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Tshegofatso Nhabe, Ntsoaki Joyce Malebo

Corresponding author: Tshegofatso Nhabe, Faculty of Health Sciences, University of Johannesburg, Gauteng, South Africa. tnhabe@uj.ac.za

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The use of matrix-assisted laser desorption ionization-time of flight-mass spectrometry (MALDI TOF-MS) to characterize bulk-tank milk isolated bacteria in Mangaung, South Africa

Tshegofatso Nhabe[1,&], Ntsoaki Joyce Malebo[2]

[1]Faculty of Health Sciences, University of Johannesburg, Gauteng, South Africa, [2]Central University of Technology, Centre for Innovation in Learning and Teaching and Learning Division, Bloemfontein, South Africa

[&]Corresponding author

Tshegofatso Nhabe, Faculty of Health Sciences, University of Johannesburg, Gauteng, South Africa

Abstract

Introduction: *spoiled milk can cause gastrointestinal illnesses. Milk spoilage is often due to the growth of psychrophilic and psychrotolerant bacteria commonly found in farm environments.* ***Methods:*** *bacteria were isolated*

from bulk tank milk. On nutrient and selective medium (chromocult and blood), serial dilutions of the milk were produced and plated. Cell morphology was used to identify isolates, and matrix-assisted laser desorption ionization-time of flight mass spectrometry was used to characterize them. ***Results:*** *utilizing matrix-assisted laser desorption ionization-time of flight mass spectrometry, 93.5% of bacteria were identified down to the species level. Six and a half percent, however, were just genus-identified.* ***Conclusion:*** *according to the findings, milk contamination originated from a common source. The results further suggest that additional sources of contamination might include milk tanks. The results demonstrate that the milk tanks' hygiene standards do not meet the criteria of South Africa's Milk and Milk Products Regulations (R1555 of 1997).*

Introduction

The nutritional richness of vitamins and minerals in milk renders it a valuable source of nutrients and proteins [1]. Businesses (shops) globally market bulk tank milk (BTM) because of its recognized nutritional value, and South Africa is among the countries where this practice is observed [2]. In terms of producing safe dairy products, the dairy business has shown to be one of the food industries that has the most success [3]. While bulk tanks are recognized for their capacity to preserve milk and control temperature, they can also result in unhygienic conditions and compromise the quality of milk [3]. According to Ouamba *et al.* [4] and Anwer *et al.* [5], there are several points in the milk production process where contamination of milk and other dairy products can occur, introducing foodborne pathogens [6,7]. This potential risk involves unhygienic practices by farm workers during equipment handling, pre-milking, and post-milking, which may occasionally lead to milk contamination [6,7]. Despite occasional outbreaks, South Africa has seen infrequent reports of isolated cases of foodborne bacteria in bulk containers of milk, resulting in sporadic episodes of food poisoning [8,9].

This indicates a significant risk of consuming milk containing harmful bacteria, increasing the likelihood of developing gastrointestinal illnesses. Research on smallholder farmers in the informal sector has focused on assessing the quality of milk in containers, the microbiological and chemical composition of milk, and relevant dairy processes [10-12]. Additionally, the studies have explored milk and food standards in the urban developing regions of South Africa. The general agreement is that milk contamination commonly stems from factors such as the environment, equipment, and inadequate health and hygienic practices of the cows [13,14]. Nyokabi *et al.* [14] indicates that contamination can have an impact on milk quality at many points in the milk supply chain; and to reduce the contamination caused by those factors, it is crucial to identify the critical control points (CCPs) and create standard operating procedures (SOP). Key tools in the dairy industry and on farms include Good Manufacturing Practices (GMP) and the Hazard Analysis and Critical Control Point (HACCP) system [15]. These concepts help minimize the risk of contamination and opportunistic infections. Furthermore, they improve the manufacturing process, enhance product quality, and ensure the safety of food intended for human consumption [15].

Methods

Study selection: the study adopted a quantitative research design to address the research objectives and problem statement.

Participants: shops selling bulk tank milk in the Mangaung region during the period from 2015 to 2016.

Study size: ten (10) businesses (shops) selling bulk-tank milk from the Mangaung Metropolitan Municipality.

