# Factors associated with *Listeria monocytogenes* contamination of cold-smoked pork products produced in Latvia and Lithuania

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**Abstract**

A total of 312 sample of sliced, vacuum packaged, cold-smoked pork from 15 meat processing plants in Latvia and Lithuania, obtained over a 15-month period from 2003 until 2004, were analyzed for the presence of *Listeria monocytogenes* at the end of their shelf-life. Overall, 120 samples (38%) tested positive for *L. monocytogenes*. Despite the long storing period, the levels of *L. monocytogenes* in cold-smoked pork products were low. Manufacturing processes were studied at seven meat processing plants. A new approach with a logistic multivariable regression model was applied to identify the main factors associated with *L. monocytogenes* contamination during the manufacturing of cold-smoked pork products. Brining by injection was a significant factor (odds ratio 10.66; $P < 0.05$) for contamination of product with *L. monocytogenes*. Moreover, long cold-smoking times (≥ 12 h) had a significant predictive value (odds ratio 24.38; $P < 0.014$) for a sample to test positive for *L. monocytogenes*. Pulsed-field gel electrophoresis results indicated that various sources of *L. monocytogenes* contamination existed over periods of time in several meat processing plants. In two meat processing plants, persistent *L. monocytogenes* strains belonging to serotypes 1/2a and 1/2c were found.

Keywords: *Listeria monocytogenes*; Prevalence; Pork; Brine injection; Cold-smoking

## 1. Introduction

Since *Listeria monocytogenes* was found to be a significant food-borne pathogen (Schlech et al., 1983), epidemiological studies have confirmed that meat products have been involved in sporadic cases and outbreaks of listeriosis in many countries during recent decades (Gellin et al., 1991; Slutsker et al., 1999; de Valk et al., 2001; Frye et al., 2002; Sim et al., 2002). Various delicatessen meat products have played important roles in outbreaks of listeriosis in France, the UK, the USA and other countries (Farber et al., 1991; Jacquet et al., 1995; McLauchlin, 1996). Meat products associated with the transmission of *L. monocytogenes* generally have been processed, have long shelf-lives, are capable of supporting the growth of *L. monocytogenes*, and are consumed without further cooking (McLauchlin, 1996).

Vacuum packaged, cold-smoked pork is a ready-to-eat (RTE) meat product commonly produced in Baltic and Eastern European countries. This product is consumed without any heat treatment, usually on sandwiches, in salads or as a cold snack. The manufacture of cold-smoked pork involves no processing steps to eliminate *L. monocytogenes*. Moreover, some manufacturers set a long shelf-life for such products, thus possibly permitting substantial growth of *L. monocytogenes*.

In this study we report for the first time the results on the prevalence of *L. monocytogenes* in sliced, vacuum packaged, cold-smoked pork products from meat processing plants in Latvia and Lithuania. A new approach was applied with a logistic multivariable regression model to identify the main factors in the manufacture of cold-

smoked pork associated with *L. monocytogenes* contamination. Furthermore, pulsed-field gel electrophoresis typing and serotyping were used to characterize *L. monocytogenes* isolates and to determine their occurrence in cold-smoked pork products from various processing plants over a period of two years.

## 2. Materials and methods

### *2.1. The manufacturing process of cold-smoked, sliced, vacuum packaged pork*

A questionnaire was distributed to obtain detailed information on manufacturing practices at seven meat processing plants. Cold-smoked pork production involves several operations (Fig. 1). After cutting, the raw meat is dry salted or dry salted and injected with brine. The salt and/ or brine may contain nitrite, nitrate, spices and/ or starter cultures. The ripening time before cold-smoking varies among manufacturers, from 7 to 21 days. The meat is maintained at ≤ 6 °C and is turned regularly. More salt can be added and manually rubbed in during this primary ripening. Before cold-smoking, excess salt is rinsed away. Cold-smoking temperatures vary among producers from 20 °C to 30 °C. The time of exposure to natural smoke also varies among companies, from 3 to 48 h. Secondary ripening takes place at some plants, for 7 to 20 days at a temperature < 14 °C, in ripening chambers with humidity controlled at 75%. Products are sliced and vacuum packaged before they are distributed to the market. The shelf-lives of the vacuum packaged products are set by the manufacturers, and range from 20 to 120 days. The manufacturing process includes a lot of handling of the product by personnel, especially during salting, ripening, slicing and vacuum packaging. This type of product is consumed without any heat treatment.

