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OCCURRENCE OF *LISTERIA MONOCYTOGENES* IN BULKED RAW MILK AND TRADITIONALLY FERMENTED DAIRY PRODUCTS IN UGANDA

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ABSTRACT

Bulked raw milk, locally processed yoghurt (LPY) and *Bongo*, a traditionally fermented dairy product sold at most informal milk cooling points in Uganda, were assessed for occurrence of *Listeria* spp. and *Listeria monocytogenes*. Total plate counts (TPC), holding temperature, pH and titratable acidity were also determined in all the milk products at the point of collection using standard methods. A total of 40 samples of bulked raw milk and 30 for each of LPY and *Bongo* were examined. *Listeria* spp. was higher in bulked raw milk than in fermented milk. *Listeria* spp. were detected in 60% of bulked raw milk, 30% of LPY and 15% of *Bongo* samples. Bulked raw milk had significantly higher ($p<0.05$) mean *Listeria* counts (3.10±0.06 $\log_{10}$ cfu mL^{-1}) than LPY and *Bongo*, 0.82±0.18 and 0.32±0.18 $\log_{10}$ cfu mL^{-1}, respectively. *L. monocytogenes* was isolated from 13 % of bulked raw milk, 3.0% of LPY but was not detectable in *Bongo*. Total plate count was significantly different ($p<0.05$) among the different milk types studied. *Bongo* had higher TPC (9.00±0.13 $\log_{10}$ cfu mL^{-1}) than bulked raw milk (8.40±0.11 $\log_{10}$ cfu mL^{-1}) and LPY (7.40±0.13 $\log_{10}$ cfu mL^{-1}). The mean total plate counts (4.90 to 9.00±0.13 $\log_{10}$ cfu mL^{-1}) of the fermented dairy products were within the acceptable limits for human consumption. The TPC for bulked raw milk (8.40±0.11 $\log_{10}$ cfu mL^{-1}) was higher than the recommended values of national and international standards. Temperature, pH and titratable acidity were significantly different ($p<0.05$) among the different milk types. Holding temperature ranged from 5.40 to 8.60^{o}C, pH was 4.20±0.04 to 6.10±0.04 whereas titratable acidity ranged from 0.22±0.01 to 089±0.01%. *Listeria* counts were not statistically predictable ($p>0.05$) from variation in the combined effect of pH, percent titratable acidity and temperature. Results of this study demonstrate a high risk associated with consumption of bulked raw milk and fermented dairy products in due to occurrence of *Listeria* spp.

Key words: Milk, yoghurt, *Bongo*, *Listeria* spp

INTRODUCTION

Listeria monocytogenes has become an issue of global concern because of its increased presence in milk and other food products [1, 2]. *L. monocytogenes* is a gram-positive psychrotrophic pathogen, which is fairly difficult to eliminate from raw and processed foods because of its ubiquitous nature [3]. In particular, the ability of the organism to grow at refrigeration temperatures [4] and on dry surfaces [5] and its ability to tolerate acidic conditions [6] make it well adapted to food environments which normally restrict bacterial growth. The primary sources of *L. monocytogenes* in milk and dairy products include the feed, bedding, vegetation, soil, animal faeces, contaminated water, diseased and unclean udders and teats, human hands and handling equipment [7, 8, 9]. It is highly virulent, causing human Listeriosis in infected individuals. The disease primarily affects pregnant women, newborns, and adults with weakened immune systems [6]. The infection is characterized by meningitis, stillbirths, abortions and severe poisoning of blood (septicemia) and brain [2, 3]. Infected pregnant women may experience only a mild, flu-like illness; however, infections during pregnancy can lead to miscarriage or stillbirth, premature delivery, or infection of the newborn.

Milk-borne disease outbreaks associated with *L. monocytogenes* have been reported in developed countries but limited information is available in most African countries, including Uganda [2, 10]. In Uganda, like other African countries where the HIV/AIDS prevalent, Listeriosis may be a silent killer among the immunity compromised individuals who may depend on milk for protein intake.

