Effect of Different Levels of Nitrite and Nitrate on the Survival of *Listeria monocytogenes* During the Manufacture of Fermented Sausage

JAANA JUNTTILA*1, JORMA HIRN1, PAULI HILL2 and ESKO NURMI1

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ABSTRACT

The fate of *L. monocytogenes* during the fermentation of Finnish fermented sausage was examined. *L. monocytogenes* was able to survive during a 21 day fermentation of sausage with levels of nitrite and salt commonly used in the meat industry today (120 ppm NaNO2 and 3.0% NaCl). Initial numbers of *Listeria* (10^3 CFU/g and 10^6 CFU/g) decreased approximately 1 log 10 CFU/g during the fermentation. Increasing the levels of nitrite/nitrate to those used 30 years ago in meat products had a marked effect on the elimination of *Listeria*. The numbers of survivors in the sausages was reduced by increasing only the levels of nitrite/nitrate. Levels of these additives with best bacteriostatic effect on *Listeria* could not be totally eliminated from highly contaminated sausage by increasing only the levels of nitrite and nitrate. Levels of these additives with best bacteriostatic effect on *Listeria* are no longer permitted in food.

Since outbreaks of listeriosis associated with coleslaw (25), milk (9), and soft cheese (13), *Listeria monocytogenes* has become a concern to the food industry. Dairy foods have been most scrutinized as vehicles for listeriosis. However, *L. monocytogenes* is a ubiquitous microorganism and it is likely that other foods will become contaminated with these bacteria. This organism has been isolated from meat products intended for cooking (4,10,18), as well as in dry sausages (18).

*L. monocytogenes* has been shown to survive during the manufacture of dry salami if initially present at levels > 10^3 CFU/g (14). There is however very little information available regarding the effects of various food additives on the capability of *Listeria* to survive in foods. Since the 1950’s the levels of nitrite and nitrate used in meat products have decreased for reasons of public health.

The purpose of this study was to examine the fate of *L. monocytogenes* during the manufacture of Finnish fermented sausage, and to evaluate differences in the manufacturing processes used during the last 30 years on the survival of these bacteria. The role of sodium nitrite and potassium nitrate combined with two levels of sodium chloride in controlling the survival of this microorganism during fermentation and drying of sausage was examined. The levels of nitrite, nitrate, and salt used in this study were based on the results of three previous studies concerning the amount and effects of these additives and various starter cultures on the quality of dry sausage (19,21,22). The levels were also selected based on the use of these chemicals in meat products during the last 30 years.

MATERIALS AND METHODS

Culture of *L. monocytogenes*

*Listeria monocytogenes* (SLCC 2375), serotype 4b was obtained from the Special Listeria Culture Collection, University of Würzburg, FRG. Inocula were prepared from cultures grown in tryptose broth (Merck) for 18 h at 37°C. After centrifugation at 6000 x g for 10 min the pellets were suspended in 0.01 M phosphate-buffered saline solution (PBS, pH 7.2), and the absorbance of the cell suspension was determined at 500 nm. The suspension was diluted appropriately in PBS, and added to ground sausage mass in a Baku type 900 mixer (Hugo Kunzi, Stuttgart, FRG) to provide final concentrations of 10^3 and 10^5 CFU/g in the first trial in order to estimate changes in the number of *L. monocytogenes* during the manufacture. Since the fate of *L. monocytogenes* was very similar with both levels of *Listeria* only one concentration (10^5 CFU/g) was used in the second trial. Enumeration was done by spread plating 0.1 ml of serial (1:10) dilutions of the inocula onto duplicate tryptose agar plates (Merck) and incubating the plates at 37°C for 48 h.

Starter culture

A commercial mixed culture of *Staphylococcus carnosus* and *Lactobacillus plantarum* (Duploferment 66, Rudolf Müller & Co, Giessen, FRG) was used in both trials. Freeze dried cultures were diluted using peptone water (0.1% peptone w/v). Resuspended cultures were inoculated into the mass to obtain final concentrations of 10^7 CFU/g of both staphylococci and lactobacilli.

Additives

In the first trial the addition of NaCl and NaNO2 was the same as in commercial dry sausages manufactured today in Finland: 120 ppm NaNO2 and 3.0% NaCl. In the second trial, 8 combinations of salt and nitrite or nitrate were used (Table 1). Effects of 1000 ppm KNO3 and 3.5% NaCl used 30 years ago (19), on *L. monocytogenes"
were compared to additions in the 1960’s (200 ppm NaNO₂) (21), today, and to very low levels of nitrite (50 ppm) (22). Nitrite and nitrate were added in 10% water solutions. Glucose was added at the level of 0.6% (w/w), and a selected spice extract and a mixture of ground spices (Helsingin Kauppiaat, Helsinki, Finland) was added at a level of 0.2% (w/w).

