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Research Note

Leuconostoc carnosum Associated with Spoilage of Refrigerated Whole Cooked Hams in Greece

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ABSTRACT

A polyphasic taxonomic approach was used to identify a major atypical group of gas-forming, arginine-negative lactic acid bacteria associated with spoilage of whole (nonsliced) refrigerated (4°C) cooked hams produced in two Greek industrial meat plants. Biochemical characterization revealed that the ham isolates shared their phenotypic properties with *Leuconostoc carnosum, Weissella viridescens,* and *Weissella hellenica.* However, gas chromatographic analysis of cellular fatty acids clearly differentiated the ham isolates from the *Weissella* spp. None of the isolates contained eicosenoic acid (n-C20:1), which is typically synthesized by *W. viridescens,* but all strains contained high amounts of C19cycl acid, which is absent in *W. hellenica* and has been found in trace amounts in *W. viridescens.* All strains had similar cellular fatty acid profiles, which were qualitatively similar to those of the cellular fatty acids of *L. carnosum.* In addition to the phenotypic and chemotaxonomic tests, three representative isolates were studied using a lactic acid bacteria database, which employs 16S and 23S *Hin*dIII restriction fragment length polymorphism patterns as operational taxonomic units in a numerical analysis. The isolate patterns were identical to those of the *L. carnosum* type strain, NCFB 2776^T. Based on the polyphasic taxonomic approach, the dominating lactic acid bacteria group was identified as *L. carnosum.*

Lactic acid bacteria (LAB) are the most important bacteria associated with spoilage of vacuum- or modified-atmosphere–packaged cured, cooked ready-to-eat meat products *(8, 17, 22).* Sliced cooked ham is a whole-muscle cured pork meat that is very popular in many countries but also is very sensitive to LAB spoilage *(6, 24).* Ham and other whole-muscle ready-to-eat cured meats are technologically different from emulsion-type sausages; hams contain more water and less fat and salt, are brine injected and then tumbled, and may be cooked at core temperatures of <70°C in boilers or steamers without smoking *(22, 25).* These processing characteristics seem to select for obligatory heterofermentive LAB, mainly *Leuconostoc* spp., which may produce gas and slime and cause rapid visible spoilage during refrigerated storage *(25, 34). Leuconostoc mesenteroides* subsp. *mesenteroides* and *Leuconostoc carnosum* were found to be the main spoilage LAB of sliced vacuum-packaged cooked ham in Greece *(24)* and Finland *(6),* respectively. In sliced Greek hams, *L. carnosum* has always been a minor part of the spoilage flora under vacuum and is usually overgrown by *L. mesenteroides.* However, the persistence of *L. carnosum* tends to increase at the precooking stage *(24)* or when the ham slices are stored in air-permeable packages *(25).* Conversely, strains of the *Lactobacillus sakei/curvatus* group dominate and *Weissella viridescens* is favored in smoked cured whole meats and emulsion sausages *(23, 25).*

During a previous study on the LAB spoilage ecology of industrially manufactured Greek ham *(24),* we isolated a major atypical group of 62 *Leuconostoc*-like bacteria that were phenotypically closer to *L. carnosum* but shared a poor sugar fermentation ability; particularly, they shared with *W. viridescens* the inability to ferment trehalose. This group of bacteria dominated the spoilage flora of whole molded hams, which served as nonsliced controls for the sliced vacuum-packaged hams prepared from the same production runs and stored at 4 or 12°C *(24).* In the sliced samples, however, those atypical *Leuconostoc*-like strains were sporadic and overgrown by postprocess contaminating *L. mesenteroides* strains during storage *(24).* More recently, similar atypical *Leuconostoc*-like isolates were again predominant on whole cooked hams but were undetectable and overgrown by *L. mesenteroides* during 4°C storage of sliced vacuum-packaged hams from another Greek industrial plant *(25).* Therefore, these unidentified *Leuconostoc*-like bacteria may be the most significant LAB causing spoilage of whole cooked hams in Greece. The present study was undertaken to resolve the taxonomy of these bacteria using a polyphasic approach *(32)* based on the combined use of biochemical and cellular fatty acid (CFA) profiles and numerical analysis of 16S and 23S *Hin*dIII restriction fragment length polymorphism (RFLP) patterns (ribotypes).

