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Isolation and characterization of *Carnobacterium*, *Lactococcus*, and *Enterococcus* spp. from cooked, modified atmosphere packaged, refrigerated, poultry meat

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Abstract

The microbiota of commercially produced, cooked and modified atmosphere packaged poultry meat was followed during storage at 3.5°C for up to 7 weeks. The dominant microbiota consisted of *Lactococcus raffinolactis* (117 isolates), *Carnobacterium divergens* (61 isolates), *Carnobacterium piscicola* (11 isolates), *Lactococcus garvieae* (four isolates), *Lactococcus lactis* (one isolate) and *Enterococcus faecalis* (three isolates). All isolates were screened for production of bacteriocins. Only *C. piscicola* isolates produced an inhibitory substance active against other lactic acid bacteria and against several *Listeria* spp. Species-specific polymerase chain reaction (PCR) primers were used for the differentiation of *Carnobacterium, L. raffinolactis*, *L. lactis*, and *L. garvieae* strains associated with the modified atmosphere packaged poultry products. No false PCR products were observed with other closely related bacterial species.

Keywords: Lactic acid bacteria; Meat; Modified atmosphere packaged; PCR; 16S rRNA

1. Introduction

Lactic acid bacteria (LAB) are the dominant microbiota in modified atmosphere packaged (MAP) meats (Shaw and Harding, 1984; Borch and Molin, 1988; McMullen and Stiles, 1989; von Holy et al., 1992). *Carnobacterium* spp., *Lactobacillus* spp., *Pediococcus* spp., and *Leuconostoc* spp. are the main genera associated with spoilage of these products. Several identification methods and keys have been proposed for different LAB (Schillinger and Lücke, 1987a; Döring et al., 1988; Montel et al., 1991); however, atypical strains are often encountered and many of these have been reclassified in new genera such as *Carnobacterium* spp. (Collins et al., 1987) and *Weissella* spp. (Collins et al., 1993), or described as new species such as *Leuconostoc gelidum* and *Leuconostoc carnosum* (Shaw and Harding, 1989).

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Recently, strains of *Lactococcus lactis*, an organism traditionally associated with dairy and vegetable products, were isolated from fermented sausages (Rodriguez et al., 1995), raw pork (Garver and Muriana, 1993) and vacuum-packed seafood (Mauguin and Novel, 1994).

Traditional classification methods based on morphology, physiology, and biochemical tests are time consuming and may be misleading. In recent years, several changes have occurred in the classification of LAB, and phylogenetic approaches to identification have become a major part of taxonomic studies (Stiles and Holzapfel, 1997). Automated nucleic acid sequencing methods and the availability of computational tools and publicly accessible genomic databases have accelerated the development of genetic-based methods for the identification of microorganisms. The identification of different species of LAB using the polymerase chain reaction (PCR) has been reported by several research groups (Klijn et al., 1991; Brooks et al., 1992; Betzl et al., 1990; Ehrmann et al., 1994; Hertel et al., 1991, 1993; Nissen et al., 1994; Tilsala-Timisjärvi and Alatossava, 1997). The standard approach has been to amplify ribosomal RNA sequences and then to confirm with species-specific DNA probes targeted to the 16S, 23S, or the 16S to 23S rRNA internal transcribed spacer regions.

This work reports the phenotypic and PCR-based identification to the species level of *Lactococcus* and *Carnobacterium* spp. isolated from cooked, ready-to-eat modified atmosphere packaged (MAP) poultry stored at 3.5°C.

2. Materials and methods

2.1. Source of organisms

Fresh chicken legs were prepared at a commercial poultry processing plant located in southwestern Ontario. Raw samples were injected with a commercial brine (sodium phosphate, sodium chloride, flavors) to between 30 and 35% of the original weight, and were steam-cooked to an internal temperature of 82°C as measured with a digital thermometer (PDT300, UEI, Beaverton, OR) inserted at the thigh joint. The cooking time was approximately 27 min. Samples were cooled to an internal temperature of 12°C by passing them through a blast freezer on a conveyor belt. The approximate cooling time was 23 min. Individual pieces were aseptically packaged in barrier foam trays with a gas impermeable lining and top film (W.R. Grace, Cryovac Division, Mississauga). The internal film liner of the tray had a moisture vapor transmission rate (MVTR) of 13.85 gm/m^2 in 24 h at 37.8°C and 100% relative humidity (RH) and an O_2 transmission rate (OTR) of 5.5 cc/m^2 in 24 h at 22.8°C and 0% RH. The top film had a MVTR of 7.69 gm/m^2 in 24 h at 37.8°C and 100% RH, and an OTR of 20 cc/m^2 in 24 h at 22.8°C and 0% RH. Packages were evacuated and back flushed with a food grade mixture of 40:60 CO_2:N_2 (Liquid Carbonic, Mississauga) using a Multivac packaging machine (Multivac Space, Sepp Hagenmüller, Wolfertschwenden, Germany). MAP samples were stored at 3.5 ± 0.5°C for up to 7 weeks and were analyzed in duplicate on a weekly basis. The study was repeated three times at one-month intervals.

