



Research Article

Phenotypic characterisation of *Listeria monocytogenes* in cow milk from three catchment areas of Goliati Area in Thyolo District, Malawi

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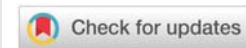
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Abstract

Background: *Listeria monocytogenes* is one of the food borne pathogens that cause an illness known as Listeriosis upon ingestion of contaminated food. Outbreaks of Listeriosis have been reported in Canada, USA, Europe, South Africa, Ghana and other countries but there is limited data on outbreaks due to *Listeria monocytogenes* and its isolation from contaminated foods in Malawi. This prompted our interest to determine the presence of *Listeria monocytogenes* in cow milk from Goliati area, Thyolo district in Malawi.

Objectives: To determine the presence and concentration of *Listeria monocytogenes* in cow milk from daily farmers around Goliati area in Thyolo district in Malawi.

Methodology: Nine raw milk samples from three bulk centers and two processed milk samples were cultured on both Nutrient Agar and Brain Heart Infusion agar as primary cultures. A pure culture of *Listeria monocytogenes* was obtained by sub culturing the primary culture on Brain Heart Infusion agar media. Colonies from the pure culture were further identified using Gram staining, Catalase test, Motility test and Beta hemolysis test on blood agar to phenotypically identify *Listeria monocytogenes*.

Results: There was a 100% growth rate of colonies suggestive of *Listeria monocytogenes* on all the nine samples. These colonies were observed to contain Gram positive, purple, short, rod shaped bacteria which was motile and catalase positive and it caused complete beta hemolysis on blood agar.

Conclusion: In this study, isolates of *Listeria monocytogenes* were phenotypically identified in cow milk that is produce from Goliath Area in Thyolo district. This is a major public health concern for milk farmers and consumers in the area and beyond. The findings from this work calls for improved hygienic practices in the handling of milk and milk products to reduce contamination.

Introduction

Milk is the fluid that is secreted by mammals for the nourishment of their offspring's. Since humans began to domesticate lactating animals, milk and milk products have been part of the human diet. It is considered one of the most complete sources of nutrients for human beings because of its diverse components such as proteins, vitamins and minerals that are important in human nutrition [1]. However due to its high nutritive value, neutral pH and high water activity, raw milk serve as an excellent growth medium for different microorganisms whose multiplication depends mainly on temperature, other competing microorganisms and their metabolic products [2]. Raw milk also creates good conditions for a variety of spoilage and thus growth of potentially pathogenic microorganisms.

Listeria monocytogenes is one of the most important pathogens of public health concerns that can contaminate milk. It is a Gram positive, facultative, intracellular, non-spore forming, motile, rod shaped bacterium that causes Listeriosis in humans, which is manifested by gastroenteritis, meningitis and meningo-encephalitis septicemia especially in people with compromised immunity, including the elderly, pregnant women and newborns [3-6]. The bacterium is widespread in nature and can survive and grow under low temperatures and pH, high salt and bile concentration, oxidative stress, carbon starvation and other adverse conditions making it a potential hazard in foods [7]. Currently 13 serotypes of *Listeria monocytogenes* have been identified with serotypes 1/2a, 1/2b, 1/2c and 4b are responsible for human Listeriosis [8].

Listeria monocytogenes has been isolated from different raw and ready to eat foods and in raw milk and dairy products in different countries [9]. Several cases of Listeriosis in humans have been reported, sometimes with high case fatality rates of up to 30% [10-12]. In Malawi, it is estimated that 23% of respondents of a survey titled 'consumers attitudes and willingness to pay for safer milk in Malawi consumed raw milk, however, there is limited data that can be used for the qualitative and quantitative assessment of the risk of *Listeria monocytogenes* infections related to the consumption of raw milk and milk products in Malawi. The objective of this study was to determine the presence of *Listeria monocytogenes* in raw milk that is produced from Goliati in Thyolo district in Malawi by isolating *Listeria monocytogenes* according to phenotypic characterization.

Materials and methods

Study location and design

Goliati is located in the southern part of Malawi with the population of approximately 28,000 people [13]; it is one of the milk producing areas. This milk is used for commercial and domestic purposes and it is distributed across Malawi by the Lilongwe dairy limited. This is a cross-sectional study where the milk samples were collected between 19th to 23rd November, 2020.

