International Journal of Food Science and Biotechnology
2024; Vol. 9, No. 2, pp. 27-39
https://doi.org/10.11648/j.ijfsb.20240902.12

Research Article

Analysis of Antibiotics in Milk from Smallholder Farmers from Kenya Shows Traces Above the Recommended Maximum Residue Limits

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Abstract

Antimicrobial resistance (AMR) is rising globally and is likely to cause more deaths because of antibiotic-resistant microbial infections and antibiotic residues in animal foods and products as a result of misuse of antibiotics in dairying. Thus, we determined the presence and quantities of sulfonamide, tetracycline, and beta-lactam drug residues in milk sampled in Nyandarua, Meru, and Kiambu counties in Kenya using the Charm TRIO® test kit and liquid chromatography (LC) coupled to the triple quadrupole mass spectroscopy (MS) (LC-MS/MS). The TRIO® test kit showed dicloxacillin as the most prevalent at 9.3%, followed by penicillin and cloxacillin at 3.7% each, and nafcillin at 0.9% among the beta-lactams. Among the tetracyclines, demeclocycline and tigecycline were the most prevalent at 10.3% each, followed by oxytetracycline at 9.3%, chlortetracycline at 7.5%, and doxycycline at 3.7%, while sulfaquinoxaline was the only sulfonamide drug detected at 4%. The LC-MS/MS detected the presence of all the tested β-lactam and tetracycline antimicrobial traces and all sulfonamide drugs except sulfamerazine in all three counties. All the tetracycline antibiotics recorded between 3 and 10.5% of antibiotic residues above the recommended Maximum Residue Levels (MRLs) in milk across all three counties, with beta-lactam antibiotics recording between 2 and 33.3%, indicating their misuse in the three counties. Sulfaquinoxaline was the only sulfonamide detected in milk samples above the recommended MRLs, indicating sulfonamides are less used in those counties. Therefore, it is important to enforce a regulatory framework to control antibiotic use in livestock to minimize potential health risks related to their traces in the foods.

Keywords

Dairy, Antibiotic Residues, Antimicrobial Resistance, Charm TRIO® Test Kit, LC-MS/MS

1. Introduction

The dairy industry is the world's largest and most rapidly growing agricultural sub-sector, employing and supporting several livelihoods, including 60 million working on farms and 400 million through the value chain, with a larger proportion in developing nations [1]. Dairy products also help in realizing food and nutrition security, for pregnant women,

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Received: 29 March 2024; **Accepted:** 16 April 2024; **Published:** 10 May 2024

breastfeeding mothers, and children below five years old. In Kenya, dairying accounts for 6-8% of the country's Gross Domestic Product (GDP) and 14% of the agricultural GDP, the second largest after beef [2]. Despite its importance, 80% of Kenya's milk production is generated on small-scale farms, presenting considerable challenges to the dairy sector, such as high production costs, the quality of raw milk, collection and cooling issues, and seasonal fluctuations in supply [3]. Nevertheless, there is a rapid rise in medium-scale farming with investment in modern and commercial dairy production. The sub-sector also employs and generates income for approximately two million people across the value chain, including youth, farmers, milk processors, employees of dairy societies, vendors, transporters, service providers, input suppliers, retailers and distributors. Nearly all Kenyans consume dairy products daily, with an average milk consumption of 115 litres per person yearly, supporting food security and nutrition [4].

As the global population rises, especially in developing countries, there is an increased need for food, including livestock protein, hence the need for a rapid and intensified dairying, which has also been found to favour the emergence of numerous diseases, including biofilm-related infections, resulting in extensive use of antibiotics to treat, prevent or control infectious diseases [5, 6]. The antibiotics ameliorate dairy cattle health and boost their product values [7], leading to improved well-being and productivity [8]. The drugs are administered orally, through feeds, water or injected directly into the udder. Globally, more antibiotics are utilized in animals than humans [9], with mastitis consuming most drugs due to its devastating economic impact on the dairy sector [10]. In the United Kingdom), summer mastitis causes a €9.03 billion loss annually in the dairy sector [11], while in Kenya, the disease is very costly and widespread in smallholder farms in major dairy regions of Embu, Kiambu and Nyandarua. Endometriosis also affects 40% of post-calving cows in Kenya, occasionally requiring antibiotic treatment [12].

The use of antibiotics in diseased cows can be very late to cure the infected animals and bring them back to health. In some cases, cattle are asymptomatic and will still eat and suckle normally, making the infections clinically non-obvious, causing farmers to miss the correct time of drug administration [13]. Moreover, some dairy cows that recover from the infections could also produce less milk for a while, making it rational to curb infectious diseases from occurring in the herd rather than treating them and minimize losses to farmers [10]. The antibiotics can also be used prophylactically in healthy dairy cows at risk of infection before the onset of any disease [6] or through metaphylaxis in animals in close contact with those diagnosed with infectious diseases [14, 12]. Over 90% of American farmers practice dry cow antibiotic therapy to eradicate the occurring intramammary infections or curb new diseases or infections [11]. Antibiotics are also used to supplement feeds to improve the growth rate of dairy cattle [5]. Recently, such growth promoters have been used broadly and uncontrollably in dairying to quickly enhance high profits by optimizing animal growth rates [10]. Dairy cows also use antibiotics as feed additives to boost the activities of the alimentary canal and eliminate competitive microorganisms that generate undesirable toxic substances or compete for nutrients in the canal [15]. The antimicrobials also offer a conducive habitat in the canal of dairy cows for effective food intake, resulting in an increased growth rate.

Despite their importance, the uncontrolled utilization of antimicrobials in dairy cattle contributes to the global rise in antimicrobial resistance (AMR), which involves the evolution of bacteria to resist antimicrobial drugs, making infections difficult to control, elevating disease spread and causing major public health concerns. These antibiotic-resistant microorganisms are pathogenic to humans and easily spread from livestock to human beings through food consumption or dispersed to the environment through animal droppings and urine. Accumulating antibiotics and antibiotic-resistant microorganisms cause an imbalance in the human microbiome and lead to prolonged and untreatable human infections, with higher medical costs and deaths [16]. The imbalance also impairs some of the functions of the human microbiota, including the production of nutrients and lack of protection from pathogenic microorganisms [17]. Currently considered a universal health emergency, AMR has destabilized the significant steps made in modern medicine and will likely cause more adverse economic implications and up to 10 million deaths by 2050 annually if not controlled [18].

