



## ORIGINAL ARTICLE

A SURVEY OF LOCAL CHEESE CONTAMINATION WITH *STREPTOCOCCI* IN KABUL CITY MARKETSSayed Arif Ahmadi<sup>1\*†</sup>, Mohammad Farzad Afshar<sup>2†</sup>, Mahyar Mokhtab<sup>3</sup>

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**Ethical considerations:** When reporting experiments on animals Observation of the ARRIVE guidelines 2.0: Updated guidelines for reporting animal research, published on July 14, 2020 (DOI: 10.1371/journal.pbio.3000410), is applied. The authors ensure that all procedures were performed in compliance with the guidelines for animal care of their institutions or with national/international guidelines.

## ABSTRACT

This study investigated the occurrence and distribution of potentially pathogenic streptococcal species in traditional cheese (semi-hard ripened cheese) sold in Kabul, a topic of significant relevance to veterinary science due to its implications for food safety, zoonotic transmission, and dairy hygiene management. To this end, cheese samples from nine regions of Kabul were analyzed for microbiological quality using Gram staining, catalase testing, hemolysis patterns, and disk sensitivity to optochin and bacitracin, supplemented by limited Analytical Profile Index (API) confirmation. Among 90 samples, 31 (34.4%) exhibited  $\alpha$ -hemolysis with optochin sensitivity, suggesting *Streptococcus pneumoniae*, while 25 (27.8%) showed  $\beta$ -hemolysis with bacitracin sensitivity, consistent with Group A *Streptococcus* (GAS). Non-hemolytic isolates (37.8%) predominated overall; API identified *Streptococcus infantarius* in a subset. Results from the chi-square test of independence illustrated that regional differences were significant ( $p < 0.001$ ): Company and Pole Sorkh had the highest optochin sensitivity, Kote Sangai had the highest bacitracin sensitivity, and three regions showed no target organisms. Standard plate counts were low ( $10^2$ – $10^3$  colony forming units (CFU·g<sup>-1</sup>)) and well below international satisfactory limits ( $\leq 10^5$  CFU·g<sup>-1</sup>), indicating generally good microbial quality. Despite low total counts, the detection of presumptive *S. pneumoniae* and GAS in specific areas highlights localized hygiene gaps.

**Keywords:** Afghanistan; cheese microbiology; Group A *Streptococci*; standard plate count; *Streptococcus pneumoniae*

## INTRODUCTION

Cheese has been produced by humans for centuries to preserve and concentrate milk, making it one of the earliest forms of processed food [1]. Cheese provides a range of essential nutrients, including fat-soluble vitamins (A, D, E, and K) and water-soluble vitamin B12, which contribute to immune function, energy metabolism, and blood cell formation [2]. It also contains diverse fatty acids—saturated, monounsaturated, and polyunsaturated—with some, like conjugated linoleic acid (CLA), showing potential anti-inflammatory and anticancer effects despite concerns linked to saturated fat intake [2, 3].

Raw milk cheeses are especially valued for their intense and robust flavor, which is stronger than that of cheeses made from pasteurized milk [1]. However, making cheese out of raw milk poses a threat to public health. In Afghanistan, such types of cheese have been made for a long time and have been reported to be contaminated with dangerous bacteria such as *Staphylococcus aureus* [4] and have been significantly associated with *Brucella* and *Coxiella burnetii* infections [5]. Indeed, worldwide outbreaks of foodborne illness have been linked to the consumption of raw milk cheese [1], such as outbreaks in Italy [6], England [7], Iran [8], and Pakistan [9].

*Streptococcus* species, a group of lactic acid bacteria, are facultative anaerobes that are catalase-negative, non-motile, non-spore-forming, and homofermentative cocci, generally consisting of Gram-positive, spherical or oval cells arranged in pairs or chains [10]. Numerous species within this genus are significant pathogens, with *S. bovis*, *S. pyogenes*, and *S. equi* subsp. *zooepidermicus* being linked to several serious medical conditions, including bacteremia, strep throat, scarlet fever, chills, hypotension, meningitis, endocarditis, sepsis, and colon cancer [11, 12]. These bacteria can enter dairy products through direct contamination during processing or via infected raw milk, particularly when hygienic practices are poor [13, 14].

