Title: COLORIMETRIC EVALUATION AND COMPARATIVE ANALYSIS OF BACTERIAL CONTAMINATION IN RAW VS. PASTEURIZED MILK USING SUDAN III-TREATED INDICATOR STRIPS

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Abstract

Milk is a vital component of the human diet but remains highly susceptible to bacterial contamination during collection, processing, and storage. This study investigates the effectiveness of a novel, colourimetric biosensor, Sudan III-treated indicator strips, for detecting bacterial contamination, specifically lipoproteins, in raw and pasteurised milk. Raw milk samples, known to harbour diverse microbial populations, were analysed and compared with pasteurised counterparts to assess the impact of heat treatment on microbial presence and lipid content. The research involved isolating bacterial strains from raw milk, identifying them through Gram staining, and extracting lipoproteins from their cell membranes. These were then subjected to colourimetric analysis using the Sudan III reagent. The results demonstrated a stronger lipid-associated colour change in raw milk samples compared to faded or absent reactions in pasteurised milk, highlighting a marked reduction in bacterial lipoproteins post-pasteurisation. This visual, rapid, and cost-effective method offers a promising alternative for microbial quality screening in dairy products, particularly in settings with limited laboratory infrastructure. The study emphasises the continued relevance of contamination detection even after pasteurisation and supports the integration of simple biosensor tools for improved dairy safety monitoring.

Keywords: Milk contamination, Sudan III, Pasteurisation, Lipoproteins, Colourimetric detection

1. Introduction:

Cow's milk is one of the most commonly consumed dairy products worldwide, but it is prone to contamination from multiple sources, such as chemical and microbial agents **(1).** This work explores the primary contaminants found in cow's milk, their origins, and their potential health impacts. It also compares the effectiveness of pasteurisation with other milk treatment methods. Despite being the most commonly used method, pasteurisation has not proven fully effective in eliminating contaminants **(2)**. Milk is exposed to microbial contaminants during collection and processing. While pasteurisation is an effective antimicrobial method that has helped reduce the incidence of many diseases, outbreaks related to pasteurised milk still occur **(3)**. These are often linked to the presence of high levels of microorganisms in raw milk, which survive the pasteurisation process, or to post-pasteurisation contamination **(4)**. A significant concern with pathogens in pasteurised milk is that they can persist without altering the milk's appearance, taste, or smell, posing a hidden health risk and revealing some limitations of pasteurisation.

