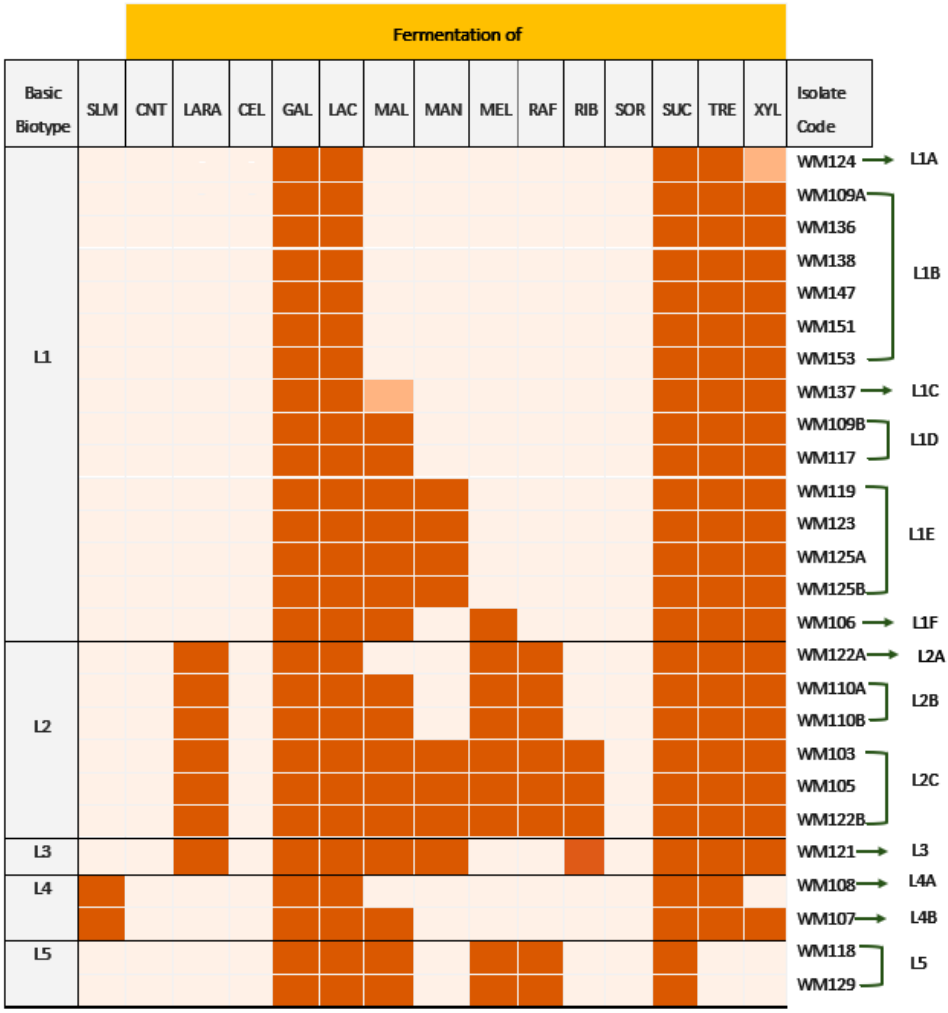

Figure 1. Basic sugar fermentation patterns and dextran-producing ability (slime; SLM) of 24 *Leuconostoc mesenteroides* (biotypes L1A to L4B) and 2 *Leuconostoc lactis* (biotype L5) isolates, obtained from terminally spoiled Greek Anthotyros whey cheeses. Red-brown boxes indicate acid production from L-arabinose (LARA), cellobiose (CEL), galactose (GAL), lactose (LAC), maltose (MAL), mannitol (MAN), melibiose (MEL), raffinose (RAF), ribose (RIB), sorbitol (SOR), sucrose (SUC), trehalose (TRE), and xylose (XYL). Light-pink boxes indicate a weak positive fermentation reaction. CNT, no acid production in MRS broth base without sugar. Uncoloured boxes, negative reaction.

To advance biotyping at strain level, 14 *Ln. mesenteroides* isolates were tested using the API 50 CHL method (Table 3). Their entire fermentation profiles, which are sorted vice versa in Table 3 (i.e., from the isolates with the richest to those with the poorest profiles), indicated that all six isolates of the homogeneous oligofermenting biotype L1B differed further from the isolates in biotypes L2C (WM105), L3 (WM121), L1E (WM119), L1F (WM106), L1D (WM117), and L4B (WM107), which formed a quite heterogeneous *Ln. mesenteroides* group of strains, in failing to ferment α-methyl-D-glucopyranoside (MDG), esculin, and turanose. The maltose-positive isolate WM137 (L1C), which also failed to ferment the above three sugars, was an intermediate strain, seemingly closer to the L1B isolates. Of note, the most oligofermenting strain, WM124 (L1A), failed to ferment D-xylose by the API method, and thus, it was moved together with strain WM108 (L4A). Lastly, we confirmed that the two trehalose-negative *Ln. lactis* isolates had an identical API 50 CHL profile, which differed from the profiles of all *Ln. mesenteroides* biotypes (Table 3). Therefore, to resolve the very high intra-species phenotypic heterogeneity of the whey

cheese spoilage *Ln. mesenteroides* isolates (Figure 1, Table 3), it was necessary to determine the subspecies-specific gene profiles by multiplex-PCR.

