MATERIALS AND METHODS

Time and Place

This research was carried out from June up to August 2008 in Laboratory of Veterinary Public Health, Faculty of Veterinary Medicine, Bogor Agricultural University (IPB) in Bogor city west Java Indonesia.

Materials

Samples collected were those of pasteurized High Temperature Short Time (HTST) liquid milk locally processed and packed in Indonesia. In Bogor City, there are several supermarkets that sell different brands of pasteurized milk from different factories in Indonesia. A total of 32 samples were collected from four different big supermarkets in Bogor City. They were in four different brands, locally manufactured and each brand was picked eight times.

Media and Chemicals

These included Listeria enrichment broth (LEB), Modified Oxford medium, Tryptose soy agar-yeast extract (TSA-YE), Tryptose soy broth-yeast extract (TSB-YE), CAMP test cultures (Staphylococcus aureus ATCC 25723, Sheep blood agar), Gram stain kit, Hydrogen peroxide (H₂O₂) 3%, Mannitol, Maltose, Rhamnose, Xylose and Nutrient agar.

Equipments

These included Cover slips, Erlenmeyer flasks (500 ml), Immersion oil (for microscope), Incubators (30°C ± 2°C and 37°C ±2°C), Refrigeric incubator (4°C±2°C) Inoculating loops (Ose), Microscope, Vortex mixer, Electric Lamp, Petri dishes, Pipettes (1.5 and 10 ml), Scissors, Autoclave, Quebec colony counter, Grease pencil or Marker and Test-tubes.

Sample Handling after Collection

Samples were placed in insulated cooler containing ice packs, which were placed on the sides, in the middle and on top of the product. More ice was always added to ensure that the temperature is within the range of 4 °C to 10 °C. Proper sterilization of all the equipments, disinfection of the outside of the packs before opening and hand washing and disinfection using alcohol (70%) before opening were done to ensure that there was no external contamination.
Qualitative Analysis of *Listeria monocytogenes*

The method used for detection of *Listeria monocytogenes* was adopted from the *Bacteriological Analytical Manual / Food and Drug Administration* (FDA 2007). The general schematic sample testing is as shown in Figure 9.

![Figure 9 Qualitative Isolation of Listeria monocytogenes](image-url)
Isolation of *Listeria monocytogenes* from dairy products was based on the FDA protocol. It begins with enrichment of the sample in *Listeria* Enrichment Broth (Oxoid M0897) a buffered medium supplemented with SR141 (contains acriflavin, nalidixic acid, and cyclohexidine as selective agents). After 24 and 48 h of incubation at 30°C, the enrichment culture was streaked to a *Listeria* selective plating media called Oxford medium (OXA) which contains polymyxin B, acriflavin, and ceftazidime. After 24 – 48h and until seven days of incubation at 37°C, suspect colonies OXA are black halo resulting from esculin hydrolysis after which the five suspect colonies were streaked into Tryptose Soy Agar with yeast extract (TSA Oxoid CM) 131 and incubated at 37°C for 24h. Presumptive *Listeria* isolates are speciated based on a standard series of biochemical tests. Typical *monocytogenes* isolates are rhamnose positive, xylose negative, and CAMP test positive with β-hemolysis enhanced in the vicinity of *Staphylococcus aureus*. The suspected colonies were also subjected to potassium hydroxide, motility test using SIM medium (Oxoid CM0435) for detecting the umbrella-like growth, Hydrogen peroxide, and mannitol. Colonies from Tryptose Soy Agar with Yeast extract were again re-streaked into Tryptose Soy Broth Broth (Figure 9).

A control positive involved picking a loop full of *Listeria monocytogenes* (Field stem – Laboratory of Veterinary Public Health, Faculty of Veterinary Medicine- IPB) and mixing it with Listeria enrichment broth in a clean and sterile erlenmeyer. A control negative involved the use of a loop full of *Staphylococcus aureus* (ATCC 25923) with Listeria enrichment broth and both controls were run in the same manner as the test samples.