Since raw milk cheese production is commonly practiced among Afghan families, this study aimed to investigate the potential presence of pathogenic *Streptococcus* species in ripened, semi-hard cheese sold across various markets in Kabul City and to analyze their microbiological quality using standard plate count (SPC). In addition, we hypothesize that the region may have a significant association with the streptococcal sensitivity pattern. To achieve these goals, a range of cheese samples was collected and subjected to biochemical testing to determine whether any zoonotic *Streptococci* were present.

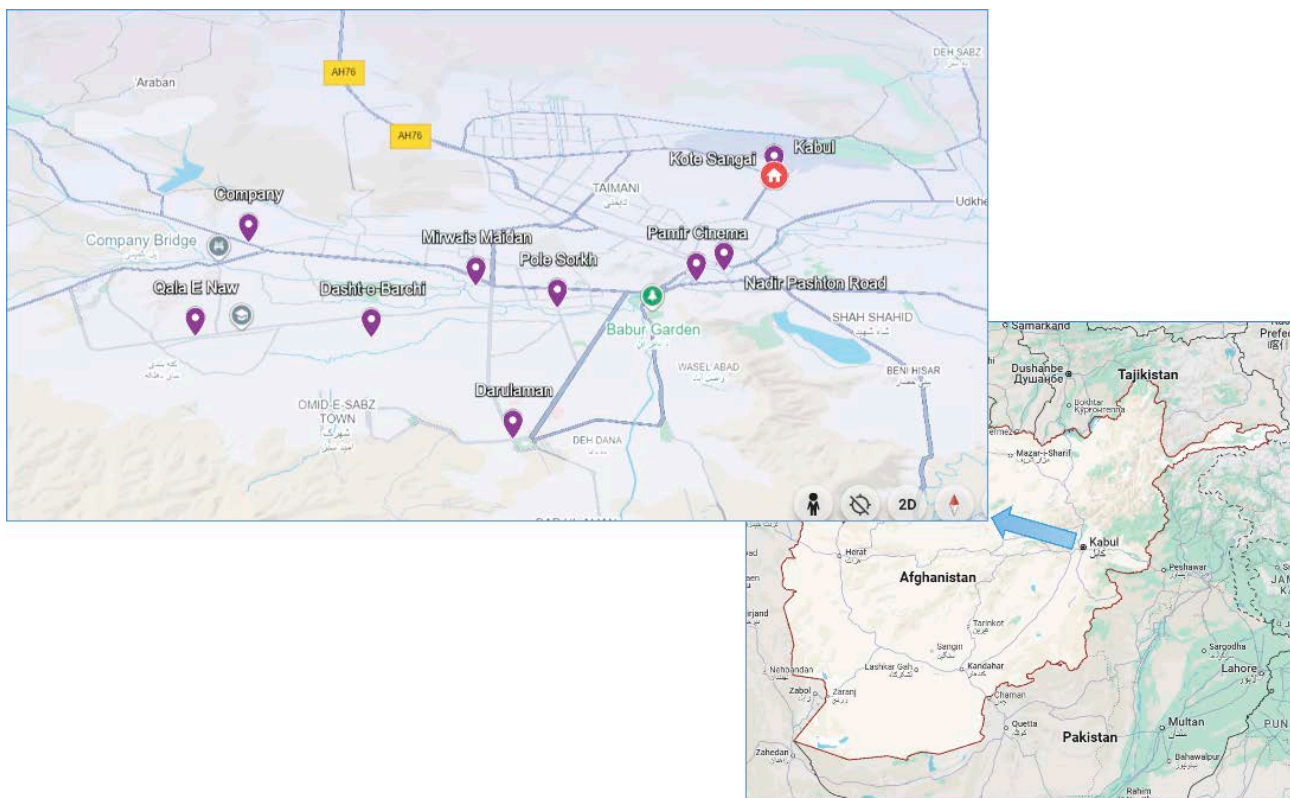


Fig. 1. Illustration of different regions in Kabul City from which the samples were gathered

