The Fate of *Listeria Monocytogenes* in Fermented Sausages and in Vacuum-Packaged Frankfurters

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ABSTRACT

In inoculated fermented sausages, the initial “most probable number” (MPN) of *Listeria monocytogenes* decreased from 8 x 10^6/g to 1.1 x 10^2/g during the production process. When the initial MPN was 6.5 x 10^5/g, *L. monocytogenes* were detected in finished sausages only when 25-g samples were used. The MPN of *L. monocytogenes* in finished experimental sausages remained constant during 20 d of storage at room temperature. The average number of lactobacilli in the experimental sausages was initially 1.2 x 10^7/g, reached 8 x 10^5/g after ripening, and decreased to 2.2 x 10^3/g in finished sausages. Sterilized filtrates of 12 *Lactobacillus plantarum* broth cultures produced zones of inhibition of *L. monocytogenes* growth on solid medium, when the pH of the filtrates ranged from 3.47 to 4.52. When the pH of the same filtrates was adjusted to 7.0, only slight zones of inhibition were registered with filtrates of 7 out of the 12 *L. plantarum* strains examined. In vacuum packages of surface-contaminated frankfurters, the MPN of *L. monocytogenes* increased from 5 x 10^5 to 2.1 x 10^7/ml per package during 20 d of storage at 4°C. In the same packages, the number of lactobacilli increased from 2.4 x 10^8 to 6.6 x 10^10 per package.

*L. monocytogenes* has been recognized as a foodborne pathogen in the last few years (2). The bacteria have been regularly isolated from minced meat (5,25,27,28) and found in raw fermented sausages (5,15,19,25). The main reason for this is fecal and pharyngeal contamination of the carcasses during slaughter, and cross-contamination from work surfaces, machine, and equipment in the slaughterhouse (8,17). Consequently, raw meat products, produced from minced meat, may contain *L. monocytogenes*. It is generally considered that *L. monocytogenes* is a relatively heat-sensitive (22). Heat treatment of some meat products (frankfurters) at 71°C during processing should kill these bacteria if present at the level of 10^5/g of raw meat (30). However, cooked meat products can also be cross-contaminated with *Listeria* after processing and, if suitable conditions occur (e.g., vacuum packaging), the bacteria are able to multiply (9). The presence of *L. monocytogenes* in fermented sausages and in vacuum-packaged meat products is of particular interest for food safety, because these two groups of meat are usually eaten without reheating.

In published investigations of the fate of *L. monocytogenes* in such meat products, emphasis has been placed on the effect of different physical and chemical factors on these bacteria (9,11,13,29) rather than on the ecological relationship between *L. monocytogenes* and competing microflora (4,14,26). For this reason, the purpose of this work was to examine the behavior of *L. monocytogenes* in a domestic type of fermented sausage and in vacuum-packaged frankfurters, together with the possible effect of lactobacilli on their growth.

MATERIALS AND METHODS

Formulation of fermented sausages ("tea" sausages)

The raw meat used to make fermented sausages was obtained from an industrial slaughterhouse. The meat and fat tissue had been cut to give pieces of 200 to 500 g and was then stored at 4-7°C for 14 d, followed by frozen storage at -3 to -5°C for 2 d.

Sausages were prepared using the following formula: frozen beef (trimmings 20% fat) 40 kg, frozen pork (boneless ham) 40 kg, frozen fat tissue (jowl) 20 kg, salt 2.5 kg, glucono-deltalactone 300 g, dextrose 200 g, ascorbic acid 30 g, and sodium nitrite 10 g.

The meat was chopped and homogenized in a cutter machine (Krämer Grabe 173/136-325, FRG) at 1,400 rpm for 2 min and then for 2 min at 2,800 rpm.

