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Behaviour of *Listeria Monocytogenes* in Pasteurization Milk during Refrigerator Storage

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Abstract
Milk is an excellent medium for the growth of microorganisms including *Listeria monocytogenes*. Some pathogenic bacteria can survive after pasteurization in milk, and these bacteria can contaminate again after storage. The purpose of this study was to determine the behavior of *L. monocyogenes* after pasteurization in refrigerator storage. Fresh milk is pasteurized at 75, 80, 85, 90 and 95 ° C for 1 minute and stored at refrigerator temperature for 1 day, 1 week and 2 weeks. Characteristics of growth and total number of *L. monocytogenes* in *Listeria* Selective agar Base medium were observed. The results showed that the total number of *L. monocytogenes* at the control temperature before pasteurization was 7.91 (log cfu / ml). At pasteurization temperatures of 75, 80 and 85 ° C for 1 min, the number of *L. monocytogenes* bacteria were decrease to 5.83, 3.82 and 1.18 (log cfu / ml), respectively, whereas the total numbers of this bacteria on pasteurization temperatures of 90 and 95 C were all listeria killed, but can grow back in refrigerator storage (4°C).

Keywords: Pasteurization, milk, Listeria monocytogenes, refrigerator storage

1. Introduction

Listeria monocytogenes has been found in raw foods of animal origin. *Listeria monocytogenes* is a Gram-positive, rod-shaped, facultative anaerobic bacterium that has been linked to food-borne illness outbreaks with an unusually high mortality rate of 20% (McLauchlin et al., 2004). Milk is a good medium for microbial growth. Cheeses are considered at risk foodstuffs (Marth and Ryser, 1990; Greenwood *et al*., 1991). *Listeria monocytogenes* is a bacterial pathogen causing outbreaks in food (food-borne bacteria) resulting in the disease listeriosis in humans and animals (Menendez et al., 1997; (Rosenow and Marth, 1987; Kasalica et al., 2011). Listeriosis causes abortions in pregnant females, neonatal sepsis, and severe infections such as septicemia and meningoencephalitis in susceptible hosts (Farber and Peterkin, 1991; Paillard*et al*., 2003). Harsoyo and Andini (2002) stated that *L. monocytogenes* is the cause of serious disease with a mortality rate of 20-30%. *Listeria monocytogenes* is widely distributed in the environment, can be found in the soil, animal feces, water and plant decay. Cattle infected with *L. monocytogenes* do not generally show symptoms but can contaminate the surrounding environment, and raw foods such as meat, dairy and other animal products. The prevalence of *L. monocytogenes* in selected samples of milk and milk product is 2.6% on average (raw bulk milk is 2.1%; raw milk from tanker trailer is 5.1%; raw milk from silo prior to pasteurization is 15%; raw milk from the balance tank of the pasteurizer is 14%; pasteurized milk is 5%; semi-finished and final product is 0%) (Navratilova *et al*., 2004).Silva *et al.* (2003) studied the occurrence of *Listeria* spp. in critical control points and the environment of the Minas Frescal cheese processing plant. This study found that

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from the total of 218 samples, thirteen were positive for Listeria;of these, 2 samples were *L. monocytogenes*.

Pasteurization is a process designed to destroy pathogenic microorganisms in raw milk (Kameni *et al.*, 2002; Elrahman*et al.*, 2013; Sarkar, 2015). The growth of *L. monocytogenes* in HTST pasteurized fresh milk is a critical point for human health. Handling of milk after milking can cause dangerous diseases (zoonoses). Precautions against the dangers of milk consumption can be dealt with by heating. Pasteurization is a heating process that uses temperatures below 100°C, aiming to deactivate enzymes and extend the shelf-life. Fresh milk is pasteurized by HTST which is expected to kill microorganisms and all unwanted pathogens, including *L. monocytogenes*. The results of the study of Nadal *et al.* (2007) stated that *L. monocytogenes* is able to survive at a temperature of 72°C for 15 seconds. The storage of pasteurized milk in a refrigerator (at 4°C) is expected to extend the shelf-life.