Sampling method and sample size

The study employed convenient sampling in the selection of the three milk bulking centres for sample collection for the following reasons; A sampling frame of all the milk bulking centres in Thyolo District was not readily available from the district council. The other reason was that these milk bulking centres were convenient in that they are close to the study laboratories, minimizing transportation cost and a time-reduction for the samples get to the laboratory.

The following bulking centres were recruited in this study; Goliati dairy bulking centre which had 450 dairy farmers within its catchment area, Namahoya dairy bulking centre which had 500 dairy farmers within its catchment area and Super dairy bulking centre which had 250 dairy farmers within its catchment area.

Each bulking centre represented a cluster of milk from dairy farmers who sold their milk at these centres. It was estimated that on average, it takes about three days for all the farmers within the catchment area of each bulking centre to supply their milk. Thus, we postulated that in an ideal situation, milk samples collected on three consecutive days from each bulking centre will represent all the milk that had been collected from the catchment area of that bulking centre. This explains why three samples were collected per each milk bulking centre. In total we had nine raw milk samples, three samples per each bulking Centre and two pasteurized milk samples processed by Lilongwe dairy company as our controls.

Sample collection

At each bulking centre, 500mLs of raw milk samples were collected per day for three consecutive days. This was done in order to capture all raw milk supplied by the farmers for each bulking centre. The samples were stored at a 4°C for transportation to the laboratory [14]. Table 1 shows a summary of the codes used to collect the samples from the three bulking areas.

Table 1: This table is showing the codes that were used in the study to represent bulking centres and the codes that were used to represent the three samples that were collected per each bulking centre.

Bulking Centre Code (code in brackets)	Samples collected and their codes
Goliati Dairy Bulking Centre (01)	01A
	01B
	01C
Namahoya Dairy Bulking Centre (02)	02A
	02B
	02C
Super Daily Bulking Centre (03)	03A
	03B
	03C
Processed milk (control samples)	C1
	C2

C=Control sample; samples collected from (01) have a prefix 01, samples collected from (02) have a prefix 02 and samples collected from (03) have a prefix 03

Sample preparation

Sample serial dilutions: 1ml of each of all the nine (9) raw milk samples was serially diluted until a dilution factor of 10^6 was reached. This means that for each sample, we had the following concentrations of the sample: 10^0 , 10^1 , 10^2 , 10^3 , 10^4 , 10^5 and 10^6 but due to the shortage of culturing plates, we randomly selected the following dilutions for primary culturing: 10^0 , 10^1 , 10^3 and 10^6 [15].

Preparation of the primary culture

0.05µl sample from each test tube of 10^0 , 10^1 , 10^3 and 10^6 dilutions were transferred to the labelled Nutrient agar (Techno PharmChem, India) and Brain heart infusion agar (Oxoid L.T.D Basingstroke, Hampshire, England) petri dishes. Sterile stick cotton swabs were used to plate the sample using quantitative streaking method. Another two plates labelled Nutrient agar and Brain heart infusion agar were used to culture the control sample which was Long life processed milk produced by the Lilongwe dairy industry, Malawi. After culturing all petri dishes, they were incubated at 36°C for 48 hours for the growth of microorganisms [16].

Preparation of the secondary (Sub) culture

Isolated colonies from the selected primary culture plates were sub cultured on Brain heart infusion agar using sterile loops. Isolation streaking technique was employed for sub culturing. Brain heart infusion agar (Oxoid L.T.D Basingstroke, Hampshire, England) was used for sub culturing to due lack of selective medium, PALCAM agar. The sub cultured petri dishes were incubated at 36°C for 48 hours for the growth of a pure culture [17].

Gram staining

Colonies were isolated from pure culture using sterile plastic inoculating loops and they were placed on clean, labelled microscope slides with a drop of normal saline (Aculife Healthcare Pvt. L.T.D – India). Colonies were mixed with normal saline to make thick smear. The prepared smear was allowed to dry in air and then the dry smear was fixed by placing the slides on a slide warmer with the smear side facing up.

