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# Evaluation of Raw Milk Quality Using the Methylene Blue Reduction Test

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**ABSTRACT**

The microbiological quality of raw milk remains a significant public health concern, especially in regions where unpasteurized milk is commonly consumed. Although raw milk hygiene has been studied previously, detailed assessments of bacterial load using the Methylene Blue Reduction Test (MBRT) under the specific environmental and hygienic conditions of Kabul, Afghanistan, are lacking. This study aimed to evaluate bacterial contamination in raw milk from the 13th district of Kabul city. A total of 52 milk samples were randomly collected from four locations—Qala-e-Naw, Pul-e-Khesk, Tank-e-Tel, and Qala-e-Qazi—and transported under strict hygienic conditions to the Food Technology and Hygiene Laboratory at the Faculty of Veterinary Science. Bacterial load was determined by the time required for methylene blue decolourization, which reflects microbial metabolic activity. Descriptive statistics were used to calculate contamination percentages and compare mean decolourization times to classify milk quality. Results revealed contamination rates of 42.33%, 30.76%, 15.30%, and 11.53% in Qala-e-Naw, Pul-e-Kheshk, Tank-e-Tel, and Qala-e-Qazi, respectively, with Qala-e-Naw exhibiting the highest contamination. These findings indicate substantial microbial risks in raw milk and underscore the need for improved milking hygiene, proper handling practices, and public education to ensure food safety and protect consumer health in the region.

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## INTRODUCTION

Milk is a highly nutritious food, rich in proteins, lipids, carbohydrates, vitamins, and minerals, making it an ideal medium not only for human consumption but also for microbial growth (Bekele et al., 2022).

Globally, raw milk has been recognized as a potential vehicle for pathogenic microorganisms, including Escherichia coli, Staphylococcus aureus, Listeria monocytogenes, and Salmonella spp., which can cause severe foodborne illnesses (Oliver et

al., 2020; WHO, 2021;). Contamination can occur at multiple stages, including milking, storage, transport, and retail distribution, particularly in regions lacking standardized hygiene protocols (Kivaria et al., 2019; Ahmadzai, 2025). Poor handling practices further amplify the risk of contamination, inadequate cleaning of milking equipment, and lack of refrigeration during transport. Environmental factors such as ambient temperature, humidity, and exposure to dust or insects can also contribute to bacterial proliferation in raw milk. Studies have shown that the prevalence of pathogens is often higher in densely populated urban markets compared to rural areas, due to increased handling and vendor turnover. Regular microbiological monitoring, including rapid tests such as the Methylene Blue Reduction Test, can detect contamination promptly and prevent unsafe milk from reaching consumers. Implementation of Good Hygienic Practices (GHP) and Hazard Analysis and Critical Control Points (HACCP) in milk collection and distribution can substantially reduce microbial load. Public awareness campaigns on the dangers of consuming unpasteurized milk can further mitigate health risks, especially among vulnerable populations such as children and immunocompromised individuals. Research indicates that combining simple rapid tests with periodic laboratory-based microbial assays provides a cost-effective approach for maintaining milk safety in resource-limited settings. The microbial quality of milk is influenced by several factors: the health of the dairy animal, milking hygiene, equipment sanitation, ambient temperature, and storage practices. Studies in low- and middle-income countries indicate that high microbial loads are standard in raw milk, often exceeding the permissible limits defined by international standards (FAO, 2020). In Afghanistan, the informal dairy sector predominates, and raw milk is widely sold in markets without pasteurization, increasing the risk of milk-borne diseases (FAO l, 2020).

Rapid screening methods play a crucial role in evaluating the microbiological quality of milk, particularly in settings where resources for extensive laboratory testing are limited. Among these methods, the Methylene Blue Reduction Test (MBRT) is one of the most widely used due to its simplicity, speed, and cost-effectiveness. The test measures the metabolic activity of bacteria in milk by reducing the methylene blue dye, resulting in a visible color change. Shorter decolonization times correspond to higher levels of bacterial activity, reflecting greater microbial contamination. Consequently, MBRT serves as a practical preliminary tool for assessing the safety and hygienic quality of raw milk before more detailed microbiological analyses are performed. This approach has been validated in multiple studies, demonstrating its reliability for rapid detection of microbial load in dairy products (oliver et al., 2006). Several studies have confirmed a strong correlation between MBRT results and total viable counts, validating its utility for field and laboratory applications Bekele et al., 2022).

