



Italian Journal of Food Safety

<https://www.pagepressjournals.org/index.php/ijfs/index>

eISSN 2239-7132

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Ital J Food Saf 2026 [Online ahead of print]

Please cite this article as:

Aouadhi C, Brahmi S, Gharbi M, et al. **Regional and seasonal influences on the microbiological quality of raw milk in northwestern Tunisia: insights into total flora, psychrotrophs, *Pseudomonas* spp., and heat-resistant spores.** *Ital J Food Saf*
doi:10.4081/ijfs.2026.14926

Submitted: 07-02-2026

Accepted: 12-06-2026

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Regional and seasonal influences on the microbiological quality of raw milk in northwestern Tunisia: insights into total flora, psychrotrophs, *Pseudomonas* spp., and heat-resistant spores

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Keywords: raw milk, microbiological quality, region, season, *Pseudomonas*.

Contributions: Chedia Aouadhi: conceptualization, data curation, investigation, software, supervision, visualization, writing - original draft. Samar Brahmi, Manel Gharbi: data curation, review and editing. Fadhlallah Garouachi: funding acquisition, project administration, review and editing. Abderrazak Maaroufi: funding acquisition, project administration, validation, review and editing.

Conflict of interest: the authors declare no conflict of interest.

Ethics approval and consent to participate: this study did not involve human participants, human tissues, or clinical samples. Therefore, ethical approval and informed consent were not required.

Availability of data and materials: all data underlying the findings are fully available.

Funding: this work was funded by the Ministry of Higher Education and Scientific Research, Tunisia (Grant no. PEJC2023-D1P14).

Acknowledgments: the authors acknowledge the support provided by the Tunisian Ministry of Higher Education and Scientific Research (LR16IPT03), as well as the northern dairy plant located in Tunisia for supplying the milk samples.

Declaration of generative artificial intelligence and artificial intelligence-assisted technologies in the writing process: during the preparation of this manuscript, we used OpenAI solely for language editing and grammar correction.

Abstract

To our knowledge, this study provides the first comprehensive investigation in Tunisia of the combined influence of region and season on the microbiological quality of raw milk collected from the governorates of Jendouba, Béja, and Kef in northwestern Tunisia. A total of 1224 samples (102/month) were analyzed over a 12-month period from March 2023 to February 2024. Significant regional and seasonal variations were observed. Total aerobic mesophilic count ranged from 10^6 to 10^7 CFU/mL in winter, autumn, and spring, reaching 8 log CFU/mL in summer. Psychrotrophic bacteria and *Pseudomonas* spp. counts were lowest in winter (5-6 log CFU/mL) but increased markedly during warmer months. Heat-resistant spore (HRS) counts ranged approximately from 0.5 to 1.47 log CFU/mL across seasons, with the highest levels observed in spring. The proportion of spore-contaminated samples increased from 58% in April to 100% in August before declining to 87% in March. Psychrotrophic flora were largely dominated by *Pseudomonas* spp. (77-86% of isolates), with the highest prevalence in autumn. Regionally, psychrotrophic loads remained high across all governorates (7.1-7.3 log CFU/mL), while *Pseudomonas* counts were significantly higher in Jendouba. HRS contamination remained relatively low (1 log CFU/mL) and consistent across regions. Correlation analysis revealed strong associations between psychrotrophs, total aerobic mesophilic count, and *Pseudomonas*, confirming their central role in raw milk spoilage. Overall, these findings highlight the pronounced impact of environmental, hygienic, and seasonal factors on raw milk quality. Improved hygiene practices, rapid cooling, and reinforcement of the cold chain, particularly during high-risk seasons, are essential strategies to enhance milk safety and extend shelf life in Tunisia's dairy sector.

Introduction

Milk quality is a critical concern for both dairy producers and consumers, as it directly impacts the safety, nutritional value, and overall quality of dairy products (Haldar *et al.*, 2022). The microbiological quality of raw milk is particularly important because it can harbor various microorganisms, some of which can lead to spoilage, foodborne illnesses, or pose risks to public health (Fusco *et al.*, 2020).

Several factors influence the microbiological quality of raw milk, including environmental conditions, animal health, farming practices, and handling procedures (Fusco *et al.*, 2020; Deddefo *et al.*, 2023). Environmental factors such as temperature, humidity, and seasonal variations have been shown to significantly affect microbial populations in milk, influencing both the types and numbers of microorganisms present (Toghdory *et al.*, 2022; Li *et al.*, 2018). The health of dairy animals also plays a crucial role in shaping the microbial profile of milk, as infected animals or those with poor health may harbor pathogens that can be transferred to milk (Batoool *et al.*, 2025). Additionally, poor handling practices, such as inadequate hygiene during milking, transportation, or storage, can introduce or promote the growth of microorganisms in milk (Bekuma and Galmessa, 2018).

In Tunisia, the quality of raw milk can exhibit significant variations due to differences in geographic regions and seasonal fluctuations. These factors are influenced by the country's diverse climate, agricultural practices, and regional differences in milk production (Attia *et al.*, 2021). For instance, the northwestern region of Tunisia, characterized by a Mediterranean climate, experiences distinct seasonal changes that can affect milk quality in terms of microbial load and composition. Despite the clear potential for regional and seasonal differences to influence raw milk microbiota, few studies have explored how these variations impact the microbiological profile of raw milk in Tunisia. This is particularly important in the northwestern region, which has its own unique environmental and agricultural characteristics that may influence milk quality.

