Leuconostoc argentinum sp. nov., Isolated from Argentine Raw Milk

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The levels of genotypic relatedness among seven strains of *Leuconostoc* spp. isolated from Argentine raw milk, *Leuconostoc lactis* DSM 20202^T (T = type strain) and DSM 20198, *Leuconostoc mesenteroides* subsp. *mesenteroides* NCDO 523^T, *Leuconostoc mesenteroides* subsp. *dextranicum* NCDO 529^T, and *Leuconostoc paramesenteroides* DSM 20288^T were determined by performing a numerical analysis of total soluble cell protein patterns and DNA-DNA hybridization data. The Argentine raw milk strains formed a tight genotypic cluster at an *r* value of ≥ 0.85 and exhibited low levels of DNA homology with the other species included in this study. These strains represent a new species within the group of leuconostocs isolated from milk or dairy products. The name *Leuconostoc argentinum* sp. nov. is proposed; the type strain is strain LL76.

The genus *Leuconostoc* contains the following species: *Leuconostoc mesenteroides* (with three subspecies, *Leuconostoc mesenteroides* subsp. *mesenteroides*, *Leuconostoc mesenteroides* subsp. *dextranicum*, and *Leuconostoc mesenteroides* subsp. *cremoris*), *Leuconostoc paramesenteroides*, *Leuconostoc lactis*, *Leuconostoc oenos* (9), *Leuconostoc amelibiosum* (14), *Leuconostoc gelidum*, *Leuconostoc carnosum* (16), *Leuconostoc citreum*, and *Leuconostoc pseudomesenteroides* (7). In a recent paper, it was suggested that *L. amelibiosum* is a later subjective synonym of *L. citreum* (17). Together with *Lactococcus* spp., strains of *L. lactis* and *L. mesenteroides* are frequently used as starter cultures for the production of buttermilk, butter, and cheese (9). Therefore, it is not surprising when *L. mesenteroides* and *L. lactis* are isolated from dairy products in higher numbers than the other *Leuconostoc* spp.

L. oenos is the only acidophilic *Leuconostoc* species (9) and therefore is easily distinguished from the other taxa. However, the nonacidophilic leuconostocs share many phenotypic characteristics (9) and are not as easily distinguished from each other. The variations in sugar fermentation patterns among strains belonging to the same species and the identical fermentation patterns recorded for strains belonging to different species (8–10) have shown that sugar fermentation is of limited value in the classification of the nonacidophilic leuconostocs. *L. mesenteroides* subsp. *mesenteroides* and *L. mesenteroides* subsp. *dextranicum* have very similar sugar fermentation patterns and cannot be differentiated on the basis of simple phenotypic characteristics (9).

Dicks et al. (5) grouped strains belonging to *L. mesenteroides*, *L. paramesenteroides*, *L. lactis*, and *L. oenos* into four genotypic clusters by performing a numerical analysis of total soluble cell protein patterns. Recently, Fantuzzi et al. (6) reclassified strains of *L. mesenteroides* subsp. *dextranicum* as *L. mesenteroides* subsp. *mesenteroides* on the basis of results obtained from a numerical analysis of total soluble cell protein patterns and DNA-DNA hybridization data. The groupings obtained from the numerical analysis of total soluble cell protein patterns corresponded well to the group-

ings obtained by using DNA-DNA hybridization data (2, 5, 6).

We studied the levels of genotypic relatedness among seven strains of *Leuconostoc* spp. isolated from Argentine raw milk and representative strains of *L. mesenteroides* subsp. *mesenteroides*, *L. mesenteroides* subsp. *dextranicum*, *L. lactis*, and *L. paramesenteroides* by performing a numerical analysis of total soluble cell protein patterns and DNA-DNA hybridization data.

MATERIALS AND METHODS

Bacterial strains and growth conditions. Strains LL81, LL80, LL127, LL64, LL76^T (T = type strain), LL100, and LL84 were isolated from Argentine raw milk and were obtained from the collection of the Laboratorio de Bacteria Lacticas, Universidad de Lujan, Buenos Aires, Argentina. *L. lactis* DSM 20202^T and DSM 20198, *L. mesenteroides* subsp. *mesenteroides* NCDO 523^T, *L. mesenteroides* subsp. *dextranicum* NCDO 529^T, and *L. paramesenteroides* DSM 20288^T were included as reference strains. All strains were cultured in MRS broth (3) at 30°C.

Numerical analysis of total soluble cell protein patterns. Cells that were 24 h old were harvested, and the proteins were extracted by using the methods described by Dicks et al. (5). Electrophoresis, densitometric tracings, photography of the gels, and a numerical analysis of normalized electropherograms were performed as described previously (5).

DNA-DNA hybridizations. Cells in the logarithmic growth phase were harvested, and DNA-DNA hybridizations were performed by using the methods described by Dellaglio et al. (1). The percentages of DNA homology were calculated by using the single-point competition technique (11).