Both pasteurised and raw cow's milk have been found to contain various pathogenic microorganisms. Contamination in raw milk can arise from factors such as mastitis in cows, poor handling practices on farms, contaminated milking equipment, water sources, cattle feed, and storage containers during transport **(5)**. Inadequate cleaning can lead to biofilm formation on instruments and increase the risk of contamination when pasteurised milk comes into contact with these surfaces **(6)**. Raw milk is more prone to contamination due to suboptimal milking, processing, storage, and transport conditions. Although there are fewer reports of microorganisms in pasteurised milk, the process may not effectively eliminate all pathogens. The main microbial contaminants include bacteria, yeasts, and Moulds. Pathogens such as *Corynebacterium*, *Staphylococcus*, *Streptococcus*, *Bacillus*, and *Micrococcus* species have been detected on dairy cattle teats, indicating that contamination can occur during milking under poor hygienic conditions. Additionally, *Salmonella typhi*, *Enterobacteriaceae*, *Pseudomonas* spp., and lactic acid bacteria have been found on milking equipment, highlighting that farm conditions may not be optimal for producing clean milk **(7)**. Typhoid and paratyphoid fever are generally recognised as food-borne and water-borne illnesses, but milk-borne infections have also been reported by *Salmonella Typhi* and *paratyphi* **(8)**. The concept of pasteurisation is named after the French scientist Louis Pasteur, who studied the spoilage of milk and other foods caused by the growth of undesirable microorganisms. Pasteurisation became a common practice in the late 1880s to combat *Mycobacterium tuberculosis*, the bacterium responsible for tuberculosis in humans, which was linked to raw milk consumption. After World War II, pasteurisation became a standard process in developed countries, aiming to eliminate pathogenic microorganisms and extend the shelf life of milk, typically to 10–14 days **(9)**. Early pasteurisation methods involved heating milk to 63–65°C for 30 minutes, followed by rapid cooling—a process known as low-temperature, long-time (LTLT) pasteurisation. While still used by small-scale processors, LTLT has largely been replaced since the 1940s by high-temperature, short-time (HTST) pasteurisation, where milk is heated to 72–74°C for at least 15 seconds using continuous-flow plate heat exchangers **(10)**. In the United States, HTST is the predominant pasteurisation method. Ultra-high temperature (UHT) treatments, which heat milk to 138–150°C for 2–6 seconds, are used for products like coffee creamers, producing nearly sterile products. Pasteurisation destroys most milk enzymes, with the loss of alkaline phosphatase (ALP) serving as an indicator to confirm proper heat treatment **(11)**. Post-pasteurisation contamination (PPC) arises from microorganisms capable of growing at refrigeration temperatures. These are mainly Gram-negative bacteria that cause spoilage and can exceed the Pasteurised Milk Ordinance (PMO) limit of 20,000 CFU/mL **(12)** before psychrotolerant spore formers begin to grow, often outcompeting them. Common PPC-causing bacteria that thrive under cold storage include psychrotolerant *Enterobacteriaceae*, coliforms, and *Pseudomonas* species **(13)**. These microbes produce enzymes that lead to undesirable changes in milk's flavour, Odor, and texture, ultimately impacting consumer satisfaction and purchase decisions **(14)**. Detecting PPC in freshly pasteurised milk processed by high-temperature, short-time methods is difficult, as contamination tends to occur sporadically and at low concentrations. Moreover, traditional indicator organisms such as coliforms represent only a small portion of total PPC. Research suggests that coliforms make up less

than 20% of the gram-negative bacteria introduced after pasteurisation, whereas *Pseudomonas*—a non-coliform genus not detected by standard coliform tests—is most frequently found in PPC milk **(15)**. To minimise PPC, dairy processors should implement testing methods that identify both coliform and non-coliform gram-negative bacteria (such as *Pseudomonas*), enabling a more accurate assessment of contamination sources. Additionally, establishing effective cleaning, sanitation, and management practices is essential to control persistent and transient PPC organisms **(16)**. Traditional microbiological techniques such as plate counting and biochemical identification, while reliable, are often time-consuming and labour-intensive. These methods require trained personnel and laboratory infrastructure, making them less feasible for quick on-site quality checks, particularly in rural dairy farms or small-scale industries **(17)**. To address these limitations, researchers have developed rapid colourimetric, biosensor-based, and molecular approaches for detecting contamination **(18)**. Among these, colourimetric methods stand out for their simplicity, affordability, and ease of visual interpretation.
Methods approved for coliform enumeration in grade 'A' pasteurised fluid milk include:

- Crystal violet tetrazolium agar (CVTA)
- Coliform plate count on violet red bile agar (VRBA)
- Petri film Coliform Count or High Sensitivity Coliform Count
- TEMPO CC-Coliform Count
- Peel Plate *E. Coli* and Coliform High-Volume Sensitivity **(19)**

Although these methods are approved for detecting coliforms, they fail to identify all instances of post-pasteurisation contamination (PPC) because they cannot detect lactose non-fermenting bacteria such as *Pseudomonas*, which make up a major portion of PPC. In contrast, pour plating using crystal violet tetrazolium agar (CVTA) demonstrated the greatest detection efficiency for PPC, as confirmed through end-of-shelf-life testing for Gram-negative bacteria, showing a strong correlation with results from the nonselective standard plate count agar ($R^2 = 0.95$) **(15)**. Physicochemical analysis of milk is conducted to assess its quality by measuring various physical and chemical properties, primarily to ensure it adheres to established standards, where key parameters include acidity (to determine lactic acid content), fat content, specific gravity (milk density), freezing point (to detect added water), and the levels of lactose, protein, and Solid Non-Fat (SNF) components, which are crucial indicators of its nutritional value and composition **(20)**.