Heat fixed smear slides were placed on staining rack over the sink and the smear was covered with the primary stain Crystal violet (Cypress Diagnostics–Belgium) which was allowed to remain on the slides for a minute. After a minute primary stain was washed away by rinsing the slides with running tap water and slides were shaken off to remove excess water. Then the smear was flooded with a Mordant gram's iodine solution (Cypress Diagnostics–Belgium) and was left for a minute. The slides were rinsed with running tap water to remove iodine solution on the slides. The decolorizing agent Acetone alcohol (Merck Chemicals (pvt) L.T.D – USA) was applied to the smear and waited for approximately 20 seconds. The slides were washed with tap water to remove acetone alcohol. Then the slides were flooded with counterstain, Safranin (Cypress Diagnostics–Belgium) and it stayed there for 20 seconds. Lastly

the slides were washed and then heat dried. After this they were observed under microscope and recorded. During microscopy, a 100x objective lens was used [18].

Catalase test

The test was executed by transferring the suspected colonies from our sub culture using sterile wire loop to labelled microscopic slides. Then one drop of 3% hydrogen peroxide (Glassworld and Chemical Suppliers, Cape Town – South Africa) was placed on the slides and observations were made and documented. This test procedure was carried out on all the samples and normal saline was used as control [19].

Haemolysis test

The test was done by isolating suspected colonies from the subculture using sterile wire loop and these were cultured on Blood agar medium (Oxoid Ltd, Basingstroke, Hampshire–England) plates. Then the plates were incubated at 36 °C for 48 hours and then observations were made and documented [20].

Motility test

The test was carried out by isolating suspected colonies using sterile wire loop and stabbing into prepared Motility Indole Ornithine (Becton, Dickinson and Company – India) test tubes. Then the tubes were incubated at 37°C for 48 hours after which observations were made and recorded [21].

Ethics review of the protocol

The study was ethically reviewed and approved by The Malawi University of Science and Technology Research and Ethics Committee (MUSTREC). It was certified under the following reference number: U10/2020/008.

Statistical analysis

The data were analysed in R. Descriptive measures of *Listeria monocytogenes* counts were made and compared across the centers and concentration levels. A test of normality of the counts was also made to check normality distribution of the data for further analysis of variance.

Results

During primary culturing, all the 3 samples per each milk bulking center were cultured at 4 different concentration levels hence there were 72 replicates with 24 replicates from each center. The mean *Listeria monocytogenes* colony count was 178 (35,600 CFU/ml of raw milk) with standard deviation of 74 counts (14,800 CFU/ml of raw milk). The mean *Listeria monocytogenes* colony count was slightly higher at center 1 than centers 2 and 3. Center 2 and 3 that had almost equal mean colony counts of *Listeria monocytogenes* in the milk samples as shown in Figure 1.

The results also indicate higher mean *Listeria monocytogenes* counts in nutrient agar of 182 counts (36,400 CFU/ml of raw milk) than in Brain heart infusion agar which had a mean *Listeria monocytogenes* colony count of 174 counts (34,800 CFU/ml of raw milk). The distribution of the counts by concentration level and the media type are shown in Figure 2.



The results indicate that an increase in the concentration in which *Listeria monocytogenes* were cultured was associated with increased growth of colonies of *Listeria monocytogenes*.

The results in Table 2, indicate that at concentration levels 10^0 and 10^{-1} (higher concentrations), the mean *Listeria monocytogenes* counts were slightly higher in nutrient agar medium than in Brain heart infusion agar. This is because Brain Heart Infusion agar is highly selective compared to nutrient agar [16,17]. The trend was different at lower concentration levels 10^{-3} and 10^{-6} where the mean counts were slightly lower in nutrient agar medium than in Brain heart infusion agar, thus this might suggest possible contamination or a technical flaw in the serial dilution method.

Further analysis was done to check if mean *Listeria monocytogenes* colony counts were significantly different at independent centers from where the milk samples were drawn. In Figure 3, are boxplots for counts at the three centers from where milk samples were obtained. The figure shows slightly higher mean *Listeria monocytogenes* colony counts for centre 1 than median *Listeria monocytogenes* colony counts for center2 and centre3 which are almost the same.