Variables: bulk tank milk selling shops that had a valid certification of acceptability to operate under conditions set under the National Health Act 61 of 2003 and comply to the regulations relating to milk and dairy products R1555 of 1997 as amended by R489 of 2001. The study period was based on the availability of operational shops in the Mangaung area.

Sample collection: during the transition from winter (August) to summer (November), eight samples (two from each store) were purchased from businesses (shops) in the Mangaung region. This included two bottles from each of the four businesses operating at that time, selected from a total list of 10. To preserve the ''cold chain temperature'' while transporting the milk from the stores to the laboratory, it was quickly transferred into sterile, dry, clean, leak-proof 500ml laboratory glass bottles, with each bottle labeled according to its corresponding shop, as also described in the study by Deka *et al.* [16]. The milk samples were examined within 24 hours of collection to inhibit the growth and multiplication of bacteria present in the milk, mirroring the approach recommended by Deka *et al.* [16].

Bias: to prevent potential bias, the culture media, equipment, and solvents underwent multiple tests.

Statistical methods

Isolation and identification of bacteria

Data analysis: to assess bacterial numbers, the standard plate count method was utilized. The milk was diluted in triplicate following Koch's serial dilution method, where the cell counts, or density decreases as the serial number increases at each stage [17]. Following the method, 25g of the milk sample was placed into a sterile test tube along with 225ml of distilled water. Subsequently, 0.1% Peptone was added to create a 10^{-1} dilution of the solution. This cultivation process adhered to the standard protocol outlined by Koch (1883) [17].

Each sample underwent serial dilutions in 9ml of sterile distilled water and was then applied in triplicate to nutrient and selective media (chromocult and blood agar-selecta-media from Thermo-Fisher Scientific) as necessary for microbial analysis. Nutrient agar was chosen due to its ability to support the growth of various non-fibrous organisms, allowing the quantification of living organisms across the entire medium. Specific media types, such as blood and chromocult, were selected for their ability to encourage the growth of particular types of organisms [17]. All samples were incubated at 37°C for 24 hours.

Confirmation of microbial isolates

Matric-assisted laser desorption ionization time-of-flight (MALDI TOF MS): for colony identification, after extraction was carried in line with the directions provided by the manufacturer, direct placement, or placement on a steel target.

Direct colony method: with a sterile pipette tip, one colony was extracted from each plate and applied as a thin film directly to a MALDI steel target. Next, 1ml of the matrix solution (acrylonitrile purified water, 20mg/ml of 3.5-dimethoxy-4-hydroxycinnamic acid) was added. Then a smeared colony was applied to the steel target using trifluoroacetic acid (TFA) (50: 50: 0.1); and then inserted in the MALDI-TOF MS for analysis after being air-dried for ten minutes [18].

Extended direct colony method: as with the direct colony approach, every strain was put on the designated plate, and then dried. The sample on the plate was then pipetted with 1.5µL of the matrix solution and added to the stain after 0.5µL of 70% formic acid, 0.5µL of acetonitrile, and the resultant mixture were dried for approximately 10 minutes at ambient temperature [18].

For the standard extraction method: bulk tank milk bacterial isolates were isolated and cultured overnight on nutrient, chromocult, and blood agar; following that, 1.2ml of 95% ethanol (Sigma-

Aldrich) was added to an inoculating loop that had been filled with cells, and the mixture was suspended using a vortex. The protein sample was then processed into a small disc after a two-minute centrifugation of the sample. The pellet was vigorously shaken with a vortex before being added to 50µL of 70% formic acid (Sigma-Aldrich) and 50µL of 100% acetonitrile to extract the proteins [18].

After allowing to dry, the proteinaceous supernatant was spotted onto the MALDI plate and coated with 1µL of saturated matrix solution from Bruker Daltonics that included 10mg/ml of hydroxycinnamic acid (HCCA) in acetonitrile-water-trifluoroacetic acid (TFA) (50: 47.5: 2.5 vol/vol/vol) (Sigma-Aldrich). The following measurement parameters were used with a microflex LT MALDI TOF mass spectrometer from Bruker Daltonics and the standard biotyping measurement instructions from the manufacturer: detection range: 2 to 20kDa, positive linear mode, 60Hz laser frequency. The finished spectrum had 240 images in each spot (40 images per grid point). The laser output was set to a level that would produce wavelengths with absolute peak intensities at their maximum ranging between 5 x $10^3$to 10^4units of measure. The accompanying MALDI BioTyper OC software (Version 3.1) was also used for analyzing the spectra (Bruker Daltonics) [19].