The commonly used antibiotics to treat microbial diseases and promote animal growth include aminoglycosides, β-lactams, sulfonamides and tetracyclines [19]. When farmers fail to adhere to the drug withdrawal period before animal slaughter or feed their livestock with feeds contaminated with antibiotic residues beyond the recommended maximum residue limits (MRLs) [20], the antibiotic residues and antibacterial-resistant microorganisms find their way into humans when consumed as food [9], leading to a long-term AMR problem [20]. The addition of antibiotics to milk and meat products to increase shelflife [21] and drinking of water containing antibiotics from wastewater treatment plants, health centres, ponds or rivers [22] also contribute to the rise in AMR. The consumption of food products adulterated with antibiotic drug residues may result in cancer, reproductive effects and hypersensitivity in humans [23] and also inhibit the microorganisms required to process cultured milk products [24]. Hence, there is an urgent need to employ analytical techniques to establish the relationships between antibiotic drug traces and the occurrence of antibiotic-resistant pathogens in milk in Kenya to improve health.

In cases where antibiotic residues find their way into the human system through the consumption of animal-based food and contaminated drinking water, it is critical to establish their presence and whether the quantities are above the recommended MRLs using chromatographic techniques [25]. Similar studies have been conducted using rapid test kits with lower sensitivity and accuracy [9, 10]. Other lateral flow

devices, such as antibiotic dipsticks, have also been used but present problems, such as the hook effect that may give false-negative results. Such limitations underscore the unreliability of rapid test kits and highlight the potential consequences of relying on methods with compromised accuracy, particularly when dealing with public health concerns like antibiotic resistance [26]. Therefore, we used the Charm TRIO® test kit and liquid chromatography (LC) coupled to the triple quadrupole mass spectroscopy (MS) (LC-MS/MS) to determine antibiotic traces in milk sampled across three counties in Kenya and showed that, unlike the TRIO® test, the LC-MS/MS was more reliable, highly selective and sensitive, enabling simultaneously and accurate detection and quantification of multiple antibiotic residues at extremely low concentrations.

2. Materials and Methods

2.1. Study Area

The research was conducted in three counties in Kenya, including Nyandarua, Meru, and Kiambu (Figure 1). We collected 107 milk samples, including 25 samples from the Engineer area located in Nyandarua County (-0.606377, 36.576318), 44 samples from Mikinduri located in Meru county (0.1199 °N, 37.8380 °E), and 38 samples from Githunguri located in Kiambu county (1.0586 ° S, 36.7779 °E). The samples were collected aseptically in 250 mL sterile sampling bottles, labelled and kept at 10 °C in an ice-packed cool box before being transported to the instrumentation laboratory at Kenya Bureau of Standards (KEBS) located in Nairobi (-1.322595616574978, 36.8349006324275) for analysis.

2.2. Antimicrobial Residues Screening Using Charm TRIO® Test

The milk samples were screened for sulphonamides, tetracyclines, and beta-lactams using Charm TRIO® test kit (Charm Sciences, Inc.).

A sterile, calibrated 25 ml micropipette was used to dispense 10 mL of the milk sample from the stock samples into a 50 ml centrifuge tube and homogenized in a shaker for 1 min. Charm TRIO® test kit was assembled according to the manufacturer's (Charm Sciences, Inc.) instructions. The tape of the TRIO test strip was peeled back to expose the sample loading well and pad and 300 uL homogenized milk sample was slowly added to the side of the well, followed by sealing, and incubation of the mixture at room temperature for 3 min, after which the strip was automatically read for all the milk samples, and all the antibiotics detected, accurately recorded. For quality control, known spiked milk samples at 50 µg/kg and 100 µg/kg of tetracycline, 2 µg/kg and 4 µg/kg of Penicillin G and 50 µg/kg and 100 µg/kg sulfamethizole were run after every 20 samples, followed by a blank sample to validate the accuracy and reliability of the test results.

2.3. Chromatographic Determination of Antibiotic Traces in the Milk Samples

Milk samples were transferred into 50 mL centrifuge tubes and centrifuged at 5, 400 g to obtain fat-free milk. After that, 2 mL of the centrifuged fat-free milk was pipetted into 50 mL plastic tubes. A 10 µL of sulfapyridine, penicillin G-d7 and methacycline purchased from Sigma Aldrich (USA), were used as the internal standards for sulfonamides, beta-lactams and tetracyclines, respectively. A 100 µL of 20% trichloroacetic acid (TCA) and 10 mL of Mc Il Vaine buffer were sequentially added to the blank, calibrating standards and samples then shaken for 10 min, followed by centrifuging at 5, 400 g for 10 min at 4°C. The supernatant was dispensed into a glass tube and ran through the OASIS PRiME HLB^{TM} column without a vacuum, followed by washing the column with 6 mL sterile water and drying under a vacuum. The solution was then eluted with 6 mL of methanol in centrifuge tubes and evaporated to dryness at 40 °C under Nitrogen gas. The antimicrobial residue solution was re-dissolved in 1, 000 µL of 5% methanol, shaken for 10 min and centrifuged at 16, 250 g for 10 min at 4°C to obtain the final extract in 1 mL vials for chromatographic analysis.

The stock solution was prepared by weighing 0.01 ± (0.0005) g of the following respective standards: beta-lactams; penicillin G, oxacillin, cloxacillin, dicloxacillin and nafcillin. Sulfonamides: sulfadiazine, sulfathiazole, sulfamerazine, sulfapyridine, sulfamethizole, sulfadimidine, sulfamethoxypyridazine, sulfachloropyridazine, sulfamethoxazole, sulfadimethoxine, sulphisoxazole, sulfadoxine, sulfamonomethoxine, and sulfaquinoxaline. Tetracyclines: tigecycline, oxytetracycline, chlortetracycline, demeclocycline, doxycycline and tigecycline standards. Each standard was dissolved separately in 10 mL methanol, to make a 1mg/ml stock solution (1000 µg/ml or 1000 ppm). For the working standards, an initial working standard was prepared by pipetting 1 mL of the stock solution into a 10 mL volumetric flask and topped up with a mixture of methanol and water (prepared in a ratio of 50:50) to form a concentration of 100 µg/ml. This was repeated for each standard. From the 100 µg/ml (100 ppm) of individual sulfonamide standard solutions, a 1 ppm mix standard solution was prepared by pipetting 500 µL of the individual standards into a 50 mL volumetric flask and then topped up with the solution of methanol and water prepared as described above and repeated for tetracyclines and beta-lactams. Appropriate five-point calibration standards of 10 ppb, 20 ppb, 50 ppb, 100 ppb, 200 ppb and 300 ppb, were prepared from the working standards through serial dilution of methanol (LC-MS grade) and water prepared in the ratio of 50:50.