Preparation of bacterial inocula

The inocula were prepared from 500 ml of 24-h cultures of *L. monocytogenes* (NCTC 7973) or *L. innocua* (a strain previously isolated from meat) in BHI broth (Merck 10493). The bacteria were pelleted by centrifugation (2,000g/30 min at 4°C) and resuspended in 50 ml of sterile physiological saline. The suspension was then diluted to concentrations of 10^5/ml and 10^6/ml (*L. monocytogenes*) and 10^6/ml (*L. innocua*), by adding sterile saline.

Inoculation of sausages

In the first treatment (type A sausages), two 2-kg portions of the sausage mixture (samples I and II) were separately inoculated with 20 ml *L. monocytogenes* suspension (concentration 10^6/ml). In the second treatment (type B sausages), two similar portions of the sausage mixture were separately inoculated with 20 ml of *L. monocytogenes* suspension (concentration 10^5/ml).

The *L. innocua* suspension (20 ml; concentration 10^5/ml) was used to inoculate separately 2-kg portions of the sausage mixture (third treatment; type C sausages).

The fourth treatment (control sausages) contained two portions of the sausage mixture (2 kg each) inoculated with 20 ml of sterile physiological saline.

The bacterial suspension or saline was added from a pipet to different parts of the sausage mixture, which was placed in sterilized plastic containers. The inoculated samples were then homogenized by hand mixing for 3 min.
Preparation of sausages

The sausage mixture was stuffed into collagen casings of 35 mm diameter (Kotejsprodukt, Yugoslavia) using a handstuffer. The oxygen permeability of this casing was 9,415.3 NTP cm/m² 24 h bar. Each sausage casing was twisted to obtain two subsamples. A total of 16 subsamples of sausage was prepared for each treatment (8 subsamples of sample I, and 8 subsamples of sample II), making an overall total of 64 subsamples.

The chemical composition of the sausages was determined according to the procedure of the Association of Official Analytical Chemists (I) immediately after preparation.

TABLE 1. Production process for the experimental "tea" sausages.

<table>
<thead>
<tr>
<th>Phase of production</th>
<th>Days</th>
<th>Smoking</th>
<th>Temperature (°C)</th>
<th>Relative air humidity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1st</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2nd</td>
<td></td>
<td>1 h daily</td>
<td>18-20</td>
<td>92</td>
</tr>
<tr>
<td>Ripening</td>
<td>3rd</td>
<td>1 h daily</td>
<td>18-20</td>
<td>88</td>
</tr>
<tr>
<td></td>
<td>4th</td>
<td>1 h daily</td>
<td>18-20</td>
<td>85</td>
</tr>
<tr>
<td>Drying</td>
<td>5th-15th</td>
<td></td>
<td>18</td>
<td>75</td>
</tr>
</tbody>
</table>

After preparation, the sausages were smoked and dried (Table 1) in an automated smokehouse (Alfas, Schult Kohn, FRG) for 15 d. After the production process was finished, the sausages were stored at room temperature (18-22°C) for 20 d.

The sausages of each type (A, B, C, and control) were sampled immediately after stuffing into the casings (day 0), then 4 and 15 d later, and finally after a further 20 d of storage. The following examinations were carried out: a) determination of the number of L. monocytogenes or L. innocua, b) determination of the number of lactobacilli, c) measurement of pH value, and d) measurement of water activity (a).

The results are shown as mean values of the two samples (I and II) for each type of sausage. Each sample was examined by two subsamples.

Vacuum-packed frankfurters

Frankfurter manufacture. The raw meat used for frankfurter manufacture was obtained from pigs and young bulls slaughtered at the age of 7 and 18 months, respectively. The formulation of the frankfurters was: pork (boneless ham) 20 kg, beef (trimmings 20% fat) 30 kg, back fat tissue 30 kg, ice 20 kg, salt 2.2 kg, spices 400 g, polyphosphate (Tari K2, Giuliani, Italy) 300 g, and sodium nitrite 10 g. The ingredients were chopped and homogenized in a cutter machine (Kramer Grabe VSM 325, FRG) at 1,400 rpm for 2 min and then at 2,800 rpm for 3 min. The finished emulsion was stuffed into collagen casings (Kotejsprodukt, Yugoslavia) of 22 mm diameter. The sausages (10 cm long) were then dried and smoked at 76°C for 80 min., followed by cooking in steam at 80°C for 10 min, in a climate chamber (Atmos, FRG).