At weekly intervals, untrained panelists (employees at the production plant) evaluated the overall acceptability of the stored MAP product which was held at 3.5°C. MAP cooked poultry samples were heated in a microwave oven prior to serving. Panelists were asked to evaluate the acceptability of poultry samples based on odor and flavor.

A sample (11 g) was aseptically cut from the joint area with a sterile scalpel (Fisher Scientific, Pittsburgh, PA) and homogenized in 99 ml of sterile 0.1% peptone water for 2 min using a Stomacher Homogenizer (Colworth Stomacher 400, Seward Medical, London). Serial dilutions were prepared in sterile 0.1% peptone water and surface plated in duplicate on standard plate count agar (SPCA, Difco) for the enumeration of mesophilic aerobes, mesophilic anaerobes and psychrotrophs. Plates for mesophiles counts were stored at 22°C for 48 h and plates for psychrotrophs counts were stored at 3.5°C for 10 days.

Colonies were selected on the basis of their appearance, from SPCA plates of the highest enumerated dilution of aerobes, anaerobes, and psychrotrophs. A total of 206 colonies was selected and checked for purity by streaking on agar plates and observing cellular morphology. Individual isolates were differentiated by observation of cellular morphology using phase-contrast microscopy, Gram-

staining, catalase, and oxidase reactions. Isolates were kept at −80°C in tryptic soy broth with 20% glycerol (w/v) for long-term storage. A total of 197 Gram-positive, catalase and oxidase-negative isolates was further identified as described below. The other nine isolates were Gram-negative, catalase-positive, oxidase-negative rods, and those were further identified using the Vitek Jr. system (Vitek Systems, bioMérieux Vitek, Hazelwood, MO) according to the manufacturer's instructions.

Microscopic examinations of the 197 Gram-positive isolates showed that 72 were short to medium rods, occurring either as single cells or in pairs while the remaining 125 isolates were single, pairs, or chains of cocci or ovoid-shaped cells.

2.2. Phenotypic identification

2.2.1. Cultures and growth conditions

Gram-positive isolates and reference strains of lactic acid bacteria were subcultured in MRS broth with acetate omitted (modified MRS, MMRS; Hammes et al., 1991) at 30°C. *Listeria* spp. were subcultured in TSA broth at 30°C.

Gram-positive isolates were classified according to the scheme shown in Fig. 1. The phenotypic tests included: phase-contrast microscopy of cell shape and arrangement, catalase and oxidase tests, motility, production of gas from glucose, growth in anaerobic conditions, production of ammonia from arginine, growth in MMRS broth at 4, 40, and 45°C, growth in MMRS broth adjusted to pH 5.0, 5.5, 9.0 and 9.5, growth in MMRS broth with 3 or 6.5% NaCl, production of dextran from sucrose, growth on acetate agar, presence of *meso*-diaminopimelic acid in the cell wall, enantiomer of lactic acid produced from glucose, growth in the presence of sodium azide and 2,3,5-triphenyltetrazolium chloride (TTC), and carbohydrate fermentation patterns (Barakat, 1998).

2.2.2. Production of antibacterial substances

All isolates were screened for the production of antibacterial substances, by the deferred antagonism method (Harris et al., 1989). Isolates were screened against *Carnobacterium* and *Enterococcus* reference strains, several strains of *L. monocytogenes* and other *Listeria* spp. (Table 1) and against 10 representative