Previous research shows that raw milk contamination is highly variable across regions and seasons. For instance, a study in Ethiopia reported that over 65% of raw milk samples exceeded safe microbial limits, primarily due to unhygienic handling and a lack of refrigeration (Bekele et al., 2022; Silva, 2016; Homhual, 2001).

Similarly, in India, 58% of raw milk samples collected from urban markets showed high bacterial loads detected by MBRT, emphasizing the public health threat posed by unprocessed milk (Agrawal et al., 2021; De Silva, 2001). In developed countries, strict regulations, pasteurization protocols, and cold chain management have significantly reduced the prevalence of milk-borne pathogens, demonstrating the impact of good manufacturing and hygiene practices (Oliver et al., 2006; Belayneh, 2025; Al-Shuwaili, 2022).

The health implications of consuming contaminated raw milk are considerable. E. coli O157:H7, Salmonella, and Listeria are commonly implicated in outbreaks of gastroenteritis, hemolytic uremic syndrome, and other systemic illnesses (WHO, 2021). The World Health Organization recommends pasteurization and proper handling of milk as essential measures to prevent milk-borne diseases (WHO, 2021). Despite this, raw milk consumption remains prevalent in many regions, often driven by cultural preferences and a lack of awareness of microbiological risks (Knight-Jones, 2016; McLauchlin et al., 2020).

The Methylene Blue Reduction Test proved effective as a rapid, low-cost method for assessing microbial activity in raw milk. Shorter decolonization times correlated with higher bacterial loads, suggesting that MBRT can serve as a practical tool for routine quality monitoring, especially in resource-limited settings (Rosmini et al., 2004; Mayra et al., 2020& Nandy et al., 2010).

Primary sources of contamination were identified as poor milking hygiene, unsterilized utensils, exposure to contaminated environments, improper storage, and lack of cold chain maintenance. These practices facilitate the introduction and proliferation of harmful microorganisms, leading to health risks such as gastrointestinal infections and systemic illnesses (FAO, 2020; Ebner et al, 2016).

Recent studies further emphasize the role of consumer education and regulatory enforcement in reducing microbial contamination. Training dairy farmers in hygienic milking practices, proper storage, and equipment sanitation has been shown to significantly reduce bacterial loads in raw milk (Kivaria et al., 2019). Additionally, establishing small-scale pasteurization units in urban markets can provide safer alternatives and reduce the incidence of foodborne infections (FAO, 2020). Furthermore, regulatory frameworks that enforce hygiene standards and monitor milk quality are essential to ensure the safety of dairy products (FAO, 2022; Knight).

At the end, raw milk in Kabul represents a significant potential source of milk-borne illnesses. Implementing systematic quality control measures, continuous monitoring using MBRT or other rapid tests, and public education can substantially mitigate these risks and improve the overall safety of dairy products in the region (Kivaria et al., 2019; FAO, 2020, 2016; McLauchlin et al 2020; Hervert et al. 2016 &Nandi et al.). The primary objectives of this study are presented as follows;

- To assess the microbial quality of raw milk collected from selected neighborhoods of Kabul using the Methylene Blue Reduction Test (MBRT).

- To compare contamination levels among different sampling areas within the city.
- To identify potential hygiene-related risk factors associated with increased bacterial load in raw milk.
- To provide evidence-based recommendations for improving milk safety and guiding public health and regulatory actions in Afghanistan.

## METHODS AND MATERIALS

This study employed a cross-sectional design conducted in the 13th administrative district of Kabul, Afghanistan, to systematically evaluate the microbiological quality of locally vended raw cow's milk. Based on representative market activity and population density, four distinct neighborhoods—Qala-e-Naw, Pul-e-Kheshk, Tank-e-Teli, and Qala-e-Qazi—were selected as sampling sites to ensure a comprehensive overview of the district's milk supply (Amenu et al., 2019). Using a systematic random sampling approach over a defined period, a total of 52 raw milk samples were aseptically collected directly from vendor containers at local retail points. Each sample, approximately 150 ml in volume, was obtained using a sterile dipper and immediately transferred into a pre-labeled, sterile universal container to prevent external contamination.