The key microbial indicators considered in this study were total aerobic mesophilic counts, psychrotrophic bacteria, *Pseudomonas* spp., and heat-resistant spores across different seasons in northwestern Tunisia. Understanding these variations is crucial for improving milk safety, as the microbial load in raw milk is directly linked to the potential for spoilage, reduced shelf life, and the risk of foodborne diseases (Aouadhi *et al.*, 2014; Aouadhi *et al.*, 2025). Psychrotrophic bacteria, which are capable of growing at low temperatures, are of particular concern in raw milk, as they can

thrive in refrigerated conditions and cause spoilage (Aouadhi *et al.*, 2025). *Pseudomonas* species, known for their ability to degrade milk proteins and fats, are also common spoilage agents that can significantly impact milk quality (Ávila *et al.*, 2025). Furthermore, the presence of heat-resistant spores, which are resilient to pasteurization, poses a unique challenge in ensuring the microbiological safety of dairy products (Aouadhi *et al.*, 2014; Kmiha *et al.*, 2017). These spores can survive pasteurization and, depending on their heat resistance, may subsequently germinate and contribute to post-processing spoilage (Aouadhi *et al.*, 2022).

The present study aims to investigate the impact of seasonal and regional variability on key microbial parameters of raw milk collected from different areas in Tunisia. The objective is to provide a scientific foundation for improving hygiene practices, optimizing dairy production processes, and reinforcing national safety standards to ensure the delivery of safe, high-quality dairy products to consumers.

Materials and Methods

Sample of raw milk

To evaluate the influence of region and season on the microbiological quality of raw cow's milk, a total of 1224 samples were collected over a 12-month period (from March 2023 to February 2024) from 34 collection centers located in the northwest of Tunisia, specifically within the governorates of Béja, Kef, and Jendouba. Each month, 102 samples were obtained, with three samples collected from each center. In total, 17 centers were located in Jendouba, 15 in Béja, and 2 in Kef. Raw milk samples were aseptically collected in sterile bottles using sterile scoops directly from the herders' tanks, immediately upon delivery to the collection centers, ensuring that the samples were representative of the milk supplied to processing facilities. The microbiological quality of the samples was assessed by enumerating total mesophilic aerobic flora, aerobic spore-forming bacteria, psychrotrophic bacteria, and *Pseudomonas* spp.

Quantification of total aerobic mesophilic count and psychrotrophic bacteria

Bacterial enumeration was performed on the same day as sample collection to prevent any changes in microbial load. Samples were serially diluted in quarter-strength Ringer's solution, and 1 mL aliquots of appropriate dilutions were plated on Plate Count Agar (PCA) to enumerate total viable bacterial counts and psychrotrophic bacteria. Plates were incubated at two different temperatures: $7\pm1^{\circ}\text{C}$ for 10 days for the enumeration of psychrotrophic bacteria and 30°C for 72 h for the enumeration of mesophilic bacteria (Aouadhi *et al.*, 2025). These incubation conditions were selected according to the optimal growth requirements of psychrotrophic and mesophilic microorganisms, respectively.

Quantification of *Pseudomonas* spp.

The enumeration of *Pseudomonas* spp. was carried out using *Pseudomonas* Agar Base. A 0.1 mL aliquot of the appropriate dilution was spread onto the surface of the medium and incubated at 21°C for 24 h, a temperature favorable for the growth of *Pseudomonas* spp. After incubation, colonies were counted and the results were expressed as $\log_{10}$ CFU/mL of raw milk.

Enumeration of heat-resistant spores

Heat-resistant spores, particularly *Bacillus* species, were enumerated following a heat treatment designed to eliminate vegetative cells and isolate surviving spores. Samples were heated at 100°C for 30 min, following protocols from Aouadhi *et al.* (2014), which are effective for inactivating vegetative bacteria while allowing spores to survive. After the heat treatment, 0.1 mL of the sample was spread onto Brain-Heart Infusion Agar (BHI-Agar, Biokar Diagnostics), supplemented with 1 mg/L of vitamin B12 (Sigma-Aldrich, St. Louis, MO, USA) to promote the growth of *Bacillus* species. The plates were incubated at 37°C for 48 hours, after which spore-forming colonies were counted.

Statistical analysis

All experiments were conducted in triplicate, and the results are expressed as mean values accompanied by standard deviations. Statistical analysis was performed using one-way ANOVA to evaluate the impact of each independent variable, employing STATGRAPHICS Centurion XV software (version 19.6.05). Mean comparisons were carried out using Fisher's least significant difference (LSD) test at a 5% significance threshold.

