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Enterococcus species dominating in fresh modified-atmosphere-packaged, marinated broiler legs are overgrown by *Carnobacterium* and *Lactobacillus* species during storage at 6 °C

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Abstract

In order to show which of the initial lactic acid bacteria (LAB) contaminants are also causing spoilage of a modified-atmosphere-packaged (MAP), marinated broiler leg product at 6 °C, LAB were enumerated and identified on the 2nd and 17th days following manufacture. A total of 8 fresh and 13 spoiled packages were studied for LAB levels. In addition, aerobic mesophilic bacteria and *Enterobacteriaceae* were determined. The average CFU/g values in the 8 fresh packages were 1.3×10^3, 9.8×10^3 and 2.6×10^2 on de Man Rogosa Sharpe agar (MRS), Plate Count Agar (PCA) and Violet Red Bile Glucose agar (VRBG), respectively. The commercial shelf life for the product had been set as 12 days, and all packages analyzed on the 17th day were deemed unfit for human consumption by sensory analysis. The corresponding CFU/g averages in the spoiled product were 1.4×10^9, 1.1×10^9 and 3.9×10^7 on MRS, PCA and VRBG agar, respectively. For characterization of LAB population, 104 colonies originating from the fresh packages and 144 colonies from the spoiled packages were randomly picked, cultured pure and identified to species level using a 16 and 23S rDNA *Hin*dIII RFLP (ribotyping) database. The results showed that enterococci (35.7% of the LAB population) were dominating in the fresh product, whereas carnobacteria (59.7%) dominated among the spoilage LAB. *Enterococcus faecalis*, *Carnobacterium piscicola* and *Carnobacterium divergens* were the main species detected. In general, when the initial LAB population is compared to the spoilage LAB, a shift from homofermentative cocci towards carnobacteria, *Lactobacillus sakei/curvatus* and heterofermentative rods is seen in this marinated product.

Keywords: Lactic acid bacteria; MAP; Broiler meat; Spoilage; Ribotyping

1. Introduction

Raw, modified-atmosphere-packaged (MAP) poultry products are consumed increasingly in Finland, and approximately 80% of these products are sold marinated. Marinating, in this context, means salting

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and mixing of the meat with water–oil-based, spiced sauces. These products have wide diversity in terms of spicing and also the type of meat used varies from carcass cuts to skinned, deboned fillet strips. For consumers, the products are very convenient because a meat dish can be prepared simply by cooking the product without any additional work.

Since there are differences between the ability of different microbes to spoil foods, the concept of specific spoilage organisms (SSO) has been adopted to describe the organisms typically causing the spoilage of a certain food type (Gram et al., 2002). As typical for cold-stored, vacuum or MAP meat products (Borch et al., 1996), lactic acid bacteria (LAB) have been the SSO in MAP, marinated broiler products. They have been reported to cause radical gaseous spoilage changes in a cold-stored, MAP, tomato-marinated broiler meat strips (Björkroth et al., 2000), and they have also been detected as the dominating bacterial group in MAP, marinated broiler meat strips at the end of the commercial shelf life (Susiluoto et al., 2002). *Carnobacterium*, *Lactobacillus* and *Leuconostoc* have been the main genera associated with spoiled or end shelf-life MAP, marinated broiler meat strips (Björkroth et al., 2000; Susiluoto et al., 2002).

Previous studies have focused on the spoilage LAB population in MAP, marinated broiler meat breast fillet strips. There is no information regarding the spoilage LAB population of skin-containing, marinated carcass cuts, nor are the levels and nature of initial LAB contaminants in these products known. Some LAB species are more potent in spoiling meat products (Borch et al., 1996; Björkroth et al., 1998, 2000). Therefore, when judging the significance of initial contamination as a cause for later spoilage, analysis must be done up to the species level. Determination of LAB level in the initial phase does not reveal if a product lot contains species causing rapid spoilage during shelf life.