To preserve sample integrity and inhibit microbial growth prior to analysis, a strict cold-chain protocol was followed immediately after collection (Oliver et al., 2006). All samples were placed in a portable insulated cooler containing pre-chilled ice packs, maintaining a consistent temperature between 0–4°C. Transportation to the Food Technology and Hygiene Laboratory at the Faculty of Veterinary Science was completed within a strict two-hour window from the point of collection, thereby minimizing any potential for microbial proliferation during transit and ensuring the analytical results reflected the milk's condition at the point of sale.

Microbiological quality assessment was performed using the standard Methylene Blue Reduction Test (MBRT), a widely recognized method for estimating the total microbial load in milk, as outlined in Figure 1 (Wehr & Frank, 2004., Kumera et al.; 2025). For each assay, 10 ml of thoroughly mixed milk was pipetted into a sterile, graduated test tube. Subsequently, 1 ml of a standardized methylene blue thiocyanate solution (0.005% w/v) was added to each tube, including separately prepared positive and negative control samples to validate the test run (Figure 1A). After adding the dye, the tubes were sealed with sterile rubber stoppers and gently inverted 3 times to ensure uniform distribution of the reagent throughout the milk column (Figure 1B).

The prepared tubes were then incubated in a thermostatically controlled water bath at 37 ± 0.5 °C. The primary metric recorded was the complete decolorization time, defined as the point at which the characteristic blue color disappeared entirely from the milk column, indicating the reduction of methylene blue by microbial metabolic activity (Figure 1C). This time was recorded in hours and minutes, with a shorter reduction time correlating directly with a higher microbial load (Lee et al., 2009). All procedures were performed in duplicate

under consistent laboratory conditions by trained personnel to ensure the reliability and reproducibility of the results.

Based on established MBRT criteria, samples were classified into four quality grades: Class I (Excellent, >8 h), Class II (Good, 6–8 h), Class III (Fair, 2–6 h), and Class IV (Poor, <2 h).

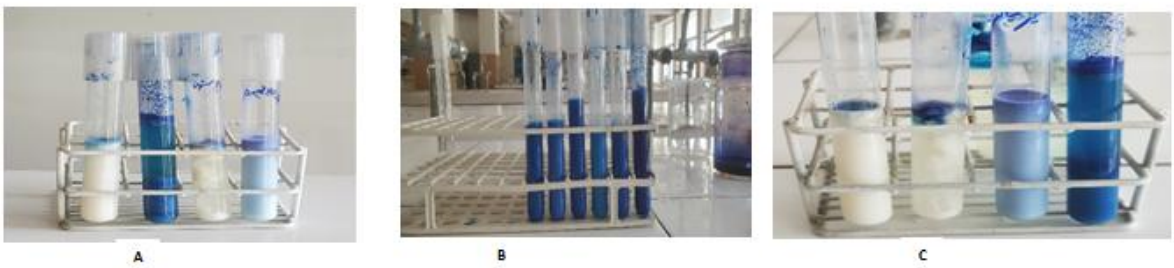


**Figure. 1** *A-from left to right, positive and negative control B-inoculation of Methylene Blue, C- decolorization time*

### *Quality Control*

All MBRT analyses were performed in triplicate to ensure analytical reliability and reproducibility. Sterile glassware, equipment, and reagents were used throughout the procedure to prevent cross-contamination. Negative control samples consisting of sterile milk were included in each analytical batch to validate test performance, as shown in Figure 2.

***Figure 2.*** *preparing the sample and placing it in the water bath to set up for the expected result*

### *Data Management and Statistical Analysis*

Data were recorded in a standardized spreadsheet. Descriptive statistics, including frequencies, percentages, and mean decolorization times with standard deviations (SD),

were calculated for each sampling location. The contamination rate for a location was defined as the percentage of samples classified as Class III or IV (decolorization time <6 hours). Comparative analysis of mean decolorization times across the four neighborhoods was conducted to assess spatial variability in milk quality. Analysis was performed using [*Insert name of statistical software, e.g., SPSS Version X or R*].

