Survival of Pathogenic Bacteria on Pork Loins as Influenced by Hot Processing and Packaging

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ABSTRACT

The impact of hot and cold processing on the survival and/or growth of Salmonella, Listeria monocytogenes, pathogenic Yersinia enterocolitica, Aeromonas hydrophila, and Campylobacter spp. on inoculated pork loins was studied. Loin pieces were inoculated with pathogen-containing feces, then vacuum packaged directly after hot boning (hot packaging), vacuum packaged after chilling for 1 d, or left unpackaged. Pathogen numbers were assayed after storage at 1 ± 1°C for 0, 1, 2, 5, and 9 d. Campylobacter numbers decreased during the storage period, but the organisms could still be recovered after 9 d storage. A lower Campylobacter survival rate was observed on unpackaged pork loin pieces. The other pathogens were less affected by packaging treatment. Numbers of L. monocytogenes increased by approximately 1-log10 CFU/cm2. Survival and growth of A. hydrophila during refrigerated storage varied between trials. In Experiment 1, numbers increased by ca. 1-log10 CFU/cm2, while levels were stable or decreased slightly in Experiment 2. Salmonella levels decreased during storage, with the greatest survival observed on hot-packaged pork. Despite the fact that pork loins were inoculated with ca. 105 CFU pathogenic Y. enterocolitica per cm2, the organism was recovered only sporadically during the storage period. This may be due to competition with the natural microflora. Existing procedures are often inadequate for recovering small numbers of pathogenic Y. enterocolitica in the presence of large numbers of competing flora. Until recovery procedures are improved, the effect of packaging treatment on pathogenic Y. enterocolitica cannot be determined.

Hot boning (HB) and processing differ from cold boning (CB) in many respects, some of which may have an impact on the microflora of the end product. A major factor is the difference in chilling and dessication rates. To limit evaporation (weight losses), HB meat is packaged immediately after boning. Packaged HB meat thus provides a warm and moist environment, an ideal medium for microbial growth. The effect of hot boning on total aerobic counts has been the object of much investigation (19), but little attention has been paid to the growth and/or survival of pathogenic bacteria.

Certainly the behavior of pathogens such as Listeria monocytogenes and Yersinia enterocolitica should be determined as these organisms are capable of growth at refrigeration temperatures. The higher relative humidity and lack of dessication associated with packaged HB meat may favor the growth of psychrotrophic meatborne pathogens. Alternatively, the survival and growth of pathogens on HB meat may be reduced as a consequence of the presence of more competing bacteria.

An additional psychrotrophic pathogen, Aeromonas hydrophila, deserves investigation because of its association with food of animal origin. A. hydrophila is increasingly recognized as a human pathogen, although a definite link between this organism and foodborne transmission is still lacking. There are only a few data on the survival of this organism under meat storage conditions.

Salmonella and Campylobacter spp. are leading causes of human foodborne disease, and meats and poultry are often implicated in illness. Salmonella, a mesophilic microorganism, does not grow at refrigeration temperatures and, therefore, is not expected to be a major concern on properly stored fresh meats. However, the difference in chilling rate between HB and CB meat may affect the survival of salmonellae. Also, survival may be influenced by the atmosphere surrounding the meat. Oosterom et al. (13) established that Campylobacter numbers on pork carcass surfaces decreased during chilling under forced ventilation. The authors further determined that survival was little affected by the chilling process, but that Campylobacter was very sensitive to drying. Concerns that immediate vacuum packaging (hot packaging) of HB meat might lead to increased survival of Campylobacter led to the investigation of van Laack et al. (20). Results indicated that hot packaging allows greater survival of Campylobacter than cold packaging the meat after overnight chilling.

All five of the aforementioned pathogens may be excreted by domestic livestock, and L. monocytogenes, Y. enterocolitica, and A. hydrophila are thought to be common in the natural environment. Hence, contamination of meat with these organisms would not be unexpected and may...
occur during slaughter, dressing, and subsequent processing operations.