*2.2. Sampling*

Vacuum packs of sliced, cold-smoked pork products were obtained from 11 stores of four supermarket chains in Latvia and Lithuania over a 15-month period during the years 2003 and 2004. A pack from each meat processing plant with product present on counter was collected. A total of 312 packs of products from eight Latvian and seven Lithuanian plants were collected (Table 1), with packs from several production lots from plants I and II being obtained. When packs were collected, the temperatures of the air in the display counters were determined. The packs were transported to the laboratory on ice and were then stored at 6 °C, before being analyzed within 5 days of the end of shelf-life stated on each pack. Each vacuum package with 70 to 150 g of sliced product was aseptically opened and a 25 g sample was taken and pummeled with 225 ml of half-strength Fraser broth (Oxoid, Basingstoke, UK) in a stomacher.

*2.3. Isolation and enumeration of L. monocytogenes*

*L. monocytogenes* was isolated according to the International Organization for Standardization method (Anonymous, 1996 a) with modification suggested by Johansson (1998). Examination for *L. monocytogenes* included two-step enrichment. All samples were incubated in half-strength Fraser broth at 30 °C for 24 h. After incubation, 0.1 ml was transferred to full-strength Fraser broth and incubated at 37 °C for 48 h. After incubation, the half- and full-strength Fraser broths were plated on PALCAM listeria selective agar (Oxoid) and *Listeria monocytogenes* blood agar (LMBA) containing Trypticase soy agar base (Difco,

Becton Dickinson, Sparks, MD, USA), 5% sterile sheep blood and, per litre, 10 g of lithium chloride (Merck KgaA, Darmstadt, Germany) and 10 mg of ceftazidime (Abtek Biologicals Ltd., Liverpool, UK). Selective agar plates were incubated at 37 ºC for 24 to 48 h. Five typical colonies were streaked from both PALCAM and LMBA onto 5% sheep blood Columbia agar (Difco) and incubated then at 37 °C for 24 h. Isolates that were β-hemolytic, catalase positive and Gram positive were presumed to be *L. monocytogenes*. Presumptive *L. monocytogenes* were confirmed using the API Listeria kit (bio Mérieux, Marcy l'Etoile, France). All confirmed *L. monocytogenes* isolates were stored at -70 °C.

Enumeration of *L. monocytogenes* was carried out according to the method of the International Organization for Standardization (Anonymous, 1996 b), for 15 of 120 positive samples produced from two of the plants (I and IV). Enumeration was performed immediately upon expiry of each product's shelf-life. The lower limit of enumeration was 10 cfu/g. The procedure included 1-h resuscitation in buffered peptone water at 20 °C, and surface plating on PALCAM and LMBA of 1.0 ml of $10^{-1}$, and 0.1 ml of each of the $10^{-2}$ and $10^{-3}$ dilutions. The plates were incubated at 37 °C for 24 to 48 h. Typical colonies were selected and plated on 5% sheep blood agar. *L. monocytogenes* was confirmed as described above.

*2.4. DNA isolation and pulsed-field gel electrophoresis (PFGE)*

A colony of *L. monocytogenes* was transferred to 5 ml of tryptic soy broth (TSB; Difco) which was incubated overnight at 37 °C. DNA isolation was performed with modifications as described by Björkroth et al. (1996) and Autio et al. (2002). After overnight

incubation, 2 ml of the culture were diluted in 5 ml PIV buffer (10 mM Tris, 1 M NaCl) and concentrated by centrifugation at 1100 × g for 15 min at 4 °C. Plugs for PFGE were prepared with concentrated cell suspension in PIV and 2% (w/v) low melting point agarose (InCert; FMC Bioproducts, Rockland, ME, USA). Cells in prepared plugs were lysed in a solution containing per ml 20 μg of RNase, 1 mg of lysozyme and 10 U of mutanolysin in lysis buffer containing 6 mM Tris, 1 M NaCl, 100 mM EDTA, 0.5% Brij-58, 0.2% sodium deoxycholate and 0.5% sodium lauroyl sarcosine, at 37 °C for 3 h with slow shaking. Lysis was continued with a 1-h wash with ESP solution containing proteinase K (Sigma) 100 μg/ml, 0.5 M EDTA and 10% sodium lauroyl sarcosine at 50 °C. ESP wash was repeated twice under the same conditions. Afterwards the plugs were washed in buffer containing 10 mM Tris and 0.1 mM EDTA at 50 °C for 1 h. A cutting enzyme *Asc*I 20 U/μl (New England BioLabs, Beverly, MA, USA) was used for digestion at 37 °C for 16 h.

