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Research Note

Lactic Acid Bacteria in Marinades Used for Modified Atmosphere Packaged Broiler Chicken Meat Products

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ABSTRACT

Lactic acid bacteria (LAB) in some marinades commonly used in Finland for modified atmosphere packaged poultry meat products were enumerated and identified to determine whether the marinades contained LAB species that cause meat spoilage. The concentrations of LAB in 51 marinade samples ranged from less than 100 to 8.0×10^5 CFU/ml. Seventeen of the samples produced LAB growth only after enrichment, and in five samples no growth was detected either by direct culturing or enrichment. Eighty-eight randomly selected isolates, 51 from the enumerated plates and 37 from enriched samples, were identified using a database of 16S and 23S rRNA gene *Hin*dIII restriction fragment length polymorphism patterns of over 300 type and references LAB strains as operational taxonomic units in numerical analyses. The predominating LAB in the enumerated samples was *Lactobacillus plantarum* (25 of 51 isolates). Eleven isolates were identified as *Lactobacillus paracasei* subsp. *paracasei,* and nine were *Lactobacillus parabuchneri.* None of these species are considered specific spoilage LAB in marinated modified atmosphere packaged poultry meat products nor have they been reported to dominate in unspoiled late-shelf-life products. These results indicate that even though marinades may contain high numbers of LAB, they are not necessarily sources of specific meat spoilage LAB. Therefore, risks associated with meat quality are not predicted by quantitative enumeration of LAB in marinades.

The consumption of broiler chicken meat products has increased notably in Finland over the past 15 years. The majority (>80%) of broiler chicken meat is sold marinated and packaged under a modified atmosphere of about 20% N_2 and 80% CO_2. Marination takes place in the poultry processing plant where raw broiler meat is mixed with acidic glucose- or sucrose-containing spiced water- and oil-based sauces. The meat is thus ready for cooking without any additional spicing.

Some lactic acid bacteria (LAB) are spoilage organisms in marinated vacuum-packaged or modified atmosphere packaged (MAP) broiler meat products. LAB dominate the bacterial populations of MAP products during the commercial shelf life because elevated CO_2, low oxygen, or anaerobic conditions and low storage temperatures inhibit the growth of aerobic gram-negative spoilage bacteria. LAB cause spoilage of MAP broiler meat products typically by producing foul-smelling compounds and gas that bulges the packages *(6).* Withdrawal of these products from the market results in both financial losses and a tarnished reputation for the producer.

Even though marinades themselves are good preservatives because of their acidity and the benzoate or sorbate usually added, marination does not lengthen the shelf life of meat but seems to result in increased numbers of bacteria *(2).* The two main reasons for this increase are the buffering capacity of meat and the high carbohydrate concentrations of marinades. When a marinade is mixed with meat, its initial pH, which is usually below 4.0, is quickly elevated to around 5.5 to 6.0 *(6, 38).* The abundance of carbohydrates used in marinades for balancing the acidic taste provides the LAB with an easily utilizable nutrient source that would not otherwise be present in raw meat. Marination also has a selective effect on LAB populations initially present in poultry. It favors the growth of some psychrotrophic LAB such as *Leuconostoc gasicomitatum (6)* and the recently described species *Lactobacillus oligofermentans (19).* These species have been associated with gas production and rapid spoilage of a marinated MAP broiler meat product *(6),* and they are dominant in the LAB populations of marinated late-shelf-life products *(10, 22, 38).*

During routine microbial quality control inspections, inspectors at meat plants have noticed that freshly marinated products sometimes contain unusually high LAB concentrations compared with nonmarinated meat. Therefore, marinades have been suspected as a source of *L. gasicomitatum* or other specific spoilage LAB. The purpose of this study was to identify LAB in some marinades commonly used for MAP broiler meat products to determine whether marinades are sources of specific spoilage LAB.