**Inoculation of *Listeria monocytogenes* in test media**

Serial dilution of the culture was done with BPW (0.1% v/v) to achieve a cell concentration of approximately 1.0 x 10^2 CFU/ml. One hundred milliliters (100 ml) of sterile milk was used as a test sample in eight clean and sterile erlenmeyer. Then a 0.1ml of it was aseptically pipetted into each of the seven erlenmeyer with codes E1 to E7, but E8 was not inoculated because it was the control sample. Then all the erlenmeyers were stored in the refrigerated incubator set at 4°C. After 24 hours, erlenmeyer E1 was removed from the refrigerator together with E8 as the control. Then 1ml of E1 was aseptically pipetted into
already prepared clean and sterile petri dishes with labels $10^0$ (in duplicate) and was also prepared using 9ml of BPW $10^{-1}$ (in duplicate), and then 1ml from E8 with labels K1 and K2. This made a total of 6 petri dishes. Then each of the petri dishes was flooded with a nutrient agar and mixed thoroughly in the form of figure 8 and then left for some time to solidify before they were transferred to the incubator that was set at 37°C and incubated for 24 to 48 hours. The control (E8) was returned to the refrigerator while E1 was removed and never used again. The next day, the colonies that grew were counted using a colony counter and the results recorded, then followed up the next day (48hrs) and recounted to check for any increment in the number of colonies, the results were also recorded. The above procedures were used for the rest of the samples until all the 7 samples were completed.

Figure 11 Milk samples for the quantitative analysis of *Listeria monocytogenes* kept in the refrigerator set at 4°C.

Data management and analysis

Program excel version 2003 (Microsoft Office excel 2003, Microsoft Office Professional Edition, 2003) and Minitab 14 a statistical software were used for collection, management and analysis of data. Descriptive statistics were used to describe the results.

RESULTS AND DISCUSSION
There was no *Listeria monocytogenes* detected in the samples tested (Table 1).

### Table 4. Presence of *Listeria monocytogenes* in pasteurized milk by the qualitative method

<table>
<thead>
<tr>
<th>Supermarket code</th>
<th>Type of sample</th>
<th>Number of Positives</th>
<th>Positive (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>G <em>(n = 8)</em></td>
<td>A1–B1–C1–D1–</td>
<td>None</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>A3–B3–C3–D3–</td>
<td></td>
<td></td>
</tr>
<tr>
<td>H <em>(n = 8)</em></td>
<td>A1–B1–C1–D1–</td>
<td>None</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>A3–B3–C3–D3–</td>
<td></td>
<td></td>
</tr>
<tr>
<td>E <em>(n = 8)</em></td>
<td>A2–B2–C2–D2–</td>
<td>None</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>A3–B3–C3–D3–</td>
<td></td>
<td></td>
</tr>
<tr>
<td>J <em>(n = 8)</em></td>
<td>A2–B2–C2–D2–</td>
<td>None</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>A3–B3–C3–D3–</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Note: Letters G, H, E and J are codes of Supermarkets, A, B, C and D are codes of samples and 1, 2, 3 and 4 are weeks during which samples were collected from the Supermarkets.

Pasteurized milk is very much known to be one of the vehicles through which *Listeria monocytogenes* is transferred into the human body. Because of this, many countries have initiated a zero tolerance policy prohibiting the sale of processed ready-to-eat food products contaminated with *Listeria monocytogenes*. This policy designates *Listeria monocytogenes* as an adulterant. Despite this policy, *Listeria monocytogenes* is constantly found in these foods. Vander-linde and Grav, (1991) found 2 (2.4%) *Listeria spp.* These numbers were attributed to secondary contamination of different supplements and different production methods. Dominguez *et al.*1985; Garcia and Vitas 2004 from Spain found a much higher incidence of *Listeria monocytogenes* in pasteurized milk, 45.3 % and 44.7 % respectively. This was far much higher than what investigators from other countries have reported. Gül *et al.* (1994) from Diyarbakir, Turkey, reported 1.1 % *Listeria monocytogenes* in pasteurized milk samples.

