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Detection of Adulteration and Contaminants in Raw Milk from Small-Scale Farms in Transylvania Using Advanced Analytical Techniques

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RESEARCH ARTICLE

Abstract
This study investigates adulteration and contamination in raw milk from small-scale farms in rural Transylvania, Romania, using advanced analytical techniques to assess safety and authenticity. A total of 100 raw milk samples (50 cows and 50 goats) were analyzed for aflatoxin M1, antibiotic residues, cryoscopic point, density, and milk substitution. Aflatoxin M1 was measured using the Symmetric M1 Lateral Flow Test Kit, revealing that 34% of cow and 26% of goat samples exceeded the EU limit of 50 ppt, with maximum concentrations of 145.35 ppt and 146.96 ppt, respectively. These levels are likely due to poor feed storage. Antibiotic residues were detected in 15 cow samples for β-lactams and in 10 samples (5 cows and 5 goats) for tetracyclines using the Symmetric BTS lateral flow test. ELISA confirmed milk substitution in 15 goat milk samples. Cryoscopic point analysis (ISO 5764:2009) and MilkoScan FT2 density testing showed non-compliance in 25% of cow and 29% of goat samples. Statistical analysis was used to evaluate significant differences and parameter relationships, including ANOVA, Tukey's test ($p \leq 0.05$), and Pearson correlation. These findings underline the need for improved monitoring, stricter compliance, and targeted support for small-scale dairy producers.

Keywords: Raw milk adulteration; analytical techniques; food safety.

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INTRODUCTION

Ensuring safety and detecting fraud in raw milk is essential for public health and consumer confidence, as well as mitigating the risks associated with consuming adulterated or falsified dairy products. Milk provides the necessary energy and nutrients for optimal growth and development and is an excellent source of high-quality protein essential for immune function, nutrient transport, and absorption (Al Mamun et al., 2021). The dairy industry is a cornerstone of the global food sector, addressing the nutritional needs of populations across age groups, from infants to the elderly (Manolica et al., 2024). However, the quality and safety of raw milk are frequently compromised by adulteration and contamination, posing significant risks to consumers and the dairy industry. Small-scale farms in Romania, particularly in rural regions like Transylvania, rely on traditional farming methods with minimal mechanization and limited access to advanced quality control measures.

Increased demand has driven growth in milk production and dairy processing,

with a strong emphasis on product safety to ensure both chemical and microbiological a strong emphasis on product safety to ensure both chemical and microbiological security. Despite these advancements, the dairy sector faces numerous challenges from farm production to consumption.

Biological, chemical, or physical hazards may be introduced into milk at various stages, intentionally or unintentionally (Haldar et al., 2022). The chemical composition and characteristics of milk are crucial in its production and processing, with variations influenced by the physiological condition of the producing animal (Nitu et al., 2021).

Milk contains key nutrients such as proteins (3.0–3.5 g/100 mL), fats (3.2–4.0 g/100 mL), carbohydrates (4.5–5.0 g/100 mL), vitamins, and minerals, which are fundamental for successful production and processing (Mehta, 2015). Water in milk exists in different forms, including free water, the main solvent; bound water, which is associated with proteins and other macromolecules; and colloidal water, which interacts with casein micelles and mineral components. Free water contains soluble components like lactose and minerals, while bound water—constituting 2–3.5% of milk—is primarily associated with proteins, including casein and albumin (Assen & Abegaz, 2024).

Adulteration, the intentional compromise of food quality by adding or replacing components with inferior substances, poses significant health risks by introducing harmful chemicals or reducing the nutrient content required for healthy development (Ghosh et al., 2022). In modern society, food adulteration is pervasive, particularly in developing countries, where it persists despite regulatory actions (Haji et al., 2023). Adulterants, often added for technical or economic purposes, compromise food safety and nutritional value, including dairy products, cereals, grains, and oils (Poonia et al., 2017). Factors driving adulteration include the strategic motives of businesses, synthetic substitutes for natural ingredients, insufficient awareness of dietary standards, increasing food demand due to population growth, and minimal investment for maximum profit. Milk adulteration and contamination are well-documented global concerns, yet studies focusing on small-scale dairy farming in Romania remain limited.

Milk is highly susceptible to adulteration due to its unique physical and chemical properties. Several factors contribute to the prevalence of milk adulteration, including supply-demand imbalances, challenges in enforcing regulatory standards, insufficient testing infrastructure, the informal nature of the dairy sector, and market competition (Raducu Branescu et al., 2023). Common adulteration practices include adding water to increase volume, which poses severe health risks when sourced from contaminated supplies (Lin et al., 2021). More advanced and difficult-to-detect fraud involves aenhance its nitrogen content. The protein content artificially increased using urea, whey, and cheese (Azad & Ahmed, 2016). The global issue of milk adulteration gained prominence following the melamine contamination scandal in Chinese infant milk products, emphasizing the vulnerability of milk to fraudulent practices, particularly in developing countries with weak monitoring systems and enforcement mechanisms (Liang et al., 2021).

This study contributes to enhancing raw milk safety standards by demonstrating the efficacy of advanced analytical techniques in detecting contamination and fraudulent practices. Integrating rapid immunoassays, ELISA, and instrumental methods provides a robust framework for quality assessment and regulatory compliance. This research highlights the need for stricter enforcement of withdrawal periods, improved feed management, and fraud prevention strategies by identifying key risks such as aflatoxin contamination, antibiotic residues, and milk adulteration. While the literature on milk adulteration and contamination in industrial dairy production is extensive, there is a paucity of research on small-scale farms in Romania. This is due to the fact that traditional farming methods, limited access to quality control, and inconsistent regulatory enforcement may increase safety risks. This gap leaves uncertainty regarding contamination levels, fraudulent practices, and compliance with EU food safety standards in these smallholder systems. To address this critical knowledge gap, the present study employs advanced analytical techniques to evaluate physicochemical properties, contamination levels, and adulteration practices in raw cow and goat milk from small-scale farms in rural Transylvania. The study's findings support the implementation of stricter quality control measures, enhanced oversight, and producer education, thereby ensuring greater transparency, traceability, and consumer confidence in raw milk production.

