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Characterization of *Leuconostoc gasicomitatum* sp. nov., Associated with Spoiled Raw Tomato-Marinated Broiler Meat Strips Packaged under Modified-Atmosphere Conditions

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Lactic acid bacteria (LAB) associated with gaseous spoilage of modified-atmosphere-packaged, raw, tomato-marinated broiler meat strips were identified on the basis of a restriction fragment length polymorphism (RFLP) (ribotyping) database containing DNAs coding for 16S and 23S rRNAs (rDNAs). A mixed LAB population dominated by a *Leuconostoc* species resembling *Leuconostoc gelidum* caused the spoilage of the product. *Lactobacillus sakei*, *Lactobacillus curvatus*, and a gram-positive rod phenotypically similar to heterofermentative *Lactobacillus* species were the other main organisms detected. An increase in pH together with the extreme bulging of packages suggested a rare LAB spoilage type called "protein swell." This spoilage is characterized by excessive production of gas due to amino acid decarboxylation, and the rise in pH is attributed to the subsequent deamination of amino acids. Protein swell has not previously been associated with any kind of meat product. A polyphasic approach, including classical phenotyping, whole-cell protein electrophoresis, 16 and 23S rDNA RFLP, 16S rDNA sequence analysis, and DNA-DNA reassociation analysis, was used for the identification of the dominant *Leuconostoc* species. In addition to the RFLP analysis, phenotyping, whole-cell protein analysis, and 16S rDNA sequence homology indicated that *L. gelidum* was most similar to the spoilage-associated species. The two spoilage strains studied possessed 98.8 and 99.0% 16S rDNA sequence homology with the *L. gelidum* type strain. DNA-DNA reassociation, however, clearly distinguished the two species. The same strains showed only 22 and 34% hybridization with the *L. gelidum* type strain. These results warrant a separate species status, and we propose the name *Leuconostoc gasicomitatum* sp. nov. for this spoilage-associated *Leuconostoc* species.

Lactic acid bacteria (LAB) are the dominant spoilage organisms in vacuum or modified-atmosphere (MA)-packaged meat products (1, 2, 8, 10, 23, 31). Spoilage is mainly caused by *Lactobacillus* (3, 4, 26, 28; W. H. Holzapfel and E. S. Gerber, Abstr. 32nd Eur. Meet. Meat Res. Workers, p. 26, 1986) or *Leuconostoc* (7, 14, 28, 43, 49) species. The activities of these organisms at stationary phase produce the compounds associated with sensory spoilage (22). Depending on the type of product, this quality deterioration usually starts 1 to 4 weeks after packaging, and it is manifested mainly as formation of sour or cheesy off odors and/or off tastes. Provided that the shelf life of the product has been estimated correctly, spoilage changes do not occur before the sell-by day. However, in the case of potent spoilage LAB and/or poor production line hygiene, severe quality faults (5, 7, 26, 27) have occurred, resulting in product recalls.

The consumption of marinated, ready-to-cook, raw poultry meat products has been increasing in Europe. As easy-to-use and low-fat food, they are favored by many consumers. In this study, we describe and characterize an unusual spoilage of MA-packaged, tomato-marinated, raw broiler meat strips. This product was manufactured at a modern large-scale processing plant, and normally, good quality was maintained at 6°C for 10 days, which had also been set as the retail shelf life. During a problematic period in the manufacture, many packages started to show bulging due to gas formation 5 days after packaging. Before the sell-by day, these packages were extensively bulged. At that time, only this tomato-marinated product was showing unusual quality fluctuation affecting several production lots.

Manufacture of the product was halted, and the packages on the market were also withdrawn. The manufacturer performed microbiological analyses covering the main groups of pathogenic and spoilage bacteria and yeasts. The only significant microbiological finding was vast numbers, up to 10^{10} CFU/g, of LAB in the product. Since very little is known about LAB spoilage in poultry products, our study set out to characterize and to identify these spoilage LAB to the species level. The LAB population was initially identified using a restriction fragment length polymorphism (RFLP) database of DNAs coding for 16S and 23S rRNAs (rDNAs). The main spoilage species was further characterized by means of classical phenotyping, cell wall analysis, and whole-cell protein analysis together with genotypic characterization analyses, including ribotyping (18), 16S rDNA sequencing, and determination of DNA-DNA homology. Based on these results, the dominant species of the spoilage population was considered novel, and we propose the name *Leuconostoc gasicomitatum* sp. nov. for it.

MATERIALS AND METHODS

Description of the product and pH, sensory, and microbiological analyses. The product was manufactured from raw, skinned broiler meat, which was cut in strips, mixed with the marinade, and packaged under MA as ca. 500-g consumer packages. The marinade contained plant oil, tomatoes, paprika, cayenne, mineral

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TABLE 1. *Leuconostoc* sensu stricto reference strains

Species	Strain
L. carnosum	LMG 11498[Ta]
L. citreum (*amelibiosum*)	LMG 9824[T]
	LMG 11417
L. fallax	CCUG 30061[Tb]
L. gelidum	LMG 9850[T]
L. lactis	CCUG 30064[T]
	LMG 7940
L. mesenteroides subsp. *cremoris*	LMG 6909[T]
	LMG 13562
L. mesenteroides subsp. *dextranicum*	LMG 6908[T]
	LMG 7954
	LMG 11318
L. mesenteroides subsp. *mesenteroides*	LMG 6893[T]
	LMG 7939
L. pseudomesenteroides	LMG 11482[T]
	LMG 11483
	LMG 11499

[a] BCCM/LMG Belgian Coordinated Collections of Microorganisms.
[b] Culture Collection of the University of Gothenburg.

salt, protein hydrosylates, starch and modified starch, natural aromas, an aroma strengthener, emulsifiers, preservatives, and a buffering additive. In routine quality control measurements, the pH of the normal product varied from 4.2 to 4.4, with pH 4.5 set as the optimal target value. The expected shelf life at 6°C was 10 days, with the day of manufacture regarded as day 0.

Six unopened packages showing clear bulging were analyzed on the last day of the shelf life. LAB were enumerated from serial 10-fold dilutions on MRS agar (Oxoid, Basingstoke, United Kingdom) and Rogosa selective *Lactobacillus* agar (Orion Diagnostica, Espoo, Finland) as described by Korkeala et al. (22). All of the plates were incubated at 25°C in an anaerobic jar with an H_2 and CO_2 generating kit (Oxoid) for 5 days. The pH was measured directly from the homogenized samples. Evaluation of odor, color, appearance, and texture of the spoiled product was performed by three trained judges, as described by Korkeala and Lindroth (21).