## MATERIALS AND METHODS

### Sample collection

In this survey, a total of 90 ripened, semi-hard cheese samples, 400 g each, were collected from various dairy product shops across Kabul City (Figure 1). To prevent contamination, all samples were aseptically collected using sterilized tubes and bags [4]. The samples were transported in iceboxes to minimize bacterial growth and were promptly delivered to the Central Diagnostic Laboratories for Animal Disease, Ministry of Agriculture and Livestock, for further microbiological analysis [15].

### Sample preparation, culture, and microscopic examination

Five grams of each cheese sample were aseptically weighed and homogenized in 9 mL of sterile sodium citrate (Sigma Aldrich, Germany) using a mortar and pestle to create a uniform suspension [16]. For the standard plate count, a ten-fold serial dilution was performed by transferring 1 mL of the suspension sequentially into 10 sterile test tubes, each containing 9 mL of sodium citrate, to achieve  $10^{-10}$  dilutions according to standard microbiological procedures. A sterile bent glass rod was used to spread 0.1 mL of diluted samples, and they were incubated at 37°C for 24 hours. After incubation, all colonies, including those of pin-point size, in standard plate count agar (SPCA) medium (HiMedia®, India) were counted [16, 17].

Blood agar plates (HiMedia®, India) were inoculated with 1 mL from each dilution and incubated at 37°C for 18–24 hours. After incubation, colonies were examined for hemolytic activity [18, 19]. Colonies showing hemolytic activity were subjected to Gram staining and examined microscopically. Gram-positive cocci appearing in chains were presumptively identified as the genus *Streptococci* [19].

### Biochemical identification within *Streptococcus* spp.

To differentiate between *Streptococcus* species, hemolytic colonies were subcultured on blood agar and incubated at 37°C for 24 hours. Optochin (5 µg) sensitivity testing was used (HiMedia®, India) to presumptively identify *Streptococcus pneumoniae* among  $\alpha$ -hemolytic isolates, with a visible zone of inhibition indicating sensitivity ( $\geq 14$  mm with a 6 mm disk containing 5 µg optochin) [20].

Bacitracin sensitivity testing using 0.04-unit disks (HiMedia®, India) was employed to presumptively identify *Streptococcus pyogenes* among  $\beta$ -hemolytic isolates, with the presence of a zone of inhibition interpreted as a positive result [18, 21]. These methods were strictly used for comparison within the *Streptococcus* genus.

Finally, to further confirm isolates to the species level, the API 20 STREP system (bioMérieux, France) were used. Colonies were suspended in sterile saline and inoculated into the API test strips following the manufacturer's instructions. The inoculated strips were incubated at 37°C for 24 hours. After incubation, color changes in the wells were recorded, and a numerical code was generated. This code was interpreted using the API database to identify the bacterial species with high precision [22].

### Statistical analysis

Statistical analysis was performed using SPSS 27 (IBM Inc., Chicago). Descriptive statistics were used to describe the sensitivity rate of antimicrobials in each region. Furthermore, A chi-square test of independence was performed to examine the association between region and *Streptococcus* sensitivity patterns (optochin sensitive or bacitracin sensitive separately) used for the presumptive identification of *Streptococci*. Standardized residuals were computed for post hoc analysis to identify cells contributing most to the overall association. Residuals with absolute values  $\geq 1.96$  were considered statistically significant at  $p < 0.05$ , indicating observed frequencies significantly different from expected. Residuals between  $\pm 1.5$  and  $\pm 1.95$  were interpreted as approaching significance.

