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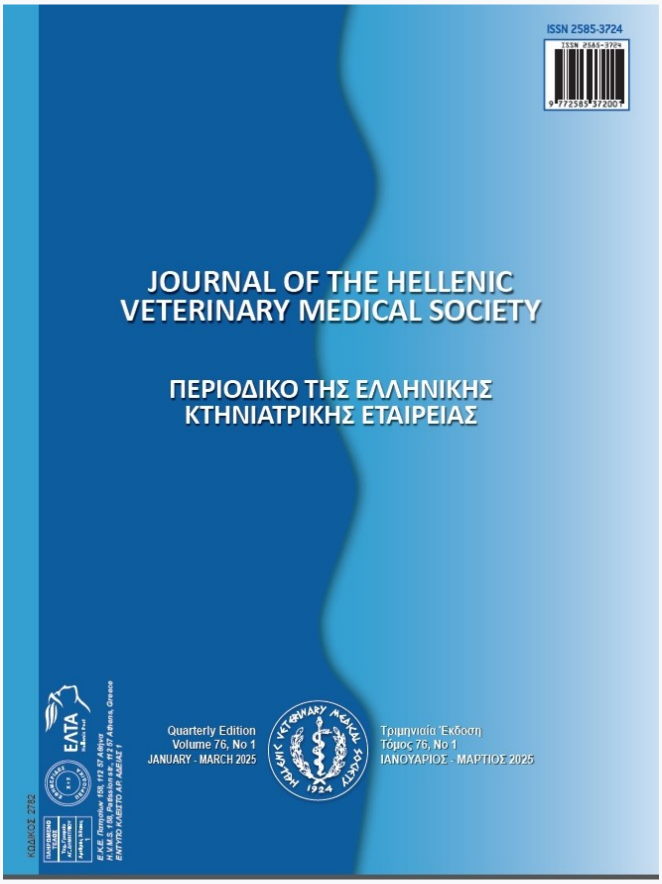

Detection of antibiotic residues in cow's milk using microbiological screening test in Souk Ahras, Algeria

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Research article
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Detection of antibiotic residues in cow's milk using microbiological screening test in Souk Ahras, Algeria

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ABSTRACT : The aim of the present study was to investigate antibiotic residues in raw and fermented cow's milk in Souk Ahras region, Algeria. A total of 78 cow's milk samples were collected, including 46 samples of raw milk from various farms and 32 samples of fermented cow's milk purchased from various points of sale. Samples were examined using a microbiological test (the European four-plate test). Results indicate that only 35 (17/46 raw milk and 18/32 fermented milk) samples were free from antibiotic residues. A high prevalence of antibiotic residues was observed in both raw and fermented milk (63,04%) and (43,75%), respectively. β-lactams and/or tetracyclines were the most detected in both raw and fermented milk, with (47,83%) and (43,75%), respectively. A rate of 30.43% for aminoglycosides, 15.22% for β-lactams and/or macrolides, and 13.04% for sulfonamides were detected in raw cow's milk. However, a rate of 18.75% for macrolides and/or β-lactams, 12,50% for aminoglycosides, and 3.12% for sulfonamides were detected in fermented milk. A rate of 62.07% of raw milk was contaminated with one family of antibiotics. Werehase (64.28%) of fermented milk was positive for two families of antibiotics. This preliminary study allowed us to outline the presence of antibiotic residues in raw and fermented cow's milk in Souk-Ahras region. Failure to adhere to good antibiotic usage practices, particularly regarding the withdrawal periods after treatment, can lead to the presence of residues in animal products, thereby exposing consumers to health risks.

***Keywords*:** Antibiotic residues; *Bacillus subtilis*; Fermented milk; Four plate test; *Micrococcus luteus*; Raw milk.