Bacterial strains and the use of strains in different phases of the study. One hundred twenty randomly picked colonies recovered from the six spoiled packages (20 isolates from each package) were purified. During the course of the study, strains were assessed in the different phases of the study as described below. The 120 spoilage isolates were all subjected to basic phenotyping and ribotyping. The ribopatterns were compared with the corresponding patterns in the LAB database of the Department of Food and Environmental Hygiene, University of Helsinki, Helsinki, Finland. These comprise patterns of all relevant spoilage LAB in the genera *Carnobacteria*, *Lactobacillus*, *Leuconostoc*, *Enterococcus*, and *Weissella* (4, 6, 7, 25). Before Southern blotting, the restriction endonuclease analysis (REA) patterns of the main spoilage species (*L. gasicomitatum* sp. nov.) were inspected visually. From these strains, possessing only two different REA patterns, four strains representing both pattern types (two of each) were chosen for further taxonomic studies. These isolates originated from three different packages and were given the following strain numbers: LMG 18811, LMG 18812, LMG 18813, and LMG 18889. The *Leuconostoc* reference strains presented in Table 1 were used during the more detailed taxonomic study dealing with the main spoilage species, and the LAB ribotyping database also contained the ribopatterns of these strains.

All of the strains were maintained in MRS broth (Difco, Detroit, Mich.) at −70°C and cultured using MRS broth or MRS agar (Oxoid).

Phenotypic characterization. All 120 isolates were Gram stained, catalase tested, streaked on Rogosa selective *Lactobacillus* agar, and studied for the production of gas from glucose (44). Further phenotypic characterization of the main spoilage species was done with strains LMG 18811, LMG 18812, LMG 18813, and LMG 18889. Production of ammonia from arginine was determined by the method of Briggs (11). Dextran formation was studied on agar containing 5% sucrose (20). Fermentation of carbohydrates was determined by use of the API 50 CHL *Lactobacillus* identification system (Biomerieux, Marcy l'Etoile, France). The ability to produce different lactic acid isomers was tested by an enzymatic method (48) utilizing Boehringer Mannheim GmbH (Mannheim, Federal Republic of Germany) D- and L-lactate dehydrogenases. The four isolates were also tested for growth in MRS broth at 4, 10, 15, and 37°C until growth was observed or at least for 21 days.

Enzymatic activities. The proteolytic activity of all 67 *L. gasicomitatum* sp. nov. isolates was tested on MRS agar supplemented with sterile skim milk to yield a 2% concentration. API ZYM (Biomerieux) was also used for the characterization of the enzymatic activities of the LMG 18811 and LMG 18812 strains.

Peptidoglycan analysis. Preparation of cell walls and determination of the peptidoglycan structure of LMG 18811 was carried out by the methods described by Schleifer and Kandler (41) with the modification of using thin-layer chromatography on cellulose sheets instead of paper chromatography. Briefly, 1 mg of freeze-dried cell walls was hydrolyzed in 0.2 ml of 4 N HCl at 100°C for 16 h (total hydrolysate) and 45 min (partial hydrolysate). Diamino acids were determined from total hydrolysate by one-dimensional chromatography in the solvent system methanol–pyridine–water–10 N HCl (320:40:70:10 [vol/vol/vol/vol]). Amino acids and peptides from total and partial hydrolysates were identified, after two-dimensional chromatography in the systems published by Schleifer and Kandler (41), by their mobilities and staining characteristics with ninhydrin spray. The resulting "fingerprints" were compared with known peptidoglycan structures.

Whole-cell protein analysis. The similarity of the main spoilage species (strains LMG 18811, LMG 18812, LMG 18813, and LMG 18889) to *Leuconostoc* sensu stricto species (Table 1) was studied by means of whole-cell protein analysis. All strains were grown for 5 days on MRS agar at 25°C in a microaerobic atmosphere (approximately 5% O_2, 10% CO_2, and 85% N_2). Preparation of cellular protein extracts and polyacrylamide gel electrophoresis were performed as described previously (36). Briefly, discontinuous gels were run overnight at constant current and temperature in a vertical slab apparatus. The separation gel was 12.6 cm long and contained 12% total acrylamide (the monomer solution contained 30% total acrylamide with 2.67% cross-linking in 0.375 M Tris-HCl [pH 8.8] and 0.1% sodium dodecyl sulfate). The stacking gel was 12 mm long and contained 5% total acrylamide (the monomer solution contained 30% total acrylamide with 2.67% cross-linking in 0.125 M Tris-HCl [pH 6.8] and 0.1% sodium dodecyl sulfate). Protein bands were stained with Coomassie blue R-250 in 50% (vol/vol) methanol and 10% (vol/vol) acetic acid. These conditions allowed the separation of proteins and peptides in the molecular weight range of 14,000 to 116,000.

Isolation of DNA, REA, and 16 and 23S rDNA RFLP (ribotyping). *Cla*I, *Eco*RI, and *Hin*dIII restriction enzymes (New England Biolabs, Beverly, Mass.) were used for ribotyping. DNA was isolated by the guanidium thiocyanate method of Pitcher et al. (35) as modified by Björkroth and Korkeala (3) by the combined lysozyme and mutanolysin (Sigma, St. Louis, Mo.) treatment. Restriction endonuclease treatment of 3 μg of DNA was done as specified by the manufacturer (New England Biolabs), and REA was performed as described previously (3). Before Southern blotting, the REA patterns were inspected visually in order to obtain preliminary information about clonal variation. Genomic blots were made using a vacuum device (Vacugene; Pharmacia, Uppsala, Sweden), and the rDNA probe for ribotyping was labeled by reverse transcription (AMV-RT [Promega, Madison, Wis.] and Dig DNA labeling kit [Roche Molecular Biochemicals, Mannheim, Germany]) as previously described by Blumberg et al. (9). Membranes were hybridized at 68°C as described by Björkroth and Korkeala (3).

Pattern analysis. The *Cla*I, *Eco*RI, and *Hin*dIII ribopatterns were compared with the corresponding patterns in the previously established LAB database. For numerical analysis, ribopatterns were scanned using a Hewlett-Packard (Boise, Idaho) ScanJet 4c/T scanner and analyzed using the BioNumerics 1.0 software package (Applied Maths, Kortrijk, Belgium). The similarities between all pairs were expressed by Dice coefficient correlation, and unweighted pair group method using arithmetic averages clustering was used for the construction of the dendrogram.

Whole-cell protein profiles were scanned using a 2202 UltroScan laser densitometer (LKB, Bromma, Sweden). The densitometric analysis, normalization, and interpolation of the protein profiles were performed with the GelCompar 4.2 software package (Applied Maths). Numerical analysis was performed using the BioNumerics 1.0 software package. The similarities between all pairs of traces were expressed by the Pearson product moment correlation coefficient converted for convenience to a percent value.