MATERIALS AND METHODS

This study was conducted in accordance with the ethical guidelines for food research, following the Codex Alimentarius (FAO/WHO) and EU Regulation (EC) No 178/2002 on food safety. Laboratory analyses were conducted in accordance with ISO 17025:2017 standards for testing and quality assurance. Ethical considerations included sample traceability, responsible data management, and adherence to best practices in food authenticity and contamination assessmen

Milk sample preparation

The milk samples utilized in this study were obtained from small-scale farmers in rural Transylvania, Romania. A total of 100 samples (50 from cows and 50 from goats) were collected to ensure a representative assessment of raw milk quality and contamination risks, enabling reliable statistical analysis and species comparisons. The milk was obtained from traditional farming practices, characterized by pasture-based livestock management, minimal mechanization, and natural feed, and processed in a local dairy factory following artisanal techniques, ensuring traceability and reflecting the unique physicochemical and microbiological characteristics of the region's milk. The samples were prepared under controlled laboratory conditions by trained analysts, following standardized protocols. The samples were homogenized using an IKA T25 Digital Ultra-Turrax mechanical homogenizer at 10,000 rpm for two minutes to ensure uniform distribution of fat, proteins, and other components, minimizing variability in subsequent analyses. The homogenization process was conducted at room temperature (25°C) to prevent alterations in sample composition and ensure consistent test results. Samples were brought to room temperature before testing to avoid any interference caused by temperature variations. The sample codification system in this study was designed to differentiate between cow and goat milk samples, with "C" followed by a number (C1–C50) representing cow milk samples and "G" followed by a number (G1–G50) representing goat milk samples, ensuring clear identification and traceability throughout the analysis.

Determination of aflatoxins

The Symmetric M1 Lateral Flow Test Kit is a lateral flow immunoassay (LFIA), a rapid diagnostic test based on immunological principles. It utilizes antibodies specific to aflatoxin M1 to detect and quantify its concentration in milk samples (Imtiaz & Yunus, 2019). The Symmetric M1 Lateral Flow Test Kit is used to quantitatively determine aflatoxin M1 in milk samples (cow, sheep, goat) within a detection range of 8–150 ppt. The required materials include the test kit, a single-channel pipette (100 µL), sterile pipette tips, and milk samples. Before testing, milk samples are homogenized and brought to room temperature. Using the pipette, 100 µL of the milk sample is dispensed into the designated sample well on the test strip. The test strip is incubated for 5–15 minutes, allowing interaction between the sample and reagents. Results are interpreted either visually or using a reader device, which scans the intensity of the test and control lines on the strip. The reader converts these signals into a numerical value, expressed in parts per trillion (ppt), ensuring precise and reliable quantification of aflatoxin M1 levels within the detection range.

Determination of antibiotics

Symmetric BTS, a competitive immunoassay-based lateral flow test, was employed to detect β-lactams, Tetracyclines, and Sulfonamides in raw cow, sheep, and goat milk, as well as pasteurized milk and milk powder, ensuring compliance with EU Maximum Residue Limits (MRLs). Milk samples were applied to the test cassette, where antibiotic residues competed with labeled antibiotics for binding to immobilize antibodies on the test strip. , which indicated negative results, while a weakened or absent test line identified positive results. Results were obtained within 90 seconds for negatives and 5 minutes for positives.

Quantification and traceability were achieved using the S-Flow reader, a portable device that provides precise numerical results and reduces user variability. The accompanying software was used for data analysis, allowing the storage, sharing, and generation of detailed reports for further evaluation. This integrated system ensured a rapid, accurate, and standardized approach to antibiotic residue detection across various milk types.

Determination of immunoglobulins

The presence of bovine immunoglobulin (IgG) in samples of goat milk was identified using the Bovino/Caprino ELISA method. This method was selected due to its high specificity, sensitivity, and ability to detect trace amounts of bovine IgG, making it particularly effective for identifying fraudulent milk substitution. Compared to alternative immunoassays or chromatographic methods, ELISA offers several advantages, including rapid analysis, cost-effectiveness, and reliability in quantifying immunoglobulins, ensuring accurate differentiation between milk types. The equipment utilized for this study included a microplate reader (450 nm), pipettes (10–1000 µL), a microcentrifuge, a vortex mixer, an incubator (37°C), and a plate washer. The reagents employed included pre-coated ELISA plates, bovino/caprino IgG standards (0.1%–5%), enzyme-conjugated secondary antibody, a substrate solution (TMB), stop solution (1N sulfuric acid), and wash buffer. The samples were subjected to a centrifugation process at 3000 rpm for 15 minutes at 4°C, separating a cream layer. These samples were subsequently diluted 1:10 with sample diluent buffer. Fifty microliters of standards (0%, 0.1%, 0.5%) and 50 µL of diluted samples were added to designated wells, followed by 50 µL of enzyme-conjugated antibody. Subsequent to an incubation period at 37°C for 45 minutes, the plates were washed with buffer, and 100 µL of substrate solution was added. The plates were then incubated at room temperature for 15 minutes, after which the substrate solution

was stopped with 50 µL of stop solution, changing the color from blue to yellow. Absorbance was measured at 450 nm using a microplate reader, and a standard curve (absorbance vs. IgG concentration) was generated to calculate IgG concentrations in the samples.

Determination of cryoscopic point

Determining milk's freezing point is a standard method for assessing purity and detecting adulteration, such as water dilution (ISO 5764:2009). The process involved using a cryoscope, a precise instrument for measuring the freezing points of liquids (ISO 5764:2009). Fresh milk samples have been collected hygienically from cows or goats to prevent contamination and thoroughly mixed before testing to ensure uniformity (Costa et al., 2021). The measurement procedure begins by introducing a 2 ml milk sample into the cryoscope, which gradually lowers the temperature until the milk begins to freeze, identifying the cryoscopic point. The natural freezing point of milk typically ranges from -0.512°C to -0.550°C. The cryoscopic point of cow's and goat's milk should be between -0.525°C and -0.550°C. Deviations from these values may indicate dilution or contamination. In addition to density, the cryoscopic point (freezing point) correlates with milk composition and can indicate potential adulteration.

Determination of density

The method for determining milk density in this procedure involved a MilkoScan FT2, a specialized instrument designed for precise analysis of milk and dairy products. The process began with collecting milk samples from various sources, such as cow or goat milk, to evaluate their density. The MilkoScan was calibrated using a reference substance, typically water, to ensure accurate measurements. Once calibrated, the milk samples were introduced into the MilkoScan. This device employs advanced analytical techniques, such as infrared spectroscopy, to measure the milk's composition and density. By analyzing the mass-to-volume ratio, the MilkoScan provided detailed data on the concentration of solids in the milk, including fat, proteins, and sugars. The instrument automatically calculated and displayed the density values, which were then recorded. Finally, the measured density values are compared with standard values from SR 2418:2008 for cow and goat milk, typically in the range of 1.028–1.035 g/mL for cow milk and 1.030–1.037 g/mL for goat milk.