3.2. Classification of the Whey Cheese Spoilage Ln. mesenteroides Isolates by Multiplex-PCR—Prevalence of Isolates with a Gene Profile Specific to Ln. mesenteroides subsp. jonggajibkimchii

The results of the multiplex-PCR analysis for the presence of *rpoB*, *araA*, *dsr*, and *sorA* in the genome of the 24 *Ln. mesenteroides* isolates, defined as profiles S1 to S5, are shown in Table 4 in comparison with the respective gene profiles of the type strains of the four subspecies, *cremoris*, *dextranicum*, *jonggajibkimchii*, and *mesenteroides*, adapted from the decision tree of the Ricciardi et al. [46] study, the three reference *Ln. mesenteroides* strains, ACA-DC 0750, ACA-DC 0493, and ACA-DC 0231 [45], and the two *Ln. lactis* control (outer species) isolates sorted last in Table 4. The PCR gene band profiles of 13/26 *Leuconostoc* spp. isolates, including *Ln. lactis* WM118, are illustrated in Figure 2; the gene band profiles of the ACA-DC strains were previously illustrated by Sioziou et al. [45].

The multiplex-PCR results confirmed that all *Ln. mesenteroides* whey cheese isolates (Table 1), as well as the three ACA-DC strains, possessed the *rpoB* gene (Table 4), which is species-specific [46]. Indeed, *rpoB* was not detected in the genome of *Ln. lactis* WM118 and WM129, which displayed blank (noband) profiles (Table 4, Figure 2), confirming the species specificity of the multiplex-PCR method. In addition to that, as we anticipated by relying on the biotyping data (Figure 1; Table 3), none of our isolates was assignable to *Ln. mesenteroides* subsp. *cremoris*, whose type strain possesses the *rpoB* gene but lacks the *araA*, *dsr*, and *sorA* genes (multiplex-PCR profile S1) from its genome (Table 4).

Unexpectedly, neither the S2 (*rpoB*/*dsr*) gene profile of the *Ln. mesenteroides* subsp. *dextranicum* type strain orthe reference ACA-DC 0493 strain was detected in any of the 24 *Ln. mesenteroides* isolates by the multiplex-PCR method, which distinguished them into two major (S3 and S5) and one minor (S4) geneprofile group, including 10, 10, and 4 whey cheese isolates, respectively (Table 4). Among them, only the minor group S4 possessed the complete gene (*rpoB*/*araA*/*dsr*/*sorA*) profile of typical *Ln. mesenteroides* subsp. *mesenteroides* strains [46], including the ACA-DC 0750 reference strain (Table 4). Unsurprisingly, the multi-fermenting isolates WM103, WM105, and WM122B in biotype L2C possessed the S4 gene profile of the subspecies *mesenteroides*. The isolate WM122A, assigned singly to the biotype L2A, belonged to the subspecies *mesenteroides*, too (Figure 2). Conversely, altogether ten phenotypically intermixed isolates, representing variable oligofermenting strain biotypes (L1A, L1D, L1E, L1F, L3, and L4B) being unable to ferment L-arabinose and/or D-raffinose (i.e., unlike the basic biotype L2) (Figure 1; Table 3), shared the S5 gene profile (*rpoB*/*dsr*/*sorA*) (Table 4; Figure 2). Thus, they were atypical 'intermediate' strains of the subspecies *mesenteroides* and *dextranicum* [45], including the reference strain *Ln. mesenteroides* subsp. *dextranicum* ACA-DC 0231 (Table 4).