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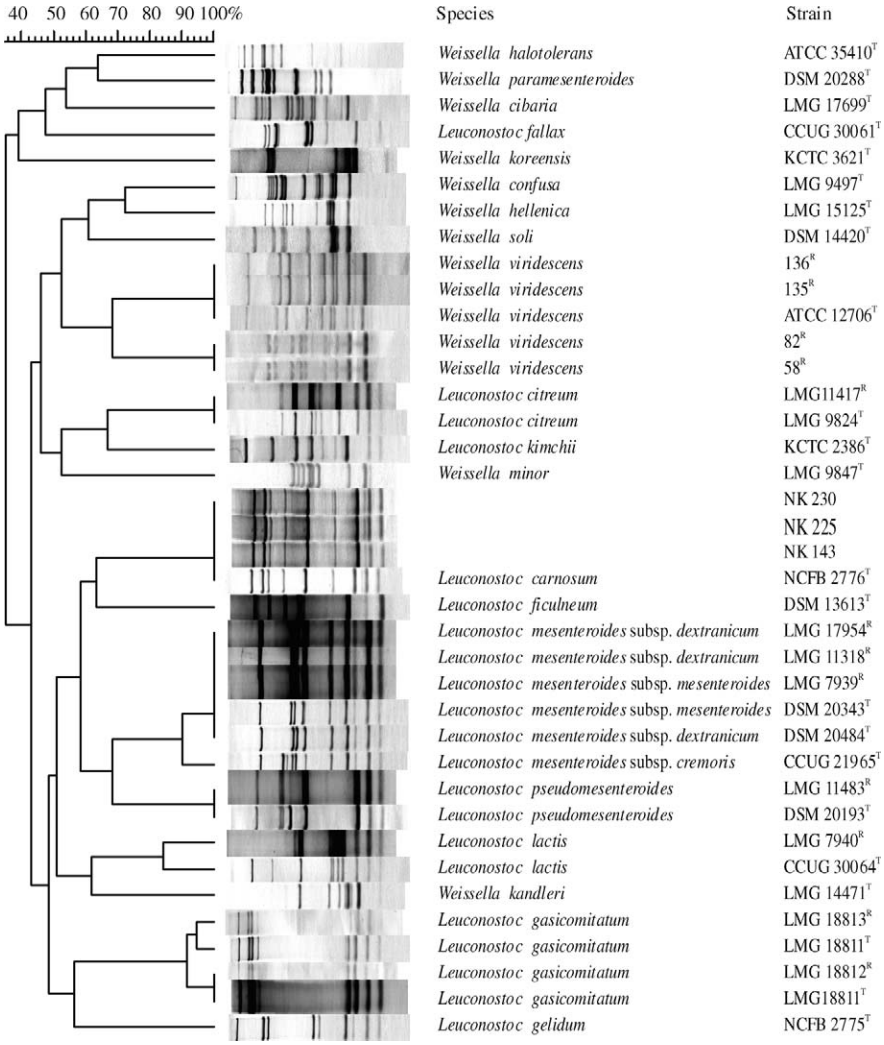

FIGURE 1. *Dendrogram based on the numerical analysis of the 16S and 23S* Hind*III RFLP patterns (ribotypes) of strains NK 143, NK 225, and NK 230 isolated from spoiled whole cooked hams and the reference strains of* Leuconostoc *and* Weissella *spp. R, reference strain; T, type strain.*