PFGE was performed with 1.0% (w/v) agarose gel (SeaKem Gold; FMC Bioproducts, Rockland, ME, USA) in buffer containing 45 mM Tris, 4.5 mM boric acid, 1 mM sodium EDTA at 8 °C in a Gene Navigator system with a hexagonal electrode (Pharmacia, Uppsala, Sweden) operated at 200 V. Pulse times ranged from 1 to 35 s for 18 h. The size of the fragments was determined with a low-range PFG marker (New England BioLabs, Beverly, MA, USA). Gels were stained with ethidium bromide. Photo images were obtained with the Alpha Imager 2000 photo documentation system (Alpha Innotech, San Leandro, CA, USA). The images were saved as TIFF files for further analysis.

*2.5. Analysis of PFGE typing results*

Macrorestriction patterns (MRP) of *Asc*I were analyzed with BioNumerics software version 4.01 (Applied Maths, Kortrijk, Belgium), and applied Dice coefficient correlation to identify similarities among PFGE types. A dendrogram was constructed with the unweighted pair group method using arithmetic averages (UPGMA). The position tolerance was set to 1.2% with the optimization value at 1.0%.

*2.6. Serotyping*

One or two *L. monocytogenes* isolates of each PFGE type were serotyped with *Listeria* antisera (Denka Seiken, Tokyo, Japan), following the instructions of the manufacturer.

*2.7. Statistical analysis*

*L. monocytogenes* prevalence and the manufacturing process data were analyzed with the Statistical Package for Social Sciences 12.0 for Windows (SPSS Inc., Chicago, IL, USA) to identify factors in the manufacturing process of cold-smoked sliced vacuum packaged pork products associated with *L. monocytogenes* contamination. All manufacturing steps were defined as variables, and analysis of variance (ANOVA) was used to determine possible significant differences ($P < 0.05$) for the prevalence of *L. monocytogenes*. A nonparametric Spearman rank order correlation coefficient with two-tailed *P* value was calculated for cross-correlations between manufacturing steps and the presence of *L. monocytogenes*. The odds ratio (OR) with a 95% confidence interval was calculated for each manufacturing step, and *L. monocytogenes* detected in cold-smoked sliced vacuum packaged

pork was set as a dependent variable in the logistic multivariable regression model. The manufacturing steps identified as most significant in the bivariate analysis were set as independent variables in the model. Predictive values of independent variables were analyzed for the dependent variable by computing the coefficient estimates (B values), *P* values for the B values, and ORs with 95% confidence intervals from the B values.

## 3. Results

### *3.1. Prevalence and enumeration of L. monocytogenes in cold-smoked sliced vacuum packaged pork products*

The prevalence of *L. monocytogenes* in sliced, vacuum packaged, cold-smoked pork products are shown in Table 1. The prevalences of *L. monocytogenes* in the products from plants I, III, IV, VII, IX, and XI were significantly higher than the prevalences in products from other plants. With products from plants I and IV, *L. monocytogenes* was not detected in 10 of the 15 analyzed samples. In products from plant I, three samples contained < 10 cfu/g, one sample, 20 cfu/g, and three samples contained 100-1000 cfu/g. With product from plant IV, *L. monocytogenes* was not detected in seven samples, and the one sample that tested positive contained 20 cfu/g.