Studies done to evaluate the thermal resistance of *L. monocytogenes* have produced conflicting results (Bearns and Girards, 1958; Beery *et al.*, 1985; Bradshaw *et al.*, 1985). *Listeria monocytogenes* is an intracellular parasite and may be present within leucocytes in contaminated milk. Some investigators suggest that an intracellular state may confer heat resistance to the organism and allow some listeriae within leukocytes to survive pasteurization. According to the report by Doyle *et al.* (1987), although *L. monocytogenes* was isolated from milk heated at 72.2^{o}C for 16.4 s, the organism was not detected in the few trials of milk heated at 76.4^{o}C for 15.4 s. The purpose of this study was to determine the effect of High Temperature Short Time (HTST) pasteurization on the survival of *L. monocytogenes* and its ability to grow in storage at refrigerator temperature.

2. Materials and Methods

2.1 Sampling and Pasteurization of Raw Milk

Raw milk samples were obtained from a dairy farm in Enrekang (about 5 hours driving distance from the laboratory), South Sulawesi, Indonesia; samples were transported using a cool box to the Biotechnology Milk Processing Technology Laboratory. All samples were placed in a sterile whirlpak bag, kept at 5^{o}C, and processed within 24 h of collection. Research was conducted at the Laboratory of Microbiology, Faculty of Animal Husbandry Hasanuddin University in Makassar, Indonesia.

Fresh milk to be pasteurized was put into test tubes, with 10 ml per tube. HTST pasteurized fresh milk was heated at 5 different temperatures (temperature 75^{o}C, 80^{o}C, 85^{o}C, 90^{o}C and 95^{o}C) at a time of 1 minute each, and 1 tube of fresh milk was used as a control (without pasteurization). Furthermore, the milk that had been pasteurized was then stored in the refrigerator at 4°C for 1 day, 1 week and 2 weeks.

2.2 Isolation and Identification of *Listeria monocytogenes*

This research used biochemical methods for listeria identification using selective media for listeria (Listeria Selektive Agar) and then a confirmation test with a biochemical characteristics test such as the sugar fermentation test, the citrate reduction or the CAMP (Christie, Atkins, Munch-Petersen) test. Milk sampling was performed by plating serial (1:10) dilution onto Listeria Selective Agar (LSA) and incubating the agar plates at 35^{o}C. Samples of 1 ml of the dilution were incorporated into a sterile petri dish

and then Listeria Selective Agar was added for storage at 45-50°C for about 15 ml. Listeria was selectively incubated after being solidified and incubated in an incubator at a temperature of 35°C for 24 hours.

2.3 Morphological observation

Colonies of bacteria in a petri dish were observed for color, nature, edge, elevation, surface, and size and color changes of media bacteria.

2.4 Gram staining

Bacteria to be colored were taken and a fixed loop was formed over the object glass; this was dripping with crystal violet and was subsequently allowed to stand for 1-2 minutes before being washed with distilled water. Then, Lugol was used and the solution was allowed to stand for 1-2 minutes before being washed with 95% alcohol to clean the sample, followed by distilled water. The mixture was given safranin and allowed to stand for 1-2 minutes before being washed with distilled water and then dried. Preparations were examined under a microscope to assess the structure, morphology and color of the cells of the bacteria.

3. Result and Discussions

3.1 Morphological characteristics

Based on the results of the study of morphological characteristics and *Listeria monosytogenes* colonies adapted to Bergey's Manual of Systematic Bacteriology (1994) can be seen in Table 1 and Figure 1. Members of the Listeria genus are short rods, facultative anaerobic, Gram positive, not forming spores and capsules, distributed individually and in form of short chains. *L. monocytogenes* creates during exponential phase a toxin called listerilysin O (hemolysin), which leads to in-vitro hemolysis on blood agar (Kasalica *et al*., 2011).