MATERIALS AND METHODS

Ham isolates. Eighteen strains of unidentified *Leuconostoc*-like bacteria associated with spoilage of whole cooked hams were included in this study. Eight of the strains (NK 124, NK 143, NK 145, NK 162, NK 164, NK 171, NK 187, and NK 196) were randomly selected to represent the major homogenous group of atypical *Leuconostoc*-like bacteria (62 isolates) described by Samelis et al. at a meat plant *(24).* The remaining 10 strains (NK 225, NK 227 through NK 231, and NK 235 through NK 238) were also randomly selected to represent a similar atypical group of *Leuconostoc*-like bacteria that were predominant (85%; 51 of 60 total LAB isolates) on whole hams at a second meat plant *(25).* In both plants, hams were made from deboned whole muscle lean pork from the hind leg *(24, 25).* Cooked ham processing and the microbiological attributes of whole hams in the first plant were discussed previously *(24).* In the second plant, raw hams in 3-kg molds were brine injected, tumbled, stuffed, and cooked in boilers to a final core temperature of 68°C *(25, 27).* After 30 and 60 days at 4°C, the spoilage flora of whole molded hams, which served as controls for the corresponding sliced samples *(25),* was dominated by LAB at average populations of 7.4 and 8.3 log CFU g^{-1}, respectively. LAB growth was accompanied by a decrease in the pH of whole hams from 6.3 to 6.1 and 5.9, respectively. After 60 days at 4°C, the molded hams accumulated gas in the package and developed a typical ''fermenting'' off-odor, a green discoloration within 1 to 2 h after opening of the packs, and a mild sour taste *(25).* Sixty LAB isolates (30 colonies per sampling day) were randomly taken from deMan Rogosa Sharpe (MRS) agar plates after 30 and 60 days of storage of whole hams at 4°C and were characterized during preliminary experiments conducted in parallel with the biochemical characterization of the primary LAB spoilage flora isolated from sliced hams *(25).* Fifty-one of the 60 isolates were similar to the atypical ham *Leuconostoc*-like strains from our previous study *(24),* and the 10 strains included in this study were chosen randomly. All 18 strains were subcultured in MRS broth at 30°C for 24 h, checked for purity by streaking on MRS agar plates, and maintained in MRS broth plus 20% glycerol at −30°C until further testing. Working cultures were kept on MRS agar slants at 4°C *(26).*

Reference strains. The type strains of the meat LAB species that were phenotypically similar to the predominant *Leuconostoc*-like bacteria from the whole cooked hams, i.e., *L. carnosum* NCFB 2776^T, *W. viridescens* NCIMB 8965^T, and *Weissella hellenica* NCFB 2973^T, were used in all experiments. Additional *Leuconostoc* and *Weissella* reference strains retrieved from the LAB database were used as operational taxonomic units for the numerical analysis of the 16S and 23S *Hin*dIII RFLP patterns (Fig. 1).

Biochemical identification. Identification of ham isolates was according to established phenotypic criteria *(23–26).* Strains were tested for cell morphology, Gram and catalase reactions, gas production from glucose, arginine hydrolysis, growth at 10, 37, and 45°C and in 8 and 10% NaCl, final culture pH at 30°C, slime

formation, and fermentation in miniplates of L-arabinose, cellobiose, galactose, lactose, maltose, mannitol, melibiose, raffinose, ribose, sucrose, sorbitol, trehalose, and xylose (Merck, Darmstadt, Germany, or Sigma, St. Louis, Mo.). The test methods were those of Samelis et al. *(26).* The configuration of lactic acid from glucose was determined enzymatically (D/L lactate kit; R-Biopharm AG, Darmstadt, Germany). All tests were done in triplicate except the sugar fermentations, which were recorded twice in miniplates at 30°C (as usually used for meat LAB *(26)*) and at 25°C. Incubation at 25°C was added to account for the psychrotrophic nature of several meat LAB species, which may restrict their fermentation ability at 30°C. In addition, the whole sugar fermentation patterns of 3 of the 18 strains (NK 143, NK 225, and NK 230), which were randomly selected for molecular identification, were determined by the API 50 CHL method (bioMérieux, Marcy l'Etoile, France) at 25°C.