Several studies have shown that the organism may survive in fermented milk for several weeks, especially if the milk was initially heavily contaminated and, more so, if the product was stored under refrigeration [11, 12]. *L. monocytogenes* has been reported to survive better in high solids yoghurt with relatively higher pH than low solids yoghurt produced under similar conditions [13]. Low pH fermented dairy products, with strong antimicrobial activity of lysozyme and other metabolites, have also been reported to pose minor inhibiting effect on growth of several strains of *L. monocytogenes* [14]. *L. monocytogenes* has been reported to possess variable heat resistance depending on conditions of medium such as pH, acidulant, fatty acid composition, storage temperature and lysozyme content [15].

In Uganda, there is an increased proliferation of uncontrolled informal milk cooling points, as well as the household and medium scale milk fermentation businesses particularly in urban areas of Kampala district. Moreover, the consumption of raw or insufficiently heat-treated milk is still practiced among some communities. Fermented dairy products, produced at small-scale, are normally produced using commercial starter cultures or by natural fermentation. The processing conditions such as incubation temperature, level of inoculum and quality of raw milk used vary from one producer to another. Despite the seemingly favorable conditions for presence of *L. monocytogenes* and other *Listeria* spp. in raw milk and dairy products, few studies have been conducted in Uganda to establish the occurrence and levels of the organisms. This study was undertaken to establish the occurrence and levels of *L.*

monocytogenes and other *Listeria* spp. in bulked raw milk, LPY and *Bongo*, a traditionally fermented dairy product, sold in Uganda.

MATERIALS AND METHODS

Sampling

Samples were obtained from five divisions of Kampala district. Bulked raw and fermented cows' milk samples were collected from milk cooling points in Central, Kawempe, Makindye, Nakawa and Rubaga divisions using stratified systematic random sampling, each division constituting a sampling stratum. The divisions were selected on the basis of the numerous milk cooling points in the areas. The cooling points were small-scale enterprises with an average cooling capacity ranging from 500 to 1500 liters. Samples (500 mL) of bulked raw cows' milk (n=40), LPY (n=30) and traditionally fermented milk, *Bongo* (n=30), were purchased from selected informal milk cooling points in each division. Traditionally fermented milk (Bongo) is made from unpasteurized milk whereas boiled milk is used for locally produced yoghurt (LPY). The numbers of the samples analysed were based on the assumed health risk associated with the products with raw milk posing a greater risk.

Bulked raw milk and *Bongo* were collected from coolers and aseptically filled in sterile screw-capped Duran glass bottles (500 mL). Locally produced yoghurt samples were purchased and collected in their original plastic packs (500 mL). Temperature of the samples was recorded at the time of sampling using a digital thermometer (HI 91541C, Singapore). Samples were coded according to type, date of collection and source, and were transported under ice to Uganda National Bureau of Standards for microbiological analysis. Levels of *Listeria* spp. and *L. monocytogenes*, total plate counts, pH and percent titratable acidity were determined within eight hours of sample collection. Each sample was tested in duplicate for consistency.

Microbiological analyses

Listeria spp. were detected and enumerated according to the procedure described by Food and Drug Administration-Bacteriological Analytical Manual (FDA-BAM) [3]. Samples (25 mL) were added to 225 mL of *Listeria* enrichment broth (LEB) (Oxoid CM862, UK). The mixture was shaken (2 minutes) and then incubated at 30^{o}C for 48 h for enrichment. The cultures from enrichment broth (0.1mL) were surface spread on Dry Oxford Agar (Merk 7004, Germany), and further incubated at 30^{o}C for 48 h.). The culture plates were then examined for grayish colonies having black halos and sunken centers [15]. Presumptive colonies from each plate that were typical of *Listeria* spp. were enumerated using a colony counting equipment (Stuart Scientific, UK). Suspect colonies (2 from each plate) were confirmed by Gram staining, catalase and oxidase tests. In order to identify *L. monocytogenes*, presumptive colonies were sub-cultured on horse blood agar (Oxoid, Basingstoke, Hampshire, UK) at 37^{o}C for 24 h and examined for presence of β-haemolysis. Cells were further tested for motility, dihydrogen sulphide (H_2S) production and glucose utilization [3, 15, 16]. The TPC was enumerated using the pour plate count technique as described by International Dairy Federation [17].