The purpose of this study was to determine which LAB species dominate the population in MAP, marinated, skin-containing broiler leg cuts during the initial phase of shelf life (2 days from the packaging) and later in the spoiled product. LAB were identified to species level using a 16 and 23S rDNA *Hin*dIII RFLP (ribotyping) database and, in case of unknown species, glucose fermentation type and morphology was determined. Our results show that enterococcal species dominating in initial storage phase are overgrown in the spoiled products during storage at 6 °C by carnobacteria, *Lactobacillus sakei/curvatus* and heterofermentative rods.

2. Materials and methods

2.1. Product description and sensory analysis

A total of 21 packages of MAP broiler meat legs from one production lot were stored at 6 °C. We analyzed 8 packages on the 2nd day and 13 packages on the 17th day following manufacture. One package consisted of three skin-containing leg cuts (approximately 1 kg), which had been salted, mixed with the marinade and packaged under MA conditions. The typical MA for broiler meat containing 80% CO_2 and 20% N_2 had been used for packaging. The marinade contained dry matter 20% (sucrose, spices, potassium sorbate, sodium benzoate, xanthan gum and guar gum and sodium chloride), 9% vegetable oil, 6% chicken fat, 5% acetic acid and 0.6% lactic acid and water. The pH of the fresh product varied between 6.2 and 6.7. The commercial shelf life of the product at 6 °C had been set at 12 days by the manufacturer.

Sensory evaluation was performed as described by Korkeala et al. (1987), omitting only the tasting of the spoiled packages.

2.2. Sample preparation for the microbial analysis

Prior to the analysis, odor, texture and consistency were checked and the amount of gas in each package noted. The purpose of checking was to ensure that none of the packages had leaked and were therefore possessing typical MA. For analysis, a sterile 22 g sample containing skin, muscle and marinade was homogenized in 198 ml of 0.1% peptone water using a Stomacher blender (NCFA, 1988). Serial 10-fold dilutions of the homogenized samples were made from 10^{-2} to 10^{-8} into 0.1% peptone water.

2.3. Aerobic plate count and detection of Enterobacteriaceae

Aerobic plate count was done using Plate Count Agar (PCA) medium (Difco Laboratories, Detroit,

MI, USA) and the plates were aerobically incubated for 3 days at 30 °C. *Enterobacteriaceae* were detected using Violet Red Bile Glucose agar (VRBG) medium (Oxoid, Basingstoke, England) and the plates were incubated aerobically at 37 °C for 1 day. Both determinations were made using the pour plate technique.

2.4. Enumeration of LAB and selection of LAB strains for species identification

Samples were plated on de Man Rogosa Sharpe agar (MRS) (Oxoid). The medium was inoculated using the spread plate technique and the plates were incubated in an anaerobic CO_2 atmosphere (Anaerogen, Oxoid; 9–13% CO_2 according to the manufacturer) at 25 °C for 5–6 days.

From each sample, 12 randomly picked colonies were cultured pure using MRS broth (Difco) and agar. Isolates were selected from the plates made with the two highest dilutions showing growth. In the case of the fresh and spoiled samples, these were 10^{-2} to 10^{-3} and 10^{-8} to 10^{-9}, respectively. This resulted in the growth of 248 LAB isolates, which were subjected to species-level identification. For DNA extraction, MRS broth was inoculated and the tubes were grown at 25 °C for 1–2 days depending on the growth rate. After incubation, cells harvested from 1.5 ml MRS broth were used for DNA extraction. The isolates were stored in MRS broth at −70 °C.