Gas chromatography of CFAs. The presence of differentiating fatty acids in the cellular lipid fraction of all 18 strains was tested according to the method of Samelis et al. *(28).* CFAs were extracted and converted into methylesters using the method of Rementzis and Samelis *(21).* Gas chromatographic analysis of the methylesters was carried out, and the reproducibility of the gas chromatography method was checked as described previously *(28).*

Numerical analysis of *Hin*dIII ribopatterns. Three randomly selected strains (NK 143, NK 225, and NK 230) were analyzed for ribotype. DNA was isolated using the modified *(4)* guanidium thiocyanate method of Pitcher et al. *(20). Hin*dIII restriction endonuclease enzyme (New England Biolabs, Beverly, Mass.) cleavage of DNA, agarose electrophoresis, genomic blotting, probe labeling *(7),* and hybridizations were performed as described by Björkroth and Korkeala *(4). Hin*dIII enzyme was chosen because it provides species-specific patterns for various meat spoilage LAB *(1, 2, 4–6, 16).* Numerical analysis of the digitized ribopatterns (ScanJet 4c/T tabletop scanner, Hewlett-Packard, Boise, Idaho) was performed with the Bionumerics 4.01 software package (Applied Maths, Kortrijk, Belgium). Based on internal controls, 1.5% position tolerance and 0.5% optimization was allowed for the bands (patterns). The similarity between all pairs was expressed by Dice coefficient correlation, and the unweighted pair-group method with arithmetic averages (UPGMA) was used for the construction of the dendrogram. The ribopatterns were compared with the corresponding patterns in the LAB database at the Department of Food and Environmental Hygiene of the University of Helsinki, which contains patterns of all meat-associated spoilage LAB in the genera *Carnobacterium, Lactobacillus, Leuconostoc, Enterococcus,* and *Weissella (1, 2, 5, 6, 15, 16).* Identification of the isolates was based on the location of the type and reference strains within the clusters.

RESULTS AND DISCUSSION

Biochemical identification. Ham isolates formed a homogeneous phenotypic group, which shared its main physiological and biochemical properties with the genus *Leuconostoc* and the arginine-negative branch of the genus *Weissella* (gram-positive, catalase-negative small coccobacilli producing gas and D-lactic acid from glucose but no ammonia from arginine) *(3, 10).* All strains grew at 4 and 10°C, but none grew at 45 or even 37°C; thus, they were considered psychrotrophic. None grew in 8 or 10% salt, and all produced slime from sucrose. The final culture pH in MRS broth ranged from 4.57 to 4.63, which was higher than that of *L. mesenteroides, L. sakei,* and other aciduric meat LAB *(26).* Fermenting ability was also poor; of the 13 sugars tested by the miniplate method, only ribose and sucrose were fermented (Table 1). Strains required at least 5 to 10 days of incubation at 30°C to show a clearly positive reaction with trehalose, and a delayed but also very weak fermentation of maltose was observed (Table 1). Because of these delays in fermentation, this group of atypical *Leuconostoc* spp. was originally reported as negative for fermentation of maltose and trehalose *(24),* because results of sugar reactions were recorded after only 5 days at 30°C *(24, 26).* When miniplates were incubated at 25°C, the ability of most strains to ferment trehalose and maltose was enhanced. In accordance with this finding, a growth temperature of 30°C has been reported to be unfavorably high for *L. carnosum* and *Leuconostoc gelidum,* which are psychrotrophs and cannot grow at 37°C *(31).* Overall, comparison of the biochemical properties of the ham isolates with those of *L. carnosum, W. viridescens,* and *W. hellenica* revealed that the ham isolates phenotypes are closer to *L. carnosum* (Table 1).