However, the most prominent finding was that all *Ln. mesenteroides* isolates encompassing the most prevalent, oligofermenting biotype L1B and the unique, dextran-forming D-xylose-negative WM108 (L4A) isolate (Figure 1; Table 3) shared the S3 (*rpoB*/*araA*/*dsr*) gene profile that is possessed by the *Ln. mesenteroides* subsp. *jonggajibkimchii* type strain (Table 4; Figure 2). The S3 profile was possessed by an additional three atypical *Ln. mesenteroides* isolates: (i) the single-strain WM137 being very similar (L1C) with the L1B isolates; and (ii) WM110A and WM110B, an isolate pair that was assigned singly (L2B) with respect to its phenotype, indicating it was another unique *Ln. mesenteroides* strain, too. The fact that this particular L-arabinose-positive, multi-fermenting strain WM110A/B shared the S3 (*rpoB*/*araA*/*dsr*) *jonggajibkimchii* profile with the L-arabinose-negative, oligofermenting L1B isolates (Table 4; Figure 2) was in sharp contrast to the high phenotypic resemblance of its biotype L2B with the typical *Ln. mesenteroides* subsp. *mesenteroides* L2C isolates (Figure 1; WM105 in Table 3).

Table 3. Comparison of the entire (API 50 CHL-based) sugar fermentation profiles of the most prevalent strain biotypes of *Leuconostoc mesenteroides* and *Leuconostoc lactis* isolated from two batches of terminally spoiled Anthotyros whey cheese after storage at 4 °C for 40 days [1].

Species Identification/ Isolate Code	Strain Biotype	LAra 4	Rib 5	DXyl 6	Gal 10	Glu 11	Fru 12	Mne 13	Man 18	MDG 21	NAG 22	Arb 24	Esc 25	Sal 26	Mal 28	Lac 29	Mel 30	Sac 31	Tre 32	Raf 35	Tur 40
Ln. mesenteroides																					
WM105	L2C	+	+	+	+	+	+	+	(+)d	+	+	(+)	+	+	+	+	+	+	+	+	+
WM121	L3	+	+	+	+	+	+	+	(+)d	+	+	(+)	+	+	+	+	-	+	+	-	+
WM119	L1E	-	-	+	+	+	+	+	(+)d	+	+	(+)	(+)	+	+	+	-	+	+	-	+
WM106	L1F	-	-	+	+	+	+	+	-	+	+	-	(+)	+	+	+	+	+	+	-	+
WM107	L4B	-	-	+	+	+	+	+	-	+	+	-	(+)	-	+	+	-	+	+	-	+
WM117	L1D	-	-	+	+	+	+	+	-	+	+	-	(+)	-	+	+	-	+	+	-	+
WM137	L1C	-	-	+	+	+	+	+	-	-	+	-	-	-	+	+	-	+	+	-	-
WM136	L1B	-	-	+	+	+	+	+	-	-	+	-	-	-	-	+	-	+	+	-	-
WM138	L1B	-	-	+	+	+	+	+	-	-	+	-	-	-	-	+	-	+	+	-	-
WM147	L1B	-	-	+	+	+	+	+	-	-	+	-	-	-	-	+	-	+	+	-	-
WM151	L1B	-	-	+	+	+	+	+	-	-	+	-	-	-	-	+	-	+	+	-	-
WM153	L1B	-	-	+	+	+	+	+	-	-	+	-	-	-	-	+	-	+	+	-	-
WM108	L4A	-	-	-	+	+	+	+	-	-	+	-	-	-	-	+	-	+	+	-	-
WM124	L1A	-	-	-	+	+	+	+	-	-	+	-	-	-	-	+	-	+	+	-	-
Ln. lactis																					
WM118	L5	-	-	-	+	+	+	+	-	-	+	-	-	-	+	+	+	+	-	+	-
WM129	L5	-	-	-	+	+	+	+	-	-	+	-	-	-	+	+	+	+	-	+	-

[1] Sugars are tabulated from left to right according to their abbreviated code names and their numerical order (1 to 49) followed in the API-50 identification strips; only the positive or weakpositive [(+)/(+)d sugar] fermentation reactions for at least one of the tested strains are profiled to allow comparison of the diverse strain biotypes; MDG, α-Methyl-D-Gluconopyranoside; NAG, N-AcetylGlucosamine. Additionally, all strains gave negative reactions with the following sugars: glycerol (1), erythritol (2), D-arabinose (3), L-xylose (7), adonitol (8), β-Methyl-D-Xylopyranoside (9), sorbose (14), rhamnose (15), dulcitol (16), inositol (17), sorbitol (19), α-Methyl-D-Mannopyranoside (20), amygdalin (23), cellobiose (27), inulin (33), melezitose (34), amidon (36), glycogen (37), xylitol (38), gentibiose (39), lyxose (41), tagatose (42), D-fucose (43), L-fucose (44), D-arbitol (45), L-arbitol (46), gluconate (47), 2-keto-gluconate (48), and 5-keto-gluconate (49). +, positive reaction; (+), weak positive reaction; -, negative reaction.

Table 4. Multiplex-PCR profiles of the 24 representative *Leuconostoc* (*Ln.*) *mesenteroides* isolates from two spoiled Anthotyros whey cheese batches relative to the presence of the *rpoB*, *araA*, *dsr*, and *sorA* in their genome in comparison with the respective profiles of the type strains of the four *Ln. mesenteroides* subspecies and three reference strains from traditional Greek cheeses and correlation with the strain biotype assignment of each isolate [1].