Table 1
Numbers and proportions of coccus-shaped lactic acid bacterium isolates distributed between different species and types identified using a 16 and 23S rRNA *Hin*dIII RFLP (ribotyping) database

Species	Ribotype	Day 2[a] from manufacture		Day 17 from manufacture	
		Number of isolates	% of total population[b]	Number of isolates	% of total population[b]
Lactococcus garvieae	I	5	4.7		
Streptococcus agalactiae	IIa	5	4.7		
	IIb	1	1.0		
Leuconostoc mesenteroides	III	1	1.0		
Pediococcus pentosaceus	IV	1	1.0		
Enterococcus gallinarum	V	4	3.8		
Enterococcus faecalis	VIa	26	25.1		
	VIb	1	1.0		
Enterococcus faecium	VII	3	2.8		
Enterococcus casseliflavus	VIIIa	1	1.0		
	VIIIb	1	1.0		
	VIIIc	1	1.0		
Lactococcus plantarum	IXa	1	1.0	2	1.4
	IXb	1	1.0	17	11.7
	IXc			1	0.7
	IXd			4	2.8
Unknown species UI	UI	1	1.0		
Unknown species UII	UIIa	2	1.9		
	UIIb	5	4.7		
	UIIc	1	1.0		
	UIId	1	1.0		
Unknown species UIII	UIII	2	1.9		
Unknown species UIV	UIVa	2	1.9		
	UIVb	3	2.8		
Unknown species UV	UV	1	1.0		
Unknown species UVI	UVIa			2	1.4
Total		70	67.3	26	18.0

All isolates originated from a modified atmosphere packaged, marinated skin-containing broiler meat leg product stored at 6 °C.

[a] The day of manufacture considered as day 0.

[b] Proportion of a species (%) counted within the isolates analyzed either on day 2 or day 17.

Table 2
Numbers and proportions of rod-shaped lactic acid bacterium isolates distributed between different species/types identified by a 16 and 23S rRNA *Hin*dIII RFLP (ribotyping) database

Species	Ribotype	Day 2[a] from manufacture		Day 17 from manufacture	
		Number of isolates	% of Total population[b]	Number of isolates	% of Total population[b]
Lactobacillus sakei subsp. *carnosus*	Xa			1	0.7
Lactobacillus sakei subsp. *sakei*	Xb			2	1.4
Carnobacterium piscicola	XIa	2	1.9	2	1.4
	XIb	4	3.8	6	4.2
	XIc	1	1.0	1	0.7
	XId			5	3.5
	XIe	4	3.8	10	6.9
	XIf	4	3.8	23	15.9
Carnobacterium divergens	XIIa			9	6.3
	XIIb	6	5.8	23	15.9
	XIIc	1	1.0	3	2.1
	XIId			2	1.4
Lactobacillus curvatus	XIIIa			1	0.7
	XIIIb			1	0.7
Carnobacterium gallinarum	XIV			2	1.4
Weissella viridescens	XV			1	0.7
Unknown species UVII	UVII	2	1.9	20	13.9
Unknown species UVIII	UVIIIa	8	7.7		
	UVIIIb	1	1.0	6	4.2
Unknown species UIX	UIX	1	1.0		
Total		34	32.7	118	82.0

All isolates originated from a modified atmosphere packaged, marinated, skin-containing broiler meat leg product stored at 6 °C.
[a] The day of manufacture considered as day 0.
[b] Proportion of a species (%) counted within the isolates analyzed either on day 2 or day 17.