Results and Discussion

Impact of the region on the quality of raw milk

Total aerobic mesophilic count

Total aerobic mesophilic count (TAMC) is a key indicator of the microbiological quality of raw milk and reflects hygiene during milking, equipment cleanliness, and storage conditions (Quigley *et al.*, 2013; O'Connell *et al.*, 2016). In this study, TAMC values in milk samples collected over a 12-month period from the Jendouba, Béja, and Kef governorates ranged between 7 log (CFU/mL) and 8 log (CFU/mL), exceeding the maximum limit of 5.4 log (CFU/mL) established by both the USPHS/FDA and the European Commission for raw milk intended for processing (USPHS/FDA, 2019; European Parliament and Council of the European Union, 2004).

As shown in Figure 1a, mean TAMC values (log CFU/mL) did not differ significantly among the three regions ($p > 0.05$). Although Jendouba showed slightly higher counts and Béja slightly lower counts, these differences were not statistically significant.

The absence of significant regional variation suggests that similar factors affecting milk hygiene and handling may operate across the surveyed areas. Comparable findings have been reported in rural dairy production systems where limitations in sanitation practices and cold chain management contribute to elevated microbial loads (Kiambi *et al.*, 2022; Ntuli *et al.*, 2023).

Psychrotrophic flora

The psychrotrophic bacterial load in raw milk samples collected over a 12-month period from the Béja, Jendouba, and Kef governorates is presented in Figure 1b. Mean counts ranged from 7.1 to 7.3 log CFU/mL, with no significant differences among regions ($p > 0.05$). These results indicate a consistently high prevalence of psychrotrophic bacteria across all sampling areas.

Psychrotrophic bacteria are able to grow under refrigeration conditions and are recognized as major spoilage microorganisms in raw milk (Quigley *et al.*, 2013). The absence of significant regional differences suggests that similar milk collection, handling, and storage practices are applied across the surveyed regions. However, the elevated psychrotrophic counts observed in all areas indicate conditions favorable to their proliferation during milk storage and transport.

These findings are consistent with previous studies reporting high psychrotrophic contamination in raw milk when cooling efficiency and cold chain management are suboptimal (O'Connell *et al.*, 2016; Yalaw *et al.*, 2024). The predominance of psychrotrophic bacteria is of technological concern because many species, particularly *Pseudomonas* spp., produce heat-stable proteases and lipases that may compromise the quality and shelf life of dairy products (Sachan *et al.*, 2018; Ávila *et al.*, 2025).

***Pseudomonas* spp. count**

The *Pseudomonas* spp. counts (log CFU/mL) measured across the three surveyed regions (Béja, Jendouba, and Kef) are presented in Figure 1c. A statistically significant difference was observed among regions ($p < 0.05$). The highest mean count was recorded in Jendouba, whereas the lowest was observed in Béja. Kef exhibited intermediate values. Multiple comparison analysis indicated that Jendouba (group B) differed significantly from Béja (group A), while Kef (group A, B) was not significantly different from either region.

The higher *Pseudomonas* counts observed in Jendouba may be associated with environmental conditions and management practices favoring bacterial proliferation. In contrast, the lower counts recorded in Béja may reflect more favorable hygiene conditions during milk production, collection,

and storage. The intermediate values observed in Kef suggest a transitional situation between the two regions.

The regional variation observed in this study is consistent with previous reports showing that microbial contamination of raw milk can be influenced by environmental factors and handling practices (Beuchat, 2002; Yap *et al.*, 2024). Given the predominance of *Pseudomonas* spp. among psychrotrophic bacteria, these findings highlight their importance as indicators of raw milk microbiological quality (Du *et al.*, 2022).

Heat-resistant spores

The mean counts of heat-resistant spores (HRS) across the three sampled governorates (Béja, Jendouba, and Kef) are presented in Figure 1d. The values ranged from approximately 1.0 to 1.3 log CFU/mL. No statistically significant differences were observed among the regions ($p > 0.05$), as indicated by the shared grouping letter “A” attributed to all three locations. These results indicate a relatively uniform distribution of HRS across the surveyed areas.

HRS are mainly represented by spore-forming bacteria belonging to the genera *Bacillus* and *Clostridium*, which are able to survive adverse environmental conditions, including heat treatment, desiccation, and exposure to sanitizing agents (Setlow, 2014; Den Besten *et al.*, 2018). The absence of significant regional variation suggests that contamination sources and management practices influencing spore occurrence may be relatively similar across the investigated regions.

Although HRS counts remained low throughout the study, their presence is relevant because bacterial spores can survive thermal processing and subsequently affect the quality and stability of dairy products (Sadiq *et al.*, 2016; Aouadhi *et al.*, 2022).

Impact of the season on the microbiological quality of milk

Seasonal variation in total aerobic mesophilic count

TAMC (log CFU/mL) measured in raw milk samples across the four seasons is presented in Figure 2a. Values ranged from approximately 7.55 to 7.85 log CFU/mL. Significant differences were observed among seasons ($p < 0.05$). The highest mean counts were recorded in autumn and spring (group B), whereas the lowest counts were observed in winter (group A). Summer belonged to group A,B, indicating intermediate levels that did not differ significantly from either extreme. These results demonstrate a significant seasonal effect on TAMC in raw milk. Higher bacterial counts were observed during spring and autumn, whereas winter samples exhibited the lowest contamination levels. Similar seasonal variations in raw milk microbiological quality have been reported previously and are generally associated with changes in environmental conditions and milk production practices throughout the year (Beuchat, 2002; Kazeminia *et al.*, 2019; Celano *et al.*, 2022).