The chromatographic separation was achieved at 40°C with an autosampler temperature of 10°C on an Agilent Zorbax column (SB-C18, 2.1 x 150 mm, 3.5 µm) and a mobile phase containing different ratios of 0.1% (vol/vol) formic acid and 40% acetoni-

trile. The flow rate was maintained at 400 µL/min and a sample injection volume of 3 µl. Each sample injection was pumped with 0.1% formic acid on pump A at 100% for 0.6 min before being subjected to pump B at 0% with 0.1% formic acid in methanol. The ratios were repeated for the same conditions for Pump A and B for intervals of 6 (5% against 95%), 7 (100% against 0%) and 8 (100% against 0%) minutes and respective percentages as shown in Table 1.

In the prepared batch, the internal standard was injected first, followed by a control test sample at concentration level four, to check for any inconsistencies or errors in the equipment. The remaining samples were then injected at intervals of 10 runs, followed by a blank sample to clean the needle and prevent sample carryover. A standard calibration curve was also analyzed after the sample run. Antibiotics were separated based on their polarity and qualitatively identified based on their retention times in comparison to known antibiotic standards. They were then quantified in the mass detector based on their masses, collision energies, fragmentation voltage, and dwell time.

2.4. Data Analysis

The prevalence of drug traces observed in the milk samples using the Charm-Trio kit was analyzed using the chi-square analysis, while their quantities were subjected to a one-way analysis of variance and their means were separated using Tukey HSD in R software ver 4.2.2 (R Development Core Team, 2020). Pearson's correlation coefficient in R was performed for the quantities of the antibiotic traces to determine possible relationships between the different antibiotic classes. The value $P \leq 0.05$ was considered significant.

3. Results

3.1. Prevalence of Beta-Lactams, Tetracyclines and Sulfonamides Using Rapid Charm Triotm Test Kit

The percentages of milk samples containing penicillin G ranged from 4.6% in Meru to 5.4% in Kiambu, with no antibiotic residue found in Nyandarua ($\chi^2 = 5.27$, $df = 2$, $P = 0.072$) using the TRIO test kit. While oxacillin was not detected using the Charm TRIO® kit in all the counties, 16% of milk samples collected in Nyandarua County contained cloxacillin, with 2.3% of samples containing nafcillin residues in the milk. The percentages of milk samples containing dicloxacillin residue differed significantly across the counties ($\chi^2 = 21.48$, df = 2, $P < 0.001$), with 11.4% detected in Meru County and 20% in Nyandarua County. All the tetracycline antibiotic drugs tested, including oxytetracycline, chlortetracycline, demeclocycline, doxycycline and tigecycline were detected by the rapid Charm TRIO® test kit in milk samples across all the counties. The number of milk samples containing oxytetracycline residue did not differ significantly across the counties ($\chi^2 = 1.55$, df = 2, $P = 0.46$). However, milk samples with the highest oxytetracycline residue were detected in Nyandarua County (12%), followed by 10.5% in Kiambu County and 6.9% in Meru County. The milk samples containing chlortetracycline residues differed significantly across all the counties assessed ($\chi^2 = 11.91$, df = 2, $P = 0.03$), with Nyandarua county recording the highest concentration of the antibiotic at 16%, followed by Kiambu county at 7.9% and Meru county at 2.3%. The percentages of milk samples containing demeclocycline also did not differ significantly ($\chi^2 = 10.37$, df = 2, $P = 0.06$) across the counties, with Nyandarua county having the highest concentration at 20%, followed by Kiambu and Meru counties at 5.3% and 2.3% respectively. The percentages of milk samples containing doxycycline were low and did not differ significantly across the surveyed counties ($\chi^2 = 1.22$, df = 2, $P = 0.544$). Kiambu recorded the highest residue concentrations, followed by Nyandarua and Meru counties at 5.3%, 4.3% and 2.0%, respectively. Though the percentages of milk samples containing tigecycline did not show differences across the three counties ($\chi^2 = 4.26$, df = 2, $P = 0.119$), the most tigecycline residue was detected in Meru county (13.7%), followed by Nyandarua (12%) and Kiambu counties (5.3%). In addition, sulfaquinoxaline was the only drug in the sulfonamide class detected in milk samples collected from Nyandarua County at 4%.

3.2. Quantity of β-Lactam Traces in Milk Samples Using LC-MS/MS

The concentrations of all the β-lactam drugs found in milk samples, including penicillin G ($F_{(2, 320)} = 13.40$, $P < 0.0001$), oxacillin ($F_{(2, 320)} = 15.88$, $P < 0.0001$), nafcillin ($F_{(2, 320)} = 10.75$, $P < 0.0001$), cloxacillin ($F_{(2, 320)} = 7.20$, $P < 0.0001$) and dicloxacillin ($F_{(2, 320)} = 12.10$, $P < 0.0001$) differed significantly across the three counties, with samples from Kiambu County containing the highest levels of penicillin G, oxacillin and nafcillin, and Nyandarua County having more cloxacillin and dicloxacillin (Figure 2). The concentration of penicillin G traces in milk samples from Kiambu was also significantly higher, with significant differences noted between Kiambu and Meru ($P < 0.0001$) and between Kiambu and Nyandarua than ($P < 0.0001$) counties, but insignificant between Meru and Nyandarua counties ($P = 0.2041$). The milk samples from Nyandarua County contained significantly higher concentrations of cloxacillin than Kiambu and Meru ($P < 0.0001$), while those between Meru and Kiambu exhibited insignificant differences ($P = 0.9961$). Similarly, milk samples from Nyandarua county contained markedly higher amounts of dicloxacillin than Meru ($P = 0.0165$) and Kiambu ($P < 0.0001$), while those between Meru and Kiambu exhibited significant differences ($P < 0.0001$) (Figure 2). Milk samples from Kiambu recorded higher concentrations of nafcillin residues than Meru ($P = 0.0108$) and Nyandarua ($P < 0.0001$), with the concentrations between Meru and Nyandarua counties also showing a high significant difference ($P < 0.0001$). Concentrations of cloxa-

cillin residues in milk also differed significantly ($P < 0.0001$) between Kiambu and Nyandarua and between Meru and Nyandarua counties, while those between Kiambu and Meru counties did not ($P = 0.9961$) (Figure 2).