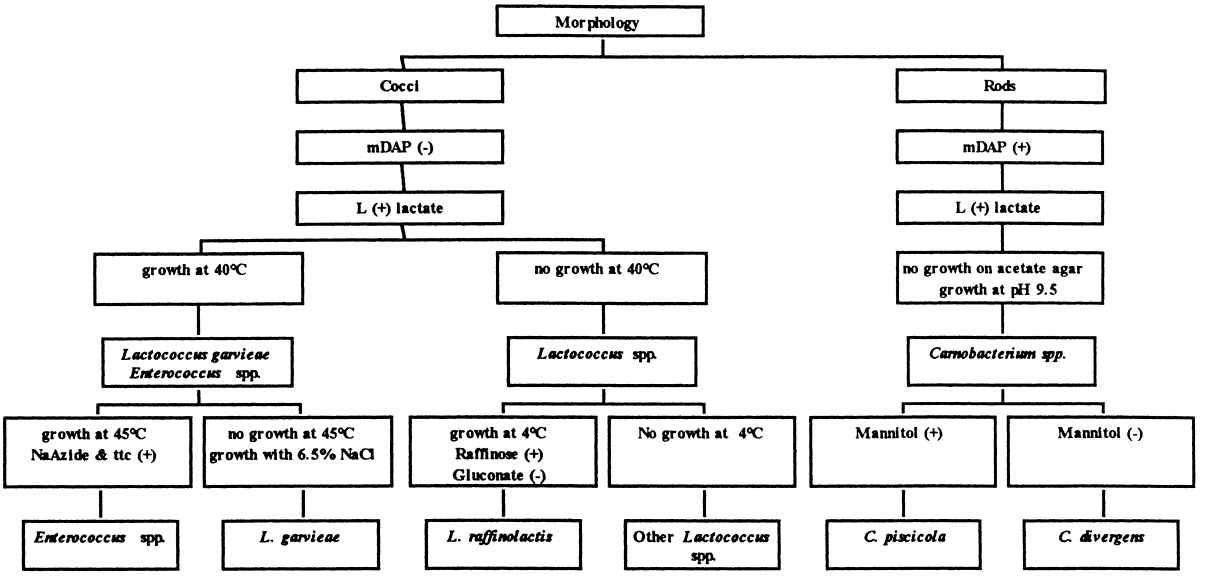

Fig. 1. Scheme used for the preliminary identification of lactic acid bacteria isolated from cooked, modified atmosphere packaged, poultry stored at 3.5°C.

Table 1
Reference strains used in this study

Strain	Reference
Bacillus cereus	ATCC 14579[a]
Brochothrix thermosphacta	ATCC 11509; type strain
Carnobacterium divergens	ATCC 35677; type strain
C. divergens	UAL 278[b]
C. divergens	LV13[c]
C. gallinarum	ATCC 49517; type strain
C. mobile	ATCC 49516; type strain
C. piscicola	UAL 26
C. piscicola	UAL 43225
C. piscicola	UAL 43224
C. piscicola	ATCC 35586; type strain
C. piscicola	LV17[c]
C. piscicola	UAL8
Enterococcus faecium	ATCC 19434; type strain
E. faecalis	ATCC 19433; type strain
E. durans	ATCC 19432; type strain
Lactobacillus buchneri	ATCC 9460; type strain
L. casei	ATCC 11578
L. sake	DSM 20017[d]
L. acidophilus	ATCC 4356; type strain
L. delbruckii	ATCC 9649
L. brevis	ATCC 3648
Lactococcus garvieae	NCFB 2155[e]; type strain
L. piscium	NCFB 2778; type strain
L. plantarum	NCFB 1869; type strain
L. raffinolactis	NCFB 617; type strain
L. lactis ssp. *lactis*	ATCC 7962
L. lactis ssp. *lactis*	ATCC 11454
L. lactis ssp. *cremoris*	ATCC 14365
Leuconostoc mesenteroides	Y105[f]
L. gelidum	UAL-187[g]
Listeria monocytogenes	LI[h] 500 ATCC 15313, ST 1/2b; rabbit
L. monocytogenes	LI 503 ATCC 19113, ST 3a; human isolate
L. monocytogenes	LI 512 Serotype (ST) 1/2b; meat isolate
L. monocytogenes	LI 514 ST 1/2b; meat isolate
L. monocytogenes	LI 521 Murray B, ST 4b; meat isolate
L. monocytogenes	LI 527 Scott A, ST 4b; patient/outbreak
L. monocytogenes	LI 531 ST 4b; Switzerland/outbreak
L. monocytogenes	LI 533 ST 4b; LCDC; coleslaw/outbreak
L. monocytogenes	LI 540 ST 1/2b; salami
L. monocytogenes	LI 564 ST 4b; cheese/outbreak
L. monocytogenes	LI 576 ST 1/2b; meat isolate
L. welshimerii	ATCC 35897; type strain
L. innocua	ATCC 33091
L. grayi	ATCC 19120; type strain
L. murrayi	ATCC 25401; type strain
L. ivanovii	ATCC 19119; type strain
Micrococcus luteus	ATCC 7468
Pediococcus acidilactici	PAC 1.0[i]
Streptococcus mitis	NCFB 2495; type strain
Weissella viridescens (previously *L. viridescens*)	ATCC 12706

[a] ATCC, American Type Culture Collection (Rockville, MD).
[b] UAL, University of Alberta Lactic Acid Bacteria Collection, University of Alberta (Edmonton, AB).
[c] Strains originally from Dr. B.G. Shaw, Institute of Food Research, Bristol UK; obtained from Dr. M.E. Stiles, University of Alberta.
[d] DSM, Deutsche Sammlung von Mikroorganismen, Braunschweig, Germany.
[e] NCFB, National Collection of Food Bacteria (Reading, UK).
[f] Héchard et al., 1992; obtained from Dr. M.E. Stiles, University of Alberta.
[g] Hastings and Stiles, 1991; obtained from Dr. M.E. Stiles, University of Alberta.
[h] *Listeria* spp. were obtained from Dr. A. Lammerding (Health of Animals Laboratory, Health Canada, Guelph, ON).
[i] Gonzalez and Kunka, 1987; obtained from Dr. M.E. Stiles, University of Alberta.

strains among the poultry isolates. The spot-on-lawn assay was used to determine the presence or absence of inhibitory activity and to determine the natures of the inhibitory substances produced (Hastings and Stiles, 1991).