All four different types of banding patterns were integrated in a single database, and numerical analyses combining the 16 and 23S rDNA RFLP data generated by means of the three different enzymes were performed by using the BioNumerics 1.0 software package. In these combined analyses, equal weight was given to each of the three banding patterns.

16S rRNA gene sequence analysis. The 16S rRNA gene was amplified with a universal primer pair (45): primer A, 5′GAGTTTGATCCTGGCTCAG3′, and primer B, 5′AGAAAGGAGGTGATCCAGCC3′. Two strains, LMG 18811 and LMG 18812, were studied. They represented the two groups detected in REA. Chromosomal DNA was isolated as for ribotyping. Amplification was performed in a Mastercycler 5330-plus thermal cycler (Eppendorf, Hamburg, Germany) using 200 ng of chromosomal DNA as a template. The PCR mixture contained 5 U of *Taq* polymerase (Promega), 5 μl of *Taq* polymerase buffer (10×; Promega), 8 μl of nucleotide mixture (dATP, dCTP, dTTP, and dGTP; 2.5 mM each), 4.0 μl of $MgCl_2$ (25 mM), 1.25 μl of primers A and B (120 pmol/μl), template DNA adjusted to 10 μl, and H_2O added to yield a total reaction volume of 50 μl. The cycles used for amplification were as described previously (45).

Sequencing of the purified PCR product (Quantum Prep PCR Kleen spin columns; Bio-Rad Laboratories, Hercules, Calif.) was performed by Sanger's dideoxynucleotide chain termination method using an ABI PRISM sequencing device (Perkin-Elmer Corp., Norwalk, Conn.) according to the manufacturer's recommendations. Sequencing was performed as two long reactions, and overlapping complementary sequences were joined by the DNASIS program (Hitachi Software, Yokohama, Japan).

Phylogenetic analysis was performed by using the GeneCompar 2.0 software package (Applied Maths). The consensus sequence and the sequences of strains

TABLE 2. Recovery of LAB on MRS and Rogosa selective *Lactobacillus* agar and pH values analyzed from six spoiled packages showing clear bulging due to gas formation

Package no.	No. of LAB (CFU/g)		pH
	MRS	Rogosa selective *Lactobacillus* agar	
1	2×10^{10}	1×10^{10}	4.9
2	4×10^{10}	4×10^{10}	4.8
3	4×10^{10}	1×10^{10}	4.7
4	1×10^{10}	2×10^{8}	4.8
5	1×10^{10}	3×10^{10}	4.8
6	9×10^{10}	3×10^{10}	5.0

belonging to the same phylogenetic group (retrieved from the National Center for Biotechnology Information GenBank data library) were aligned. The accession numbers of the 16S rDNA sequences used are as follows: *Leuconostoc argentinum* LMG 18543^T, AF175403; *Leuconostoc carnosum* LMG 11498^T, X95997; *Leuconostoc citreum* LMG 9824^T, X53963 and S78390; *Leuconostoc fallax* LMG 13177^T, S63851; *Leuconostoc gelidum* LMG 9850^T, S63851; *Leuconostoc lactis* LMG 8894^T, M23031 and M23032; *Leuconostoc mesenteroides* subsp. *cremoris* LMG 6909^T, M23034; *Leuconostoc mesenteroides* subsp. *mesenteroides* LMG 6893^T, M23035; *Leuconostoc pseudomesenteroides* LMG 11482^T, X95979; and *Weissella paramesenteroides* LMG 9852^T, X95982.

DNA base composition and DNA-DNA hybridization. DNA was isolated from two spoilage isolates (LMG 18811 and LMG 18812), *L. gelidum* type strain NCFB 2775, and the *Leuconostoc mesenteroides* subsp. *dextranicum* type strain DSM 20484. *L. gelidum* was selected because it had the highest similarity to the main spoilage species according to some phenotyping schemas, RFLP analysis, whole-cell protein analysis, and 16S rDNA sequencing. *L. mesenteroides* subsp. *dextranicum* was chosen on the basis of API CH 50 *Lactobacillus* identification results, and it was also used as a control species representing *Leuconostoc* sensu stricto.

For large-scale DNA isolation, the modified (3) guanidium thiocyanate method of Pitcher et al. (35) was scaled up 10-fold. Cells from 200 ml of a well-grown MRS broth culture were used for each isolation batch. DNA from one batch was dissolved overnight in 1 ml of TE 10:1 (10 mM Tris, 1 mM EDTA, pH 8.0). RNase A (Sigma) was added to provide a concentration of 125 μg/ml, and the solution was incubated at 37°C with gentle shaking for 1 h. Following the 1-h incubation, proteinase K (Sigma) was added to provide a concentration of 0.5 mg/ml, and incubation at 37°C was continued for at least 6 h. DNA was precipitated as described by Pitcher (35) and dissolved in 1 ml of 0.1× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate). When dissolved, the SSC concentration of a sample was adjusted with 20× SSC to 1× SSC.

Purified DNA was dialyzed twice overnight at 4°C using a 12,000- to 14,000-Da-pore-size membrane (Medicell International Ltd., London, United Kingdom). The first dialysis was carried out against 1× SSC–EDTA (10 mM), and the second was carried out against 1× SSC. DNA was fragmented two times in a French pressure cell (SML Aminco; Colora Messtechnik GMBH, Lorch, Germany) at about 1.5×10^6 Pa. Before reassociation, it was dialyzed once more overnight at 4°C against 2× SSC.

The DNA base composition (moles percent G+C) was estimated by the thermal-denaturation method (12), and the DNA homology values were determined from renaturation rates using a Gilford Response spectrophotometer (Giba Corning Diagnostics Corp., Gilford Systems, Oberlin, Ohio).

Nucleotide sequence accession numbers. The approximately 1,500-bp sequences of the 16S ribosomal genes of strains LMG 18811 and LMG 18812 have been deposited in the GenBank data library with accession numbers AF231131 and AF231132, respectively.

RESULTS

Microbiological and sensorial qualities of the product. Table 2 shows the results of microbial enumeration on MRS and Rogosa selective *Lactobacillus* agars and corresponding pH values obtained from the six packages. An increase in the pH of the product, very atypical for LAB spoilage, was detected. Instead of the normal pH values, ranging from 4.2 to 4.4, values from 4.7 to 5.0 were detected. All of the packages were deemed unfit for human consumption by all three judges. They were all described as clearly bulged, and the smell of the product was described as pungent and very unpleasant. The consistency and texture of the product was, however, normal, and no color changes were visible.