Statistical analysis

All experiments were conducted in triplicate, with results expressed as the mean ± standard deviation (SD). The analysis of variance (ANOVA) and Tukey's test ($p \leq 0.05$) were employed to assess the differences in aflatoxin M1, antibiotics, cryoscopic point, and density. JASP Team software(2025), Version 0.19.3 was used for statistical analysis. The Pearson correlation coefficient between cryoscopic point and density in cow and goat milk samples was calculated using the Regression module of JASP Team software (JASP Team Version 0.19.3 ; Wagenmakers, 2025).

RESULTS AND DISCUSSIONS

Aflatoxins

Aflatoxins are toxic compounds in various agricultural products such as cereal grains, peanuts, dried fruits, and cottonseeds, particularly during harvest and post-harvest processing (Mollayusefian et al., 2021). These toxins are mainly produced by *Aspergillus flavus* and *Aspergillus parasiticus*, with a minor contribution from *Aspergillus nomius*, under hot and humid conditions (Iqbal et al., 2017). Of the twenty recognized types of aflatoxins, aflatoxin B1 (AFB1) is the most prevalent and highly carcinogenic (Tadesse et al., 2020).

Livestock consuming AFB1-contaminated feed metabolize it into aflatoxin M1 (AFM1), which is excreted in milk about 12 hours after intake, with levels diminishing within 72 hours (Alahlah et al., 2020). Milk and dairy products, being highly susceptible to AFM1 contamination, pose significant health risks, including hepatotoxicity, mutagenicity, and carcinogenicity. Milk is a nearly complete food, essential for various age groups, particularly infants and children. However, due to the heat stability of AFM1, it resists standard thermal treatments like pasteurization or sterilization (Mollayusefian et al., 2021). Regulatory bodies have set maximum limits for AFM1 in milk to minimize these risks, with the EU and US FDA limits at 50 ng/L and 500 ng/L, respectively.

As presented in Table 1, the analysis of aflatoxin M1 (AFM1) in 100 milk samples, comprising 50 cow milk and 50 goat milk samples, revealed that 34% of cow samples and 26% of goat samples of the total samples exceeded the maximum permissible limit of 50 ppt, classifying them as nonconforming.

Table 1. Aflatoxins results for cow and goat milk samples

Cow milk samples	Aflatoxin M1 (ppt)	Goat milk samples	Aflatoxin M1 (ppt)	Cow milk samples	Aflatoxin M1 (ppt)	Goat milk samples	Aflatoxin M1 (ppt)
C1	**73.66±0.04^{a}**	**G1**	<50^{a}	**C26**	<50^{b}	**G26**	**101.47±0.06^{c}**
C2	<50^{b}	**G2**	<50^{a}	**C27**	<50^{b}	**G27**	<50^{a}
C3	**73.65±0.05^{a}**	**G3**	**74.92±0.08^{b}**	**C28**	<50^{b}	**G28**	<50^{a}
C4	<50^{b}	**G4**	**119.21±0.4^{c}**	**C29**	<50^{b}	**G29**	**110.37±0.08^{c}**
C5	**110.49±0.09^{c}**	**G5**	<50^{a}	**C30**	**51.13±0.06^{a}**	**G30**	<50^{a}
C6	<50^{b}	**G6**	<50^{a}	**C31**	<50^{b}	**G31**	<50^{a}
C7	<50^{b}	**G7**	<50^{a}	**C32**	**126.34±0.1^{d}**	**G32**	<50^{a}
C8	**65.46± 0.06^{a}**	**G8**	<50^{a}	**C33**	<50^{b}	**G33**	<50^{a}
C9	**135.02±0.8^{d}**	**G9**	<50^{a}	**C34**	**131.85±0.2^{d}**	**G34**	<50^{a}
C10	<50^{b}	**G10**	**96.56±0.05^{bc}**	**C35**	**68.73±0.06^{a}**	**G35**	<50^{a}
C11	**145.35± 0.9^{d}**	**G11**	<50^{a}	**C36**	<50^{b}	**G36**	<50^{a}
C12	<50^{b}	**G12**	**101.11±0.05^{c}**	**C37**	<50^{b}	**G37**	<50^{a}
C13	**110.52±0.07^{c}**	**G13**	<50^{a}	**C38**	**102.39±0.7^{c}**	**G38**	<50^{a}
C14	**98.67±0.04^{c}**	**G14**	**71.98±0.3^{b}**	**C39**	**126.7±0.2^{d}**	**G39**	<50^{a}
C15	<50^{b}	**G15**	<50^{a}	**C40**	<50^{b}	**G40**	<50^{a}
C16	<50^{b}	**G16**	**130.34±0.3^{d}**	**C41**	<50^{b}	**G41**	<50^{a}
C17	<50^{b}	**G17**	**136.43± 0.4^{d}**	**C42**	<50^{b}	**G42**	<50^{a}
C18	<50^{b}	**G18**	<50^{a}	**C43**	<50^{b}	**G43**	<50^{a}
C19	**78.87± 0.03^{a}**	**G19**	<50^{a}	**C44**	<50^{b}	**G44**	<50^{a}
C20	<50^{b}	**G20**	**138.56±0.5^{d}**	**C45**	<50^{b}	**G45**	<50^{a}
C21	<50^{b}	**G21**	<50^{a}	**C46**	<50^{b}	**G46**	<50^{a}
C22	<50^{b}	**G22**	<50^{a}	**C47**	**88.06±0.09^{ac}**	**G47**	<50^{a}
C23	<50^{b}	**G23**	**97.19±0.07^{bc}**	**C48**	<50^{b}	**G48**	**121.73 ±0.2^{d}**
C24	<50^{b}	**G24**	<50^{a}	**C49**	<50^{b}	**G49**	<50^{a}
C25	<50^{b}	**G25**	<50^{a}	**C50**	**94.35±0.07^{ac}**	**G50**	**146.96± 0.3^{d}**

Note: The results are expressed as the mean value of the three replicates ± the standard deviation (SD); different letters in superscript within the same column indicate significant differences (Tukey test, $p \leq 0.05$).

Among the cow milk samples, nonconforming values ranged from 73.65 ppt to 110.49 ppt, while in goat milk samples, values ranged from 74.92 ppt to 119.21 ppt. The remaining 66% of the samples displayed AFM1 levels below the limit (<50 ppt), indicating compliance with regulatory standards, as shown in Figure 1. These findings suggest potential contamination, likely stemming from poor feed storage practices, fungal toxins such as *Aspergillus flavus* and *A. parasiticus* exposure, or inadequate quality control measures in small-scale dairy operations. The

results highlight the necessity for regular monitoring and intervention to mitigate the risks associated with AFM1 contamination.