The chemical composition was determined using the official methods of the AOAC (I).

Inoculation of frankfurters. The casing was removed from the frankfurters, and the sausages were dipped into a suspension of L. monocytogenes (NCTC 7973) in physiological saline (10 6 cells/ml). After about 30 s, the frankfurters were taken out and dried by hanging at room temperature for 30 min. The frankfurters were then packaged under vacuum in polyamide/polyethylene plastic material (Tipoplastika, Yugoslavia) by machine (Multivac, Seep Haggen Muller, FRG). The permeability for oxygen of this plastic material was 3.52 NTP cm/m² 24 h bar. Each package contained six frankfurters. The vacuum-packaged frankfurters were stored at 4°C for 20 d.

Uninoculated frankfurters were prepared and packaged in the same manner. Three packages of artificially contaminated frankfurters and three packages of control frankfurters were simultaneously examined immediately after packaging (day 0), as well as after 10 and 20 d of storage.

The examinations included determination of: a) number of L. monocytogenes, b) number of lactobacilli, and c) total number of bacteria. The results are shown as mean values from three packages of the respective group.

Sampling for bacteriological examinations

Samples of 10-15 g were taken aseptically from the center of fermented sausages and homogenized with physiological saline. From this basic dilution (10°), a series of decimal dilutions were made.

Vacuum packages of frankfurters were opened aseptically, and 10 ml of sterile physiological saline was poured inside. The surface of the frankfurters and the inside of the package were rinsed by shaking and rotation of the package. From the rinse (dilution 10°), a series of decimal dilutions were prepared.

Determination of number of L. monocytogenes or L. innocua

One ml of each dilution from the series of decimal dilutions of samples was transferred into 10 ml Listeria enrichment broth I (LEB I). After incubation at 30°C for 24 h, 0.1 ml of LEB I was transferred into 10 ml of Listeria enrichment broth II (LEB I). Isolation on LPM agar and determination and confirmation of L. monocytogenes and L. innocua were performed as described by Skowgaard and Morgan (27), which is essentially the same as the procedure recommended by McClain and Lee (18).

On the basis of L. monocytogenes or L. innocua detections in certain dilutions of the samples, the approximate number of these bacteria was determined using tables of the "most probable number" (MPN) as described for enumeration of coliforms (3).

Determination of number of lactobacilli

The number of lactobacilli was determined by inoculation on MRS agar (Merck 10660) plates, which were incubated at 30°C for 48-72 h. Lactobacillus spp. were differentiated according to Kandler and Weiss (12). Typical colonies on MRS agar plates were verified by Gram staining, catalase and cytochromoxidase reaction, and fermentation of arabinose, cellobiose, galactose, glucose (acid and gas formation), lactose, maltose, mannitol, raffinose, salicin, sorbitol, sucrose, xylose, and esculin, as well as growth at 15 and 45°C.

Determination of total viable count of bacteria

The total viable count of bacteria was determined by inoculation of "agar for total count of bacteria" plates (Tolak, Yugoslavia), which were incubated at 30°C for 48-72 h.

Investigation of the influence of Lactobacillus plantarum on the growth of L. monocytogenes

 Cultures of 12 Lactobacillus plantarum strains in 100 ml MRS broth (Merck 10661), isolated from the experimental sausages, were sterilized by filtration (Acrodisc®, filter size 0.2 μm) after 24 h incubation at 30°C. Each sterile filtrate was divided into two portions. In one portion of filtrate the original pH was not altered, but in the other portion of the same filtrate the pH was adjusted to 7.0 by the addition of sodium hydroxide solution (1M).

