

Research Note

Leuconostoc Spoilage of Vacuum-Packaged Vegetable Sausages

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ABSTRACT

The present study was conducted to assess the role of lactic acid bacteria (LAB) in spoilage of a vacuum-packaged vegetable sausage product. This spoilage problem was characterized by formation of gas and slime, and was limiting the shelf life of the product. To investigate the LAB populations, LAB were enumerated in vegetable sausages graded as either spoiled or acceptable. From these vegetable sausages, 110 prevailing LAB isolates were recovered and identified using an LAB ribotyping database, which uses *Hind*III restriction fragment length polymorphism patterns of the 16S and 23S rRNA genes as operational taxonomic units. Finally, to determine the effects of the prevailing LAB on the sensory properties of the product, fresh vegetable sausages were inoculated with six LAB strains. The results revealed that *Leuconostoc gelidum*, *Leuconostoc gasicomitatum*, and *Leuconostoc mesenteroides* were the predominant LAB in the commercial vegetable sausages. The inoculation of these LAB onto vegetable sausages resulted in the formation of gas, slime, and a sour off-odor. Based on these findings, *L. gelidum*, *L. gasicomitatum*, and *L. mesenteroides* were responsible for spoilage of the vegetable sausage product.

To obtain safe and stable food products with reasonable shelf lives, perishable foods are often stored at refrigeration temperatures and packaged under vacuum or CO₂-enriched atmospheres to inhibit the microbial growth. During refrigerated storage, psychrotrophic lactic acid bacteria (LAB) frequently become predominant in the microbial populations of anaerobically packaged foods. Sometimes, the growth of LAB becomes a problem, as undesirable changes develop because of their metabolic activities. Spoilage because of growth of LAB is well documented in cold-stored, vacuum-packaged, or modified atmosphere-packaged meat, poultry and processed meat products (3, 7, 8, 11, 20, 27, 29, 31). In these products, LAB spoilage is often characterized by deteriorations in odor, flavor, color, or appearance.

Recently, microbial spoilage characterized by gas and slime formation in a vacuum-packaged vegetable sausage product became a main concern of the manufacturer. The processing of the vegetable sausages included a cooking treatment, after which the sausages were chilled, peeled, packaged, and distributed under refrigeration (<8°C). The manufacturer was convinced that the spoiled lots were exposed to neither processing failures nor temperature abuse. However, the results of the preliminary microbial analyses suggested that LAB, found at considerably high numbers (>log 9 CFU/g) in the spoiled vegetable sausage lots, were responsible for the problem.

In general, little is known about microbial spoilage of cooked, vacuum-packaged, vegetable-based foods. Therefore, the purpose of the present study was to identify the predominant LAB associated with the vacuum-packaged vegetable

sausages, and to determine whether these LAB have detrimental effects on the sensory properties of the product.

MATERIALS AND METHODS

Vegetable sausage product. During a problematic production period, the manufacturer delivered to our laboratory 10 packages of vegetable sausages. Each package contained three sausages (ca. 100 g). Five of the packages were graded as spoiled due to gas and slime formation, whereas as the rest of them were of acceptable sensory quality. The spoiled packages were received 21 ($n = 2$) or 31 ($n = 3$) days postprocessing, and the packages of good quality 5 ($n = 2$), 9 ($n = 2$), or 15 ($n = 1$) days postprocessing. The manufacturer-defined shelf life was 24 days.

The vegetable sausages consisted of the following ingredients: minimally processed carrots (56%), rapeseed oil, cheese, cream, egg yolk powder, dehydrated potato, potato starch, spices, stabilizers, ascorbic acid, NaCl (2.0%), sucrose, and potassium sorbate. These ingredients were mixed and emulsified, stuffed into casings, cooked to core temperature of 82°C, cooled to 8°C, peeled, packaged under vacuum, and stored below 8°C.

LAB analysis and pH measurements. A 25-g sample from each package was homogenized with 225 ml of peptone saline solution (PSS; distilled water with 0.85% NaCl and 0.1% peptone) for 1 min in a stomacher blender (Lab Blender 400, London, UK). Serial dilutions were made in PSS, and appropriate dilutions were surface plated on deMan, Rogosa and Sharpe (MRS) agar, pH 6.2 (Oxoid, Basingstoke, UK). After 5 days of anaerobic (AnaeroGen, Oxoid) incubation at 25°C, LAB were enumerated. For the samples with LAB numbers above 1,000 CFU/g, 10 to 20 colonies were randomly picked from the MRS plates of the highest sample dilutions. The isolates were cultured to purity using MRS broth (Difco, Becton Dickinson, Sparks, Md.) and agar, and subjected to ribopattern-based identification. The stock cultures of the isolates were archived in MRS broth at -70°C.