The aim of this investigation was to determine what effect packaging treatment had on the survival of Campylobacter spp., Salmonella, pathogenic Y. enterocolitica, L. monocytogenes, and A. hydrophila on pork loins stored at 1 ± 1°C. Three packaging treatments were examined: hot packaged (HP), cold packaged (CP), and unpackaged. Examination of unpackaged pork was necessary because this is the manner in which conventionally processed pork primals are typically distributed in The Netherlands. Pork loin surfaces were smeared with pathogen-inoculated feces in an attempt to simulate "natural contamination", and pork loin pieces were chilled overnight in the cooler of a slaughterhouse. Packaged and unpackaged pork loin pieces were then removed to the laboratory and held at 1 ± 1°C for 9 d.

MATERIALS AND METHODS

Preparation of inoculum and inoculation of feces

Strains used were as follows: A. hydrophila WA 8 and WA10 (water isolates; RIVM, Biltlhoen, The Netherlands); S. typhimurium V49 (meat isolate; Department of the Science of Food of Animal Origin, Utrecht, The Netherlands); L. monocytogenes 890620 and 128901 (meat isolates; Department of the Science of Food of Animal Origin); Campylobacter jejuni 81116 (human isolate; Department of Veterinary Bacteriology, University of Utrecht, The Netherlands) and LA32 (human isolate; RIVM) and Campylobacter coli C59 and C63 (pig and chicken isolates, Department of Veterinary Bacteriology), and Y. enterocolitica serotypes O:3 and O:9 (L. de Zutter, University of Ghent, Belgium). With the exception of Campylobacter, stock cultures of each organism were subcultured at least twice on brain heart infusion (BHI) agar before being used for inoculum preparation. Y. enterocolitica, L. monocytogenes, and A. hydrophila were incubated at 28°C for 24-36 h; S. typhimurium was incubated at 37°C for 48 h. Campylobacter spp. were subcultured onto blood agar (Oxoid #CM272, #SR84, and #SR48) and incubated at 37°C for 48 h under microaerobic conditions (BBL Gas-pak envelope, without catalyst; Cockeysville, MD).

Prior to inoculum preparation, Y. enterocolitica, A. hydrophila, and L. monocytogenes were grown in BHI broth (Oxoid #CM 225) for 36 h at 10°C. S. typhimurium was grown in BHI broth at 37°C for 6 h, and Campylobacter spp. were grown in Brucella broth (BBL) at 37°C for 36 h under microaerobic conditions. Previous experiments had shown that these incubation conditions yielded cells in log-phase growth. For each genus, cells of the various strains were combined in a cocktail. Cells were centrifuged at 1500 x g for 20 mn, then suspended in 0.85% saline (PS; used in Experiment 1) or BHI broth (Experiment 2). Cell numbers were estimated spectrophotometrically at 500 nm or, in the case of Campylobacter, microscopically with the aid of a counting chamber. Bacterial suspensions were diluted in PS in Experiment 1 and in 1% buffered peptone water (BPW) in Experiment 2. Inoculum levels were determined by spread plating onto blood agar (Campylobacter spp.) or BHI agar (other pathogens) and incubating at 37°C for 48 h (Campylobacter spp.) or 24 h (other pathogens).

On the morning of the experiment, ca. 500 g of fresh feces was collected from two hogs. The feces were inoculated with a suspension of mixed pathogens so that the desired contamination levels would be achieved when 1 g of feces was used to inoculate 100 cm² meat surface. Target inoculum levels were 10³-10⁴ CFU/cm² for Aeromonas and Yersinia, 10⁴ CFU/cm² for Salmonella and Listeria, and 10⁵ CFU/cm² for Campylobacter. Inoculated feces were placed in a double-layer sterile plastic bag, kneaded thoroughly to mix, and transported to the slaughterhouse.

Preparation and inoculation of pork loins

Two experiments (Experiments 1 and 2) were performed; procedures used in the two experiments were nearly identical. Twelve carcasses were used in each experiment. In Experiment 1, carcasses with pH >6.2 at 45 mn postmortem were selected. In Experiment 2, no selection criterion was used and the incidence of the pale-soft-exudative condition was assessed at 1 d postmortem. In both experiments, the meat pH was measured at 9 d postmortem. No pale-soft-exudative or dark-firm-dry meat samples were used in either of the experiments.