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TABLE 1. *LAB concentrations in marinades, pH values, number of samples studied, and isolates selected for identification*

Marinade type	LAB concn (CFU/ml)	Marinade pH	No. of marinade samples	No. of isolates selected for identification	
				From direct culture	After enrichment
A (sweet and sour)	$1.1–8.0 \times 10^5$	4.2	4	16	4
B (rosé)	2.0×10^{2a}	4.2	1	3	1
C (honey)	$<100–9.0 \times 10^{2a}$	3.7–4.2	11	13	10
D (garlic)	$<100–1.0 \times 10^{2a}$	4.3–4.4	5	1	3
E (tomato)	$<100–1.6 \times 10^{3a}$	3.7–3.8	9	18	10
F (curry-apple)	$<100^a$	3.7–3.8	1		1
G (vegetable)	$<100^a$	4.1	1		1
H (lemon)	$<100–3.0 \times 10^{2a}$	3.7–3.8	3		4
I (gorgonzola)	$<100^a$	4.2–4.3	3		1
J (spicy hot)	1.0×10^{2a}	4.9	1		1
K (tangerine)	$<100–1.0 \times 10^{2a}$	4.1–4.2	3		1
L (pesto)[b]	<100	3.9–4.1	2		
M (barbeque)[b]	<100	4.4	1		
N (Italian)[b]	$<100–1.0 \times 10^{2a}$	4.1	2		
O (gravy)[c]	<100	4.4	2		
P (herb)[c]	<100	4.0	1		
Q (Mexican)[c]	<100	3.8	1		
Total			51	51	37

[a] Estimated values.
[b] Colonies obtained after enrichment did not survive further culturing.
[c] No growth detected.

MATERIALS AND METHODS

Marinade samples. Fifty-one samples of marinades in plastic containers were transported from the production plant to our laboratory in cool boxes. Samples were stored at 6°C and analyzed within 8 days of arrival. Common ingredients in all 17 varieties of marinades were water, vegetable oil, sucrose or glucose, citric or acetic acid, sodium chloride, potassium sorbate, sodium benzoate, xanthan gum, and guar gum. Different types of marinades also contained different spices. Table 1 lists the marinade types studied. The exact combinations of spices in the marinades was not identified because of trademark protections.

Growth conditions of bacteria and selection of colonies for species identification. The pH of all samples was measured in the undiluted marinades prior to microbiological analyses. A sterile 22-ml sample was homogenized in 198 ml of 0.1% peptone water in a stomacher blender *(25).* Ten-fold dilutions of the homogenized samples were made from 10^{-2} to 10^{-4}. The method employed in this study was the Nordic Committee on Food Analysis determination of lactic acid bacteria in meat and meat products *(26),* except that the incubation temperature of the samples was 25°C instead of 20°C. All well-formed colonies were counted on plates with 25 to 250 colonies, and the number of LAB per 1 ml of sample was calculated. Gram staining and catalase testing were performed on the colonies to differentiate LAB from staphylococci, which were excluded. All samples were also enriched in deMan Rogosa Sharpe broth containing 1% sorbic acid as a yeast inhibitor (MRS-S broth; Difco, Becton Dickinson, Sparks, Md.). For enrichment, 9 ml of broth was inoculated with 1 ml of undiluted marinade and incubated at 25°C for 2 to 4 days, after which 10 μl of the broth was spread onto MRS-S agar plates. These plates were then incubated at 25°C for 3 to 5 days and observed for colony growth.

A total of 88 isolates were subjected to species-level identification. The number of samples analyzed per marinade type, number of isolates from directly cultured samples, and number of isolates from enriched samples are shown in Table 1.

For DNA extraction, MRS broth was inoculated and grown at 25°C for 1 to 2 days depending on the growth rate. Cells harvested from 1.5 ml of this MRS broth were used for DNA extraction. The isolates were stored at −70°C in MRS broth.