## RESULTS

Gram staining, catalase testing, and hemolysis patterns combined with disk sensitivity testing (optochin and bacitracin) were used for the presumptive identification of streptococcal isolates from nine regions. Out of 90 total samples, 31 (34.4%) showed  $\alpha$ -hemolysis and were sensitive to optochin, indicating a moderate presence of presumptive *Streptococcus pneumoniae*. Meanwhile, 25 samples (27.8%) exhibited  $\beta$ -hemolysis and bacitracin sensitivity, suggesting a putative low presence of Group A *Streptococci* (GAS). A notable 34 samples (37.8%) displayed no hemolysis, implying a higher proportion of

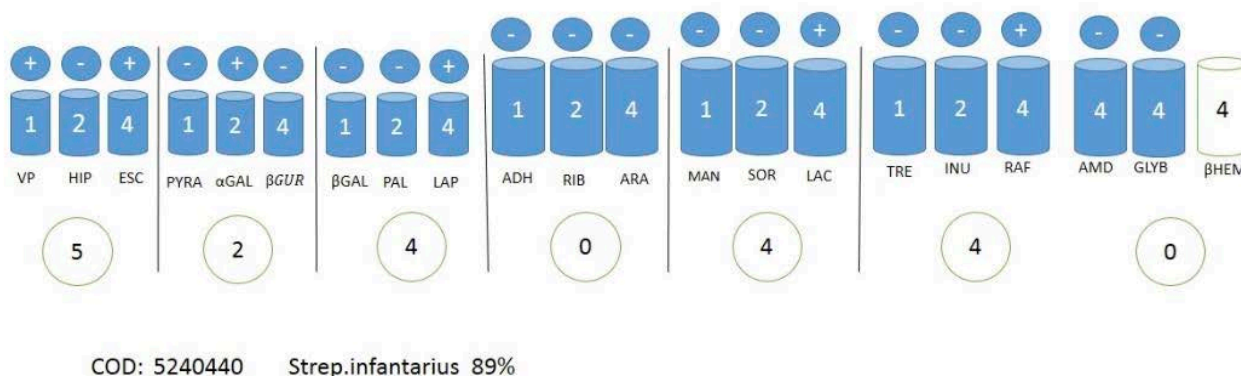


Fig. 2. Identification of non-hemolytic-looking isolates on blood agar to the species level using the API system

non-hemolytic bacteria. Due to limited access to the API system, it was only possible to test 4 samples (detected from Kote Sangai and Mirwais Maidan). The species of these non-hemolytic-looking samples was found to be *S. infantarius* (Figures 2 and 3).

Finally, it was found that there was a significant association between the region and the distribution of isolates showing optochin or bacitracin sensitivity patterns used for presumptive species differentiation ( $p < 0.001$ ). Post-hoc analysis of standardized residuals indicated that cheese samples from Kote Sangai had a significantly higher frequency of bacitracin-sensitive isolates (presumptive presence of *S. pyogenes*) than expected (standardized residual = +2.3). For optochin sensitivity (presumptive presence of *S. pneumoniae*), samples from Company showed a higher-than-expected frequency, with a residual approaching significance (+1.9). Conversely, Dasht-e-Barchi, Qala-e-Naw, and Darulaman each showed lower-than-expected frequencies of optochin sensitivity, with residuals of -1.9, suggesting a marginal trend toward underrepresentation in these regions.

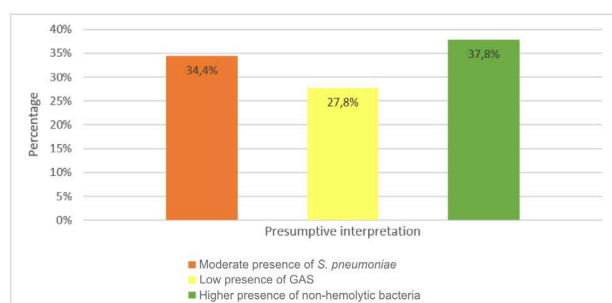


Fig. 3. Presumptive identification of streptococcal isolates based on hemolysis patterns and disk sensitivity to bacitracin and optochin