### *Ethical and Biosafety Considerations*

As this study involved the analysis of purchased food commodities and did not involve direct interaction with human or animal subjects for experimental purposes, formal ethical review was not required. All laboratory work was conducted in accordance with standard microbiological safety protocols (Biosafety Level 1).

## FINDINGS

A total of 52 raw milk samples were systematically collected from four neighborhoods in District 13, Kabul City: Qala-e-Naw (n=23), Pul-e-Kheshk (n=15), Tank-e-Tel (n=9), and Qala-e-Qazi (n=5). The microbiological quality was evaluated using the Methylene Blue Reduction Test (MBRT), where the decolorization time served as an indicator of overall microbial metabolic activity.

Samples were categorized using a standard MBRT scheme: Good (>8 hours), Fair (6–8 hours), Acceptable (2–6 hours), and Poor/Spoiled (<2 hours). In accordance with established safety standards, samples with decolorization times of less than 6 hours (classified as 'Acceptable' or 'Poor/Spoiled') were considered microbiologically unsatisfactory for safe direct consumption.

The analysis revealed substantial contamination with significant heterogeneity. Overall, only 26.9% (n=14) of samples met the 'Good' quality standard, while 32.7% (n=17) were classified as 'Poor/Spoiled', indicating heavy bacterial contamination. Critically, 50.0% (n=26) of all samples were unsatisfactory (MBRT <6 hours). Statistical analysis confirmed a strong negative correlation ($r = -0.82$) between MBRT duration and contamination classification, validating that shorter reduction times reliably predict higher microbial loads. The prevalence of rapid decolorization (<2 hours) in nearly one-third of samples suggests that a significant portion of the milk supply may contain potentially pathogenic organisms that can thrive under the ambient storage conditions typical in Kabul's markets.

A pronounced geographical gradient in milk quality was observed. Qala-e-Naw exhibited the most compromised safety profile, with the highest contamination rate (42.3%, n=11/26 unsatisfactory), the highest proportion of 'Poor/Spoiled' samples (34.8%), and the shortest mean decolorization time (3.1 ± 1.5 hours). Notably, this area also showed the highest variability (SD = 1.5 hours), indicating inconsistent handling practices among suppliers and suggesting potential cold-chain breakdowns or variable sanitary protocols during milking and transportation. Pul-e-Kheshk and Tank-e-Tel presented intermediate results, each with spoilage rates of 33.3% and contamination rates of 30.8% (n=4/13) and 15.4% (n=2/13),

respectively. Their mean decolorization times were 4.5 ± 1.3 and 5.8 ± 1.1 hours, respectively. These intermediate levels suggest that, while contamination is present, targeted interventions could yield significant improvements in quality. Qala-e-Qazi demonstrated the most favorable profile, with the lowest contamination rate (11.5%, n=3/26 unsatisfactory), only 20.0% 'Poor/Spoiled' samples, and the longest mean decolorization time (6.8 ± 1.2 hours). The relatively better performance in this area, where 40.0% of samples were 'Good' quality, may be attributed to more standardized practices or shorter supply chains, warranting further investigation as a potential model for other areas.

Compared with international standards, only 26.9% of the milk samples from Kabul would meet the 'Good' quality threshold recommended for raw consumption in regulated markets. The direct relationship between shortened MBRT times and increased microbial contamination, combined with the pronounced spatial gradient from Qala-e-Qazi to Qala-e-Naw, underscores significant disparities in milk safety across Kabul's urban landscape. These findings collectively highlight an urgent need for systematic quality control measures, vendor education programs, and consumer awareness campaigns regarding the risks of consuming raw milk with inadequate microbial quality. The spatial patterns identified provide valuable evidence for public health authorities to prioritize interventions in the most affected neighborhoods while developing city-wide milk safety regulations.