To confirm sugar fermentation reactions and incubation temperature effects, we tested strains NK 143, NK 225, and NK 230 with the API 50 CHL strips incubated at 25°C. All three fermented D-glucose, D-fructose, and gluconate in addition to ribose and sucrose (Table 1). Fermentation of trehalose and maltose was clearly positive in strains NK 225 and NK 230 but remained weak and delayed in strain NK 143. Two of the strains also fermented D-mannose, *N*-acetyloglucosamine, salicin, and cellobiose and hydrolyzed esculin. None fermented glycerol, erythritol, D-arabinose, L-arabinose, D-xylose, L-xylose, adonitol, α-methyl-xyloside, galactose, L-sorbose, rhamnose, dulcitol, inositol, mannitol, sorbitol, α-methyl-D-mannoside, α-methyl-D-glucoside, amygdalin, arbutin, lactose, melibiose, inulin, melezitose, D-raffinose, amidon, glucogen, xylitol, α-gentiobose, D-turanose, D-lyxose, D-tagatose, D-fucose, L-fucose, D-arabitol, L-arabitol, 2-keto-gluconate, and 5-keto-gluconate. The above reactions suggested that these ham spoilage isolates were *L. carnosum.* However, this species is reported as gluconate negative *(31),* whereas our strains were gluconate positive, like *W. hellenica* (Table 1). Thus, it was necessary to confirm the biochemical classification by using more advanced identification tools.

CFA profiles. Samelis et al. *(28)* reported that *W. hellenica* and *W. viridescens* are differentiated from *Weissella paramesenteroides* and meat-associated *Leuconostoc* spp. by their CFA profiles. Although both *W. hellenica* and *W. viridescens* contain zero to low (<3% of total CFA) amounts of C19 cycl, *W. paramesenteroides, L. mesenteroides, L. carnosum,* and *L. gelidum* contain adequate (>5 to 10% of total CFA) to large amounts (>10 to 30% of total CFA) of C19 cycl *(28, 31).* In addition, *W. viridescens* synthesizes eicosenoic (n-C20:1) acid, which was not found in any other *Leuconostoc* or arginine-negative *Weissella (28, 31).* On this basis, the CFA profiles of the 18 atypical ham isolates were determined and compared with those of biochemically close type strains (Table 2). Unlike the type

TABLE 1. *Close phenotypic similarity between atypical* Leuconostoc*-like bacteria isolated from Greek whole cooked hams and* Leuconostoc carnosum *and the arginine-negative species* Weissella hellenica *and* Weissella viridescens[a]

Characteristic	Ham isolates	*Leuconostoc carnosum*[b]	*Weissella hellenica*[c]	*Weissella viridescens*[d]
Acid produced from:				
L-Arabinose	−	−	+	−
Cellobiose	−	V	−	−
Galactose	−	−	−/((+))d	−
Gluconate	+	−	+	−/(+)
Maltose	((+))d	V	+	+
Mannose	V	V	+	+
Melibiose	−	V	−/((+))d	−
Ribose	+	V	−	−/((+))
Salicin	V	V	−	−
Sucrose	+	+	+	V
Trehalose	+d/(+)d	+	+	V
Dextran formation	+	V	−	−
Lactic acid configuration	D	D	D	DL

[a] All three reference species and the ham isolates fermented glucose and fructose. None fermented lactose, mannitol, raffinose, sorbitol, and xylose. +, 90% or more of strains were positive; −, 90% or more of strains were negative; V, 11 to 89% of strains were positive; d, delayed reaction; ( ), weak reaction; (( )), very weak reaction.

[b] Data from Shaw and Harding *(31).* The type strain NCFB 2776^T produces slime from sucrose and is positive with ribose and mannose but negative with cellobiose, maltose, melibiose, and salicin.

[c] Data from Collins et al. *(10). W. hellenica* strains may show very weak and delayed reactions with galactose and melibiose.

