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Evaluation of the effects of fermentation of buffalo curd and acidity on survival kinetics of *Listeria monocytogenes*

VS Jayamanne[1] and U Samarajeewa[2]
[1] Dept of Food Science & Technology, Faculty of Agriculture, University of Ruhuna, Kamburupitiya, Sri Lanka
[2] Dept of Food Science & Technology, Faculty of Agriculture, University of Peradeniya, Peradeniya, Sri Lanka

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ABSTRACT

***Listeria monocytogenes*, a food-borne pathogen often found in milk and milk products, causes listeriosis in pregnant women, newborns, elderly and immuno-suppressed people. There have been reports on survival of *Listeria* in various milk products in the world, but comparable studies on the survival of *Listeria* in buffalo curd have not been reported. Therefore, the present study investigated the effects of fermenting buffalo milk and acidity on survival kinetics of *L. monocytogenes*. *L. monocytogenes* FSTLC2 and lactic acid starter cultures were aseptically introduced to boiled and cooled buffalo milk (fat 6%, protein 4%) and the mix was allowed to ferment at ambient temperature (26 ± 2°C) for 18h. The *Listeria* count on Modified Oxford Agar (MOX; Oxoid Ltd.), lactic acid bacteria count on MRS Agar (Oxoid Ltd.), pH and titratable acidity were determined in the beginning and at 2h intervals during fermentation. The effect of pH/acidity on *Listeria* was determined by introducing *Listeria* to buffalo milk with pH values (pH 4.0, 4.5, 5.0, 5.5) adjusted using 88% lactic acid (BDH Chemicals) and enumerating *Listeria* on MOX Agar at 12h intervals for 96h. It was observed in the present study that *Listeria* count decreased over time and after 16h of fermentation of buffalo milk at ambient temperature, *Listeria* cannot be detected on MOX Agar. The pH value decreased from 6.8 to 4.1 and titratable acidity (lactic acid %) increased from 0% to 1.2 % during fermentation. Fermentation appeared to be an effective preservation technique in eliminating *Listeria* in buffalo curd. The pH value of 5.5 appeared to be the critical pH for inactivation of *Listeria* as no growth of *Listeria* was observed below pH 5.5. The total inactivation of *Listeria* in buffalo milk appeared to be due to lowering of pH coupled with increasing titratable acidity as well as action of bacteriocins, especially Nisin, produced by lactic acid bacteria during fermentation. It is clear that fermentation is an effective tool in inactivating *Listeria* in buffalo milk.**

Keywords: *Listeria monocytogenes*, Buffalo curd, Fermentation, Inactivation, Acidity

INTRODUCTION

Listeria monocytogenes, which is a Gram-positive, facultatively anaerobic, cold tolerant, salt tolerant, non-spore forming and non-acid fast rod often found in soil and water, and faeces of animals, is a relatively recent food-borne pathogen reported mostly from developed countries (Maijala *et al*. 2001; Karakolev 2009). *L. monocytogenes* is a food -borne pathogen of high concern to the food industry as its ubiquitous occurrence in the environment can lead to contamination of foods. The toxic substance produced during the exponential growth phase of *L. monocytogenes* is designated listeriolysin O (hemolysin), which causes hemolysis of blood agar in in-vitro studies. When *L. monocytogenes* is contracted through the oral route, it apparently colonizes the intestinal tract by mechanisms that are poorly understood. From the intestinal tract, the organism invades tissues including the placenta in pregnant women, and enters the blood stream, from which it reaches other susceptible body tissues. Listeriosis, the illness caused by this bacterium, affects mostly pregnant women, elderly, newborns, and the immuno-suppressed adults due to AIDS, alcoholism, diabetes, cancer, cardiovascular disease, kidney disease, renal transplant, and corticosteroid therapy (Rocourt *et al*. 2000). When susceptible adults contract the disease, meningitis and septicemia are the most commonly recognized symptoms. The non-pregnant healthy individuals who are not immuno-suppressed are fairly resistant to infection by *L. monocytogenes*. Infected pregnant women may experience only mild flu-like illness, and the illness can be transmitted from mother to the fetus through the placenta resulting in abortion, premature birth, stillbirth, or serious health problems for the newborn child. The mortality rate of the disease is approximately 25% (USDA 1999).