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INTRODUCTION

Veterinary drugs are wide-spectrum antibiotics utilized in livestock and poultry farms for growth-promoting, therapeutic, and preventive purposes (Alimohammadi et al., 2020; Liu et al., 2024). There are three ways to provide antibiotics to animals: topically, parenterally, or orally. Antibiotics are generally eliminated from an animal's body through urine and, to a lesser extent, excrement (Bacanlı & Başaran, 2019). However, the intensive use of antibiotics may lead to the release of significant portions of antibiotics in foodstuffs such as milk, chicken liver, eggs, and meat (Treiber and Beranek-Knauer 2021; Mouliom Mouiche et al., 2024). If inappropriate practices are applied, the risk of contamination with antibiotic residues is higher; the risk of carcinogenicity, allergic reactions, and imbalances in the intestinal microflora can be registered (Merhi et al., 2023; Mouliom Mouiche et al., 2024). Antibiotic residues may cause serious health problems, contribute to the failure of antibiotic therapy, and be the primary causes of antimicrobial resistance (Ouabdesselam et al., 2020). Maximum residue limits (MRLs) have been set to guarantee that antibiotic residue levels in dairy products remain within safe and acceptable limits for human consumption. However, several studies have reported the underestimation and disregard of the danger posed by these residues (Niu et al., 2023; Liu et al., 2024).

Products that are targeted for food consumption have to be controlled. Several countries have prohibited the residue of veterinary drugs. Despite this, some people illegally administer these drugs to animals, including cows, to enhance milk production for economic gain (Parmar et al., 2021). The emergence of increased contamination levels of antibiotic residues in milk contributes to certain damage to public health (Chen et al., 2019).

Milk is the most consumed product in Algeria. A balanced combination of proteins, lipids, and carbs can be found in cow's milk. It is necessary as food because of its accessibility and low cost (Onyinye et al., 2020).

Microbiological and immunological assays have been widely described for milk analysis; they have been initially developed to detect β-lactams and tetracyclines (Merhi et al., 2023; Liu et al., 2024). The choice is also based on their short time of analysis and low cost (Wu et al., 2021; Butovskaya et al., 2023). However, confirmation of the results obtained using sensitive methods such as HPLC (high performance liquid chromatography) is required in order to determine the type of antibiotic residues and their concentration in the milk (Baazize-Ammi et al., 2019; Alnassrallah et al., 2022; Yazdanpanah et al., 2023).

Few data and studies on this subject are available in Algeria (Baazize-Ammi et al., 2019; Ouabdesselam et al., 2020; Zeghilet et al., 2022). Therefore, the purpose of this work was to assess antibiotic residues in raw and fermented cow's milk produced in our area using the four-plate test in order to have some information about the abusive use of veterinary drugs in dairy cattle.

MATERIALS AND METHODS

Study area and sample collection

In this prospective study, a random sampling methodology was performed. A total of 78 cow's milk samples were collected. Among them, 46 samples of raw milk intended for sale were collected from three dairy farms, whereas 32 samples of fermented cow's milk were purchased from dairy shops in Souk Ahras, Algeria, from January 2024 to March 2024. The samples were collected in sterile laminated polyesthylene test tubes after registration and specifications, and then transported into an ice box.

Reference strains

The detection of antibiotic residues in milk was conducted using the European four-plate test (FPT). This qualitative microbiological method is based on inhibition zone formation around samples in four culture media with different pHs, utilizing specific reference bacterial strains (Onyinye et al., 2020).

The efficacy of this method lies in its capacity to detect antibiotic levels exceeding the recommended maximum residue limits (MRLs) for various antibiotic families, including β-lactams, tetracyclines, macrolides, aminoglycosides, and sulfonamides (Titouche et al., 2022). Bacteria used in this study were *Bacillus subtilis* ATCC 6633 and *Micrococcus luteus* ATCC 9341, as previously described (Alimohammadi et al., 2020). These bacteria were cultured in a nutrient agar medium for revivification and incubated at 37 °C for 24 h.