Normality assumptions and analysis of variance

Data were checked for normality distribution assumptions. The data were independently obtained from the three

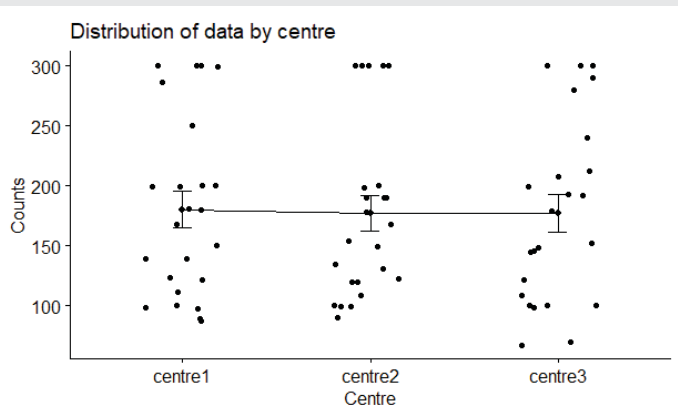


Figure 1: Mean counts of *Listeria monocytogenes* by Centre of the sample.

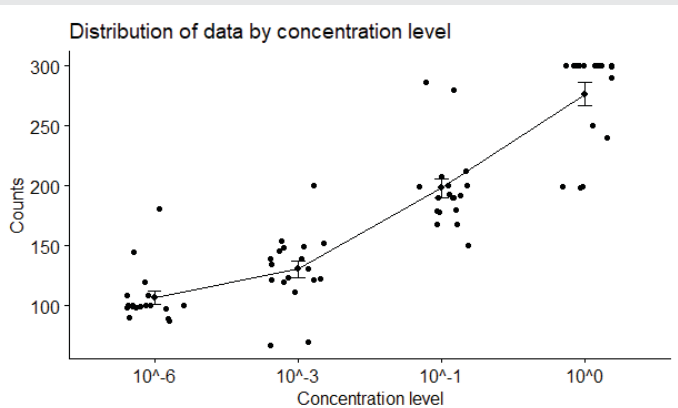


Figure 2: Mean *Listeria monocytogenes* counts by concentration level of medium.

Table 2: Mean *Listeria monocytogenes* colony counts by media and concentration level.

Concentration	Medium	
	Nutrient agar	Brain heart infusion agar
	Mean <i>Listeria monocytogenes</i> counts	
10^0	292.2 (58,440 CFU/ml)	260.9 (52,180 CFU/ml)
10^{-1}	199.4 (39,880 CFU/ml)	196.6 (39,320 CFU/ml)
10^{-3}	129 (25,800 CFU/ml)	131.9 (26,380 CFU/ml)
10^{-6}	105.7 (21,140 CFU/ml)	107.8 (21,560 CFU/ml)

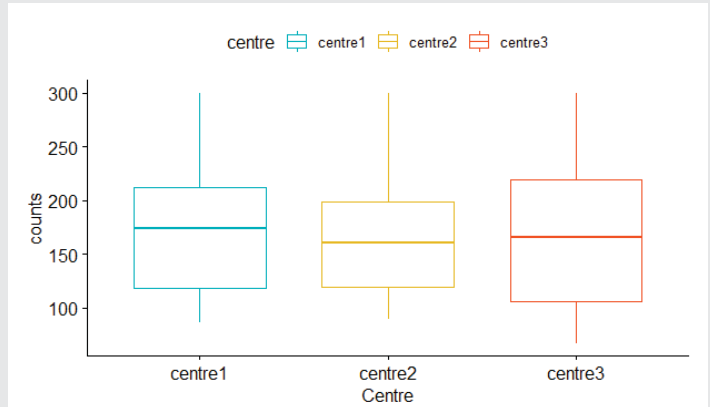


Figure 3: Boxplots for *Listeria monocytogenes* at the three centres.

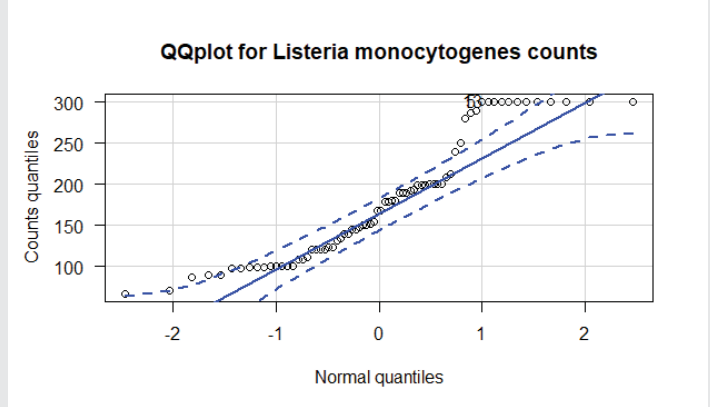


Figure 4: Testing normality distribution of *Listeria monocytogenes* counts.

centres. Here, the only assumptions checked were Normality distribution assumptions of data and homogeneity of variance assumptions using Leven’s test.