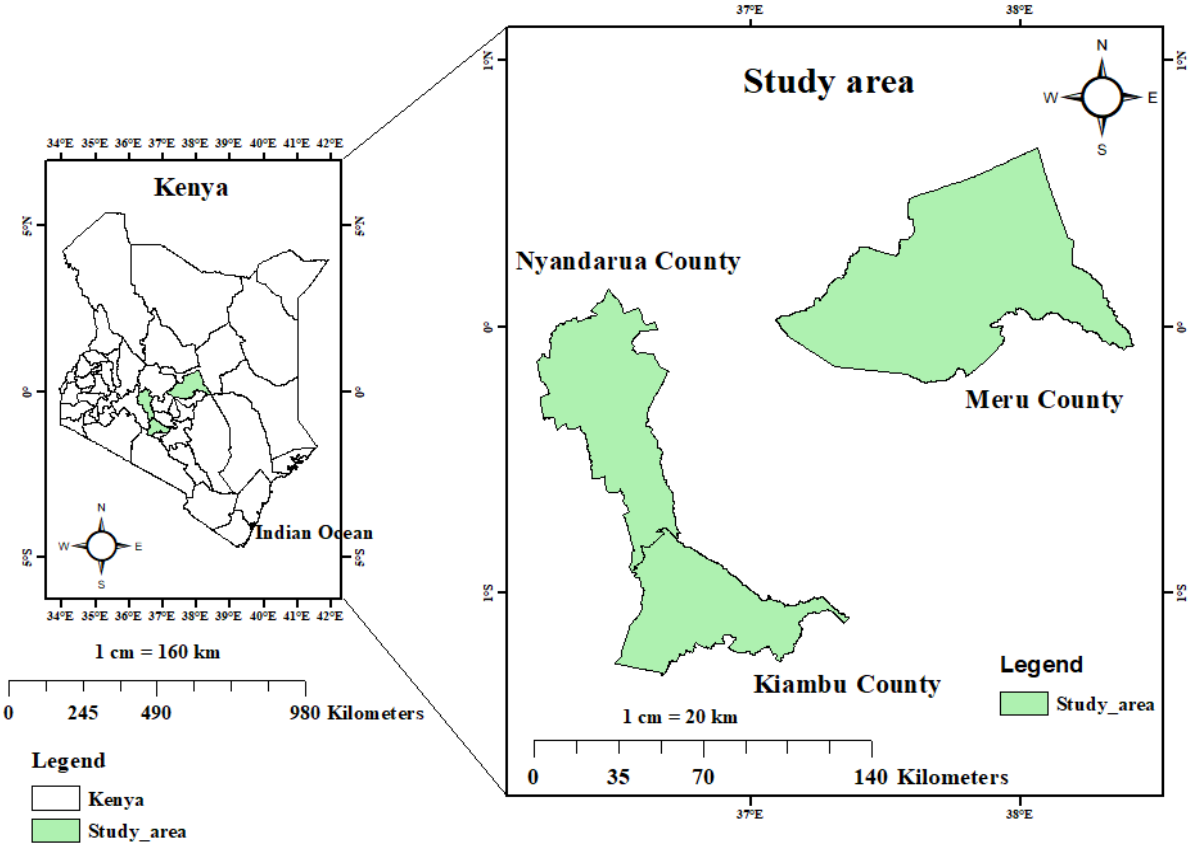

Figure 1. *Map of Kenya showing the counties of study.*

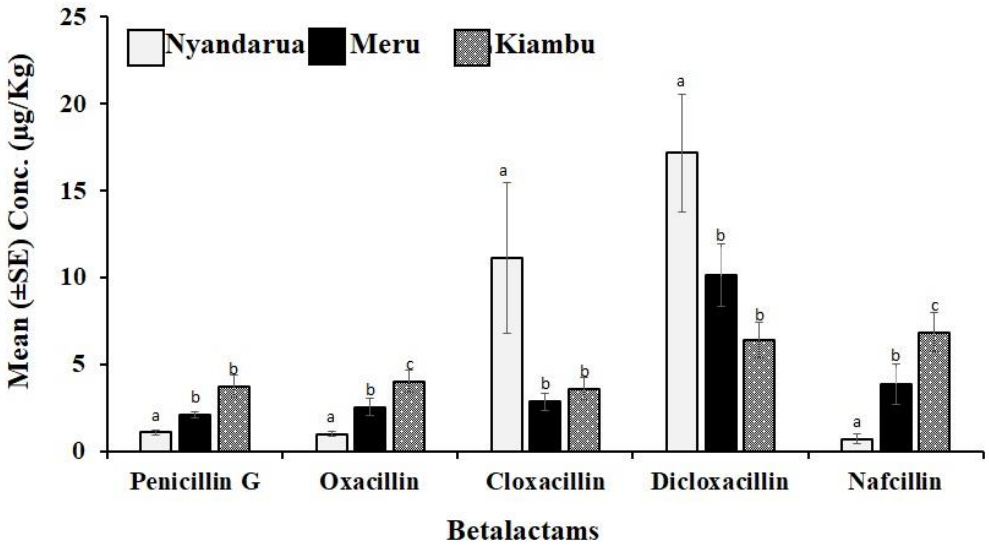

Figure 2. *The concentrations of beta-lactam drugs in milk from Nyandarua, Meru and Kiambu counties. The error bars show the standard deviations in the concentrations of beta-lactam drug residues from the three counties. $P < 0.05$ was considered significant. Means followed by identical letters on the error bars show that they are not statistically different ($P > 0.05$).*

3.3. Quantity of Tetracycline Traces in Milk Samples Using LC-MS/MS

Among the tetracyclines, the concentrations of oxytetracycline ($F_{(2, 320)}$ = 0.92, P = 0.4537) and doxycycline ($F_{(2, 320)}$ =1.17, P = 0.3256) showed no significant differences across all three counties (Figure 3). However, the concentrations of chlortetracycline residues differed significantly ($F_{(2, 320)}$ = 4.64, P = 0.0012) in milk sampled across the three counties, with the quantities from Nyandarua county markedly higher than Meru (P < 0.0001) and Kiambu (P = 0.0032) counties and those between Kiambu and Meru counties showing insignificant difference (P = 0.6098). The concentrations of demeclocycline ($F_{(2, 320)}$ = 6.21, P < 0.0001) and tigecycline ($F_{(2, 320)}$ = 4.32, P = 0.0021) residues also differed significantly in milk sampled across the three counties. Thus, milk from Nyandarua county contained more demeclocycline residues than Meru (P = 0.0003) and Kiambu (P < 0.0001), with the antibiotic traces observed in Kiambu and Meru counties (P = 0.4771) insignificantly different. Milk samples collected in Nyandarua County also contained more tetracycline residues than in Meru (P = 0.0008) and Kiambu (P = 0.0005) counties, with the tigecycline traces observed between Kiambu and Meru, showing no significant differences (P = 0.8560) (Figure 3).