Physical and chemical analyses

The pH of samples was measured using an analogue pH meter (WPA-C18, UK) with a combined glass electrode. The percent titratable acidity was expressed as percent lactic acid and determined by titration of 10 mL sample aliquots against 0.1N sodium hydroxide solution using phenolphthalein indicator [18]. The temperature of samples was measured with a digital thermometer (HI 91541C, Singapore).

Statistical analyses

Data for microbial counts was normalized by expressing as $\log_{10}$ cfu mL^{-1} and summary statistics calculated for all continuous variables. Parametric data was subjected to analysis of variance and means separated using the Fischer's Least Significant Difference (LSD) test. The linear regression approach was used to relate temperature, pH and percent titratable acidity with the $\log_{10}$ cfu mL^{-1} of bacterial counts. Data weere analyzed using Genstat 5 computer software, Release 3.2 (PC/Windows NT, Lawes Agricultural Trust, Rothamsted Agricultural Station, UK [19].

RESULTS

Total plate counts (TPC) were significantly different ($p<0.05$) among the different milk product types studied. *Bongo* had higher TPC (9.00±0.13 $\log_{10}$ cfu mL^{-1}) than raw milk (8.40±0.11 $\log_{10}$ cfu mL^{-1}) and LPY (7.40±0.13 $\log_{10}$ cfu mL^{-1}). *Listeria* spp. were recovered from the bulked raw and fermented milks sold by the informal milk cooling points of Kampala district (Table 1). Occurrence of *Listeria* spp. was higher in bulked raw milk than in fermented milk. Out of the 40 samples of bulked raw milk, 60% tested positive for *Listeria* spp. For LPY and *Bongo*, 30% and 15% of the samples respectively, were found to be contaminated with the organisms. *L. monocytogenes* was similarly isolated from both bulked raw milk (13%) and LPY (3.0%) but its occurrence was not detected ($p< 0.01$) in *Bongo*. Raw milk had significantly higher ($p<0.05$) mean *Listeria* spp counts (3.10±0.06 $\log_{10}$ cfu mL^{-1}) than LPY and *Bongo*, 0.82±0.18 and 0.32±0.18 $\log_{10}$ cfu mL^{-1}, respectively (Table 1). Raw milk had significantly higher ($p<0.05$) mean *Listeria* spp counts than other milk product types, (Raw milk 3.10±0.06 $\log_{10}$ cfu mL^{-1}, LPY, 0.82±0.18 og_{10} cfu mL^{-1} and *Bongo*, 0.32±0.18 $\log_{10}$ cfu mL^{-1},) (Table 1).

The holding temperature, pH and percent titratable acidity of raw milk, LPY and *Bongo* obtained from milk cooling points in Kampala district were significantly different ($p<0.05$) among the milk product types sampled (Table 2). Holding temperature ranged from 5.40 to 8.60^{o}C, pH was 4.20 to 6.10 while titratable acidity was in the range of 0.22 to 0.89%. Regression analysis indicated that variability in the mean *Listeria spp (*counts was not predictable ($p> 0.05$) from variation in sample pH ($R^2 = 0.26$) and temperature ($R^2 = 0.05$). In addition, variation in *Listeria* counts was not statistically predictable ($p>0.05$) from variation in the combined effect of pH, percent titratable acidity and temperature.

DISCUSSION

The TPC of bulked raw milk was higher than the acceptable standard ($2x10^6$ cfu mL^{-1}). Total viable count is an important criterion for evaluating the microbial quality of various foods and also the degree of freshness of food [20]. In addition, the holding temperature of milk (> 4^oC) was favorable for microbial growth and may be a contributing factor to high TPC values observed [20]. The use of contaminated water during milking, aerial contamination from dust, contaminated milkers' hands and inadequately cleaned containers are likely sources of contamination of the raw milk [20]. Total plate count of the fermented milk products was above the acceptable level for fresh raw milk (10^5cfu mL^{-1}) [13, 21].