At the regional level, Company and Pole Sorkh showed the highest  $\alpha$ -hemolytic activity with 7/10 (70%) optochin-sensitive isolates each, suggesting a strong likelihood of *S. pneumoniae* colonization. Pamir Cinema and Nadir Pashton Road exhibited moderate optochin sensitivity (5/10, 50%) and moderate bacitracin sensitivity (5/10, 50%), indicating potential mixed populations of *S. pneumoniae* and GAS. Kote Sangai demonstrated a high  $\beta$ -hemolytic bacitracin sensitivity (7/10, 75%), strongly pointing toward a predominant presence of GAS. In contrast,  $\alpha$ -hemolysis and optochin sensitivity were lower in this region (2/10, 20%), suggesting a smaller proportion of tentative *S. pneumoniae*. Regions such as Darulaman, Dasht-e-Barchi, and Qala-e-Naw showed no hemolysis and no sensitivity to either antibiotic disk (0/10), indicating the absence of the target organisms. In Mirwais Maidan, moderate optochin sensitivity (5/10, 50%) and low bacitracin sensitivity (2/10, 20%) were observed, alongside a considerable proportion (3/10, 30%) of non-hemolytic samples. Overall, these findings highlight significant regional variations in streptococcal colonization patterns, with some areas dominated by presumptive *S. pneumoniae*, others by presumptive GAS, and several regions showing a predominance of non-hemolytic bacterial species. The overall means of standard plate count revealed that the samples gathered from Company had the highest CFU·g<sup>-1</sup>:  $6.0104 \times 10^2 \pm 0.44$ , followed by Kote Sangai ( $5.2104 \times 10^2 \pm 0.41$ ) and Mirwais Maidan ( $5.0104 \times 10^2 \pm 0.52$ ), while no colony was detected from Darulaman, Dasht-e-Barchi, and Qala-e-Naw (Table 1). Thus, the standard plate counts across regions were uniformly low. These counts (on the order of  $10^2$ – $10^3$  CFU·g<sup>-1</sup>) are well below common international “satisfactory” thresholds for total aerobic mesophilic counts in ready-to-eat dairy products (e.g.,  $\leq 1 \times 10^5$

CFU·g<sup>-1</sup> as per established microbiological criteria frameworks), indicating generally good microbial quality and low overall contamination levels in the positive samples [23].

## DISCUSSION

This study investigated the prevalence and distribution of streptococcal species in dairy products collected from nine regions, using classical microbiological methods complemented by limited confirmatory identification with the API system. The findings demonstrate notable heterogeneity in hemolysis patterns and overall bacterial load across regions, highlighting the variable colonization and potential public health risks associated with streptococcal contamination in locally sourced products.

The predominance of optochin sensitivity observed in multiple regions (Company, Pole-Sorkh, and Mirwais Maidan) contrasted with a relatively lower frequency of bacitracin sensitivity. The predominance of  $\alpha$ -hemolytic, optochin-sensitive isolates in the mentioned regions strongly suggests the presumptive presence of *S. pneumoniae* in these regions. This finding is significant given the pathogenic potential of *S. pneumoniae* as a leading cause

of pneumonia, meningitis, and septicemia worldwide, especially in populations with limited access to healthcare and vaccination [24].

On the other hand, Kote Sangai showed a predominance of  $\beta$ -hemolytic, bacitracin-sensitive isolates, consistent with the potential presence of Group A *Streptococci* (GAS). The presence of *S. pyogenes*, a member of GAS, in dairy products has remained a concern due to its ability to cause pharyngitis, skin infections, and invasive diseases such as necrotizing fasciitis [25]. The relatively high frequency of GAS in this region may reflect differences in hygienic practices, handling procedures, or local reservoirs of infection. Furthermore, detection of bacitracin sensitivity may reflect regional variations in antibiotic application or bacterial strain heterogeneity, paralleling findings by Perez-Trallero [26] and Katla et al. [27]. Besides, other Gram-positive bacteria (*Staphylococcus aureus*) have been detected in cheese in Kabul city (4.28%), which depicts the signs of lack of hygienic standards as stated in the study as well [4]. This mirrors patterns reported in both regional and global settings, pointing toward a concerning trend in dairy microbiology that carries significant public health implications.