DNA isolation, ribotyping, and identification of strains based on numerical analysis of ribopatterns. DNA was isolated using a modified *(7)* method of Pitcher et al. *(27)* with cell lysis solution containing mutanolysin (250 U/ml; Sigma, St. Louis, Mo.) and RNase in addition to lysozyme (25 mg/ml; Sigma). DNA samples (8 μg) were cleaved with the *Hin*dIII restriction enzyme (New England Biolabs, Beverly, Mass.) as recommended by the manufacturer. *Hin*dIII provides species-specific restriction patterns for various spoilage LAB within the genera *Carnobacterium, Lactobacillus, Lactococcus, Leuconostoc, Pediococcus,* and *Weissella (4, 6, 9, 10–12, 19).* DNA fragments were separated by agarose gel electrophoresis, and the resulting fingerprint patterns were transferred onto nylon membranes via Southern blotting in a vacuum blotting device (Vacugene, Pharmacia, Uppsala, Sweden). Ribotyping was performed using cDNA probe reverse transcription (AMV-RT, Promega, Madison, Wis.) from 16S and 23S rRNA, and fragments were labeled with digoxigenin using the Dig DNA Labeling Kit (Roche Molecular Biochemicals, Mannheim, Germany) as described by Blumberg et al. *(13).*

Membranes were hybridized at 58°C overnight, and the digoxigenin label was detected as recommended by the manufacturer. After scanning the membranes (ScanJet 4c/T tabletop scanner, HP, Boise, Idaho), the *Hin*dIII ribopatterns were numerically analyzed with BioNumerics 4.1 software (Applied Maths, Kortrijk, Belgium). The similarities between all pairs were expressed with the Dice coefficient correlation. Clustering by the unweighted pair-group method with arithmetic averages was used in the construction of a dendrogram. The ribotype patterns were compared

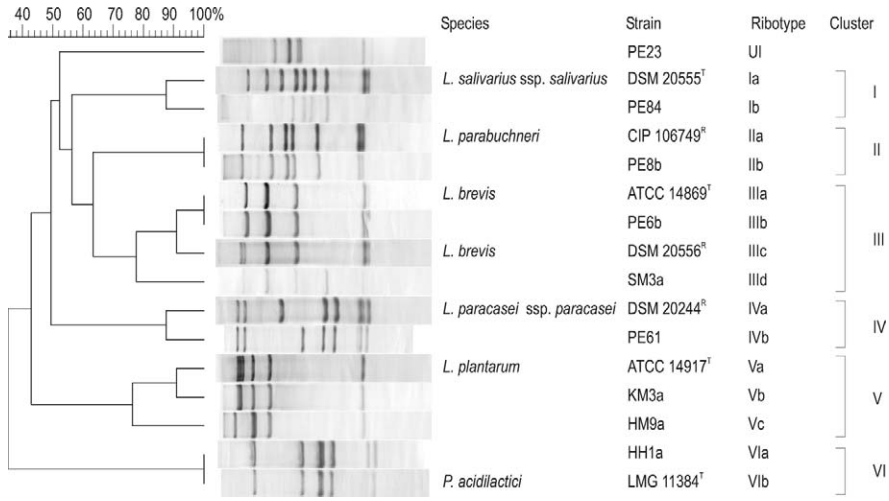

FIGURE 1. *Numerical analysis of* Hind*III ribopatterns, showing all different ribotypes obtained from the LAB detected in the marinades and the culture collection strains with similar patterns.*

with corresponding patterns found in the LAB database of the Department of Food and Environmental Hygiene (University of Helsinki). The isolates were identified based on the locations of type strains within the clusters. The reliability of the cluster method for distinguishing between different species has been evaluated in several polyphasic taxonomy studies of LAB *(5, 6, 8, 12, 18, 19).*

RESULTS AND DISCUSSION

The pH values and LAB concentrations in each marinade type are shown in Table 1. Three isolates were catalase positive and were excluded from these results. These strains were identified as staphylococci by 16S rRNA sequencing (data not shown). No growth was detected in five marinade samples either by direct culturing or enrichment. Colonies obtained from four marinade samples after enrichment did not survive further culturing.