Milk and milk products have received much attention because they have been reported to harbor *L. monocytogenes* to a much higher extent compared to other foods. *L. monocytogenes* has been isolated from cow milk, goat milk, cheese, and pasteurized milk in Sri Lanka (Jyamanne and Samarajeewa

*Corresponding author : *vsjayamanne@fst.ruh.ac.lk*
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2001). There have been reports of *Listeria* outbreaks associated with dairy products (Farber and Peterkin 1991). Cow milk has been implicated in food-borne fatal listeriosis. The ability of *L. monocytogenes* strains to proliferate in raw milk even under refrigerated condition is well documented.

Though milk and milk products were reported to transmit food-borne listeriosis, only two studies have been reported in Sri Lanka to ascertain the safety of the dairy products. In a survey on *Listeria* in foods, it was demonstrated that the percentage of *L. monocytogenes* positive samples was highest among vegetables (49%), second in chicken (34%), and lowest in dairy products (26%) (Gunasena *et al*. 1995). They further observed that 5 pasteurized milk (31%), 3 raw milk (25%) and 4 ice cream (33%) samples contained *L. monocytogenes*. In a similar study, of the samples tested (265), 39 samples (15%) contained virulent *L. monocytogenes.* Cow milk (29%), goat milk (27%), pasteurized milk (17%), and cheese (33%) samples contained virulent strains of *L. monocytogenes* (Jayamanne & Samarajeewa 2001). They further observed the complete absence of *Listeria* in buffalo curd. However, no studies on the effect of fermenting buffalo milk on survival of *Listeria* have been carried out. Therefore, the present study investigated the survival kinetics of *Listeria* in fermenting buffalo milk and at different pH values at ambient temperature (26 ± 2^{o}C).

MATERIALS AND METHODS

Two major experiments were carried out to determine the effect of fermentation of buffalo curd and added acidity on the survival kinetics of *L. monocytogenes*.

Effect of fermentation of buffalo curd on the survival kinetics of *L. monocytogenes*

Buffalo milk (fat ~6%, protein ~4%) was aseptically collected and was boiled for 5 min to destroy all the organisms. Boiled buffalo milk (250ml) was poured into sterile clay pots. Boiled buffalo milk was first tested for *Listeria* using the FDA Listeria Enrichment Broth (LEB; Oxoid Ltd., Basingstoke, UK) and Modified Oxford Agar medium (MOX; Oxoid Ltd.) in order to ensure that milk was not contaminated with *Listeria*.

In the enumeration process of the organism, *Listeria* in milk was first enriched in FDA Listeria Enrichment Broth. Samples (25ml) were blended with LEB (225ml) at 12,000rpm for 2min in a Waring blender. The resulting solution was incubated at 35^{o}C for 48h for selective enrichment and the selective isolation of *Listeria* was done in MOX. Enriched samples were next streaked onto MOX Agar plates and incubated at 35^{o}C and examined for colonies with black halos after 24, 48 and 72h. Single colonies growing on the MOX medium were isolated and transferred to MOX agar slants in McCartney bottles and incubated at 35^{o}C until there was sufficient growth. The MOX agar slants were stored in the refrigerator at 3 ± 2^{o}C pending confirmatory morphological and biochemical tests. MOX agar slants were sub-cultured at 3-month intervals to ensure the viability of organisms. The tests [microscopy on wet mount, Gram staining, catalase test, methyl red (MR) test, Vogus-Proskauer (VP) test, H_2S production, color changes in Triple Sugar Iron Agar, color changes in Urea Agar, hydrolysis of aesculin and CAMP test] were carried out to confirm the presence of *Listeria* in the food samples.

Three Sub experiments were carried out to determine the effect of fermentation of buffalo curd on survival kinetics of *L. monocytogenes*.

An inoculum of starter culture (2%) from a day old buffalo curd pot was introduced to boiled buffalo milk (250ml) and allowed to ferment at ambient temperature ($26\pm2\ ^{o}$C). The total lactic acid bacteria on MRS Agar (Oxoid Ltd.), pH value, and titratable acidity in the beginning and at 2h intervals, were recorded during fermentation. The pH values were determined using a portable pH meter (Model HI8424; Hanna Instruments, Milan, Italy). The pH meter was calibrated using standard pH buffers 4.0 and 7.0 before use. The titratable acidity was determined by titrating 10g of fermented milk (mixed with 10ml of hot water) against 0.1M NaOH to a phenolphthalein (0.5%) end point and expressed as percent lactic acid according to the Standard formulae (AOAC 1985).