Results

Participants: businesses (shops) selling bulk-tank milk in the Mangaung region. The selection of these shops was since environmental health practitioners inspect the premises only twice a year, as mandated by the South African legislation. However, this infrequent inspection schedule may lead some shop owners to become complacent, neglecting proper precautionary measures in cleaning, disinfection, and sterilization. This negligence creates an environment that compromises milk quality, promotes microbial growth, and increases the risk of foodborne illnesses upon consumption.

Sample methods: the choice of sampling methods was based on the National Health Act 61 of 2003 and regulations relating to milk and dairy products (R1555 of 1997 as amended by R489 of 2001).

Variables: analysis of bacteria and assessment of the antibiotic tolerance in isolated milk samples.

Descriptive data: Table 1 indicates a failure to comply with regulation R1555 of 1997 (R489) as most of the 10^{-1}, 10^{-2}, 10^{-3} serial dilution plate results exceeded a countable number (over 30,000 colonies). However, Table 2 demonstrates compliance with regulation R1555 of 1997 (R489 of 2001) as the 10^{-1}, 10^{-2}, 10^{-3} serial dilution plate results were less than 100. Lastly, Table 3 results illustrate the effectiveness of MALDI TOF MS in identifying the genus and species names of bacteria. However, some outcomes were inconclusive, likely due to the system's bacterial data not matching the relevant microorganism. Consequently, the MALDI TOF MS system requires updating, considering the discovery of new pathogens each year.

Outcome data: nearly all the bacteria found in the milk samples examined in the study were psychrotrophic. Known sources of psychrotrophic bacteria include livestock bedding, grass, milking machines, udder contamination, and milk tank lines around dairies [20-22].

Main results: the research suggests that the contamination of the milk might have occurred at the farm rather than at the point of sale. In farm environments, psychrotrophic bacteria can form biofilms on different milk storage and processing equipment, serving as persistent sources of microbial contamination due to their potential for bio-transfer. Furthermore, there is a link between unclean bulk tanks and the recontamination of milk in bulk tanks at businesses (shops), as the identified bacteria commonly thrive in refrigerated conditions, leading to product spoilage [21,22].

The study identified isolated bacteria using nutrient agar, selective media (chromocult and blood agar), and MALDI TOF-MS. It is important to mention that on blood and chromocult media, colonies appeared in shades of pink, navy blue, or dark purple. Research indicates that members of the enterobacteriaceae family, *Serratia* species (sp.), and *Pseudomonas* sp., among others, produce acid resulting in colonies appearing dark purple or navy blue, as described in the literature [22,23]. Moreover, biochemical tests and laboratory materials used for BIO203 reveal that certain lactose-fermenting bacteria form flat, black colonies, distinguishing them from non-fermenters like *Pseudomonas* pathogens [24]. All 25 isolates were identified at both the genus and species levels using MALDI TOF MS (Table 3).

Discussion

The aim of the study was to isolate, quantify, and identify bacteria from bulk tank milk collected from shops. The findings of the study were evaluated based on the criteria outlined in R1555 of 1997, as amended by R489 of 2001 [25]. According to these regulations, the standard plate count should not surpass 50,000 colony-forming units per milliliter (cfu/ml) in a standard plate count test, and the plate count must fall within the range of 30-300cfu/ml for milk intended for direct consumption [25].

The findings additionally illustrated the presence of total viable microorganisms, with certain plates displaying colony-forming units on chromocult agar below 30 (purple), while others exhibited colony-forming units exceeding 30,000, with some forming a white biofilm on the agar. These observations were noted in the representative samples collected in November. According to Machado *et al.* [26] warmer temperatures are known to accelerate the reproduction of microorganisms. Without proper precautions, such as thorough cleaning of bulk tanks and equipment, changing animal bedding, and ensuring a cold storage temperature after milking, there is an increased risk of milk contamination [27]. Furthermore, contamination and spoilage can occur if fresh milk from the cows is not separated from leftover milk inside the tank. It is therefore important to discard leftover milk and add a fresh batch to a tank that is clean and disinfected [27].