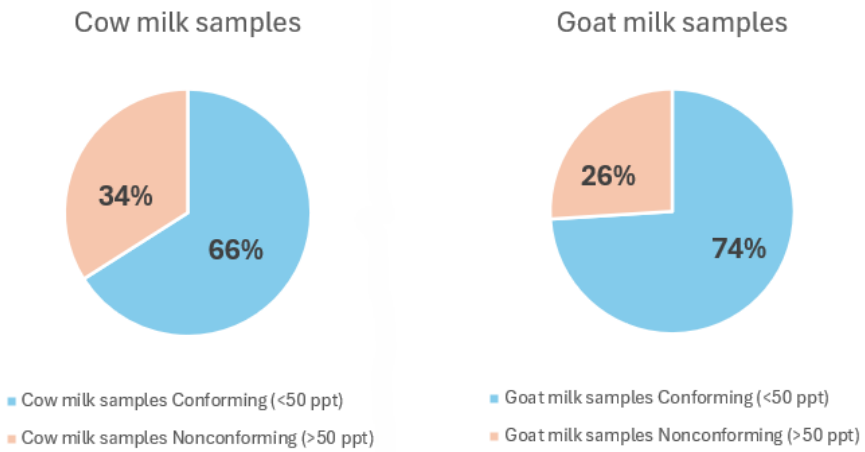


Figure 1. The proportion of conforming and nonconforming Aflatoxin M1 levels in cow and goat milk samples.

Compared with other studies, the findings align with global trends in AFM1 contamination. For example, a systematic review analyzing global data from 1988 to 2020 reported that AFM1 contamination in milk remains a widespread issue, with prevalence varying significantly across regions and time periods. The study emphasized the critical need for continuous monitoring and effective mitigation strategies to reduce contamination risks (Salari et al., 2020). Similarly, a study conducted in northwest Iran found that 25.4% of milk samples exceeded the EU's maximum permissible limit of 50 ppt, with concentrations reaching up to 0.893 µg/L, highlighting the regional variability in AFM1 contamination (Ismail et al., 2016).

Further supporting these findings, a 20-year review of AFM1 contamination data revealed that contamination remains a persistent global issue, particularly in regions with inadequate regulatory enforcement. This review recommended stronger agricultural practices and regulatory frameworks to combat contamination (Malissiova et al., 2024). The 34% respectively 26% nonconformity rate observed in this study reflects a higher contamination rate than in some other regions. Still, it remains consistent with findings in areas where small-scale farming and limited resources are prevalent. The higher contamination levels in goat milk than cow milk can be attributed to specific farm management practices, feeding habits, and regulatory oversight. According to the study, 34% respectively 26% of the total milk samples exceeded the EU regulatory limit for aflatoxin M1 (50 ppt), with the highest levels recorded being 145.35 ppt in cows' milk and 146.96 ppt in goats' milk. The study suggests that these high levels of contamination are likely related to inadequate feed storage, which promotes the growth of aflatoxin-producing fungi such as *Aspergillus flavus* and *Aspergillus parasiticus*. Because goats are more commonly raised in extensive systems with access to diverse and often uncontrolled feed sources, they are more exposed to contaminated feed than cows, which are typically fed controlled diets with regulated storage conditions.

Antibiotics

Annually, the global consumption of antibiotics in livestock amounts to 63,151 tons, with a standard deviation of ±1,560 tons (Van Boeckel et al., 2015). Within the domain of animal husbandry, antibiotics are utilized for therapeutic and prophylactic purposes. Extensive research has demonstrated that a significant proportion (30%–70%) of antibiotics is released into the environment in an unaltered state, thereby retaining their potential antimicrobial activity (Polianciuc et al., 2020). Milk, a widely consumed and nutritionally valuable food, often contains antibiotic residues due to improper use in treating animal diseases and indiscriminate use as feed additives, raising public health concerns (Ventola, 2015).

Antibiotic residues and the prevalence of antibiotic-resistant bacteria in raw cow and goat milk pose significant public health concerns, underscoring the need for rigorous monitoring and regulation. Studies have demonstrated that milk is frequently contaminated with antibiotic residues, with β-lactam antibiotics being the most commonly detected (Sachi et al., 2019). Research on raw milk samples has identified varying contamination levels, with cow milk generally showing higher rates than goat milk.

The presence of antibiotic-resistant strains, such as *Staphylococcus aureus*, further emphasizes the potential health risks to consumers (Tamendjari et al., 2021). A growing body of research, including studies from Algeria and South Africa, has revealed that inadequate control measures in dairy farming contribute to the persistence of these

residues in milk. This emerging concern underscores the need for enhanced screening techniques and more stringent regulations to ensure the safety and quality of raw milk products (Sachi et al., 2019).

In the analysis of 100 raw milk samples (Table 2), the presence of β-lactams was detected in 15 samples of raw cow's milk (C1, C2, C10, C14, C22, C23, C26, C30, C35, C39, C42, C44, C45, C46, and C50), while no goat milk samples exhibited a positive response for β-lactams. Conversely, tetracyclines were identified in five samples of cow milk (C7, C12, C17, C33, and C48) and in five samples of goat milk (G4, G11, G15, G23, and G46), confirming their presence in both milk types. No sulfonamides were detected in any samples, indicating their absence in the analyzed raw milk.

Table 2. Presence of antibiotics in cow and goat milk samples

Samples	β-lactame	Tetracyclines	Sulfonamides	Samples	β-lactame	Tetracyclines	Sulfonamides
	+(poz)/-(neg)	+(poz)/-(neg)	+(poz)/ (neg)		+(poz)/-(neg)	+(poz)/-(neg)	+(poz)/-(neg)
C1	**+**	-	-	**G1**	-	-	-
C2	**+**	-	-	**G2**	-	-	-
C3	-	-	-	**G3**	-	-	-
C4	-	-	-	**G4**	-	**+**	-
C5	-	-	-	**G5**	-	-	-
C6	-	-	-	**G6**	-	-	-
C7	-	**+**	-	**G7**	-	-	-
C8	-	-	-	**G8**	-	-	-
C9	-	-	-	**G9**	-	-	-
C10	**+**	-	-	**G10**	-	-	-
C11	-	-	-	**G11**	-	**+**	-
C12	-	**+**	-	**G12**	-	-	-
C13	-	-	-	**G13**	-	-	-
C14	**+**	-	-	**G14**	-	-	-
C15	-	-	-	**G15**	-	**+**	-
C16	-	-	-	**G16**	-	-	-
C17	-	**+**	-	**G17**	-	-	-
C18	-	-	-	**G18**	-	-	-
C19	-	-	-	**G19**	-	-	-
C20	-	-	-	**G20**	-	-	-
C21	-	-	-	**G21**	-	-	-
C22	**+**	-	-	**G22**	-	-	-
C23	**+**	-	-	**G23**	-	**+**	-
C24	-	-	-	**G24**	-	-	-
C25	-	-	-	**G25**	-	-	-
C26	**+**	-	-	**G26**	-	-	-
C27	-	-	-	**G27**	-	-	-
C28	-	-	-	**G28**	-	-	-
C29	-	-	-	**G29**	-	-	-
C30	**+**	-	-	**G30**	-	-	-