Species Identification	Strain Code	Target Genes Detected by Multiplex-PCR				Subspecies Identification	Reference	Multiplex Profile	Basic Biotype	Updated Biotype
Reference strains (literature/our previous data)		*rpoB*	*araA*	*dsr*	*sorA*					
Ln. mesenteroides	ATCC 19254^T	+	-	-	-	*cremoris*	[46]	S1	NA	NA
Ln. mesenteroides	DSM 20484^T	+	-	+	-	*dextranicum*	[46]	S2	NA	NA
Ln. mesenteroides	DRC1506^T	+	+	+	-	*jonggajibkimchii*	[46]	S3	NA	NA
Ln. mesenteroides	ATCC 8293^T	+	+	+	+	*mesenteroides*	[46]	S4	NA	NA
Ln. mesenteroides	ACA-DC 0750	+	+	+	+	*mesenteroides*	[45]	S4	NA	NA
Ln. mesenteroides	ACA-DC 0493	+	-	+	-	*dextranicum*	[45]	S2	NA	NA
Ln. mesenteroides	ACA-DC 0231	+	-	+	+	*dextranicum* (atypical)	[45]	S5	NA	NA
Anthotyros cheese strains							This study			
Ln. mesenteroides	WM124	+	-	+	+	*dextranicum* (atypical)		S5	L1	L1A
Ln. mesenteroides	WM109A	+	+	+	-	*jonggajibkimchii*		S3	L1	L1B
Ln. mesenteroides	WM136	+	+	+	-	*jonggajibkimchii*		S3	L1	L1B
Ln. mesenteroides	WM138	+	+	+	-	*jonggajibkimchii*		S3	L1	L1B
Ln. mesenteroides	WM147	+	+	+	-	*jonggajibkimchii*		S3	L1	L1B
Ln. mesenteroides	WM151	+	+	+	-	*jonggajibkimchii*		S3	L1	L1B
Ln. mesenteroides	WM153	+	+	+	-	*jonggajibkimchii*		S3	L1	L1B
Ln. mesenteroides	WM137	+	+	+	-	*jonggajibkimchii*		S3	L1	L1C
Ln. mesenteroides	WM109B	+	-	+	+	*dextranicum* (atypical)		S5	L1	L1D
Ln. mesenteroides	WM117	+	-	+	+	*dextranicum* (atypical)		S5	L1	L1D
Ln. mesenteroides	WM119	+	-	+	+	*dextranicum* (atypical)		S5	L1	L1E
Ln. mesenteroides	WM123	+	-	+	+	*dextranicum* (atypical)		S5	L1	L1E
Ln. mesenteroides	WM125A	+	-	+	+	*dextranicum* (atypical)		S5	L1	L1E
Ln. mesenteroides	WM125B	+	-	+	+	*dextranicum* (atypical)		S5	L1	L1E
Ln. mesenteroides	WM106	+	-	+	+	*dextranicum* (atypical)		S5	L1	L1F
Ln. mesenteroides	WM122A	+	+	+	+	*mesenteroides*		S4	L2	L2A
Ln. mesenteroides	WM110A	+	+	+	-	*jonggajibkimchii*		S3	L2	L2B
Ln. mesenteroides	WM110B	+	+	+	-	*jonggajibkimchii*		S3	L2	L2B
Ln. mesenteroides	WM103	+	+	+	+	*mesenteroides*		S4	L2	L2C
Ln. mesenteroides	WM105	+	+	+	+	*mesenteroides*		S4	L2	L2C

Table 4. *Cont.*

Species Identification	Strain Code	Target Genes Detected by Multiplex-PCR				Subspecies Identification	Reference	Multiplex Profile	Basic Biotype	Updated Biotype
Ln. mesenteroides	WM122B	+	+	+	+	*mesenteroides*		S4	L2	L2C
Ln. mesenteroides	WM121	+	-	+	+	*dextranicum* (atypical)		S5	L3	L3
Ln. mesenteroides	WM108	+	+	+	-	*jonggajibkimchii*		S3	L4	L4A
Ln. mesenteroides	WM107	+	-	+	+	*dextranicum* (atypical)		S5	L4	L4B
Ln. lactis	WM118	-	-	-	-	None (N/A)		No bands	L5	L5
Ln. lactis	WM129	-	-	-	-	None (N/A)		No bands	L5	L5

[1] The *Ln. lactis* isolates coded WM118 and WM129 were used as negative (outer species) controls in the multiplex-PCR analysis. NA, not analyzed in the course of this study. +, presence of the target gene in the strain's genome; -, absence of the target gene in the strain's genome.