Microbiological test

The culture medium used for screening was Mueller Hinton Agar, prepared according to the manufacturer's instructions and then heated with a magnetic stirrer until boiling. The pH was adjusted to 6, 7.2, and 8 using a digital pH meter with sodium hydroxide and acetic acid. The mixture was sterilized in an autoclave at 121 °C for 15 minutes. After cooling to 45° C, 25 cc of the culture medium was poured into each Petri dish. A suspension of 0.5 McFarland of *Bacillus subtilis* and *Micrococcus luteus* was then prepared in 9 mL of physiological saline solution. *Micrococcus luteus* was cultured at pH 8, while *Bacillus subtilis* was cultured at pH levels of 6, 7.2, and 8, using cotton swabs under sterile conditions to inoculate the plate surface (Alimohammadi et al., 2020). Samples were then impregnated onto blotting discs (9 mm), and placed on Mueller Hinton agar, and the disc impregnated with the control solution containing the known antibiotic was placed at the center of the plate. Plates were then incubated at 37°C for 24 hours for *Micrococcus luteus* and at 30°C for 24 hours for *Bacillus subtilis*. The blank disc soaked in the control solution should exhibit an inhibition zone. These solutions, containing known antibiotics (Penicillin, and erythromycine) serve as positive controls, and ensure regular adherence to operating conditions, prepared as previously described (Butovskaya et al., 2023). Samples were considered positive for antibiotic residues if the inhibition zone was not less than 2 mm. The possible classes of antimicrobials detected are β-lactams and/or tetracyclines at pH 6; sulfonamides at pH 7.2, and aminoglycosides at pH 8 with *Bacillus subtilis*. Additionally, β-lactams and/or macrolides are detected at pH 8 with *Micrococcus luteus* (Titouche et al., 2022).

Data Analysis

Descriptive statistics were utilized to outline the fundamental characteristics of the data in this study.

RESULTS

Antibiotic residues in raw and fermented cow's milk

The study shows that out of the 46 raw cow milk samples analyzed, 29 samples were contaminated with antibiotic residues. The overall contamination rate was 63.04% (Table 1).

Regarding fermented cow's milk, the study shows that out of 32 samples analyzed, 14 were contaminated with antibiotic residues. The overall contamination rate was 43.75% (Table 1).

Possible families of antimicrobials detected in raw milk using FPT

This study shows that out of 29 positive samples of raw milk analyzed, 22 samples were contaminated with β-lactams and/or tetracyclines, resulting in a contamination rate of 47.83%. This was followed by 14 samples contaminated with aminoglycosides, with a rate of 30.43% and, then seven samples contaminated with macrolides and/or β-lactams, resulting in a rate of 15.22%. Finally, there were six positive samples for sulfonamides, with a rate of 13.04% (Figure 1).

Possible families of antimicrobials detected in fermented cow's milk using FPT

In fermented cow's milk, β-lactams and/or tetracyclines exhibited the highest prevalence among antibiotic families, accounting for 43.75%. This was followed by 8 samples contaminated with aminoglycosides, with a rate of 12,50% and, then six samples contaminated with macrolides and/or β-lactams, resulting in a rate of 18.75%. Sulfonamides had the lowest occurrence at 3.12% (Figure 1).