[d] Data from Kandler and Weiss *(14)* and Collins et al. *(10).* The type strain NCIMB 8965^T tested in our laboratory was weakly positive with ribose and gluconate. This species is described as ribose and gluconate positive in the 8th edition of the *Bergey's Manual,* but these reactions were not confirmed by Kandler and Weiss *(14).*

strain NCIMB 8965 of *W. viridescens,* all ham isolates contained C19 cycl but no C20:1 in their CFA fraction. The ham isolates were also clearly differentiated from *W. hellenica* by possession of C19 cycl (Table 2). Overall, their CFA profiles were similar to each other and to those of *Leuconostoc* sensu stricto, which have most CFAs in common with *W. paramesenteroides (28, 30).* Specifically, all strains contained qualitatively the same CFAs as does *L. carnosum (31)* but at different proportions (Table 2). Although 40% of the meat strains originally described as *L. carnosum* contained 3.5 to 5.5% C17 acid, type strain NCFB 2776^T does not *(31).* Consequently, the only major qualitative differences in CFA profile between our ham strains and strains of *L. carnosum* is their high C18:0 content (Table 2) versus its absence in the *L. carnosum* species description *(31).* However, this discrepancy and quantitative differences in the CFA proportions may be due to variations in the basal media used to culture the strains before CFA extraction *(21).* Shaw and Harding *(31)* used trypticase soy broth, whereas we used MRS broth with Tween 80, a source of oleic acid that may affect the synthesis of CFAs with 18 and 19 carbon atoms *(13).*

16S and 23S *Hin*dIII RFLP patterns. Figure 1 shows the dendrogram obtained from the numerical analysis of strains NK 143, NK 225, and NK 230 and the reference *Leuconostoc* and *Weissella* strains. The patterns of these three isolates are identical to that of the *L. carnosum* type strain NCFB 2776^T and can be clearly distinguished from those of the other species (Fig. 1). Therefore, these three isolates were considered strains of *L. carnosum.*

Applied aspects. A polyphasic taxonomic approach was used to verify that the major group of atypical *Leuconostoc*-like bacteria that dominated the spoilage flora of refrigerated (4°C) whole cooked hams produced in Greece *(24, 25)* were *L. carnosum.* This bacterium and other *Leuconostoc* spp. often are the major or dominant members of the spoilage LAB of fresh, marinated, or even cured meat products *(2, 8, 11, 29, 31, 34). L. carnosum* in particular has been responsible for the spoilage of sliced Finnish cooked hams in vacuum packs *(6).* However, in sliced Greek hams *L. carnosum* has always been among the LAB species partially or fully overgrown during storage by postprocess contaminating strains of *L. mesenteroides* subsp. *mesenteroides* and *L. sakei (22, 24, 25).* In accordance with our findings for commercial hams, Vermeiren et al. *(33)* found that *L. carnosum* grew very slowly compared with *L. mesenteroides* subsp. *mesenteroides* when inoculated on a model cooked ham stored anaerobically at 7°C. It seems, therefore, that the growth potential of *L. carnosum* in commercial cooked hams may be high when postprocess contamination with *L. mesenteroides* is avoided. Apparently, the lack of slicing and further handling of slices in the plant cutting room prevented whole molded Greek hams from being contaminated with *L. mesenteroides*, enabling *L. carnosum* to grow and dominate after 30 to 60 days of storage. Additional in-plant studies are required to determine the

TABLE 2. *Relative percent cellular fatty acid composition of 18 atypical* Leuconostoc carnosum *strains isolated from whole cooked hams and type strains of* L. carnosum *and phenotypically closely related arginine-negative* Weissella *spp.*[a]