The research study findings have shown that psychrotrophic bacteria dominated over mesophilic microorganisms, as they can endure cold temperatures [27]. Moreover, they are more likely to proliferate in milk when the cold chain temperature (4°C) is not maintained [27,28]. The increased bacterial numbers may potentially originate from poor water quality on the farm. Unclean water can adversely impact detergents, requiring larger quantities for effective cleaning due to binding. To maintain bacterial counts, the water on the dairy farm must adhere to regulations and meet drinkable quality standards [29].

While 5% of the milk samples met the standards, most milk in the study area was found to be contaminated. The identified bacteria suggest that this contamination may have been caused by udder infections and inadequate cleaning and storage practices, as documented in previous studies [26-29]. Moreover, there might have been a break in the cold chain during the transportation of milk from the farm to the businesses (shops). This indicates a disregard for the stipulations outlined in the regulations relating to milk and dairy products, R1555 of 1997 as amended by R489 of 2001, where milk with 200,000 colony-forming units (CFU) or more per milliliter in the standard plate count method could result in spoilage. While somatic cell counts were not the focus of the present study, related research suggests that mastitis impacts the overall plate count of milk, with infected cows potentially releasing significant quantities of pathogens into the milk supply [30]. The specific bacteria causing the infection, its stage, and the percentage of infected herds all influence the milk production volume [30]. Despite various studies confirming the presence of bacteria in farm tank milk, the

microbial composition of the milk remains unknown by the time it reaches customers [31,32].

MALDI TOF MS results: the differences in identification rates across various studies can be attributed to variations in growth factors, sample preparation, the number of reference strains, BioTyper software versions, and study designs [33,34]. The standard extraction process comprised approximately 13 steps, requiring 30 minutes for the analysis of 25 samples. According to Haider *et al.* [34] and Adam *et al.* [35] the direct colony approach offers simplicity and user-friendliness, as it only involves 4 steps for the complete identification of 25 samples.

In the study, the most identified colonies were gram-negative bacteria belonging to the genera *Enterobacter*, *Hafnia*, *Lelliottia*, *Serratia*, and *Pseudomonas* species. Notably, *Pseudomonas* showed a higher identification rate compared to other species. Additionally, there were inconclusive results, as indicated in Table 3. In MALDI protein profiling-based biomarker research, the objective is to identify intensity level variations between case and control samples, and the reliability of peak intensities is crucial [36,37].

The identified gram-negative bacteria belonging to the enterobacteriaceae family, like *Enterobacter*, *Hafnia*, and *Lelliottia*, are frequently present in environments like soil, plants, feed, and water [38]. As reported by Lee *et al.* [38], these bacteria are primarily identified in lactating machines, milk pipelines, coolers, dirty udders, and reproductive organs. Moreover, they are also detected in litter and stall floors, possibly acting as opportunistic mastitis pathogens.

The pseudomonadaceae family emerged as predominant in this study, representing a species with significant effects on milk quality. This type of bacteria can produce mucilage and induce protein coagulation, resulting in the generation of heat-stable lipases and proteases that contribute to milk imperfections, including bitterness and putrefaction [39-41]. The elevated bacterial counts in tank milk could be attributed to the prevalence of these environmental microorganisms, which may infiltrate the milking system through contaminated water, manure, and inadequately cleaned milking equipment [42]. Sebela's study [39] also suggests that *Pseudomonas* infection outbreaks can sometimes be linked to unhygienic housing and bedding conditions.

Additional insights from Sebela´s study [39] also propose that milk contamination may stem from the farm and bulk tanks that are not properly cleaned and disinfected in accordance with the manufacturer's guidelines, as evidenced by the identified bacteria in this study. All the bacteria mentioned in the study have the capacity to contaminate dairy products, posing risks to consumer health and leading to foodborne illnesses [42,43]. Additionally, these bacteria play a significant role in the spoilage of milk and dairy products, with some acting as opportunistic pathogenic bacteria that result in substantial financial losses in the agricultural and food sector.