2.3. Identification of lactococci and carnobacteria using species-specific PCR primers

2.3.1. Sequence analysis

16S rRNA sequences were retrieved from the sequence databases at the National Center for Biotechnology Information (NCBI) using the RETRIEVE E-mail server (Bethesda, MD). Primer similarity searches were performed using the NCBI BLAST E-mail server (Altschul et al., 1990). The PC/GENE® (IntelliGenetics, Mountain View, CA) program was used for sequence alignments to determine variable regions of the 16S rRNA.

2.3.2. Bacterial strains

The reference strains used in this study are listed in Table 1. Reference strains were maintained on APT agar (Difco) plates at 4°C and transferred monthly. For DNA extraction, bacteria were cultured overnight in APT broth or agar at 22°C for *Brochothrix thermosphacta*, and 30°C for all other strains.

2.3.3. PCR primers

Primer sequences, orientations and annealing positions are summarized in Table 2. Primers were obtained from a commercial supplier (MOBIX, McMaster University, Hamilton, ON). A universal forward primer, 27f, and three reverse primers, Cdi, Cmo, and Cpg, were used to amplify target regions (198–199 bp) of the 16S rDNA of *C. divergens*, *C. mobile*, and *C. piscicola*/*C. gallinarum*, respectively. The species-specific reverse primers were designed from the domains of least homology among species of carnobacteria. Primers Cdi and Cmo were previously used by Brooks et al. (1992) for the identification of *Carnobacterium* spp. by DNA hybridization. *C. piscicola* and *C. gallinarum* share more than 96% homology in the 16S rRNA and the Cpg primer will amplify sequences from both *C. piscicola* and *C. gallinarum*. To differentiate between the two species, a forward primer, Cga, was designed for *C. gallinarum*. Cga and Cpg were used to amplify a 128-bp region of the 16S rRNA of *C. gallinarum*.

For the identification of *Lactococcus* spp., forward primer 27f and three species-specific reverse primers were used to amplify target sequences of *L. lactis*, *L. garvieae*, and *L. raffinolactis*. Primer pairs 27f–Lla (for *L. lactis*), 27f–Lga (for *L. garvieae*) and 27f–Lra (for *L. raffinolactis*) should give products of 87, 90, and 203 bp, respectively. Primers Lla and Lga were described by Klijn et al. (1991).

To ascertain the presence of genetic material in the crude DNA extracts, PCR reactions were set up using 16S rDNA-targeted universal primers 27-forward (27f) and 100-reverse (100r).

2.3.4. Amplification of target DNA

Taq DNA polymerase, amplification buffers, and deoxynucleoside triphosphates were purchased from Boehringer-Mannheim (Mannheim, Germany).

Table 2
Sequences and positions of the universal and specific primers used for PCR

PCR primer	Primer sequence (5′ to 3′)	Position[a]	Orientation
27f	AGAGTTTGATCMTGGCTCAG[b]	8–27	Forward
100r	ACTCACCCGTTCGCYRCTC[b]	100–118	Reverse
Cdi[c]	GCGACCATGCGGTCACTTGAA	185–206	Reverse
Cga	GGAAAGCTTNCTTTCTAACC[b]	77–97	Forward
Cmo	TCCACCAGGAGGTGGTTGGAGT	184–206	Reverse
Cpg	GAATCATGCGATTCCTGAAAC	184–205	Reverse
Lla[d]	CAGTCGGTACAAGTACCAAC	72–91	Reverse
Lga[d]	CATAAAAATAGCAAGCTATC	75–94	Reverse
Lra	TGTCGAATATGCATCCAAC	189–207	Reverse

[a] *E. coli* numbering system.
[b] Mixed mers symbols M = (A,C); Y = (C,T); R = (A,G); N = (A,C,G,T).
[c] Primer sequence similar to that of Brooks et al. (1992).
[d] Primer sequences similar or identical to those of Klijn et al. (1991).

For DNA amplification, 50 μl of overnight cultures (in APT broth) were pelleted and suspended in 50 μl of sterile, ultrapure water, or colonies from agar plates were suspended in 50 μl of sterile, ultrapure water. PCR amplifications were carried out in 25-μl volumes, with the following reagents: 1× *Taq* buffer, 0.1 mM of each dNTP, 25 ng of each primer, 0.5 μl of bacterial suspension, and 0.5 U of *Taq* DNA polymerase. Following an initial denaturation step of the template DNA at 99°C for 5 min, the remaining reaction components were added and the mixtures were subjected to 35 successive cycles of denaturation (94°C, 30 s), annealing (55°C, 45 s), and extension (72°C, 1 min) in an automated DNA thermal cycler (GeneAmp® PCR system 2400; Perkin-Elmer Cetus, Norwalk, CT). The amplification reactions were terminated by an elongation step of 3 min at 72°C. The reaction mixture was visualized on a 3% agarose gel in Tris–acetate buffer containing ethidium bromide (Sigma), and photographed on an UV transilluminator (2011 Macrovue Transilluminator, LKB, Bromma, Sweden).