LAB population associated with the spoiled product. Table 3 shows the division of the 120 isolates into different species and groups of species based on the LAB ribotyping database. An organism possessing typical lower molecular bands for leuconostocs in *Hin*dIII ribopatterns (Fig. 1C) was found to dominate (67 of 120) in the product. These isolates were all gram-positive, catalase-negative oval cocci, produced gas from glucose, and did not grow on Rogosa agar. They possessed identical ribopatterns, showing, however, two different types in the REA patterns. The distribution of the isolates between these two REA types was almost even.

The two other major species associated with the product were *Lactobacillus curvatus* (32 of 120) and *Lactobacillus sakei* (16 of 120). Isolates possessing ribotypes identical to the *L. sakei* or *L. curvatus* patterns in the database (no new pattern types were detected) were gram-positive rods or coccoid rods; all grew on Rogosa agar, were catalase negative, and did not produce gas from glucose. Three of the 120 isolates were identified as *Leuconostoc* sensu stricto species, one *L. carnosum* and two *L. gelidum*. They all shared identical ribopatterns with the corresponding *Leuconostoc* type strain, were oval cocci, produced gas from glucose, and did not grow on Rogosa selective *Lactobacillus* agar. Twelve isolates could not be identified with the existing ribotyping database. They were all gram-positive rods growing on Rogosa selective *Lactobacillus* agar, produced gas from glucose, and shared identical ribopatterns. They did not have any similarity to the ribopatterns of *Leuconostoc brevis*, *Leuconostoc buchneri*, *Leuconostoc collinoides*, *Leuconostoc fermentum*, *Leuconostoc fructivorans*, or *Leuconostoc hilgardii* type strains. This was also the case in respect to the patterns of *Carnobacterium divergens*, *Carnobacterium piscicola*, *Carnobacterium mobile*, and *Carnobacterium gallinarum* type strains.

Phenotypic reactions of the main spoilage species. LMG 18811, LMG 18812, LMG 18813, and LMG 18889 strains showed typical reactions for the genus *Leuconostoc*. They did not produce ammonia from arginine, did not grow in the presence of 6.5 to 12% NaCl, and synthesized only D-(−)-lactic acid from glucose. They all grew at 4 and 15°C but not at 37 or 45°C. Growth was already slower at 30°C, and during the study, 25°C was observed as an optimum temperature for growth in MRS. All four strains produced excessive slime from sucrose and fermented L-arabinose, ribose, D-xylose, glucose, fructose, mannose, α-methyl-D-glucoside, *N*-acetyl-glucosamine, esculin, cellobiose, maltose, melibiose, sucrose, trehalose, raffinose, gentiobiose, turanose, and 5-keto-gluconate. LMG 18811, LMG 18813, and LMG 18889 also fermented galactose and gluconate. Glycerol, erythritol, D-arabinose, L-xylose, adonitol, β-methyl-D-xyloside, sorbose, rhamnose, dulcitol, inositol, mannitol, sorbitol, α-methyl-D-mannoside, amygdalin, arbutin,

TABLE 3. Species division of 120 LAB isolates originating from six packages (20/package) of MA-packaged raw marinated broiler meat strips according a *Cla*I, *Eco*RI, and *Hin*dIII ribopattern database

Package no.	No. of isolates					
	Leuconostoc spp.	*L. carnosum*	*L. gelidum*	*L. sakei*	*L. curvatus*	Unidentified
1	4	1	2	4	5	4
2	9			4	5	2
3	10			3	5	2
4	15			4		1
5	12			1	6	1
6	7				11	2
Total	57	1	2	16	32	12

