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*Research Article*

# *Listeria* Contamination in Milk-Processing Chain and Proficiency in *Listeria monocytogenes* Decontamination of Small-Scale Milk Retailers

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*Listeria* spp. is an important foodborne bacterium. This microorganism can be discarded from milk using high temperatures such as pasteurization. The milk-processing methods of many small-scale retailers lack quality control. This study was to survey *Listeria* contamination at the farm and retailer levels. The retailers were to be interviewed for knowledge, attitude, and practice as well. Finally, we were to determine the heating processes employed to decontaminate microorganisms by the retailers using a reference strain of *L. monocytogenes*. Milk samples were collected from milk-collecting centers and small-scale retailers. In clinical trial, the processing measures were proved for the proficiency in *L. monocytogenes* decontamination. One out of 99 farms presented *Listeria* contamination, confirmed to *L. marthii*. Fifty small-scale retailers participated in the second part, including 13 males and 37 females. No *Listeria* spp. but *Staphylococcus* spp. and *Bacillus* spp. were identified in the processed milk. Data analyses revealed that the location of the retailer was significantly associated with the volume they routinely ordered per lot and the milk-processing time the retailers used to treat milk. Knowledge on raw milk contamination is significantly associated with the stocking or processing of the whole milk lot. Processing measures presale were significantly influenced by the gender of the retailer. The male retailer reportedly spent less time treating milk compared to their female counterparts. To assess the efficacy of the processing methods, a trial using *L. monocytogenes* as a reference strain was conducted. Interestingly, no *L. monocytogenes* was detected after sample treatment, but other microorganisms such as S. epidermidis, S. warneri, and Escherichia coli were found, suggesting potential issues with cross-contamination. In conclusion, while the trial implied that the retailers' processes were effective in *L. monocytogenes* decontamination, the study highlighted inappropriate practices and the risk of cross-contamination. Continuous monitoring of product safety in small-scale milk retailers is imperative to ensure consumer well-being.

## 1. Introduction

Foodborne diseases are a major global problem. International institutes such as the World Health Organization (WHO) and the Centers for Disease Control and Prevention (CDC) are concerned about listeriosis because of the infectious and severe nature of the disease [1, 2]. Listeriosis is a fatal foodborne disease that can be caused by *Listeria* spp., especially *L. monocytogenes*. However, listeriosis has a low incidence in humans and animals [3–5]. *Listeria* spp. most likely contaminate raw milk, cheese, seafood, and frozen foods. At the farm level, *Listeria* spp. are contagious bacteria. Transmission normally occurs during milking and from the environment [6–8]. The prevalence of *Listeria* spp. in raw milk was reported in a wide range such as 3–7% in North America, 0–50% in Europe, and about 5% in Iran [9–11]. In

Thailand, there was not the report of *Listeria* contamination in raw milk [12]. Unpasteurized or improper processed milk caused three out of 36 outbreaks of listeriosis. The illness has many forms, gastroenteritis, septicaemia, and meningitis, for example. Pregnant women and their neonates, 65 years or older people, and immunosuppressed patients were susceptible groups for the disease [1, 2]. According to WHO, the risk group should become aware of consumption of fresh or improperly treated milk [3, 13, 14].

Raw milk is a substantial risk to human health; therefore, milk should be heated before consumption [9]. Several small-scale retailers presently buy untreated milk directly and treat it at home with processes that might not follow the protocol or be substandard. The chance of *Listeria* spp. infection increases owing to inefficient processes [15].

Size of dairy farms in Thailand varies from less than 10 up to over 100 milking cows per farm. Almost all farms were crossbred with a high percentage of Holstein Friesians. The annual raw milk averaged 1,200 tons between 2017 and 2022, related to the production of 11 kg/cow/day, approximately [16] The whole raw milk has supported the consumption within the country. Milk normally is sold as pasteurization, UHT, and sterilization products, but some are uncontrollably heated and sold in small retailers. The product from milk retailers is highly risky because the quality of milk processing is not controlled. *Listeria* spp. can contaminate in all processes to which the condition is proper for bacterial growth, even packaging or storage after heat treatment [7, 17].

This study was to operate into three sections. The first and second objectives were to survey *Listeria* contamination in fresh milk from farms and milk from small-scale retailers. The retailers were additionally interviewed for knowledge, attitude, and practice. For the final part, we were to determine the heating processes employed to decontaminate microorganisms by the retailers using a reference strain of *L. monocytogenes*.