Seasonal variation in psychrotrophic bacterial count

Psychrotrophic bacterial count in raw milk varied significantly among seasons ($p < 0.05$), ranging from approximately 7.0 to 7.5 log CFU/mL (Figure 2b). The lowest counts were recorded in winter (group A), whereas the highest values were observed in summer (group C). Autumn and spring showed intermediate values and did not differ significantly from either extreme. These results indicate a marked seasonal influence on psychrotrophic bacterial populations in raw milk. Higher counts were observed during the warmer months, whereas lower levels were detected during winter. Similar seasonal trends have been reported in previous studies investigating psychrotrophic contamination in dairy systems (Boubendir *et al.*, 2015). Given their ability to produce heat-stable enzymes, psychrotrophic bacteria are considered important contributors to milk spoilage and reduced shelf life of dairy products (Aouadhi *et al.*, 2025).

*Seasonal variation in *Pseudomonas* prevalence*

The seasonal distribution of *Pseudomonas* spp. is presented in Figure 2c, with percentages ranging from 82% to 86%. The highest proportion was observed during autumn (approximately 85.5%), which was significantly higher ($p < 0.05$) than those recorded in spring, summer, and winter. No significant differences were detected among the latter seasons, as indicated by the shared grouping letter “A”.

These results indicate that *Pseudomonas* spp. constituted the dominant fraction of the psychrotrophic microbiota throughout the year, with only limited seasonal variation. The predominance of *Pseudomonas* spp. in raw milk is consistent with previous studies reporting this genus as one of the major psychrotrophic spoilage bacteria in refrigerated milk and dairy environments (Quigley *et al.*, 2013; Yuan *et al.*, 2019; Ávila *et al.*, 2025). The higher proportion observed during autumn suggests a seasonal effect on the relative abundance of *Pseudomonas* spp. in raw milk.

Seasonal variation in heat-resistant spores

The seasonal distribution of HRS (log CFU/mL) in raw milk is presented in Figure 2d. The results reveal statistically significant differences ($p < 0.05$) among the seasons, with clear groupings indicated by different letters above the bars. The lowest count was observed in autumn, with a mean value of around 0.5 log CFU/mL and classified as group A. Spring showed the highest values followed by summer (groups C and B, respectively), while winter fell within the B, C group, indicating no significant difference from either summer or spring.

These results suggest a heterogeneous seasonal distribution of thermoresistant spore-forming bacteria, which could be influenced by a variety of environmental and management factors, including soil contamination, feeding practices, hygiene levels, and climatic variations (Scheldeman *et al.*, 2005; Setlow, 2014). The elevated counts observed in some samples may be due to increased spore contamination from soil and bedding material during wetter conditions, or the use of silage-based feeding, which has been previously associated with spore contamination in dairy systems (Borreani *et al.*, 2019; Den Besten *et al.*, 2018).

HRS are of particular concern due to their resistance to heat treatments such as pasteurization and even UHT treatment. Their survival and subsequent germination can lead to milk spoilage, biofilm formation, and reduced shelf life, as well as posing a risk to consumer health if pathogenic species are present (Velázquez-Ordoñez *et al.*, 2019; Sadiq *et al.*, 2016). The findings underline the importance of implementing season-specific control strategies, including improved cleaning protocols, udder hygiene, and raw milk screening, especially during high-risk periods.

Monthly variation in the microbiological quality of milk

The monthly evolution of microbial contamination in raw milk was evaluated using multiple range tests based on the 95% LSD method. The results revealed clear and statistically significant monthly variations across all microbial groups (Figure 3).

The total aerobic mesophilic count ranged from 7.49 log₁₀ CFU/mL (January) to 7.77 log₁₀ CFU/mL (March), with most values from late spring to autumn forming a statistically homogeneous group (group B). January, November, and February exhibited relatively lower values (groups A and AB), suggesting better microbiological quality in winter. These results are consistent with previous studies reporting that lower temperatures, reduced transportation times, and improved cold chain efficiency during winter can limit bacterial proliferation in raw milk (Vithanage *et al.*, 2016; Celano *et al.*, 2022).

HRS, representing heat-resistant spore-forming bacteria, showed the most pronounced monthly variability. The lowest levels (0.387 log₁₀ CFU/mL) were observed in April-June (group A), while the highest value (1.47 log₁₀ CFU/mL) occurred in October (group E), indicating a marked seasonal increase. This pattern may reflect environmental spore accumulation, increased soil contamination, and enhanced biofilm persistence in dairy equipment under suboptimal sanitation conditions (Gopal *et al.*, 2015; Lopez-Brea *et al.*, 2017; Setlow, 2014). These organisms are of particular concern for UHT and long shelf-life dairy products due to their resistance to thermal processing.