### *3.2. Factors associated with L. monocytogenes contamination in the manufacture of cold-smoked sliced vacuum packaged pork products in seven meat processing plants*

All production steps were studied separately for plants I, II, III, IV, V, VI, and VII, that produce similar products in which the prevalences of *L. monocytogenes* varied from 0 to 67%. Production processes were divided into manufacturing steps and analyzed as separate variables. ANOVA showed significantly higher prevalence ($P < 0.05$) of *L. monocytogenes* in the products of meat processing plants I, III, IV, and VII than in products from plants II, V, and VI. The presence of *L. monocytogenes* correlated with the use of brining injections and cold-smoking time (Table 2). Based on bivariate correlations, the four most significant variables, brining procedures, cold-smoking time, cold-smoking temperature, and total time at temperature between 10 °C and 30 °C, were selected for use in a multivariate logistic regression model. Brining injections were observed as a significant ($P < 0.05$) factor in product contamination with *L. monocytogenes* (Table 3). A long cold-smoking time (≥ 12 h) also had a significant ($P < 0.014$) predictive value for testing positive for *L. monocytogenes*, whereas cold-smoking at 24 to 30 °C for 8 to 48 h carried a significant ($P < 0.002$) predictive value for testing negative for *L. monocytogenes*.

### *3.3. Genotyping with PFGE and serotyping*

All 120 *L. monocytogenes* isolates were typed using PFGE. Based on genetic similarity, 27 different PFGE types were detected and grouped then into two genetic lineages (Fig. 2). Genetic lineage I contained 95% of all *L. monocytogenes* isolates belonging to serotypes 1/2a and 1/2c, while the remaining 5% of isolates in genetic lineage II, belonged to serotypes 1/2b, 3b, and 4b. Overall, 32% of *L. monocytogenes* isolates were recovered from only one meat processing plant. More than three PFGE types were found in the products from meat processing plants I, II, III, VII, IX, and XI (Table 4). *L. monocytogenes* isolates

obtained from Latvian plants (n = 99) and Lithuanian plants (n = 21) were grouped into 22 and 10 different PFGE types, respectively. The most common PFGE type 19 was recovered from Latvian plants I, II, III, and VI and Lithuanian plants IX and XI. PFGE type 19 was found in seven of eight production lots from meat processing plant I, and in four production lots from meat processing plant II (Table 5). *L. monocytogenes* PFGE types 10, 19, and 20 were recovered over a 9-month period from lots from plant I. PFGE types 12, 15, and 19 were recovered repeatedly, during a shorter period, from plant II. *L. monocytogenes* contamination in product from plants I and II was continuous throughout production. However, the prevalence levels of *L. monocytogenes* differed significantly ($P < 0.05$) between plants I and II.

## 4. Discussion

The prevalences of *L. monocytogenes* in cold-smoked pork products varied from 0 to 67% in Latvian products and from 10 to 73% in Lithuanian products. Regardless of how long products were stored, levels of *L. monocytogenes* at the ends of manufacturers' shelf-lives were mostly < 100 cfu/g. This suggests that cold-smoked pork products contain inhibitors of *L. monocytogenes*. The wide use of starter cultures in the manufacture of cold-smoked pork products may explain the low levels of *L. monocytogenes*. The use of starter cultures in the production of meat products is one of the most important factors for controlling *L. monocytogenes* growth (Farber et al., 1991; Ingham et al., 2004; Thevenot et al., 2005).

The manufacture of cold-smoked pork involves no processing steps to eliminate *L. monocytogenes*, thus contamination of the raw meat and contamination during processing

can both contribute to *L. monocytogenes* in the finished product. Several investigators have shown that the occurrence of *L. monocytogenes* in the plant environment and on processing surfaces has a significant positive relationship with the presence of this organism in finished products (Chasseignaux et al., 2001; Heir et al., 2004).

The multivariate logistic regression model indicated that brining, cold-smoking time, and cold-smoking temperature were factors affecting product contamination with *L. monocytogenes*. Autio et al. (1999) previously showed that injected brines were an important source of the *L. monocytogenes* found in cold-smoked rainbow trout. Recirculation of contaminated brine through injection machines may also increase dissemination of *L. monocytogenes* during production (Autio et al., 1999; Gailey et al., 2003; Greer et al., 2004). Eradication programs, including disassembly of machines, thorough cleaning, and disinfection of all equipment components have been successful in eliminating *L. monocytogenes* from brining equipment (Autio et al., 1999).

A long cold-smoking time (≥ 12 h) was also predictive of a sample testing positive for *L. monocytogenes*, but use of a cold-smoking temperature between 24 °C to 30 °C may reduce *L. monocytogenes* contamination. Eklund et al. (1995) observed that cold-smoking temperatures of 22 °C to 30 °C decreased surface populations of *L. monocytogenes* in cold-smoked fish products. However, *L. monocytogenes* injected into the flesh increased during cold-smoking at these temperatures; and Rørvik et al. (2000) found that *L. monocytogenes* injected with brine can grow during cold-smoking. Thus, prolonged cold-smoking may allow the growth of *L. monocytogenes* in cold-smoked pork if contaminated brine has been injected prior to cold-smoking.