## 2. Materials and Methods

*2.1. Study Sample Description.* Milk was collected from three collecting milk centers, Kamphaeng Saen Dairy Co-operative, Nakhon Pathom Dairy Co-operative, and Nong Pho Dairy Co-operative at the farm level. These three centers are in the central part of Thailand and received fresh milk from 3,000 farms in total. The raw milk, which passes quality check, is to be distributed to large-scale commercial dairy manufacturers or processed by the milk-collecting centers. Both the large-scale dairy manufacturer and the milk-collecting center treat milk by the standard measures and sell it as commercial products. However, there is still the small-scale milk retailer, which produces and sells unpasteurized products in the local area. They usually buy raw milk from dairy farms directly and treat milk with their own measures.

*2.2. Study Design.* This study was designed to explore the possibility of the contamination of *L. monocytogenes* in the small milk-processing line. The first part focused on the raw milk from dairy farms. The next step explored the product from the small-scale milk retailers. Additionally, the perception of milk safety was interviewed by the questionnaire of knowledge, attitude, and practice. Their practices in treating milk were concluded to design the treatment groups in the trial. The last step was to confirm the retailer's treatments to see if the methods were able to decontaminate *L. monocytogenes* from milk.

*2.3. Sample Size and Sampling Techniques.* The sample number was run by ProMESA 2.3.0.2 (INTA & Massey University, Castelar, Argentina). The sample size in the farm level was calculated based on the disease detection at 3% prevalence of *Listeria* spp. [8] and population size of 3,000 farms. The sample numbers, weighted by the total number of registered farms, of Kamphaeng Saen Dairy Co-op, Nakhon Pathom Dairy Co-op, and Nong Pho Dairy Co-op, were 33, 23, and 43 samples, respectively. A simple random sampling method was used for selecting the dairy farms.

In the retailer level, the number of samples was 50 retailers, which was based on the population size of 500 shops and 5% of prevalence in pasteurized milk [18]. The retailers were selected by a purposive sampling method from five provinces, which surrounded the positive farm from the first survey. The samples were in Nakhon Pathom, Ratchaburi, and Bangkok for 11 retailers each, whereas those from Kanchanaburi and Suphanburi were nine and eight retailers, respectively.

*2.4. Sample Collection and Transportation.* Both farm and retailer levels collected at least 30 mL milk in a sterile container. The samples were kept in a cool box during transportation to the Laboratory, Faculty of Veterinary Medicine, Kasetsart University, Thailand. Milk was to be kept at 4°C and analysis of *Listeria* spp. within 24 h.

*2.5. Questionnaire Survey Description.* The retailers were asked for consent to an interview. The questionnaire was about their knowledge, attitude, and practice on milk safety. The retailers who responded to the questionnaires included 72% women and 28% men. The qualification of approximately 80% of the respondents fell under a bachelor's degree. The data from the questionnaire were to be used in data analysis and design of the treatment groups in the trial.

The retailers treated milk without monitoring temperature and time. Their processes were completed by noticing the appearance of heated milk. The retailer's processes were classified into (1) using double boiling until bubbles formed, (2) using double boiling until a film layer formed, (3) using double boiling for 2 min (in 100°C water), (4) using direct heating until bubbles formed, and (5) using direct heating until a film layer formed. Table 1 shows the data of temperature and time of each process from the trial.

*2.6. Experiment Description.* The trial consisted of five treatments, following the retailer's processes (Table 1), and control groups as pasteurization of 63°C for 30 min and 72°C

Table 1: Milk temperature during process and processing time for each treatment.

| Measures* | Temperature (°C) | Processing time (min) |
|---|---|---|
| Double boiling until bubbles formed | 78 | 19 |
| Double boiling until a film layer formed | 83 | 24 |
| Double boiling for 2 min (in 100°C water) | 75 | 2 |
| Direct heating until bubbles formed | 88 | 14 |
| Direct heating until a film layer formed | 90 | 35 |

*Each treatment operated in 1 liters of milk.

for 15 s. Sterile milk with added *L. monocytogenes* (ATCC® 51414™, American Type Culture Collection, VA, USA) were used in the experiment. Each treatment was assessed with $10^5$, $10^3$, and $10^1$ colony forming units [CFU]/mL of *L. monocytogenes*. The sample was collected in duplicates at three time points: pretreatment, post-treatment, and 30 min post-treatment.

*2.7. Bacteriological Test.* Vidas® LDUO (bioMérieux, Marcy-l'Étoile, France), based on an enzyme-linked fluorescent assay (validated by AFNOR/ISO16140 (BIO 12/12–07/04)), was used in qualitatively screening *Listeria* spp. and *L. monocytogenes* contamination in the farm level. The positive samples from Vidas® LDUO and the samples from the retailer level were cultured on ALOA® One Day (bioMérieux, Marcy-l'Étoile, France). ALOA® One Day is the alternative method to detect the contamination of *Listeria* spp. in foods and environment samples, based on chromogenic agar. This analysis technique follows ISO 11290-1:2017.