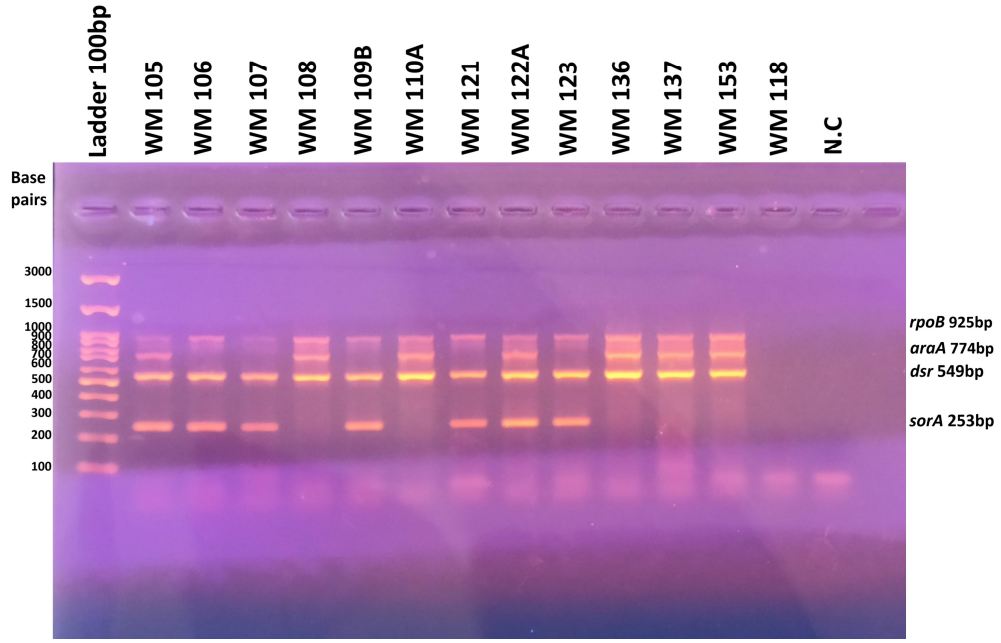

Figure 2. Multiplex-PCR profiles of 12 *Leuconostoc mesenteroides* (WM) isolates from terminally spoiled Greek Anthotyros whey cheeses. Lane 1: Nippon Genetics ready-to-use DNA ladder, 100 to 3000 bp fragments; Lanes 2–13: *Ln. mesenteroides* WM isolates; Lane 14: *Leuconostoc lactis* WM118 (negative control strain); L15: negative control (no bacterial DNA). At 925 bp is the *rpoB* gene band; at 774 bp is the *araA* gene band; at 549 bp is the *dsr* gene band; at 253 bp is the *sorA* gene band.

4. Discussion

Apart from being phenotypically intermixed, the first three food (dairy)-associated subspecies of *Ln. mesenteroides*, namely *cremoris*, *dextranicum*, and *mesenteroides* (i.e., defined before the introduction of molecular typing methods) [50], share very high genotypic relatedness and interrelationships, which cause difficulties in differentiating between them [36,51]. Additionally, it is well documented that the subspecies *dextranicum* and *mesenteroides* encompass atypical slime (dextran)-negative strains that display variable sugar fermentation patterns [46]; based on their phenotype/s, such *Ln. mesenteroides* variants can easily be misclassified with the closelyrelated *Ln. lactis* and *Ln. pseudomesenteroides* or even as *Weissella paramesenteroides* [52–54]. Generally, the species and mainly the subspecies delineation of newly isolated, autochthonous (beneficial or spoilage) *Ln. mesenteroides* strains from traditional dairy, meat, or plant foods is a challenge [24,37,54], including the newest fourth subspecies, *jonggajibkimchii*, which is phenotypically and genomically intermixed, too [23,43]. Therefore, over time, various molecular identification and typing techniques, such as 16S rRNA gene sequencing, RAPD-PCR, rep-PCR, multiplex-PCR, species-specific PCR, 16S PCR-RFLP, PFGE, ARDRA, MLST, and partial sequencing of housekeeping genes [22,25,26,33,36,37,53,55–59] and, more recently, MALDI-TOF MS profiling [27,33,60], have been proposed to differentiate *Ln. mesenteroides* and its subspecies. While all the above techniques have shown success in distinguishing the type/reference or native strains tested at the species level (i.e., from *Ln. pseudomesenteroides*, *Ln. lactis*, and *Ln. citreum*), most of them, including 16S rDNA sequencing, have been insufficient to classify *Ln. mesenteroides* at the subspecies level, despite the valid strain profiling differentiations provided [36,53,57,60]. Recent studies have demonstrated that more powerful WGS analyses provide robust support [23,43,51]; however, these approaches cannot be applied routinely in food laboratories due to the specific expertise and tools required [46]. Conversely, the faster and readily applicable multiplex-PCR approach used in this study was successful in differentiating all representative *Ln. mesenteroides* isolates from the two *Ln. lactis* (outer species) isolates, in alignment with their preceding basic phenotypic