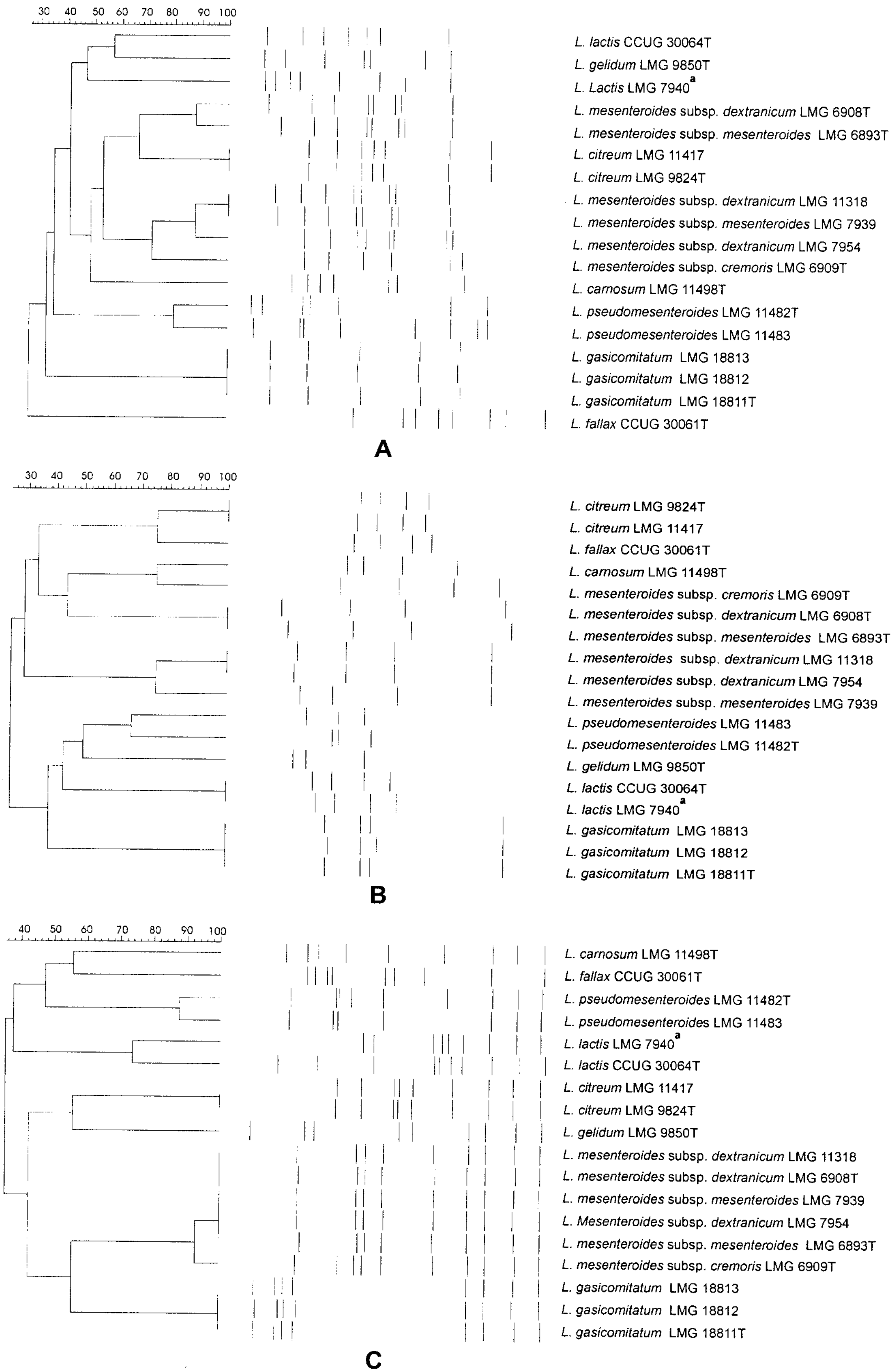

FIG. 1. Ribopatterns and numerical analysis of the patterns presented as dendrograms. Patterns and dendrograms generated by using *Cla*I (A), *Eco*RI (B), and *Hin*dIII (C) restriction enzymes are shown. The left sides of the banding patterns show large molecular sizes (23 kbp), and the right sides show small molecular sizes (500 bp). [a], the *L. argentinum* type strain showed ribopatterns identical to those of *L. lactis* LMG 7940. Scales from 30 to 100 show percentile similarity values for the patterns.

TABLE 4. Main differences in sugar fermentation between *L. carnosum*,[a] *L. gelidum*,[a] and *L. gasicomitatum* sp. nov.

Sugar	Fermentation[b]		
	L. carnosum	*L. gelidum*	*L. gasicomitatum* sp. nov.
Amygdalin	−	+	−
L-Arabinose	−	+	+
Arbutin	−	+	−
Raffinose	−	+	+
D-Xylose	−	+	+
Salicin	−/+[c]	+	−

[a] Reactions adapted from Shaw and Harding (43).
[b] −, no fermentation; +, fermentation takes place.
[c] Most strains do not ferment salicin.

salicin, lactose, inulin, melezitose, starch, glycogen, xylitol, D-lyxose, D-tagatose, fucose, arabitol, and 2-keto-gluconate were not fermented. The API CHL *Lactobacillus* system identified these isolates with an extremely good identification level (99.9%) as *L. mesenteroides* subsp. *dextranicum*. Table 4 shows the key carbohydrate fermentation reactions among the phylogenetically associated *L. carnosum*, *L. gelidum*, and the strains representing the main spoilage group.

Peptidoglycan type of the main spoilage species. The purified cell walls of LMG 18811 contain, besides muramic acid and glucosamine, the amino acids lysine, glutamic acid, and alanine in a molar ratio of 1:1:4, respectively. The fingerprints of the partial hydrolysate were compatible only with the peptidoglycan type A3α, L-Lys–L-Ala–L-Ala.

Enzymatic activities. None of the 67 *L. gasicomitatum* sp. nov. isolates changed the appearance of the skim milk-supplemented MRS agar. According to API ZYM analysis, both LMG 18811 and LMG 18812 showed the presence of β-galactosidase activity. LMG 18811 also showed esterase (C_4), esterase lipase (C_8), lipase (C_{14}), acid phosphatase, and naphthol-AS-BI-phosphohydrolase activities.

Numerical analyses of the main spoilage species based on ribopatterns and whole-cell protein patterns. Figure 1 shows the dendrograms and banding patterns of the main spoilage species and the reference strains based on *Cla*I, *Eco*RI, and *Hin*dIII ribotypes, respectively. Figure 2 shows a dendrogram obtained by combining the pattern information of all three ribotypes into one numerical analysis. The result of the numerical analysis of the whole-cell protein patterns is shown in Fig. 3, and the combined information from all of the ribopatterns and whole-cell protein analysis is presented as a dendrogram in Fig. 4.

The three spoilage isolates and the reference strains of the *Leuconostoc* species formed distinct clusters in the dendrograms based on the *Hin*dIII ribopatterns (Fig. 1C) and the protein patterns (Fig. 3), indicating that these techniques generated species-specific patterns. In the dendrograms generated from numerical comparison of *Cla*I and *Eco*RI ribopatterns, only *L. citreum*, *L. pseudomesenteroides*, and the spoilage isolates formed distinct species-specific clusters (Fig. 1A and B). When equal weight is given to all three types of *Leuconostoc* ribopatterns, the analysis combining this information also resulted in distinct species-specific clusters (Fig. 2).

The *L. gelidum* type strain had the highest similarity to the spoilage isolates in the numerical analyses of combined RFLP patterns (Fig. 2) as well as in the whole-cell protein profiles (Fig. 3). In respect to the subdivision of *L. mesenteroides*, none of the numerical analyses performed correlated with the current subspecies division of the genus. The *L. argentinum* type strain was found to possess the same ribopatterns as the *L. lactis* LMG 7940 strain, and in the dendrogram based on the numerical analysis of whole-cell protein patterns, it also formed a tight cluster with *L. lactis* strains (Fig. 3). Such a close association was only seen among strains of a single species.

Phylogenetic analyses based on 16S rDNA sequence. Table 5 shows the sequence homologies of strains LMG 18811 and LMG 18812 compared with the *Leuconostoc* sensu stricto spe-

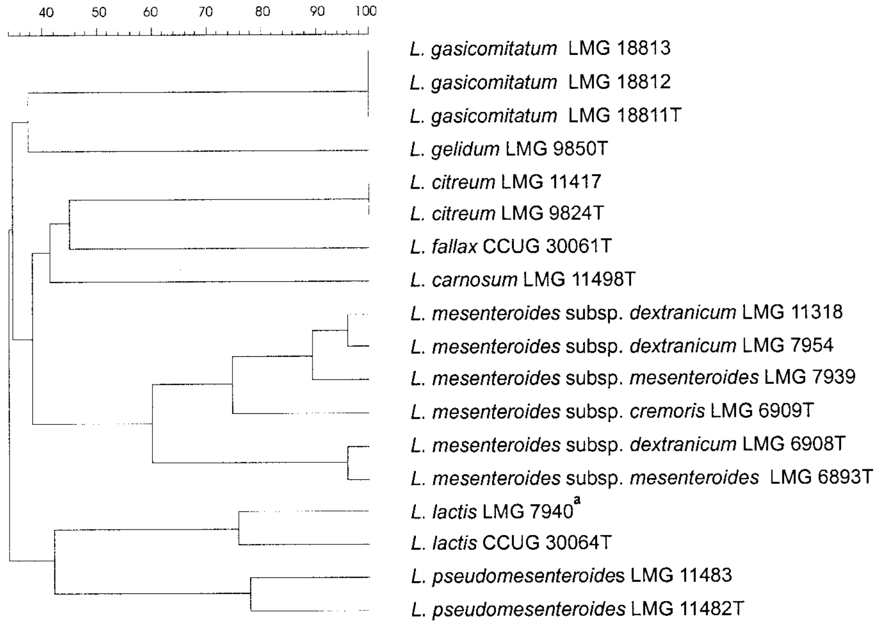

FIG. 2. Dendrogram obtained by combining the pattern information of *Cla*I, *Eco*RI, and *Hin*dIII ribotypes into one numerical analysis. [a], the *L. argentinum* type strain showed ribopatterns identical to those of *L. lactis* LMG 7940. The scale from 40 to 100 shows percentile similarity values.