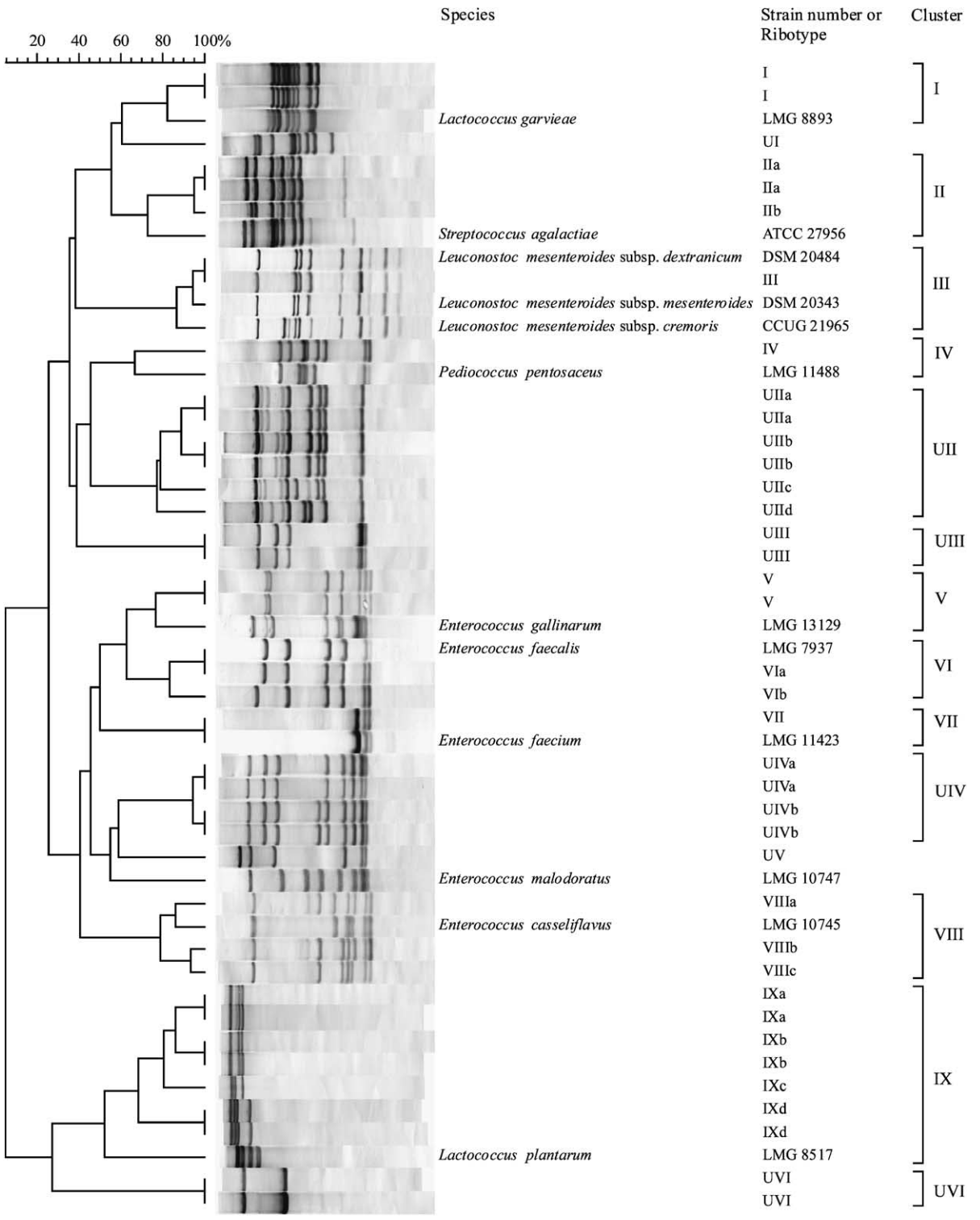
2.5. 16 and 23S rDNA *Hind*III RFLP, ribotyping

DNA was isolated by using a modified (Björkroth and Korkeala, 1996a) guanidium thiocyanate method of Pitcher et al. (1989). In this modification, the cell lysis solution contains mutanolysin (250 U/ml; Sigma, St. Louis, MO, USA) in addition to lysozyme (25 mg/ml, Sigma). Restriction endonuclease treatment of 8 μg of DNA was carried out by using *Hind*III restriction enzyme (New England Biolabs, Beverly, MA, USA) as specified by the manufacturer. *Hin*dIII was chosen because it has been found to provide species-specific patterns for various spoilage LAB (Björkroth and Korkeala, 1996b, 1997; Björkroth et al., 1998, 2000). REA was performed as described earlier (Björkroth and Korkeala, 1996a). 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, WI, USA) and Dig DNA Labeling Kit (Roche Molecular Biochemicals, Mannheim, Germany) as previously described by Blumberg et al. (1991). Membranes were hybridized at 58 °C overnight and the detection of the digoxigenin label was performed as recommended by Roche Molecular Biochemicals.