C31	-	-	-	**G31**	-	-	-
C32	-	-	-	**G32**	-	-	-
C33	-	**+**	-	**G33**	-	-	-
C34	-	-	-	**G34**	-	-	-
C35	**+**	-	-	**G35**	-	-	-
C36	-	-	-	**G36**	-	-	-
C37	-	-	-	**G37**	-	-	-
C38	-	-	-	**G38**	-	-	-
C39	**+**	-	-	**G39**	-	-	-
C40	-	-	-	**G40**	-	-	-
C41	-	-	-	**G41**	-	-	-
C42	**+**	-	-	**G42**	-	-	-
C43	-	-	-	**G43**	-	-	-
C44	**+**	-	-	**G44**	-	-	-
C45	**+**	-	-	**G45**	-	-	-
C46	**+**	-	-	**G46**	-	**+**	-
C47	-	-	-	**G47**	-	-	-
C48	-	**+**	-	**G48**	-	-	-
C49	-	-	-	**G49**	-	-	-
C50	**+**	-	-	**G50**	-	-	-

Note: Positive results: +(poz); negative results: -(neg). The results are expressed in terms of the positive or negative value of the three replicates of each sample.

The presence of β-lactams and tetracyclines in certain samples indicates improper antibiotic usage in livestock, raising concerns regarding public health risks and adherence to food safety regulations. Conversely, the absence of sulfonamides suggests they may be utilized less frequently or more stringently regulated in the examined regions. In addition to mycotoxins, the study identified higher levels of antibiotic residues in goat milk than cow milk. Five goat milk samples contained tetracyclines, while 15 samples of cow milk exhibited the presence of β-lactam residues. The presence of antibiotic residues suggests potential issues in farm management, including poor veterinary oversight, noncompliance with withdrawal periods, and the misuse of antibiotics in disease prevention. Given the absence of well-developed management systems on small-scale goat farms, these farms may exhibit less adherence to proper medication withdrawal times and hygiene protocols, resulting in a higher likelihood of residues in milk.

Immunoglobulins

The substitution of higher-value milk types (e.g., goat, sheep, buffalo) with lower-cost cow milk is a form of adulteration driven by seasonal variations in milk production and the limited availability of high quality milk (Ionescu et al., 2023).

The milk composition from diverse farm animal species demonstrates considerable variation in physicochemical properties, encompassing protein and fat content, minerals, vitamins, enzymes, and other components (Mafra et al., 2022). A critical parameter for distinguishing between milk types is the polymorphism of caseins, which are key technological constituents influencing the suitability of raw milk for industrial processing. To prevent fraudulent substitution of goat and sheep milk with cow milk, it is essential to establish robust analytical methodologies capable of detecting such adulteration and ensuring accurate labeling to protect consumers from misrepresentation (Momtaz et al., 2023).

The ELISA method for Bovino/Caprino is a highly sensitive and specific immunoenzymatic assay used to detect the presence of bovine (cow) or caprine (goat) milk in milk samples from other species, particularly sheep. This method is widely employed to identify fraudulent substitution in milk products and ensure authenticity. The ELISA

method—Bovino/Caprino—relies on the specific binding of antibodies to immunoglobulins (IgG) unique to bovine or caprine milk. The antigen-antibody interaction is identified and amplified using an enzyme-conjugated antibody, which reacts with a substrate to detect a color change. The intensity of the resulting color is directly proportional to the concentration of bovine or caprine IgG present in the sample, enabling precise quantification (Abedini et al., 2023).

Table 3. Immunoglobulin values for goat milk

Samples	Bovino		Samples	Bovino	
	+(poz)/-(neg)	%		+(poz)/-(neg)	%
G1	-	≤ 0.1 [a]	**G26**	-	≤ 0.1 [a]
G2	**+**	**0.85±0.02[b]**	**G27**	-	≤ 0.1 [a]
G3	**+**	**0.15±0.01[c]**	**G28**	**+**	**0.55±0.01[d]**
G4	**+**	**0.33±0.02[d]**	**G29**	-	≤ 0.1 [a]
G5	-	≤ 0.1 [a]	**G30**	-	≤ 0.1 [a]
G6	-	≤ 0.1 [a]	**G31**	-	≤ 0.1 [a]
G7	-	≤ 0.1 [a]	**G32**	-	≤ 0.1 [a]
G8	-	≤ 0.1 [a]	**G33**	**+**	**0.46 ±0.02[d]**
G9	-	≤ 0.1 [a]	**G34**	-	≤ 0.1 [a]
G10	-	≤ 0.1 [a]	**G35**	-	≤ 0.1 [a]
G11	-	≤ 0.1 [a]	**G36**	-	≤ 0.1 [a]
G12	-	≤ 0.1 [a]	**G37**	**+**	**0.15± 0.02[c]**
G13	**+**	**0.17 ±0.01[c]**	**G38**	-	≤ 0.1 [a]
G14	-	≤ 0.1 [a]	**G39**	-	≤ 0.1 [a]
G15	-	≤ 0.1 [a]	**G40**	-	≤ 0.1 [a]
G16	**+**	**0.26±0.03[c]**	**G41**	-	≤ 0.1 [a]
G17	**+**	**0.74±0.03[b]**	**G42**	**+**	**0.22±0.02[c]**
G18	-	≤ 0.1 [a]	**G43**	-	≤ 0.1 [a]
G19	-	≤ 0.1 [a]	**G44**	-	≤ 0.1 [a]
G20	-	≤ 0.1 [a]	**G45**	**+**	**0.33± 0.03[d]**
G21	**+**	**0.15± 0.01[c]**	**G46**	-	≤ 0.1 [a]
G22	-	≤ 0.1 [a]	**G47**	-	≤ 0.1 [a]
G23	-	≤ 0.1[a]	**G48**	**+**	**0.39±0.03[d]**
G24	-	≤ 0.1 [a]	**G49**	**+**	**0.42±0.03[d]**
G25	**+**	**0.16±0.01[c]**	**G50**	-	≤ 0.1[a]

Note: Positive results: +(poz); negative results: -(neg). The results are expressed in terms of the positive or negative value of the three replicates of each sample. The procentual results are expressed as the mean value of the three replicates ± the standard deviation (SD); different letters in superscript within the same column indicate significant differences (Tukey test, $p \leq 0.05$).