Limitations: the observations indicate that certain shops are not following proper food safety handling practices. Both dairy farms and milk-selling shops show a lack of adherence to crucial food safety precautions. This emphasizes the necessity for more frequent inspections by environmental health practitioners, as stipulated in the National Health Act 61 of 2003 (Norms and Standards for Environmental Premises and Acceptable Monitoring Standards for Environmental Health Practitioners), which currently mandates inspections only twice a year [13]. This implies a deficiency in legislation enforcement, highlighting that food safety training and awareness campaigns are not given priority in these establishments.

Interpretation: considering the limitations in the data and scope of this research, it is essential for future studies to focus on improving methods for reporting on food safety and hygiene in shops selling bulk tank milk. Despite the biannual

inspections by environmental health practitioners, the study's results indicate the need for additional inspections to ensure compliance and prevent the consumption of unsafe milk by the community, which is often associated with gastrointestinal and foodborne illnesses.

Generalisability: the presence of environmental organisms identified in this study may be linked to the practice of farm workers milking unhygienic and unclean cows. Various microorganisms found in the udders of cows have the potential to cause mastitis, impacting the herd. The indication of these bacteria suggests the possible existence of bacterial biofilms in the pipes and milking equipment, emphasizing the need to clean the milking system. Funding for this study was provided by the Free State´ the Department of Education, South Africa, and the National Research Foundation at the Central University of Technology.

Conclusion

Consuming bulk-tank milk with elevated bacterial counts can lead to foodborne infections, and the severity of the consequences may vary depending on the amount of milk ingested. Individuals with weakened immune systems, such as pregnant women, the elderly, children, and those with conditions like cancer and Human Immunodeficiency Virus (HIV), face an increased risk. However, these risk factors can change depending on the situation and the individuals involved. Poor-quality milk can negatively impact consumer health and result in economic consequences, including increased hospital and healthcare expenses, reduced productivity, and a long-term decline in the quality of life. The findings of this study highlight that both dairy farms and businesses (shops) have the potential to produce hazardous substances, emphasizing the need for stronger regulatory control over the dairy industry by relevant authorities.

What is known about this topic

- *Among the causes of bulk tank milk contamination are dirty cow udders, dirty bulk tanks in shops, dirty machinery, and equipment in abattoirs/farms;*
- *Coliforms, enterobacteriaceae, and pseudomonadaceae bacteria are commonly used to demonstrate unsafe food handling, processing, or post-processing;*
- *Due to the improper use of chemical disinfectants, several coliform and psychotropic bacteria have antibiotic resistance.*

What this study adds

- *The research emphasizes the need for increased and regular food hygiene and safety inspections by environmental health practitioners in the Mangaung region, aiming to minimize or potentially eliminate the spread of diseases through food, additionally,*
- *it recognizes the importance of educating farm employees and business (shop) owners on maintaining a clean environment on the farm and in shops, along with adopting appropriate hygiene practices.*

Competing interests

The authors declare no competing interests.

Authors' contributions

Tshegofatso Nhabe was responsible for the design, sample collection, and analysis of the proposal; the research was conceived and supervised by Ntsoaki Joyce Malebo, who also conducted edits and reviewed the data collection and analysis. All the authors read and approved the final version of this manuscript.

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Tables

Table 1: standard plate count in samples taken in summer (2015)

Table 2: standard plate count in samples taken in winter (2015)

Table 3: matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI TOF-MS) direct colony and extraction method isolates

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Table 1: standard plate count in samples taken in summer (2015)

Sample 1	**10^1**	**10^2**	**10^3**	**Colony morphology**
Nutrient Agar	TMTC	TMTC	TMTC	A white biofilm formed
Chromocult	TMTC	TMTC	TMTC	Pink colonies formed
Blood Agar	130	110	99	Big clear colonies formed, together with pink flaky colonies
Sample 2				
Nutrient Agar	126	59	TFTC	Big white colonies formed
Chromocult	189	45	No growth	Yellowish big colonies formed
Blood Agar	152	33	No growth	Pink colonies formed
Sample 3				
Nutrient Agar	194	TFTC	No growth	White colonies formed
Chromocult	156	TFTC	TFCT	Yellow, white, and navy-blue colonies formed
Blood Agar	TMTC	TFTC	TFTC	Yellow biofilm formed at 10^1
Sample 4				
Nutrient Agar	TMTC	TMTC	TMTC	A white biofilm formed
Chromocult	TMTC	160	34	Blue, pink, and clear yellow colonies formed
Blood Agar	TMTC	TMTC	TMTC	Clear yellow, and flaky colonies formed