Preparation of sausage

Sausage was prepared from frozen beef and pork, which were thawed at 2°C for one d before manufacture. The meat was ground using a 200 L Graemer-Greber cutter, in which salt, sugar, and spices were added during rotation. A 15 kg batch was prepared in the first trial, and a mass of 27 kg in the second. The batches were then divided into five and nine parts respectively; the weight of each being 3 kg.

Start cultures were added during a regrinding in an ice-cooled Rasant K cutter (Sedellmann, Stuttgart, FRG). Mixed sausage batches were then moved to a Baku type 900 mixer (Hugo Kunzi, Stuttgart, FRG), where L. monocytogenes inocula were added. Controls without Listeria inoculation were prepared in both trials. Then after mixing for 1 min, NaNO₂ and/or KNO₃ were added, and the masses were mixed for 1 min.

The sausage masses were stuffed in fibrous casings with a diameter of 70 mm (Visko; Hanko, Finland) using a hand stuffer. The sausages, each weighing about 300 g, were tied by hand.

The sausages were pre-dried in the Autoterm (Waxweiler, FRG) for 2 d at 23°C. The relative humidity was 95%. During the next 5 d the sausages were smoked in the Autoterm (Waxweiler) at 20 to 22°C and the humidity was decreased to 80%. After the smoking, they were kept for 1 week at 18°C and 75 to 80% humidity, and then at 10°C and 50% humidity for another week to complete the 21 d manufacture.

Sausage sampling and isolation of Listeria

Two samples of each type were sampled from different parts of the Autoterm (Waxweiler) at the time of manufacture and on the 3rd, 7th, 14th, and 21st day of fermentation. Duplicate composite samples, aseptically taken from different locations of each sausage were macerated in a stomacher (Seward Medical, London, UK) for 2 min after adding 225 ml of Listeria enrichment broth (LEB) (3) in a sterile stomacher bag (Seward Medical). Samples were then diluted in 0.01 M PBS, and 0.1 ml portions were spread plated onto duplicate plates of LPM-phenylethanol-moxalactam (LPM)-agar (16). The plates were incubated at 30°C and L. monocytogenes colonies were counted with 45° oblique transillumination (15) after 18 and 48 h incubation. One ml of each dilution was also added to 9 ml of LEB, and enriched and sampled as described by McClain & Lee (17).

From each plate three colonies typical of L. monocytogenes were sub-cultured and confirmed biochemically based on the criteria of Seeliger & Jones (28), and serologically according to the Seeliger-Donker-Voet antigenic scheme (27).

Chemical analyses and pH determination

pH-values, nitrite and nitrate were determined according to the standard methods of Finnish Standards Association - International Organization for Standardization (SFS-ISO) (6,7,8). Water activity (a_w) was determined with Vaisala' Humicamp HMP 31UT Instrument (Vaisala Oy, Helsinki, Finland) after equilibrating the samples at 20°C for 2 h. The determination time was 5 min.

RESULTS

In the first trial L. monocytogenes was able to survive during the manufacture of fermented sausage with levels of food additives commonly used in the meat industry today (120 ppm NaNO₂ and 3.0% NaCl). Numbers of survivors were enumerated by direct plating on LPM-agar, and during a 21 d fermentation listeriae decreased approximately 1 log 10 CFU/g. The fate of L. monocytogenes during fermentation was very similar with both levels of Listeria inoculation (10⁵ and 10⁷ CFU/g) (data not shown). Therefore, only one concentration (10⁵ CFU/g) was used in the second trial.

L. monocytogenes was isolated by direct plating from all samples tested in the second trial (Table 2). A remarkable decrease in the number of L. monocytogenes was detected in the sausage with initial addition of 1000 ppm KNO₃. The number of Listeria decreased 3.3 log 10 CFU/g during the fermentation of 3 weeks and a decrease of approximately 2.0 log 10 CFU/g was noted after 7 d manufacture.

With the addition of 200 ppm NaNO₂ and 300 ppm KNO₃, the number of L. monocytogenes decreased less than 1 log 10 CFU/g during the first week of fermentation and drying, but in 3 weeks the number of survivors had decreased 2.0 log 10 CFU/g. When the level of nitrite was ≤ 200 ppm the decrease was slower, and the survivors were reduced approximately 1-1.5 log 10 CFU/g after the 3 weeks of ripening. Increasing the salt concentration from 3.0 to 3.5% in the sausages did not essentially affect the survival of Listeria. No listeriae were detected in uninoculated control sausages.

Water activity (a_w) of the sausages was 0.88 ± 0.01 after 21 d fermentation, and the pH values decreased from 5.7 to 4.6 ± 0.05 in 3 weeks. The contents of nitrite ranged from 0.5 ppm (sausage 2) to 42.0 ppm (sausage 8) after the fermentation of 3 weeks. Nitrate was not detected in sausage 2 after 21 d, and the highest levels (49.5 ppm) were found in sausage 8.