In the numerical analysis of *Hin*dIII ribopatterns of the strains, six distinctive clusters were obtained (Fig. 1). One strain did not cluster with any reference strain in the database and therefore could not be identified. The dominant species in the directly cultured marinades were *Lactobacillus plantarum, Lactobacillus paracasei* subsp. *paracasei, Lactobacillus parabuchneri,* and *Lactobacillus brevis* (Table 2). Some researchers have proposed that the species *Lactobacillus casei* and *L. paracasei* are members of the same taxon and should be united within the name *L. casei,* rejecting the name *L. paracasei* altogether *(15).* The second most common LAB species found in these marinades may therefore be called *L. casei* in the future. The group of LAB species represented by the 37 strains from the enriched samples was almost the same as the species detected in the directly cultured samples. Only the unknown isolate and all isolates identified as *L. brevis* and *Pediococcus acidilactici* were picked from the enriched samples, whereas *L. plantarum, L. p. paracasei,* and *L. parabuchneri* were found both in the directly cultured and the enriched samples.

TABLE 2. *Number of isolates per LAB species in marinades*

LAB species	Marinade type: A	B	C	D	E	F	G	H	I	J	K	Total	Total from enriched samples
Lactobacillus plantarum	19	3	11	2								35	10
L. paracasei ssp. *paracasei*	1	1	5		11	1	1					20	9
L. parabuchneri			5		9							14	5
L. brevis				2	2			3				7	7
L. casei			2		1				1			4	3
L. salivarius ssp. *salivarius*					1							1	
L. reuteri					1							1	
Pediococcus acidilactici										1	1	2	2
Unknown							1					1	1
Total	20	4	23	4	28	1	1	4	1	1	1	85	37

In products similar to marinades with low pH (3.5 to 4.0), such as salad dressings, citrus products, and vinegar-based preserves, a common spoilage LAB is *Lactobacillus fructivorans (28). L. brevis* and *L. plantarum* have occasionally been isolated from these products *(20, 24, 35, 36).* Based on the results from our study, *L. p. paracasei* (i.e., *L. casei*) also can be acid tolerant enough to thrive in products such as marinades. However, the ability of this species to spoil marinades is not known. The sources of the LAB in our marinades are probably spices and other ingredients of plant origin. *L. plantarum* has been isolated from molasses *(39),* grapes *(40),* wheat flour *(14),* wine *(17),* and various plant materials (leaves, stems, and flowers) *(23). L. p. paracasei* originates from fermented grape juice *(1)* and grape must and wine *(30). L. brevis* has been found in wine *(17, 31),* apple cider *(17),* and wheat flour *(14). Lactobacillus salivarius* subsp. *salivarius* (represented by a single isolate in this study) is commonly found in the intestinal tracts of humans and other animals *(32)* such as horses *(33, 41),* birds *(16, 34),* and dogs, cats, and pigs *(29, 37).* However, all LAB are widespread in nature and are found in a wide variety of niches *(17, 21).*

The LAB species detected in these marinades are not

among the specific spoilage LAB of marinated MAP broiler meat products, such as *Lactobacillus sakei, Lactobacillus curvatus, L. gasicomitatum, L. oligofermentans, Carnobacterium maltaromaticum* (*piscicola*) and *Carnobacterium divergens (3, 6, 10, 19, 38).* Based on these results, the source of spoilage LAB in marinated broiler meat products is likely to be something other than the marinade; the most obvious possibility is the production environment or the microbiome of the poultry. Because the marinade volume used in these products accounts for approximately 30% of the weight of the final poultry product, LAB concentrations as high as those found in marinade type A (the sweet and sour marinade) may result in product LAB concentrations of 10^5 CFU/g immediately after packaging. According to some manufacturers, the initial concentrations of LAB or of total bacteria should not exceed 10^4 CFU/g in freshly prepared products. Thus, even though the marinade LAB contamination may not result in spoilage of the meat product, the presence or LAB may cause a false alarm in the routine quality control inspection. These results highlight the fact that quantitative LAB enumeration is not sufficient for evaluating risks associated with specific spoilage LAB contamination and that further research is needed to identify the origins of the specific spoilage LAB of poultry meat products.

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