In Figure 4, the normality assumption was validated through a QQ-plot as most data points are within the reference line. The Leven’s test validates the equality of variance assumptions ($p\text{-value}=0.8734$).

The analysis of variance results indicate that the mean *Listeria monocytogenes* counts were not significantly different at the three centres ($p\text{-value}=0.987$). However, at different concentration levels, the mean *Listeria monocytogenes* colony counts are significantly different ($p\text{-value}<0.0001$). Turkeys multiple pairwise comparison showed that the only pair whose mean counts were not significantly different were concentration levels 10^{-6} and 10^{-3} with adjusted ($p\text{-value}=0.1331$). The mean *Listeria monocytogenes* colony counts were significantly



different at the rest of the pairs of concentration levels (p -value<0.0001).

Comparison of primary culture results with sub-culture results

Samples that were randomly selected for sub-culturing in Brain heart infusion agar indicate a mean *Listeria monocytogenes* count of 101.2 (20,440 CFU/ml) at concentration level of $10^{\wedge}-6$ and 99.8 counts (19,960 CFU/ml) at concentration level $10^{\wedge}-3$. When these results were compared to primary culturing counts obtained from nutrient agar and brain heart infusion agar media at concentration levels $10^{\wedge}-3$ and $10^{\wedge}-6$, it shows that the mean *Listeria monocytogenes* colony counts in sub-culturing are lower than mean counts in primary culturing. This is due to the fact that primary cultures are prone to microbial contamination compared to secondary cultures hence the noted higher mean counts in primary cultures [22]. Different from these results, standard milk samples that were obtained from market places showed lower mean *Listeria monocytogenes* colony counts of 55 and 64 in nutrient agar and Brain heart infusion agar respectively. This is inevitable as milk processing which include pasteurization has been shown to result in log10 or more reduction of *Listeria monocytogenes* [23] Table 3.

Results of Gram staining test, Haemolysis test, Motility test and Catalase test for isolates from secondary (sub) culture per each sample

Thus, Tables 4-7 indicates that the isolates that were obtained and microbiologically analysed were gram positive, catalase-positive, orthinine negative, indo negative but motile. These isolates also exhibited beta haemolysis on blood agar suggestive of *Listeria monocytogenes*.

There was also growth of colonies on control samples whose isolates were exhibiting phenotypic features that were similar to the isolates from the raw milk.

Table 3: Mean *Listeria monocytogenes* colony counts at concentration levels $10^{\wedge}-3$ and $10^{\wedge}-6$ in primary and sub-culture.

Culture	Sample concentration levels	
	$10^{\wedge}-3$	$10^{\wedge}-6$
Primary	131.9 (26,380 CFU/ml)	107.8 (21,560 CFU/ml)
Sub-culture	101.2 (20,440 CFU/ml)	99.8 (19,960 CFU/ml)

Table 4: Gram staining results for isolates from secondary (sub) culture per each sample.

samples	Morphology
01A	Purple, single and rod-shaped cells
01B	Purple, single and rod-shaped cells
01C	Blue, single and rod-shaped cells
02A	Purple, single and rod-shaped cells
02B	Purple, single and rod-shaped cells
02C	Purple, single and rod-shaped cells
03A	Purple, single and rod-shaped cells
03B	Blue, single and rod-shaped cells
03C	Purple, single and rod-shaped cells
C1	Purple, single and rod-shaped cells
C2	Purple, single and rod-shaped cells

Table 5: Showing intrinsic character of target colonies isolated from secondary (sub) culture of each sample on blood agar.

Samples	Results
01A	Beta-hemolysis seen
01B	Beta-hemolysis seen
01C	Beta-hemolysis seen
02A	Beta-hemolysis seen
02B	Beta-hemolysis seen
02C	Beta-hemolysis seen
03A	Beta-hemolysis seen
03B	Beta-hemolysis seen
03C	Beta-hemolysis seen
C1	Beta-hemolysis seen
C2	Beta-hemolysis seen

Table 6: Showing results of the motility test on target colonies isolated from secondary (sub) culture of each sample.