DNA base composition. DNA was isolated and purified by using the technique described by Dicks et al. (5). The G+C content was calculated by using the method of Marmur and Doty (12).

Biochemical and physiological tests. The biochemical and physiological characteristics were determined by using the methods described by Sharpe (15). Carbohydrate fermentation characteristics were determined by using the API

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TABLE 1. Levels of genotypic relatedness among strains isolated from Argentine raw milk and reference strains

Strain	Cluster ^a	% DNA homology with:			
		Strain DSM 20202 ^T	Strain NCDO 523 ^T	Strain NCDO 529 ^T	Strain LL76 ^T
DSM 20202 ^T	I	100	8	6	35
LL81	II	36	10	12	96
LL80	II	40	11	9	97
LL127	II	31	5	8	68
LL64	II	30	10	5	83
LL76 ^T	II	39	9	11	100
LL100	II	10	7	10	75
LL84	II	27	7	3	69
LL62	NC ^b	11	68	60	ND ^c
NCDO 523 ^T	III	10	100	79	ND
LL104	III	12	89	73	ND
LL122	III	6	83	75	ND
NCDO 529 ^T	III	17	81	100	ND
LL97	III	9	78	91	ND
LL218	III	20	86	106	ND
LL116	III	8	71	78	ND
LL56	III	7	78	80	ND
LL106	NC	9	60	65	ND
DSM 20288 ^T	NC	0	10	18	0

^a Clusters were determined by performing a numerical analysis of the total soluble cell protein patterns.

^b NC, not clustered.

^c ND, not determined.

50CHL system (La Balme Les Grottes, Montalieu Vercieu, France); the API 50CHL results were recorded after 48 h.

RESULTS AND DISCUSSION

The seven strains isolated from Argentine raw milk grouped in one cluster and exhibited low levels of DNA homology to *L. lactis* DSM 20202^T, *L. mesenteroides* subsp. *mesenteroides* NCDO 523^T, and *L. mesenteroides* subsp. *dextranicum* NCDO 529^T (Table 1). *L. paramesenteroides* DSM 20288^T exhibited no DNA homology with strain LL76^T in cluster II (Table 1). The levels of genotypic relatedness between the strains in cluster II and the strains in cluster III, which have been classified previously as *L. mesenteroides* subsp. *mesenteroides* and *L. mesenteroides* subsp. *dextranicum* (6), are shown in Fig. 1 and Table 1.

Strains LL81, LL80, LL127, LL64, LL76^T, LL100, and LL84 formed a tight cluster at an *r* value of ≥ 0.85 (Fig. 1, cluster II). The overall protein profiles of these strains were similar to each other but different from those of all other strains included in this study. The strains in cluster II exhibited high levels of DNA homology (68 to 97%) with strain LL76^T, which was selected as the representative strain of cluster II (Table 1). Low levels of DNA homology were obtained between the strains belonging to cluster II and *L. mesenteroides* subsp. *mesenteroides* NCDO 523^T (5 to 11%) and between the strains belonging to cluster II and *L. mesenteroides* subsp. *dextranicum* NCDO 529^T (3 to 12%) (Table 1). Furthermore, the results obtained by restriction enzyme digestion of the genomic DNAs of strains LL64 and LL84 clearly distinguished the cluster II strains from the strains belonging to *L. lactis* (cluster I) and *L. mesenteroides* (Fig. 1, cluster III) (6).

Cluster II strains exhibited low levels of DNA homology (10 to 40%) with the type strain of *L. lactis* (strain DSM 20202^T) (Table 1). This finding was confirmed by the different protein profiles obtained for the cluster II strains and cluster

I strains *L. lactis* DSM 20202^T and DSM 20198 (Fig. 1). The type strain of *L. paramesenteroides* (strain DSM 20288^T) exhibited no DNA homology with cluster II strain LL76^T (Table 1).

On the basis of the results obtained from the numerical analysis of the total soluble cell protein patterns (Fig. 1), DNA-DNA hybridizations (Table 1), and restriction enzyme digestions of the genomic DNA (6), strains LL81, LL80, LL127, LL64, LL76^T, LL100, and LL84 are regarded as members of a new species in the genus *Leuconostoc*. We propose the name *Leuconostoc argentinum* sp. nov. for these organisms.

Characteristics that are useful for differentiating *L. argentinum* from previously described taxa are shown in Table 2.

The results obtained from the API 50CHL system analysis yielded valuable information regarding the carbohydrate fermentation profile of *L. argentinum*. However, the overall sugar fermentation profiles of *L. argentinum* and the other *Leuconostoc* spp. included in this study were very similar, indicating that the API 50CHL system is of little value as an identification method, as reported previously for many other lactic acid bacteria (4, 7, 8, 13, 18, 19).