The counts of *Listeria* spp. in bulked raw milk were higher than that reported (10^2 cfu mL^{-1}) [22]. The concentration of *L. monocytogenes* less than 100cfu/mL can be considered to be of low risk to consumers, although the possibility of infection from low numbers of *L. monocytogenes*, especially among the most susceptible population groups (young, old, pregnant, immunocompromised) cannot be discounted. Variation in *Listeria* spp counts in dairy products is largely attributed to use of different isolation protocols and source of the samples [23]. The occurrence of *L. monocytogenes* (12.5%) in bulked raw milk was similar to that reported in other countries [4, 24]. Raw milk, which is vended by informal cooling points in Kampala district, is often transported from farms in plastic cans, usually at ambient temperature (25 to 30^oC). Plastic is rather difficult to clean, absorbs extraneous flavors and is conducive for the formation of biofilms. *L. monocytogenes* has been reported to adhere to polymeric materials, including plastic and stainless steel, especially at temperatures greater than 30^oC and low pH (4 to 7) [25]. Iodophors, hypochlorite, quaternary ammonium compounds are common sanitizers used for cleaning of many dairy utensils and equipment. A number of these compounds provide inadequate reduction in numbers of *Listeria* cells that adhere to milk biofilms [26]. Consequently, the inadequate cleaning of the containers may led to contamination of the subsequent batch of the product. These practices are common in some milk handling facilities in Uganda.

The incidence of *L. monocytogenes* in LPY (3.0%) was, as expected, less frequent and generally lower than that reported [4]. Contamination of traditionally fermented dairy products with *Listeria* spp. has been reported in North Africa with incidences of 10% for *raib*, 6% for *Ibens* and 20% for *Jben* [4]. This has been attributed to the differences in hygiene conditions during milking, handling, processing and storage of raw and fermented dairy products [8]. Studies have also suggested that raw milk and fermented dairy products may be contaminated with *L. monocytogenes* and other *Listeria* spp. from the environment, diseased udders, ingredients and packaging materials [6, 27]. Pasteurization as a thermal process may be used to reduce food borne pathogens including Listeria but studies have shown that it can survive the pasteurization conditions. *L. monocytogenes* is a major concern to manufacturers worldwide due to the resistance of the pathogen to a number of food preservation practices, in particular, the ability of the organism to grow and survive in food environments which are restrictive to bacterial growth [6]. Consequently, control of this bacterium is a significant challenge for the food manufacturer. *L. monocytogenes* can survive the minimum low-temperature, long-time pasteurization treatment required by the U.S. Food and Drug Administration for milk. *L.*

monocytogenes has been reported to survive the minimum high-temperature, short-time treatment (71.7 degrees C, 15 s) required by the U.S. Food and Drug Administration for pasteurizing milk [28]. In simulated studies close to natural situation, viable *L. monocytogenes* was recovered from cow's milk injected with *L. monocytogenes* after pasteurization (HTST treatment) at 71.7 C for 15 seconds, although not after treatment at 76.4 C-77.8 C for 15 seconds. This survival was attributed in part to protection of *L. monocytogenes* within leukocytes in milk [28]. Potentially inadequate thermal processing has been associated with sporadic listeriosis outbreaks. Heat shocked cells of *L. monocytogenes* can remain heat resistant after being held for 24 h at 4°C [28]

Although pasteurization can kill the pathogens in the milk, contamination can still occur during packaging. Improperly performed pasteurization and the occurrence of contamination after pasteurization are the most likely explanations for the presence of *L. monocytogenes* in LPY [4]. Where HIV/AIDS is prevalent, Listeriosis may be a silent killer among the immunity compromised individuals who may depend on milk for protein intake.

The low counts observed in *Bongo* may be attributed to the low pH and other antimicrobial compounds such as bacteriocins produced by lactic acid bacteria. Also, the type of acid and the storage temperature have a marked effect on the ability of *Listeria* to survive and grow at low pH. On the other hand, the presence of *Listeria* spp. in *Bongo* may be attributed to contamination from raw milk, the starter culture inoculum and slow rate of acid formation and pH decline. A number of studies have demonstrated that *L. monocytogenes* is more acid tolerant than most food-borne pathogens, although the sensitivity of the organism to organic acids varies with the nature of the acidulant used [29] *Listeria* spp. prefers to grow at pH 7-8 but they will grow in the range pH 5-10 and may survive and grow in material with a pH as low as 4.4.