characterization and 16S rRNA gene identification [21]. Additionally, a highly constant genotypic differentiation of the 24 biochemically diverse *Ln. mesenteroides* isolates in three distinct multiplex-PCR profiles, S3 (41.7% of the isolates), S4 (16.6%), and S5 (41.7%), was achieved: the atypical S3 and S4 isolates were more homogeneous biochemically and matched the subspecies *jonggajibkimchii* and *mesenteroides* type strains, respectively, whereas the S5 isolates represented an intermixed group of several atypical strain biotypes seemingly closer to the subspecies *dextranicum* (Table 4). The fact that none of the autochthonous whey cheese spoilage *Ln. mesenteroides* isolates possessed the *cremoris* S1 profile, deficient of the *araA*, *dsr*, and *sorA* genes (Table 4), was an important confirmatory finding, particularly with regard to the six slime-negative and oligofermenting strain biotypes L1A to L1F (Figure 1), all grown at 37 °C. It is well documented that the subspecies *cremoris* strains neither grow at 37 °C (having an optimal between 18 °C and 25 °C) nor produce dextran from sucrose [24,39]. Additionally, all of them ferment a limited number of carbohydrates compared to the subspecies *mesenteroides* and *dextranicum* encompassing dextran-producing strains, capable of growth at 37 °C, including the type strains ATCC 8293^T (DSM 20343^T) and ATCC 19255^T (DSM 20484^T), respectively [39,49]. Specifically, all *cremoris* strains ferment glucose and lactose, while the fermentation of galactose and maltose is strain-specific, being positive and negative, respectively, in most strains [39]. All other basic sugars, including the key taxonomic ones L-arabinose, cellobiose, trehalose, and D-xylose (Figure 1), are not fermented by the ATCC 19254^T (DSM 20346^T) strain or any other typical *cremoris* strain, although sucrose-fermenting mutants have been reported since 1966 [22,39,49]. Gu et al. [22] highlighted that the subsp. *cremoris* strains do not ferment aesculin, salicin, and melibiose either. Moreover, later comparative studies based on advanced pan-genomic and transcriptomic analyses confirmed that the type strains of the four *Ln. mesenteroides* subspecies shared very high 16S rRNA gene sequence similarities (>99.72%) and could not be differentiated by this method, which is inappropriate to infer the phylogenetic relationships of *Ln. mesenteroides* strains [23,36,43,51]. In accordance with the literature, we could not identify the subspecies of our autochthonous whey cheese spoilage *Ln. mesenteroides* isolates by the Sanger 16S rRNA gene sequencing method either [21], which prompted us to follow the multiplex-PCR approach of Ricciardi et al. [46].

Ricciardi et al. [46] reported that sequence analysis of the *rpoB* gene and comparison of the *rpoB* amino acid sequences clearly separated the subspecies *cremoris* group but were not conclusive for the other strains. Specifically, in the phylogenetic tree of partial *rpoB* gene sequences retrieved from 57 *Ln. mesenteroides* strains and 21 published *Ln. mesenteroides* genomes (i.e., strain P45 was excluded), the *cremoris* ATCC 19254^T cluster, being deficient of the *araA*, *dsr*, and *sorA* genes, included five strains only. Consistent with the lack of the above genes, all five *cremoris* strains displayed three subspecies-specific 'negative' phenotypic traits (no acid production from arabinose; no slime (dextran) formation; no growth at 37 °C). At least one of them, however, the Irish *cremoris* DPC 3944 (MK574700) strain from artisanal cheese, was found to ferment cellobiose, maltose, ribose, sucrose, trehalose, and xylose [46]. Because the DPC 3944 strain phenotype, seemingly genotyped with the subsp. *cremoris* by molecular tools in addition to the 16S rRNA gene sequencing, was similar to our atypical, slime-negative subsp. *dextranicum* isolates in biotypes L1D–L1F (Figure 1), autochthonous *Ln. mesenteroides* subsp. *cremoris* strain genotypes with enriched sugar fermentation patterns may also occur in traditional Greek cheeses to be misidentified as *Ln. mesenteroides* subsp. *dextranicum* strain genotypes with an oligofermenting phenotype, such as that of the respective NCDO 529 (ATCC19255^T = DSM20484^T) type strain originally defined by Garvie [49,50].