3.4. Quantity of Sulfonamide Traces in Milk Samples Using LC-MS/MS

Among the 14 sulfonamide drugs quantified using LC-MS, the traces of 11 varied significantly across the three counties (P < 0.001), with sulfapyridine ($F_{(2, 320)}$ = 1.04, P = 0.3872) and sulfaquinoxaline ($F_{(2, 320)}$ = 0.35, P = 0.8458) exhibiting insignificant differences, while sulfamerazine was not detected (Figure 4). The sulfadiazine drug residues in milk samples differed significantly across the three counties ($F_{(2, 320)}$ = 12.74, P < 0.0001), with the concentrations of the drug in milk from Kiambu and Meru markedly higher than Nyandarua (P < 0.0001), and those from Meru slightly more than Kiambu (P = 0.0214). The concentrations of sulfathiazole in the milk samples also differed significantly ($F_{(2, 320)}$ = 25.03, P < 0.0001) across the three counties, with those from Kiambu and Meru considerably higher than Nyandarua (P < 0.0001), and those from Kiambu and Meru counties exhibiting insignificant changes (P = 0.7311) (Figure 4). The concentration of sulfamethizole also differed significantly ($F_{(2, 320)}$ = 15.09, P < 0.0001) in milk samples across the three counties. However, the equal amounts of sulfamethizole drug residues found in the milk samples collected in Kiambu and Nyandarua counties (P = 0.8232) were considerably higher than those from Meru (P < 0.0001). Though the concentrations of sulfadimidine drug residues showed significant differences ($F_{(2, 320)}$ =7.18, P < 0.0001) across the three counties, the amounts of the drug in milk samples collected in Kiambu (P < 0.0001) and Meru (P = 0.0003) were impressively higher than Nyandarua, with no differences in milk samples between Kiambu and Meru county (P = 0.2015) (Figure 4).

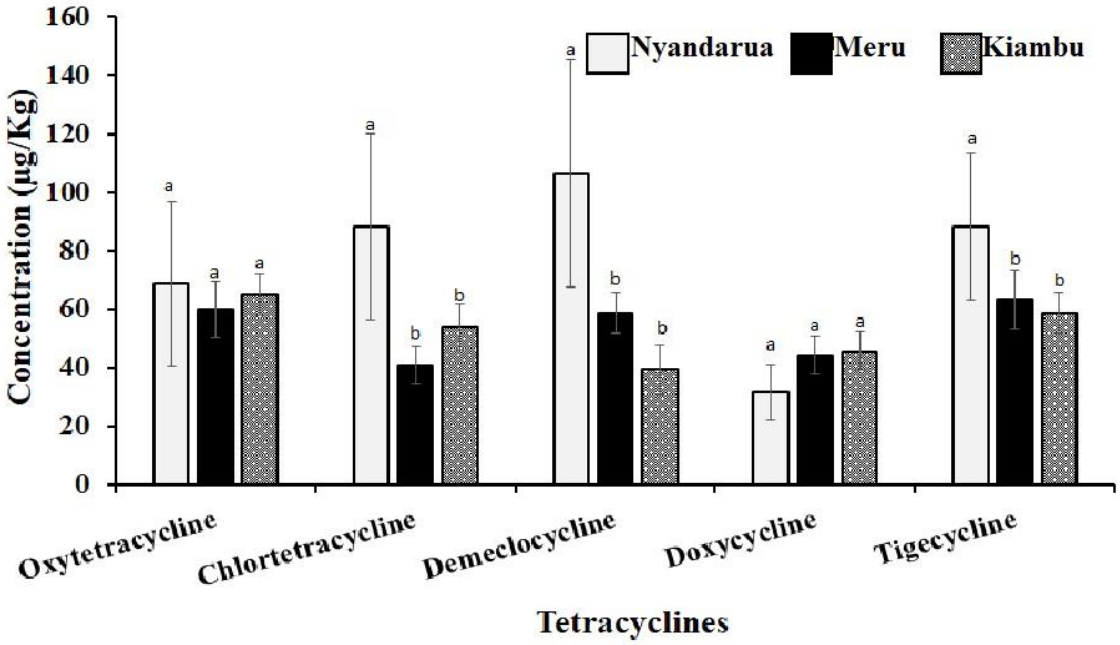

Figure 3. *The concentrations of tetracycline drugs in milk collected from Nyandarua, Meru, and Kiambu counties. The error bars show the standard deviations in the concentrations of tetracycline drug residues from three counties. P < 0.05 was considered significant. Means followed by identical letters on the error bars show that they are not statistically different (P > 0.05).*

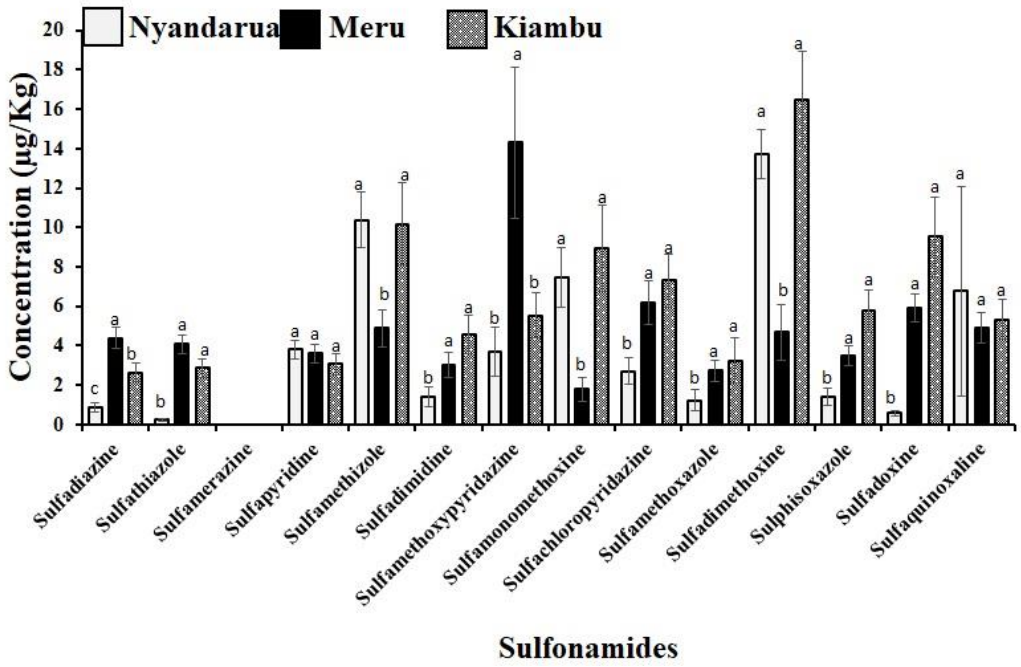

Figure 4. *Graphical representation of the concentration of sulfonamide drugs in milk from three different counties. The error bars show the standard deviations in the concentrations of sulfonamide drug residues from three counties. $P < 0.05$ was considered significant. Means followed by identical letters on the error bars show that they are not statistically different ($P > 0.05$).*