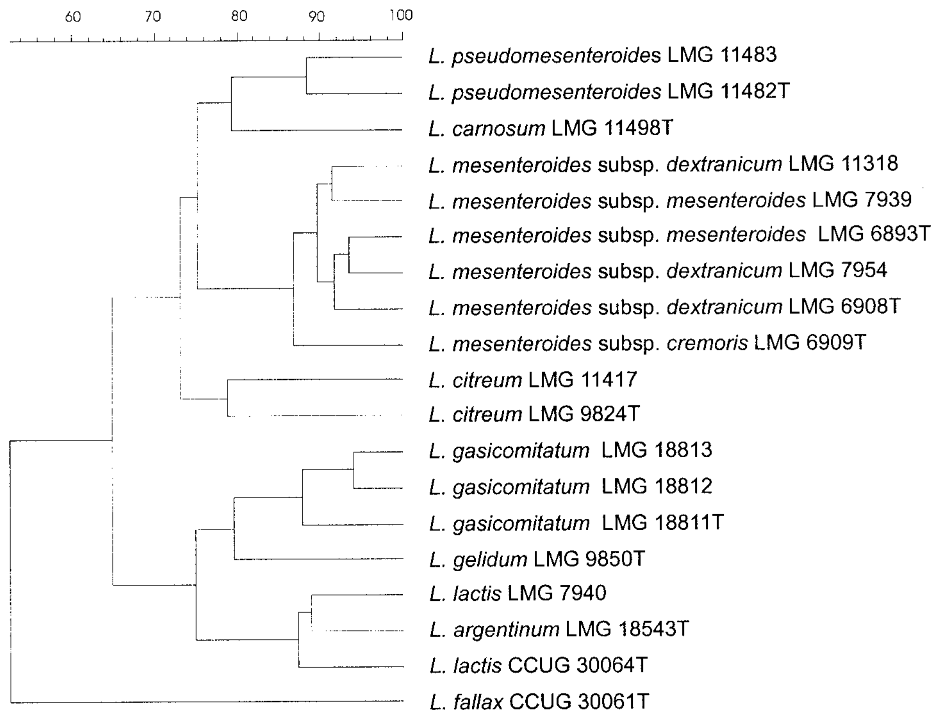

FIG. 3. Numerical analysis of whole-cell protein patterns presented as a dendrogram. The scale from 60 to 100 shows percentile similarity values.

cies. The two strains, LMG 18811 and 18812, showed 16S sequence homology of 99.3%, and the highest homology, 99.0 and 98.8%, respectively, was exhibited with the *L. gelidum* type strain.

DNA base composition and DNA-DNA hybridization results. The following DNA homology values were obtained in DNA-DNA reassociation: LMG 18811 × LMG 18812, 100%; LMG 18811 × *L. gelidum* LMG 18297T, 22%; LMG 18812 × *L. gelidum* LMG 18297^T, 34%; and LMG 18812 × *L. mesenteroides* subsp. *dextranicum* LMG 6908^T, 33%. The DNA G+C contents of strains LMG 18811 and LMG 18812 are 37 and 38 mol%, respectively.

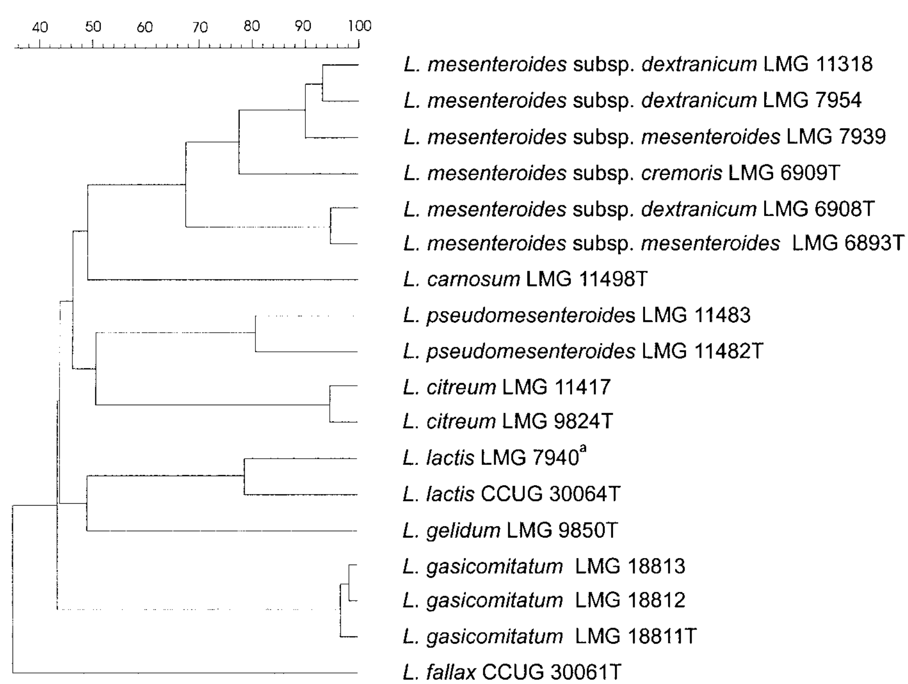

FIG. 4. Combined information from *Cla*I, *Eco*RI, and *Hin*dIII ribopatterns and whole-cell protein patterns presented as a dendrogram. [a], the *L. argentinum* type strain showed ribopatterns identical to those of *L. lactis* LMG 7940. The scale from 40 to 100 shows percentile similarity values.