2.6. Numerical analysis of *Hind*III ribopatterns

The membranes were scanned with a Hewlett-Packard ScanJet 4c/T tabletop scanner (Boise, ID, USA). Numerical analysis of the ribopatterns was

Fig. 1. Schematic patterns of *Hin*dIII ribopatterns and the numerical analysis of the patterns presented as a dendrogram of coccus-shaped LAB from modified-atmosphere-packaged, marinated broiler leg cuts. Left side of the banding patterns, high molecular masses <23 kbp and right the low molecular mass >1000 bp.


Species
Strain number or Ribotype
Cluster
20 40 60 80 100%
I
I
Lactococcus garvieae
LMG 8893
UI
IIa
IIa
IIb
Streptococcus agalactiae
ATCC 27956
Leuconostoc mesenteroides subsp. dextranicum
DSM 20484
III
Leuconostoc mesenteroides subsp. mesenteroides
DSM 20343
Leuconostoc mesenteroides subsp. cremoris
CCUG 21965
IV
Pediococcus pentosaceus
LMG 11488
UIIa
UIIa
UIIb
UIIb
UIIc
UIId
UIII
UIII
V
V
Enterococcus gallinarum
LMG 13129
Enterococcus faecalis
LMG 7937
VIa
VIb
VII
Enterococcus faecium
LMG 11423
UIVa
UIVa
UIVb
UIVb
UV
Enterococcus malodoratus
LMG 10747
VIIIa
Enterococcus casseliflavus
LMG 10745
VIIIb
VIIIc
IXa
IXa
IXb
IXb
IXc
IXd
IXd
Lactococcus plantarum
LMG 8517
UVI
UVI
I
II
III
IV
UII
UIII
V
VI
VII
UIV
VIII
IX
UVI

performed using the Bionumerics 2.0 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