Samples	Results
01A	Orthinine negative, Indo negative and Motile
01B	Orthinine negative, Indo negative and Motile
01C	Orthinine negative, Indo negative and Motile
02A	Orthinine negative, Indo negative and Motile
02B	Orthinine negative, Indo negative and Motile
02C	Orthinine negative, Indo negative and Motile
03A	Orthinine negative, Indo negative and Motile
03B	Orthinine negative, Indo negative and Motile
03C	Orthinine negative, Indo negative and Motile
C1	Orthinine negative, Indo negative and Motile
C2	Orthinine negative, Indo negative and Motile

Table 7: Showing results of Catalase Test on target colonies isolated from secondary (sub) culture of each sample.

Sample	Results
1A	Oxygen bubbles seen
1B	Oxygen bubbles seen
1C	Oxygen bubbles seen
2A	Oxygen bubbles seen
2B	Oxygen bubbles seen
2C	Oxygen bubbles seen
3A	Oxygen bubbles seen
3B	Oxygen bubbles seen
3C	Oxygen bubbles seen
C1	Oxygen bubbles seen
C2	Oxygen bubbles seen

Discussion

The colony forming units found in this study which were enumerated from all the raw milk samples and the control samples used in this study were above 10,000 CFU/ml and according to the European legislation containing microbiological food safety criteria for *Listeria monocytogenes* which stipulates that the presence of more than 100 CFU/ml of

Listeria monocytogenes is injurious to health, hence this means that this finding requires special attention from policy makers and milk producers to ensure the production of safe and quality milk [24].

There was no statistical significant difference in the mean colony forming units per milliliter (CFU/ml) across the three milk bulking centers suggesting that the level of contamination was almost equal across the centers and probably they employ similar milk handling procedures when collecting milk from farmers.

It has been shown that there is a 100% growth rate of colony forming units suggestive of *Listeria monocytogenes* in all the three milk bulking centers. This is not a true reflection of the actual individual level prevalence as bulk tank raw milk represented a cluster of milk from individual farmers within the catchment area of each bulk tank. Thus, the contamination of raw milk with *Listeria monocytogenes* is attributed to the entire catchment area supplying the bulk centre. This explains why this finding is different from the prevalence's that have been reported in several studies; the prevalence was reported at 41.6% in Syria [25], the prevalence was reported at 6.5% in the USA [26], the prevalence was reported at an average of 6.8% in Iran [27-29], the prevalence was reported at 5.9% in Morocco [30] and the prevalence was reported at 1.4% in Latvia [31]. It is essential to note that these prevalence's were based on samples of individual farmers contrary to the design that was used in this study.

The phenotypic findings that have been reported in this study are similar to those that were reported from samples of Kerman, Iran [2]. In order to ascertain specific species or strains of *Listeria monocytogenes*, they employed genotypic techniques which were not used in this study due to lack of reagents. This explains why we only managed to phenotypically isolate *Listeria monocytogenes*.

The isolation of *Listeria monocytogenes* in this study shows that there is contamination of raw milk with *Listeria monocytogenes*. The following risk factors have implicated in necessitating raw milk contamination with *Listeria monocytogenes*; defective disinfection of teats before milking, lack of correct management of barn and silage, insufficient hygiene practice in the environment and a low level of cleanliness among cows [32]. Milk handling processes during storage and transportation have also been shown to contribute to milk contamination with various microorganisms [33]. Despite showing the presence of *Listeria monocytogenes*, this study did not focus on the local factors that leads to milk contamination with this microorganism.

In conclusion, it is essential to appreciate that there is *Listeria monocytogenes* contamination of raw milk in all the three catchments areas that supplied milk to the three milk bulking centres. The isolates are a cluster or group level finding suggesting that individual level studies are needed to ascertain the exact prevalence of *Listeria monocytogenes* contamination. There is also a need to do studies that will focus on genotypic and virulent profiling of the strains of *Listeria monocytogenes*

that are in circulation in these catchment areas and also ascertain the factors that facilitate local contamination of milk with *Listeria monocytogenes*. This will have an impact on public health planning and interventions.

Authors contributions

CM and AM were involved in the study conceptualisation, sample collection, laboratory analysis and interpretation of results.

YK and MK were involved in providing technical guidance in the laboratory.

DM was involved in statistical analysis and interpretation

GB, MM, PAMN, AGM, WM and PC were involved in the reviewing of the entire study from the stage of the proposal to this final article.

TEM was the overall supervisor of the study.

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