All loins of the hog carcasses were hot boned at 40-45 mn postmortem, then skin and backfat were removed from the dorsal surfaces. The dorsal sides of all loins were inoculated by smearing the feces over the entire side at a rate of 1 g feces per 100 cm². Eight of the 24 loins were randomly selected, cut into six pieces of ca. 300 g, immediately vacuum packaged (polyamide/polyethylene vacuum bags with oxygen permeability of 25-30 ml/m², 24 h, measured at 23°C and 75% relative humidity, Wolff, Walsrode, Germany), and chilled at 2 ± 2°C (HP). Remaining loins were chilled overnight at 2 ± 2°C. The following morning, eight of these loins were cut into six pieces and vacuum packaged (CP). The remaining eight loins were left unpackaged throughout the remainder of the experiment (cold unpackaged loins; CU). Packaged and unpackaged loins were then transported to the laboratory and stored in a refrigerated incubator at 1 ± 1°C.

Sampling procedures and bacterial isolation

Immediately following inoculation (day 0) and on days 1, 2, 5, and 9 after inoculation, eight loin pieces from each treatment group (HP, CP, CU) were sampled using a destructive method (17). Tissue samples (15 cm² total surface area) from each loin piece were placed in a sterile bag, then refrigerated for ≤1.5 h. Forty-five milliliters of diluent (PS for Experiment 1, BPW for Experiment 2) was added to each sample and samples were homogenized in a stomacher for 2 mn. Serial (10-fold) dilutions of this sample were used to inoculate a "3 tube" MPN (most probable number) series for each pathogen.

Microbiological assays

Y. enterocolitica. A MPN series was set up by adding 1 ml from serial dilutions of the tissue samples to 9 ml of Igusangan ticarcillin and potassium chlorate broth (21). After incubation for 48 h at 23°C, a loopful of this enrichment was streaked onto SSDC agar (Salmonella, Shigella Deoxycholate Calcium agar; ref 211 B Merck #11443). Subsequent incubations were at 28°C in order to retain virulence plasmids. Confirmation of suspect colonies was done by testing five typical colonies from plates prepared from the highest dilution in a series; if these isolates were not Yersinia, then five colonies from the next-highest dilution were tested. If these colonies were not typical of Yersinia, colonies off plates from the next-highest dilution were tested. Suspect colonies were streaked onto BHI agar, tested for gram reaction, and then inoculated into Kligler’s iron agar, Simmons’ citrate agar, and urea broth. Serological confirmation was done with O:3 and O:9 antisera (Eco-Bio, Genk, Belgium).

L. monocytogenes. Isolation principally followed the revised U.S. Department of Agriculture-Food Safety Inspection Service (USDA-FSIS) protocol (18). One milliliter from serial dilutions of the tissue samples was added to 9 ml of University of Vermont medium (Difco #0223-17). After incubation at 30°C for 24 h, 0.1 ml was transferred to 10 ml of Fraser broth. After incubation at 37°C for 24 and 48 h, broth from tubes exhibiting darkening was streaked onto modified Oxford agar, and plates were incubated at 37°C for 48 h. Suspect colonies, taken from plates streaked from...
the highest dilution of the enrichment broth (as described for *Yersinia*), were tested for catalase production and streaked onto BHI agar. Confirmation tests included β hemolysis on 5% sheep blood agar and umbrella motility (in Difco motility test medium, Detroit, MI) when grown at 20°C.

Campylobacter. One milliliter from sample serial dilutions was added to 9 ml of Preston broth (ref 1; Oxoid #CM67, #SR117, #SR84, #SR48) and incubated at 42°C for 24 h under microaerobic conditions (BBL Gas-pak envelope, without catalyst). Enrichments were streaked onto Preston agar (ref 1; Oxoid #CM689, #SR117, #SR84, #SR48), and plates were incubated at 42°C for 48 h under microaerobic conditions. Suspect colonies from the highest dilution were streaked onto blood agar (Oxoid #CM271, #SR84, #SR48) and incubated as described for Preston agar. Isolates were tested for catalase and oxidase production, as well as darting motility, H₂S production in BHI agar. Confirmation tests included p hemolysis on 5% sheep blood agar (Oxoid #CM329) and incubated at 37°C for 48 h. Colonies with morphology typical of *Salmonella* were streaked to green agar (Oxoid #CM 329) and incubated at 37°C for 48 h. Colonies. Upon further characterization, most of these isolates were capable of citrate utilization and, thus, were not *Y. enterocolitica*.