3. Results

When samples of cooked poultry were aseptically packaged in 20:80 CO_2:N_2 immediately after coming out of the oven and prior to cooling, or immediately after exiting the blast freezer, microbiological counts were below the detection limit (<100 CFU/g) for all populations enumerated throughout 7 weeks of storage at 3.5°C (data not shown). Microbial populations were only observed on poultry samples that were aseptically collected at the packaging line situated downstream of the blast freezer. Initial populations were typically below or near the detection limit and increased during the storage period.

Sensory spoilage indicators were not detected in cooked MAP poultry meat after 7 weeks of storage at 3.5°C, when levels of background microbiota were 10^8 CFU/g. No off-odors were detected in samples that were left at 3.5°C for an additional month. Panelists reported that poultry meat samples had an acceptable appearance, odor, and flavor during the storage period; however, most samples did not taste as 'fresh' after 21 days of storage. Samples were deemed acceptable by panelists, even following achievement of maximal population numbers (10^8 CFU/g).

3.1. Phenotypic identification of poultry isolates

A total of 206 isolates from MAP, cooked poultry samples was selected for taxonomic characterization. Of nine Gram-negative organisms, eight were identified as *Serratia liquefaciens*, and one as *Pantoea agglomerans* (synonym for *Enterobacter agglomerans*; Holt et al., 1994a; *Bergey's Manual of Determinative Bacteriology*).

Gram-positive isolates (197) were subdivided to the genus level according to the scheme shown in Fig. 1. Organisms were identified to the species level according to the differential properties listed in Bergey's Manual of Determinative Bacteriology (Holt et al., 1994b,c).

3.1.1. Characterization of Gram-positive rods

Isolates were slender rods, occurring singly, in pairs or short chains. Pairs of cells often formed a broad-angle V-shape. All isolates were facultatively anaerobic, had *meso*-DAP in the cell wall and produced only the L-(+)-lactic acid enantiomer. They were differentiated from lactobacilli by growth at pH 9.5 in MMRS broth and by their inability to grow on acetate agar. No growth occurred at pH 5.0 in MMRS broth, but organisms grew well in 3% NaCl and more weakly in 6.5% NaCl. Growth in MMRS broth was observed at 3.5°C within 7 days, while no growth occurred at 40°C within 1 week. Isolates were classified as *C. divergens* (61 isolates) or *C. piscicola* (11 isolates) based on carbohydrate utilization patterns, most importantly mannitol fermentation (Montel et al., 1991). Gas production was observed in all *C. divergens* isolates, however, this property was inconsistent.

3.1.2. Characterization of Gram-positive cocci

A few of the isolates were small spherical cocci occurring singly or in pairs, while the majority were ovoid, occurring singly, in pairs or in short to long chains. Morphology was not easy to determine by phase-contrast microscopy, and it was necessary to resort to scanning electron microscopy to accurately determine morphology. Organisms were facultative anaerobes, did not have *meso*-DAP in the cell wall and produced only L-(+)-lactic acid. No gas pro-

duction was observed. Based on these characteristics, they were classified as either *Enterococcus* spp. or *Lactococcus* spp. The majority of isolates, 118 strains out of 125, did not grow at 40°C and were included in the *Lactococcus* genera. All but one of these presumptive lactococci grew within 10 days in broth at 3.5°C. The single isolate was classified as *L. lactis*, while the remaining 117 isolates were identified as *L. raffinolactis*, based on carbohydrate fermentation patterns. Although their growth characteristics were similar to those of *L. piscium*, they were differentiated from this organism by their inability to ferment gluconate. Presumptive *L. raffinolactis* isolates did not grow at pH 5.0, in 6.5% NaCl, or on acetate agar. Growth was observed in 3% NaCl and at pH 9.0. Isolates varied in their ability to ferment xylose, lactose, arabinose, amygdalin, melezitose, mannitol, and inulin. The remaining seven of the 125 isolates grew well at 40°C but not at 3.5°C. Of these seven isolates, three grew at 45°C but not at 50°C. Based on carbohydrate fermentation patterns and ability to grow in the presence of sodium azide and TTC, those three isolates were identified as *Enterococcus faecalis*, while the remaining four were identified as *Lactococcus garvieae*.

L. raffinolactis isolates were collected from all three trials, and they were the predominant isolates in the third trial.

3.1.3. Production of inhibitory substances

C. piscicola isolates produced an antibacterial substance active against reference strains of carnobacteria, enterococci and *Listeria*. The activity was not affected by treatment with catalase or by heating at 64°C. The activity of the inhibitory agents was lost when the supernatant was treated with pronase, indicating that inhibition was due to a proteinaceous agent.