The results of the serial dilution plate count at 10^{-1}, 10^{-2}, and 10^{-3} showed predominantly high counts, exceeding 30,000 colonies, indicating non-compliance with Regulation R1555 of 1997 (R489 of 2001). TMTC: too many to count; TFTC: too few to count

Table 2: standard plate count in samples taken in winter (2015)				
Sample 1	**10^1**	**10^2**	**10^3**	**Colony morphology**
Blood Agar	40	19	10	White and flat, dry pink colonies formed
Chromocult	24	4	2	Yellow, white and flat, dry pink colonies formed
Nutrient Agar	61	47	33	White colonies formed
Sample 2				
Blood Agar	93	31	22	Yellow colonies formed
Chromocult	24	22	17	Navy blue and yellow colonies formed
Nutrient Agar	64	34	20	White colonies formed
Sample 3				
Blood Agar	24	20	16	Yellow colonies formed
Chromocult	38	10	5	Purple colonies
Nutrient Agar	27	10	7	White colonies formed
Sample 4				
Blood Agar	46	46	40	White colonies formed
Chromocult	8	5	3	Purple colonies formed
Nutrient Agar	27	10	9	White colonies formed
The outcome results of the serial dilution at 10^{-1}, 10^{-2}, and 10^{-3} indicated counts below 100, demonstrating compliance to Regulation R1555 of 1997 (R489 of 2001)				

Table 3: matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI TOF-MS) direct colony and extraction method isolates

Sample ID	Organism (colony direct method)	Organism (extraction method)
1	*Lelliotia amnigena*	*Lelliotia amnigena*
2	*Pseudomonas ludensis*	*Pseudomonas ludensis*
3	Inconclusive	Inconclusive
4	*Pseudomonas ludensis*	*Pseudomonas ludensis*
5	*Pseudomonas ludensis*	*Pseudomonas ludensis*
6	*Serratia liquefaciens*	*Serratia liquefaciens*
7	*Pseudomonas oleovorans*	*Pseudomonas*
8	*Pseudomonas oleovorans*	*Pseudomonas*
9	*Serratia liquefaciens*	*Serratia*
10	Inconclusive	Inconclusive
11	Inconclusive	*Pseudomonas corrugata*
12	*Pseudomonas ludensis*	*Pseudomonas ludensis*
13	*Pseudomonas ludensis*	*Pseudomonas ludensis*
14	Inconclusive	*Pseudomonas corrugata*
15	*Pseudomonas taetrolens*	*Pseudomonas taetrolens*
16	*Pseudomonas ludensis*	*Pseudomonas ludensis*
17	Inconclusive	*Enterobacter caancerogenus*
18	*Pseudomonas fragi*	*Pseudomonas fragi*
19	*Pseudomonas ludensis*	*Pseudomonas ludensis*
20	*Lelliotia amnigena*	*Lelliotia amnigena*
21	*Enterobacter cloacae*	*Enterobacter cloacae*
22	Inconclusive	Inconclusive
23	*Lelliotia amnigena*	*Lelliotia amnigena*
24	*Serratia amnigena*	*Serratia liquefaciens*
25	*Hafnia alvei*	*Hafnia alvei*
20	*Lelliotia amnigena*	*Lelliotia amnigena*
21	*Enterobacter cloacae*	*Enterobacter cloacae*
22	Inconclusive	Inconclusive
23	*Lelliotia amnigena*	*Lelliotia amnigena*
24	*Serratia amnigena*	*Serratia liquefaciens*
25	*Hafnia alvei*	*Hafnia alvei*

Results indicate that MALDI TOF MS was accurate for identifying the genus and species names of bacteria. Other results, however, were inconclusive, which is likely to be the result of the system's bacterial data not matching the relevant microorganism. As a result, since new microorganisms are discovered every year, the MALDI TOF MS system needs to be updated