Immunoglobulins (Igs) in bovine and caprine milk are pivotal in passive immunity transfer, particularly to neonates. Accurate quantification of these Igs is imperative for evaluating colostrum quality and ensuring sufficient immune protection. Due to its specificity and sensitivity, the enzyme-linked immunosorbent assay (ELISA) is a widely employed method for this purpose. For instance, an indirect competitive ELISA has been developed to detect bovine IgG in milk, identifying milk adulteration and ensuring product authenticity (Ma et al., 2021). Furthermore, ELISA has been utilized to assess IgG concentrations in bovine colostrum and milk, thereby facilitating the evaluation of colostrum quality and the efficacy of passive immunity transfer. The ELISA method was employed to analyze goat milk samples to ascertain the presence of bovine milk (see Table 3). Among the fifty goat milk samples

that were subjected to analysis, thirty-five exhibited no presence of cow milk (IgG concentration ≤ 0.1%). In contrast, fifteen (G2, G3, G4, G13, G16, G17 Conversely, fifteen samples (G2, G3, G4, G13, G16, G17, G21, G25, G28, G33, G37, G42, G45, G48, and G49) exhibited varying concentrations of bovine milk, ranging from 0.15% to 0.85%.The Bovino/Caprino method effectively identified fraudulent substitution in goat milk samples. These findings underscore the significance of this analytical method in ensuring proper labeling and preventing economic and ethical concerns associated with milk adulteration.

Cryoscopic point and density

The cryoscopic point, or freezing point, is the temperature at which milk undergoes solidification under controlled conditions. It is a critical indicator of milk quality and authenticity, with pure milk exhibiting an average freezing point of approximately -0.530°C. This temperature is influenced by solutes such as water, sugars, and salts. A higher freezing point indicates water adulteration, as dilution raises the temperature closer to 0°C (Murphy, 2022). This method has gained significant traction within the dairy industry, where it is employed as a standard practice for the detection of adulteration. Extant research has demonstrated the efficacy of this approach in accurately differentiating between pure and adulterated milk (Şahin et al., 2021).

The term "milk density" is defined as the mass per unit volume. This density indicates the quality and composition of the milk, influenced by factors such as fat content, solids concentration, and temperature. Cow milk typically exhibits a density of 1.028–1.035 g/mL, while goat milk ranges from 1.030–1.037 g/mL due to variations in fat and protein content (STAS 6347-73). A reduced density is indicative of adulteration, such as the addition of water. Deviations from standard density may also indicate alterations in fat content or the presence of additives. (Fox et al., 2017). Density analysis is an integral component of quality control measures. Such analysis is critical in ensuring regulatory compliance and verifying the authenticity of milk products (Chalupa-Krebzdak et al., 2018).

The analysis of freezing point and density measurements is of paramount importance in detecting adulteration in milk and the assurance of its quality. Water addition raises the freezing point and lowers the density, making combining these parameters a reliable approach to identifying dilution and maintaining dairy standards; Poonia et al., 2017).

Table 4 presents cryoscopic point and density measurements for cow and goat milk samples. Our findings indicate that 75% of cow and 71% of goat milk samples were conforming, with cryoscopic points and densities within standard ranges. The high percentage of nonconforming samples—25% in cow milk and 29% in goat milk—suggests potential adulteration or compositional anomalies, likely due to water dilution, nutrient imbalances, or fraudulent substitution. These deviations signify vulnerabilities in the supply chain, from farm management to milk processing, compounded by inconsistent monitoring and testing capabilities. Without stringent regulations and enhanced producer education, small-scale farms remain vulnerable to food safety violations. The small-scale dairy industry in Romania is particularly susceptible to contamination due to a confluence of factors, including substandard infrastructure, conventional farming methodologies, and deficient regulatory oversight. As the study indicates, small-scale farms frequently lack advanced quality control measures, resulting in elevated contamination risks from substandard feed storage, improper antibiotic use, and adulteration.