2. Methodology:

2.1 Collection of Raw Milk

The study commenced on March 4, 2025. Raw milk samples were aseptically collected from a nearby cow farm and divided into two sterile 50 mL tubes to prevent contamination. Samples were stored at a controlled temperature until further processing, following the aseptic sampling guidelines described by **APHA (21).**

2.2 Characterisation of Microorganisms

2.2.1 Preparation of Raw Milk for Inoculation

Raw milk (10 mL) was mixed with an equal volume of sterile autoclaved water and homogenised on an orbital shaker (100 rpm, 37 °C, 15 min) as per methods modified from **(22)**.

2.2.2 Inoculation by Spread and Pour Plate Method:

Microbial inoculation was carried out using standard spread and pour plate techniques on nutrient agar (HI Media, Mumbai, India), as outlined in the procedures of **(23)**. For spread plate, 0.1 mL of sample was spread on pre-solidified agar plates; for pour plate, 1 mL of sample was mixed with molten agar (~45 °C) and allowed to solidify. Plates were incubated at 37 °C for 24–48 h.

2.2.3 Isolation of Pure Cultures:

Distinct colonies differing in morphology were subcultured using a sterile inoculating loop into

nutrient broth **(HI Media, Mumbai)** and incubated at 37 °C for 24 h to obtain pure isolates, following standard microbiological procedures **(23)**.

2.2.4 Gram Staining:
Gram staining was performed according to the classical Hucker method as described in **(24)**,**(23)**. Smears were heat-fixed, stained sequentially with crystal violet, Gram's iodine, alcohol, and safranin, and observed under oil immersion (100×) for Gram reaction and cell morphology.

2.3 Isolation of Surface Proteins from Microbial Culture

2.3.1 Precipitation by Ammonium Sulphate:
Pure cultures grown in nutrient broth were centrifuged (8000 rpm, 10 min, 4 °C) and washed with phosphate-buffered saline (PBS). The pellet was resuspended in lysis buffer, and solid ammonium sulphate was slowly added to 80% saturation with constant stirring at 4 °C. Samples were kept overnight for protein precipitation following the procedure of **(25)**.
2.3.2Precipitation by Centrifugation:
Alternatively, bacterial suspensions were centrifuged (10 000–12 000 rpm, 15–20 min, 4 °C) to separate proteins. The supernatant containing soluble molecules, including lipopolysaccharides (LPS), was collected for further processing as per procedures modified from **(26)**.

2.4 Identification of Lipoproteins by Sudan III Pre-Loaded Strips
2.4.1 Preparation of Sudan III Strips:
Sudan III (0.5% w/v) was dissolved in isopropanol **(Merck India Ltd.)**, filtered, and used to soak sterile filter-paper strips, which were air-dried and stored in light-protected containers until use. The procedure followed lipid-staining principles described by **(27)**.
2.4.2 Protein Loading and Reaction:
Isolated protein samples (20–30 µL) were applied to sterile strips, air-dried, and then reacted with Sudan III strips. The presence of lipid-containing proteins was indicated by an orange-red colouration, confirming lipoprotein content **(28)**.

2.5 Comparative Analysis of Pasteurised and Raw Milk Using Sudan III Strips:
Equal volumes of raw and pasteurised milk (20–30 µL) were spotted onto sterile strips and pressed onto Sudan III-pre-loaded strips for 5–10 min at room temperature. Lipoproteins were detected through orange-red staining, and colour intensity before and after pasteurisation was compared following methods adapted from **(29)** to assess heat-induced lipid alteration.

3. Results:

3.1 Samples of Milk Collected
Samples of milk were collected from two sources as shown in Table 1. Each sample was analysed for microbial and physicochemical properties following aseptic collection procedures.