TABLE 5. Homology values for a 1,491-nucleotide region of 16S rDNA

Species	Homology (%) to:											
	1	2	3	4	5	6	7	8	9	10	11	12
1. *Leuconostoc* sp. strain LMG 18811	100											
2. *Leuconostoc* sp. strain LMG 18812	99.3	100										
3. *L. argentinum* LMG 18534^T	97.1	97.1	100									
4. *L. carnosum* LMG 11498^T	98.3	98.5	97.5	100								
5. *L. citreum* LMG 9824^T	97.5	97.6	98.7	97.4	100							
6. *L. fallax* LMG 13177^T	93.6	93.7	92.4	94.3	94.5	100						
7. *L. gelidum* LMG 9850^T	98.8	99.0	97.1	98.3	97.4	93.5	100					
8. *L. lactis* LMG 8894^T	97.3	97.6	99.3	97.3	98.5	93.6	97.6	100				
9. *L. mesenteroides* subsp. *cremoris* LMG 6909^T	97.7	98.0	97.7	97.8	97.7	94.6	97.9	98.2	100			
10. *L. mesenteroides* subsp. *mesenteroides* LMG 6893^T	97.7	98.0	97.5	97.8	97.7	94.6	97.9	98.2	100	100		
11. *L. pseudomesenteroides* LMG 11482^T	97.9	98.0	97.8	97.8	97.5	94.6	98.0	98.0	99.5	99.5	100	
12. *W. paramesenteroides* LMG 9852^T	91.2	91.4	90.3	91.0	91.2	92.4	90.9	91.3	91.8	91.8	91.7	100

DISCUSSION

Gaseous deterioration caused by LAB has mainly been associated with highly acidic foods, such as fermented vegetables (16, 29) or acetic acid preserves (6, 24), but it may also affect meat products (10). Even though LAB have also been found as the dominant spoilage organisms in vacuum- or MA-packaged poultry products (34, 39, 40), the strains have never been identified to the species level. Gaseous deterioration as an LAB spoilage type in vacuum- or MA-packaged poultry products has not been reported previously. It was not surprising to find *L. sakei* and *L. curvatus* strains in the poultry product studied here. These species are very typical for all meat products (3, 4, 26, 28; Holzapfel and Gerber, Abstr. 32nd Eur. Meet. Meat Res. Workers) and might have been the *Lactobacillus* sp. population detected in the previous studies dealing with vacuum- or MA-packaged poultry products (17, 33, 39, 40). *L. carnosum* and *L. gelidum* are also quite common species occurring in vacuum- or MA-packaged cold-stored meat products (43, 49). Two species occurring in the spoiled poultry product were unusual LAB species for meat products. *L. gasicomitatum* sp. nov. was the main spoilage species characterized in this study, and the identification of the gram-positive rod-shaped organism will be carried out as a separate study.

In addition to the novel species, this LAB spoilage also showed unique properties. Normally, in a case of clear LAB spoilage, the pH of the product decreases due to lactic acid formation, but in this case an increase of pH was detected. This type of LAB spoilage was first reported by Meyer (30) in canned fish marinades. He called it "protein swell" and distinguished it from "carbohydrate swell," where increased acidity and CO_2 formation result from heterofermentative utilization of glucose. In protein swell, proteins are decomposed by proteolytic enzyme action, and the subsequent decarboxylation of amino acids leads to enhanced CO_2 production. Therefore, the LAB having an effect on gas production in protein swell may also possess homofermentative glucose metabolism. Decrease in acidity related to protein swell has been attributed to production of ammonia by bacterial deamination of amino acids.

Protein swell has also been reported to affect anchovy-stuffed olives (19), but to our knowledge there are no previous reports of this type of spoilage affecting any type of meat product. The previous studies of protein swell have associated the main component of the spoilage LAB with the decarboxylation reaction (19, 30) and considered protein hydrolysis to be due to endogenous fish enzymes. The initial hydrolysis of muscle proteins has also been attributed to endogenous enzymes, mainly cathepsins, and the bacterial activity has been associated with the degradation of oligopeptides and free amino acids (32, 46). The proteolytic systems of various meat-related LAB are poorly known, and the abilities of *L. sakei* and *L. curvatus* to degrade myofibrillar proteins have only recently been studied (15, 37). These species have been shown to possess peptidase activity and also to express strong amino acid metabolism (15, 33, 37, 38), and even though they were not the major components of the spoilage population, they may have played a major role in this case.

Leuconostocs have not yet been detected as dominant species in protein swell, which makes their predominance intriguing. *Leuconostoc* species produce gas (CO_2) during normal glucose fermentation, and in this case, the extreme bulging may have resulted from complicated interaction between various LAB species and the endogenous muscle-associated enzymes. Whether the *Leuconostoc* component alone, or in association with the endogenous muscle proteinases, could induce the gaseous spoilage remains unknown. There are no data on the proteolytic systems of meat-associated leuconostocs. The main *Leuconostoc* component did not show proteolytic activity on the skim milk-supplemented MRS agar, but due to the substrate specificity of proteolytic systems, more complicated techniques should be used for the evaluation of the proteolytic effect on myofibrillar proteins. In the spoiled product, the LAB counts were exceptionally high (10^{10} CFU/g). Two factors, the marinade and the small rise in pH, may have played major roles in facilitating the growth of LAB. The marinade had a tomato base, which contains growth stimulants for some LAB (50). The plant was simultaneously processing poultry strips in other marinades, such as honey based, but only the tomato-marinated product showed gaseous deterioration. The carbohydrates and protein hydrolysates in the marinade may have provided nutrients facilitating pronounced growth. This spoilage problem was overcome by stabilizing the marinade pH with another type of additive. Apparently this change had an effect on the growth of leuconostocs, which are generally not as acid tolerant as *Lactobacillus* species.

Leuconostoc sensu stricto comprises *L. argentinum*, *L. carnosum*, *L. citreum*, *L. gelidum*, *L. lactis*, *L. mesenteroides* (three subspecies: *cremoris*, *dextranicum*, and *mesenteroides*), and *L. pseudomesenteroides*, showing 97 to 99% 16S rDNA sequence homology. In addition, an atypical leuconostoc, *L. fallax*, possessing 94 to 95% 16S rDNA homology with the other sensu stricto species, has been described. Our results show high 16S rDNA sequence homology between *Leuconostoc* sensu stricto and the main spoilage species, clearly assigning it to the genus *Leuconostoc*. The highest 16S rDNA sequence homology (98.8 and 99.0%) was displayed with *L. gelidum*, and the lowest was with *L. fallax* (93.6 and 93.7%). According to these data, *L. gasicomitatum* sp. nov. is in the same evolutionary branch as *L. gelidum* and *L. carnosum*.

In addition to the 16S rDNA sequence homology, whole-cell protein analysis and combined 16 and 23S rDNA RFLP showed *L. gelidum* to possess the highest similarity to *L. gasicomitatum* sp. nov. According to the phenotyping schema of Villiani et al. (47), this species should be regarded as *L. gelidum*, whereas API CHL analysis identified it as *L. mesenteroides* subsp. *dextranicum*. DNA-DNA reassociation experiments with *L. gelidum* and *L. mesenteroides* subsp. *dextranicum* showed, however, that the spoilage strains clearly represent a different species. Considering all of the results, status as a novel species is warranted. The results show that the identification of leuconostocs may demand special methods. It is difficult to distinguish between *L. carnosum* and *L. gelidum*, and as can be seen from the phenotypic reactions, identification of the new species follows the same lines. These three species have similar growth temperature characteristics and share the same peptidoglycan type, and only some of the carbohydrate fermentation reactions provide differences among them (Table 3). Due to the variability seen in the sugar fermentation reactions within a *Leuconostoc* species (13), these reactions are not, however, absolute. The conserved nature of the genes encoding 16S RNA in the genus *Leuconostoc* does not enable species identification based on sequence comparison of complete 16S rDNA. Therefore, DNA-DNA reassociation has been considered to be the only reliable method to distinguish *L. carnosum* from *L. gelidum* (13).