Psychrotrophic counts ranged from 6.93 log₁₀ CFU/mL in January (group A) to 7.47 log₁₀ CFU/mL in September (group E), with a gradual increase from winter to late summer. Intermediate values were observed during spring (April-June; Group BCD). As cold-adapted microorganisms, psychrotrophs can proliferate even under refrigeration, particularly when cooling is delayed or insufficient. Their seasonal increase is likely associated with prolonged storage time and inadequate

initial chilling (D'Amico and Donnelly, 2010; Quigley *et al.*, 2013). Their enzymatic activity, particularly proteases and lipases, contributes significantly to milk spoilage.

Pseudomonas spp. followed a similar trend, with the lowest counts recorded in January ($5.60 \log_{10}$ CFU/mL; group A) and increasing up to $6.13 \log_{10}$ CFU/mL during April-June (group D). These bacteria are of major technological concern due to their ability to produce heat-stable enzymes and form biofilms that enhance persistence in dairy environments (Martin *et al.*, 2016; Zhang *et al.*, 2019). Despite being psychrotrophic, their prevalence increases during warmer months, likely due to higher environmental exposure and reduced cold chain efficiency. Notably, *Pseudomonas* constituted the dominant fraction of the psychrotrophic population (77-84%), with peaks observed in early summer and late autumn, confirming their central role in raw milk spoilage (Figure 4).

Winter months consistently showed the lowest microbial contamination, whereas late summer and autumn exhibited the highest levels. These findings confirm that ambient temperature fluctuations, storage duration, and cold chain reliability are key determinants of microbial proliferation in raw milk. Furthermore, both psychrotrophic and spore-forming bacteria, due to their resistance to processing and sanitation stresses, require targeted control strategies in dairy production systems intended for long shelf-life products.

Correlation analysis of microbiological parameters

The Pearson correlation coefficients among the different microbiological parameters are presented in Figure 5. Moderate to strong positive correlations were observed between psychrotrophs and total aerobic mesophilic count ($r=0.65$), psychrotrophs and *Pseudomonas* spp. ($r=0.65$), *Pseudomonas* spp. and the percentage of *Pseudomonas* ($r=0.55$), and total count and *Pseudomonas* spp. ($r=0.51$). These correlations indicate that higher *Pseudomonas* counts were associated with higher total aerobic mesophilic and psychrotrophic counts. The co-occurrence of elevated psychrotrophic and *Pseudomonas* levels indicates that these groups may share similar ecological niches or be influenced by common environmental factors, particularly temperature, moisture, and hygiene conditions during milk handling and storage (Chiellini *et al.*, 2019; Quigley *et al.*, 2013).

In contrast, weak or negative correlations were observed between heat-resistant spore-forming bacteria and the other microbial groups, with correlation coefficients ranging from -0.02 to -0.13 . This likely reflects the distinct ecological behavior of spore-forming bacteria, which are capable of surviving under adverse conditions rather than actively proliferating, and therefore respond differently to environmental fluctuations compared to vegetative cells (Gauvry *et al.*, 2017; Setlow, 2014). The absence of strong correlations between spore-formers and total or psychrotrophic counts suggests that their presence is more stochastic and related to specific contamination sources such as soil, feed, and equipment rather than overall microbial growth dynamics.

These findings highlight important microbial interrelationships that can help guide monitoring and control strategies in dairy safety programs. In particular, *Pseudomonas* spp. may serve as a useful indicator organism, given its consistent association with both total viable counts and psychrotrophic populations.

Recommendations for milk quality improvement

Based on the findings of this study, several practical measures can be proposed to improve the microbiological quality of raw milk and reduce spoilage-related risks along the dairy chain. First, strengthening milking hygiene at the farm level is essential. This includes proper udder cleaning and drying before milking, regular disinfection of milking equipment, and training of farm workers in good hygienic practices. Given the consistently high total viable counts and psychrotrophic bacteria observed, these basic measures represent a critical control point in raw milk production.

Second, rapid cooling of raw milk immediately after milking should be prioritized. Psychrotrophic bacteria, particularly *Pseudomonas* spp., are capable of proliferating even under refrigeration when cooling is delayed. Therefore, maintaining milk at $\leq 4^{\circ}\text{C}$ and minimizing the time between milking and cooling are essential to limit bacterial growth.

Third, improving the efficiency and reliability of the cold chain during collection and transport is necessary. The observed seasonal patterns, with higher microbial loads during warmer months, highlight the need for strengthened temperature control, particularly during summer and transitional periods.

Fourth, targeted control of spore-forming bacteria should be considered. Although present at lower levels, heat-resistant spores remain a concern for UHT and long shelf-life dairy products. The implementation of spore-targeted sanitation strategies, improved cleaning-in-place (CIP) systems, and, where applicable, advanced technologies such as high-pressure processing may help reduce contamination.

Finally, routine microbiological monitoring programs should be implemented, focusing on key indicator organisms such as total viable counts and *Pseudomonas* spp. This would allow early detection of hygiene failures and better management of variability in raw milk quality across regions and seasons.

Overall, an integrated approach combining improved hygiene practices, cold chain optimization, and targeted microbial control strategies is required to improve the microbiological quality of raw milk, extend shelf life, and improve the technological quality of dairy products.