Campylobacter numbers on the HP loins decreased by less than 1-log₁₀ CFU/cm² during storage while a larger decrease was seen on CP loins (Table 1). *Campylobacter* on CU pork showed the greatest decline in numbers. Even after 9 d of refrigerated storage, however, *Campylobacter* was still detectable, even on the CU pork.

Numbers of *L. monocytogenes* increased by ca. 1 log₁₀ during refrigerated storage regardless of packaging treatment (Table 1). *Listeria* numbers appeared to increase fastest on HP pork, then stabilized around day 5. In contrast, *L. monocytogenes* populations on the CP and CU meat gradually increased throughout the 9-d storage period.

Table 1 presents data on levels of *Campylobacter* spp., *L. monocytogenes*, and *A. hydrophila* on pork loin pieces during 9 d of refrigerated storage. Recovery of *Salmonella* and *Y. enterocolitica* was problematic throughout the entire experiment, and data were so scarce that they are not included in Table 1. Pathogenic *Y. enterocolitica* was recovered only occasionally during Experiment 1. Great difficulties were encountered with the recovery of pathogenic *Y. enterocolitica* because SSDC plates from all MPN dilutions were crowded with *Yersinia*-like colonies. Upon further characterization, most of these isolates were capable of citrate utilization and, thus, were not *Y. enterocolitica*.

**RESULTS**

As it was our aim to follow the survival of various pathogens on inoculated pork loin pieces over time, targeted inoculum levels for each pathogen represented a compromise between realistic contamination levels and numbers appropriate for detecting growth or inactivation of a particular pathogen. Accordingly, targeted inoculum levels ranged from 10⁶ CFU/cm² for *Campylobacter* (an organism expected to die off) to 10⁴ CFU/cm² for *Salmonella* and *Listeria*, and 10³ CFU/cm² for *Y. enterocolitica* and *A. hydrophila* (organisms capable of growth during refrigerated storage).

**Experiment 1**

Spectrophotometric estimation of inoculum levels worked relatively well with *A. hydrophila*, *L. monocytogenes*, and *Salmonella*, and the targeted levels were added to the feces. Unexpectedly, the MPN dilution series expected to confirm the number of salmonellae present in the feces did not detect the organism. Numbers of *Campylobacter* and *Y. enterocolitica* added to the feces were below target levels. As a result, these organisms were not recovered from samples taken on day 0 and day 1 as the MPN series were set according to the targeted inoculum levels. Once the MPN dilution series for *Campylobacter* was adjusted, recovery of this organism was accomplished.

**Experiment 2**

Spectrophotometric estimation of inoculum levels worked relatively well with *A. hydrophila*, *L. monocytogenes*, and *Salmonella*, and the targeted levels were added to the feces. Confirmation tests included β hemolysis on 5% sheep blood agar and umbrella motility (in Difco motility test medium, Detroit, MI) when grown at 20°C.

Campylobacter. One milliliter from sample serial dilutions was added to 9 ml of Preston broth (ref 1; Oxoid #CM67, #SR117, #SR84, #SR48) and incubated at 42°C for 24 h under microaerobic conditions (BBL Gas-pak envelope, without catalyst). Enrichments were streaked onto Preston agar (ref 1; Oxoid #CM689, #SR117, #SR84, #SR48), and plates were incubated at 42°C for 48 h under microaerobic conditions. Suspect colonies from the highest dilution were streaked onto blood agar (Oxoid #CM271, #SR84, #SR48) and incubated as described for Preston agar. Isolates were tested for catalase and oxidase production, as well as darting motility, H₂S production in Brucella broth/cysteine with lead acetate strips, and nitrate reduction. In Experiment 1, isolates were also tested for hippurate hydrolysis.

A. hydrophila. A MPN enrichment series with alkaline peptone water (0.1% peptone, pH 8.4-8.6; 16) was used. Enrichments were incubated for 16-20 h at 23°C, then streaked onto starch-ampicillin agar (15), and incubated at 28°C for 24 h. After incubation, plates representing the highest dilution were flooded with ca. 5 ml Lugol iodine solution. Amylase-positive colonies were confirmed by Gram stain, catalase and oxidase tests, and DNase production (Experiment 1) or gram-negative tube (Experiment 2; 11).