The samples from the experiment were cultured on ALOA® One Day and brain heart infusion (BHI) agar for identifying and enumerating *Listeria* spp. and *L. monocytogenes*. The typical appearance of *Listeria* spp. and *L. monocytogenes* on ALOA® One Day was blue-green colonies and blue green colonies with opaque, respectively. *Listeria* spp. grow on BHI agar as white colonies.

The typical colonies from both ALOA® and BHI agar were cultured on Trypticase Soy Agar (TSA) to prepare the colonies for VITEK® MS (bioMerieux, Marcy-l'Etoile, France), based on matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) technology. VITEK® MS reported as genus with/without species according to their mass-to-charge (m/z) comparing the database. The confidence level presented the certainty of the result. *E. coli* ATCC 8739 was used as a positive control.

*2.8. Data Analysis.* Milk volume per lot between provinces was compared by using the Kruskal–Wallis test. The associations between variables were analyzed by the chi-squared test and Fisher's exact test using Stata 13.1 (Stata Corp LLC, College Station, TX, USA). The statistical significance was set at the significance level of 0.05.

## 3. Results

*3.1. Farm Level.* Ninety-nine samples of bulk tank milk were collected from three locations. All samples were negative for *L. monocytogenes*, but 1% was positive for *L. marthii* from the Nong Pho milk-collecting center, Ratchaburi province. The contaminating species were confirmed as *L. marthii* at a confidence value of 98.7%.

*3.2. Retailer Level.* Fifty milk samples from small-scale retailers were detected for the contamination of *Listeria* spp. however, VITEK® MS reported *Staphylococcus* spp. and *Bacillus* spp. contamination.

The questionnaire results suggested that 56% of individuals had knowledge regarding the severity of raw milk consumption, bacterial contamination in raw milk, and the necessity for treatment before consumption. Almost all retailers identified diarrhea as the most common illness associated with untreated milk. Other consequences included vomiting, fever, headache, convulsions, and flatulence. Notably, the results showed that 4% of individuals lacked awareness on the health impacts of untreated milk.

Regarding milk treatment, 90% of respondents knew that bacteria could be killed by heating milk. Less than 10% understood the pasteurization process, but 25% of respondents could explain it correctly. Unfortunately, 10% of milk retailers believed that milk did not require processing before sale.

The data on the practices revealed that approximately half of the responders ordered milk daily, and 6% ordered weekly or at longer intervals. Dairy farms were the primary source of raw milk for 42% of retailers, and the rest received milk from cooperations or intermediaries. The median milk volume ordered for each lot was approximately 20 kg, and Q1 and Q3 were 10 and 40 kg, respectively. Thirty-five percent of retailers processed whole milk on the day of reception. Over 50% stored the nonprocessed milk under cold conditions and 10% froze milk. A mere 10% of the processed milk was subjected to pasteurization at controlled temperature and time presale. The remaining retailers only checked the appearance of milk whilst heating to finish the treatment; they did not follow a time-controlled heating.

The data from questionnaires suggested that 62% of retailers heated milk for less than 30 min. The daily leftover-processed milk was not resold by 20% of retailers. Over 50% of the retailers kept previously heated milk under cold storage, and approximately 15% froze milk. The major health concern associated with this process was gastrointestinal illness. However, only a few respondents were aware of fever and neurological problems associated with the illness.

The results of the association test are presented in Table 2. The location of retail was associated with the volume of milk ordered. Retails in Bangkok significantly stocked a larger volume ($p < 0.05$).

Table 2: Test of association using the chi-squared test and Fisher's exact test.

| Variables | Explanatory variables | $Chi^2$ | $P$ value |
|---|---|---|---|
| Raw milk management | Sex | — | 0.32 |
| | Province | — | 0.07 |
| | Knowledge of milk contamination | 7.26 | 0.007 |
| | Knowledge of bacterial virulence | — | 0.70 |
| Milk treatment | Sex | 10.38 | 0.001 |
| | Province | 9.88 | 0.04 |
| | Knowledge of milk contamination | 0.004 | 0.95 |
| | Knowledge of bacterial virulence | — | 0.15 |
| | Knowledge of pasteurization | — | 0.10 |
| Processed milk management | Sex | — | 0.13 |
| | Province | — | 0.39 |
| | Knowledge of milk contamination | — | 0.73 |
| | Knowledge of bacterial virulence | — | 0.09 |

Additionally, knowledge about contamination was significantly associated with the measures for managing milk after reception ($p < 0.05$). The knowledge about the methods by which retailers processed whole milk in a lot instead of stocking raw milk was six-fold higher than those who lacked relevant knowledge. The sex of the retailer was not associated with the knowledge that milk treatment could reduce contamination, but the decontamination practices did. Men were 18 times less likely to heat milk for less than 30 min than women ($p < 0.01$). The practices significantly depended on the location of the retailers ($p < 0.05$). Retailers in Bangkok were five times more likely to treat milk improperly compared to those in other locations.