Similarly, the levels of sulfamethoxypyridazine in milk samples showed significant differences across the three counties ($F_{(2, 320)}$ =14.77, $P < 0.0001$), with those detected in milk samples from Kiambu and Nyandarua insignificantly different ($P = 0.7523$), but markedly lower than from Meru ($P < 0.0001$). The concentration of sulfamonomethoxine drug was also found to be significantly different ($F_{(2, 320)} = 9.83$, $P < 0.0001$) in the milk samples across the three counties, with those from Meru considerably lower ($P < 0.0001$) than those found in Nyandarua and Kiambu, which indicated insignificant differences among themselves ($P = 0.9868$). The amounts of sulfachloropyridazine differed significantly ($F_{(2, 320)} = 6.11$, $P < 0.0001$) across the three counties, with those from Kiambu ($P = 0.003$) and Meru ($P < 0.0001$) considerably higher than Nyandarua. However, those between Kiambu and Meru counties indicated similarity in the drug levels ($P = 0.1963$). The levels of sulfamethoxazole in milk samples differed significantly ($F_{(2, 320)} = 4.02$, $P = 0.0034$) across the three counties, with considerably higher amounts of residues in Kiambu ($P = 0.0004$), and Meru ($P = 0.0038$) than Nyandarua, while those in Kiambu and Meru counties did not indicate significant differences ($P = 0.6890$). The levels of sulfadimethoxine drug differed significantly ($F_{(2, 320)} = 24.46$, $P < 0.0001$) in milk samples across the three counties. However, the equal amounts of sulfadimethoxine drug residues found in the milk samples collected in Kiambu and Nyandarua counties ($P = 0.0646$) were considerably higher than those from Meru ($P < 0.0001$).

The concentration of sulphisoxazole in milk differed significantly ($F_{(2, 320)} = 7.87$, $P < 0.0001$) across the three counties. The equal amounts of sulphisoxazole drug residues found in the milk samples collected in Kiambu and Meru counties ($P = 0.2845$), were considerably higher than those from Nyandarua county ($P < 0.0001$). The levels of sulfadoxine ($F_{(2, 320)} = 15.95$, $P < 0.0001$) equally differed significantly across the three counties. While Nyandarua County ($P < 0.0001$) recorded the lowest levels of sulfadoxine residues, slightly higher levels of drug residues with equal values ($P = 0.0023$) were also noted than between Kiambu and Meru.

3.5. Correlation Coefficients for the Strength of the Relationship Between Tetracycline and Beta-Lactam Antibiotics

Nafcillin showed a weak but positive correlation with penicillin G and a strong correlation with oxacillin (Table 2). Dicloxacillin showed a strong and positive correlation with cloxacillin and a weak but positive correlation with tigecycline. Dicloxacillin also showed a strong and negative correlation with oxacillin and a weak but negative correlation with penicillin G. Cloxacillin strongly and negatively correlated with doxycycline and weakly but negatively with penicillin G. Oxacillin had a very strong and positive correlation with penicillin G and a weak but negative correlation with tetracycline. Besides oxacillin, penicillin G also slightly but positively correlated with doxycycline. Tetracycline had a very strong and positive correlation with oxytetracycline, chlortetracycline, demeclocycline and doxycycline, while doxycycline correlated strongly and positively with only oxytetracycline, chlortetracycline and demeclocycline.

Demeclocycline also positively correlated with oxytetracycline and chlortetracycline, while chlortetracycline correlated positively and strongly with oxytetracycline (Table 2). There were no significant correlations between sulfonamides and tetracyclines and with β-lactams.

3.6. Prevalence of Antibiotic Residues Exceeding the Maximum Residue Limits

Approximately 33.3% of milk samples from Kiambu County recorded the highest number of penicillin G residues above the recommended MRLs (Table 3), while about 2% of milk samples contained cloxacillin residues above the recommended MRLs. Dicloxacillin was also detected above the recommended MRLs in 8.7% and 16% of the milk samples collected at Meru and Nyandarua, respectively. However, other β-lactams quantified contained residues within the recommended MRLs (Table 3). The quantities of tetracycline traces in the milk samples varied widely, with those above the recommended MRLs ranging from 2 – 10.5% across the three counties. In Kiambu County, tetracycline was the least detected residue above the recommended MRL, followed by doxycycline, chlortetracycline, demeclocycline, and oxytetracycline (Table 3). In Nyandarua County, doxycycline, chlortetracycline and demeclocycline were the least, while tigecycline was the most detected residues in milk samples, with Meru County recording the highest amounts of tigecycline, chlortetracycline and demeclocycline residues above the recommended MRLs. Among the sulfonamides, only sulfaquinoxaline was used above the recommended MRL in 2% of milk samples collected in Nyandarua County. Sulfamerazine antibiotic residues remained undetected across all the counties.

Table 1. *The linear gradient for the liquid chromatography.*

Time (Minutes)	Pump A (0.1% formic acid)	Pump B (0.1% formic acid in methanol)
Initial	100	0
0.6	100	0
6.0	5	95
6.0	5	95
7.0	100	0
8.0	100	0

Table 2. *Pearson's correlation coefficients for tetracycline and β-lactam.*

	Oxy	Chlo	Dem	Dox	Tige	Pen	Oxa	Clo	Dicl	Nafc
Oxy	1.000	0.672**	0.702**	0.430***	0.610***	-0.044ns	-0.048 ns	-0.093 ns	-0.021 ns	0.037 ns
Chlo		1.000	0.852***	0.573***	0.827***	-0.049 ns	-0.068 ns	-0.039 ns	-0.001 ns	0.074 ns
Dem			1.000	0.586***	0.850***	-0.043 ns	-0.040 ns	0.030 ns	0.065 ns	0.075 ns
Dox				1.000	0.573***	0.121*	0.045 ns	-0.145***	-0.046 ns	0.023 ns
Tige					1.000	-0.013 ns	-0.125*	-0.024 ns	0.110*	0.013 ns
Pen						1.000	0.163***	-0.109*	-0.142*	0.116*
Oxa							1.000	-0.050ns	-0.211***	0.503***
Clo								1.000	0.466***	-0.107 ns
Dicl									1.000	-0.084 ns
Nafc										1.000

Key: Oxy= oxytetracycline; Chlo= chlortetracycline; Dem= demeclocycline; Dox= doxycycline; Tige= tigecycline; Pen= penicillin G; Oxa= oxacillin; Clo= cloxacillin; Dicl= dicloxacillin; Nafc= nafcillin; ns= not significant at $p \leq 0.05$; *** = significant at $p < 0.001$ and *= significant at $p \leq 0.05$.