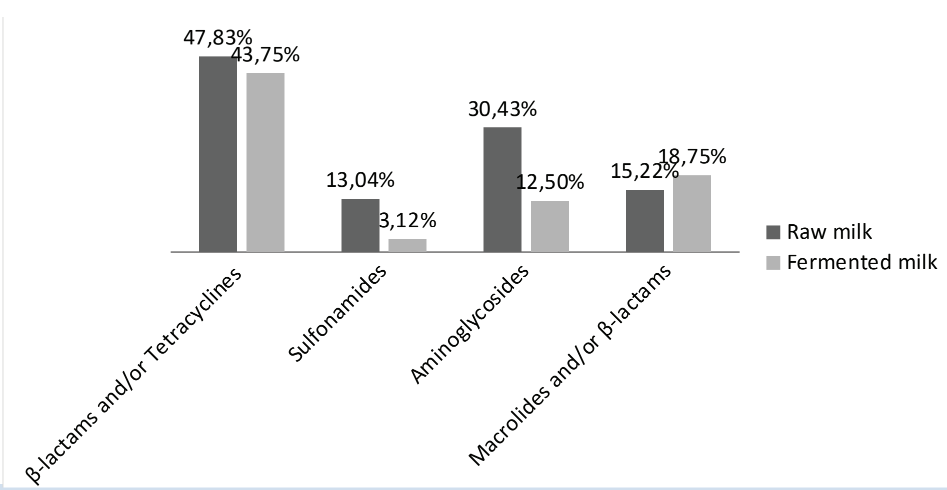

Figure 1. Possible families of antimicrobials detected in raw and fermented cow's milk.

Table 1. The rate of antibiotic residues contamination in raw and fermented milk.

Analysis of antibiotic residues	Milk samples	
	Raw milk (%)	**Fermented milk (%)**
Number of tested samples	46	32
Contaminated with antibiotic residues	29 (63.04%)	14 (43.75%)
Free from antibiotic residues	17 (36.96%)	18 (56.25%)

Table 2. Frequency of contamination of raw and fermented cow's milk by antibiotic residues.

Analysis of antibiotic residues	Milk samples	
	Raw milk (%)	**Fermented milk (%)**
Contaminated samples	29	14
With one family of antibiotics	18 (62.07%)	4 (28.57%)
With two families of antibiotics	4 (13.79%)	9 (64.28%)
With three families of antibiotics	5 (17.24%)	1 (7.14%)
With four families of antibiotics	2 (6.90%)	0 (0%)

Frequency of contamination of raw and fermented cow's milk by antibiotic residues

Our analysis of antibiotic residue detection frequency revealed that in raw cow's milk, 18 out of 29 samples (62.07%) tested positive for one family of antibiotics, 13.79% for two families, 17.24% for three families, and 6.90% for four families. In contrast, in fermented cow's milk, 28.57% of the samples tested positive for one family of antibiotics, 64,28% tested positive for two families,and finally 7.14% tested positive for three families (Table 2).

DISCUSSION

The abusive and uncontrolled use of antibiotics in animal-derived foodstuffs such as milk poses a threat to human health. To effectively monitor a high volume of milk samples for residues exceeding the levels specified by community regulations, low cost screening methods are required (Layada et al., 2016). In practical applications, microbiological screening methods are predominantly utilized due to their superior cost-effectiveness compared to physical-chemical detection methods. The microbial inhibitor tests, widely available commercially, are the most commonly used (Layada et al., 2016; Titouche et al., 2022).

In this study, the European four-plate test (FPT) was chosen as a tool for screening antibiotic residues in milk samples collected in Souk Ahras, Algeria. This qualitative microbiological method is based on inhibition zone formation around samples in four culture media with different pH levels, employing reference bacterial strains (Alimohammadi et al., 2020). The pH of the medium influences the activity of certain antimicrobial substances. For example, aminopenicillins and tetracyclines become more effective in acidic conditions, while macrolides, and aminoglycosides are more active in alkaline conditions. The mechanisms behind the effect of pH on antimicrobial activity are not fully understood and vary between drugs. However, some argue that screening tests, particularly plate tests, detect antimicrobial presence only when their concentrations exceed the maximum residue limits (MRLs) (Yamada et al., 1981; Titouche et al., 2022).

The main results obtained in this study revealed that 63.04% of the raw milk samples were positive. Furthermore, of the fermented cow's milk samples collected, 43.75% were positive. Our study was carried out in the winter, and antibiotic residues are more prevalent in the winter compared to other seasons. This can be explained by the weather and the practice of housing livestock indoors. The cold weather and shorter days of winter result in livestock being kept indoors more frequently, which increases the incidence of disease and subsequently leads to higher antibiotic usage for treatment (Alimohammadi et al., 2020).