None of the retailers sold unprocessed milk. The products were treated by double boiling or direct heating before selling. The retailers prepared milk by double boiling and direct heating without checking the temperature and processing time. The appearances, i.e., bubbles or layers formed after heating were used to notice that the products were ready to sell.

*3.3. Trial Level.* In the trial, the typical colony of *L. monocytogenes* was not shown from any processes post-treatment and 30 min post-treatment; however, the white colonies grew on BHI agar (Table 3). Neither of colonies on BHI agar confirmed the presence of *L. monocytogenes* contamination. Vitek® MS showed *S. epidermidis*, *S. warneri*, and *E. coli* at the confidence level of 99.9%.

Table 3: Concentration (CFU/mL) of bacteria, growing on BHI agar after treatment.

| *L. monocytogenes* added in milk (CFU/mL) | Time[a] | Processes[b] | | | | |
|---|---|---|---|---|---|---|
| | | 1 | 2 | 3 | 4 | 5 |
| 10 | T1 | 15 | 0 | 0 | 0 | 0 |
| | T2 | 0 | 10 | 0 | 0 | 10 |
| $10^3$ | T1 | 0 | 15 | 0 | 15 | 0 |
| | T2 | 0 | 0 | 5 | 5 | 75 |
| $10^5$ | T1 | 0 | 5 | 0 | 0 | 0 |
| | T2 | 0 | 0 | 0 | 0 | 5 |

[a]T1: post-treatment, T2: 30 min post-treatment. [b]1: using double boiling until bubbles formed, 2: using double boiling until a film layer formed, 3: using double boiling for 1–2 min, 4: using direct heating until bubbles formed, and 5: using direct heating until a film layer formed.

## 4. Discussion

This study reported 1% of *Listeria* contamination in bulk tank milk and identified as *L. marthii*. The first isolation of this bacterium was in 2010 from the environmental samples [19]. This microorganism was classified to be "Listeria sensu strictu" as same as *L. monocytogenes*. The bacteria in this group share common characteristics. *L. marthii* was not globally distributed; however, the members in the "Listeria sensu strictu" group probably identified from healthy animals and in animal-origin food [20, 21]. The presence of this microorganism might imply that the environment of dairy farms in the study area was suitable for the survival of *Listeria* spp. *L. marthii* was reported to be of no risk to human and animal health. This species was just found in 2010. Its characteristic and severity should be concerned continually [20].

*Listeria* spp. contamination incidence in bulk tank milk was notably low in this study; however, the contamination occurrence differed depending on the location [8, 9]. *L. monocytogenes* appearance in raw milk was reported to spread from indigenous silage. Therefore, farmers should carefully consider hygiene practices and high-quality feeds [8, 22]. Improper practices during milking and postmilking on the farm level (especially for small-size farms) could cause differences in these occurrences [8, 14, 23]. Fresh milk from farms could be the source of the health risk from *Listeria* spp. if people consumed raw or unpasteurized milk [13, 14, 24].

Additionally, bacteria could remain in milk in cases of improper cooling, crosscontamination during handling, packaging, or storage [13, 25]. *Listeria* spp. can still exist in food even after refrigeration because the organism can survive at low temperatures [25].

The leukocytes in milk would degrade if the raw or improper pasteurized milk were stored at 4°C for over 3 days. As a result of this deterioration, the number of heat-resistant *L. monocytogenes* could increase [26]. Even though the small-scale retailers were suspected of treating milk

improperly, the result in this study was not to identify *Listeria* spp. contamination. The current result differed from the earlier articles. The authors in [15] reported approximately 18% *Listeria* spp. contamination in boiled milk. *Listeria* prevalence, even in pasteurized milk, ranged from 5 to 40% [27, 28]. Raw milk consumption or improper processing is the cause of the contamination in ready-to-drink milk [15, 29].