Salmonella. For the MPN, serial dilutions of the tissue homogenate in PS (Exp. 1) or BPW (Exp. 2) were incubated at 37°C for 18-22 h. Then 0.1 ml was added to 10 ml of Rappaport-Vassiliadis medium (Oxoid #CM669) and incubated at 42°C. After 24 and 48 h, the enrichment was streaked onto phenol red brilliant green agar (Oxoid #CM 329) and incubated at 37°C for 48 h. Colonies with morphology typical of *Salmonella* were streaked to BHI agar, and after testing with polyvalent O serum (RIVM, Bilthoven, The Netherlands), were inoculated into triple sugar iron slants.

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A. hydrophila grew well on all pork loins, though numbers increased most rapidly on HP pork loins. Packaging treatment had little influence on the growth of A. hydrophila.

**Experiment 2**

Due to the difficulties encountered with the recovery of *Y. enterocolitica* during Experiment 1, we did not attempt to recover this organism in Experiment 2. *Y. enterocolitica* was, however, inoculated onto the pork loin pieces as in Experiment 1. In an attempt to improve *Salmonella* recovery, tissue samples were homogenized and preenriched in BPW rather than in PS as was done in Experiment 1. Sample preenrichment in BPW is a well-accepted technique for recovery of *salmonellae* (4).

For all pathogens (*L. monocytogenes*, *A. hydrophila*, *Campylobacter* spp., *Salmonella*, and *Y. enterocolitica*), numbers of bacteria inoculated into the feces approximated the target levels. The results of Experiment 2 are presented in Table 2. Numbers of *Campylobacter* decreased during storage, but the decrease was more gradual and less drastic than in Experiment 1. The first day of unpackaged storage (seen in CP and CU treatments) resulted in the largest decrease in *Campylobacter* numbers. As seen in Experiment 1, survival of *Campylobacter* was poorest on CU pork. *L. monocytogenes* results paralleled those of Experiment 1, with approximately a 1-log increase in numbers across all packaging treatments.

In sharp contrast to Experiment 1, numbers of *A. hydrophila* remained nearly constant or even decreased during refrigerated storage in Experiment 2. Levels of *A. hydrophila* especially decreased on CU pork.

Recovery of *Salmonella* was more successful than in Experiment 1. Numbers decreased on all pork loins, but the decrease was smallest on HP pork. *Salmonella* recovery was variable early in the experiment; on days 1 and 2, as few as one loin piece out of 8 yielded the organism.

### DISCUSSION

Both experiments demonstrate that *Campylobacter* survived on inoculated pork during refrigerated storage. Even on unpackaged pork, *Campylobacter* was capable of surviving a 9-d refrigerated storage period. Our results confirm those of van Laack et al. (20) who reported better survival of *Campylobacter* on hot-packaged pork compared to pork packaged after chilling. This may be due to more moisture retention and the higher water activity present at the surface of hot-packaged meat. Also, an immediate decrease in oxygen tension due to vacuum packaging of actively respiring meat may have contributed to the survival of this microaerophilic organism.

We observed much greater survival of *Campylobacter* on unpackaged pork than would have been predicted based on the report of Oosterom et al. (13). It should be realized that the present study is a model study, and some of the conditions may have enhanced survival of *Campylobacter*. For instance, we used artificially contaminated feces to inoculate the meat surface, whereas Oosterom et al. (13) spread naturally contaminated feces on pork skin.

<table>
<thead>
<tr>
<th>Sampling</th>
<th>Packaging treatment</th>
<th>Average log_{10} CFU/cm²&lt;sup&gt;a&lt;/sup&gt;</th>
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<tr>
<td></td>
<td>C. spp.</td>
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<td>4.64 (6/8)&lt;sup&gt;b&lt;/sup&gt;</td>
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<td>HP</td>
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<td></td>
<td>CP</td>
<td>1.31 (6/8)</td>
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<sup>a</sup> Average of MPN values from 'positive' samples.

<sup>b</sup> The number between brackets indicates the proportion of samples from which a given pathogen was recovered.