DISCUSSION

In the first trial the fate of L. monocytogenes during manufacture of sausage was similar to that described by Johnson et al. (14) who demonstrated the survival of this microorganism in hard salami. The growth and multiplica-

<table>
<thead>
<tr>
<th>Sausage</th>
<th>NaCl (%)</th>
<th>NaNO₂ (ppm)</th>
<th>KNO₃ (ppm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>3.0</td>
<td>50</td>
<td>-</td>
</tr>
<tr>
<td>2</td>
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<tr>
<td>8</td>
<td>3.5</td>
<td>1000</td>
<td>-</td>
</tr>
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</table>

*Levels of food additives used today.

According to Nurmi (1966).

According to Niinivaara (1955).
tion of *L. monocytogenes* was prevented but the organism did survive during the fermentation, although in decreased numbers.

The pH values (4.6 ± 0.05) and the a<sub>0</sub> (0.88 ± 0.01) of prepared sausages are commonly found in commercial Finnish fermented sausage today. *L. monocytogenes* grows over a wide pH range (5.0-9.6) (26), and although growth does not occur below pH 5.0 the organisms can survive e.g. in silage, even at pH 4.0 or lower (5,12). Therefore survival of *Listeria* in dry sausage with pH values slightly lower than 5.0 is likely, and thus a low pH value provides no guarantee of a *Listeria*-free product.

*L. monocytogenes* is quite tolerant to sodium chloride. Since these bacteria have been reported to grow in 10% NaCl (26), the addition of 3.0-3.5% salt used in dry sausage manufacture cannot be expected to eliminate *Listeria* from contaminated products. Increasing the salt concentration from 3.0 to 3.5% does not markedly decrease the number of survivors.

Meat starter cultures composed of lactobacilli have inhibitory effects against many pathogens and function as effective preservatives against undesirable microorganisms (1,20,30). When associated with *Lactobacillus plantarum* the number of *L. monocytogenes* in ground beef has been reported to decrease (11). Acid production of lactobacilli may be an inhibitory mechanism also against *L. monocytogenes* in fermented sausage.

The results of the second trial show that increasing levels of nitrite and nitrate in sausage has a marked effect on the elimination of *Listeria*. Levels of these additives used 30 years ago in meat products have best bacteriostatic effect on *L. monocytogenes*, but can not totally eliminate these bacteria from highly contaminated sausages. These levels of nitrite and nitrate are not permitted any longer in food.

It has been suggested that the antimicrobial activity of NaNO<sub>2</sub> against *Listeria* at levels within current regulations can be achieved only with ≥ 3% NaCl and pH ≤ 5.5 at refrigeration temperatures; warmer temperatures may decrease the inhibitory effects (29). Therefore, the fermentation temperatures of sausage which are even higher today than some years ago cannot be expected to be optimal for the bacteriostatic effect of nitrite against *Listeria*. Increasing only the levels of nitrite in sausage is not effective enough in controlling the growth and survival of these microorganisms.

The growth of *L. monocytogenes* in sausage was apparently suppressed by the synergism of salt, nitrite, and pH. It seems, however, that unlike some other pathogens, such as *Yersinia enterocolitica* (23,24), *L. monocytogenes* cannot totally be eliminated from fermented meat products with the levels and combinations of food additives most commonly used in the meat industry today.

Although no outbreaks of listeriosis associated with the consumption of meat have been reported, *L. monocytogenes* has been isolated from both raw meat (4,10,18), and dry sausage (18). More research needs to be done on the interaction of different factors, such as starter cultures and various food additives on controlling the survival of *L. monocytogenes* in dry sausage and other meat products.

**REFERENCES**


**TABLE 2. Numbers of *L. monocytogenes* during a 21 d manufacture of Finnish fermented sausage.**

<table>
<thead>
<tr>
<th>Days of fermentation</th>
<th>1&lt;sup&gt;a&lt;/sup&gt;</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
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<tr>
<td>L. monocytogenes (log&lt;sub&gt;10&lt;/sub&gt; CFU/g)&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1.570</td>
<td>1.585</td>
<td>1.562</td>
<td>1.581</td>
<td>1.578</td>
<td>1.593</td>
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<td>1.510</td>
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<td>1.520</td>
<td>1.520</td>
<td>1.500</td>
<td>1.508</td>
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<tr>
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<td>1.484</td>
<td>1.484</td>
<td>1.484</td>
<td>1.483</td>
<td>1.431</td>
<td>1.374</td>
<td>1.260</td>
</tr>
</tbody>
</table>

<sup>a</sup>Mean values from duplicate samples of two sausages.

<sup>b</sup>Combinations of sodium nitrite, potassium nitrate, and sodium chloride used in the manufacture of each sausage is shown in Table 1.