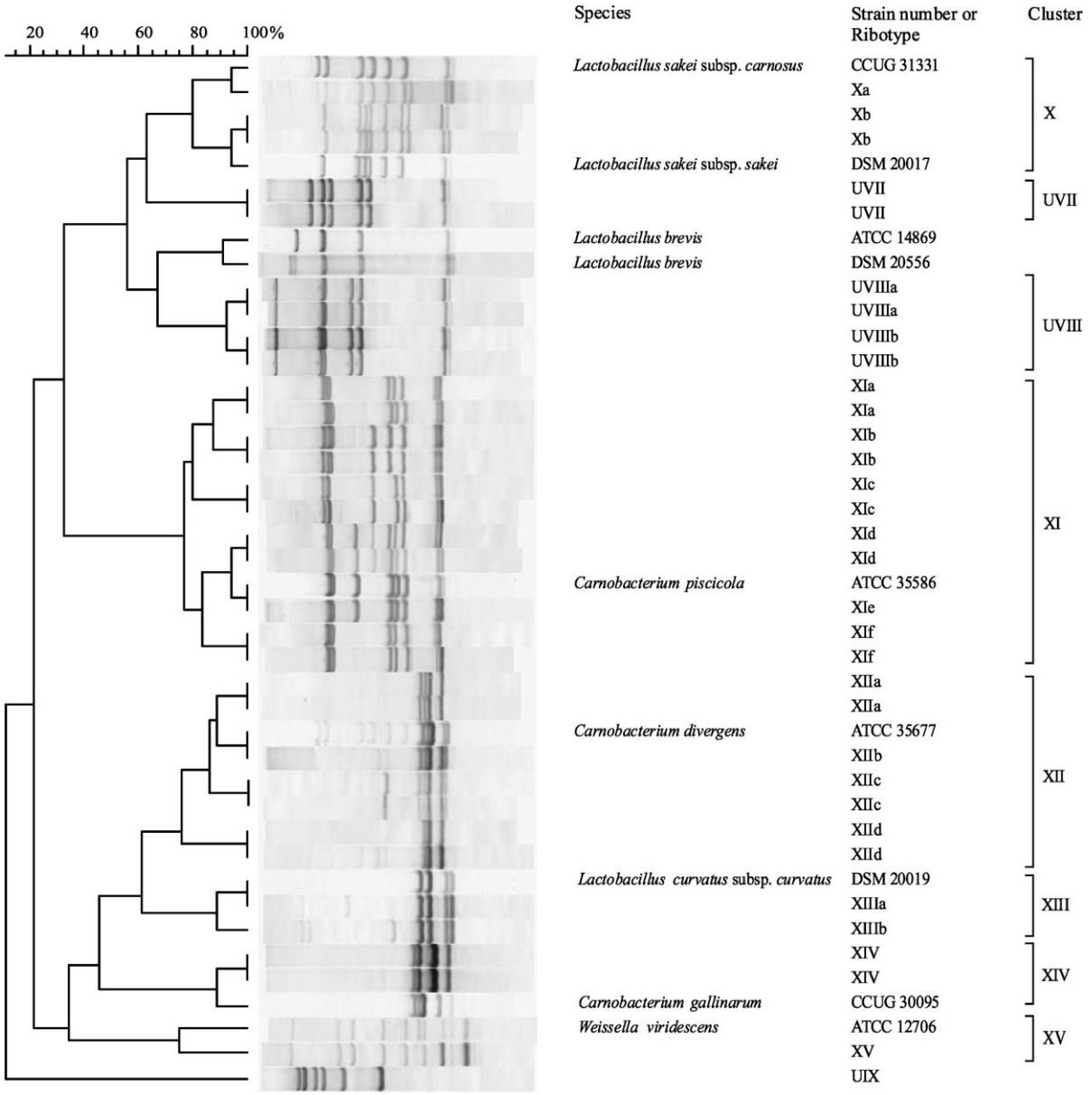


Fig. 2. Schematic patterns of *Hin*dIII ribopatterns and the numerical analysis of the patterns presented as a dendrogram of rod-shaped LAB from modified-atmosphere-packaged, marinated broiler leg cuts. Left side of the banding patterns, high molecular masses <23 kbp and right the low molecular mass >1000 bp.

Environmental Hygiene, University of Helsinki, Finland. It comprises patterns of all relevant spoilage LAB of the genera *Carnobacterium*, *Lactobacillus*, *Leuconostoc*, *Enterococcus* and *Weissella* (Björkroth and Korkeala, 1996b, 1997; Björkroth et al., 1998, 2000; Lyhs et al., 1999). Identification of the isolates was made on the basis of locations of the type strains in the clusters, taking into account pattern similarity levels seen in previous studies utilizing this database.

2.7. Phenotypic determinations

Representative strains (1–5) from all clusters were Gram-stained and tested for catalase activity. In the case of clusters remaining unknown but containing five or more isolates, 1–2 strains from each unknown type were tested for glucose fermentation type using the method of Schillinger and Lücke (1987).

3. Results

The average CFU/g values in the eight fresh packages were 1.3×10^3, 9.8×10^3 and 2.6×10^2 on MRS, PCA and VRBG media, respectively. These eight packages were all excellent or of good quality according to the sensory test, whereas all packages studied on the 17th day were deemed unfit for human consumption based on scores that evaluated their appearance, texture and smell. The corresponding CFU/g averages in the spoiled, 17-day-old products were 1.4×10^9, 1.1×10^9 and 3.9×10^7 on MRS, PCA and VRBG agar, respectively.