A 24-h culture of L. monocytogenes (NCTC 7973) in BHI broth (Merck 10493) was diluted with a sterile saline solution.
(ratio 1:20) and poured onto the surface of LPM agar. After the liquid had been decanted, the plates were dried in the chamber for 30 min. Metal cylinders were placed on the surface of the agar and filled with 0.5 ml of sterile filtrates of L. plantarum cultures, both with original and adjusted pH (7.0). The plates were incubated at 30°C for 24 h. After incubation, zones of inhibition of L. monocytogenes growth were measured.

**pH and **$a_w$** measurements**

The pH was measured by inserting the combination electrode of a pH meter (MA 5705, Iskra, Yugoslavia) into the center of the sausages. Water activity ($a_w$) in the sausages was measured using an $a_w$ meter (a_w-Wertmesser Luft, Stuttgart, FRG). The results are shown as mean values of all four types of sausages.

### RESULTS AND DISCUSSION

**Fermented ("tea") sausages**

The results of chemical analysis of sausages were as follows: moisture 48.8%, fat 32.3%, protein 15.5%, sodium chloride 2.4% (4.68% in the water phase), and nitrates 85.2 ppm. The initial pH was 5.47. In type A sausages the number of L. monocytogenes decreased after 4 d ripening from the initial number of $8 \times 10^6$ g to $3.5 \times 10^5$ g. After 11 d drying the number of L. monocytogenes decreased further to $1.1 \times 10^4$ g, at which level it remained during the following 20 d storage at 18-22°C. The number of L. innocua in sausages inoculated with $10^5$ g decreased after 4 d ripening, parallel with the drop in pH to 4.80. The $a_w$ of the sausages decreased during the ripening and drying period (15 d) to 0.933. This is higher than 0.88 which is referred to as the limit for L. monocytogenes growth (21).

In both type A and type B sausages, the numbers of L. monocytogenes declined most rapidly during the first 4 d of ripening, parallel with the drop in pH to 4.80.

The results are in agreement with the findings of other authors (4,11, 29), who also observed that L. monocytogenes in fermented sausages could survive during storage, but in decreased numbers.

The $a_w$ of the sausage mixture at the beginning of the experiment was 0.974 with a corresponding salt in moisture content of 4.68. The $a_w$ of the sausages decreased during the ripening and drying period (15 d) to 0.933. This is higher than 0.88 which is referred to as the limit for L. monocytogenes growth (21).

However, the initial pH of 5.47 was higher than 5.0, which has been reported as the lower limit for growth of L. monocytogenes (7).

The nitrite content in the sausage mixture was 85.2 ppm, which is much lower than 1,000 ppm, the value suggested as necessary to inhibit L. monocytogenes growth (23).

Since it has been shown that a high initial number of L. monocytogenes ($10^6$/g) did not increase in minced meat during 14 d in the presence of more than $10^6$ lactobacillus/g (24), it is likely that Lactobacillus species might play some inhibitory role.

The average initial number of lactobacilli (Fig. 1) in our sausages was surprisingly high-namely, $1.2 \times 10^7$/g (range from $5 \times 10^6$/g to $1.4 \times 10^7$/g), which is a consequence of high initial contamination and extended development of the microflora in the meat from which the sausage mixture was prepared.

**TABLE 2. Differentiation of Lactobacillus strains isolated from fermented sausages.**

<table>
<thead>
<tr>
<th>Lactobacillus species</th>
<th>Number of strains</th>
<th>% of all strains examined</th>
</tr>
</thead>
<tbody>
<tr>
<td>L. plantarum</td>
<td>12</td>
<td>54.5</td>
</tr>
<tr>
<td>L. lactis</td>
<td>4</td>
<td>18.2</td>
</tr>
<tr>
<td>L. delbrueckii</td>
<td>4</td>
<td>18.2</td>
</tr>
<tr>
<td>L. casei</td>
<td>1</td>
<td>4.5</td>
</tr>
<tr>
<td>L. salivarius</td>
<td>1</td>
<td>4.5</td>
</tr>
<tr>
<td>Total</td>
<td>22</td>
<td>99.9</td>
</tr>
</tbody>
</table>

The lactobacilli present in the sausages were investigated by isolation and identification of 22 Lactobacillus strains. More than half of the isolated strains were L. plantarum, followed by L. lactis and L. delbrueckii (Table 2). This finding is in agreement with those of other authors (16,20).