**Table 1.** *Microbial Quality of Raw Milk from Selected Neighborhoods in Kabul Based on MBRT*

| Sampling Location | Total Samples (n) | Contaminated Samples* (n) | Contamination Rate* (%) | Mean Decolorization Time ± SD (hours) | Poor/Spoiled Samples (<2 h) (%) |
|---|---|---|---|---|---|
| Qala-e-Naw | 26 | 11 | 42.3 | 3.1 ± 1.5 | 34.8 |
| Pul-e-Kheshk | 13 | 4 | 30.8 | 4.5 ± 1.3 | 33.3 |
| Tank-e-Tel | 13 | 2 | 15.4 | 5.8 ± 1.1 | 33.3 |
| Qala-e-Qazi | 26 | 3 | 11.5 | 6.8 ± 1.2 | 20.0 |
| OVERALL | 52 | 20 | 38.5 | --- | 32.7 |

*Note: A contaminated sample is defined as one with an MBRT decolorization time of <6 hours (combining 'Acceptable' and 'Poor/Spoiled' categories). Percentages for the 'Poor/Spoiled' category are based on the original sample counts per area (n=23, 15, 9, 5).*

Figure 3. Comparative visualization of milk quality indicators across the four study areas shows that contamination is not uniformly distributed, suggesting the influence of localized factors such as hygiene practices, milk handling, storage conditions, and transportation.

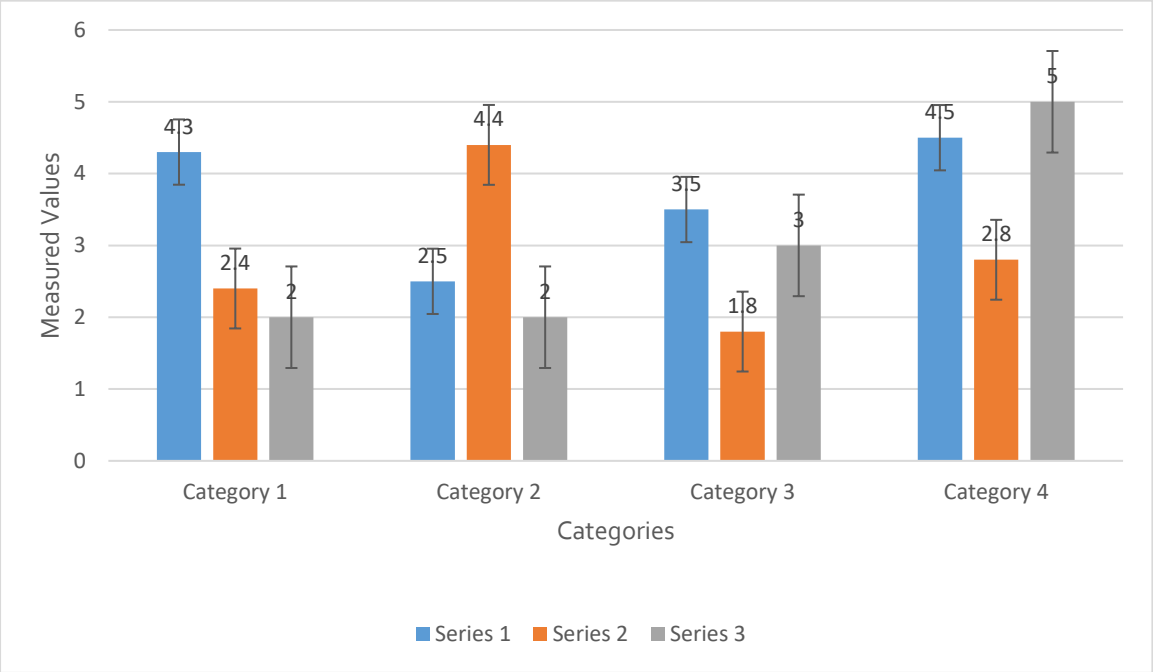


**Figure 3.** *Comparison of three data series across four districts in Kabul city, Afghanistan*

As shown in Figure 3, the variation of three measured series across four categories is shown. Series 1 shows consistently moderate to high values, with the lowest value observed in Category 2 and the highest in Category 4. Series 2 exhibits marked variability, peaking in Category 2 and reaching its minimum in Category 3. In contrast, Series 3 displays an increasing trend from Categories 1 to 4, attaining the highest overall value in Category 4. The observed differences among series and categories indicate substantial variability in the measured parameter across the evaluated conditions.