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The pH of each sample homogenate was measured using an inoLab pH 720 pH meter (WTW GmbH, Weilheim, Germany).

Ribotyping. Ribotyping, or 16S and 23S rRNA gene restriction fragment length polymorphism, was performed according to the method of Regnault et al. (26), which utilizes an oligonucleotide probe mixture of five universal probes targeting to conserved regions of genes encoding 16S and 23S rRNA. For ribotyping, DNA was isolated using a modified (4) method of Pitcher et al. (25) with cell lysis solution containing mutanolysin, ribonuclease (RNase) and lysozyme (all from Sigma, St. Louis, Mo.). Genomic DNA (8 µg) was cleaved with *Hind*III restriction endonuclease (New England Biolabs, Beverly, Mass.), and the DNA fragments were separated by gel electrophoresis using a digoxigenin-labeled DNA Molecular Weight Marker II (Roche Diagnostics, Penzberg, Germany) as a size standard. The resulting fragment patterns were transferred onto a nylon membrane by Southern blotting, using a vacuum blotting apparatus (VacuGene, Pharmacia, Uppsala, Sweden). The fragments were hybridized with a mixture of five digoxigenin-labeled oligonucleotide probes as described by Regnault et al. (26). The probes were commercially synthesized and digoxigenin labeled by Oligos Etc., Inc. (Wilsonville, Oreg.). After hybridization at 53°C and stringency washes, the hybridized banding patterns (ribopatterns) were detected with alkaline phosphatase-coupled anti-digoxigenin antibodies (Roche Diagnostics), and color development with (nitroblue tetrazolium chloride and 5-bromo-4-chloro-3-indolyl-phosphate) substrate (Roche Diagnostics).

Numerical analysis of ribopatterns and identification of LAB. The LAB database of the Department of Food and Environmental Hygiene, University of Helsinki, Finland, contains 16S and 23S rRNA gene *Hind*III restriction fragment length polymorphism patterns (ribopatterns) of approximately 7,000 LAB including 350 ribopatterns of type and reference strains of relevant food-associated LAB (2–5, 7, 17, 21–23, 30, 31). The patterns of these strains are used as operational taxonomic units in numerical analysis resulting in the formation of clusters. Based on the locations of the type and reference strains in these clusters, isolates are considered to represent the corresponding LAB species. Species specificities of these clusters have been demonstrated in several independent polyphasic taxonomy studies of LAB (3, 6, 14–18).

The ribopatterns were scanned, converted to tagged image file format images and imported into the BioNumerics database (version 4.61 Applied Maths, Sint-Martens-Latem, Belgium). Ribopatterns were made comparable using DNA molecular weight markers. Dice coefficients were used for estimating similarity between the patterns, and the unweighted pair group method with arithmetic mean clustering algorithm was used for constructing a dendrogram.

Determination of spoilage potentials. Six LAB strains were separately inoculated onto fresh vegetable sausages to determine the spoilage potentials of these strains. In addition, these strains were inoculated as a mixture to study whether bacterial interactions are required for spoilage reactions. Five of the inoculants originated from the commercial samples and were chosen based on their ribopatterns to represent the prevalent LAB. In addition, *Leuconostoc gasicomitatum* LMG 18811^T, a strain previously associated with spoilage of meat and fish products (4, 23, 31), was included. Each strain was precultured in MRS broth, and an inoculation culture was prepared in PSS. The cultures were diluted according their OD₆₀₀ values to achieve a final cell density of approximately 10⁵ CFU/ml. To prepare the six-strain mixture, MRS broth cultures were combined before dilution in PSS.

Vegetable sausages, processed the day before, were obtained

TABLE 1. Details of commercial, vacuum-packaged vegetable sausages

Storage time (days) ^a	Packages (n)	Sensory quality	LAB count (log CFU/g)	pH
5	2	Acceptable	<3.0	6.4 ^b
9	1	Acceptable	4.2	6.4
15	2	Acceptable	7.3 ± 0.3 ^c	6.3 ^b
21	2	Unacceptable	8.8 ± 0.1 ^c	5.1 ^b
31	3	Unacceptable	9.4 ± 0.2 ^c	5.1 ^b

^a Time of sampling after manufacturing.

^b Data are presented as means (SD < 0.2).