3.2. Identification of lactococci and carnobacteria using species-specific PCR primers

The results for PCR amplification reactions using universal primers and species-specific primers are summarized in Tables 3 and 4. Amplification products using universal primers were obtained with all reference strains tested (Table 1). The PCR products expected are 199 bp in length for *C. divergens*, 198 bp for *C. mobile* and *C. piscicola*, and 128 bp for *C. gallinarum* (with the Cga–Cpg primer pair). Expected product sizes are 87 bp for *L. lactis* ssp. *lactis*, 90 bp for *L. garvieae*, and 203 bp for *L. raffinolactis*. No false positives were observed with any of the other bacterial species tested. Figs. 2 and 3 illustrate the results obtained with the *Carnobacterium* and *Lactococcus* species-specific primers, respectively.

Results of the PCR-amplification reactions from presumptive *Carnobacterium* poultry meat isolates were consistent with the biochemical test results. With mannitol-negative strains, identified as *C. divergens*, a PCR product was observed only with the 27-Cdi primer pair, while for mannitol-positive isolates identified as *C. piscicola*, a PCR product was obtained exclusively with the 27-Cpg primer pair.

Table 3
Differentiation of *Carnobacterium* species using 16S rDNA-targeted PCR primers

Species	No. tested	Primer pairs				
		27f–Cdi	Cga–Cpg	27f–Cmo	27f–Cpg	27f–100r
Carnobacterium divergens[a]	3	+	−	−	−	+
C. gallinarum[a]	1	−	+	−	+	+
C. mobile[a]	1	−	−	+	−	+
C. piscicola[a]	6	−	−	−	+	+
Other species tested[b]	26	−	−	−	−	+
Poultry isolates						
C. divergens	20	+	−	−	−	+
C. piscicola	11	−	−	−	+	+

[a,b] Reference strains (Table 1).

Table 4
Differentiation of *Lactococcus* species using 16S rDNA-targeted PCR primers

Species	No. tested	Primer pairs			
		27f–Lla	27f–Lga	27f–Lra	27f–100r
Lactococcus lactis ssp. *cremoris*[a]	1	−	−	−	+
Lactococcus lactis ssp. *lactis*[a]	3	+	−	−	+
L. garvieae[a]	1	−	+	−	+
L. raffinolactis[a]	1	−	−	+	+
Other species tested[b]		−	−	−	+
Poultry isolates					
L. lactis	1	−	−	−	+
L. garvieae	3	−	+	−	+
L. raffinolactis	30	−	−	+	+

[a,b] Reference strains (Table 1).

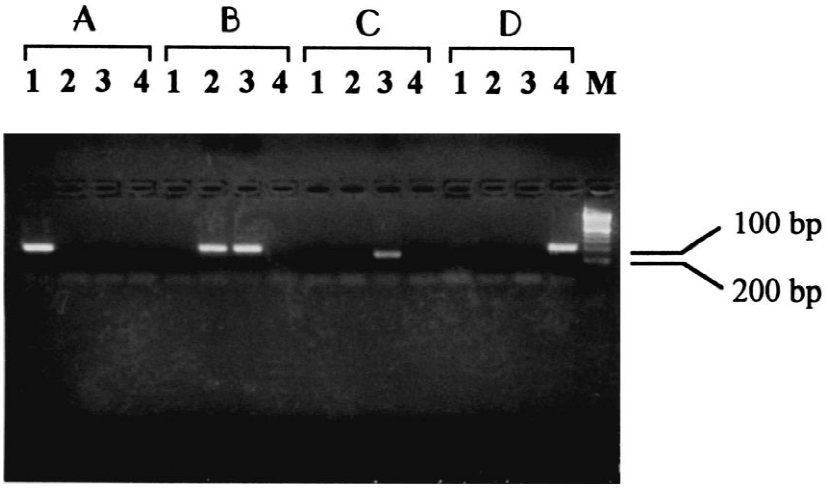

Fig. 2. PCR products from *Carnobacterium* reference strains, obtained using 16S rRNA-targeted, species-specific primers. Series: (A) 27f–Cdi primer pair; (B) 27f–Cpg primer pair; (C) Cga–Cpg primer pair; (D) 27f–Cmo primer pair. Lanes: (1) *C. divergens* ATCC 35677; (2) *C. piscicola* ATCC 35586; (3) *C. gallinarum* ATCC 49517; (4) *C. mobile* ATCC 49516; (M) 100-bp DNA ladder.

Representative poultry isolates identified as *L. raffinolactis* gave PCR products of identical size to the product obtained with the type strain of *L. raffinolactis*. Similarly, the identity of suspect *L. garvieae* was confirmed using primer pair 27f–Lga. No product was observed with the poultry isolate identified as *L. lactis* using primer pair 27f–Lla. When control strains of *L. lactis* ssp. *lactis* and one strain of *L. lactis* ssp. *cremoris* were tested with this primer pair, only *L. lactis* ssp. *lactis* strains were positive. *L. lactis* ssp. *hordniae* was not tested. However, the work of Klijn et al. (1991) indicates that primer Lla is specific to *L. lactis* ssp. *lactis*.