Table 4. Cryoscopic point and density of cow and goat milk samples

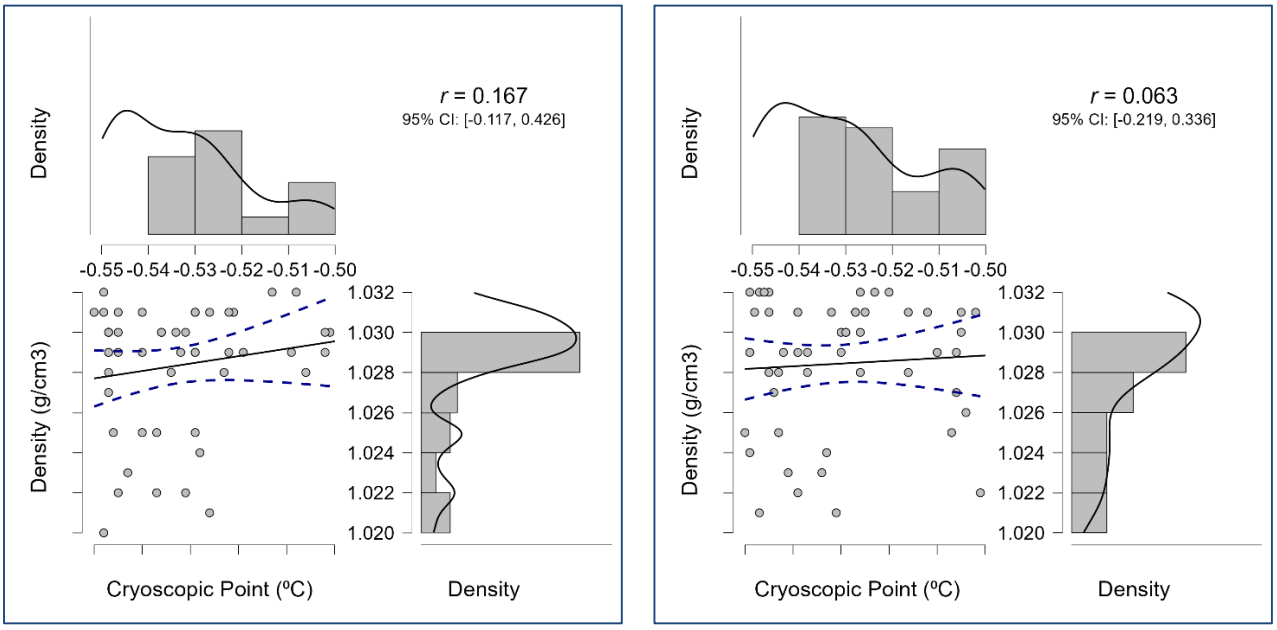
Cow milk samples	Cryoscopic Point (°C)	Density (g/cm^3)	Goat milk samples	Cryoscopic Point (°C)	Density (g/cm^3)
C1	**-0.528±0.01^a**	**1.024±0.01^a**	**G1**	-0.529±0.02^a	1.030**±0.01**^{a}
C2	**-0.546±0.01^b**	**1.025±0.02^b**	**G2**	-0.539**±0.01**^{b}	1.031**±0.03**^{b}
C3	-0.547**±0.02**^{b}	1.030**±0.05**^{c}	**G3**	-0.530**±0.05**^{a}	1.030**±0.04**^{a}
C4	-0.547**±0.02**^{b}	1.029**±0.02**^{c}	**G4**	-0.527**±0.06**^{a}	1.031**±0.02**^{b}
C5	**-0.519±0.03^c**	**1.029±0.05^c**	**G5**	-0.545**±0.03**^{c}	1.028**±0.06**^{c}
C6	-0.534**±0.05**^{d}	1.028**±0.03**^{d}	**G6**	-0.520**±0.01**^{d}	1.032**±0.05**^{b}
C7	-0.548**± 0.02**^{b}	1.031**±0.01**^{e}	**G7**	-0.510**±0.05**^{e}	1.029**±0.04**^{a}
C8	-0.545**±0.06**^{b}	1.031**±0.05**^{d}	**G8**	-0.547**±0.07**^{c}	1.032**±0.09**^{b}
C9	**-0.540±0.08^b**	**1.025±0.09^b**	**G9**	-0.537**±0.02**^{b}	1.028**±0.01**^{c}
C10	**-0.543±0.08^b**	**1.023±0.07^a**	**G10**	-0.506**±0.01**^{e}	1.027**±0.07**^{c}
C11	-0.526**±0.03**^{a}	1.031**±0.02**^{d}	**G11**	**-0.547± 0.05**^{c}	**1.021±0.03^d**

C12	-0.529**±0.01**[a]	1.031 **±0.01**[d]	**G12**	-0.512**±0.02**[d]	1.031**±0.06**[b]
C13	**-0.529±0.07**[a]	**1.025±0.02**[b]	**G13**	-0.549**±0.03**[c]	1.029**±0.02**[a]
C14	-0.529**± 0.08**[a]	1.029 **±0.08**[c]	**G14**	**-0.543±0.05**[c]	**1.025±0.01**[e]
C15	**-0.501±0.06**[e]	**1.030 ±0.03**[c]	**G15**	**-0.549±0.02**[c]	**1.024±0.09**[f]
C16	**-0.523±0.03**[a]	**1.028±0.08**[d]	**G16**	-0.523**±0.06**[a]	1.032**±0.04**[b]
C17	-0.529**±0.01** [a]	1.031**±0.01**[d]	**G17**	-0.502**±0.04**[e]	1.031**±0.05**[b]
C18	**-0.531±0.01**[d]	**1.022±0.09**[f]	**G18**	-0.545**±0.08**[c]	1.032**±0.08**[b]
C19	**-0.548±0.03**[b]	**1.020±0.08** [f]	**G19**	**-0.507±0.01**[e]	**1.025±0.06**[e]
C20	**-0.540±0.02**[b]	**1.029±0.08**[c]	**G20**	-0.526**±0.07**[a]	1.030**±0.04**[a]
C21	-0.547**±0.08**[b]	1.030**±0.07**[c]	**G21**	-0.537**±0.05**[b]	1.029 **±0.01**[a]
C22	-0.547**±0.06** [b]	1.028**± 0.02**[d]	**G22**	-0.506**± 0.01**[e]	1.029**±0.07**[a]
C23	-0.548**±0.03**[b]	1.032**±0.09**[d]	**G23**	-0.516**±0.04**[d]	1.031**±0.02**[b]
C24	-0.536**±0.01**[d]	1.030**±0.05**[c]	**G24**	-0.516**±0.03**[d]	1.028 **±0.05**[c]
C25	**-0.513±0.01**[c]	**1.032± 0.03**[d]	**G25**	-0.544**±0.02**[c]	1.027**±0.06**[c]
C26	-0.533**±0.08**[d]	1.030**±0.01**[c]	**G26**	**-0.550±0.01**[c]	**1.025±0.05**[e]
C27	**-0.537±0.08**[d]	**1.025±0.08**[b]	**G27**	-0.505**±0.07**[e]	1.031**±0.01**[b]
C28	-0.547**± 0.01**[b]	1.030**±0.07**[c]	**G28**	-0.532**±0.06**[b]	1.031**±0.03**[b]
C29	-0.531**±0.07**[d]	1.030**±0.02**[c]	**G29**	**-0.504±0.03**[e]	**1.026±0.09**[e]
C30	**-0.508±0.06**[e]	**1.032±0.08**[d]	**G30**	-0.549**±0.02**[c]	1.032**±0.08**[b]
C31	-0.547**±0.02**[b]	1.029**±0.09**[c]	**G31**	-0.526**±0.05**[a]	1.028**±0.02**[c]
C32	-0.545**±0.01**[b]	1.030**±0.08**[c]	**G32**	-0.526**±0.04**[a]	1.032**±0.07**[b]
C33	**-0.502±0.02**[e]	**1.029±0.05**[c]	**G33**	**-0.534±0.01**[b]	**1.023±0.04**[f]
C34	**-0.509 ±0.01**[d]	**1.029±0.03**[c]	**G34**	**-0.539±0.06**[b]	**1.022±0.06**[d]
C35	-0.529**±0.07**[a]	1.029**±0.09**[c]	**G35**	-0.542**±0.02**[c]	1.029**±0.01**[a]
C36	**-0.545 ±0.08**[b]	**1.022±0.01**[f]	**G36**	-0.543**±0.09**[c]	1.028**±0.05**[c]
C37	-0.550**±0.07**[b]	1.031**±0.07**[d]	**G37**	-0.539**± 0.04**[b]	1.029**±0.04**[a]
C38	**-0.526± 0.08**[a]	**1.021±0.03**[f]	**G38**	-0.546**±0.02**[c]	1.032**± 0.01**[b]
C39	-0.547**± 0.03**[b]	1.030**±0.09**[c]	**G39**	-0.549**±0.03**[c]	1.029**± 0.05**[a]
C40	**-0.502 ±0.01**[d]	**1.030± 0.01**[c]	**G40**	**-0.533±0.06**[b]	**1.024±0.08**[f]
C41	**-0.522± 0.06**[a]	**1.029±0.02**[c]	**G41**	-0.530**±0.05**[a]	1.029 **±0.03**[a]
C42	-0.540**±0.03**[b]	1.031**±0.05**[d]	**G42**	-0.548**±0.01**[c]	1.031**±0.02**[b]
C43	-0.545**± 0.02**[b]	1.029**±0.07**[c]	**G43**	**-0.541±0.03**[c]	**1.023±0.07**[f]
C44	**-0.537± 0.01**[d]	**1.022±0.01**[f]	**G44**	**-0.531± 0.02**[b]	**1.021±0.06**[d]
C45	**-0.521± 0.02**[a]	**1.031± 0.01**[d]	**G45**	-0.527**±0.04**[a]	1.031**±0.01**[b]
C46	-0.536**±0.08**[d]	1.030**± 0.02**[c]	**G46**	-0.545**±0.06**[c]	1.031**±0.03**[b]
C47	**-0.506±0.06**[d]	**1.028±0.03**[d]	**G47**	-0.529**±0.01**[a]	1.030**± 0.02**[a]
C48	**-0.522± 0.08**[a]	**1.031±0.01**[d]	**G48**	**-0.501±0.05**[e]	**1.022±0.04**[d]
C49	**-0.547±0.01**[b]	**1.027±0.05**[d]	**G49**	-0.505**±0.03**[e]	1.030**±0.04**[a]
C50	-0.532 **±0.03**[d]	1.029**± 0.02**[c]	**G50**	-0.525**±0.02**[a]	1.031**±0.01**[b]