Conclusions

This study highlights the significant influence of both region and season on the microbiological quality of raw milk in northwestern Tunisia. Elevated total viable counts and psychrotrophic bacteria across all regions underline persistent challenges related to hygiene and cold chain management, while regional differences in *Pseudomonas* spp. may be associated with variations in environmental conditions and farm-level practices. Seasonal patterns, characterized by higher microbial loads in autumn and spring and lower levels in winter, confirm the strong effect of climatic conditions on milk microbiology. Correlation analysis further identified *Pseudomonas* spp. as a key indicator of microbial quality and spoilage risk. Overall, these findings emphasize the need to strengthen milking hygiene practices, improve rapid cooling and refrigeration systems, and enhance farmer training, particularly in regions and during periods associated with higher microbiological risks. Such targeted interventions are essential to improve the safety, technological quality, and market value of raw milk in Tunisia.

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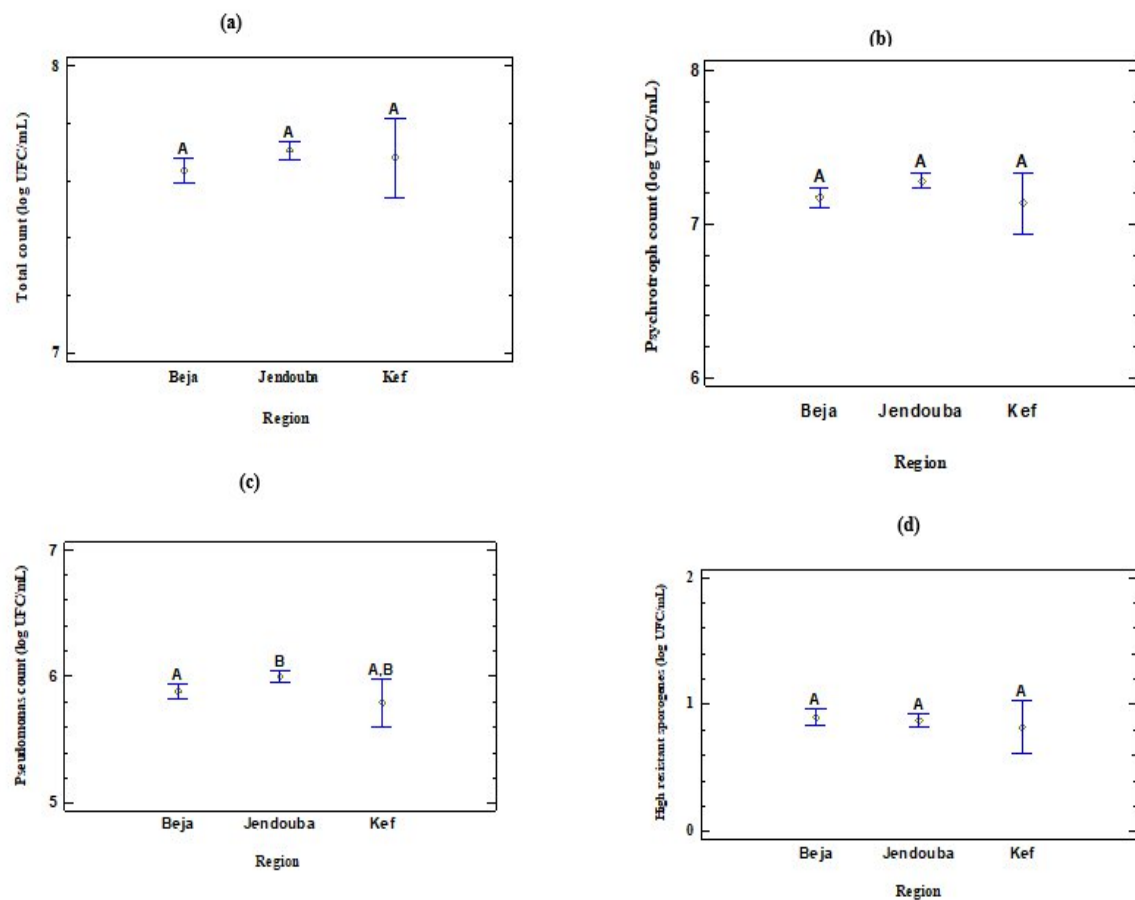


Figure 1. Effect of region on the microbiological quality of raw milk, a) Total aerobic mesophilic count; b) psychrotrophic bacterial count; c) *Pseudomonas* spp. count; (d) heat-resistant spores. Means with different letters are different ($p < 0.05$).