^c Data are presented as means ± SD.

directly from the processing plant. The sausages were placed into individual transparent, oxygen-impermeable pouches identical to those used for vacuum packaging of the commercial product. Quadruplicate sausages were surface inoculated with 1 ml of appropriate dilution. Four sausages were treated with 1 ml of PSS, thus serving as uninoculated controls. The sausages were allowed to rest for 15 min at 6°C before vacuum packaging (Multivac A 300/16, Sepp. Hagenmüller KG, Wolfertschwenden, Germany). The packages were identified by randomly assigned codes, stored in the dark at 8°C, and examined daily for visible spoilage. After a 12-day storage period, a three-member sensory panel was asked to evaluate the severities of gas and slime formation. The panelists used a three-point scale (1 = no defect, 2 = mild defect, 3 = severe defect), in which 1 was considered as the point of acceptability. Furthermore, the panelists were asked to evaluate the odor after opening each package and to describe potential off-odors associated with the samples.

In addition to the sensory analyses, LAB counts and pH were determined for each sample as described previously.

RESULTS

Table 1 shows the LAB numbers and the pH values in the 10 commercial, vegetable sausage packages. To identify the predominant LAB, 110 isolates were recovered from the sausage samples, and subjected to ribotyping-based identification. Of these isolates, 82 were recovered from the spoiled samples, and 28 from samples of good quality. The numerical analysis of the ribopatterns (Fig. 1) resulted in formation of seven clusters, and grouped the LAB strains from vegetable sausages together with either the *L. gasicomitatum* LMG 18811, *Leuconostoc gelidum* NCFB 2775, or *Leuconostoc mesenteroides* DSM 20343 type strains. Table 2 lists the distribution of the 110 LAB isolates into different species and ribopatterns.

By a reference to their various ribopatterns, the following five strains were selected for the inoculation experiment: *L. gasicomitatum* 16-1 and 3-1 (Fig. 1: ribotypes I and II), *L. gelidum* 1-2 and 15-9 (Fig. 1: ribotypes III and IV), and *L. mesenteroides* 3-4 (Fig. 1: ribotype VI). Additionally, *L. gasicomitatum* LMG 18811^T (Fig. 1: ribotype I) was included in the inoculation experiment. The results from the sensory evaluation revealed that these six LAB strains, both alone and as a mixture, caused gas or slime formation in vegetable sausages (Table 3). The LAB treated samples had also a sour off-odor, which the panel described as “vinegary.” Furthermore, the deteriorations in the treat-

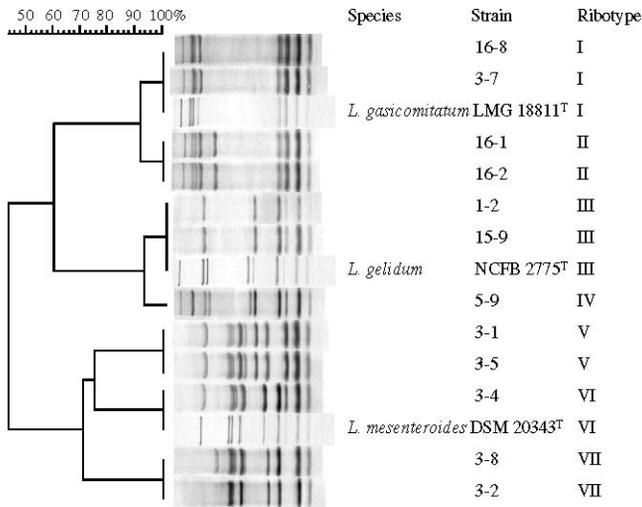


FIGURE 1. Numerical analysis of HindIII ribopatterns, showing the seven different ribotypes obtained from the LAB detected in the vegetable sausages, and the culture collection type strains with similar patterns.

ed samples were accompanied with increased acidification and LAB counts (Table 3). In contrast to the treated sausages, the sensory panel found the appearance and the odor of the nontreated controls acceptable (Table 3).

DISCUSSION

L. gelidum, *L. gasicomitatum*, and *L. mesenteroides* were the predominant LAB in the vacuum-packaged vegetable sausage product. The inoculation experiments revealed that these LAB were able to produce gas, slime, and vinegary off-odors in vacuum-packaged sausages, and thus were responsible for the premature spoilage of the commercial product. Previously, *L. gelidum*, *L. gasicomitatum*, and *L. mesenteroides*, as well as *Leuconostoc carnosum* have been implicated in spoilage of meat and poultry, including heat-processed meat products (3, 7, 8, 11, 19, 27, 31, 33). In addition to spoilage of meat, the findings of this study indicate that psychrotrophic leuconostocs may cause severe deteriorations in cold-stored, cooked vegetable-based products.