PFGE typing results showed a high genetic diversity of *L. monocytogenes* isolates obtained from cold-smoked pork products from several plants. This may indicate the existence of various sources of contamination at different production stages in the cold-smoked pork processing environment. In addition to the non-persistent strains, three persistent PFGE types were detected over several months in each of plants I and II. Previous studies have shown that persistent *L. monocytogenes* strains can often be found in various parts of food processing equipment (Rørvik et al., 1995; Nesbakken et al., 1996; Lundén et al., 2002). Contamination with *L. monocytogenes* in processing plants most often results from strains persisting, possibly for years, within the plant (Miettinen et al., 1999; Senczek et al., 2000; Hoffman et al., 2003; Lundén et al., 2003). Lundén et al. (2002) concluded that processing lines, including complex equipment with poor hygienic design, may harbor persistent *L. monocytogenes* strains, thus leading to continuous contamination of finished products.

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## FIGURE LEGENDS

**Fig. 1.** Generic process flow diagram for the manufacture of sliced, vacuum packaged, cold-smoked pork.

**Fig. 2.** Dendrogram demonstrating the genetic similarity among PFGE (*Asc*I) restriction profiles of *Listeria monocytogenes* isolated from sliced, vacuum packaged, cold-smoked pork products from Latvian and Lithuanian meat processing plants.

1 Table 1. Prevalence of *Listeria monocytogenes* in cold-smoked sliced vacuum packaged pork

2 products originating from 15 meat processing plants in Latvia and Lithuania in 2003- 2004

| Country | Plant | Manufacturer's shelf-life (days) | No. of production lots tested | No. of packs tested | No. of positive packs (%) |
|---|---|---|---|---|---|
| Latvia | I | 120 | 8 | 95 | 48 (51)[a] |
| | II | 90 | 10 | 73 | 17 (23) |
| | III | 30 | 2 | 25 | 10 (40)[a] |
| | IV | 20 | 2 | 15 | 7 (47)[a] |
| | V | 90 | 3 | 14 | 0 (0) |
| | VI | 60 | 3 | 17 | 1 (6) |
| | VII | 60 | 3 | 24 | 16 (67)[a] |
| | VIII | ND [b] | | 4 | 0 (0) |
| Subtotal | | | | 267 | 99 (37) |
| Lithuania | IX | ND | 4 | 13 | 9 (69)[a] |
| | X | ND | 2 | 10 | 1 (10) |
| | XI | ND | 1 | 11 | 8 (73)[a] |
| | Other (XII-XV) | ND | | 11 | 3 (27) |
| Subtotal | | | | 45 | 21 (47) |
| Total | | | | 312 | 120 (38) |

3 [a] Prevalence is significantly higher than in product without the superscript.

4 [b] ND, no data available.

Table 2. Analysis of bivariate Spearman rank order correlation coefficients with two-tailed *P* values and odds ratios with 95% confidence intervals for correlations between various manufacturing steps (variables) and the presence of *Listeria monocytogenes* in cold-smoked sliced vacuum packaged pork products

| Manufacturing step (variable value) | Presence of *L. monocytogenes* | |
|---|---|---|
| | *r (P)* | OR (95% CI) |
| Salting procedures: use of brine injection (Yes vs. No) | 0.22 (< 0.001) | 2.56 (1.53-4.30) |
| Primary ripening time (> 10 days vs. ≤ 10 days) | 0.11 (0.072) | 1.61 (0.96-2.63) |
| Cold-smoking time (≥ 12 hours vs. < 12 hours) | 0.21 (< 0.001) | 2.50 (1.49-4.17) |
| Cold-smoking temperature (19 °C to 23 °C vs. 24°C to 30 °C) | 0.11 (0.059) | 1.62 (0.98-2.69) |
| Secondary ripening time (> 7 days vs. ≤ 7 days) | 0.10 (0.079) | 1.59 (0.95-2.63) |
| Total time at ”risk temperature” 10 °C to 30 °C (> 48 hours vs. ≤ 48 hours) | 0.11 (0.059) | 1.64 (0.99-2.70) |