In a related study to develop data on the risk of listeriosis to support a science-based strategy for addressing *Listeria monocytogenes* in foods in the United States, eight categories of ready-to-eat foods were collected over 14 to 23 months from retail markets at Maryland and northern California FoodNet sites. The product categories included luncheon meats, deli salads, fresh soft "Hispanic-style" cheeses, bagged salads, blue-veined and soft mold-ripened cheeses, smoked
seafood, pasteurized milk and seafood salads. The presence and levels of *L. monocytogenes* in the samples were determined by rapid DNA-based assays in combination with culture methods. Of the 31,705 samples tested, 577 were positive. The overall prevalence was 1.82%, with prevalence ranging from 0.17 to 4.7% among the product categories (Chen *et al*., 2003).

In Ankara, Turkey, Farber *et al.* (1988) reported (18.2%) of raw milk contained *Listeria monocytogenes*. Sharif and Tunail (1991) tested pasteurized milk samples and reported that all were negative for *Listeria monocytogenes*. This research also found no sample positive to *Listeria monocytogenes*. The result is in agreement with the findings of other researchers in other countries. Based on all the findings mentioned above, it can be concluded that *Listeria monocytogenes* has a very low occurrence in foods. However, in this research, this does not mean that all the samples were free of *Listeria monocytogenes*, maybe other factors may have played a big role.

Mesophilic aerobic bacteria and lactobacilla overwhelming flora that were seen may prevent proliferation of *Listeria*. Some authors reported that *Lactobacillus sake* (Lb.706) and similar spp, produce bacteriosin and play role in inhibiting proliferation of *Listeria* spp (Weis 1989; Johnson *et al*., 1989). Stopforth *et al.* (2005) reported that *L. monocytogenes* does not proliferate in food with high total microorganism numbers. Inhibitory effect on the growth of *L. monocytogenes* is mainly related to the microbiological composition of the raw milk, in terms of thermophilic *Lactobacillus* and yeast. Inhibition of *L. monocytogenes* is probably due to the interrelationship between microbiological and chemical factors.

Besides this the control of *L. monocytogenes* in pasteurized milk via HACCP is focused on the selection of raw milk and the control of the processing, packaging, distribution and storage conditions. Although the pathogen is effectively controlled during pasteurization, its presence in the finished product is possible as a result of post-pasteurization contamination from sources in the plant environment. At present, however, it is presumed that *L. monocytogenes* is killed by heating to 72°C at least 15 seconds.
Growth of inoculated *Listeria monocytogenes* in a commercial sterile whole milk product was monitored at 4°C for a period of one week and the results obtained were tabulated and a growth curve drawn (Figure 12).

![Growth curve for *Listeria monocytogenes* in commercial sterile milk stored in the refrigerator at 4°C for 7 days.](image)

**Figure 12** Growth curve for *Listeria monocytogenes* in commercial sterile milk stored in the refrigerator at 4°C for 7 days.

**Table 5** Total Colony counts for *Listeria monocytogenes* in commercial sterile milk stored in the refrigerator at 4°C for 7 days.

<table>
<thead>
<tr>
<th>Sample</th>
<th>No. of days under storage at 4°C</th>
<th>Colony counts (cfu/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>E1</td>
<td>1</td>
<td>5.9 X 10^2</td>
</tr>
<tr>
<td>E2</td>
<td>2</td>
<td>1.0 X 10^3</td>
</tr>
<tr>
<td>E3</td>
<td>3</td>
<td>7.0 X 10^4</td>
</tr>
<tr>
<td>E4</td>
<td>4</td>
<td>1.0 X 10^5</td>
</tr>
<tr>
<td>E5</td>
<td>5</td>
<td>7.4 X 10^5</td>
</tr>
<tr>
<td>E6</td>
<td>6</td>
<td>2.5 X 10^6</td>
</tr>
<tr>
<td>E7</td>
<td>7</td>
<td>2.8 X 10^6</td>
</tr>
</tbody>
</table>