4. Discussion

Phenotypic and PCR-based characterization of the dominant microbiota indicated that strains of *L. raffinolactis*, an organism seldom isolated or encountered in the literature, were present in samples of cooked, modified atmosphere packaged poultry in

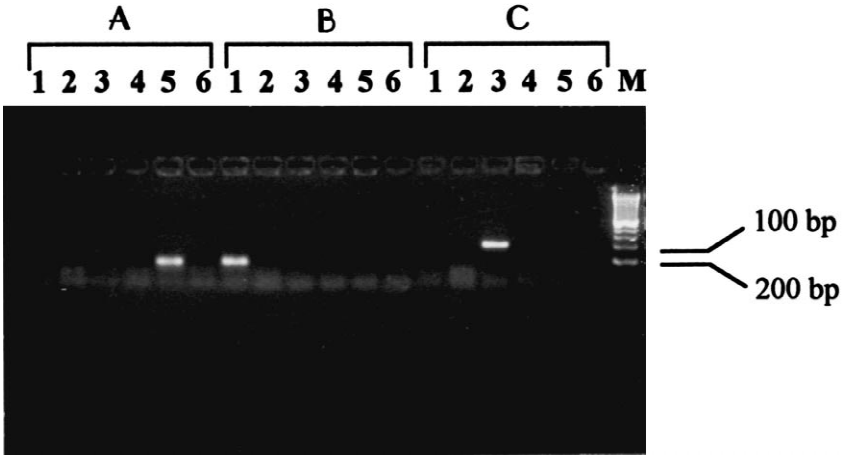

Fig. 3. PCR products from *Lactococcus* reference strains, obtained using 16S rRNA-targeted, species-specific primers. Series: (A) 27f–Lga primer pair; (B) 27f–Lla primer pair; (C) 27f–Lra primer pair. Lanes: (1) *L. lactis* ssp. *cremoris* ATCC 14365; (2) *L. lactis* ssp. *lactis* ATCC 7962; (3) *L. raffinolactis* NCFB 617; (4) *L. piscium* NCFB 2778; (5) *L. garvieae* NCFB 2155; (6) *L. plantarum* ATCC 1869; (M) 100-bp DNA ladder.

all trials. They were predominant in samples from trial three by the third week of storage. These organisms were only isolated from cooked poultry samples that were collected at the packaging line after cooling. Samples aseptically collected directly out of the cooker prior to cooling or at the exit of the blast freezer before the packaging line did not develop a microbiota during storage. The presence and dominance of *L. raffinolactis* does not appear to be a chance occurrence. These data suggest that post-cooking contamination occurred at the packaging line and *L. raffinolactis* was part of the environmental microbiota of this processing facility.

Schillinger and Lücke (1987b) documented the isolation of *L. raffinolactis* from raw, vacuum-packed (VP) beef and pork meat and noted the highly competitive behavior of that species when inoculated on VP raw meat with other LAB. It was those authors' belief that the microbiota of the cutting room influenced the development of the microbiota on the meat as *L. raffinolactis* were dominant in VP raw beef taken from one cutting room, but were seldom isolated from meat samples obtained from three other cutting rooms.

The scarcity of reports about the presence of *Lactococcus* spp. in meat products could be due to a failure to look for them and a belief that the organisms are associated with dairy and vegetable products. Isolates may be classified as 'atypical' strains or remain unidentified. The morphology of lactococci may be a factor that hinders their proper identification. Phase-contrast microscopic examination of *L. raffinolactis* isolates indicated that ovoid cells may be mistaken for rods. Morphology of selected strains of presumptive *L. raffinolactis* were confirmed by electron microscopy. Mauguin and Novel (1994) reported similar problems when identifying *Lactococcus* spp. from seafood.

A review of the taxonomy and identification of LAB indicates that reports of 'atypical' organisms are not uncommon. Until recently, *Carnobacterium* spp., previously classified as 'atypical lactobacilli' (Shaw and Harding, 1985), were believed to be associated with MAP meat products and fish (Hiu et al., 1984; Baya et al., 1991). Millière et al. (1994) reported their isolation from the surface of mould-ripened soft-cheeses. In this study, *C. divergens* strains (61) were isolated from poultry samples in the first and second trial, while *C. piscicola* (11) isolates were present in samples from the third trial. Presence of a specific *Carnobacterium* spp. was not as consistent as the presence of *L. raffinolactis* organisms. Isolates of *C. divergens* from trials one and two had identical carbohydrate fermentation patterns, which may imply that the same strain was present on the different samples of poultry.

L. garvieae and *E. faecalis* strains were only isolated from poultry samples at the end of the first

week of storage in trial three. The absence of these organisms from subsequent packages in trial three could be explained by their mesophilic nature which would have prevented them from growing at the storage temperature of the poultry products. Whether enterococci and species of lactococci other than *L. raffinolactis* are more common on meat products cannot be inferred from the results of this study.