Tables 1 and 2 show the distribution of the isolates between coccus- or rod-shaped LAB species detected 2 and/or 17 days from manufacture. The tables also show the distribution of isolates between the different ribotypes obtained. Figs. 1 and 2 present the ribopatterns and cluster division, which was used for species delineation. The majority of the species detected in the fresh packages belonged either to the genera *Enterococcus*, *Lactococcus*, *Pediococcus* or *Streptococcus* and, with the exception of *Lactococcus plantarum*, they were not detected in the spoiled packages. *Carnobacterium piscicola* and *Carnobacterium divergens* isolates formed 21.1% of the initial contamination, whereas 58.3% of the isolates characterized from the spoilage population consisted of these species. *Carnobacterium gallinarum*, *L. sakei*, *L. curvatus* and *Weissella viridescens* strains were not detected among the initial contaminants. These species constituted 5.6% of the spoilage LAB population.

A total of nine clusters remained unidentified. Six of these (UI–UV and UIX) were associated only with the fresh packages and they were all cocci with the exception of one rod-shaped isolate possessing type UIX. The strains tested for glucose fermentation type from cluster UII showed the homolactic fermentation type. If these isolates belong to a known species, they are likely to belong to the genera of *Enterococcus*, *Lactococcus* or *Streptococcus* since our database contains all *Pediococcus* and *Leuconostoc* species commonly associated with food spoilage. The rod-shaped isolates tested for glucose fermentation type from clusters UVII and UVIII both possessed heterolactic fermentation pattern and therefore belong either to the genera of *Lactobacillus* or *Weissella*. These may well represent novel species, because all *Weissella* species and all known spoilage-causing lactobacilli are included in our database. Patterns of *Lactobacillus brevis* strains were most similar with UVIIIa and b (Fig. 2). Types UVII and UVIIIb were detected both from fresh and spoiled packages. UVII type was strongly associated with spoilage; the strains formed 13.9% of the spoilage population.

4. Discussion

As in many previous studies dealing with cold-stored, MAP meat products (Borch et al., 1996), LAB were also the dominating spoilage organisms in this MAP, marinated broiler leg product. Compared to the levels of *Enterobacteriaceae*, LAB growth was approximately two logarithmic units higher. However, in the initial population, LAB did not dominate since growth on PCA (9.8×10^3 CFU/g) was higher than on MRS agar (1.3×10^3 CFU/g).

Of the LAB in the fresh product, 67.3% were cocci. *Enterococcus faecalis* formed 26.1% of the initial population. The proportions of other enterococci, *Enterococcus casseliflavus*, *Enterococcus faecium* and *Enterococcus gallinarum* were clearly lower at 3.0%, 2.8% and 3.8%, respectively. The high proportion of enterococci (35.7%) in the fresh product strongly suggests fecal contamination during slaugh-

ter. *E. faecalis*, *E. faecium*, *Enterococcus durans* and *Enterococcus hirae* have previously been associated with the gut microbiom of poultry (Devriese et al., 1991). However, the manufacturing slaughterhouse under study is very modern and it applies good hygienic manufacturing practice. With the current poultry slaughtering lines, fecal contamination during scalding, defeathering and evisceration seems unavoidable. Entrapment of bacteria in the skin crevices and feather follicles after defeathering makes the removal of bacteria very difficult in later processing stages (Notermans and Kampelmacher, 1974; Benedict et al., 1991). Enterococci have also been considered as significant psychrotrophic spoilage organisms (Jay, 2000). Our results show that these bacteria were poor competitors in the cold-stored, MAP broiler product; they were not at all detected in the spoilage population. Therefore, even if low levels of enterococci were obtained in fresh products, it cannot be used as a criterion of good shelf life quality.

The other cocci detected in the fresh product may reflect plant-originated contamination or may be a part of the normal poultry skin bacterial population. Since there is no prior knowledge regarding the skin LAB populations of chicken or broiler, the origin of these species cannot be concluded. *L. plantarum* was the only coccoid species detected in both fresh and spoiled samples. This species was originally characterized from frozen peas, but unfortunately there is no information regarding its other habitats. It has not previously been associated with poultry products or meat spoilage, but this study shows it grows in MAP broiler meat during storage at 6 °C.