L. monocytogenes FTSLC2 (University of Peradeniya Culture Collection), previously isolated from raw cow milk, from the culture collection was enriched in LEB at 35^{o}C for 48h, transferred to Tryptose Broth (10ml) and incubated for 48h at 35^{o}C. A loopful of enriched *Listeria* culture was aseptically transferred to McCartney bottles containing 20ml of Tryptose Broth (Oxoid Ltd.), incubated for another 24h at 35^{o}C. After 24h of incubation in Tryptose Broth, the *Listeria* cells at the late stationary phase were harvested by centrifugation at 6,000 × g for 20min. *Listeria* was introduced to boiled and cooled buffalo milk (250ml) in clay pots to have an initial *Listeria* population of approximately 10^{5}cfu/ml, and it was obtained by the surface plate count method on MOX Agar. The *Listeria* count on MOX Agar and pH values were recorded in the beginning and at two-hour intervals of

the fermenting milk solution at ambient temperature (26±2°C).

The inoculum (2%) of previously fermented curd was aseptically introduced to boiled buffalo milk. *L. monocytogenes* was also introduced to boiled buffalo milk (250ml) at the same time to have an initial *Listeria* population of 10^5cfu/ml. The *Listeria* count on MOX Agar, lactic acid bacteria count on MRS Agar, pH, titratable acidity of the fermenting milk solution at ambient temperature (26±2°C) were taken simultaneously in the beginning, and at two-hour intervals during fermentation. Logarithmic survival number of *Listeria,* total lactic acid bacterial count, and pH values (Y axis) were plotted against time (X axis).

It can be hypothesized that the pH/acidity plays a major role in inactivating *Listeria* in buffalo milk and lactic acid is the acid that is formed during fermentation. Therefore, a separate experiment was carried out to determine the effect of different concentrations of lactic acid on the survival of *Listeria* in buffalo milk.

Determination of the effect of pH on the survival of *L. monocytogenes*

L. monocytogenes FSTLC2 (University of Peradeniya Culture Collection), previously isolated from raw cow milk, from the stored culture collection was enriched in LEB at 35°C for 48h. A loopful of the enriched *Listeria* culture was aseptically transferred to Tryptose Broth and incubated at 35°C for further 48h. After 24h of incubation in Tryptose Broth, the *Listeria* cells at the late stationary phase were harvested by centrifugation at 6000 × g for 20 min. Buffalo milk was boiled for 5 min to destroy microorganisms. Boiled buffalo milk (250 ml each) was aseptically transferred to four Erlenmeyer flasks. Acidity of boiled milk in Erlenmeyer flasks was adjusted using 80% lactic acid (BDH Chemicals, UK) to obtain different pH values such as 5.5, 5.0, 4.5 and 4.0. *Listeria* was introduced to boiled and cooled buffalo milk (250ml) to have an initial *Listeria* population of approximately 10^5cfu/ml. A surface count of *Listeria* on MOX Agar was taken in duplicate at 12h intervals for a period of 96 h at ambient temperature (26±2°C). A graph of the log survival number of *Listeria* (Y axis) against time (X axis) was plotted.

RESULTS AND DISCUSSION

Fermentation appeared to be an effective tool in eliminating *Listeria* from buffalo milk. After 16h of fermentation of buffalo milk at ambient temperature *Listeria* was not detected on MOX Agar.

Effect of fermentation of buffalo milk on growth dynamics of *L. monocytogenes*

It was observed that when the inoculum from previously fermenting buffalo curd was introduced at 2% concentration to boiled buffalo milk and incubated at room temperature (26±2°C), the lactic bacteria started to proliferate and reached its maximum (approximately 10^6cfu/ml) after 14h of fermentation (Figure. 1). The pH value decreased from 6.8 to 4.1 during 14h fermentation. It can be safely assumed that almost all the bacteria in the inoculum of previously fermented curd are lactic acid bacteria, because during fermentation the growth of other organisms is inhibited by decreasing pH, increasing titratable acidity and bacteriocins. The total plate count on MRS Agar in the fermenting buffalo curd solution could almost represent the lactic acid bacteria.

When *L. monocytogenes* FSTLC2 was introduced at a population of 10^5cfu/ml to boiled buffalo milk after cooling to room temperature, *Listeria* count decreased with time, and after 16h of fermentation

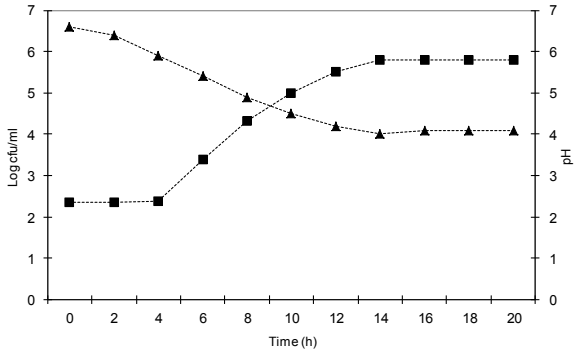

Figure 1: Total lactic acid bacteria count on MRS Agar (■) and changes in pH (▲) in buffalo curd during fermentation at ambient temperature (26±2 °C)