Bulked raw milk was slightly more acidic with mean pH values below the normal range of 6.6 to 6.8 and percent titratable acidity (0.22 to 0.89%) above the normal range of 0.14 to 0.16% [18]. The low pH and high percent titratable acidity values of bulked raw milk may be attributed to microbial activity (TPC > 6.00 $\log_{10}$ cfu mL^{-1}) [20]. In many parts of Uganda, raw milk is frequently delivered to cooling points under ambient temperature (25-30^{o}C), which favors microbial growth [13]. The mean percent titratable acidity and pH of LPY were close to the normal values of 0.9% and 4.2, respectively [30]. However, for *Bongo*, much lower pH values were observed at relatively low percent titratable acidity levels (Table 2)**.** This is probably due to a lower buffering capacity of the system. Foods undergoing a natural fermentation are reported to posess relatively lower buffering capacity [31]. Food systems with a low buffering capacity require relatively less acid to achieve the same pH values and, therefore, less protective against microbial proliferation.

CONCLUSION AND RECOMMENDATION

Raw milk, locally processed yoghurt and *Bongo* sold in Kampala district present a health risk given their high levels of *L. monocytogenes*. Risk assessment of *Listeria* spp. from farm-to-table under the Ugandan production and processing environment is, therefore, required. The study has demonstrated the occurrence and survival of *Listeria monocytogenes* in fermented and refrigerated, packaged milk products such as *Bongo* and LPY. *Listeria monocytogenes*

grows under low-oxygen conditions that prevail in packaged foods. The presence of *L. monocytogenes* in LPY and *Bongo* may suggest post contamination, survival of pasteurization and tolerance to acidic conditions. Besides, there is need to identify other *Listeria* spp. in the various dairy products in Uganda. Further studies should also focus on identifying the specific sources of *Listeria* spp. in both small- and large-scale milk processing plants. This may be an important step in risk assessment and controlling the incidence of these organisms in raw milk and fermented dairy products in Uganda. The high levels of Listeria in raw milk and its products pose a public health risk to humans. Pasteurization should be encouraged to minimize the risk especially when processing traditionally fermented dairy products.

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Table 1: Occurrence of *Listeria* spp. and *L. monocytogenes* in bulked raw and fermented milks sold in Kampala district

Milk product	TPC (log_{10} cfu mL^{-1})	Samples tested for *Listeria* spp.* (%)	*Listeria* spp counts (log_{10}cfu mL^{-1})	*L. monocytogenes** (%)
Raw milk	8.40 ± 0.11^{b}	60	3.10 ± 0.06^{a}	13
LPY	7.40 ± 0.13^{c}	30	0.82 ± 0.18^{b}	03
Bongo *(TF)*	9.00 ± 0.13^{a}	15	0.32 ± 0.18^{c}	00

* Percentage of samples that tested positive for the organism. LPY: Locally processed yoghurt; TF: Traditionally fermented; TPC: Total plate count. TPC and Listeria spp count values are means ± standard deviations of duplicate determination. Values of TPC and listeria counts were found significantly different ($p<0.05$) for values in columns with different superscripts.

Table 2: Physico-chemical properties of bulked raw and fermented milk sold by informal milk cooling points in Kampala district

Milk type	pH	Titratable acidity (%)	Temperature (^{o}C)
Raw milk	6.10±0.04^{a}	0.22±0.01^{a}	6.90±0.01^{a}
LPY	4.20±0.04^{b}	0.89±0.01^{b}	5.40±0.40^{b}
Bongo	4.40±0.04^{c}	0.69±0.01^{c}	8.60±0.40^{c}

LPY: Locally processed yoghurt. Values are means ± standard deviation of duplicate determination. Values in columns with different superscripts are significantly different ($p<0.05$).

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