Overall, it is not always possible to differentiate between the L-arabinose-negative *Ln. mesenteroides* subspecies *cremoris* and *dextranicum* on a phenotypic level [39], particularly when the latter dairy strains are atypical in failing to produce dextran (slime) from sucrose although they possess the *dsr* gene [46], as all representative whey cheese spoilage isolates of *Ln. mesenteroides*, except of the WM107 and WM108 strains, do (Figure 1; Table 4). Altogether, 20 strains with the atypical S5 (*dsr*/*sorA*) *dextranicum* profile vs. an additional

39 strains with the typical S4 (*araA*/*dsr*/*sorA*) profile of *Ln. mesenteroides* subsp. *mesenteroides* ATCC 8293^T clustered variably and quite ambiguously on the basis of their partial *rpoB* gene sequences; in total, five ambiguous clusters in addition to the four clusters comprising the respective type strains of the *Ln. mesenteroides* subspecies were detected by Ricciardi et al. [46]. Surprisingly, ACA-DC 0493 was among four *dsr*/*sorA* strains in the mes_ATCC8293^T cluster of Ricciardi et al. [46], whereas in our present (Table 4) and previous [45] studies, it was found to possess the S2 (*dsr*) gene profile of *Ln. mesenteroides* subsp. *dextranicum* DSM 20484^T. While this multiplex-PCR discrepancy regarding the ACA-DC 0493 strain cannot be addressed without further inter-laboratory testing, it needs to be stressed that only the type strain (DSM 20484^T = LMG 6908^T) of the subspecies *dextranicum* was a single *dsr* gene possessor among the 78 *Ln. mesenteroides* strains studied by Ricciardi et al. [46]. In our opinion, that finding is of prominent importance with respect to the LAB ecology of the artisanal cheeses overall and corroborates the absence of typical (*dsr*) *Ln. mesenteroides* subsp. *dextranicum* strains contrary to the prevalence of atypical (*dsr*/*sorA*) intermediate *dextranicum* and/or *mesenteroides* strains among the 24 representative isolates herein (Table 4), among 10 similar isolates from Epirus raw sheep milk [45], and probably among the total 92 *Ln. mesenteroides* isolates from four spoiled Anthotyros whey cheese batches [21].

The absence of *araA* and thus the L-arabinose-negative fermentation reaction of the subsp. *dextranicum* strains remain key traits for their separation from the subsp. *mesenteroides* and *jonggajibkimchii* strains [46]. Therefore, the fact that the type strain of *Ln. mesenteroides* subsp. *dextranicum* KACC 12315^T produced acid from L-arabinose by the API 50 CHL method, according to the fermentation data tabulated in the taxonomic study defining *Ln. mesenteroides* subsp. *jonggajibkimchii* subsp. nov. [23], is a critical contradiction with Bergey's Manual tabulation [39]. Nevertheless, for many of the *Ln. mesenteroides* strains analyzed by Ricciardi et al. [46], the presence of *dsr* and *araA* did not reflect the production of dextran from sucrose and acid from arabinose, as it was respectively shown in the present study for 22 out of the total 24 *dsr*-positive *Ln. mesenteroides* isolates and, most profoundly, for 8 *araA*-positive isolates in the oligofermenting L1B, L1C, and L4A biotypes (Figure 1) possessing the S3 multiplex-PCR profile of *Ln. mesenteroides* subsp. *jonggajibkimhcii* DRC 1506^T, deficient of the *sorA* gene only (Table 4). Notably, only 3 out of the 74 *Ln. mesenteroides* reference strains that had the expected *araA*/*dsr* profile of the subsp. *jonggajibkimchii* did the most ambiguous clustering, i.e., clearly outside of the jon_DRC 1506^T cluster, which instead included an additional 10 strains, all of them sharing the typical *araA*/*dsr*/*sorA* profile of the subspecies *mesenteroides* [46].

Altogether, the above literature data suggest that *Ln. mesenteroides* strains with the *araA*/*dsr* profile of our atypical subsp. *jonggajibkimchii*-like strains have been rarely isolated so far, at least from dairy foods, and may also be genotyped closer to either typical subsp. *mesenteroides* or subsp. *dextranicum* strains. Ricciardi et al. [46] highlighted the above discrepancies and concluded that none of the phenotypic and genotypic traits (including the *sorA* gene) separated the former two subspecies. The authors opinioned that contradictive clustering or taxonomic inaccuracies among strains may occur because the proposal of *Ln. mesenteroides* subsp. *jonggajibkimchii* subsp. nov. by Juan et al. [23] was based on the description of a single strain, DRC 1506^T. Therefore, it is necessary to analyze additional subsp. *jonggajibkimchii* as well as much more subsp. *dextranicum* strains, comparatively to the subsp. *mesenteroides* strains, before asserting the presence of this newest fourth subspecies [46]. Robust genome-based approaches supported by comparative metabolic diversity studies are constantly required for an accurate subspecies discrimination of *Ln. mesenteroides* strains [36,43,51], particularly of novel artisan cheese isolates seemingly assigned to the subsp. *jonggajibkimchii* by the combined use of MALDI-TOF MS and *pheS* gene sequencing analysis [27] or with more advanced taxonomic tools, such as digital DNA-DNA hybridization (dDDH) using the DSMZ type strain genome server (TYGS) analysis [29]. The complex phylogeny of the ubiquitous *Ln. mesenteroides* and its closelyrelated *Ln. suionicum*, *Ln. litchii*, *Ln. pseudomesenteroides*, and *Ln. falkenbergense* has