Table 3. Multivariate analysis by logistic multiple regression model in which *Listeria monocytogenes* detected is the dependent variable[a]

| Model or variable | Model-fitting information | | | | Parameter estimates | | | |
|---|---|---|---|---|---|---|---|---|
| | -2 Log likelihood | Chi square | df | *P* | B | Exp (B) (95% confidence interval) | SE for B | *P* |
| Models | | | | | | | | |
| Intercept only | 66.643 | | | | | | | |
| Final | 21.743 | 44.900 | 6 | 0.001 | | | | |
| Variables | | | | | | | | |
| Intercept | | | | | -5.966 | | 2.207 | 0.007 |
| Use of brine injection (Yes vs. No) | | | | | 2.36 | 10.66 (1.21-93.69) | 1.10 | 0.033 |
| Cold smoking time (≥ 12 h vs. < 12 h) | | | | | 3.19 | 24.38 (1.91-310.32) | 1.29 | 0.014 |
| Cold-smoking temperature (24 ℃ to 30 ℃ vs. 19 ℃ to 23 ℃) | | | | | -3.46 | 0.031 (0.03-0.28) | 1.11 | 0.002 |

[a] Only significant ($P < 0.05$) variables from multivariate analysis by logistic multiple regression model are presented.

Table 4. *L. monocytogenes* pulsed field gel electrophoresis (PFGE) types and serotypes detected in products from meat processing plants in Latvia and Lithuania

| Country | Plant | Number of isolates | Number of PFGE types | Serotypes (no. of isolates) |
|---|---|---|---|---|
| Latvia | I | 48 | 9 | 1/2a (36), 1/2b (8), 1/2c (3), 4b (1) |
| | II | 17 | 7 | 1/2a (14), 1/2c (3) |
| | III | 10 | 6 | 1/2a (8), 1/2c (2) |
| | IV | 7 | 3 | 1/2a (5), 1/2c (2) |
| | VI | 1 | 1 | 1/2a (1) |
| | VII | 16 | 5 | 1/2a (16) |
| Lithuania | IX | 9 | 4 | 1/2a (9) |
| | X | 1 | 1 | 1/2a (1) |
| | XI | 8 | 4 | 1/2a (4), 1/2c (4) |
| | Other (XIV-XV) | 3 | 3 | 1/2a (2), 3b (1) |
| Total | | 120 | 27 | 1/2a (96), 1/2b (3), 1/2c (19), 3b (1), 4b (1) |

1 Table 5. Distribution of pulsed field gel electrophoresis (PFGE) types of *Listeria monocytogenes* in

2 production lots from two meat processing plants

| Plant | PFGE type | No. of isolates | Production lot | Isolation month/ year |
|---|---|---|---|---|
| I [a] | 13 | 1 | IL 8 | 01/2004 |
| | 12 | 1 | IL 5 | 07/2003 |
| | 10 | 5 | IL 1, IL 2, IL 4, IL 5 | 01, 06, 07/2003 |
| | 19 | 25 | IL 1, IL 2, IL 3, IL 4, IL 5, IL 6, IL7 | 01, 06, 07, 09/2003 |
| | 20 | 7 | IL 2, IL 5, IL 7 | 06, 07, 09/2003 |
| | 21 | 1 | IL 8 | 01/2004 |
| | 22 | 4 | IL 2 | 06/2003 |
| | 25 | 3 | IL 8 | 01/2004 |
| | 27 | 1 | IL 6 | 07/2003 |
| Total | 9 | 48 | | |
| II | 12 | 2 | IIL 2, IIL 3 | 07/2003; 01/2004 |
| | 2 | 1 | IIL 3 | 01/2004 |
| | 7 | 1 | IIL 2 | 07/2003 |
| | 19 | 7 | IIL 1, IIL2, IIL 3, IIL 4 | 06, 07/2003; 01, 02/2004 |
| | 15 | 3 | IIL 3, IIL 4 | 01, 02/2004 |
| | 17 | 2 | IIL 3 | 01/2004 |
| | 22 | 1 | IIL 1 | 06/2003 |
| Total | 7 | 17 | | |

3 [a] Prevalence of *L. monocytogenes* in product is significantly higher ($P < 0.05$) than in meat plant II.