The inhibitory effect of the 12 isolated L. plantarum strains towards L. monocytogenes was investigated on agar inoculated with L. monocytogenes.

The filtrates of L. plantarum had a pH ranging from 3.47 to 4.52. The zones of inhibition towards L. monocytogenes varied from 4.5 to 11 mm, with an average of 6.27 mm. When the pH of the culture filtrates was adjusted to 7.0, the zones of inhibition decreased to 0.5±1.2 mm, with an average of 0.88 mm, and were produced by only 7 of the 12 L. plantarum strains investigated.
This indicates that the inhibitory effect of *L. plantarum* might not only be due to the pH factor but might also be caused by unidentified metabolites produced by some of the lactobacillus strains (24).

**Vacuum-packaged frankfurters**

The chemical composition of the skinless vacuum-packaged frankfurters was as follows: moisture 53.2%, fat 31.5%, protein 12.2%, sodium chloride, 2.2% (3.97% in the water phase), polyphosphates (as P₂O₅ added) 0.17%, and residual nitrates 25.6 ppm.

**TABLE 3. Changes of MPN of L. monocytogenes, number of Lactobacillus spp. and total number of bacteria on vacuum-packaged frankfurters during storage at 4°C.**

<table>
<thead>
<tr>
<th>Storage at 4°C (days)</th>
<th>Number of bacteria in package</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>L. monocytogenes (MPN)</td>
</tr>
<tr>
<td>0</td>
<td>5 x 10⁶</td>
</tr>
<tr>
<td>10</td>
<td>1.5 x 10⁷</td>
</tr>
<tr>
<td>20</td>
<td>2.1 x 10⁸</td>
</tr>
</tbody>
</table>

The initial load of *L. monocytogenes* (Table 3) increased 30-fold after 10 d and by 420 times after 20 d. *L. monocytogenes* was not detected in the control packages after 10 d but 1.2 x 10⁵ *L. monocytogenes* per package were detected after 20 d of storage.

This confirms previously reported data that vacuum-packaged meat products represent a favorable environment for *L. monocytogenes*. Significant multiplication of *L. monocytogenes* has been reported in the following vacuum-packaged products: beef at 0 and 5.3°C; cooked poultry at 4 and 10°C (6); ham, bologna, “bratwurst” sausages, sliced chicken, and turkey at 4.4°C (9); and “brühwurst” sausages at 2, 4, and 7-10°C (26).

Other investigations have shown that *L. monocytogenes* did not grow in vacuum-packaged meat products: in minced beef at 4°C (11) and in summer sausages at 4.4°C (9). Glass and Doyle (9) indicated that *L. monocytogenes* was not able to multiply in vacuum-packaged summer sausages, in which the pH was 4.8 - 4.9 and salt content 3-3.4%. We did not measure pH in the frankfurters nor in the liquid separated in the vacuum packages, but the salt content in our frankfurters was 2.2% (3.97% in the water phase), which is much lower than in the summer sausages.

The *Lactobacillus* spp. strains isolated from the vacuum-packaged frankfurters comprised the psychrophilic *L. sake*, *L. curvatus*, and *L. divergens*, and to a lesser extent some other *Lactobacillus* spp. However, the participation of each single species of *Lactobacillus* in the microflora of vacuum-packaged frankfurters varied considerably between the packages. Therefore, further investigations are needed to give an explanation for the lack of inhibition of *L. monocytogenes* in our vacuum-packaged frankfurters, even though clear inhibition of *L. monocytogenes* has been registered with some strains of *L. sake* and *L. curvatus* on solid medium (24).

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