Sample No.	Samples Collected	Temperature at Collection	Colour of Milk	Quantity Collected
1	Raw milk	37 °C	White	50 mL
2	Pasteurized milk	4 °C	White	50 mL

Table 1. Samples of Milk Collected

3.2 Preparation of Raw Milk for Inoculation

Raw milk was collected aseptically and diluted with sterile distilled water in a 1:1 ratio. The mixture was placed in an orbital shaker and maintained at 37 °C and 100 rpm for 30 minutes to promote microbial activation (Fig. 1). After incubation, a slight colour change from white to yellowish-white was observed, indicating microbial metabolic activity.



Figure 1. Thoroughly mixed raw milk with sterile water in an orbital shaker.

3.3 Inoculation by Spread and Pour Plate Methods

For the preparation of nutrient agar (NA), 5.75 g of NA powder was dissolved in 250 mL of distilled water, corresponding to the standard concentration of 23 g/L. Samples were inoculated using spread and pour plate techniques and incubated at 37 °C for 24 hours (Fig. 2, Fig. 3). After incubation, visible colonies developed on the agar plates (Fig. 4).

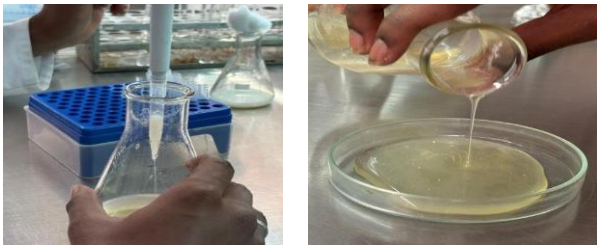

Figure 2. Inoculation by pour plate method



Figure 3. Inoculation by spread plate method

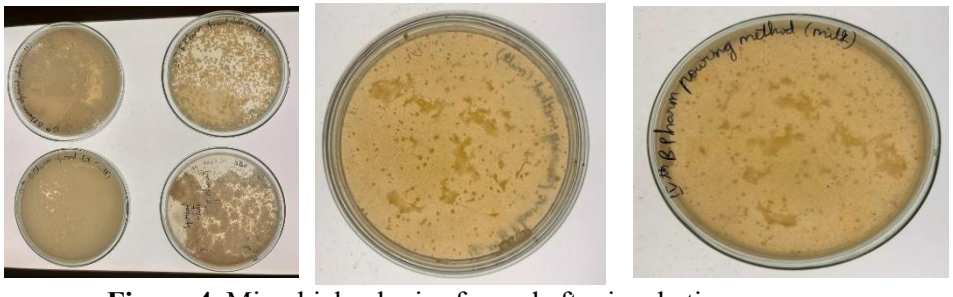

Figure 4. Microbial colonies formed after incubation

Six distinct colony types were observed and characterised morphologically as shown in Table 2.

Colony Code	Colour	Shape	Margin	Elevation	Surface	Size
P1	White	Circular	Smooth	Convex	Smooth	Small
P2	Cream	Irregular	Lobate	Raised	Rough	Medium
P3	Yellow	Circular	Entire	Flat	Shiny	Large
P4	White	Irregular	Undulate	Raised	Dull	Medium
P5	Cream	Circular	Entire	Convex	Smooth	Small
P6	Pale Yellow	Circular	Smooth	Flat	Shiny	Medium

Table 2. Morphological Characteristics of Colonies

The colonies showed variation in colour, size, margin, and surface characteristics, indicating the presence of multiple bacterial species in the raw milk sample.

3.4 Isolation of Pure Cultures

Distinct colonies were subcultured into nutrient broth and incubated at 37 °C for 24 hours. Growth in broth culture was indicated by turbidity, sediment formation, or pellicle development. Morphological observations of the broth cultures are presented in Table 3.

Source Colony Code	Appearance in Broth
P1	Turbid
P2	Turbid with Sediment
P3	Clear with Pellicle
P4	Turbid
P5	Turbid
P6	Turbid with Flocculent
P7	Slightly Turbid

Table 3. Morphological Characteristics of Broth Cultures

3.5 Identification of Microorganisms by Gram Staining

Gram staining was performed on all seven bacterial isolates using crystal violet, Gram's iodine, decolourizer, and safranin reagents (Fig. 5).