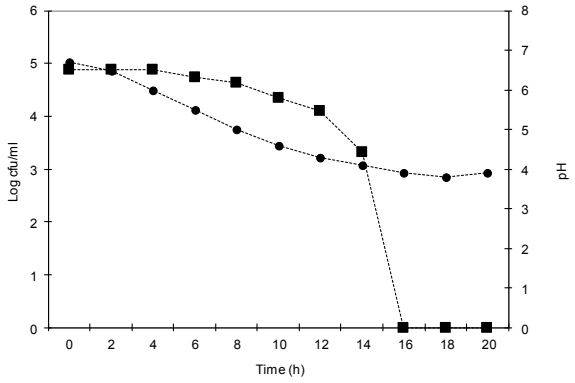

Figure 2: Survival of *L. monocytogenes* FSTLC2 (■) and changes in pH (●) during fermentation of buffalo milk at ambient temperature (26±2 °C)

with an inoculum of fermenting curd at ambient temperature, *Listeria* cannot be detected on MOX Agar (Figure. 2). In the fermentation process, the pH decreased from 6.8 to 4.1. It is evident that at pH 5.5, the *Listeria* count begins to drop sharply (Fig. 2). The pH value 5.5 appeared to be the critical point for inactivation of *Listeria* in fermenting buffalo milk. When both the total bacterial and *Listeria* counts were taken simultaneously in fermenting buffalo curd, an inverse relationship between the total bacterial count and *L. monocytogenes* was apparent (Figure. 3). It was observed that when the total bacterial count increases, the *Listeria* count decreases, and the pH decreases from 6.8 to 4.1 (Figure. 3). It appears that fermentation eliminates *Listeria* from buffalo curd. This could be mainly due to lowering of pH and increasing acidity. Although the pH decrease in later stages of fermentation was small compared to the rapid drop during initial fermentation, the titratable acidity increased steadily from 0% to 1.2%. The combination of low pH and increasing titratable acidity would result in increasing levels of undissociated acid, which is more harmful to microorganisms and is clearly a factor in the rapidly decreasing listerial population towards the end of fermentation (Figure. 3) (Adams & Moss 2000). Additionally, production of antilisterial substances by lactic acid bacteria, and the action of natural lactoperoxidase system present in milk may be playing a contributory role. Of the three factors, the lowering of pH coupled with increasing acidity could be the most important factor in inhibiting the *Listeria* in milk.

It was reported that inhibition of *L. monocytogenes* in fermented milk products is associated with the decrease in pH (<4.0) (Zuniga *et al.* 1995). Other micro-flora of fermented milk products is also reported to increase the death rate of *L. monocytogenes* during storage at 20°C (Stanczak *et al*. 1997).

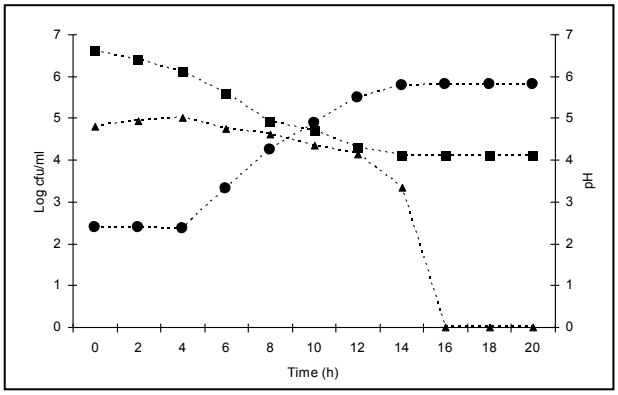

Figure 3: Population level of *L. monocytogenes* FSTLC2 on MOX (▲), total lactic acid bacteria count on MRS Agar (●) and changes in pH (■) during fermentation of buffalo milk at ambient temperature (26±2° C)

Lactic acid bacteria and their metabolic products have a direct impact on *L. monocytogenes*. The lactic acid bacteria compete with other organisms for nutrients and they produce antibacterial compounds including weak acids, hydrogen peroxide, diacetyl and bacteriocins (for example nisin and pediocin). A large number of bacteriocins from lactic acid bacteria are active against *L. monocytogenes*. It was reported that these bacteriocins usually destabilize the cytoplasmic membrane of sensitive cells, increase membrane permeability, and dissipate the proton motive force by forming water-filled transmembrane pores or channels (Jack *et al*. 1995). Nisin, a bacteriocin produced by certain strains of *Lactococcus* species has proven to be useful in suppressing the growth of *L. monocytogenes* (Mohamed *et al.* 1980). Further, *L. lactis* is a bacterial species that is normally present in the inoculum used to produce buffalo curd.