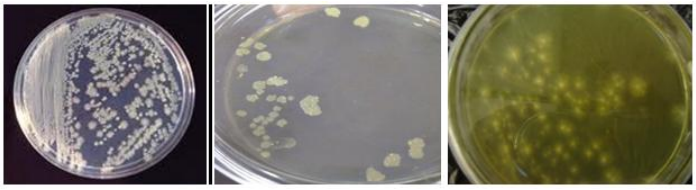

Figure 1. Colonies of Listeria monocytogenes on Listeria Selective Agar (LSA)

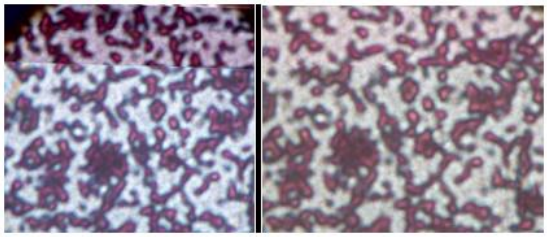

Figure 2. Listeria monocytogenes morphology with Gram staining

Identification of isolates obtained on LSA was evaluated by observation of colonial morphology, cellular morphology and hemolytic reaction in sheep blood (5% v / v) agar and then compared with the characteristics of *Listeria monocytogenes* described in Bergeys manual (Figure 1 and 2). Figure 1. shows that the colony of *Listeria monocytogenes* was colony color from white to yellowish and opaque, a serrated edge indicating that this bacterium form longer rods with long filaments. Figure 2 shows that the isolate bacteria of HTST pasteurized milk made through Gram staining are Gram positive, do not form spores and capsules, are individually distributed and form short chains, some of which form V and Y, indicated the isolates are *Listeria monocytogenes*. In the motility test using indole media showed that this isolate is mobile when incubated at 25 ° C, this is because these bacteria form the peritrichous flagella (Kasalica *et al*., 2011), this also causes the colony to form a filament.

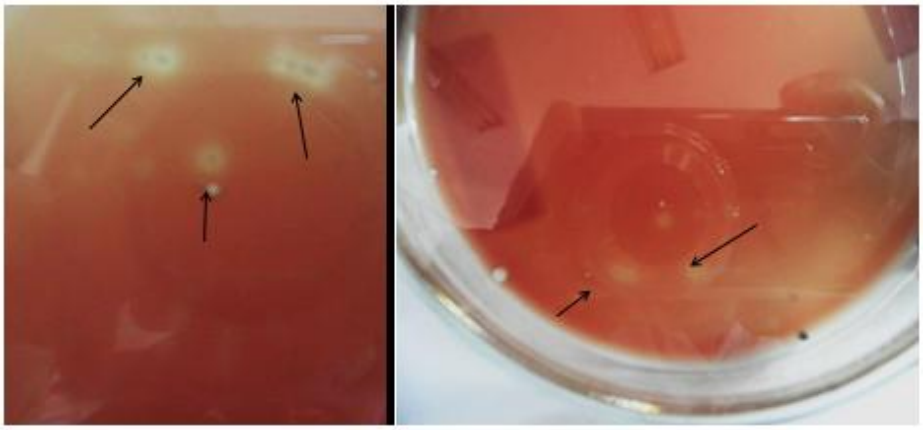

Figure 3. Colonies of Listeria monocytogenes form a zone of hemolysis (β- hemolysis) in blood sheep agar

Listeria monocytogenes creates during exponential phase a toxin called listeriolysin O (hemolysin, which leads to in vitro hemolysin on blood agar (Kasalica *et al*., 2011) with narrow zone of hemolysis around colonies.

