



Microbiological and Biochemical Characterization of Pasteurized Cow's Milk

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Information	Abstract
Keywords: Pasteurized milk ,Bacteria, Spores, Bacillus, Heat-resistant Correspondence: mohamedhaddouche240@gmail.com	Algeria is considered one of the largest milk consumers in the Maghreb region. Spore-forming bacteria, particularly those of the <i>Bacillus</i> genus, are among the most significant contaminants in milk. The objective of this study was to evaluate the microbiological quality of pasteurized cow's milk. Five samples were collected from a dairy in the Ain Temouchent region and analyzed at an agri-food analysis laboratory in the same area. The results indicated that the total aerobic mesophilic flora ranged from 14×10^2 to 61×10^2 CFU/mL, while heat-resistant flora counts ranged from 110 to 350 CFU/mL. A total of 14 bacterial strains were isolated. Phenotypic identification revealed that 85.71% of the isolates were Gram-positive and 14.29% were Gram-negative. Additionally, 71.42% of the strains were bacilli and 28.58% were cocci. Enzymatic profiling of the bacilli showed that 50% were capable of hydrolyzing starch (amylolytic activity), 70% could hydrolyze casein (proteolytic activity), and 20% exhibited lecithinase activity. Further biochemical testing using the API 20E test kit identified four bacterial species: <i>Geobacillus thermoglucosidasius</i>, <i>Bacillus cereus</i>, <i>Bacillus licheniformis</i>, and <i>Bacillus subtilis</i>. These findings, in line with previous research, confirm that pasteurization does not effectively eliminate heat-resistant endospores, underscoring the need for improved control measures in dairy processing.

1. Introduction

Dairy products, including milk, cheese, yogurt, and butter, are integral to human nutrition, providing essential nutrients such as proteins, carbohydrates, lipids, minerals, and vitamins. In Algeria, dairy consumption is notably high, with per capita intake averaging 145 liters annually, surpassing regional figures in Tunisia (90 liters) and Morocco (66 liters) (Sustainable Agri Trade, 2024). Despite this, Algeria's domestic milk production meets only about 56% of its needs, resulting in a substantial reliance on imported milk powder (Dairy Business Africa, 2024).

Spore-forming bacteria, especially those belonging to the *Bacillus* genus, are significant milk contaminants. Due to their strong resistance to heat, *Bacillus* spores can withstand processes like pasteurization and may cause spoilage in the finished dairy product (Kmiha et al., 2017). Species such as *Bacillus licheniformis*, *Bacillus cereus*, and *Bacillus subtilis*, are common spoilage agents in dairy products. Their spoilage activity is primarily attributed to the enzymatic functions of these contaminating strains (Christieans & Zagorec, 2013, p. 52).

In Algeria, reconstituted pasteurized milk, a widely consumed beverage, is typically subjected to high temperature pasteurization at 85°C for 5 to 10 minutes. However, despite this treatment, bacterial contamination levels often remain high, leading consumers to reboil the milk before consumption (Malek et al., 2012).

This study aims to evaluate the microbiological quality of pasteurized cow's milk by screening for the presence and diversity of spore-forming bacteria in products from a dairy processing facility in the Ain Temouchent region. By identifying and characterizing these microorganisms, this work seeks to support improved safety and shelf life of dairy products in Algeria.

2. Materials and Methods

2.1. Sampling Methodology

Five samples of pasteurized cow's milk were collected in October 2024 from a dairy located in the Ain Temouchent region of western Algeria. The samples, packaged in pasteurized milk bags, were stored in a cooler during transport to the Agri-Food Analysis Laboratory in Ain Temouchent. Upon arrival, they were stored at 5 °C for three days prior to microbiological and biochemical analyses.

2.2. Enumeration of Total Aerobic Mesophilic Flora

Serial dilutions were prepared from 10^{-1} to 10^{-8} by transferring 1 mL of the stock solution into 9 mL of Ringer's solution. From each dilution, 1 mL was aseptically transferred to a Petri dish containing Plate Count Agar (PCA), followed by gentle mixing of the medium. The plates were incubated at 37°C for 72 hours. Only plates with 30 to 300 colonies were considered for colony-forming unit (CFU) enumeration.