In raw milk, the highest antibiotic residues belong to β-lactams and/or tetracyclines (47.83%), and aminoglycosides (30.43%). This contamination can be attributed to the use of these antibiotics, which are known for their broad spectrum of action against various diseases and their low toxicity. The low cost of β-lactams in Algeria ensures their widespread availability. Consequently, private farmers in remote areas often use penicillin without veterinary supervision (Layada et al., 2016). Similar studies conducted in our country, employing either the same methods or more advanced ones, exhibit a diversity of results (Baazize-Ammi et al., 2019; Ouabdesselam et al., 2020; Meklati et al., 2022; Zeghilet et al., 2022). Although these studies use another microbiological screening test such as *Premi®Test* or *Delvotest®*, the presence of tetracyclines and β-lactams remains the most frequently detected (Meklati et al., 2022). On the other hand, lower incidence was observed in 117 samples of raw cow's milk and 33 samples of raw goat's milk. The milk test results indicated contamination in 12.6% of the samples. β-lactams and tetracyclines were detected in 26.32% and 15.79% of the samples analyzed, respectively (Baazize-Ammi et al., 2019).

In our study, in fermented cow's milk, β-lactams and/or tetracyclines are also the most prevalent antibiotic family, comprising 43.75%. This contamination can be attributed to the widespread and improper use of veterinary antibiotics, as well as the disregard for the necessary time interval between administering antibiotics to animals and collecting milk. Nonetheless, higher rates have been reported recently from Béjaia, Penicillin residues and/or tetracyclines initially accounted for 75% of the positive milk sample contamination, whereas macrolides and/or aminoglycosides residues were detected in only 25% of the positive samples tested (Ouabdesselam et al., 2020). On the other hand, lower occurrence rates were reported in Constantine. The findings reveal that 13 samples (10.66%) were found positive for antibiotic residues: 12 (9.84%) for β-lactams (10 (8.20%) from raw milk and two (1.64%) from fermented milk samples), and only one (0.82%) for tetracyclines in a raw milk sample (Zeghilet et al., 2022).

The detection of antimicrobials at different pH levels in the study suggests that different classes of antimicrobials were administered to cows at the same time. These differences may arise from several factors, including compliance with withdrawal periods, concentration and type of antibiotics, inadequate identification of cows under treatment, and farmers' insufficient awareness regarding the residual effects of antibiotic residues in milk on human health (Sachi et al., 2019). Additionally, their selection is influenced by their short time of analysis and low cost (Wu et al 2021; Butovskaya et al 2023). Nevertheless, verifying the results with sensitive techniques such as high performance liquid chromatography (HPLC) is necessary to identify the type and concentration of antibiotic residues in the milk ((Baazize-Ammi et al., 2019; Alnassrallah et al., 2022; Yazdanpanah et al., 2023).

CONCLUSION

The findings of this study clearly demonstrate a significant level of contamination in raw and fermented cow's milk samples. All sampled products from both raw and fermented cow's milk contained antibiotic residues. The contamination rate is higher in raw milk compared to fermented milk. This contamination can be attributed to the widespread and improper use of veterinary medications, as well as the disregard for the necessary time interval between administering antibiotics to animals and collecting milk. This also indicates a deficiency in public health regulations and provides evidence of negligent antibiotic usage in livestock, both of which pose a risk to public health. Specific measures need to be implemented, including respecting withdrawal periods before milk collection and establishing regular and systematic screening of milk for antibiotic residues before consumption or processing. Additionally, further research needs to be conducted in order to identify antibiotic residues in milk using analytical methods such as high-performance liquid chromatography. This approach will not only identify the type of antibiotic residues present but also determine their concentration in the collected milk.

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CONFLICT OF INTEREST

The authors have declared no conflicts of interest for this article.

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