Bacteriocin-producing carnobacteria have been extensively studied (Ahn and Stiles, 1990; Schillinger and Holzapfel, 1990; Buchanan and Klawitter, 1992) and are often isolated from meat products. All *C. piscicola* isolated from the cooked MAP poultry meat produced an inhibitory substance active against enterococci, other carnobacteria, and several strains of *L. monocytogenes*. Much interest has been directed towards the use of bacteriocin-producing carnobacteria as 'protective' cultures in food products. Although carnobacteria grew faster than *L. raffinolactis* isolates at refrigeration temperature (4°C) in a liquid medium and *C. piscicola* isolates produce inhibitory substances, these properties did not give them a competitive advantage over *L. raffinolactis* strains in the third trial. Schillinger and Lücke (1987b) demonstrated that *L. raffinolactis* isolates were the most competitive of the species when *L. raffinolactis*, *L. sake* and *C. divergens* were inoculated onto raw meat prior to vacuum-packaging and storage at 2°C for 30 days.

The method used in the current study to select 'representative' colonies from total aerobic plate counts based on color and colony appearance was subjective and depended on the judgement of the investigator. Different species of lactic acid bacteria often look similar on a general-purpose media such as plate count agar. Anyone familiar with the intensive labor required in traditional identification methods would understand the necessity for more rapid and more reliable identification methods, such as nucleic acid-based techniques. In the past years, several species of lactic acid bacteria have been reported to occur in various 'unexpected' food matrices and other habitats, and display uncharted growth patterns, leading to 'atypical' strains being reported in almost every taxonomy paper. The use of DNA-based techniques to identify bacterial organisms is becoming increasingly popular and LAB classification will be more easily achieved. For example, the use of 16S rRNA-targeted oligonucleotide probes in hybridization experiments would allow screening of whole plates within a fraction of the time required for traditional identification. Unfortunately, these methods are still restricted to large, well-equipped laboratories with a comprehensive database of oligonucleotide probes for the identification of lactic acid bacteria.

Similarity searches of the species-specific primers used for identification of *Carnobacterium* spp. showed that only the target organisms had a 100% identity with the specific primer used, with the exception of the Cpg primer. *C. gallinarum* and *C. piscicola* share $>96\%$ homology in their 16S rRNA sequences and the reverse Cpg primer anneals to both species. The Cga primer allowed the differentiation of *C. gallinarum* from *C. piscicola*. This method was very successful in differentiating between species of carnobacteria within 3 h and was based on a modification of the procedure described by Brooks et al. (1992). Those investigators used a semi-universal primer and a *Carnobacterium* genus-specific primer to amplify a 259-bp region of the 16S rDNA, followed by hybridization with species-specific probes to the PCR products. However, when aligned with sequences from other species using the BLAST E-mail server, the genus-specific primer used by Brooks et al. (1992) showed 100% match with several bacterial genera including *Vagococcus* spp., *Enterococcus* spp., and *Listeria* spp. The PCR primers used were not specific enough to differentiate *Carnobacterium* spp. from other bacterial strains. However, hybridization of species-specific oligonucleotide probes to the PCR products allowed differentiation among *C. divergens*, *C. mobile*, and *C. piscicola*/*C. gallinarum*. The latter two species could not be differentiated by this technique. Nissen et al. (1994) were able to identify carnobacteria at the genus level by nucleic acid hybridization using 16S rRNA-targeted genus-specific oligonucleotide probes. The probes showed 100% identity only with *Carnobacterium* spp.

Over the past decade, several genetic-based techniques have been proposed for the identification of *Lactococcus* spp., although interest has focused mainly on the identification of *L. lactis* species due to their importance in food processing applications. Betzl et al. (1990) used 23S rRNA-targeted oligonucleotide probes for the identification of different subspecies of *L. lactis* by colony hybridization.

Later, Beimfohr et al. (1993) used 23S rRNA-targeted probes labeled with a fluorescent compound to identify single, whole cells of *L. lactis* by epi-fluorescence microscopy. Klijn et al. (1991) used a PCR amplification step of the V1 region of lactococci, followed by hybridization of species-specific 16S rRNA-targeted oligonucleotide probes against the PCR products. This approach specifically differentiated between species of lactococci, but again requires a PCR amplification of the target region followed by dot-blot hybridization procedures. To confirm the identity of the lactococci isolated from poultry, we used one universal forward primer, and three species-specific primers. Poultry isolates that were classified as *L. lactis*, *L. garvieae*, or *L. raffinolactis* did not have biochemical profiles identical to the respective type strains and were classified based on closest match profile. Such classification methods are very time consuming and may be misleading. The method presented in this study is very specific and results can be obtained within 3–4 h. Although *L. piscium* and *L. plantarum* were not tested, an analysis of the 16S rRNA sequences of these two species shows that species-specific primers can be easily designed.

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