The statistically significant association between region and the distribution of isolates showing optochin or bac-

**Table 1. Hemolysis pattern, disk test results, and interpretation of streptococcal isolates from different regions**

| Region             | Presumed Hemolysis  | Disk Test Applied | Result (No. & % of sensitive samples) | Overall mean of CFU·g <sup>-1</sup> |
|--------------------|---------------------|-------------------|---------------------------------------|-------------------------------------|
| Company            | $\alpha$ -hemolysis | Optochin          | 7/10 (70)                             | $6.0104 \times 10^2 \pm 0.44$       |
|                    | $\beta$ -hemolysis  | Bacitracin        | 3/10 (30)                             |                                     |
| Darulaman          | No hemolysis / none | N/A <sup>a</sup>  | 0/10 (0)                              | Nil <sup>b</sup>                    |
| Dasht-e-Barchi     | No hemolysis / none | N/A               | 0/10 (0)                              | Nil                                 |
| Qala-e-Naw         | No hemolysis / none | N/A               | 0/10 (0)                              | Nil                                 |
| Pamir Cinema       | $\alpha$ -hemolysis | Optochin          | 5/10 (50)                             | $4.7104 \times 10^2 \pm 0.21$       |
|                    | $\beta$ -hemolysis  | Bacitracin        | 5/10 (50)                             |                                     |
| Pole Sorkh         | $\alpha$ -hemolysis | Optochin          | 7/10 (70)                             | $4.0104 \times 10^2 \pm 0.17$       |
|                    | $\beta$ -hemolysis  | Bacitracin        | 3/10 (30)                             |                                     |
| Nadir Pashton Road | $\alpha$ -hemolysis | Optochin          | 5/10 (50)                             | $4.8104 \times 10^2 \pm 0.23$       |
|                    | $\beta$ -hemolysis  | Bacitracin        | 5/10 (50)                             |                                     |
| Mirwais Maidan     | $\alpha$ -hemolysis | Optochin          | 5/10 (50)                             | $5.0104 \times 10^2 \pm 0.52$       |
|                    | $\beta$ -hemolysis  | Bacitracin        | 2/10 (20)                             |                                     |
|                    | No hemolysis        | N/A               | 3/10 (30)                             |                                     |
|                    | $\beta$ -hemolysis  | Bacitracin        | 7/10 (75)                             |                                     |
| Kote Sangai        | $\alpha$ -hemolysis | Optochin          | 2/10 (20)                             | $5.2104 \times 10^2 \pm 0.41$       |
|                    | No hemolysis        | N/A               | 1/10 (5)                              |                                     |

<sup>a</sup>: Not applicable.

<sup>b</sup>: No bacterial growth or no colonies detected in the samples from the respective areas.

itracin sensitivity patterns used for presumptive species differentiation categories ( $p < 0.001$ ) indicates that local environmental and production factors influence contamination and presence of various streptococcal species. The higher-than-expected bacitracin sensitivity in Kote-Sangai and increased optochin sensitivity in Company and Pole Sorkh may reflect localized differences in the presence of a specific type of *Streptococci* and cheese handling practices, which warrant further investigation. Meanwhile, the marginal underrepresentation of optochin and bacitracin sensitivity in regions such as Dasht-e-Barchi, Darulaman, and Qala-e-Naw suggests differential impacts of local hygiene practices, processing methods, or pasteurization, aligning with international evidence underscoring how proper sanitary measures mitigate microbial contamination [28]. Similarly, one study in Herat province of Afghanistan reported the presence of antibiotic residues (14.1%) in milk collected from shops and open markets of Herat city with the highest residue proportion related to beta-lactam antibiotics (12.8%) [15]. Thus, the presence of antibiotic residues previously reported in raw milk from Herat suggests an underlying selective pressure that may influence the occurrence of streptococcal species such as *S. pyogenes*. In particular, sustained antimicrobial exposure may enrich resistance mechanisms, including Bce-type transporters that detect bacitracin and promote efflux or inactivation, thereby reducing its inhibitory effect on cell wall synthesis [29]. Comparative studies further reinforce the relevance of these findings; for instance, one study in Iraq reported the presence of *S. pneumoniae* from cheese [30].