We cultured *Staphylococcus* spp. and *Bacillus* spp. at the retailer level. The *Bacillus* spp. were heat-resistant species that could be killed in a wide range of temperatures and time [30]. *Bacillus* spp. spores may resist heat treatment. Spores from some bacilli species can be isolated in milk, even after sterilization [30, 31]. Additionally, spores are commonly found in the environment, including soil, dust, air, and surfaces [32].

*Staphylococcus* spp. are normal microbiota on the human skin and mucous membranes [33]; however, its contamination possibly resulted in crosscontamination. Many studies have elucidated the contamination of *Staphylococcus* spp., especially *S. aureus*, and its severity on human health [17, 34, 35]. Pasteurization products were identified as the source of *S. aureus* contamination. The incidence of *S. aureus* contamination in pasteurization was 4% in China, while that in South Africa was high, up to 20% [36, 37].

Milk from the retailers needed to be concerned about safety, even with no *Listeria* spp. being shown from milk. The bacteria in milk might be the result of crosscontamination from the environment and human or improper processing steps. The sample collection was the limitation in this part. Milk from retailers was analyzed only one time in the different period of the sample collection at the farm level. The result was like the snapshot situation of *Listeria* contamination.

The location was associated with the milk volume of each lot that the retailer ordered and their pasteurization practices. The order volume is related to the number of dairy farms in the area. Retailers in Bangkok may have a larger stock than other locations because of the transport limitations. The magnitude of milk treatment was unexpectedly related to location. People with unawareness of the risk of milk-borne pathogens were twice as likely to contract abdominal illness than those who were aware [38].

Earlier articles showed the risk of consuming raw milk as well as the products produced from unpasteurized milk on human health [4, 8, 39]. With quality-uncontrolled processes, public perceptions of the product safety were certainly doubtful. Surprisingly, we had reason to say that the five milk-processing measures effectively destroyed *L. monocytogenes* because of no appearance of *L. monocytogenes*. The trial revealed that all processes reached the pasteurization condition including temperature and time; however, the temperature and processing time in this study might not be the same as the retailers because each person might consider stopping heating differently.

*L. monocytogenes* would be reduced to greater than $\log_{10}$ 6.9 $mL^{-1}$ after heating at 65.5°C for 15 s [26]. However, the authors in [40] reported that low temperature, long-time pasteurization at 63°C for 30 min or high temperature, and short time could make a negligible $\log_{10}$ 2 $mL^{-1}$ reduction. This variation implied the possibility of failure in milk treatment. The low pathogen load following treatment could also be attributed to the lack of detectable *Listeria* spp. in the culture.

In the experiment, we isolated *S. epidermidis, S. warneri,* and *E. coli,* which may result from crosscontamination. *Staphylococcus* spp. is usually found on the skin and surfaces [32]. *E. coli* is a human pathogen that causes gastrointestinal illness. Typically, the source is the host intestine; however, *E. coli* can persist on surfaces [1, 41]. Pasteurized milk was the source of up to 9% *E. coli* contamination [42]. Milk processed with inadequate measures presented approximately two-fold higher *E. coli* numbers than adequate treatment measures [43]. According to crosscontamination suspected, the processes after heating treatment were strictly hygienic [2, 17, 42, 44].

This study collected samples from farms and retailers only one time. The incidence of *Listeria* contamination might be underestimated. The retailers notice the bubble or film layer to complete the milk heating process that the temperature and time might highly deviate from person to person. The temperature and processing time in the trial might differ from the retailers used. *Listeria monocytogenes* in the experiment were lastly interrupted by other bacteria from crosscontamination.

## 5. Conclusions

In conclusion, on a farm level, which was the main source of raw milk, we observed 1% *Listeria* incidence, whereas no identification of *Listeria* spp. was observed on the retailer level and postprocessed milk in the trial. The presence of *Listeria* spp. at the farm level warns people about the risk of raw or improperly treated milk to human health, even low incidence of *Listeria*. Milk should be processed following the method of pasteurization. The retailers should prevent crosscontamination during other steps after treatment as well. Finally, the responsible organizations should incessantly educate the milk retailers in adequate protocols as well as routinely check their product safety.

## Data Availability

The datasets of the current research are available at https://doi.org/10.5281/zenodo.7014148.

## Disclosure

The preprint is readily available from https://www.researchsquare.com/article/rs-1998662/v1 [45].

## Conflicts of Interest

The authors declare that they have no conflicts of interest.

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## Supplementary Materials

Questionnaire in the topic: "*Listeria* contamination in milk-processing chain and proficiency in *Listeria monocytogenes* decontamination of small-scale milk retailers" was presented as supplementary data. (*Supplementary Materials*)

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