<sup>c</sup> <1.48 CFU/cm², exact MPN could not be determined because the lowest dilution for MPN assessment was 10², and plates from that dilution did not yield the pathogen indicated.
Our results indicated that growth of *L. monocytogenes* can occur on refrigerated pork in the presence of numerous competitors. There is a dearth of published studies on the growth and survival of *L. monocytogenes* on packaged pork, and the literature contains conflicting data on the growth of *L. monocytogenes* on beef. Growth of *L. monocytogenes* on vacuum-packaged, high-pH beef has been reported (6), but ground beef inoculated with the organism did not support growth over a 14-d storage period (9). Grau and Vanderlinde (7) observed that *L. monocytogenes* could grow on vacuum-packaged inoculated beef held at 0°C, but 11 weeks of storage were necessary for numbers to increase 10-fold. The faster growth rate observed during the present experiments may be due to the pork substrate; Khan et al. (10) reported faster growth of *L. monocytogenes* in pork sarcoplasmic solutions (‘drip’) than in drip from beef or lamb. Also, as demonstrated by Hart et al. (8), the temperature at which the inoculum is grown may strongly influence the survival and growth after inoculation. In the present study, we used cells of a fresh culture grown at 10°C. As most *Listeria* contamination is thought to occur during chilling and further processing, it is very likely that those bacteria are temperature adjusted and, therefore, best suited for survival and possibly growth on meats.

We observed great differences in the survival of *A. hydrophila* between our two experiments. While we do not know what caused these conflicting results, one possible explanation is the natural competitive flora present in the hog feces. We made no attempt to standardize the feces used for inoculation in the two experiments, nor did we investigate the profile of the naturally occurring flora. It is also possible that the natural microflora on the pork loins used in Experiment 2 was more antagonistic towards *A. hydrophila* than the microorganisms present on the loins used in Experiment 1. Palumbo (14) observed that the growth of *A. hydrophila* was diminished by the presence of naturally occurring meat microflora. We observed that starch ampicillin agar plates contained mainly *A. hydrophila* in Experiment 1, but plates in Experiment 2 were crowded with non-Aeromonas organisms.

We did not observe any appreciable effect of packaging on the survival and/or growth of *A. hydrophila*. Buchanan and Palumbo (2) have suggested that reducing the O₂ concentration in refrigerated packages of meat enhances the growth of *A. hydrophila* by suppressing the growth of *Pseudomonas*. This would imply better growth of *A. hydrophila* on packaged versus unpackaged meat.

In Experiment 1, physiological saline was used as diluent and preenrichment medium for *Salmonella*. This may account for some of the difficulties we experienced in the recovery of this organism. In Experiment 2, we used BPW as diluent which resulted in more recoveries of *Salmonella*; recovery was still very variable, however. Judging from the amount of competitive flora present on the phenol red brilliant green agar plates, it is apparent that BPW preenrichment followed by selective enrichment in Rappaport-Vassiliadis medium and plating onto phenol red brilliant green agar is not optimal for the recovery of *Salmonella*. Given this situation, we cannot be sure if the observed decrease in recovery of salmonellae over time is due to cell death or interference by competitors. In any case, *Salmonella* numbers appeared to decrease during refrigerated storage, though the decrease was less dramatic in the HP pork. In an extensive review on *Salmonella*, D’Aoust (3) concluded that growth of salmonellae on food products may occur at temperatures as low as 2°C.

We encountered considerable difficulty in recovering pathogenic *Y. enterocolitica* from inoculated pork. This has been our general experience in the recovery of *Y. enterocolitica* from meats in the Netherlands. Profuse growth is present on the SSDC plates, and a considerable proportion of the colonies has similar morphology to pathogenic *Y. enterocolitica*, but the number of colonies that would have to be picked before a *Y. enterocolitica* is selected is prohibitive. A number of the non- *Yersinia* organisms we have recovered on SSDC appear to be similar to *Enterobacter agglomerans* and *Serratia liquefaciens*, but these organisms have not been completely characterized. Based on a comparison of cultural methods and DNA hybridization studies, Nesbakken et al. (12) concluded that use of conventional cultural isolation procedures may greatly underestimate pathogenic *Y. enterocolitica* in pork products. They attributed this to the low numbers of pathogenic *Y. enterocolitica* in pork products and overgrowth by competitors during cultural enrichment. Puikushima and Gomyoda (5) have reported that growth of pathogenic *Y. enterocolitica* in inoculated ground pork is inhibited by the natural microbial flora, especially environmental strains of *Yersinia* and *Haemalia alvei*.

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