Interestingly, non-hemolytic isolates comprised the largest proportion overall (37.8%). API testing of a subset of four samples identified *S. infantarius*, a member of the *Streptococcus bovis*/*Streptococcus equinus* complex, which has been associated with fermented dairy products in Africa and Asia [31]. While often considered commensal or involved in traditional fermentation, *S. infantarius* has also been implicated in endocarditis and other opportunistic infections [32]. Its detection suggests that traditional dairy practices in Kabul may foster colonization by non-pathogenic or opportunistic *Streptococci*, a finding consistent with studies documenting *S. infantarius* dominance in raw and fermented dairy products in resource-limited settings [33]. Regions such as Darulaman, Dasht-e-Barchi, and Qala-e-Naw demonstrated no bacterial growth, which may indicate either effective hygienic

practices during milk handling or environmental conditions less conducive to streptococcal survival. One limitation of the current results, however, is that only 4 isolates could be identified as *S. infantarius* using the API kit and therefore, these results are indicative and not definitive.

The standard plate count results provide reassuring evidence of generally acceptable microbiological quality across the positive samples. The CFU measurements ranging from approximately  $4.01 \times 10^2$  to  $6.01 \times 10^2$  per gram in bacitracin-resistant samples fall well below established international thresholds for ready-to-eat dairy products ( $\leq 1 \times 10^5$  CFU·g<sup>-1</sup>) [34, 35] and indicate the good quality and generally hygienic production of cheese, which is in contrast of previous findings in Herat and Kabul reporting low-hygiene dairy products [4, 17, 15]. However, the combined low standard plate counts together with the regional differentiation in hemolysis and disk sensitivity profiles underscore localized patterns of streptococcal occurrence rather than widespread heavy contamination; this supports a risk-based focus on areas (e.g., Company and Kote Sangai) where presumptive pathogens co-occur with detectable bacterial burden.

Future work should expand confirmatory identification beyond biochemical testing, incorporating molecular approaches such as 16S rRNA sequencing or MALDI-TOF for more accurate species-level classification since these results are considered presumptive. In addition, safety assessment, exploration of virulence genes, and antimicrobial susceptibility testing against a set of antibiotics would help clarify potential resistance patterns in these isolates. Such studies are crucial for developing region-specific risk mitigation strategies, especially in areas like Kote Sangai and Company, where contamination appears most pronounced.

## CONCLUSION

This study revealed notable regional variation in streptococcal contamination of ripened, semi-hard cheese in Kabul, with Company and Pole Sorkh showing a predominance of presumptive *S. pneumoniae* and Kote Sangai dominated by Group A *Streptococci*. Although overall bacterial loads were low and within international microbiological quality standards, the presence of potential pathogens in specific areas highlights localized hygiene and

handling deficiencies. The detection of *S. infantarius* further underscores the influence of traditional dairy practices on microbial profiles. Targeted interventions, improved hygienic measures, and molecular confirmation of isolates are warranted to better assess public health risks and guide region-specific control strategies.

### Data Availability Statement

The raw data of this article will be made available by the authors upon request.

### Ethical Statement

No ethical approval was necessary for this study.

### Conflict of Interest

There was no conflict of interest among the authors.

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### Generative AI Statement

No generative AI or AI-assisted technologies were used in the writing process of this study.

### Authors' Contributions

All authors contributed to the study conception and design. The conceptualization was performed by Sayed Arif Ahmadi (SAA) and Mohammad Farzad Afshar (MFA). The methodology was developed by SAA and MFA. Data collection and analysis were performed by SAA, MFA, and Mahyar Mokhtab (MM). Supervision of the study was carried out by SAA. The original draft of the manuscript was written by SAA, MFA, and MM. The manuscript was reviewed and edited by MFA and MM. All authors read and approved the final manuscript.

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