not been fully resolved yet [51]. In this context, our present dairy study provides novel *Ln. mesenteroides* isolates possessing the rare *jonggajibkimchii* (*araA*/*dsr*) profile, which, apart from lacking *sorA*, display an atypical oligofermenting pattern (Figure 1; Table 3) compared to the DRC 1506^{T}, which, among others, ferments L-arabinose, aesculin, salicin, mannitol, ribose, and raffinose, and produces dextran (slime) from sucrose [23]. Because all *Ln. mesenteroides* (*araA*/*dsr*) isolates in the strain biotypes L1B, L1C, and L4A were not retrieved as autochthonous non-starter LAB from traditionally fermented and ripened cheeses, but they evolved as competitive predominant psychrotrophic LAB spoilers in a fresh (non-fermented) whey cheese during refrigerated storage, they may be free-living, D-xylose-positive strains of plant origin having adapted to raw milk niches by shifting their active-gene sugar fermentation pathways accordingly [43,51]. In support of this hypothesis, two similar *Ln. mesenteroides* (*araA*/*dsr*) strains (KFM3 and KFM9) were isolated, for the first time, from bulk Greek raw sheep milk [45].

5. Conclusions

In conclusion, at least 8 of the 24 diverse representative *Ln. mesenteroides* whey cheese isolates with the atypical novel strain phenotypes L1B, L1C, and L4A, classified as *Ln. mesenteroides* subsp. *jonggajibkimchii*, according to their multiplex-PCR (*araA*/*dsr*) profile (Table 4), deserve further investigations, based on WGS and transcriptomic analyses, to resolve their taxonomy and metabolic features. Future studies are also required to validate the actual spoilage potential and safety of all *Ln. mesenteroides* strain biotypes/genotypes retrieved from Anthotyros whey cheese [21] in association with their bioprotective and probiotic potential, as it was done for other 'two-faced' *Ln. mesenteroides* strains from food (dairy) microecosystems [34,40,44]. The present psychrotrophic *Ln. mesenteroides* strains promoted an unmonitored, batch-dependent natural acidification (pH 5.5 to $\leq$ 5.0), which ceased the growth of Gram-negative bacteria in the spoiling vacuum-packaged Anthotyros whey cheeses from day 15 to day 40 at 4 °C [21], without causing blowing [6] or strong flavor defects, suggesting minor in situ CO_2 formation and enzymatic activity [8,61]. In parallel, they controlled the growth of inoculated *Listeria monocytogenes*, whose viability declined in most batches during storage [62], suggesting the presence of bacteriocinogenic (Bac+) *Ln. mesenteroides* or other psychrotrophic Bac+ LAB strains. Particularly, the atypical *jonggajibkimchii* (*araA*/*dsr*) strain biotypes may have low spoilage potential and also inhibit coexisting spoilage or pathogenic bacteria at low storage temperatures to be intentionally added as probiotic biopreservatives, like *Carnobacterium* spp. or *Lacticaseibacillus casei*, in fresh (whey) cheeses [20,63,64]. Relevant studies on defined Bac+ *Ln. mesenteroides* or other *Leuconostoc* spp. strain applications remain scarce in the dairy industry [65,66]. Therefore, fresh whey cheese inoculation studies with present *Ln. mesenteroides* strains, preselected for their technological, functional, and safety traits [35], should be conducted. A recent review by Dimov [67] does not encompass *Leuconostoc*/*Ln. mesenteroides* among the most concerning LAB (excluding *Enterococcus* spp.) of controversial (probiotic vs. pathogenic) nature actively participating in cheese ripening. However, previous reports have associated *Leuconostoc*, mostly *Ln. mesenteroides* strains, with clinical infections as opportunistic pathogens and multi-antibiotic resistance [24,40,68]. Therefore, a thorough safety evaluation is a prerequisite for applying any of the present natural *Ln. mesenteroides* whey cheese strains as a biopreservative and/or probiotic adjunct culture in Greek dairy foods.

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