Figure 5 Gram Staining Characteristics of Microorganisms

The microscopic characteristics of the stained microorganisms are summarised in Table 4.

Source Broth Code	Gram Reaction	Shape	Arrangement
B1	Gram-negative	Rods	Scattered
B2	Gram-negative	Rods	Scattered
B3	Gram-negative	Cocci	Diplococci
B4	Gram-positive	Rods	Chains
B5	Gram-negative	Rods	Chains
B6	Gram-negative	Cocci	Clusters
B7	Gram-positive	Rods	Chains

Table 4. The microscopic characteristics of the stained microorganisms

Surface proteins were isolated using two precipitation techniques: ammonium sulphate precipitation and centrifugation. After centrifugation at 10,000 rpm for 15–20 minutes, the supernatant and pellet fractions were collected. Protein concentration was estimated using the Lowry's colourimetric method, and results are presented in Table 5.

Sample Code	Protein Content (%)
B4	66
B7	83

Table 5. Protein Concentration of Selected Samples

The colour intensity observed during Lowry's assay was proportional to the protein concentration, confirming successful protein precipitation.

3.6 Identification of Lipoproteins Using Sudan III Pre-Loaded Strips

Lipoproteins were identified using two approaches: (a) preloading Sudan III onto filter paper strips, and (b) preloading isolated protein samples. Upon reaction, a visible colour change from colourless to orange-red confirmed the presence of lipid components within the protein samples.
Under visible light, lipid-rich areas appeared red-orange, while under UV light (360 nm), Sudan III-stained lipoproteins emitted bright orange-red fluorescence, enhancing sensitivity for lipid detection.

3.7 Comparative Analysis of Pasteurised and Raw Milk Using Sudan III Pre-Loaded Strips

A comparative study was conducted to assess the lipid content before and after pasteurisation using Sudan III staining. Results are shown in Table 6.

Sample No.	Sample	Colour Before Pasteurisation	Colour After Pasteurisation
1	Raw Milk – 1	Dark red-orange	Faded red
2	Raw Milk – 2	Dark red-orange	No colour change

Table 6. Colour Intensity Before and After Pasteurisation

The reduction in staining intensity after pasteurisation indicated partial or complete alteration of lipid components due to heat treatment.

4. Discussion:

The microbial and biochemical characteristics of raw milk indicated the presence of diverse bacterial populations, predominantly Gram-negative species such as *Pseudomonas* and *Enterobacteriaceae*, which are known post-pasteurisation contaminants. The strong Sudan III reaction in raw milk confirmed the abundance of lipid-rich microbial components and milk fat globules.

Pasteurisation reduced the colour intensity in Sudan III staining, signifying structural modification of lipids and loss of microbial lipoproteins. Similar findings have been reported by Atherton (2000) and Fox & McSweeney (2015), who noted that heat treatment disrupts lipid membranes and alters protein–lipid complexes. The results demonstrate that while pasteurisation effectively lowers microbial load, residual lipid-associated structures can serve as indirect indicators of contamination or heat impact. Therefore, Sudan III-based colourimetric analysis provides a simple qualitative method to compare lipid integrity in milk before and after heat treatment.

5. Conclusion:

This study successfully demonstrates that Sudan III–treated indicator strips can effectively detect bacterial contamination in milk by exploiting the affinity of the dye toward microbial lipoproteins. The method provides a simple, rapid, and visually interpretable alternative to conventional microbiological techniques. Raw milk consistently showed stronger colour development than pasteurised milk, confirming a higher microbial load. Pasteurisation significantly reduced contamination, though not eliminating post-process risk. Given its low cost, minimal technical requirements, and immediate results, the Sudan III colourimetric test holds significant promise for routine quality control in dairy industries and on-farm monitoring, particularly in regions lacking advanced laboratory facilities.

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7. Conflict of interest:

The authors declared no conflict of interest.

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