2.3. Enumeration of Heat-Resistant Flora

A stock solution of the milk samples was heat-treated at 80°C for 10 minutes to eliminate non-heat-resistant organisms. Serial dilutions were then prepared up to 10^{-8} using Ringer's solution. From each dilution, 100 µL was spread onto the surface of two PCA plates. The plates were incubated at 37°C for 48 hours.

Following incubation, 5 to 7 morphologically distinct colonies were selected from each plate for isolation. Each selected colony was streaked onto nutrient agar and incubated at 37°C for 24 hours. This process was repeated to obtain pure isolates.

2.4. Phenotypic Identification of Heat-Resistant Strains

2.4.1. Morphological Characterization of Isolates

A. Macroscopic Characteristics

Colony morphology, including size, shape, surface texture, opacity, consistency, and color, was evaluated after incubation on nutrient agar at 37°C for 24–48 hours.

B. Microscopic Characteristics

Cell morphology, arrangement, and Gram reaction were assessed using Gram staining of young cultures, examined under an optical microscope.

2.4.2. Biochemical Characterization of Isolates

A. Amylolytic Activity

Starch hydrolysis was tested by streaking isolates onto starch agar. Plates were incubated at 37°C and observed every 24 hours for up to 72 hours. Iodine (Lugol's solution) was used to reveal hydrolysis zones. A clear halo around the colonies indicated starch degradation; areas with intact starch turned brown.

B. Proteolytic Activity

- **Casein Hydrolysis**

Isolates were streaked onto milk agar and incubated at 37°C for up to 72 hours. A clear halo around the colonies indicated casein degradation.

- **Lecithin Hydrolysis**

An emulsion of 10 mL egg yolk in 90 mL of Tryptone Soy Agar (TSA) was prepared and poured into Petri dishes at ~45°C. After solidification, strains were streaked centrally. Plates were incubated at 37°C for 24–72 hours. The appearance of a clear zone around the streak indicated lecithinase activity.

2.5. Characterization Using the API 20E Test Kit

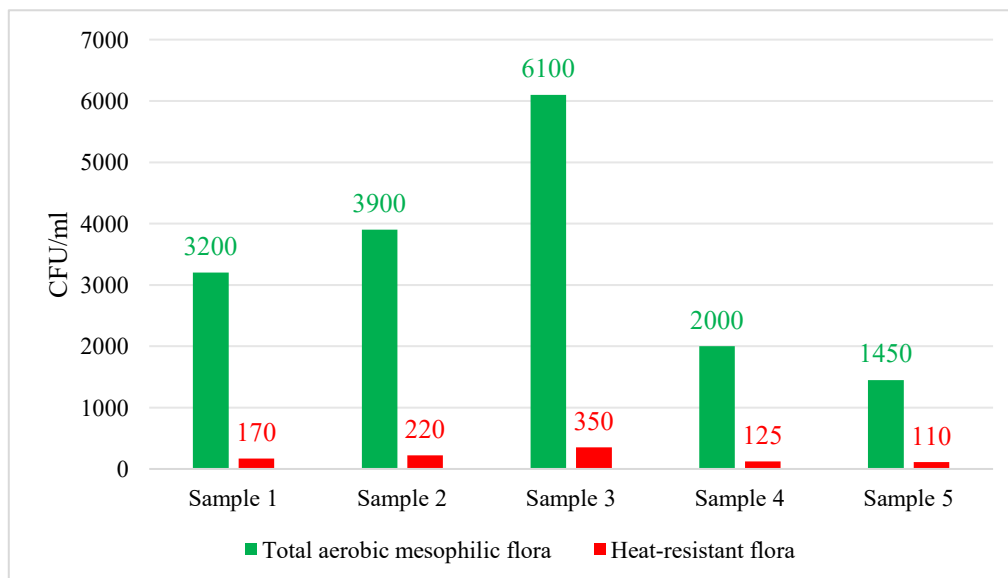
Bacterial suspensions were prepared in 0.85% physiological saline from 18-hour cultures. These suspensions were inoculated into the microtubes of the API 20E strip and incubated at 37°C for 24 hours. Biochemical reactions were determined by spontaneous color changes or the addition of specific reagents.