3.2 Behaviour of Listeria on milk pasteurization

HTST pasteurization at various temperatures and for 1 minute does not guarantee the death of *L. monocytogenes*. *Listeria monocytogenes* was the highest level of bacteria at the control temperature, with a result of 7.91 cfu/ml (Figure 1). Pasteurization temperatures of 75, 80 and 85^{o}C contained bacterial suspected to be *L. monocytogenes,* at levels of 5.83, 3.82 and 1.18 cfu/ ml, respectively. At 90 and 95°C, no *L. monocytogenes* grew. This is in accordance with the SNI, which states that *Listeria* sp. must be zero (0) to indicate that there is no *L. monocytogenes* growing on milk.

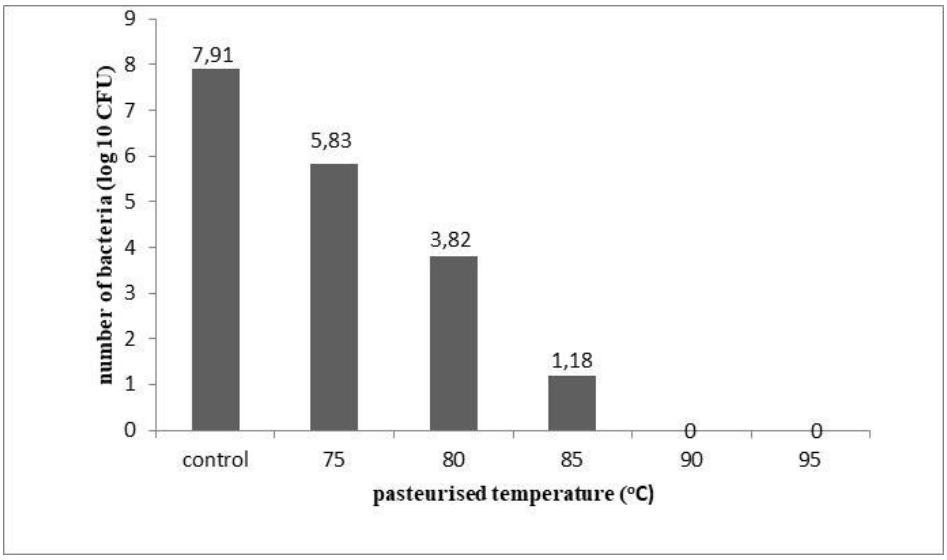

Fig 1. Number of L. monocytogenes bacteria

Pasteurization is expected to kill pathogenic bacteria that exist in fresh milk. Murdiati *et al.* (2004) stated that the goal of pasteurization is to eliminate pathogenic microbes that are dangerous human health without changing the taste, consistency and nutritional content of milk. According to Hobss and Roberts (1997), the aim of pasteurization is to kill pathogenic bacteria and non-pathogenic bacteria (decay or destruction), as well as to improve milk quality.

Figure 1 shows that the number of *L. monocytogenes*-controlled bacteria in fresh milk was higher because fresh milk is not heated; this means that bacteria can grow in milk because this is a suitable medium for bacterial growth. Heating the milk will cause death and suppress the growth of bacteria. The results of Malaka *et al.* (2014) showed that the levels of suspected *Listeria* sp. in fresh milk in South Sulawesi exceeded 3.0×10^6. In fresh milk, microorganisms can be contaminated from the skin of the livestock, feed, air, water and equipment used for rinsing and the storage of feed.

The higher the pasteurization temperature, the lower the number of *L. monocytogenes* (Figure 1). This is consistent with the opinion of Fox and Cameron (1989), that pasteurization at a temperature of 71-75°C for 15 seconds only kills 95% of the bacteria present in the milk so that the quality is greatly influenced by the treatment after heating. One factor that may affect the presence of *L.monocytogenes* in milk is contamination after pasteurization.