The observations in the present study suggest that fermentation is a useful food preservation technique for elimination of *L. monocytogenes*. Fermentation appears to be able to eliminate an initial *Listeria* population of approximately 10^5cfu/ml in buffalo curd. It is also notable that none of the buffalo curd or yoghurt sample tested in a previous study (Jayamanne & Samarajeewa 2001), contained *Listeria* indicating the effectiveness of fermentation in inhibiting *Listeria* growth. The consumption of fermented buffalo curd is therefore safe with regard to human listeriosis.

Effect of pH on survival of *L. monocytogenes*

It was observed that an initial *L. monocytogenes* FSTLC2 population of 10^5 cfu/ml survived only 48 h at pH 4.0, when *Listeria* was introduced to boiled buffalo milk after adjusting pH using lactic acid (Figure. 4). Furthermore, maintenance of a population level of *L. monocytogenes* in milk at pH 5.5

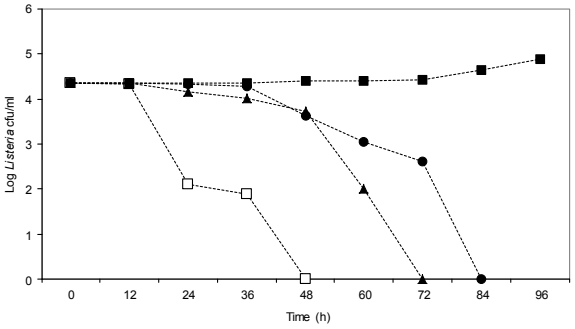

Figure 4: Survival of *L. monocytogenes* FSTLC2 in bovine milk at pH values of 4.0 (□), 4.5 (▲), 5.0 (●) and 5.5 (■) at ambient temperature (26±2°C)

was observed (Figure. 4). The minimum pH value for survival of *Listeria* appears to be 5.5, and pH values below 5.5, inhibit the growth of *Listeria* (Figure. 4). Lactic acid, which is reported to have deleterious effects on *Listeria,* was used to acidify the medium. It is reported that at the same pH, the relative anti-microbial activity caused by added acids follows the order of acetic acid> lactic acid> citric acid> malic acid> HCl (Sorrells *et al.* 1989). In comparable studies on effect of pH, it was reported that *L. monocytogenes* can only grow at pH values from 5.6 to 9.6, with optimal growth occurring at neutral to slightly alkaline pH values (Petran and Zotolla 1989). It was also reported that *L. monocytogenes* failed to grow in Dextrose (glucose) Broth at pH less than 5.6 after 2-3 days of incubation at 37^{o}C, and in addition, routine subculturing from the medium was nolonger successful (Seeliger 1961).

All evidence, from the present study and reported work suggest the possibility of using pH as a mean to control *Listeria* activity in milk. It appears that the observed inactivation of *Listeria* in buffalo curd could be due to the pH/acidity and bacteriocins produced by lactic acid bacteria, and the natural lactoperoxidase system, because a pH of 4.0 alone took 48h to inactivate *Listeria* (Figure. 4)

The apparent inability of *L. monocytogenes* to survive at pH values below 5.5, both observed in the present study and reported by other scientists, could be a useful tool in food product formulations. It appears that *Listeria* can withstand low and high temperatures, tolerate salt, grow anaerobically, and adapt to various environmental stresses. But, in general *Listeria* does not appear to be able to withstand low pH or acidity. This phenomenon can be made use of in food product developments where all other growth parameters are less effective. It is also evident that lactic acid is suited to acidify food formulations in order to arrest *Listeria* growth.

CONCLUSIONS

Fermentation appeared to be an effective preservation technique to eliminate *Listeria* in buffalo curd. The pH value of 5.5 appeared to be the critical pH for inactivation of *Listeria* as no growth of *Listeria* was observed below pH 5.5. The total inactivation of *Listeria* in buffalo milk may be due to lowering of pH coupled with increased acidity as well as action of bacteriocins produced by lactic acid bacteria during fermentation. *Lactococcus lactis* in the buffalo curd culture appeared to produce nisin that has an inhibitory effect on *Listeria*. Further, lowering of pH in food can be an effective method to arrest listerial growth in milk and milk products.

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