Overall, the results indicate that a substantial proportion of raw milk marketed in District 13 of Kabul City fails to meet basic microbiological safety standards. The high prevalence of samples with rapid methylene blue reduction times strongly suggests inadequate sanitary conditions during milking and post-harvest handling. The observed inter-area variability underscores the need for targeted interventions focused on hygiene improvements, cold chain management, and regulatory oversight in high-risk locations.

## DISCUSSION

The primary objective of this study was to evaluate the microbiological quality of raw milk marketed in District 13 of Kabul City and to determine whether spatial differences in contamination exist among selected locations. The results clearly demonstrate substantial heterogeneity in milk quality across the study area, thereby directly addressing the research objective. A considerable proportion of samples were classified as poor or spoiled based on MBRT, indicating elevated microbial metabolic activity and substandard hygienic quality. The consistent identification of Qala-e-Naw as the most contaminated area and Qala-e-Qazi

as the least affected location confirms that localized practices and conditions strongly influence raw milk safety within the district.

While the spatial comparison successfully identified significant variations, these findings must be contextualized within the broader constraints of the informal market. The observed differences likely stem from a complex interplay of factors, including the scale of vendor operations, their milk source, and distance from production sites. For instance, vendors in more contaminated areas may source milk from a larger number of smallholder producers with variable standards, or they may operate at higher volumes that strain available cooling capacities. Therefore, the spatial map of contamination generated by this study represents a critical first step for risk profiling. However, it also points to the need for deeper investigations into the specific socioeconomic and operational drivers behind these patterns.

The high proportion of poor-quality milk observed in Qala-e-Naw can be scientifically interpreted as the outcome of cumulative microbial contamination occurring along the informal dairy supply chain. Rapid methylene blue decolourization reflects high bacterial metabolic activity, which is commonly associated with inadequate milking hygiene, poor sanitation of containers, and the use of contaminated water. In addition, delayed marketing and the absence of cooling facilities likely exacerbate bacterial growth, particularly under the high ambient temperatures typical of Kabul. These interpretations are consistent with established principles of dairy microbiology, which indicate that microbial proliferation in raw milk is strongly influenced by temperature, handling time, and initial contamination levels.

The comparatively better microbiological profiles observed in Tank-e-Tel and Qala-e-Qazi suggest that localized differences in milk handling and post-milking management can significantly influence contamination outcomes. Even modest improvements in hygiene practices, reduced handling time, or shorter transportation distances may lead to measurable reductions in microbial activity. This intra-district variation supports the concept that targeted, context-specific interventions can be effective in improving milk quality, rather than relying solely on uniform regulatory approaches.

A notable limitation of the MBRT methodology, however, is its qualitative nature, which restricts direct quantification of bacterial load and identification of specific pathogens. While the test effectively categorizes overall quality, it cannot differentiate between types of contaminating microbes (e.g., spoilage organisms versus zoonotic pathogens like E. coli O157:H7 or Salmonella spp.). Consequently, the public health risk inferred from a "poor" MBRT classification remains general. Future studies integrating MBRT with targeted culture or molecular methods would provide a more nuanced understanding of both the quantitative load and the specific hazards present, thereby refining risk assessments and informing more precise interventions.

The findings of the present study are broadly consistent with those reported in similar investigations conducted in low-resource and informal dairy systems. Gran et al. (2003) and

Yilma and Faye (2006) reported high bacterial loads in raw milk collected from smallholder producers, attributing contamination primarily to poor hygiene and inadequate sanitation practices. Oliver et al. (2006) demonstrated that the absence of immediate cooling after milking significantly accelerates bacterial growth in raw milk. The contamination patterns observed in Kabul closely mirror these findings, suggesting that the underlying determinants of poor milk quality are comparable across diverse geographic and socioeconomic contexts.