Note: The results are expressed as the mean value of the three replicates ± the standard deviation (SD); different letters in superscript within the same column indicate significant differences (Tukey test, $p \leq 0.05$).

The mean cryoscopic point of cow milk was determined to be approximately -0.540°C, with densities ranging from 1.028 to 1.035 g/mL. In comparison, goat milk exhibited a slightly higher mean cryoscopic point of around -0.509°C, with densities ranging from 1.030 to 1.037 g/mL. These findings are consistent with prior research, as Ceniti et al. (2023) reported typical freezing points for cow milk between -0.57°C and -0.53°C, while (Otwinowska-Mindur et al., 2017) established an average freezing point of -0.530°C. In a similar vein, observed that goat milk generally has a higher freezing point (less negative) than cow milk, a finding that is consistent with the results of this study. Concerning density, the measured values are consistent with the extant literature Fox et al. (2015) found that the density of cow milk typically ranges from 1.028 to 1.035 g/mL, while goat milk densities are slightly higher, ranging from 1.030 to 1.037 g/mL.

The observed disparities in cryoscopic point and density between cow and goat milk indicate compositional variations, particularly in fat, protein, non-fat solids, and mineral content. The higher density and slightly less negative cryoscopic point in goat milk can be attributed to its greater non-fat solids and protein content, which impact the physicochemical properties of milk. These parameters are critical for evaluating milk quality and detecting adulteration, underscoring the significance of cryoscopic point and density analysis in ensuring the integrity of dairy products. Figure 2 (a) and (b) demonstrate a noticeable correlation between the cryoscopic point and density in both cow and goat milk samples. The cow milk samples displayed a more concentrated distribution (Pearson's r: 0.167), with a cryoscopic point ranging from -0.55°C to -0.50°C and density between 1.020 and 1.032 g/cm^3, suggesting a stable composition. In contrast, goat milk samples exhibited a lower Pearson's r: 0.063, with slightly higher density (1.025–1.037 g/cm^3) and a broader cryoscopic range, indicative of higher non-fat solids and greater compositional variability influenced by factors such as feeding practices and lactation stage. These parameters are critical for detecting milk adulteration and assessing milk authenticity, underscoring the pronounced physicochemical distinctions between cow and goat milk.



(a) (b)

Figure 2. Correlation between cryoscopic point and density in cow (a) and goat (b) milk samples.

CONCLUSIONS

This study provides a comprehensive and data-driven evaluation of raw cow and goat milk from small-scale farms in Transylvania. The quantitative analysis of aflatoxin M1 using lateral flow assays revealed that 34% of cow milk samples and 26% of goat milk samples exceeded the EU regulatory limit of 50 ppt, with peak concentrations reaching 145.35 ppt in cow milk and 146.96 ppt in goat milk. These findings suggest that inadequate feed storage practices are a probable cause of contamination and underscore the necessity for enhanced feed management and mycotoxin control measures at the farm level. Antibiotic residue testing identified the presence of β-lactams in 15 samples of cow milk and tetracyclines in 5 samples of cow and goat milk. The absence of sulfonamides, while notable, suggests potential inconsistencies in adherence to veterinary withdrawal periods, particularly in

smallholder systems lacking formal oversight. Furthermore, species authenticity testing via ELISA revealed the presence of bovine immunoglobulins in 15 of the 50 goat milk samples (30%), with substitution levels ranging from 0.15% to 0.85%, thereby confirming cases of milk mixing. Concurrently, cryoscopic point and density measurements revealed that 25% of cow milk and 29% of goat milk samples fell outside the established standards, suggesting the possibility of water dilution or natural variability. A weak correlation was observed between the cryoscopic point and density in both cow and goat milk samples, with the Pearson's r for cow milk samples (0.167) being slightly higher than that for goat milk samples (0.063), so the factors influencing the cryoscopic point and milk density are not strongly correlated between them. The findings of this study collectively underscore the efficacy of a multi-analytical approach for raw milk quality control in traditional dairy systems. This studyidentifies critical gaps in compliance and provides a framework for risk-based monitoring. Future research endeavors should prioritize the development of rapid, cost-effective diagnostic tools and focus on scalable solutions to support smallholder farmers. The reinforcement of quality assurance practices is imperative for safeguarding consumer trust, ensuring regulatory alignment, and enhancing the resilience of artisanal dairy production.

Author Contributions: C.-M.C. conceived and designed the analysis; A.M. collected the data; G.R.B. contributed data or analysis tools; R.-A.M.-I. wrote the paper; A.-I.M. performed the analysis; M.I.L. reviewed and edited the manuscript.

Conflicts of Interest

The authors declare that they do not have any conflict of interest.

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