In this study, we used ribotyping and whole-cell protein analysis for the characterization of *Leuconostoc* strains. Numerical analysis of total cellular proteins is a generally accepted tool for speciation of bacteria, and we have also previously used ribotyping for LAB identification (4, 6, 7, 25) with good results. Whole-cell protein analysis, and particularly *Hin*dIII ribotyping, provided species-specific clustering results for the *Leuconostoc* reference strains and the new taxon (Fig. 1C and 3). *Hin*dIII digestion resulted in evenly distributed banding patterns, providing a reliable matrix for numerical analysis. When *Eco*RI was used, only a small number of high-molecular-weight fragments were obtained (Fig. 1B), subjecting the numerical analysis to errors due to the limited differences in the mobilities of these fragments. The banding patterns created by *Cla*I were densely located within each other (Fig. 1A) and thus were not optimal for numerical analysis. The locations of the rDNA genes in many of the *Leuconostoc* species seem to be very conserved, providing only little variation between different strains. In our previous study dealing with *L. carnosum*, 29 different *Sma*I macrorestriction patterns showed the same ribotype (7). Here also, spoilage isolates possessing different REA patterns yielded the same *Cla*I, *Eco*RI, and *Hin*dIII ribotypes. Due to the highly consistent *Hin*dIII ribopatterns, ribotyping is a good tool for *Leuconostoc* identification, but none of the three enzymes allowed good strain typing results. It can be concluded that numerical analysis of protein patterns and *Hin*dIII-based ribopatterns can both be used for the identification of *Leuconostoc* species. The only limiting factor currently associated with these approaches is the lack of well-characterized reference strains. This is the case especially in respect to *L. gelidum*.

Our study also shows that the species-specific clustering obtained in numerical analyses of 16 and 23S rDNA RFLP and whole-cell protein patterns results in clusters which do not correlate with phylogenic branches based on 16S rDNA homology. When the phylogeny of *Leuconostoc* sensu stricto is placed under more precise scrutiny, three evolutionary branches are distinguished on the basis of 16S rDNA homology (42). One of these branches contains *L. carnosum* and *L. gelidum*, another contains *L. citreum* and *L. lactis*, and the third contains *L. mesenteroides* and *L. pseudomesenteroides*. As can be seen in patterns 1 to 4, the species-specific clusters never reflect the phylogenic branching of the 16S rDNA tree. Figures 2 and 3 also show that the dendrograms of whole-cell protein patterns and the combined 16 and 23S rDNA RFLP are different even though both techniques provide species-specific clustering. The consensus of both techniques results in another type of dendrogram (Fig. 4). This shows clearly that the percentile values of different numerical analyses should not be considered comparable and that these techniques should be used for species-level identification and not for deducing phylogeny.

The *L. argentinum* type strain possessed ribopatterns that were identical and whole-cell protein profiles that were almost identical to those of *L. lactis* LMG 7940. Such a close association was otherwise seen only among strains belonging to the same species. E. Falsen, curator of the Culture Collection of the University of Gothenburg (personal communication), has also observed similarity between *L. argentinum* and *L. lactis* strains. He detected protein profile similarities between the *L. argentinum* and *L. lactis* type strains and also similar API profiles (API rapidID32strep, API 50 CHL, and API ZYM). If the *L. argentinum* type strain is authentic, *L. argentinum* may be a doubtful species. The high 16S rDNA sequence homology (99.3%) that the two type strains show also supports this doubt.

The polyphasic approach used in this study showed clearly that the major spoilage species was a *Leuconostoc* sp. possessing the highest similarity to *L. gelidum*. The low homology values in DNA-DNA reassociation experiments clearly distinguished it from *L. gelidum*. Based on these results, this species is considered to be a distinct, novel *Leuconostoc* species for which we propose the name *L. gasicomitatum*.

Description of *L. gasicomitatum* sp. nov. *Leuconostoc gasicomitatum* (ga.si.co.mi.ta′tum. N. L. neut. n. *gasium* gas, L. neut. adj. *comitatum* accompanied, N. L. adj. *gasicomitatum* accompanied by gas, referring to the association with gaseous spoilage). Gram-positive, nonmotile, and non-spore-forming spherical or oval cells, 0.5 to 1 μm in diameter. Colonies are small, grayish white, and catalase negative. Growth occurs at 4 and 15°C, is slow at 30°C, and does not occur at 37°C. Heterofermentative; produces gas from glucose. More than 95% of the produced lactate is the D-(−)-isomer. Arginine is not hydrolyzed. Slime is produced from sucrose. Does not grow in the presence of 6.5 to 12% NaCl. The peptidoglycan type is A3α, L-Lys–L-Ala–L-Ala. L-arabinose, ribose, D-xylose, glucose, fructose, mannose, α-methyl-D-glucoside, *N*-acetylglucosamine, esculin, cellobiose, maltose, melibiose, sucrose, trehalose, raffinose, gentiobiose, turanose, and 5-keto-gluconate were fermented. Some isolates ferment galactose and gluconate. Glycerol, erythritol, D-arabinose, L-xylose, adonitol, β-methyl-D-xyloside, galactose, sorbose, rhamnose, dulcitol, inositol, mannitol, sorbitol, α-methyl-D-mannoside, amygdalin, arbutin, salicin, lactose, inulin, melezitose, starch, glycogen, xylitol, D-lyxose, D-tagatose, fucose, arabitol, gluconate, and 2-keto-gluconate were not fermented. β-Galactosidase positive.

The G+C content of the type strain is 37%, determined by the thermal-denaturation method. Isolated from MA-packaged, tomato-marinated broiler meat strips showing extreme gaseous spoilage.

The type strain is LMG 18811 (= TB 1-10). The description of the type strain corresponds to that of the species with the exception that esterase (C_4), esterase lipase (C_8), lipase (C_{14}), acid phosphatase, and naphthol-AS-BI-phosphohydrolase activities are also present. Ferments galactose and gluconate. The type strain and strains LMG 18812, LMG 18813, and LMG 18889 have been deposited in the BCCM/LMG Bacteria Collection.

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