C. piscicola and *C. divergens* have often been associated with meat and fish sources. The most studied habitat of *C. divergens* and *C. piscicola* appears to be unprocessed, refrigerated red meat. These two *Carnobacterium* species have been isolated from minced meat (Holzapfel and Gerber, 1983; Shaw and Harding, 1984, 1985), refrigerated, vacuum-packaged unprocessed beef, pork and lamb (Borch and Molin, 1988; Hitchener et al., 1982; Schillinger and Lücke, 1986, 1987; Shaw and Harding, 1984, 1985), and CO_2-packed pork (Borch and Molin, 1988). They have also been reported to dominate in cooked, MAP, refrigerated, poultry meat (Barakat et al., 2000), but to our knowledge, they have not been detected to dominate the spoilage population of raw, MAP poultry products. Among the initial 1.3×10^3 CFU/g LAB contaminants, these two species formed 21.1% of the isolates collected from the plates of the two highest dilutions, whereas the corresponding part in the 1.4×10^9 CFU/g level spoilage population was 58.3%. These results show that both *C. divergens* and *C. piscicola* possess good growth capabilities in MAP, marinated broiler legs. They have also been detected in the late shelf life population of MAP, marinated broiler meat strips (Susiluoto et al., 2002), but not as the dominating component. *Leuconostoc gasicomitatum* was detected as the dominating species in MA, marinated broiler meat strip products (Björkroth et al., 2000; Susiluoto et al., 2002), and it is noteworthy that this species was not detected during the current study. These differences may be due to the higher pH of the broiler leg product and/or the presence of skin in this product.

L. sakei and *L. curvatus* have often been associated with spoilage of vacuum and MAP meat products. They were also detected in the spoilage population of the tomato-marinated broiler meat strip products (Björkroth et al., 2000) and in the late shelf life population of many MAP, marinated broiler meat strip products (Susiluoto et al., 2002). In our study, these species were not detected among the initial contaminants, but they were detected among the isolates obtained from the plates made with dilutions of 10^{-8} and 10^{-9} as was the case with *W. viridescens*. *W. viridescens* has been associated with greening of vacuum-packaged meat products (Niven and Evans, 1957) but not with spoiled MAP poultry, and the unknown rod-shaped species UVII was also detected in the spoilage population of the tomato-marinated product (Björkroth et al., 2000). It possesses heterofermentative glucose metabolism, and currently the taxonomy of this organism is being studied in order to determine whether it belongs to the genus *Lactobacillus* or *Weissella*. UVIII strains clustered next to the *L. brevis* strains (Fig. 2), and if this species is very diverse these strains may belong to *L. brevis* species.

Based on our results, the spoilage LAB population in this MAP, marinated, skin-containing broiler meat product is dominated by carnobacteria, but the population is also very diverse. In order to find means for avoiding LAB contamination and/or reducing the growth of the spoilage bacteria, it should be evaluated whether all of these species and strains types are

equally responsible for the sensory spoilage changes. Despite LAB being the dominant group in the spoiled product, *Enterobacteriaceae* levels, detected approximately two logarithmic units lower than LAB, may also play a role in the spoilage process. The great bacterial diversity, together with the small initial contamination level, makes finding of the ways to limit the LAB growth in these products extremely challenging.

5. Conclusion

Enterococci were the dominating (35.7%) initial LAB contaminants in a MAP, skin-containing, marinated broiler leg product, whereas carnobacteria were the dominating (59.7%) contaminants among spoilage LAB population. In general, when the initial LAB population of this product is compared to the LAB associated with the spoiled product, a shift from homofermentative cocci towards carnobacteria, *Lactobacillus sakei/curvatus* and heterofermentative rods is observed.

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