Based on the results of this study (Figure 1), it is known that there are a large number of *L. monocytogenes* bacteria in raw milk and that milk pasteurized at temperatures of 75, 80 and 85°C for 1 minute should not be consumed. Milk pasteurized at 90 and 95°C for 1 minute in this research is feasible for consumption because, based on previous calculations, the *L. monocytogenes* estimated bacteria count is 0. This is in accordance with the Indonesian National Standard (2000) which stated that standard bacterial contamination of *Listeria* sp. in fresh milk and pasteurized milk should not exist or that

the amount of *Listeria monocytogenes* should be zero (0). Navratilova *et al.* (2004) gave the same result,with positive samples of *L. monocytogenes* derived from HTST pasteurized milk at a temperature of 72.6°C for 15 seconds; the possibility of this microbe existing in milk due to secondary contamination after pasteurization was stated. Nadal *et al.* (2007) also found that *L. monocytogenes* was able to survive the treatment of milk pasteurized by HTST at a temperature of 72°C for 15 seconds. *Listeria monocytogenes* still survives in HTST pasteurized milk from a temperature of 72-85°C.

The storage of pasteurized milk at refrigerator temperature indicates the growth of *L. monocytogenes* (Fig. 2). Pasteurization at 90 and 95°C showed no *L. monocytogenes*after 1 day of storage, but after 1 and 2 weeks of storage there was a suspected level of *L. monocytogenes*.This gives an indication that pasteurization only causes this bacterium to become dormant. The longer the milk is stored, the more *Listeria* sp. are found, indicating that these bacteria grow in cold storage.

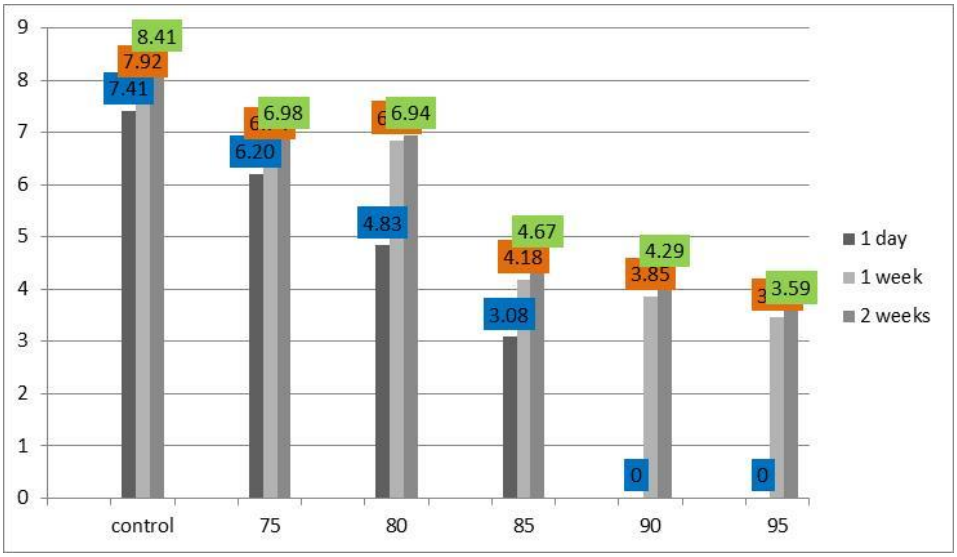

Fig 2. Listeria monocytogenes growth in Refrigerator Storage

Another possibility for the existence of *L. monocytogenes* in pasteurized milk is contamination after pasteurization. This is in accordance with the opinion of Sudarwanto (2012), who stated that pathogenic bacteria will die following a perfect pasteurization process. An outbreak of listeriosis due to the consumption of pasteurized milk is associated with contamination after pasteurization (Schaack and Marth, 1988). Al-Nabulsi *et al.* studied the behavior of *Listeria monocytogenes* during the fermentation and storage of camel milk, and found that the viability of *L. monocytogenes* was not affected during the fermentation of camel milk at 43°C for 5 h for Lactic Acid Bacteria, but it was significantly reduced during storage at 4 or 10°C.