Strain[b]	% of total fatty acids							
	C14:0	C16:0	C16:1	C17:0	C18:0	C18:1	C19cycl	C20:1
Ham isolates[b]								
NK 124	7.59	22.31	7.14	7.18	5.99	17.06	32.73	0.00
NK 143	6.22	22.29	7.43	6.08	15.76	22.62	19.60	0.00
NK 145	5.20	20.84	5.27	5.27	23.76	17.57	22.09	0.00
NK 162	5.88	20.66	7.15	3.76	11.10	28.36	23.09	0.00
NK 164	5.99	19.69	7.87	3.26	8.47	24.51	30.21	0.00
NK 171	5.00	20.34	7.10	2.74	13.70	27.12	24.00	0.00
NK 187	5.25	21.80	6.69	8.95	16.52	21.23	19.56	0.00
NK 196	5.56	19.33	6.72	3.43	11.93	22.50	30.53	0.00
NK 225	7.89	21.84	9.39	5.04	7.82	29.85	18.17	0.00
NK 227	10.44	19.25	9.56	4.68	3.50	17.84	34.73	0.00
NK 228	6.18	22.44	7.56	4.43	19.18	22.92	17.29	0.00
NK 229	5.09	22.52	6.90	3.93	16.54	24.69	20.33	0.00
NK 230	5.54	18.47	6.13	5.98	10.99	20.45	32.44	0.00
NK 231	5.42	19.30	6.16	3.77	16.19	25.95	23.21	0.00
NK 235	5.04	22.29	6.16	4.44	15.74	25.67	20.66	0.00
NK 236	4.46	24.70	5.74	5.18	14.17	28.16	17.59	0.00
NK 237	3.59	24.16	5.87	3.60	17.14	28.89	16.75	0.00
NK 238	5.30	23.15	5.80	1.91	15.58	29.21	19.05	0.00
L. carnosum								
NCFB 2776[Tc]	5.50	34.50	18.50	0.00	0.00	35.50	4.50	0.00
W. viridescens								
NCIMB 8965[Td]	1.09	9.37	7.46	1.83	5.94	58.71	0.00	14.95
W. hellenica								
NCFB 2973[T]	2.83	18.62	7.32	3.26	3.38	64.59	0.00	0.00

[a] Each percentage is the average of four repetitions (chromotograms): two cultures per strain × two extracts per culture (coefficient of variation < 5%).

[b] Strains NK 124 through NK 196 are part of the atypical *Leuconostoc*-like group of 62 ham isolates reported by Samelis et al. *(24).* Strains NK 225 through NK 238 are similar atypical isolates obtained from whole cooked hams at another meat plant by Samelis et al. *(25).*

[c] NCFB, National Collection of Food Bacteria, Reading, UK.

[d] NCIMB, National Collection of Industrial and Marine Bacteria, Aberdeen, UK.

exact route of *L. carnosum* contamination of whole cooked hams. This LAB is psychrotrophic, potentially oligotrophic, and primarily associated with fresh chilled meat *(31).* Samelis et al. *(24)* reported a 20% persistence of typical *L. carnosum* strains among the LAB present on raw pork meat after it was butchered for making hams. Thus, a few *L. carnosum* cells might have survived boiling of whole hams or might have contaminated these hams after cooking, e.g., during cooling, if the molds had not been tightened firmly or had become loose during the cooking operations. *Carnobacterium* spp. were predominant in raw tumbled hams prior to molding; however, these bacteria were minimized after cooking *(24).* Thus, boiling of hams might have been selective in inactivating the carnobacteria *(24),* which are more heat sensitive *(19)* than *L. mesenteroides, L. sakei, L. curvatus,* most *W. viridescens (9, 12, 18, 19),* and potentially *L. carnosum.* Unfortunately, the existing data do not allow for direct comparison of the in situ thermal resistance of *L. carnosum* with that of *Carnobacterium* or other *Leuconostoc* and *Weissella* species in ready-to-eat meats. Further research is therefore needed to determine *D*-values of the main meat spoilage LAB under the same cooking conditions in commercial or model cured meat products.

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