The defects developed in commercial vegetable sausages were similar to those typically described in association with LAB spoilage of processed meat products (8, 19, 29, 33). In processed meats, the growth of spoilage LAB is probably favored by the presence of added carbohydrates (1, 24, 28). Similarly, the vegetable sausages contained sucrose as well as other ingredients rich in carbohydrates (carrots, potato starch), thus providing *Leuconostoc* spp., with fermentable substrates for growth. *Leuconostoc* spp. are heterofermentative organisms, and their basic pathway of glucose conversion yields organic acids and CO₂. These metabolites accumulate, giving rise to formation of sour off-odors and flavors, and swelling of the package through gas production. Furthermore, the ability of *Leuconostoc* spp. to produce dextran (slime) from sucrose may result in unacceptable slime formation in sucrose-containing foods such as in processed meat and fish products (11, 13, 23, 28).

Identification of the spoilage organisms is essential since it guides the manufacturer in determining strategies

TABLE 2. LAB species distribution, ribotypes, and numbers of isolates detected in vacuum-packaged vegetable sausages

Species	Ribotype	No. of isolates	
		Spoiled samples (n = 5)	Acceptable samples (n = 3)
<i>L. gasicomitatum</i>	I	10	4
	II	14	
<i>L. gelidum</i>	III	56	18
	IV		1
<i>L. mesenteroides</i>	V	2	3
	VI	4	
	VII		2

to prevent spoilage problem. During the vegetable sausage manufacturing, the heat treatment is the most effective hurdle inactivating the majority of the vegetative cells, including psychrotrophic *Leuconostoc* spp. (12, 28). Therefore, the presence of *Leuconostoc* spp. in the vegetable sausages is primarily related to postcooking contamination. Previous studies dealing with psychrotrophic spoilage LAB contamination in meat processing plants have highlighted the role of airborne contamination (7, 32). Although sources of LAB contamination in vegetable sausage processing were not addressed in this study, the spoilage problem in vegetable sausages likely arose from airborne LAB contamination, since raw materials, such as unprocessed vegetables, were occasionally handled close to the packaging area. Unprocessed carrots, such as those included in the vegetable sausage formulation, have been shown to contain *L. gelidum*, *L. gasicomitatum*, and *L. mesenteroides* (9, 10, 23). Therefore, carrots are likely to contaminate the processing environment with those LAB species responsible for spoilage of the vegetable sausages. Similar to the latter, Lyhs et al. (23) considered carrots as the primary source of *L. gelidum* and *L. gasicomitatum*, which caused slimy spoilage in a fish preserve.

TABLE 3. Characteristics of vacuum-packaged vegetable sausages without or after being inoculated with lactic acid bacteria strains, and stored at 8°C

Inoculum	Mean scores for sensory defect ^a		LAB (log CFU/g) ^b	pH ^c
	Gas	Slime		
<i>L. gasicomitatum</i> 3-7	1.7	2.1	7.8 ± 0.2	5.4
<i>L. gasicomitatum</i> 16-1	2.0	2.5	8.9 ± 0.1	4.8
<i>L. gasicomitatum</i> LMG 18811 ^T	2.3	2.2	8.0 ± 0.1	5.3
<i>L. gelidum</i> 1-2	2.8	3.0	9.0 ± 0.1	5.1
<i>L. gelidum</i> 15-9	2.8	3.0	9.1 ± 0.2	5.2
<i>L. mesenteroides</i> 3-4	2.1	3.0	9.1 ± 0.1	5.0
Mixture of six strains	2.3	3.0	8.8 ± 0.3	4.9
Nontreated control	1.0	1.0	7.1 ± 0.3	6.1

^a Scores for sensory defects were judged from none, 1; to clear defect, 2; to severe defect, 3.

^b Data are presented as means ± SD of quadruplicate samples.

^c Data are presented as mean of quadruplicate samples (SD ≤ 0.1).

In conclusion, our findings reveal that *L. gelidum*, *L. gasicomitatum*, and *L. mesenteroides* are specific spoilage organisms of vacuum-packaged, cooked vegetable sausages. Taking knowledge of psychrotrophic LAB into account, the strategy to prevent spoilage problems in vegetable sausages should focus on strict precautions to control recontamination of the finished product. Furthermore, replacing minimally processed carrots with blanched carrots in vegetable sausage formulation might also help to minimize the risk of recontamination of the cooked product.

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