Table 3. The range of concentrations of different antibiotic traces in milk samples collected in Kiambu, Nyandarua and Meru counties.

Antibiotic	Class	MRL *(ug/kg)	Kiambu[$]	Nyandarua[$]	Meru[$]
Penicillin G	β-lactam	4	0.23-15.79 (33.3%)	1.15-2.68	0.23-4.33
Oxacillin	β-lactam	30	0.02-9.98	0.13-2.36	0.03-9.98
Cloxacillin	β-lactam	30	0.07-13.05	0.62-98.32 (2%)	0.21-16.59
Dicloxacillin	β-lactam	30	0.02-19.08	3.76-83.74 (16%)	0.01-54.77 (8.7%)
Nafcillin	β-lactam	30	0.03-21.16	0.00-6.56	0.03-21.14
Oxytetracycline	Tetracycline	100	2.48-154.78 (10.5%)	3.03-546.73 (5%)	2.76-187.33 (5%)
Chlortetracycline	Tetracycline	100	7.87-138.48 (8%)	4.95-810.59 (3%)	3.08-152.12 (10.5%
Demeclocycline	Tetracycline	100	2.34-178.99 (8%)	9.22-1022.25 (3%)	3.25-115.68 (10.5%)
Doxycycline	Tetracycline	100	2.04-117.56 (5%)	4.53-246.89 (3%)	5.07-148.55 (3%)
Tigecycline	Tetracycline	100	2.11-162.39 (3%)	37.02-671.30 (10.5%)	3.42-187.48 (10.5%)
Sulfadiazine	Sulfonamide	100	0.11-8.19	0.00-5.14	0.11-9.64
Sulfathiazole	Sulfonamide	100	0.21-9.05	0.13-1.29	0.03-9.14
Sulfamerazine	Sulfonamide	100	Not detected	Not detected	Not detected
Sulfapyridine	Sulfonamide	100	0.15-9.45	0.31-7.92	0.22-9.44
Sulfamethazine	Sulfonamide	100	0.02-39.46	0.41-26.98	0.11-17.56
Sulfadimidine	Sulfonamide	100	0.08-19.73	0.12-11.99	0.02-12.54
Sulfamethoxypyridizine	Sulfonamide	100	0.22-22.74	0.21-21.88	0.11-73.04
Sulfamonomethoxine	Sulfonamide	100	0.23-34.04	4.22-21.56	0.07-9.99
Sulfachloropyridizine	Sulfonamide	100	0.23-28.44	0.19-14.95	1.11-25.99
Sulfamethoxazole	Sulfonamide	100	0.22-27.33	0.04-12.94	0.11-13.09
Sulfadimethoxine	Sulfonamide	100	0.32-43.27	2.22-27.42	0.11-36.66
Sulfisoxazole	Sulfonamide	100	0.02-22.47	0.11-7.33	0.37-8.98
Sulfadoxine	Sulfonamide	100	0.17-31.36	0.11-2.88	0.76-15.28
Sulfaquinoxaline	Sulfonamide	100	0.17-20.75	0.42-134.06 (2%)	1.22-14.65

*Indicates the antibiotics' recommended maximum residue limit (MRL) based on the Codex Alimentarius Commission [27]. [$]The brackets indicate the percentage prevalence of beta-lactams, tetracyclines and sulfonamides above the recommended MRLs.

4. Discussion

Milk is a widely consumed food with significant nutritional benefits for humans. However, its consumption in the presence of antimicrobial residues contributes to increased AMR [28], posing serious health concerns, such as allergies and cancers and food safety [29, 30], emphasizing the necessity for rigorous and dependable methods for detecting antibiotic traces in milk [31]. In this study, we used the rapid Charm TRIO® test kit and LC-MS to identify and quantify antimicrobial traces in milk samples collected in Kiambu, Nyandarua and Meru counties. The rapid Charm TRIO® test kit is a competitive multiplex immuno-receptor assay that concurrently detects different antibiotic classes [32]. Across the three counties, the tetracycline drugs were the most prevalent antimicrobial residues recorded using the TRIO® test kit in most milk samples, indicating its frequent usage in cattle management than β-lactams and sulfonamides. Milk samples from Nyandarua County also had a high prevalence of traces of oxytetracyclines, chlortetracycline, and demeclocycline, with those from Kiambu and Meru counties more prevalent in doxycycline and tigecycline. This could be due to the high population of dairy cows in the counties [34] utilizing the drugs in disease management. Besides, oxytetracyclines are also the most commonly used antibiotics in cattle farms in Kenya [33], which agrees with our findings.

Beta-lactams are widely used in dairy farming, especially

in treating mastitis, and their residues have been frequently identified [13, 35]. In this study, 11.4% and 20% of milk samples from Meru and Nyandarua counties contained dicloxacillin residues, making it the most prevalent β-lactam antibiotic. The milk screening for the antibiotic residues indicated that the county affected the type and prevalence of the antibiotics, with milk samples from Meru and Kiambu counties containing only dicloxacillin residues and those from Nyandarua counties only lacking penicillin G and oxacillin. However, the rapid test kit failed to detect sulfonamide drug residues except for sulfaquinoxaline at 4%. The inability to detect sulfonamides could have been due to low concentrations of the drug residues below the detection limits of the TRIO® test kit [36]. The Charm TRIO® test uses antibodies specific to each antibiotic family to bind to the antibiotic residues in the milk, forming a complex that the test can detect and can be affected by fat, protein and other substances in the milk, leading to false-positive or false-negative results [31].