3. Results

3.1. Enumeration of Total Aerobic Mesophilic Flora and Heat-Resistant Flora

The aerobic mesophilic bacterial counts in the five milk samples ranged from 1450 to 6100 CFU/mL. Heat-resistant bacterial counts ranged from 110 to 350 CFU/mL (**Figure 1**).

Figure 1. Enumeration results of total aerobic mesophilic flora and heat-resistant flora in pasteurized cow's milk samples.



3.2. Phenotypic Identification of Heat-Resistant Strains

A. Morphological Characteristics of Isolates

Colonies exhibiting distinct macroscopic appearances were observed after 48 to 72 hours of incubation on PCA medium. Following purification through repeated streaking, 14 isolates were selected from nutrient agar based on predefined morphological criteria.

Microscopic examination after Gram staining revealed that the majority of isolates were Gram-positive (85.71%), while 14.29% were Gram-negative. The bacterial cells displayed various shapes and arrangements. Among the isolates, 71.42% were bacilli and 28.58% were cocci (Table 1).

Table 1. Microscopic characteristics of heat-resistant bacterial isolates.

Strains	Gram	Shapes	Arrangements
1	-	Coccus	Clusters
2	-	Coccus	Chains and irregular clusters
3	+	Coccus	Pairs and clusters
4	+	Bacillus	Clusters
5	+	Bacillus	Chains
6	+	Bacillus	Chains
7	+	Coccus	Chains and irregular clusters
8	+	Bacillus	Chains

9	+	Bacillus	Chains
10	+	Bacillus	Pairs and chains
11	+	Bacillus	Pairs
12	+	Bacillus	Chains
13	+	Bacillus	Chains
14	+	Bacillus	Clusters

B. Biochemical Characteristics of Isolates

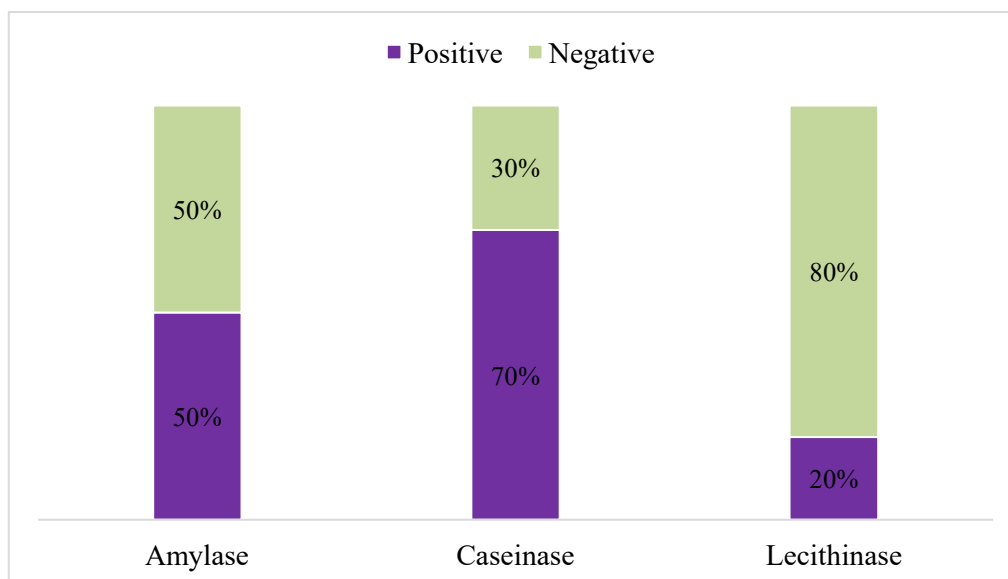
The results of the proteolytic and amylolytic activity tests are presented in **Figure 2**.

The starch hydrolysis assay showed that 50% of the isolates produced amylase, indicating starch-degrading capability, while the remaining 50% showed no activity.