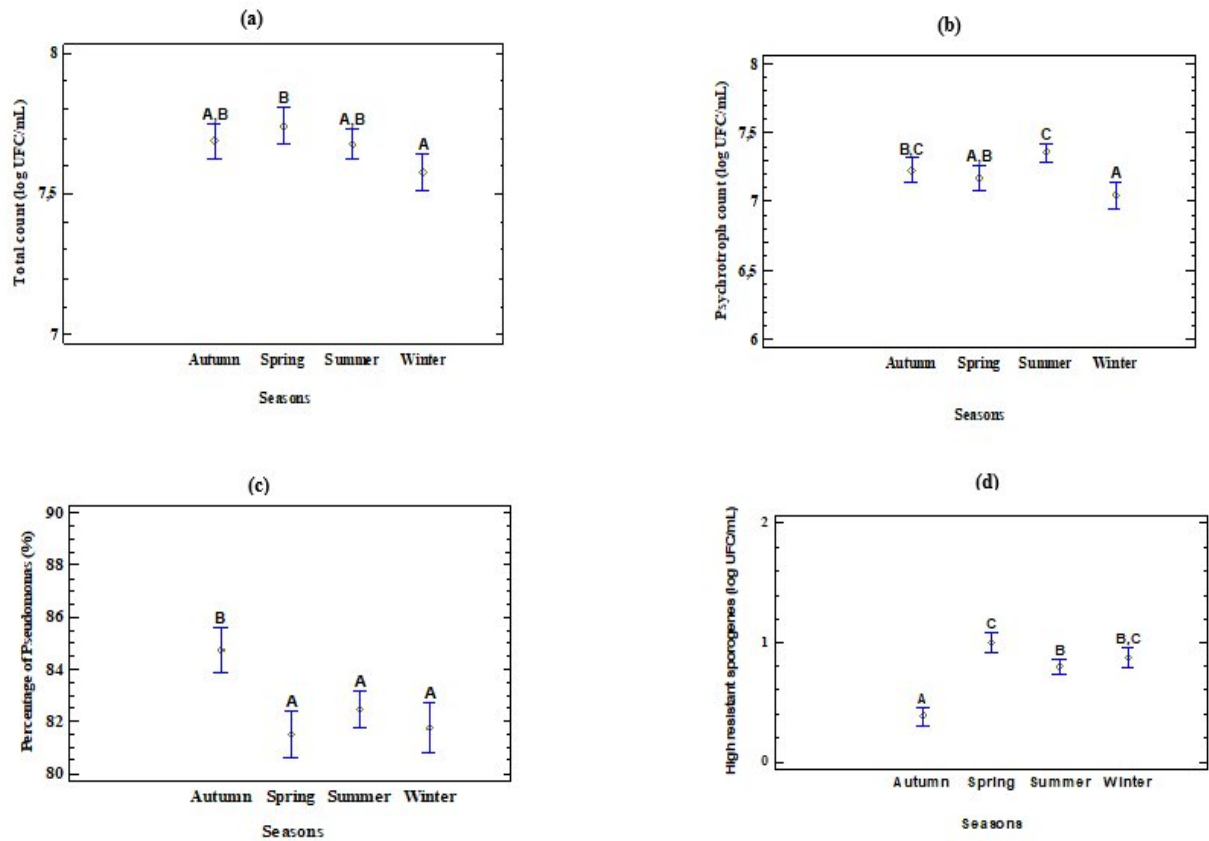


Figure 2. Effect of sampling season on the microbiological quality of raw milk. a) Total aerobic mesophilic count; b) Psychrotrophic count; c) *Pseudomonas* spp. percentage; d) heat-resistant spores. Means with different letters are different ($p < 0.05$).

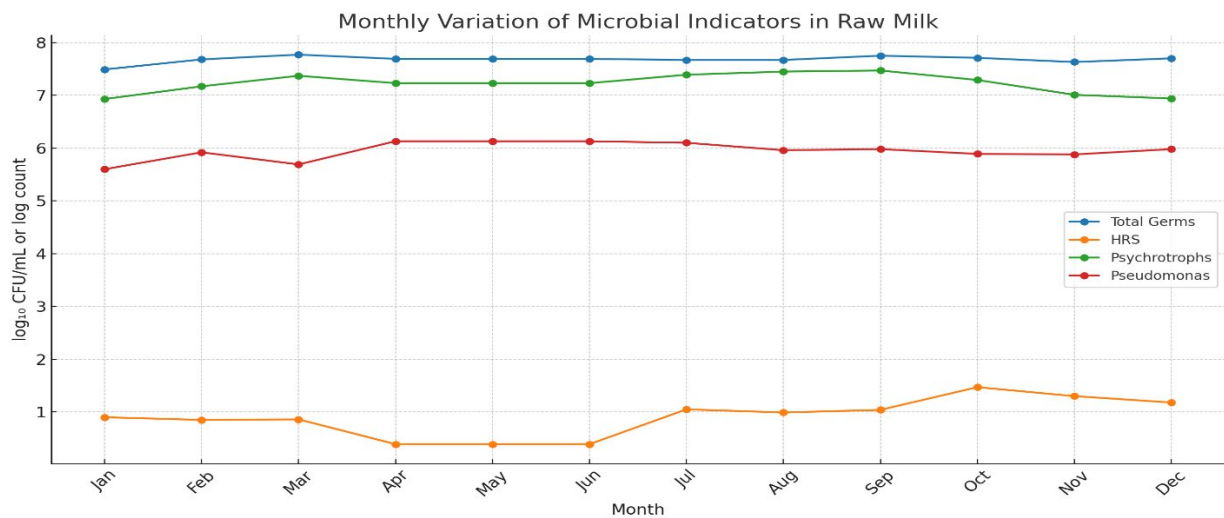


Figure 3. Monthly variation in microbial indicators in raw milk.