Because of the low sensitivity of the Charm TRIO® test kit and its inability to quantify antibiotic traces, we used the LC-MS/MS to confirm the presence and quantities of antibiotic residues in the milk, with the prevalence of antibiotic residues detected by LC-MS/MS increasingly higher than those found using the rapid Charm TRIO® test kit. The LC-MS/MS detected the presence of β-lactam and tetracycline antimicrobial traces and all sulfonamide drugs tested except sulfamerazine in all three counties, which could be due to its higher precision and accuracy compared to rapid tests [26, 37, 38]. The LC-MS/MS results also showed a strong correlation between some β-lactam and tetracycline drugs and none with sulfonamides, indicating that farmers in the three counties used more than one antibiotic drug to treat cows. The strong positive correlation observed between the five tetracycline antibiotics, however, shows the frequent usage of these antibiotics alone or in combination. There was no apparent correlation between penicillin and tetracycline except for the strong correlation between doxycycline and cloxacillin and a weak but positive correlation between doxycycline and penicillin, tigecycline and oxacillin, and tigecycline and dicloxacillin. This correlation shows the usage of these antibiotics in combination to overcome resistance, as has been reported for penicillin G and oxytetracycline in cattle treatment [39]. No relationship was observed between a few beta-lactam drugs, including between nafcillin and dicloxacillin, nafcillin and cloxacillin, and between cloxacillin and oxacillin.

According to KEBS regulations, cow milk must meet the Codex MRLs for veterinary medications in milk [29] to help safeguard the health of consumers. However, in this study, milk samples from Kiambu recorded penicillin G residues of about 15 µg/kg higher than the recommended 4 µg/kg, corroborating earlier studies [40], which found 13% of milk samples with penicillin G residues exceeding the recommended MRLs in Kericho, Nakuru and Kiambu counties in Kenya. Nyandarua recorded dicloxacillin levels as high as 83.74 µg/kg, with Meru recording 54.77 µg/kg, above the recommended 30 µg/kg. Cloxacillin residues in Nyandarua County also recorded levels as high as 98.32 µg/kg. However, nafcillin and oxacillin were within the recommended MRLs, similar to studies conducted in northern-central Algeria [26]. Therefore, it is important to enforce a regulatory framework to ensure that antibiotic use in livestock, is controlled to minimize potential health risks related to antimicrobial traces in the food supply.

All the antibiotics in the class of tetracyclines recorded between 3 and 10.5% of antibiotic residues used above the recommended MRLs in milk across all three counties. These results conflicted with those of Kosgey *et al*. [41], where residues of tetracyclines in Eldoret, Kenya, were below or within the recommended MRLs. The difference could be due to differences in geographical regions, causing different usages of antibiotics. Results from this study also contradicted those conducted in Nakuru County where tetracycline antibiotics were not confirmed using HPLC [12]. The peaked incidence of tetracyclines could be due to its broad-spectrum function. Tetracyclines are also commonly used by farmers and can be administered through food and water, parenterally, or by intramammary infusion and even small amounts may remain persistent in animals after administration which may then be excreted in milk [42, 43]. In Kibera, about 67% of milk samples contained *E. coli*, resistant to tetracyclines [9] (Brown *et al*., 2020), which shows that persistent infections due to antibiotic-resistant bacteria could necessitate frequent use of these antibiotics in a bid to clear infections. In Indonesia, HPLC detected the presence of chlortetracycline, oxytetracycline, and tetracycline in milk, with only 3.45% of samples exceeding chlortetracycline MRL [44]. Though only 2% of milk samples contained sulfaquinoxaline above the recommended MRLs, all other sulfonamides were found within the recommended MRLs, agreeing with studies conducted earlier in Kenya with only 0.4% [8] and 4.1% [12] prevalence, indicating they were less used in the sampled counties.

According to Brown *et al*. [9] and Myers *et al*. [45] antimicrobial drug use in animal health management promotes drug use globally, leading to the rising threat of AMR to humans. Besides posing the danger of causing AMR, the occurrence of antibiotic drugs in milk can cause food allergenicity, cancer, and reproductive disorders and be toxic to the bone marrow and liver among consumers. Since the distribution of veterinary antimicrobials is not controlled due to the lack of regulation of veterinary pharmaceuticals in most African territories [46], limiting antimicrobial traces in milk will require a multifaceted approach, including educating producers on the importance of responsible usage of antibiotics and the risks of antibiotic traces in milk to ensure stricter oversight of the drug sales and withdrawal times [9]. This will also include setting clear and enforceable regulations to strengthen the surveillance of antimicrobial traces and AMR in animal food products and increasing awareness and concern about AMR and its dissemination pathways among policymakers, veterinary officials, and the public.

5. Conclusion

The rapid Charm Trio™ Test kit detected antibiotic traces in milk, highlighting its efficacy due to its rapidness and ability to detect up to three antibiotic classes, however, the kit was less sensitive in the detection of sulfonamides since sulfonamides have a higher limit of detection compared to tetracyclines and Betalactams. Confirmatory tests analysis revealed two key findings: First, some milk samples that tested negative on rapid tests still recorded antibiotic residues. Secondly, milk from all counties contained all types of antibiotics under study except sulfamerazine. However, all tetracyclines, some beta-lactams (penicillin G, cloxacillin and dicloxacillin) and only sulfaquinoxaline among sulfonamides recorded levels above tolerable maximum residue limits, highlighting the high sensitivity of LC-MS/MS as a confirmatory method.

Tetracycline residues exceeded recommended MRLs in a significant portion of samples, possibly due to its broad-spectrum function. Sulfonamide residues were generally within recommended limits, suggesting lesser usage in the sampled counties.

Tetracycline drugs were most prevalent, indicating their frequent use in cattle management, especially oxytetracyclines, which aligns with their common usage in Kenya. Beta-lactams, particularly dicloxacillin, were prevalent, possibly due to their use in treating mastitis in dairy farming.

Abbreviations

AMR: Antimicrobial Resistance
GDP: Gross Domestic Product
LC-MS/MS: Liquid Chromatography Triple Quadrupole Mass Spectroscopy
MRLs: Maximum Residue Levels
TCA: Trichloroacetic Acid

Ethical Considerations

Approval was sought from the Kenyatta University Ethical Committee and the National Commission for Science and Technology (NACOSTI).

Author Contributions

All authors contributed to the study's conception and design. Bonnita Odeny Aluoch performed material preparation, data collection and analysis, and wrote the first version of the manuscript. All authors helped to write the final version of the manuscript. The authors read and approved the final manuscript.

Funding

The authors declare that no funds, grants, or other support were received during the preparation of this manuscript.

Conflicts of Interest

The authors declare no conflicts of interest.

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