In the casein hydrolysis test, 70% of the isolates were positive for caseinase production, whereas 30% did not exhibit proteolytic activity.

The lecithin hydrolysis test revealed that 20% of the isolates produced lecithinase, while 80% showed no enzymatic activity.

Figure 2. Enzymatic activity profiles of heat-resistant bacterial isolates (amylase, caseinase, and lecithinase).



3.3. API 20E Test Kit Results

Additional biochemical identification was performed using the API 20E test kit, leading to the identification of four bacterial species: *Geobacillus thermoglucosidasius*,

Bacillus cereus, *Bacillus licheniformis*, and *Bacillus subtilis*. The isolate distribution is as follows:

- S6, S8, and S10: *Geobacillus thermoglucosidasius*
- S5 and S13: *Bacillus cereus*
- S4, S9, S11, and S12: *Bacillus licheniformis*
- S14: *Bacillus subtilis*

4. Discussion

The results showed that the total aerobic mesophilic flora ranged between 1.4×10^3 and 6.1×10^3 CFU/mL. According to Calvo and Olano (1992), total mesophilic flora in milk collected under appropriate hygienic conditions should not exceed 10^3 to 10^4 CFU/mL.

The counts of heat-resistant flora ranged from 110 to 350 CFU/mL. Minerals naturally present in milk, such as magnesium, calcium, and potassium, play an important role in sporulation and may promote the formation of *thermophilic bacilli* spores during dairy processing (Burgess et al., 2010).

A study by Aladhadh and Abdallah (2025) reported that the mean total bacterial count after three days of storage was 3.75×10^4 CFU/mL in raw cow's milk and 0.5×10^3 CFU/mL in pasteurized cow's milk. After 17 days of storage, the bacterial count increased to 3.15×10^6 CFU/mL in raw milk and 4.06×10^6 CFU/mL in pasteurized milk.

From the five pasteurized milk samples analyzed, 14 bacterial strains were isolated. Gram staining and microscopic analysis revealed that 85.71% of the isolates were Gram-positive, while 14.29% were Gram-negative. Furthermore, 71.42% of the isolates were bacilli, and 28.58% were cocci. Bacilli are typically Gram-positive and capable of forming heat-resistant spores; however, certain species can appear Gram-negative as they age (Turnbull, 1996).

Biochemical testing showed that 50% of the isolates exhibited amylolytic activity, 70% showed caseinolytic activity, and 20% were positive for lecithin hydrolysis. Amylases in milk exist in two forms: α -amylase (dextrinizing activity) and β -amylase

(saccharifying activity). β -amylase is more heat-resistant, with 90% inactivation occurring after heating at 65°C for 225 seconds or at 80°C for 26.8 seconds (Deeth, 2021).

Lecithinase and caseinase activities are recognized as indicators of potential food toxicity (Sharaf et al., 2014).

Further biochemical identification via API 20E confirmed the presence of *Geobacillus thermoglucosidasius*, *Bacillus cereus*, *Bacillus licheniformis*, and *Bacillus subtilis*. Similar findings were reported by Rückert et al. (2004), who identified *Bacillus licheniformis* and *Geobacillus stearothermophilus*. Biofilms formed by mesophilic strains of the *Bacillus cereus* group have been shown to resist standard cleaning protocols and contribute to persistent contamination of pasteurized milk and dairy equipment (Malek, 2019).

Enzymes such as hydrolases secreted by *Bacillus subtilis* are primary agents of spoilage in pasteurized milk. They degrade milk proteins and lipids, resulting in off-flavors and increased acidity (Yang et al., 2023).

5. Conclusion

Milk is a nutrient-rich medium that provides microorganisms with favorable conditions for growth. Our results, consistent with previous research, demonstrate that pasteurization is not entirely effective at eliminating heat-resistant bacterial endospores.

Given the increasing concerns surrounding food safety and quality, a deeper understanding of microbial traits related to food origin, identity, and resilience is essential. This knowledge can inform improved strategies for controlling spores, ultimately helping to reduce microbial spoilage and minimize food loss.

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