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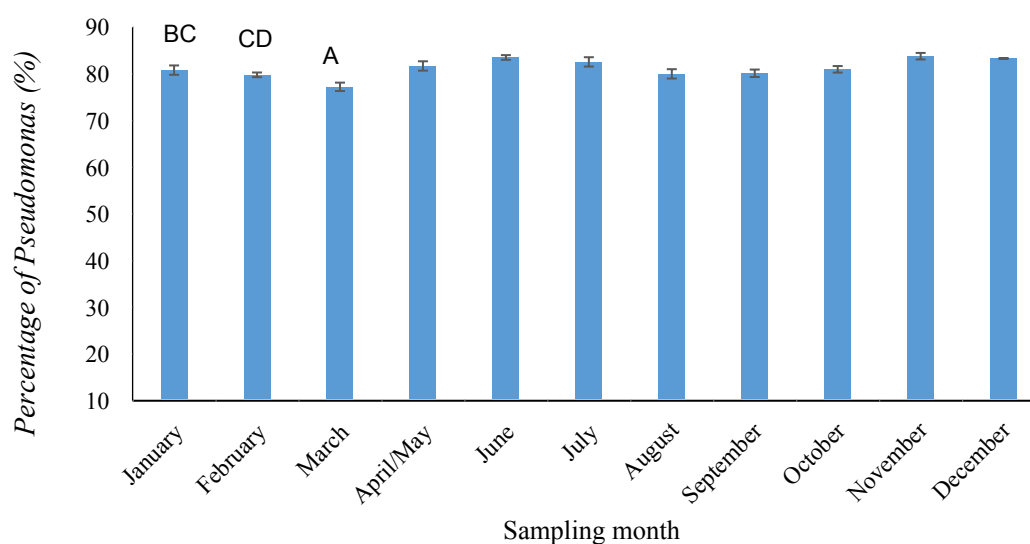


Figure 4. Effect of sampling month on the percentage of *Pseudomonas* spp. in raw milk. Means with different letters are different ($p < 0.05$).

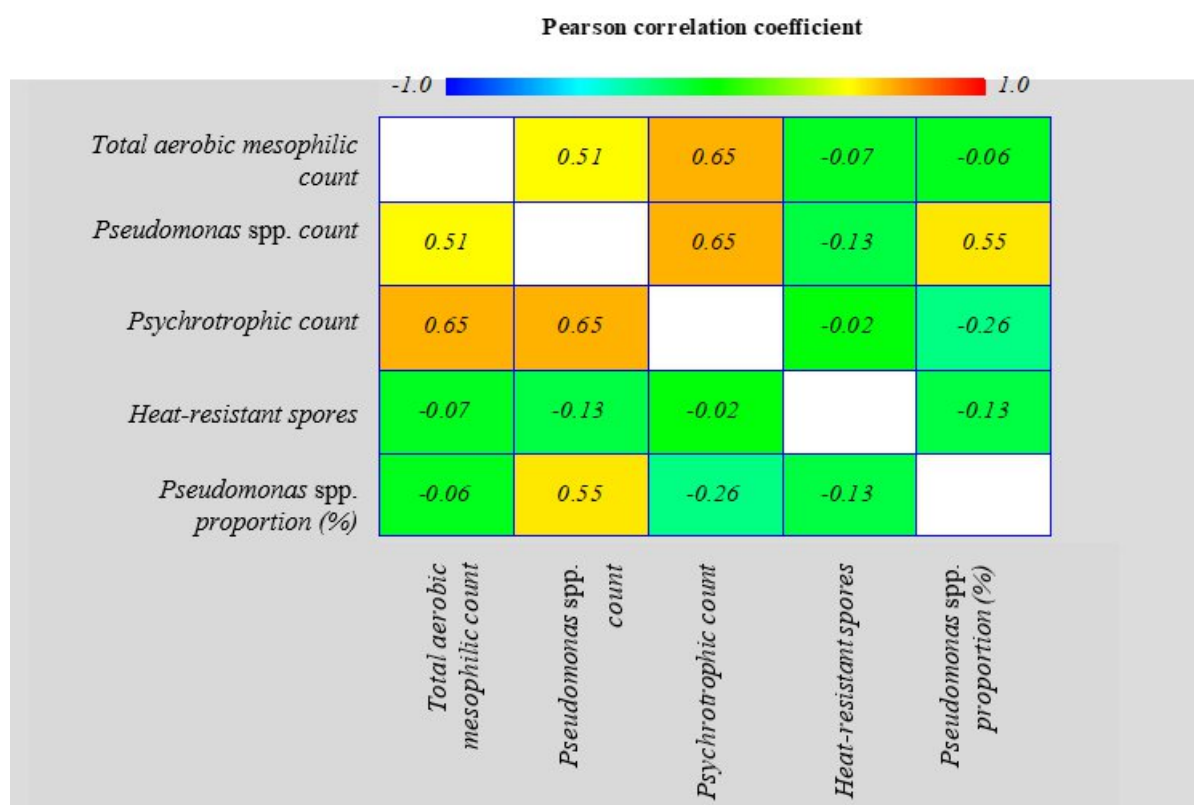


Figure 5. Pearson correlation matrix among microbial indicators in raw milk.