According to Kusumawati (2000), cooked food, and heated and properly stored food, will be safe to eat because *L. monocytogenes* dies at 75°C. Chotiah (2006) stated that the main factors that determine the quality of milk following treatment with pasteurization

are raw materials, processing and packaging.

The pasteurization of milk is generally combined with the cooling method as a preservation technique. The cooling method, at a maximum temperature of 10°C, extends the shelf-life of pasteurized milk (Setya, 2012). According to Sanjaya *et al.* (2009) milk should be stored in a refrigerator for no more than 7 days. The presence of the contamination of *L. monocytogenes* in fresh milk and HTST pasteurized milk during refrigerator storage is due to the nature of *L. monocytogenes* that can grow at low temperatures. This is in accordance with the opinion of Sudarwanto (2012), which states that *L. monocytogenes* is a bacteria that can often cause disease outbreaks due to the consumption of pasteurized milk, because the bacteria are contaminants that can grow at refrigeration temperature. This is supported by Kusumawati (2000), who stated that the potential of *L. monocytogenes* to cause food poisoning is due to its ability to grow in cold storage temperatures (1-10°C) as a psychophysical bacteria and is even able to survive at frozen storage temperatures. Silva *et al.* (2003) stated that *L. monocytogenes* is psychotrophic and can grow at low temperatures.

In addition to the ability to grow at low temperatures, the presence of *L. monocytogenes* during storage may be affected by the presence of Polymorph Nuclear Leucocyte (PMNL) in milk as a factor supporting the cross-contamination after pasteurization and sampling. It can be seen that although *L. monocytogenes* died after heating at 90 and 95°C for 1 minute, it was then detected again after 1 week of storage in the refrigerator (Figure 2). According to Doyle *et al.* (1987), *L. monocytogenes* in PMNL is resistant to pasteurization,so the degradation of PMNL and *Listeria* sp. in milk after being stored in the refrigerator for 3 - 4 days causes the bacteria to regain sensitivity to heating. Therefore, in the case of *Listeria* contamination, it can be safe for consumption after repeated heating (Tindalization), but this causes the nutritional value of the milk to decrease.

The disease caused by consuming milk contaminated with *L. monocytogenes* is listeriosis. Symptoms of listeriosis include septicemia, meningitis or meningoencephalitis, encephalitis and uterine or cervical infection in pregnant women, which can result in spontaneous abortion (second/third trimester) or the baby being born and dying soon after. Kusumawati (2000) states that the symptoms of listeriosis begin with influenza and prolonged fever. Listeriosis is characterized by symptoms of the gastrointestinal tract, namely nausea, vomiting, and diarrhea.

One way of handling the effort to preserve milk is by treatment with moderate heating or pasteurization (Sofos, 1993). Prevention efforts that can be performed include avoiding the consumption of raw food, pasteurizing with HTST first in accordance with Good Manufacturing Procedures (GMPs) and applying sanitation hygiene and aseptic technique in every food handling.

Minea *et al.* (2005) analyzed 196 samples of dairy products in 9 dairy processing plants in 5 countries of the Moldova territory; in total, 20.4% were identified as being contaminated with *Listeria* sp., of which 3.57% were *Listeria monocytogenes*.The highest frequency of *Listeria monocytogenes* contamination was registered in raw milk (10.53%) and brining maturated cheeses (9.67%). Minea *et al.* (2005) concluded that the contamination indicates that *Listeria monocytogenes* is commonly in the dairy industrial environment.

4. Conclusion

Listeria monocytogenesis is resistant to warming at 75, 80 and 85oC for 1 minute, but increasing the heating temperature causes an increase in bacterial death. *Listeria monocytogenes* can not survive after warming at 90 and 95oC. *Listeria monocytogenes* can grow in refrigerator storage temperature (4oC) and cross-contamination can occur after pasteurization and during processing

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