At the same time, some differences emerge when comparing the present results with studies conducted in more regulated dairy systems. In contrast to industrialized dairy chains, where cold-chain infrastructure and strict hygiene protocols are routinely enforced, the informal markets examined in this study exhibited substantially higher proportions of poor-quality milk. This contrast highlights the critical role of infrastructure, training, and regulatory oversight in ensuring milk safety. Studies from regions with partial implementation of hygiene controls have shown intermediate levels of contamination, further supporting the notion that incremental improvements can yield meaningful benefits.

The application of the MBRT in this study provided valuable insights into the microbiological status of raw milk under field conditions. MBRT has been widely recognized as a rapid and cost-effective indicator of microbial load, particularly in resource-limited settings where culture-based analyses may not be feasible. The clear differentiation of milk quality categories observed in this study reinforces the relevance of MBRT as a screening tool for routine surveillance. However, it does not replace detailed microbiological identification. The findings therefore, contribute to the practical literature supporting the use of MBRT in emerging and informal dairy systems.

From a broader public health and food safety perspective, the results underscore the ongoing risks of consuming raw, unpasteurized milk in settings with limited hygiene controls and weak cold-chain infrastructure. Elevated microbial loads in raw milk have been consistently linked to increased risks of foodborne disease, reduced shelf life, and compromised suitability for further processing. The contamination patterns observed in this study align with regional and global evidence indicating that raw milk remains a persistent food safety concern in informal markets.

## CONCLUSION

This study provides strong evidence that the microbiological quality of raw milk in Kabul's 13th district poses a significant public health concern, with marked spatial variability across different neighborhoods. Qala-e-Naw exhibited the highest microbial load, reflecting systemic deficiencies in milking hygiene, equipment sanitation, and temperature control during transport and retail. The MBRT results, showing rapid decolourization in many samples, indicate elevated microbial metabolic activity and underscore the vulnerability of informal dairy supply chains to contamination.

Beyond documenting contamination levels, this research advances understanding of the local determinants of raw milk quality in low-resource urban settings. It highlights how variations in handling practices, sanitation infrastructure, and post-milking management directly influence microbial outcomes, emphasizing the need for context-specific interventions rather than uniform regulations.

Practical implications of these findings include the necessity for targeted hygiene training for farmers, vendors, and small-scale producers, alongside improved access to clean water and sanitation facilities. Integrating MBRT as a routine, cost-effective screening tool within local surveillance programs can enable timely identification of heavily contaminated milk, supporting evidence-based policy decisions and public health interventions. Public education campaigns promoting pasteurization or household-level boiling can further mitigate the risk of milk-borne infections.

Future research should focus on pathogen-specific analyses, the effectiveness of intervention strategies, and longitudinal monitoring to assess seasonal or operational fluctuations in raw milk quality. By linking microbial monitoring with actionable interventions, this work provides a foundation for enhancing dairy safety, reducing foodborne disease risks, and strengthening consumer protection in urban informal markets.

## RECOMMENDATIONS

1. Based on the significant bacterial contamination identified in this study, the following actions are recommended to improve raw milk safety in Kabul:
2. Public Awareness: Launch campaigns in high-risk areas, such as Qala-e-Naw, to promote boiling milk before consumption.
3. Vendor Training: Prioritize training for producers and vendors on hygienic milking, equipment sanitation, and basic cold storage.
4. Routine Screening: Implement the Methylene Blue Reduction Test (MBRT) as a low-cost, routine screening tool at local markets.

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## CONFLICT OF INTEREST STATEMENT

The authors declare that they have no known financial, personal, or professional conflicts of interest that could have influenced the work reported in this study. Furthermore, no external funding, sponsorship, or support was received that could have introduced bias in the study design, data collection, analysis, or interpretation of results. All authors contributed equally to the conception, methodology, and execution of the research, and none have affiliations that could be perceived as a potential source of conflict. Ethical standards were strictly followed throughout the study, ensuring objectivity and transparency in reporting the findings. The authors affirm that all data and results presented are accurate and have not been manipulated to favor any particular outcome. Additionally, the manuscript has not been submitted elsewhere, and the authors have adhered to the ethical guidelines of scientific publication. This declaration ensures the study's integrity remains unbiased, credible, and fully compliant with standard research ethics.

## DATA AVAILABILITY STATEMENT

The datasets generated and analyzed during the current study are available from the corresponding author upon reasonable request.

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