Description of *Leuconostoc argentinum* sp. nov. *Leuconostoc argentinum* (ar.gen.ti'num. L. adj. *argentinum*, pertaining to Argentine). Gram-positive cocci. Nonmotile, non-sporulating, catalase negative, and oxidase negative. Spherical and sometimes lenticular cells occur usually in pairs and short chains. Colonies on MRS agar are round, smooth, greyish white, and usually small (less than 1.00 mm in diameter). Growth occurs at temperatures between 10 and 39°C; the optimum temperature is 27 to 30°C. Facultatively anaerobic. Heterofermentative, producing gas from glucose. More than 97% of the lactate is produced as the D(-) isomer. Arginine and esculin are not hydrolyzed. Voges-Proskauer negative. Indole is not formed, and nitrates are not reduced. Polysaccharides are not produced from sucrose. Milk is coagulated without added yeast extract, but

FIG. 1. Dendrogram and normalized photographs of protein gels of *L. argentinum* sp. nov., *L. lactis*, *L. mesenteroides* subsp. *mesenteroides*, *L. mesenteroides* subsp. *dextransicum*, and *L. paramesenteroides*.

TABLE 2. Diagnostic characteristics of subspecies and species belonging to the genus *Leuconostoc*^a

Characteristic	<i>L. mesenteroides</i> subsp. <i>mesenteroides</i>	<i>L. mesenteroides</i> subsp. <i>dextranicum</i>	<i>L. mesenteroides</i> subsp. <i>cremoris</i>	<i>L. paramesenteroides</i>	<i>L. lactis</i>	<i>L. oenos</i>	<i>L. gelidum</i>	<i>L. carnosum</i>	<i>L. pseudomesenteroides</i>	<i>L. citreum</i>	<i>L. amelibiosum</i>	<i>L. argentinum</i>
Acid produced from:	+	-	-	d	-	-	+	-	d	+	+	d
Arabinose	d	-	-	(d)	-	-	+	-	d	+	+	-
Arbutin	d	d	-	+	+	+	+	+	ND	ND	ND	ND
Cellulose	+	+	-	+	+	+	+	+	+	+	+	+
Fructose	+	d	d	+	+	+	+	+	d	+	+	+
Galactose	d	+	d	+	+	+	+	+	+	+	+	+
Lactose	+	+	d	+	+	+	+	+	+	+	+	+
Maltose	d	d	-	-	d	d	+	+	d	+	+	+
Salicin	d	+	-	+	+	+	+	+	+	+	+	+
Sucrose	+	+	-	+	+	+	+	+	+	+	+	+
Trehalose	+	+	-	+	+	+	+	+	+	+	+	+
Xylose	d	d	-	d	-	-	+	+	+	+	+	d
Hydrolysis of esculin	d	d	-	d	-	-	+	+	d	+	+	-
Dextran formation	+	+	-	-	-	-	+	+	ND	ND	+	-
Growth at pH 4.8	-	-	-	d	-	-	+	+	ND	ND	+	ND
Requirement for TjF ^b	-	-	-	-	-	-	+	+	-	-	-	-
Growth in 10% ethanol	-	-	-	-	-	-	+	+	ND	ND	ND	ND
NAD-dependent G-6-PDH present ^c	+	+	+	+	+	+	-	-	ND	ND	ND	ND
Growth at 37°C	d	+	-	d	+	+	-	-	+	d	ND	+

^a +, 90% or more of the strains are positive; -, 90% or more of the strains are negative; d, 11 to 98% of the strains are positive; (d), delayed reaction; ND, not determined. Data for *L. mesenteroides*, *L. paramesenteroides*, *L. lactis* and *L. oenos* are from reference 9; data for *L. gelidum* and *L. carnosum* are from reference 16; data for *L. pseudomesenteroides* and *L. citreum* are from reference 7; and data for *L. amelibiosum* are from reference 14.

^b TjF, tomato juice factor.

^c G-6-PDH, glucose-6-phosphate dehydrogenase.

only after extended incubation (48 h). All strains produce acid from galactose, D-glucose, D-mannose, maltose, N-acetylglucosamine, lactose, melibiose, and sucrose. Most strains produce acid from D-fructose and D-raffinose. Some strains produce acid from L-arabinose, D-xylose, mannitol, α -methyl-D-glucoside, cellobiose, trehalose, β -gentiobiose, and D-turanose. Acid is not produced from ribose, amygdalin, arbutin, salicin, D-tagatose, glycerol, erythritol, D-arabinose, L-xylose, adonitol, β -methyl-xyloside, sorbose, rhamnose, dulcitol, inositol, sorbitol, α -methyl-D-mannoside, inulin, melezitose, starch, glycogen, xylitol, D-lyxose, D-fucose, L-fucose, D-arabitol, gluconate, 2-ketogluconate, and 5-ketogluconate.

The G+C content of the DNA of the type strain is 40.5 mol% (as determined by the thermal denaturation method).

Isolated from Argentine raw milk. Represents 7 to 10% of the *Leuconostoc* spp. isolated.

The type strain is strain LL76. The description of the type strain corresponds to the description of the species, except that acid is typically produced from α -methyl-D-glucoside, trehalose, and D-turanose and acid is not produced from L-arabinose, D-xylose, cellobiose, and β -gentiobiose.

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