*Listeria monocytogenes* maintained its population relatively well in sterile milk stored at 4°C as seen in figure 12. This is because it is a psychrotrophic
organism which grows at lower temperatures. On the first, second, third and fourth day there was no significant increase in the number of colony forming units. However, on the fifth day a dramatic increase in the number of colony forming units was observed. On the sixth day, a slight decrease in colony forming units was noted if compared with the fifth day. However on the seventh day, the population of colony forming units shot up. From the growth curve it can be noted that increase in number days has a strong relationship with the increase in number of *Listeria monocytogenes*. Delignette – Muller *et al.* (2005) reported a linear relationship between and growth of *Listeria monocytogenes*. The reasoning for this kind of graph is likely an initial shock to the cells which may make them harder to recover.

Baranyi *et al.* (1995) reported that the predictions for *Brochothrix thermosphacta* growth were good when the temperature profile contained step changes from 17 – 25°C down to 5°C, but the predictions no longer held for steps changes down to 4°C. In this study the author also attributes this observation to the alteration of the physiological state of the organism caused by the sudden cold shock which may result in additional lag phase. This initial shock may also be due to the lack of growth compounds in the media associated with these results. The cells become easier to recover as they adapt to the environment at the later stages. This model provides the dairy industry with a useful tool for effective management and optimization of product safety and can lead to more realistic estimations of pasteurized-milk related safety risks. It can therefore be presumed that *Listeria monocytogenes* concentrations on the fifth, sixth and seventh day of milk storage in the refrigerator at 4°C can be quite dangerous to human health in both the immune-compromised and the healthy individuals. A population of 10³ cells is already enough to cause serious *listeriosis* in the non-healthy individuals (Invasive form) and in healthy individuals (non – invasive form) since it requires just over 10⁵ cells.

Doyle *et al.* (2001) reported that *L. monocytogenes* grows in pasteurized milk, with the numbers increasing 10-fold in 7 days at 4°C. Therefore, fluid milk that becomes contaminated after pasteurization and stored under refrigeration temperature may attain very high populations of *L. monocytogenes* after one
week. Therefore temperature abuse may further enhance the multiplication of bacterial cells. In relation to the above condition, Hayes (1996) noted that the organism achieved a significant increase on the fourth and the fifth day while this research obtained a drastic increase from the fifth day, the numbers increasing unreasonably high on the sixth day. However, on the seventh day there was no difference in the number cfus if compared with those counted on the sixth day. This could have been due to exhaustion of nutrients in the media and also lack of space for multiplication. Therefore, the public health importance and the sanitary measures for the control of the organism in ready-to-eat foods that have longer shelf-lives and stored under refrigeration is very much important.

CONCLUSIONS AND RECOMMENDATIONS

Conclusion
All the samples collected from the four different Supermarkets in Bogor City were negative to *Listeria monocytogenes* based on the FDA method for the isolation and detection of *Listeria monocytogenes* in foods. This means that the results are in agreement with hypothesis which states that there is no *Listeria monocytogenes* in pasteurized milk sold in Bogor City because of the effectiveness of the pasteurization process and the fact that there is no post-pasteurization contamination. The risk assessment reinforces past conclusions that food borne listeriosis is a moderately rare although it can lead to severe disease.

*Listeria monocytogenes* is capable of resisting cold temperatures, that is to say the 4°C cannot inactivate the organism, though other organisms cannot survive. This model also illustrates that *L. monocytogenes* can multiply to very high numbers following storage under this temperature.

**Recommendation**

A survey should be done on samples from traditional markets to identify *monocytogenes*. Other available new methods with a much higher sensitivity like PCR together with rapid tests should be used. Every effort should be made to ensure that ready-to-eat foods with longer shelf – life are free of *Listeria monocytogenes* since few cells can multiply to very high numbers under refrigeration temperature.

The results of this assessment will assist government in the evaluation of the adequacy and focus of current programs, help in the development of new programs to ensure that these programs protect the public health and to evaluate the effectiveness of new strategies to minimize the public health impact of food-borne listeria.

**REFERENCES**


